

“Species” Radiations of Symbiotic Dinoflagellates in the Atlantic and Indo-Pacific Since the Miocene-Pliocene Transition

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Endosymbiotic dinoflagellates, or “zooxanthellae,” are required for the survival of a diverse community of invertebrates that construct and dominate shallow, tropical coral reef ecosystems. Molecular systematics applied to this once understudied symbiont partner, *Symbiodinium* spp., divide the group into divergent lineages or subgeneric “clades.” Within each clade, numerous closely related “types,” or species, exhibit distinctive host taxon, geographic, and/or environmental distributions. This diversity is greatest in clade C, which dominates the Indo-Pacific host fauna and shares dominance in the Atlantic-Caribbean with clade B. Two “living” ancestors in this group, C1 and C3, are common to both the Indo-Pacific and Atlantic-Caribbean. With these exceptions, each ocean possesses a diverse clade C assemblage that appears to have independently evolved (adaptively radiated) through host specialization and allopatric differentiation. This phylogeographic evidence suggests that a worldwide selective sweep of C1/C3, or their progenitor, must have occurred before both oceans separated. The probable timing of this event corresponds with the major climactic changes and low CO₂ levels of the late Miocene and/or early Pliocene. Subsequent bursts of diversification have proceeded in each ocean since this transition. An ecoevolutionary expansion to numerous and taxonomically diverse hosts by a select host-generalist symbiont followed by the onset of rapid diversification suggests a radical process through which coral-algal symbioses respond and persist through the vicissitudes of planetary climate change.

Introduction

Symbiotic invertebrates dominate the underwater landscapes of coral reef ecosystems and contribute substantially to primary productivity and reef framework construction (Veron 1995). The harboring of intracellular, phototrophic dinoflagellates (zooxanthellae) is credited for the long-term success and dominance of these animals in shallow, tropical, nutrient-poor environments since the Triassic (Muscatine and Porter 1997; Stanley 2003). Mass coral bleaching and mortality has called greater attention to the global ecological importance and sensitivity of these symbioses (Brown 1997; Fitt et al. 2001). Coral communities exposed to temperatures of 1 or 2 degrees above normal summer highs may lose their symbionts (bleaching). If the episode is prolonged or more extreme, mass coral mortality and ecosystem degradation follows (Wilkinson 2000). This instability is inconsistent with the resiliency of symbiotic corals to major climate changes over geological time. Investigations into the ecology, biogeography, and genetic relatedness among symbionts can reveal evolutionary processes operating between host and symbiont, knowledge that may resolve this paradox.

Physiological, ecological, and evolutionary studies on symbiotic invertebrates, especially corals, were initially hampered because of difficulties in classifying the symbiont partner, *Symbiodinium* spp. The advent and application of culturing techniques revealed morphological, physiological, and genetic differences among these *Symbiodinium* spp. (Schoenberg and Trench 1980; Trench 1993, 1997; LaJeunesse 2001). Recent developments in molecular techniques and analyses of DNA sequences provided estimates of genetic divergence among these symbionts and created a basic scheme for their classification (Rowan and Powers 1991). Such genetic approaches

to symbiont identification have begun a renaissance in our understanding of ecological and evolutionary relations between numerous host taxa and their symbiotic partners (reviewed in Baker [2003]).

Symbiodinium taxonomy is founded primarily on molecular phylogenetics (LaJeunesse 2001; Baker 2003). Nuclear (rDNA) and chloroplast (cpDNA) ribosomal DNA phylogenies divide the genus into at least eight highly divergent subgeneric lineages, or “clades,” designated “A” through “H” (Rowan and Powers 1991; LaJeunesse 2001; Pawlowski et al. 2001; Santos et al. 2002; Baker 2003; Pochon, LaJeunesse, and Pawlowski 2004). While initially useful, these taxonomic designations have had limited utility as markers for ecological investigations (Rowan and Powers 1991; Baker and Rowan 1997; Rowan et al. 1997; Savage, Trapido-Rosenthal, and Douglas 2002). For example, the majority of symbiotic invertebrates (approximately 95% of genera sampled to date) associate with clade C *Symbiodinium* spp. in the Indo-Pacific (Baker 2003; LaJeunesse et al. 2004). This conservative “clade-level” taxonomy provides little information from an ecological standpoint (Baker and Rowan 1997; Loh, Carter, Hoegh-Guldberg 1998). The search for greater taxonomic resolution has led to the development and employment of genetic markers that resolve diversity below the “clade” level (e.g., ITS 2 [LaJeunesse 2001], ITS 1 [Van Oppen et al. 2001], microsatellites and flanking sequences [Santos et al. 2004], and DNA fingerprinting [Goulet and Coffroth 2003]). Improved identification of the symbiont partner has revealed important ecological and evolutionary patterns not evident at higher taxonomic ranks.

Diversity within a “clade” is enumerated by small differences in ITS, LSU, and plastid gene sequences (approximately 0.2% to 10.0% [cf. Baker 2003]). Initially, some differences were thought to represent intraspecific variability. However, these assumptions changed once it became obvious that populations characterized by a single base substitution exhibited distinctive environmental, geographic, and/or host taxa distributions (LaJeunesse 2001, 2002; Baker 2003; Rodriguez-Lanetty, Krupp, and Weis.

Key words: adaptive radiation, climate change, corals, molecular clock, phylogeography, protist evolution, *Symbiodinium*, symbiosis.

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2004). The most prevalent *Symbiodinium* group, clade C, comprises the greatest number of ecologically distinctive “types,” or species. This assemblage associates with a broad host taxonomy, including the three major cnidarian classes, tridacnid bivalves, soritid foraminifera, acoel flat worms (O. Barneah et al., personal communication), and marine ciliates (Lobban et al. 2002). The ecological breadth, host community dominance, and wide geographic distribution of clade C underscore its appropriateness for deducing general mechanisms of *Symbiodinium* evolution.

This paper presents a phylogeographic synthesis of clade C and B *Symbiodinium* (Avice 2000). Relying on phylogenetic, geographic, and ecological data, I deduce that episodes of expansion by a few successful “opportunistic” symbionts and compensatory displacement or loss in *Symbiodinium* diversity occurs in response to periods of major climatic upheaval. Furthermore, rapid diversification, driven by host specialization and geographic isolation, proceeds during intervals of relative climatic stability. To reconstruct the timing of these episodes in the evolutionary history between “zooxanthellae” and their coral hosts (Arbogast et al. 2002), a preliminary molecular clock is calibrated.

Methods

Sample Collection and Processing

The clade C *Symbiodinium* diversity analyzed here originates from approximately 1,500 host individuals comprising nearly 100 host genera. Collections were made in the Caribbean (Bahamas, Florida keys, Mexico-Yucatan, and Belize [LaJeunesse 2002; LaJeunesse, unpublished data; LaJeunesse and Warner, unpublished data.]), southern west Pacific (southern Heron Island, central Rib and Feather reefs, and northern Lizard Island Great Barrier Reef [GBR], Australia [LaJeunesse et al. 2003; LaJeunesse et al. 2004; Baker and LaJeunesse, unpublished data.]), northern west Pacific (Zamami Island near Okinawa Japan [LaJeunesse et al. 2004]), central Pacific (Oahu, Hawaii [LaJeunesse et al. 2005]), eastern Pacific (Iglesias-Prieto et al. 2004; LaJeunesse and Reyes-Bonilla, unpublished data; Baker and LaJeunesse, unpublished data), western Indian Ocean (Kenya [Baker and LaJeunesse, unpublished data], and Red Sea (Baker and LaJeunesse, unpublished data; LaJeunesse and Barneah, unpublished data) were combined into one data set and aligned. Clade B *Symbiodinium* diversity, introduced in the *Discussion*, was characterized from the same Caribbean locations mentioned above.

The tissues from host colony fragments (or individuals) ranging from 1 to 5 cm² were processed in a manner as described previously (Baker and Rowan 1997; LaJeunesse 2002; LaJeunesse et al. 2003). Each pellet of isolated algae or intact host fragment was preserved in 95% EtOH or a DNA preservation buffer consisting of 20% DMSO and 0.25 M EDTA in NaCl-saturated water (Seutin, White, and Boag 1991) and stored at 4°C until DNA was extracted.

Genetic Analyses

Because this work is mostly a review of published genetic data, the reader is referred to the primary papers that

detail the methods of DNA extraction, PCR-DGGE, and sequencing (LaJeunesse 2002, LaJeunesse et al. 2003, 2004, 2005). Each “type” corresponds with a fingerprint of the ITS 2 region based on PCR-denaturing gradient gel electrophoresis (DGGE) and direct sequencing of the brightest diagnostic bands from each fingerprint (LaJeunesse 2001, 2002, and table 1 Supplementary Material online). For organizational and taxonomic purposes, I have classified these “types” alphanumerically, and they are listed in table 1 Supplementary Material online. For each “type” an alphanumeric code is provided along with a GenBank accession number and corresponding ecological data that includes relative range of geographic distribution, frequency of occurrence, host partner(s) (recorded at the genus level), irradiance (depth), and location of collection. A classification based on genetic and ecological grounds is deemed sufficient and more pragmatic and than a classification scheme strictly adhering to biological and/or morphological species concepts (LaJeunesse 2001, 2002; Santos et al. 2004; Finlay 2004).

Past work indicates that the tandem array of ITS 2 in *Symbiodinium* spp. usually contains one dominant sequence. Occasionally, two or more dominant intragenomic variants co-occur. When these “variants” are present as bright bands that clearly distinguish a particular fingerprint from all others, they are designated by a lowercase letter. The relative ratios of two or more intragenomic variants appear to be fixed within the genome of *Symbiodinium* spp., and their reproducibility in PCR-DGGE fingerprinting has been demonstrated (LaJeunesse 2002; LaJeunesse et al. 2004). The redundancy of using PCR-DGGE with direct sequencing of excised bands avoids placing emphasis on rare intragenomic variants and also eliminates cloning and/or sequencing artifacts (Speksnijder et al. 2001) and limits human error.

Phylogenetic Reconstructions

The ITS 2 sequences of approximately 78 Indo-Pacific and 37 Atlantic-Caribbean clade C “types” were compiled and aligned (table 1 in Supplementary Material online; a nexus file of aligned sequences is available from the author upon request). Sister lineages to clade C represented by clade H (Pochon, LaJeunesse, and Pawlowski 2004; formally phylotype Fr1 [*sensu* Pawlowski et al. 2001]) and *Symbiodinium kawagutii* in clade F (formally part of Fr 5 [*sensu* Pawlowski et al. 2001; LaJeunesse 2001]) were used as out-groups (GenBank sequences AJ291520 and AF333515). Phylogenies were estimated under maximum-parsimony (MP) and neighbor-joining (NJ) criteria using PAUP* version 4.10 (Swofford 2000). For MP, each continuous sequence insertion/deletion (indel) was treated as a single character change under the method of parsimony. For NJ analyses, a best-fit model of base substitutions, HKY+G (with a distribution shape parameter, $\alpha = 1.14$; base frequencies A = 0.316, C = 0.246, G = 0.237, and T = 0.200), was identified using MODELTEST version 3.06 (Posada and Crandall 1998). Out-groups were then removed and MP was repeated to produce unrooted phylogenies. Bayesian analyses were implemented using the MrBayes version 3.0b4 software (Huelsenbeck and Ronquist 2001).

One million generations were run under the HKY+G models of sequence evolution, beginning with an unspecified tree topology and no defined prior probabilities. The log probability reached stationarity at approximately 75,000 generations. This burn-in was then discarded and the posterior probabilities calculated. Given the high number of aligned sequences, a bootstrap resampling using MP was limited to 100 replicates for assessing internal branch support (Felsenstein 1985). Low bootstrap values reflect the high proportion of invariant characters in the sequence alignment and not ambiguity in these data (out of 366 aligned characters, 211 were invariable and only 70 were parsimony informative).

Calculating a Molecular Clock

A relative rate test (RTT) was performed using the RRTree program version 1.1.1 (Robinson-Rechavi and Huchon 2000) to determine rate constancy between clade C assemblages in the Indo-Pacific and Atlantic-Caribbean. Pairwise comparisons of observed base substitutions (parsimony) and inferred substitutions (distance; based on the HKY+G model of evolution [Philippe et al. 1994; Rokas et al. 2002]) was conducted among clade C *Symbiodinium* and the out-group taxa from clades F and H.

Based on the unrooted phylogenetic analyses described above, sequences common to both oceans, C1, C3, C21/C3d, and C1c/C45, formed an “ancestral core” from which most others, if not all, have evolved (i.e., adaptively radiated). Each base substitution and indel differing from the consensus sequence of the ancestral core was given a divergence value of 1. A total value was calculated for each “type.” In taxa that contained more than one intragenomic sequence variant, an average value was used. For example, a total value of 0.5 was assigned to symbionts whose genome contained two codominant ITS 2 sequences (viewed as two repeatedly co-occurring bands on a PCR-DGGE fingerprint), one ancestral sequence (e.g., C1) and the second containing a single derived change (base substitution or indel) (e.g., C1b). For clusters, or micro-radiations, near branch termini (e.g., C8, C15, and C31) the average “distance” for all the related types was calculated, and this value was used to represent the entire cluster.

Molecular clock calculations of substitutions per site per year for clade C symbionts were based on an average ITS 2 length of 192 bp (LaJeunesse 2001). Estimates of the final closure of the Isthmus of Panama (3.1 to 3.5 MYA [Coates and Obando 1996]) and dates for distinctive Caribbean coral and benthic foraminifera assemblages evolution (6 to 9 MYA [Collins, Budd, and Coates 1996]) were used to calibrate a molecular clock.

Results

Phylogeny of Clade C ITS “Types”

Phylogenetic reconstructions using MP, NJ, and Bayesian methods produced largely unresolved polytomies. A basic topology remained consistent between each reconstruction method. Figure 1a is a MP reconstruction that used phylogenetically informative indels, which the

other methods, employing PAUP* or MrBayes software, did not incorporate. The presence and absence of indels represent phylogenetically valuable character traits and their incorporation was important.

Statistical support for many of the subclades presented in figure 1a was low. The limited sequence length, high number of invariant characters, and large number of taxa placed limitations on the bootstrapping support for most internal branches. Bayesian posterior probabilities (only posterior probabilities greater than 95 % are shown) strongly supported some internal branches; however, without the ability to incorporate indels, Bayesian analyses were restricted to base substitutions. For example, all members of the major subclade represented by C21 and dominated by symbionts found in the montiporids contain a 5-base deletion. A progression of intermediate “types” linking C3 with divergent “types” within the C21 subclade support the hypothesis that this subclade has evolved from C3 (fig. 1b). Partial data from ITS 1 sequences also support the *Montipora* subclade division (Van Oppen 2004).

Many *Symbiodinium* spp. genomes contain co-dominant intragenomic variants. When present, they rarely differ by more than 1 or 2 bases (or indels). Concerted evolution may prohibit substantial divergence between two variant copies. Replacement/conversion of the ancestral sequence by copies of the more derived sequence must ultimately take place. Among the “types” reported to have codominant intragenomic ITS sequences, only one possible crossover from the C1 to the C3 radiation is known. Type C3m from Hawaii contains the ancestral sequence C3 and a codominant paralog, “m,” that groups with the C1 radiation (fig. 2a). This case may represent sexual recombination and/or reticulate evolution or is simply an example of homoplasmy.

Tree topologies based on different phylogenetic methods differed in minor ways and depended on the out-group (data not shown). Slight variation in the exact point where the out-group branch joined the clade C polytomy, or when a particular type grouped with either the C1 or C3 radiation, were probably products of long-branch attraction (Felsenstein 1978; Huelsenbeck 1997).

Most evidence supports an ancestral position of C1 and C3. All rooted and unrooted phylogenetic reconstructions place these “types” at the center, or base, of the polytomy (figs. 1a, 2a, and 2b). The combination of ecological attributes (e.g., wide host range [LaJeunesse et al. 2003]), biogeographic evidence (pandemic in their distribution [LaJeunesse 2001; LaJeunesse et al. 2003, 2004; this paper]), and the phylogenetic relations mentioned above (ancestral sequences to polytomies of derived host-specific and/or rare sequence types) indicate that C3 and C1 are the “living” ancestors from which most of the present clade C diversity has evolved.

Separate Radiations in the Indo-Pacific and Atlantic-Caribbean

A small number of sequences, C1, C3, C1c/C45 (Pacific/Caribbean), and C21/C3d, occur in both the Indo-Pacific and western Atlantic-Caribbean. Interestingly, they form a closely related sequence core at the center of a mostly

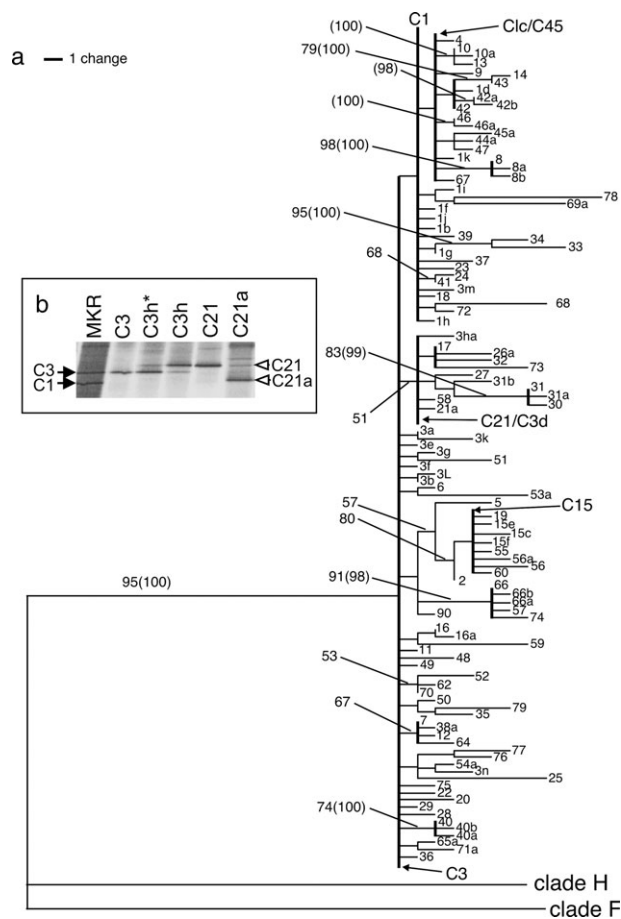


FIG. 1.—(a) Maximum-parsimony reconstruction of clade C *Symbiodinium* ITS 2 diversity. Neighbor-joining (distance) and Bayesian methods (indels omitted) yielded similar tree topologies. This polytomy in DNA sequence divergence is separated by long branches from representative sequences from sister clades, H (*sensu* Pochon, LaJeunesse, and Pawlowski, 2004) and F (LaJeunesse 2001). No intermediates between these clades are known. Widely distributed symbiont types (e.g., C1 and C3 represented by bold vertical lines) are host generalists and are ancestral to many host-specific and/or regionally endemic species. Cycles or pulses of diversification are suggested by the topology of this phylogeny. Such a process involves host generalists giving rise to a wide diversity of ecologically different “types.” Further diversification has occurred in “younger,” less host-specialized and geographically widespread, members of this polytomy (e.g., C15). Values indicated for each internal branch node are bootstrap estimates based on 100 resamplings and Bayesian posterior probabilities greater than 95% (in parentheses). Because of the low number of phylogenetically informative characters, many internal nodes lacked statistical support. (b) Some internal branches are supported by the existence of living intermediates. For example, the conversion from a genome dominated by the ITS “C3” sequence to one with a derived sequence, C21a, can be tracked over a geographic range from southern Great barrier Reef (GBR) to the northern west Pacific. Types C3h (C3h*) and C21 occur commonly on the central and southern GBR respectively; C21a occurs in the northern hemisphere, present in a variety of coral taxa around Okinawa. All display similar ecological niches where they are most commonly found (LaJeunesse et al. 2004).

undifferentiated polytomy (fig. 2a and b). Branching (radiating) from this core are numerous forms that exist in the Indo-Pacific (fig. 2a) or Atlantic-Caribbean (fig. 2b). Each taxon possesses a discreet geographic distribution, host-specificity, and/or depth (irradiance) zonation. Examples of large subclades whose members associate with the coral genera *Porites* and *Montipora* and family pocillopor-

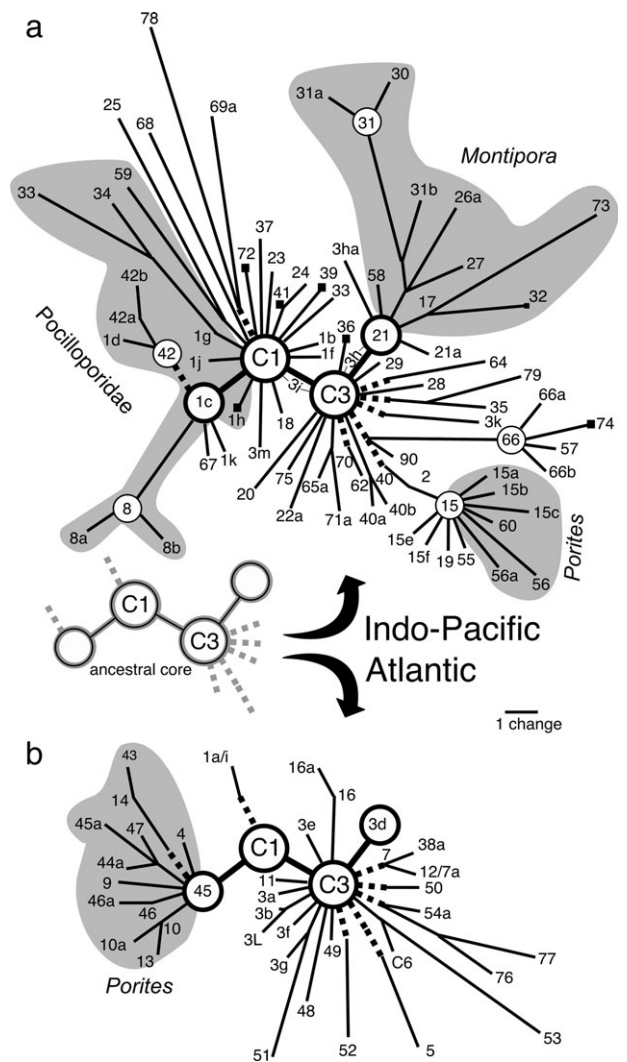


FIG. 2.—Independent radiations of clade C *Symbiodinium* in the (a) Indo-Pacific and (b) Atlantic-Caribbean. A small number of closely related progenitor sequences constitute the “ancestral core.” Little or no sequence overlap exists between radiations from each ocean. Branches with broken lines extending from the “ancestral” core are instances of overlap but may represent homoplasy. The independence of each radiation is exemplified by the separate Indo-Pacific and Caribbean “*Porites*” subclades. Phylogeographic patterns indicate this lineage underwent a worldwide expansion during the late Miocene and/or early Pliocene. Each radiation was redrawn from an unrooted phylogeny based on MP (an indel is equivalent to one difference). The topologies of these unrooted phylogenies are similar to their rooted versions. Solid squares were placed on the branch termini of “types” characterized from the western Indian Ocean/Red sea province of the Indo-Pacific.

idae (*Pocillopora* and *Stylophora*) are highlighted (fig. 2a and b). Independent subclades of *Symbiodinium* spp. have evolved for *Porites*, a host genus common to both oceans (figs. 2a, 2b, 3a, and 3b). Furthermore, “types” comprising each subclade have characteristic geographic distributions within each ocean (fig. 3a and b; [LaJeunesse 2002; LaJeunesse et al. 2004, 2005]).

A comparison between distance estimates and parsimony support assumptions that base substitutions in the ITS 2 region are not saturated for clade C *Symbiodinium* (fig. 4).

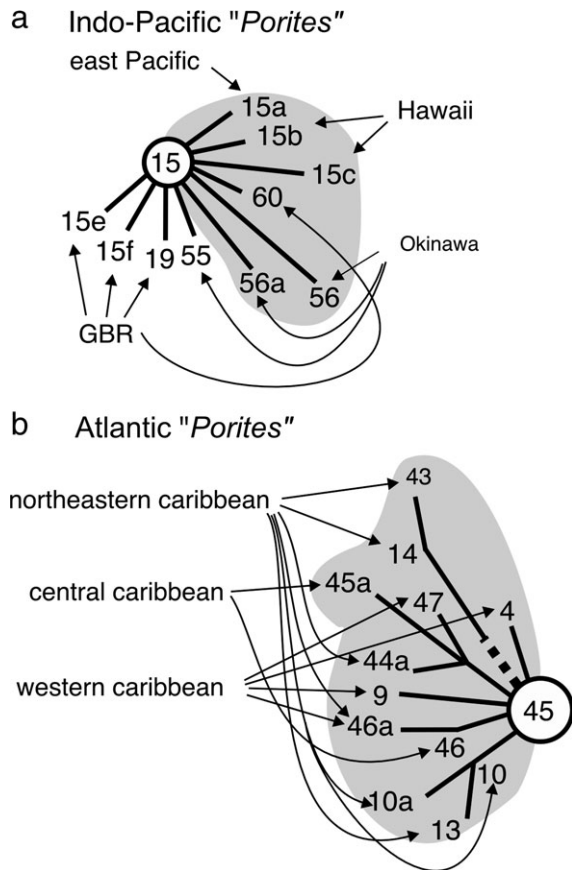


FIG. 3.—(a) Indo-Pacific and (b) Atlantic-Caribbean “poritid” subclades contain host specialized and/or rare types characteristic of certain geographic regions (subclades are redrawn from figures 2a and b, respectively). *Porites* vertically transmit symbionts via the egg, a life history trait important but not essential for the evolution of host-specialized *Symbiodinium* spp. The separate evolution of these subclades demonstrate the importance of host specialization and geographic isolation in driving *Symbiodinium* diversification. “Type” C15 associates with *Porites* spp. throughout the Indo-Pacific. On the Great Barrier Reef, it also occurs in several taxonomically diverse host taxa (see text for details). Some “types” from each “poritid” subclade associate with nonporitid hosts and are presumed examples of host-range expansions and/or host shifts (cf. table 1 in Supplementary Material online).

Therefore, differences based on parsimony are proportionally equivalent to distance values that assume a model of nucleotide substitution (HKY with a gamma distribution shape parameter, $\alpha = 1.14$). By using parsimony changes as a metric for divergence, indels could be utilized (one continuous indel was scored equivalent to 1 bp substitution). A total “divergence” value was calculated for each “type” based on the number of substitutions (and indels) different from the consensus sequence of the ancestral core (several examples are given in figure 5b).

The number of ecological “types” and the amount of “divergence” each has from the ancestral core are shown for the Indo-Pacific and Atlantic-Caribbean assemblages (fig. 5a). A linear trend line based on combined numbers from both assemblages (open circles) is shown. As divergence from the ancestral core increases, fewer and fewer “types” exist (fig. 5a). The overall divergence of each assemblage was not statistically different as determined by relative rate tests ($P = 0.736$, using out-groups from clade

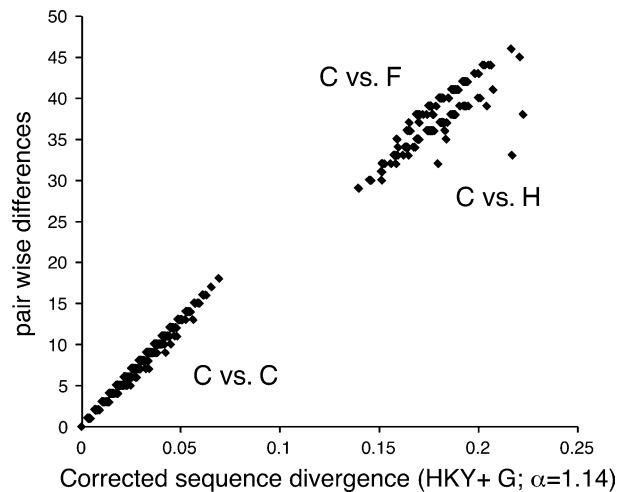


FIG. 4.—Pairwise comparison of sequence divergence based on the number of observed differences (parsimony) with divergence estimates corrected by an optimized model of DNA evolution (distance estimates). Observed and estimated values are proportionally equivalent for within-clade C comparisons (C versus C) and indicate that the ITS2 sequences within the clade C radiation are not saturated (number of differences is equivalent to number of changes). The ITS 2 region is close to saturation for interclade comparisons (e.g., C versus H, C versus F).

H and clade F under Kimura two-parameter [K-2] model of base substitution rates; $P = 0.753$, clade H alone using K-2; $P = 0.732$, clade H alone using Jukes-Cantor distance estimates; $P = 0.308$, when C1, C3, C1c/C45, and C21/C1d were used as “out-groups” under K-2). Therefore, the overall pattern of diversification and rate of molecular evolution has progressed similarly in each radiation.

Nevertheless, these radiations possess different characteristics. The average divergence from the ancestral core for Indo-Pacific and Atlantic-Caribbean radiations are 3.1 and 2.2, respectively, with a combined average of 2.7 changes. The greater average divergence value and overall number of different “types” found in the Pacific radiation can be explained by several factors. The Caribbean is a much smaller region than the Indo-Pacific and lacks corals in the genus *Montipora* and most pocilloporidae (except for *Madracis* spp.). Hence, an independent divergence of specific symbionts associated with these corals, similar to that observed for *Porites*, was never possible. The smaller area encompassed by the Atlantic-Caribbean has probably limited allopatric differentiation among C “types” in this region (cf. LaJeunesse et al. 2004). Finally, the ITS 2 in certain “types” has probably diversified faster (or slower) than others. For example, Pacific anemones (actiniaria) and pocilloporid corals were found to associate with rare and/or host-specific lineages that may have undergone rapid evolution (e.g., C25, C68, C69a and C34, C35, C78, C79, respectively [fig. 2a]).

Molecular Clock Calibrations

The phylogenetic and geographical data provided above present an opportunity for calibrating a molecular clock for ITS 2 evolution for *Symbiodinium*. There are many pitfalls in calculating rates of molecular evolution, and perhaps most critical is the choice of times when

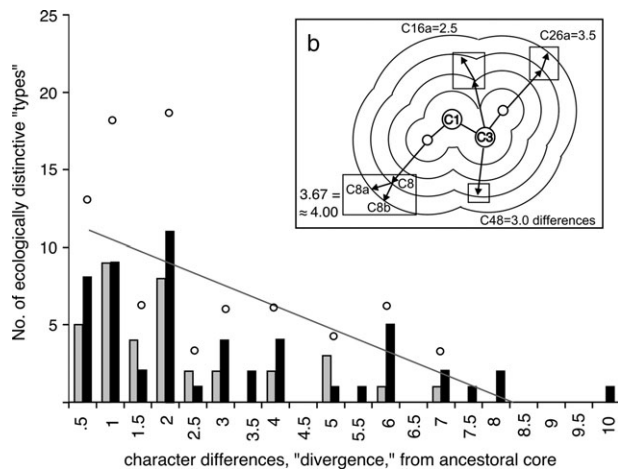


FIG. 5.—(a) The number of “types” and their divergence from the ancestral core from the Indo-Pacific (black columns) and Atlantic-Caribbean (gray columns) assemblages. (b) Divergence was calculated by totaling the number of character changes that distinguished each “type” from a consensus ancestral sequence. A linear trend line based on the combined values from each assemblage (open circles) is shown. In general, less divergent “types” are considered younger, evolving later than “types” more distant from the ancestral core. Some rate heterogeneity probably exists among “types” within each radiation.

diversification first began (Knowlton and Weigt 1998; Arbogast et al. 2002). The extensive distributional range of C1 and C3 must have been established before the Caribbean was isolated. The nearly independent radiation of Atlantic-Caribbean and Indo-Pacific clade C *Symbiodinium* spp. (figs. 2a and b) indicates that these symbiont lineages began diversifying near the time when biotic exchange between each ocean was lost or highly diminished.

Several dates were chosen to provide a range of estimates. Times predicted for the formation of the Central American isthmus, 3.1 and 3.5 MYA (Coates and Obando 1996), were used as a conservative reference point of divergence. It should be emphasized that dispersal barriers began developing during the late Miocene. By the time of the early Pliocene (ca. 4.7 to 4.2 MYA), the change in circulation of surface currents was a major barrier to dispersal (Chaisson and Ravelo 2000; Haug et al. 2001). Therefore, it is unreasonable to assume that these assemblages began radiating only after complete closure was achieved (Knowlton and Weigt 1998; Marko 2002). Divergence in fossil assemblages between Pacific and Caribbean communities had started as early as 12.9 to 11.8 MYA (Duque-Caro 1990). However, significant change and turnover in diversity did not occur in Caribbean faunal communities until 9 to 6 MYA as the Central American seaway shoaled and constricted (Collins, Budd, and Coates 1996). Therefore, branch termini most distant from the ancestral core are hypothesized to represent those *Symbiodinium* spp. populations that began diverging as, or soon after, connectivity was lost (as early as 9 to 6 MYA) but before the final closure (3.5 to 3.1 MYA). Combining these earlier dates of 6 to 9 MYA with the “oldest” of the clade C radiation (predicted by the x -intercept of the trend line in figure 5a) provides a second point of calibration.

The average divergence among clade C from the ancestral core (2.7 differences) divided by time estimates

for the closure of the Isthmus of Panama (between 3.5 and 3.1 MYA) calibrates a clock between 0.89 and 0.77 changes per Myr (or between 1.15 and 1.3 Myr per change, or between 4.0×10^{-9} and 4.5×10^{-9} substitutions site⁻¹year⁻¹ [average number of changes]/[ITS 2 length of 192 bp \times age of closure]). The clock rate is correspondingly slower, (between 0.4 and 0.3 differences per Myr, or between 2.2 and 3.3 Myr per change, or between 1.6×10^{-9} and 2.3×10^{-9} substitutions site⁻¹year⁻¹) if average divergence values are divided by older dates estimated for the divergence of Caribbean and Pacific fossil communities (9 to 6 MYA). Realistically, the most “derived” and the putatively oldest “types” (characterized by 8 differences indicated by the x -intercept of the trend line in figure 5b) should be matched with this 9 to 6 MYA horizon. This calculates a rate of between 0.88 and 1.33 differences per Myr (or between 0.75 and 1.13 changes per Myr, or between 4.6×10^{-9} and 6.9×10^{-9} substitutions site⁻¹year⁻¹). A combined range of 0.75 and 1.3 Myr per change and/or difference will be used for calculations made in the *Discussion* (the upper estimate is based on the absolute closure, with the average clade C divergence, and the lower estimate calculated from the oldest “types,” with the earliest record of fossil community divergence caused by the uplift).

Discussion

The following phylogeographic and ecological observations can be used to explain *Symbiodinium* evolution and how their symbioses respond to climate change: (1) Ecological dominance among clades differs among oceans (Baker and Rowan 1997; Baker 2003; LaJeunesse et al. 2003) (2) Closely related symbionts are found in unrelated or distantly related hosts (Rowan and Powers 1991; Carlos et al. 1999) (3) Whereas there is no correlation with host and symbiont phylogenies at the scale of clade, high host specificity and coevolution are evident at lower taxonomic ranks (LaJeunesse 2002, Diekmann et al. 2003, LaJeunesse et al. 2003, 2004, 2005) (fig. 2a and b) (4) Widespread host generalists are the “living” ancestors to numerous host-specialized, rare and/or regionally endemic “species” (figs. 2a, 2b, 3a, and 3b) (5) Certain partner combinations correlate with physical-environmental conditions such as irradiance and temperature (Rowan et al. 1997; Baker and Rowan 1997; LaJeunesse and Trench 2000; Rodriguez-Lanetty et al. 2001) (6) Generational shifts in host-symbiont associations involving numerous host taxa are possible (LaJeunesse et al. 2004) (7) Long internal branches with no intermediates separate *Symbiodinium* clades comprising clusters of closely related taxa, a pattern that signifies an expansion/radiation and/or recovery from a bottleneck event in recent geological time (figs. 1, 2a, and 2b).

Symbiodinium spp. Diversification and Speciation

Host specialization in conjunction with allopatric differentiation drives the fine-scale evolution of *Symbiodinium*. The proportionately high number of host-specific

“types” and the existence of several well-developed subclades within clade C emphasize the importance of host-symbiont specificity in the evolution of new *Symbiodinium* “species” (e.g., fig. 2a and b). Additionally, regional endemism, characteristic of many “types,” indicates that barriers of dispersal exist (even within the Caribbean) and that geographic isolation promotes differentiation (cf. LaJeunesse et al. 2004).

The phylogenetic position of host-generalist symbionts suggests that these populations are the source from which specialized endemic “species” arise (fig. 2a and b). Smaller radiations originating from a number of more derived taxa exist at various positions in the phylogenies of figures 1, 2a, and 2b. For example, C15 represents a derived, and putatively “younger,” “type” from which a number of endemic host-specific “types” have evolved (fig. 3a). C15 itself has a wide geographic distribution and is found throughout much of the Indo-Pacific, predominantly in corals of the genus *Porites*. On the GBR, where more extensive sampling has occurred, C15 associates with particular species of hydrozoa, foraminifera, and alcyonaria, as well as with *Montipora digitata* (table 1 in Supplementary Material online). Although C15 is not as generalized as C1 or C3 and is limited to the Indo-Pacific, its ecological success has led to an evolutionary radiation. This, and other examples, suggests that cycles, or pulses, of diversification occur involving host generalists giving rise to mostly host-specialized forms. Of these, a select few become widely distributed, develop a greater host-range capacity, and, in turn, undergo diversification.

The DNA sequence-similarity cluster of clade C could represent one massive metapopulation diversifying, then homogenizing, over millions of years in response to genetic connectivity and environmental conditions (Veron 1995). This possibility is not supported by present genetic data. Albeit limited, the data indicate that populations with different ITS sequences are on independent evolutionary trajectories (Rodriguez-Lanetty 2003; Santos et al. 2004). Population-level studies may eventually resolve the amount of divergence required before barriers to genetic exchange between *Symbiodinium* spp. evolve (Santos et al. 2004).

How then do these host-specialized lineages evolve from host-generalist populations? The primary habitats where most of these dinoflagellates proliferate (i.e., bloom) are in cell cytoplasm of host tissues, environments where resident symbiont populations are under intense selection pressure from biotic and abiotic factors (Moulder 1979). Natural selection via host selectivity, symbiont competition, and/or external environmental conditions may generate small founder populations susceptible to genetic drift and/or “genetic revolutions” (Carson 1968; Mayr 1970; Templeton 1980). Possibly the use of DNA sequences that resolve finer-scale differences (Santos et al. 2004) and/or the incorporation of population genetic markers may ultimately describe the microevolutionary processes, or “genetic revolutions,” that lead to divergence and “speciation” among *Symbiodinium* spp. populations. Interestingly, genetic resolution below the ITS level classifies entities that exhibit even greater host specificity and more narrowly defined geographic distributions (Santos et al. 2002, 2004; Goulet and Coffroth 2003).

The microevolutionary processes proposed to explain the divergence of bacterial ecotypes may help provide a conceptual template for investigating *Symbiodinium* evolution. Successive selective sweeps are implicated in the evolution of host-specialized symbionts. Because they are primarily asexual, *Symbiodinium* spp. are presumably subject to the same selective sweeps that maintain genetic similarity within bacterial ecotypes (Cohan 2001; Finlay 2004). During episodes of intense selection, a bacterial strain carrying a beneficial mutation out-competes other members of the “ecotype”(cluster) to extinction (Palys, Nakamura, and Cohan 1997; Cohan 2001). Thus, genetic diversity within an ecotype is periodically eliminated or “purged.” Repeated selection events lead to genetic divergence away from other ecotypes.

“Living Fossils”: Punctuated Equilibria in *Symbiodinium* spp. Evolution

Numerous observations in the fossil record in addition to recent molecular data indicate that genetic divergence, or change, can be constrained for millions of years (Gould and Eldridge 1977; Soltis et al. 2002). The “types” C1 and C3 form the epicenter, or ancestral core, of separate “species” radiations in the Atlantic-Caribbean and Indo-Pacific (fig. 2a and b). They are the only “types” found in both regions, and it suggests that their ribosomal sequences have remained unchanged at least since the mid-Pliocene when the uplift of the Central American isthmus occurred (3.1 to 3.5 MYA). These ancestors are perhaps comparable to living molecular fossils (Soltis et al. 2002). *Symbiodinium* are primarily clonal organisms, but evidence for high allelic diversity and no linkage disequilibrium suggests that sexual reproduction occurs in these protists (if only intermittently [Baillie et al. 2000; LaJeunesse 2001; Santos et al. 2002]). Genetic recombination, especially within these large host-generalist populations, may have prevented sequence divergence between respective C1 and C3 populations present in each ocean basin (Kimura 1979). Ultimately, explaining the apparent stasis in divergence within these “types” requires further investigation.

Climate Change Triggers Major Shifts in Ecological Dominance Among *Symbiodinium* spp.

Why are clade C *Symbiodinium* so ecologically dominant around the world today? This question might be answered by determining what factors promoted the success of C1 and C3 (or their progenitor). They are both prevalent the Indo-Pacific and Atlantic, which argues for their ascent to dominance before connectivity between each region was lost. Major turnover events in the evolution of floral and faunal communities are often attributed to shifts in physical-environmental conditions associated with climate change. The substantial oscillation in temperature and reduction in CO₂ known to have occurred at the end Miocene/early Pliocene, 1 and 3 Myr before the final closure of the Central American seaway, could have initiated a symbiont expansion/bottleneck involving C1 and C3 or their progenitor.

