Vol. 325: 59–71, 2006 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published November 7

Pelagic photosymbiosis: rDNA assessment of diversity and evolution of dinoflagellate symbionts and planktonic foraminiferal hosts

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ABSTRACT: We present large subunit (LSU) and internal transcribed spacer (ITS) rDNA based phylogenies of symbiotic dinoflagellates retrieved from single-cell planktonic foraminifera collected around the world. All modern foraminiferal species involved in such symbiosis are included in our analyses. The pelagic symbiotic dinoflagellates form a monophyletic group sister to the *Symbiodinium* species complex found in coastal-benthic environments. The pelagic symbionts are descendants of free-living species and, together with the coastal-benthic *Symbiodinium* spp., they originated from the early Mesozoic suessiacean family represented by the extant *Polarella glacialis*. Out of hundreds of single planktonic foraminifera examined, 21 unique pelagic symbiont ribotypes were recognized, which could be divided into 2 main clades and 4 genetic subgroups. We observed an absence of specificity between the symbiont genetic types and the host genetic and morphological species. A few foraminifera even harbored dinoflagellates of more than one genetic subgroup. This genetic flexibility may be constrained by the fast life cycles of pelagic single-cell hosts, which acquire symbionts de novo from the ambient water at each generation. The obligatory transitional free-living stage of pelagic symbionts prior to acquisition by foraminiferal hosts may also explain their significantly lower rates of DNA substitution in comparison to their coastal-benthic relatives. We propose that the open ocean ecosystem has maintained photosymbioses involving a relatively low genetic diversity, but an extreme flexibility in the relationships between both partners, which also preserved their ancestral ability for independent life.

KEY WORDS: Symbiosis · Planktonic foraminifer · Dinoflagellate · Open ocean · Suessiales

INTRODUCTION

Marine symbiosis involving heterotrophic hosts and photosynthesizing dinoflagellate algae (often referred to as zooxanthellae) results in a holosymbiont unit with advantages of both strategies (Rowan 1998, Buddemeier et al. 2004). Photosymbiosis is especially important in oligotrophic oceans, and zooxanthellae are hosted by a variety of marine invertebrates including (among others) corals, sponges, and foraminifera (Carlos et al. 1999, LaJeunesse 2001, Pochon et al. 2001, Baker 2003, Santos et al. 2003). Symbiosis is key to the success of coral reefs over geological times, and may be a major mechanism that enables corals to survive varying climate conditions (Stanley & Swart 1995, Buddemeier et al. 2004). However, dinoflagellate based photosymbiosis is not confined to coastal reef environments. Symbiosis between pelagic foraminifera and dinoflagellates is abundant in surface waters of the world ocean, and plays a prominent role in oligotrophic open ocean ecology. In this study, we assess phylogenetic relationships among pelagic foraminiferan symbionts, and examine host-symbiont specificity, flexibility and co-evolution.

Dinoflagellate endosymbiosis has probably affected marine ecology since at least the Early Mesozoic. Dinoflagellates underwent a radiation at that time, and most of the morphological features found in modern dinoflagellates already existed by the early Cretaceous (Fensome et al. 1996). Co-evolution with scleractinian corals, whose modern symbiotic dinoflagellates of the genus *Symbiodinium* resemble the Late Triassic genus

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Suessia, likely contributed to Early Mesozoic dinoflagellate radiation (Fensome et al. 1996). This is supported by geochemical evidence that at least some Triassic corals were symbiont-bearing (Stanley & Swart 1995). In planktonic foraminifera, the earliest record of photosymbiosis comes from the stable isotope composition of fossil tests from the Late Cretaceous (Houston & Huber 1998, Houston et al. 1999). A rapid radiation of the symbiont-bearing foraminifera species occurred in the Paleocene and was followed by stepwise extinction of the group in the Oligocene (Kelly et al. 1996, Norris 1996, Wade 2004). According to $\delta^{18}O$ and $\delta^{13}C$ measurements in planktonic foraminiferal shells, the proliferation of modern pelagic symbioses (mainly within the genus *Globigerinoides* and *Orbulina*) occurred only in the Early Miocene (Norris 1996). The dinoflagellate fossil record comprises organic-walled cysts produced by some members of the group. Discovery of *Polarella glacialis* (Buck et al. 1992, Montresor et al. 1999), a free-living cyst-forming dinoflagellate resembling the fossil *Suessia* spp. thought to be extinct since the Mesozoic, underlines limitations of the fossil record in reconstructing the evolution of dinoflagellate symbiosis.

In modern oceans, the most intensively studied photosymbioses are those found in coral reefs. In these coastal and mostly benthic ecosystems, the endosymbiotic *Symbiodinium* spp. enhances coral metabolism and eventually calcification by providing fixed carbon through photosynthesis, while corals provide protection and vital inorganic nutrients to their symbionts. In coral reefs, molecular methods uncovered an astounding diversity of *Symbiodinium* spp. phylotypes (LaJeunesse 2001, Pawlowski et al. 2001, Santos et al. 2002, Baker 2003, Rodriguez-Lanetty 2003, Pochon et al. 2006). This diversity may assist corals in acclimation to changing conditions (Buddemeier & Fautin 1993, Rowan et al. 1997, Baker 2001, Baker et al. 2004, Rowan 2004). Corals of the same species in different geographic locations and under different physical conditions may harbor different *Symbiodinium* spp. phylotypes, and even within a single coral colony different symbiont phylotypes may be found in line with micro-ecological gradients (Rowan 1998, Baker 2003). Furthermore, the symbiont community in a coral colony may undergo significant population changes following events of exposure to extreme temperatures or light intensity (Trench 1993, Baker et al. 2004, Little et al. 2004).

Based on rDNA phylogenies and RFLP patterns, the *Symbiodinium* species complex is often divided into 'clades' comprising many phylotypes (Rowan 1998, Pawlowski et al. 2001, Baker 2003, Pochon et al. 2006). While differences in physiology among *Symbiodinium* spp. clades (Fabricius et al. 2004, Rowan 2004) and phylotypes (Tchernov et al. 2004) have been indicated, the biological and ecological significance of the present rDNA-based taxonomic division is yet unclear (Savage et al. 2002). The considerable and confusing biodiversity of *Symbiodinium* spp., coupled with the complex nature of reef ecology and the wide diversity and taxonomic ambiguities of scleractinian corals (Romano & Palumbi 1996, Romano & Cairns 2000, Stanley & