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Populations of *Symbiodinium muscatinei* Show Strong Biogeographic Structuring in the Intertidal Anemone *Anthopleura elegantissima*

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Abstract. Among temperate cnidarian symbioses, the partnership between the intertidal anemone *Anthopleura elegantissima* and its dinoflagellate and chlorophyte symbionts is one of the most well characterized. Biogeographic, reciprocal transplant, and physiological studies have convincingly demonstrated a relationship between environmental factors such as temperature and irradiance and the distribution of symbionts from both algal phyla. However, little is known about the fine-scale diversity or biogeographic distribution within symbiont lineages of this anemone. We used sequence information from the mitochondrial cytochrome *b* and chloroplast 23S ribosomal genes and restriction fragment length polymorphism data from the 18S nuclear ribosomal gene to characterize the *Symbiodinium* populations in tentacles clipped from 105 anemones at 14 sites along the entire California coast, spanning about 1200 km. Our results show the presence of at least three primary biogeographic regions with breaks around Cape Mendocino and Monterey Bay, each dominated by a different *Symbiodinium muscatinei* genotype. Sharp clines suggest limited gene flow between adjacent regions. Few sampling locations or individual anemones showed symbiont diversity at either organellar locus within the limits of our detection method, while sequence analysis of cloned nr18S polymerase chain reaction product suggests that nuclear pseudogenes may underlie intra-host diversity observed at that locus.

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Introduction

The temperate intertidal anemone *Anthopleura elegantissima* Brandt, 1835 hosts both dinoflagellates (zooxanthellae of the genus *Symbiodinium*) and chlorophytes (zoochlorellae; *Elliptochloris marina* [Letsch *et al.*, 2009]) at levels that vary predictably over both local differences in microhabitat and regional gradients of light, temperature, tidal height, and latitude (Secord and Augustine, 2000; Secord and Muller-Parker, 2005). These anemones acquire their microbial partners horizontally each generation, experience a much greater range of tidal and seasonal variation in temperature and irradiance than their tropical relatives, and are distributed over a broad latitudinal range from Alaska to Baja California. Zooxanthellate anemones are most frequently found in areas with brighter light and warmer temperatures, at higher tidal elevations, and at lower latitudes, suggesting possible fitness consequences to partner type in different environments (Secord and Augustine, 2000). Differences in symbiont photosynthetic efficiency (Verde and McCloskey, 2001, 2002) and population dynamics (Saunders and Muller-Parker, 1997), translocation of carbon to the host (Bergschneider and Muller-Parker, 2008), and even differential predation by symbiont type (Augustine and Muller-Parker, 1998) all support this hypothesis, though the specific mechanisms driving symbiont distribution in natural populations remain unclear. While aposymbiotic adults have been shown to acquire symbiont populations appropriate to local conditions (Secord and Muller-Parker, 2005), previously established symbiont populations are relatively stable in the face of environmental perturbation (Saunders and Muller-Parker, 1997; Secord and Muller-Parker, 2005; Verde and McCloskey, 2007), especially compared to seasonal dynamics observed in tropical species (Muller-Parker, 1987; Venn *et al.*, 2008).

A. elegantissima has also been reported to harbor multiple types of *Symbiodinium*. LaJeunesse and Trench (2000) reported the presence of two *Symbiodinium* types in *A. elegantissima.* Using denaturing-gel gradient electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) analysis of symbiont nuclear ribosomal DNA, they found a single B-clade type (designated *S. muscatinei* nom nud, LaJeunesse and Trench, 2000) to be ubiquitous among sampled individuals, and a second E-clade type (designated *S. californium*) co-inhabiting anemones in the three southernmost sites. Subsequent studies have investigated potential factors underlying this observed distribution, including differential thermal tolerances of the two types of dinoflagellate (Muller-Parker *et al.*, 2007) and variations in larval uptake efficacy, which may be a proxy for specificity (Schwarz *et al.*, 2002). However, these studies have been hampered by an inability to directly control for symbiont type, as *S. muscatinei* has not been isolated in culture, and the E-clade *S. californium* has not been described in anemones independently from the *S. muscatinei*.

The patterns of diversity observed in *A. elegantissima* symbionts indicate that local environmental conditions play an important role in structuring the ecology of this interaction, but little is known about the fine-scale distribution and connectivity among populations of either partner. In marine systems subject to strong oceanographic currents, panmixis of highly dispersive populations may limit local adaptation (Palumbi, 1992; Kawecki and Ebert, 2004). While few studies have examined the dispersal potential of temperate cnidarian symbionts, tropical and subtropical coral species host *Symbiodinium* populations that range in connectivity from thousands of kilometers (Magalon *et al.*, 2006) to isolation on the scale of tens of kilometers (Santos *et al.*, 2003a; Howells *et al.*, 2009). Recent data on high-latitude *Symbiodinium* populations in the Pacific suggest a trend toward greater symbiont endemicity and host specificity in these regions relative to tropical habitats (Wicks *et al.*, 2010), possibly due to strong gradients in selection imposed by a more seasonally variable environment.

To help elucidate the factors governing *Symbiodinium* distribution in *A. elegantissima*, we expanded the study by LaJeunesse and Trench (2000) using a finer sampling scale over the reported transition zone and a multilocus, sequence-based analysis for greater resolution of diversity.

Materials and Methods

Collections

Tentacles were clipped from intertidal *Anthopleura elegantissima* at 14 locations along the California coast during May of 2005 (northern and central locations) and June of 2006 (southern locations; Table 1). Samples were collected haphazardly at low- to mid-tide, though an effort was made to sample across microhabitats at each site to maximize sampled symbiont diversity. To prevent accidental sampling of the solitary congener *A. sola*, only individuals from clonal aggregations were sampled, and they were chosen from different clonal aggregations to avoid resampling clones. Tissue samples were preserved in 70% ethanol.

Table 1

Sampling locations

Location	Anemones sampled (no.)	Latitude	Longitude
Pelican Point (PP)	5*	N41°57′	W124°12′
Wilson Creek (WC)	8	N41°36′	W124°07′
Trinidad Head (TH)	9	N41°02′	W124°08′
Humboldt Bay (HB)	7	N40°46′	W124°14′
Mattole Road (MR)	9	N40°24′	W124°23′
Shelter Cove (SC)	8	N40°01′	W124°04′
Albion (AL)	4	N39°16′	W123°47′
Point Arena (PA)	9	N38°54′	W123°43′
Salt Point (SP)	7	N38°33′	W123°20′
Bodega Bay (BB)	5	N38°19′	W123°04′
Pacific Grove (PG)	10	N36°37′	W121°37′
San Luis Obispo (SL)	7	N35°10′	W120°43′
Los Angeles (LA)	7	N33°42′	W118°17′
San Diego (SD)	7	N32°51′	W117°16′

Tentacle clips taken from anemones in May 2005 (PP to PG) and June 2006 (SL, LA, and SD).

* Nine anemones were sampled from Pelican Point, but four appeared to contain zoochlorellate symbionts and did not yield positive amplifications using *Symbiodinium*-specific primers.

We chose the initial set of 11 locations sampled in 2005 so that the region of California coastline spanning the shift in symbiont composition reported by LaJeunesse and Trench (2000) was covered more-or-less evenly, resulting in an average distance of about 60 km between sites; an additional three southern locations were sampled opportunistically at lower resolution (≈150 km between sites) the following year. The northern locations (Pelican Point to Bodega Bay) span much of the Mendocinian/Oregonian biogeographic province (provinces *sensu* Blanchette *et al.,* 2008), and are characterized by high rock cover and populations of the mussel *Mytilus californianus*, *Chthamalus* barnacles, and red algae (Blanchette *et al.*, 2008). Pacific Grove (PG) and San Luis Obispo (SB) both fall in the Montereyan province, with representative fauna roughly similar to that of the Mendocinian province and higher proportions of crustose coralline algae and bare rock. The southernmost two sites fall into the Southern Californian (LA) and Ensenadian (SD) provinces, and are characterized by higher proportions of bare rock. Of relevance to this study, the solitary congeners *A. xanthogrammica* and *A. sola* were observed north and south of PG, respectively, with all three species abundant at PG; these observations are

consistent with previous descriptions of their ranges (Smith and Potts, 1987; Pearse and Francis, 2000).

Molecular characterization

We extracted total genomic DNA from two or three tentacles per anemone by using NucleoSpin (Machery-Nagel) column extraction kits according to manufacturer instructions. We amplified three loci, using *Symbiodinium*-specific primers to determine symbiont composition for each sample. A fragment (about 1700 bp) of small-subunit (18S) nuclear ribosomal DNA (nrDNA) was amplified using the SS5 and SS3Z primers of Rowan and Powers (Rowan and Powers, 1991b) and the following reaction conditions: 3 min initial denaturing at 94 °C, followed by 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min, followed by a 10-min final extension. A fragment (about 700 bp) of chloroplast 23S (cp23S) ribosomal DNA was amplified using the 23S1 and 23S2 primers of Zhang *et al.* (2000) and the following reaction conditions: 3-min initial denaturing at 94 °C, followed by 35–40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a 5-min final extension. Finally, an approximately 900-bp fragment of the mitochondrial cytochrome *b* (cyt *b*) gene was amplified using the Dinocob1F and Dinocob1R primers of Zhang *et al.* (2005) and the following reaction conditions: 1–min initial denaturing at 94 °C, followed by 35 cycles of 95 °C for 45 s, 50°C for 45 s, and 72 °C for 1 min, followed by a 7-min final extension. Negative controls were included in each amplification to avoid contamination.

18S nrDNA amplicons were restriction-digested using αTaqI and following the protocol of LaJeunesse and Trench (2000), with digested fragments electrophoresed in 3% agarose gels and visualized with ethidium bromide. Cytochrome *b* and chloroplast 23S amplicons were direct-sequenced using respective amplification primers and a Big Dye Terminator Cycle Sequence kit (Applied Biosystems, Inc.), then analyzed on an Applied Biosystems 3100 genetic analyzer.

Because we were unsuccessful at sequencing 18s nrDNA directly from polymerase chain reaction (PCR) product, we cloned PCR product from two anemones (MR1 and PG2) with mixed RFLP signatures into chemically competent *E. coli* using the pGEM-T kit (Promega) according to manufacturer instructions. Ninety-six colonies from each sample were amplified using T7f and M13r primers and screened for size on a 1% agarose gel. We then sequenced colonies containing inserts by using the above protocol and a combination of T7f, M13r, SS5 and SS3Z primers, depending on amplified fragment length.

Analysis

All experimental sequences were aligned and cleaned by hand in Sequencher 4.5 (Gene Codes Corp.). Haplotype assignment was done manually. Mixed *Symbiodinium* haplotype populations were scored by eye by examining polymorphic nucleotide positions in the chromatogram trace for perceptible double peaks; to be counted as mixed, the subordinate peak had to (1) be greater than 10% of the height of the dominant peak, (2) be substantially higher than any background fluorescence, and (3) represent a nucleotide dominant at that position in at least one other sample. This method is likely to overlook mixed populations that are dominated by a single type.

Population genetic parameters were calculated from cp23S and cyt *b* haplotypes by using the online version of GENEPOP (Raymond and Rousset, 1995). Anemones harboring detectably mixed populations at these two loci were scored as two separate haploid individuals in the data matrix.

Phylogenetic analysis of cp23S haplotypes was performed using an alignment of the four haplotypes found in this study with representative *Symbiodinium* cp23S sequences from GenBank; sequences were aligned using default parameters in MUSCLE (Edgar, 2004). Four hypervariable regions were removed to reduce problems associated with ambiguous alignment of highly divergent sequences; analysis was performed exclusively on the resulting 392-bp alignment. Maximum parsimony (MP) phylogenies were computed in PAUP 4.0b10 (Swofford, 2002) using a heuristic search with 10 random addition Wagner tree replicates and Tree-Bisection-Reconnection (TBR), with gaps treated as missing data; support was calculated using 1000 full-heuristic bootstrap replicates. The maximum likelihood (ML) phylogeny was computed in PAUP using the same heuristic search parameters and a model of nucleotide evolution (TrN + I + γ) chosen in Modeltest 3.7 (Posada and Crandall, 1998). Maximum-likelihood bootstrap values were calculated using 1000 bootstrap replicates.

Results

We characterized the symbiont populations from 105 anemones from 14 locations along the coast of California (Fig. 1), with sample sizes ranging from 4 to 10 individual anemones per location. Amplification using *Symbiodinium*-specific primers was successful in all cases, except for four anemones from the northernmost site (PP), which from their bright green color appeared to be hosting only zoochlorellae.

RFLP data from 18S nrDNA showed a large amount of variation, primarily due to extensive secondary banding. Electrophoresis of PCR product yielded a band of about 1800 bp in all samples, with most samples from the central and southern coast (MR, SC, SP, BB, PG, SB, LA, and SD) showing a fainter and slightly smaller second band. After αTaqI digestion, all samples displayed three bright bands in

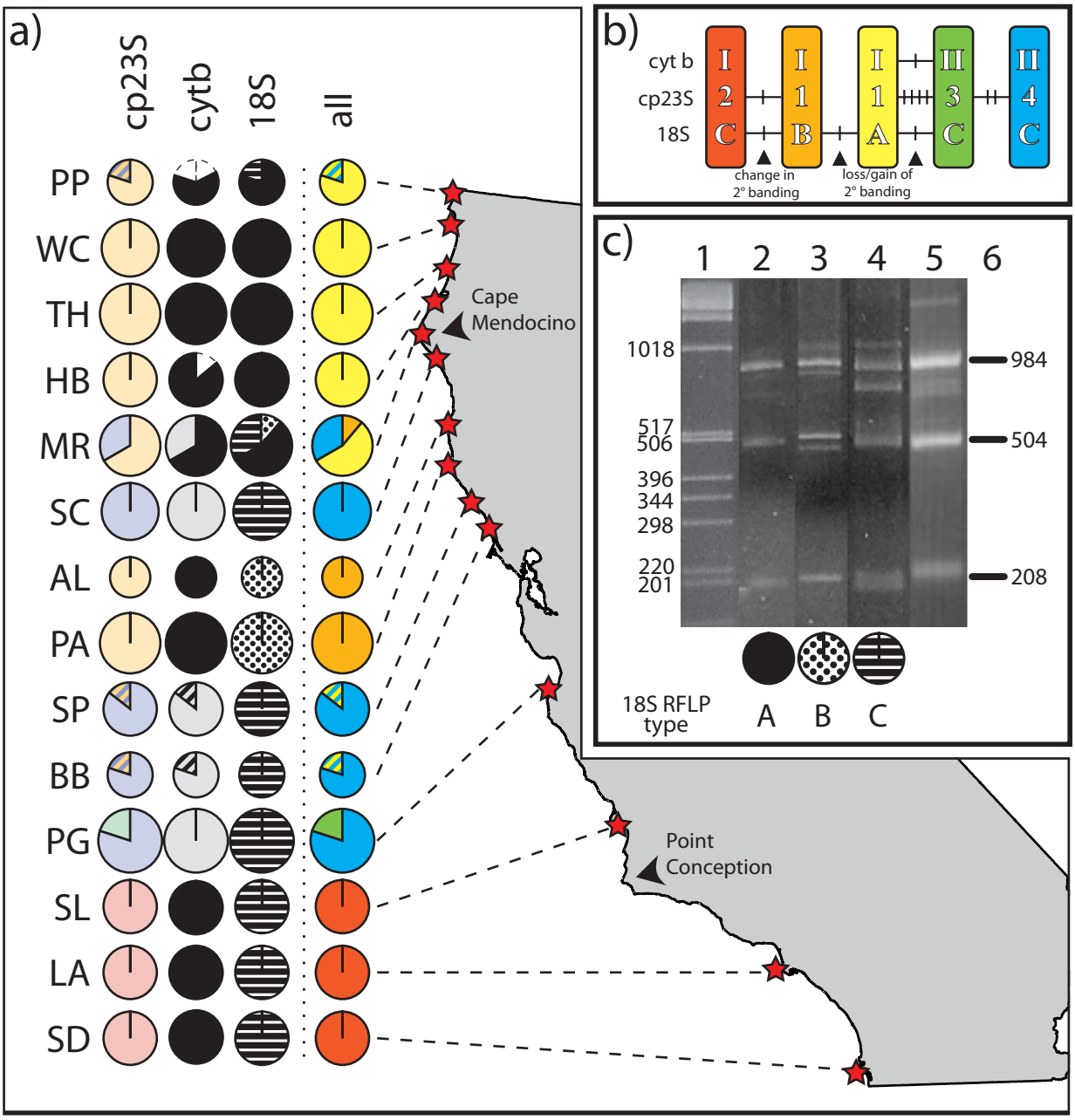

Figure 1. (a) Distribution of genotypes in *Symbiodinium* sp. associated with *Anthopleura elegantissima.* Each pie chart represents haplotypes from one locus and sampling location; pie size corresponds to number of anemones sampled (min = 4, max = 10). Missing wedges (marked by dashed border) indicate the three samples for which we were unable to sequence cyt *b.* Rows are labeled with two-letter location codes given in Table 1, and columns with the respective genetic locus. Colors correspond to unique multilocus haplotypes as in (b); crosshatched blue and yellow wedges represent anemones harboring detectable levels of both cp23S haplotypes 1 and 3. Black and grey wedges in the second column similarly represent cyt *b* haplotypes I and II respectively; crosshatched patterns denote presence of both. 18S wedges are patterned according to the αTaqI restriction fragment pattern. (b) Minimum spanning multilocus haplotype network. Hatches represents SNPs or contiguous indels at cp23S and cyt *b* loci, or the indicated changes in RFLP banding pattern for 18S. (c) Representative 18S αTaqI RFLP patterns. Lanes are digitally combined from multiple gel images; restriction profiles observed in this study are marked with a circle patterned as in (a). *1.* 1kb ladder. *2.* "Northern" Type A profile, showing the three bright bands at 984, 504, and 208 bp typical for B-clade *Symbiodinium*. *3.* "Transition" Type B profile; note secondary banding. *4.* "Southern" Type C profile, with bright secondary bands at ≈740 and ≈1100 bp. *5.* "Southern" 18S αTaqI RFLP profile reproduced from LaJeunesse and Trench (2000). *6.* Typical B-clade profile, after Rowan and Powers (1991a).

a typical B-clade pattern (Rowan and Powers, 1991a) at about 950, 500, and 250 bp. Except from the northernmost sites (PP, WC, TH, and HB) and two individuals from the central coast (PG3 + 4), samples also displayed diverse secondary banding. Secondary band sizes did not match previously described restriction patterns from other *Symbiodinium* clades (Rowan and Powers, 1991a), but they did fall into three main types that were mostly consistent within sampling locations (designated A, B, and C; see Fig. 1c).

Cloning the 18S locus yielded only a few complete sequences (defined as including regions expected to come immediately internal to the amplification primers) for each sample, resulting in a total of 12 sequences for MR1 and 6 sequences for PG2. In addition to these full-length sequences, cloned fragments showed extensive variation in sequence length, including large deletions up to 445 bp; only two clones yielded identical sequences. A maximum parsimony analysis of these sequences and one to two representatives each of clades A–H showed all 19 cloned sequences grouping with other B-clade *Symbiodinium* sequences (data not shown).

Cytochrome *b* sequencing revealed two *Symbiodinium* genotypes (Types I and II; Genbank Accessions JF927798 and JF927798, respectively), differing by a single nucleotide transversion (C/G) near the 3′ end of the amplified fragment. Sixty-four anemones yielded Type I and thirty-one yielded Type II cyt *b Symbiodinium* sequence; two samples from the central coast showed a double peak in the chromatogram at that position, suggesting the presence of both symbiont types; and three did not give usable sequence after three attempts. Anemones hosting Type I cyt *b Symbiodinium* were distributed primarily at the northern and southern extremes of the sampling range, with Type II abundant at only five sampling locations in the central coast (Fig. 1a). Only one sampling location, MR, had significant representation of both sequence types. F_{ST} among populations for cytochrome *b* was 0.89.

Sequences from the chloroplast 23S rDNA locus correlated with those from cyt *b*, but showed more nucleotide diversity: there was a total of seven nucleotide differences (six single nucleotide polymorphisms and one indel) among four haplotypes (Types 1–4, Fig. 1; Genbank Accessions JF927794–7, respectively). All these differences were synapomorphic over a maximum-parsimony tree, permitting a simple minimum-spanning haplotype network to be constructed by hand (Fig. 1b). One haplotype (Type 3) was rare, occurring only in two anemones from Pacific Grove (PG3 + 4). The remaining three haplotypes followed a similar distribution to the cyt *b* sequences, with cp23S Type 3 or 4 present in all anemones with Type II cyt *b* (Fig. 1a). Anemones hosting Type I cyt *b* sequences always yielded either Type 1 or Type 2 cp23S sequence, with Type 1 in the north and Type 2 in the south. Both anemones hosting symbiont populations heterologous for cyt *b* also showed cp23S chromatograms heterologous for Type 2 and 4 haplotypes, as did an anemone from the northernmost sampling location for which we were unable to sequence cyt *b*. Multiple haplotypes were not detectable in any other chromatograms. F_{ST} among populations for the cp23S locus was 0.87. Phylogenetic analysis of the four cp23S haplotypes placed them with a high degree of support (95%/82% MP/ML bootstrap support) as a monophyletic sister group to the rest of the B clade (Fig. 2), consistent with the placement of *S. muscatinei* by LaJeunesse and Trench (2000). The maximum parsimony 50% majority-rule consensus tree was consistent with the maximum likelihood phylogeny.

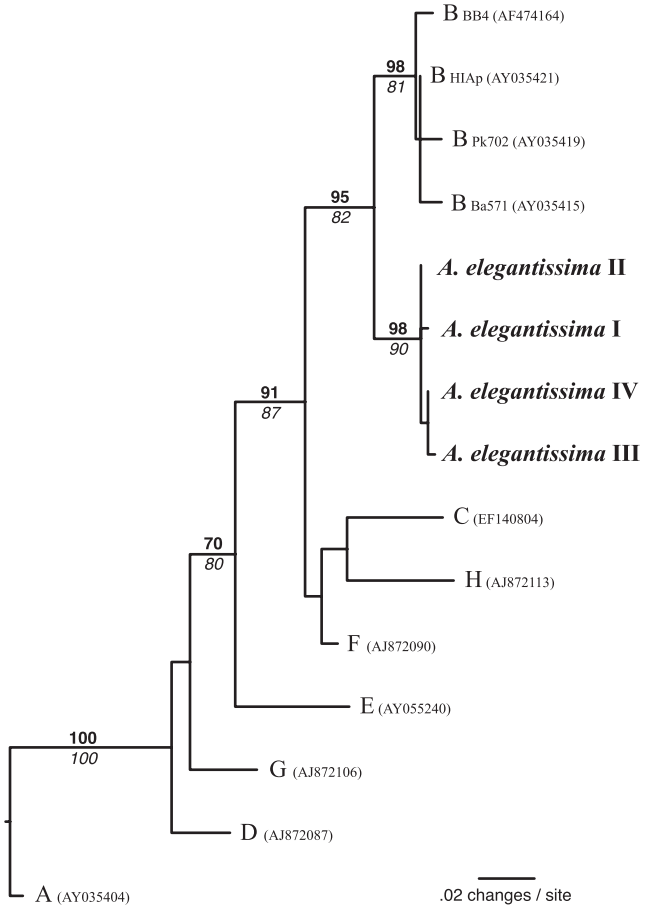

Figure 2. Maximum likelihood phylogeny of cp23S gene. Phylogeny computed using a TrN + I + γ model of nucleotide evolution. Bootstrap values greater than or equal to 70% are given above (bold, maximum parsimony) and below (italic, maximum likelihood) branches. Terminals are labeled with clade type and representative GenBank accession number; B-clade terminals are also labeled with the subtype as identified in the GenBank description.

In summary, a total of five multilocus genotypes were detected (Fig. 3). At the northernmost sites (PP, WC, TH, and HB), almost all *Symbiodinium* sequences were Type I-1-A (cyt *b* Type I, cp23S Type 1, 18S RFLP pattern A). At two sites in the central coast (AL and PA), all samples were

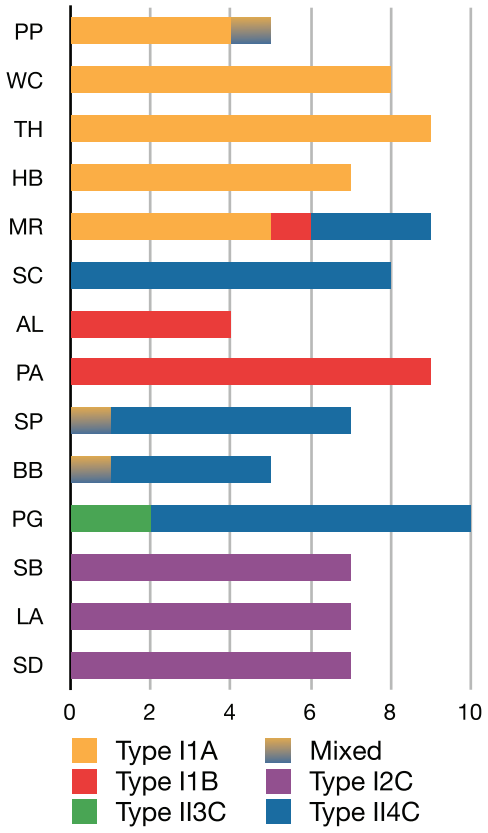

Figure 3. Distribution of *Symbiodinium* multilocus genotypes in tentacles of anemones at each sampling site. Sampling location codes as in Table 1; from top of figure, sites are ordered north to south, and length of bars corresponds to number of individual anemones sampled per site. Type codes correspond to cyt *b* (I or II), cp23S (1–4), and 18S RFLP (A, B, and C) haplotypes. Three individual anemones appeared to host *Symbiodinium* populations mixed for both cyt *b* (I and II) and cp23S (1 and 4) loci.

identical but for a different pattern of secondary banding in the 18S RFLP analysis (Type I-1-B). Central coast sites (SC, SP, BB, and PG) were dominated by the divergent Type II-4-C, with two anemones from PG hosting *Symbiodinium* with a unique cp23S sequence (multilocus haplotype II-3-C). Uniquely, *Symbiodinium* from three major genotype groups were found at the Mattole Road sampling site (five individual anemones hosted Type I-1-A, one Type I-1-B, and three Type II-4-C). Southern sites (SB, LA, and SD) hosted only Type I-2-C, which was identical to the northern Type I-1-A except for the 18S RFLP pattern and a single nucleotide polymorphism in cp23S. Additionally, a single individual each from sites BB, SP, and PP appeared to host mixed *Symbiodinium* populations.

Discussion

In this study, we identified five distinct *Symbiodinium* multilocus genotypes associated with *Anthopleura elegantissma* in California, distributed in a highly structured pattern along the coast. No evidence was found of the previously described E-clade *S. californium*, though the cp23S sequences we did find formed a monophyletic group sister to the B-clade of *Symbiodinium*, consistent with the placement of *S. muscatinei* described previously in the *Anthopleura* system (LaJeunesse and Trench, 2000) (Fig. 2). The five multilocus genotypes were distributed among northern, central, and southern provinces, with sharp clines indicated by complete genotype turnover between adjacent sampling locations at our sampling resolution of about 100 km. Our results open the possibility that within-clade diversity may have important adaptive consequences in *A. elegantissima*, and provide the necessary fine-scale phylogeographic data for well-controlled follow-up studies on symbiont functional differences.

Pseudogenes and S. californium

Despite direct-sequencing two organellar loci as well as performing RFLP analysis and cloning of the nuclear 18S gene, we were unable to detect the presence of the E-clade *Symbiodinium californium* described by LaJeunesse and Trench (2000). Given the limited sampling depth of our cloning effort and the very limited sensitivity of direct sequencing and RFLP to detect variation within a sample, it is entirely possible that *S. californium* was present in low abundance in our samples, but was simply undetected. Alternatively, heterogeneously distributed or temporally variable intra-host variation in symbiont populations—previously reported for *Symbiodinium* in tropical corals (Rowan *et al.*, 1997; van Oppen *et al.*, 2001; Ulstrup and Van Oppen, 2003) and anemones (Venn *et al.*, 2008), and for populations of zoochlorellae and zooxanthellae in *Anthopleura* (Secord and Augustine, 2000)—may have systematically biased our samples, which were taken exclusively from tentacles in spring and summer.

Nonetheless, the presence of extensive secondary banding in RFLP profiles and the large amount of sequence length variability detected among cloned 18S sequences suggest the presence of pseudogene diversity at that locus among southern- and central-type *S. muscatinei*, coincident with the range reported for *S. californium.* Purported nuclear ribosomal pseudogenes and sequence variants have been observed within *Symbiodinium* before (Santos *et al.*, 2003b; Kirk *et al.*, 2005; Thornhill *et al.*, 2007), potentially confounding cloning-based diversity estimates and complicating analysis (Thornhill *et al.*, 2007; Stat *et al.*, 2009). In the current study, the extent of intragenomic variation appears to have confounded even the less sensitive RFLP- and direct-sequencing-based methods.

S. californium was reportedly isolated in culture from southern *A. elegantissima* after repeated unsuccessful attempts (T. LaJeunesse, Penn State University; pers. comm.). Subsequently, most study of *S. californium* has been per-

formed on this culture, with only a single study (LaJeunesse and Trench, 2000) inferring its presence in field-collected *Anthopleura* on the basis of RFLP analysis. Is it possible that previous reports of *S. californium* found in association with wild *A. elegantissima*, which were based exclusively on RFLP and DGGE analysis of the nuclear ribosomal locus, were misled by intragenomic pseudogene diversity? The presence of extraneous RFLP banding in anemones from southern sites inferred by LaJeunesse and Trench (2000) to host *S. californium* and the absence of these bands in northern sites suggest that this hypothesis is at least plausible. The few other E-clade *Symbiodinium* described to date are free-living (Santos *et al.*, 2002); given the apparent resistance of *S. muscatinei* to culture (LaJeunesse and Trench, 2000), it is possible that a low-abundance free-living or parasitic E-clade dinoflagellate was accidentally introduced to the original stock, misrepresenting the natural symbiont population (Santos *et al.*, 2001). Unfortunately, descriptions of the initial culture conditions for *S. californium* #343 are not found in either of the two studies typically referenced as its source (Banaszak *et al.*, 1993; Trench and Blank, 1987).

If the long-standing connection between *S. californium* and *A. elegantissima* does prove spurious, it would help explain perplexing results in several previous studies. Schwarz *et al*. (2002), for instance, found *S. californium* unable to establish intracellular associations in larval anemones, even after a significant number of dinoflagellate cells had been ingested. Banaczak and Trench (1995) found that cultured *S. californium* developed multilayer cell walls after chronic exposure to UV, while *S. microadriaticum*, both in culture and *in symbio*, as well as *Symbiodinium* cells in southern anemones presumed to contain *S. californium*, did not. Our results, while not conclusive, at least suggest that the current understanding of *A. elegantissima* as hosting a dual B- and E-clade symbiosis should be critically reevaluated.

Symbiodinium *population connectivity*

The strongly structured biogeography of closely related *Symbiodinium* genotypes described in this study suggests that some combination of selection and limited dispersal is driving dinoflagellate symbiont distribution in *A. elegantissima* at the regional scale. Although our data here are insufficient to separate the relative contributions of dispersal limitation and selection, average larval dispersal distance in marine organisms can, as a general rule of thumb, be approximated to be one-third the clinal width under moderate levels of selection (Sotka and Palumbi, 2006). The mean distance between populations fixed for different symbiont types in this study is about 110 km. Thus, an average dispersal distance of 30 km or less with moderate selection on symbiont type could produce these clines.