The Miocene/Pliocene transition was a time of global ecological change, marked by faunal and floral turnovers attributed to increased seasonality and greater aridity (Janis 1993). Extensive cooling and sea-level regression occurred during the Messinian stage of the end Miocene and was followed by an abrupt warming and sea-level transgression in the early Pliocene (ca. 7.2 to 5.3 MYA [Adams et al. 1977; Haq, Hardenbol, and Vail 1987]). These climate changes were accompanied by the lowest atmospheric CO₂ concentrations since the Permo-Carboniferous glaciation, 325 to 260 MYA (<500 p.p.m.v. [cf. Beerling 2002]). Major floral shifts in tropical and subtropical communities to C₄-photosynthesizing plants occurred in response to these changes 6 to 8 MYA (Cerling et al. 1997). As important primary producers in tropical marine ecosystems, coralline red algae also suffered their greatest Cenozoic extinctions in the late Miocene/early Pliocene (Aguirre, Riding, and Braga 2000).

Concurrent with this significant turnover in terrestrial (Janis 1993) and marine biota (Collins, Budd, and Coates 1996), a major episode of symbiont replacement or “switching” appears to have occurred involving numerous symbiotic reef invertebrates. This scenario involving the displacement of “previous” symbionts by an “opportunistic,” and/or successful host-generalist symbiont across numerous taxonomically diverse invertebrates over wide geographic areas would explain the general lack of congruence between *Symbiodinium* and host phylogenies and why so many distantly related hosts associate with closely related symbionts (Rowan and Powers 1991; Carlos et al. 1999).

The potential does exist for rapid changes in coral-dinoflagellate associations involving taxonomically diverse hosts over wide geographic distances. Generational shifts in symbiont type can occur in hosts whose larvae must acquire symbionts from environmental pools (mostly involving broadcast spawners [LaJeunesse et al. 2004]). Hosts exhibiting vertical transmission (brooders) do not demonstrate this level of “flexibility.” Nevertheless, the massive ecological expansion and success of the C1-C3 progenitor during the late Miocene/early Pliocene included invertebrates exhibiting both horizontal acquisition and vertical symbiont transmission. The possible physiological innovation that led to the success of C1 and C3, or their progenitor, in so many different hosts needs further study (cf. Iglesias-Prieto and Trench 1997).

At a fundamental level, competitive dominance must be important in the ecological and evolutionary success of certain *Symbiodinium* spp. Because most symbiont “types” occupy a distinctive ecological niche, ecological theory would predict that minimal competition occurs between them. Competition would be more intense between clonal lines that are specialized to the same host taxon (Goulet and Coffroth 2003). Indeed, subclades of host-specialized “types” have been maintained for millions of years (e.g., separate *Porites* subclades are found in both the Atlantic and Indo-Pacific [fig. 2a and b], indicating that partner recombination (“switching”) or host-range expansion occurs infrequently (Buddemeier and Fautin 1993; Baker 2003; LaJeunesse et al. 2003; Van Oppen 2004). Boundaries that may limit or prevent direct

competition between higher taxonomic ranks could break down during major physical-environmental change. Under this scenario, host-specialized lineages (e.g., “*Montipora*” clade [fig. 2a]) would become vulnerable to competition and displacement by an “opportunistic,” or successful, host generalist. Given the relatively few divergent clades (A through H) in existence today, changes in climate that correlate with the boundaries between geological epochs must eventually eliminate most *Symbiodinium* microdiversity, including most host-specialized lineages.

The progenitors of the clade C radiation (i.e., C1 and C3) experienced a magnitude of success that is unique among intracellular symbioses. Symbiont replacements have been reported in other endosymbiotic associations (Piercey-Normore and DePriest 2001; Lefèvre et al. 2004), but such examples involved a narrow group of related host taxa. “Switching” has been related to a host’s change in nutritional habits and/or symbiont competition but not directly to changes in climate. To substantiate that such wide-sweeping replacements and turnover among *Symbiodinium* spp. are probably driven by the vicissitudes of planetary climate change, a second example is offered.

Pleistocene Radiation of Clade B in the Caribbean

An ecoevolutionary expansion of clade B *Symbiodinium* may have occurred during the late Pliocene and/or early Pleistocene in the Caribbean. The coral reef community of symbiotic invertebrates in the Atlantic-Caribbean is unusual because a large proportion of them associate with clade B *Symbiodinium* (>50 % genera [Baker and Rowan 1997; LaJeunesse 2002; Baker 2003]). The MP phylogenies presented in figure 6a (unrooted) and b (rooted) indicate that an intense diversification has also occurred in this group. B1 and B19 are ancestral to numerous host-specific “types” (fig. 6a and b). This diverse “species” assemblage, however, is found only in the western Atlantic (B1 exists in the west, central, and east Pacific).

Physical-environmental conditions of the Pliocene/Pleistocene transition (2 to 4 MYA) likely facilitated the regional spread of clade B generalists in the Caribbean. Between 1 and 2 MYA, the Caribbean had experienced its most severe coral extinctions since the beginning of the Cenozoic (Budd 2000), whereas west Pacific reefs were evidently spared (Veron and Kelley 1988). The closure of the Central American isthmus followed by substantial oscillations in sea surface temperature associated with increased glaciation in the northern hemisphere (Ruddiman and Raymo 1988) may have promoted an ecological expansion and radiation of these symbionts (LaJeunesse et al. 2003).

Molecular clock calculations support the scenario of a clade B expansion at the Pliocene/Pleistocene boundary, 1 to 2 Myr after the complete closure of the Central American seaway. Assuming ITS 2 rate homogeneity between clades B and C, the average age of divergent host-specific and/or rare types directly related to B1 (1.6 changes) is calculated between 1.2 and 2.1 MYA (assuming divergence range of 0.75 to 1.3 changes per Myr). This radiation appears to be younger than the ecologically rare, less diverse group