These estimates differ substantially from population genetic data available for the host anemone. In a study based on 16 allozyme loci, McFadden *et al.* (1997) found a very low (though significant) level of genetic differentiation among populations of *A. elegantissima* ($\phi_{st} = 0.061$, $P < 0.05$), in line with other intertidal invertebrates with long-lived, feeding larvae (Kelly and Palumbi, 2010). More recently, *A. elegantissima* has been found to be monomorphic at the mitochondrial COI locus over the range examined in this study (R. P. Kelly, pers. comm; though note that anthozoans have very low levels of intraspecific mitochondrial variation [Shearer *et al.*, 2002]).

By comparison, the very high level of genetic differentiation demonstrated here among symbionts (combined loci $\phi_{st} = 0.88$) may suggest that host and symbiont populations are distributing relatively independently along the coast. Under one possible scenario, motile aposymbiotic host larvae disperse, then acquire symbionts derived from sessile adults at or near the location of settlement. Although little is known about the natural history of *Anthopleura* larvae in the wild, laboratory studies have shown that aposymbiotic larvae readily form associations with nonmotile symbionts naturally egested by adult anemones, lending some support to this hypothesis (Schwarz *et al.*, 2002). Fitness differences among different symbiont genotypes may also bias symbiont populations within individual anemones toward more locally adapted types, as has been proposed regarding competing A- and B-clade *Symbiodinium* populations in the tropical anemone *Condylactis gigantea* (Venn *et al.*, 2008), as well as in shifting populations of dinoflagellate and chlorophyte symbionts in *A. elegantissima* itself (Secord and Muller-Parker, 2005). Another possibility is that the widespread distribution of neutral alleles in *A. elegantissima* (McFadden *et al.*, 1997) masks substantial heterogeneity at adaptive loci (Pespeni *et al.*, 2010), and that scales of functionally different genetic divergence are similar in hosts and symbionts.

Our findings add to a growing body of literature suggesting that horizontally transmitted *Symbiodinium* have limited population connectivity relative to their hosts (Howells *et al.*, 2009; Santos *et al.*, 2003a). Further investigation of *A. elegantissima* symbiont population genetics using more variable markers would permit a more direct comparison to the above studies and clarify the dynamics underlying the observed distribution.

Symbiont biogeography and evolution

The five multilocus genotypes described in this study were distributed in three relatively sharply defined biogeographic provinces: the northernmost extending south roughly to Cape Mendocino, the central continuing to Point Conception, and the southern to the boundary of our sampling near San Diego (Fig. 1). These provinces do show

some mosaic structure: north of Humboldt Bay in northern California, our samples are dominated by a single *Symbiodinium* multilocus genotype (Fig. 3). Just south of Cape Mendocino, at our sampling sites at Mattole Road and Shelter Cove, there are substantial numbers of a second genotype more common in the central coast. The subsequent stretch of coast contains a multilocus genotype identical to the northern type at cyt *b* and cp23S but with a unique 18S RFLP pattern, after which the population is again dominated by the central type (Fig. 3). There is some possibility that data from the three southern sites are affected by a temporal confound, as these locations were sampled a year after northern and central-coast sites (Table 1). Although wholesale exchange of symbiont genotype over an entire region would be surprising, temporal changes in a within-clade symbiont population deserve attention in future studies of this *Anthopleura* system.

A biogeographic break around Cape Mendocino is consistent with data from other intertidal invertebrates (Kelly and Palumbi, 2010) and subtidal fish (Burford and Bernardi, 2008; Sivasundar and Palumbi, 2010), and may be derived from an important oceanographic barrier to dispersal in these species. This area is home to a major upwelling jet that brings cold water to the surface, reducing the pH of surface waters (Feely *et al*., 2008). From Cape Mendocino south, upwelling is stronger and more consistent than it is to the north (see Sivasundar and Palumbi, 2010, for discussion).

Environmental changes may also be important to anemones across this area: Cape Mendocino marks the major transition between zooxanthellate and zoochlorellate symbiont populations in the congeneric *A. xanthogrammica* (Secord and Augustine, 2000), which is thought to be largely mediated by environmental factors. Under an assumption of selection gradients that vary monotonically with latitude, the mosaic distribution of northern and central genotypes along the north-central coast of California might argue against environmental effects on *Symbiodinium* genotype. However, intertidal environments are strikingly controlled by tide and solar heating, and these have been shown to vary in a patchwork fashion along the west coast of the United States (Helmuth *et al.*, 2006). As the relative contributions of oceanographic factors and terrestrial conditions to fitness in these mid- to upper-intertidal anemones are poorly constrained, the hypothesis that the northern and central *Symbiodiunium* genotypes we describe here are controlled by intertidal environmental conditions remains to be tested.

The environmental pattern might be further complicated by the unique evolutionary landscape afforded by flexible, horizontally transmitted symbioses among several species of hosts. The *Anthopleura* species complex on the eastern Pacific coast contains at least three closely related anemones known to harbor *Symbiodinium*. Of these, *A. elegantissima* spans the widest geographic range and is the only species known to reproduce clonally, *A. xanthogrammica* is more abundant in the north, and *A. sola* more abundant in the south (Pearse and Francis, 2000). The presence of multiple host lineages, each representing a slightly different symbiont microhabitat due to differences in body size or placement (Secord and Augustine, 2000) or partner specificity (Bates *et al.*, 2010), could conceivably permit the divergence of symbionts in sympatric host populations; subsequent host switching and range expansion by a symbiont lineage might then lead to the pattern described here.

The *Anthopleura* system was one of the first among cnidaria for which relationships between symbiont type, physiology, environmental parameters, and distribution could be empirically demonstrated (Muscatine and Hand, 1958). The data we present here are the first to show finer-grained, intra-clade-level dinoflagellate diversity in *Anthopleura*. The highly structured biogeography suggests several hypotheses about selective pressures, dispersal, and genetic inheritance, and tests of the physiological differences among the *Symbiodinium* genotypes would greatly help elucidate the role of environment on symbiont abundance. Future research characterizing the fine-scale phylogeographic diversity of *Symbiodinium* from the entire *Anthopleura* genus over the extent of its range should help to clarify the evolutionary history of the relationship, while analysis for both host and symbiont of more variable markers such as microsatellites (Pettay and LaJeunesse, 2007; Andras *et al.*, 2009) or even the 18S pseudogenes suggested above would help to elucidate the population genetic parameters underlying their respective distributions.

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Literature Cited

Andras, J. P., N. L. Kirk, M. A. Coffroth, and C. D. Harvell. 2009. Isolation and characterization of microsatellite loci in *Symbiodinium* B1/B184, the dinoflagellate symbiont of the Caribbean sea fan coral, *Gorgonia ventalina*. *Mol. Ecol. Resour.* **9:** 989–993.