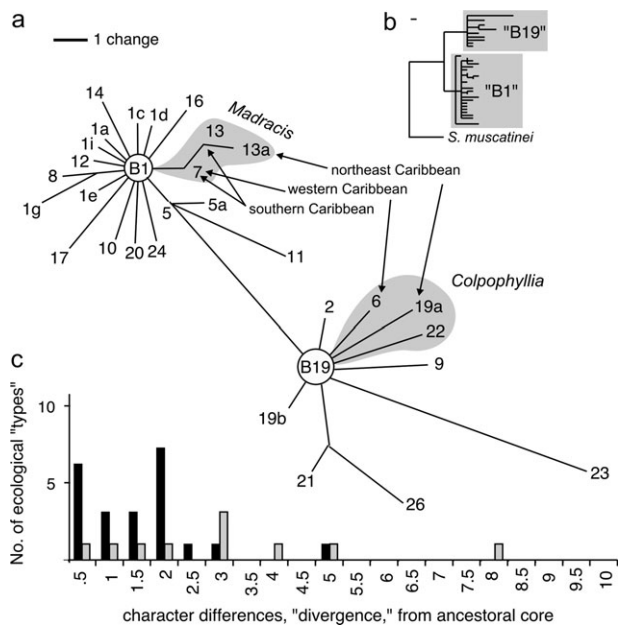


FIG. 6.—(a) Unrooted and (b) rooted MP phylogenies of clade B diversity from the Caribbean. This lineage divides into two major subclades characterized by “types” B1 and B19 that are ancestral to each radiation. As with clade C, most derived “types” have defined host, depth and/or geographic ranges. For example, the symbionts found in the corals *Madracis* and *Colpophyllia* have distinctive regional distributions. B1 is also found in the Indo-Pacific, yet there is no indication that any kind of diversification has occurred in this region (Baker 2003). (c) The distribution in divergence values of “types” radiating from B1 (gray columns) and B19 (black columns) are compared. A molecular clock rate of between 0.75 and 1.3 changes/Myr places the average age of the B1 radiation in the early to mid-Pleistocene (1.2 to 2.1 MYA). The greater average divergence found for the B19 radiation may indicate that it is older, or that evolutionary rates differ between them. The rise to ecological dominance of clade B in the Caribbean is attributed to the onset of Northern hemisphere glaciation, beginning during the Pliocene/Pleistocene transition (4 to 2 MYA). Analyses of these data are as was described for clade C.

comprising the B19 radiation (2.3 to 4.0 MYA based on an average of 3.1 changes [fig. 6c]). The B19 radiation may have begun earlier, nearer to the times calculated for clade C, or, alternatively, differences in age could be explained by rate heterogeneity in DNA substitution between these subclades.

Relevance and Application of a Molecular Clock

In the absence of fossil evidence, development of an accurate molecular clock would be useful for reconstructing the timing of important events in evolutionary history between corals and their zooxanthellae (Arbogast et al. 2002). Although there are many uncertainties and assumptions made when calculating rates of molecular evolution, findings of this paper compare similarly with other estimates in the literature. Surprisingly, the full range of values from 1.6×10^{-9} to 6.9×10^{-9} substitutions site⁻¹ year⁻¹ estimated for *Symbiodinium* are similar to, and fall within, the range of estimations made for numerous woody and herbaceous plants (reviewed in Richardson et al. [2001]) and for certain insects (Bargues et al. 2000). Detailed analyses of the secondary structure of ITS rDNA sequences may lead to

improved rate estimates of evolutionary change (S. R. Santos and T. C. LaJeunesse, unpublished data).

Divergence times accepted for west Pacific and Indian Ocean marine communities can be tested by comparing the divergence of clade C endemics identified from the western Indian Ocean/Red Sea with nearest relatives in the west Pacific. Using clock rates of between 0.75 and 1.3 Myr per base substitution, their divergence is estimated at between 0.4 and 2.6 MYA (cf. fig. 2a). These times roughly correspond with previous valuations of biogeographic partitioning between the Indian and Pacific Oceans (Benzie 1999). Although such preliminary comparisons support the predictive accuracy of this molecular clock calibration, a more thorough characterization and comparison of the *Symbiodinium* communities from the western Indian Ocean/Red Sea and west Pacific should be conducted.

Determining whether a clade C molecular clock can be applied to *Symbiodinium* from different clades will require comparison of metabolic rates, generation times, and DNA repair efficiency between these lineages (Martin and Palumbi 1993). Differences in historic population sizes influence rates of evolution, as exemplified by the genetic stasis of C1 and C3 over millions of years, may significantly confound long-term rate homogeneity among clades (Arbogast et al. 2002). There are indications that clade B nuclear and chloroplast rDNA genes have evolved more slowly relative to rates in other clades (Santos et al. 2002). Nevertheless, the correspondence of the clade C clock with phylogenetic and geographic data on clade B diversity indicate that evolutionary rates are similar, at least over limited time scales.

Conclusions and Implications

The Miocene/Pliocene expansion-radiation involving progenitors of the clade C radiation have generalized implications for the long-term coevolution between coral and dinoflagellate lineages. Based on the principles of uniformitarianism, major climatic changes that we know occurred earlier in geological time (e.g., late Oligocene) probably induced similar widespread shifts and subsequent radiations in symbiont diversity involving numerous host-specific lineages (Mayr 1970; Futuyma and Moreno 1988). Diversification would then proceed until the next major paleoclimatic cycle caused another shift across the host community to a new dominant symbiont “type” or clade (e.g., clade B in the Caribbean).

Coral-dinoflagellate symbioses are important components of the biosphere and are important indicators of environmental change in shallow tropical ecosystems (Hoegh-Guldberg 1999). The geologic resiliency, persistence, and success of hermatypic corals could be explained, in part, by drastic turnovers in symbiont communities to major climate change. Over the past 2 decades, pollution and exploitation by humans and abnormal spikes in sea-surface temperature have adversely impacted coral reef communities around the world (Wilkinson 2000). How these systems may respond to the rapid and major global warming predicted over the coming century is uncertain but largely dependent on whether coral-algal symbioses can adjust to decadal rather than millennial rates of climate change

(Hoegh-Guldberg, Loh, and Jones 2002). Increases in the frequency of different kinds of “zooxanthellae,” and their spread to numerous hosts, may harbingers a new episode in the turnover of *Symbiodinium* diversity (Baker et al. 2004).

Supplementary Material

Table 1 in Supplementary Material online contains the following information.

Diversity of *Symbiodinium* clade C based on ITS2 sequence data from PCR-DGGE fingerprinting of samples from approximately 1,500 host individuals. Each alphanumeric “type” has a characteristic fingerprint that corresponds to a particular geographic and ecological distribution. Alphanumeric labeling refers to the symbiont clade (uppercase letter), the ITS type (number), and presence of a characteristic codominant intragenomic sequence in the ribosomal array (lowercase letter). Many of these “types” were observed under repeated sampling from numerous host individuals and/or from host species taken from different reef systems over wide geographic distances (see *Methods* for specific collection locals and relevant citations). Distributions are coded as G (global), widest distribution, identified in all the major regions surveyed (e.g., Indo-Pacific and Atlantic); O (ocean wide), distributed throughout a particular ocean/sea, (e.g., Caribbean); P (provincial), distribution to a particular region within an ocean/sea (e.g., eastern Pacific); L (localized), found in more than one individual or host species collected from a particular reef system (e.g., Belize’s barrier reef or Florida Keys); and S (single), found only once but, usually, based on a single sample of a particular host species. The frequency of occurrence (in parentheses) of each “type” in the host taxa listed to the right is categorized as A (abundant), C (common) and R (rare) and may change depending on geographic location and depth of collection. Location abbreviations are given next to host taxa where a particular symbiont was identified: ep = eastern Pacific, cp = central Pacific, wp = western Pacific, wi = western Indian Ocean, rs = Red Sea, nc = north Caribbean, cc = central Caribbean, and wc = western Caribbean. These distribution-abundance characterizations are strictly preliminary and many will likely change with further sampling of host diversity, in new regions, and from a greater number of host individuals.

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