Augustine, L., and G. Muller-Parker. 1998. Selective predation by the mosshead sculpin *Clinocottus globiceps* on the sea anemone *Anthopleura elegantissima* and its two algal symbionts. *Limnol. Oceanogr.* **43:** 711–715.

Banaszak, A. T., and R. K. Trench. 1995. Effects of ultraviolet (UV) radiation on marine microalgal-invertebrate symbioses. 1. Response of the algal symbionts in culture and in hospite. *J. Exp. Mar. Biol. Ecol.* **194:** 213–232.

Banaszak, A. T., R. Iglesias-Prieto, and R. K. Trench. 1993. *Scrippsiella velellae* sp. nov. (Peridiniales) and *Gloeodinium viscum* sp. nov. (Phytodiniales), dinoflagellate symbionts of 2 hydrozoans (Cnidaria). *J. Phycol.* **29:** 517–528.

Bates, A. E., L. Mclean, P. Laing, L. A. Raeburn, and C. Hare. 2010. Distribution patterns of Zoochlorellae and Zooxanthellae hosted by two Pacific Northeast anemones, *Anthopleura elegantissima* and *A. xanthogrammica*. *Biol. Bull.* **218:** 237–247.

Bergschneider, H., and G. Muller-Parker. 2008. Nutritional role of two algal symbionts in the temperate sea anemone *Anthopleura elegantissima* Brandt. *Biol. Bull.* **215:** 73–88.

Blanchette, C. A., C. M. Miner, P. T. Raimondi, D. Lohse, K. E. K. Heady, and B. R. Broitman. 2008. Biogeographical patterns of rocky intertidal communities along the Pacific coast of North America. *J. Biogeogr.* **35:** 1593–1607.

Burford, M. O., and G. Bernardi. 2008. Incipient speciation within a subgenus of rockfish (*Sebastosomus*) provides evidence of recent radiations within an ancient species flock. *Mar. Biol.* **154:** 701–717.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32:** 1792–1797.

Feely, R. A., C. L. Sabine, J. M. Hernandez-Ayon, D. Ianson, and B. Hales. 2008. Evidence for upwelling of corrosive "acidified' water onto the continental shelf. *Science* **320:** 1490–1492.

Helmuth, B., B. R. Broitman, C. A. Blanchette, S. Gilman, P. Halpin, C. D. G. Harley, M. J. O'Donnell, G. E. Hofmann, B. Menge, and D. Strickland. 2006. Mosaic patterns of thermal stress in the rocky intertidal zone: implications for climate change. *Ecol. Monogr.* **76:** 461–479.

Howells, E., M. Oppen, and B. Willis. 2009. High genetic differentiation and cross-shelf patterns of genetic diversity among Great Barrier Reef populations of *Symbiodinium*. *Coral Reefs* **28:** 215–225.

Kawecki, T., and D. Ebert. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* **7:** 1225–1241.

Kelly, R. P., and S. R. Palumbi. 2010. Genetic structure among 50 species of the Northeastern Pacific rocky intertidal community. *PLoS One* **5:** e8594.

Kirk, N. L., J. R. Ward, and M. A. Coffroth. 2005. Stable *Symbiodinium* composition in the sea fan *Gorgonia ventalina* during temperature and disease stress. *Biol. Bull.* **209:** 227–234.

LaJeunesse, T. C., and R. K. Trench. 2000. Biogeography of two species of *Symbiodinium* (Freudenthal) inhabiting the intertidal sea anemone *Anthopleura elegantissima* (Brandt). *Biol. Bull.* **199:** 126–134.

Letsch, M. R., G. Muller-Parker, T. Friedl, and L. A. Lewis. 2009. *Elliptochloris marina* sp. nov. (Trebouxiophyceae, Chlorophyta), symbiotic green alga of the temperate Pacifc sea anemones *Anthopleura xanthogrammica* and *A. elegantissima* (Anthozoa, Cnidaria). *J. Phycol.* **45:** 1127–1135.

Magalon, H., E. Baudry, A. Husté, M. Adjeroud, and M. Veuille. 2006. High genetic diversity of the symbiotic dinoflagellates in the coral *Pocillopora meandrina* from the South Pacific. *Mar. Biol.* **148:** 913–922.

McFadden, C. S., R. K. Grosberg, B. B. Cameron, D. P. Karlton, and D. Secord. 1997. Genetic relationships within and between clonal and solitary forms of the sea anemone *Anthopleura elegantissima* revisited: evidence for the existence of two species. *Mar. Biol.* **128:** 127–139.

Muller-Parker, G. 1987. Seasonal variation in light-shade adaptation of natural populations of the symbiotic sea anemone *Aiptasia pulchella* (Carlgren, 1943) in Hawaii. *J. Exp. Mar. Biol. Ecol.* **112:** 165–183.

Muller-Parker, G., J. Pierce-Cravens, and B. Bingham. 2007. Broad thermal tolerance of the symbiotic dinoflagellate *Symbiodinium muscatinei* (Dinophyta) in the sea anemone *Anthopleura elegantissima* (Cnidaria) from northern latitudes. *J. Phycol.* **43:** 25–31.

Muscatine, L., and C. Hand. 1958. Direct evidence for the transfer of materials from symbiotic algae to the tissues of a coelenterate. *Proc. Natl. Acad. Sci. USA* **44:** 1259–1263.

Palumbi, S. 1992. Marine speciation on a small planet. *Trends Ecol. Evol.* **7:** 114–118.

Pearse, V., and L. Francis. 2000. *Anthopleura sola*, a new species, solitary sibling species to the aggregating sea anemone, *A. elegantissima* (Cnidaria : Anthozoa : Actiniaria : Actiniidae). *Proc. Biol. Soc. Wash.* **113:** 596–608.

Pespeni, M., T. Oliver, M. Manier, and S. Palumbi. 2010. Restriction Site Tiling Analysis: Accurate discovery and quantitative genotyping of genome-wide polymorphisms using nucleotide arrays. *Genome Biol*. **11:** R44.

Pettay, D. T., and T. LaJeunesse. 2007. Microsatellites from clade B *Symbiodinium* spp. specialized for Caribbean corals in the genus *Madracis*. *Mol. Ecol. Notes* **7:** 1271–1274.

Posada, D., and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Raymond, M., and F. Rousset. 1995. GENEPOP (Version 1.2) - Population-genetics software for exact tests and ecumenicism. *J. Hered.* **86:** 248–249.

Rowan, R., and D. A. Powers. 1991a. A molecular genetic classification of Zooxanthellae and the evolution of animal-algal symbioses. *Science* **251:** 1348–1351.

Rowan, R., and D. A. Powers. 1991b. Molecular genetic identification of symbiotic dinoflagellates (Zooxanthellae). *Mar. Ecol. Prog. Ser*. **71:** 65–73.

Rowan, R., N. Knowlton, A. Baker, and J. Jara. 1997. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* **388:** 265–269.

Santos, S. R., D. J. Taylor, and M. A. Coffroth. 2001. Genetic comparisons of freshly isolated versus cultured symbiotic dinoflagellates: implications for extrapolating to the intact symbiosis. *J. Phycol.* **37:** 900–912.

Santos, S. R., D. J. Taylor, R. A. Kinzie, M. Hidaka, K. Sakai, and M. A. Coffroth. 2002. Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. *Mol. Phylogenet. Evol.* **23:** 97–111.

Santos, S. R., C. Gutierrez-Rodriguez, H. R. Lasker, and M. A. Coffroth. 2003a. *Symbiodinium* sp. associations in the gorgonian *Pseudopterogorgia elisabethae* in the Bahamas: high levels of genetic variability and population structure in symbiotic dinoflagellates. *Mar. Biol.* **143:** 111–120.

Santos, S. R., R. A. Kinzie, K. Sakai, and M. A. Coffroth. 2003b. Molecular characterization of nuclear small subunit (18S)-rDNA pseudogenes in a symbiotic dinoflagellate (*Symbiodinium*, Dinophyta). *J. Eukaryot. Microbiol.* **50:** 417–421.

Saunders, B., and G. Muller-Parker. 1997. The effects of temperature and light on two algal populations in the temperate sea anemone *Anthopleura elegantissima* (Brandt, 1835). *J. Exp. Mar. Biol. Ecol.* **211:** 213–224.

Schwarz, J., V. Weis, and D. Potts. 2002. Feeding behavior and acquisition of zooxanthellae by planula larvae of the sea anemone *Anthopleura elegantissima*. *Mar. Biol.* **140:** 471–478.

Secord, D., and L. Augustine. 2000. Biogeography and microhabitat variation in temperate algal-invertebrate symbioses: zooxanthellae and zoochlorellae in two Pacific intertidal sea anemones, *Anthopleura elegantissima* and *A. xanthogrammica*. *Invertebr. Biol.* **119:** 139–146.

Secord, D., and G. Muller-Parker. 2005. Symbiont distribution along

a light gradient within an intertidal cave. *Limnol. Oceanogr.* **50:** 272–278.

Shearer, T. L., M. J. H. van Oppen, S. L. Romano, and G. Worheide. 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Mol. Ecol.* **11:** 2475–2487.

Sivasundar, A., and S. Palumbi. 2010. Life history, ecology and the biogeography of strong genetic breaks among 15 species of Pacific rockfish, *Sebastes*. *Mar. Biol.* **157:** 1433–1452.

Smith, B. L., and D. C. Potts. 1987. Clonal and solitary anemones (*Anthopleura*) of western North America—population genetics and systematics. *Mar. Biol.* **94:** 537–546.

Sotka, E. E., and S. Palumbi. 2006. The use of genetic clines to estimate dispersal distances of marine larvae. *Ecology* **87:** 1094–1103.

Stat, M., X. Pochon, R. Cowie, and R. Gates. 2009. Specificity in communities of *Symbiodinium* in corals from Johnston Atoll. *Mar. Ecol. Prog. Ser.* **386:** 83–96.

Swofford, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, MA.

Thornhill, D., T. Lajeunesse, and S. Santos. 2007. Measuring rDNA diversity in eukaryotic microbial systems: how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. *Mol. Ecol.* **16:** 5326–5340.

Trench, R. K., and R. J. Blank. 1987. *Symbiodinium microadriaticum* Freudenthal, *Symbiodinium goreauii* sp. nov., *Symbiodinium kawagutii* sp. nov., and *Symbiodinium pilosum* sp. nov.—Gymnodinioid dinoflagellate symbionts of marine invertebrates. *J. Phycol.* **23:** 469–481.

Ulstrup, K., and M. Van Oppen. 2003. Geographic and habitat partitioning of genetically distinct zooxanthellae (*Symbiodinium*) in *Acropora* corals on the Great Barrier Reef. *Mol. Ecol.* **12:** 3477–3484.

van Oppen, M. J. H., F. P. Palstra, A. M. T. Piquet, and D. J. Miller. 2001. Patterns of coral-dinoflagellate associations in *Acropora*: significance of local availability and physiology of *Symbiodinium* strains and host-symbiont selectivity. *Proc. R. Soc. Lond. B Biol. Sci.* **268:** 1759–1767.

Venn, A. A., J. E. Loram, H. G. Trapido-Rosenthal, D. A. Joyce, and A. Douglas. 2008. Importance of time and place: patterns in abundance of *Symbiodinium* clades A and B in the tropical sea anemone *Condylactis gigantea*. *Biol. Bull.* **215:** 243–252.

Verde, E. A., and L. R. McCloskey. 2001. A comparative analysis of the photobiology of Zooxanthellae and Zoochlorellae symbiotic with the temperate clonal anemone *Anthopleura elegantissima* (Brandt). I. Effect of temperature. *Mar. Biol.* **138:** 477–489.

Verde, E. A., and L. R. McCloskey. 2002. A comparative analysis of the photobiology of Zooxanthellae and Zoochlorellae symbiotic with the temperate clonal anemone *Anthopleura elegantissima* (Brandt). II. Effect of light intensity. *Mar. Biol.* **141:**225–239.

Verde, E. A., and L. R. McCloskey. 2007. A comparative analysis of the photobiology of Zooxanthellae and Zoochlorellae symbiotic with the temperate clonal anemone *Anthopleura elegantissima* (Brandt). III. Seasonal effects of natural light and temperature on photosynthesis and respiration. *Mar. Biol.* **152:** 775–792.

Wicks, L. C., E. Sampayo, and J. P. A. Gardner, and S. K. Davy. 2010. Local endemicity and high diversity characterise high-latitude coral–*Symbiodinium* partnerships. *Coral Reefs* **29:** 989–1003.

Zhang, H., D. Bhattacharya, and S. Lin. 2005. Phylogeny of dinoflagellates based on mitochondrial cytochrome *b* and nuclear small subunit rDNA sequence comparisons. *J. Phycol.* **41:** 411–420.

Zhang, Z. D., B. R. Green, and T. Cavalier-Smith. 2000. Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. *J. Mol. Evol.* **51:** 26–40.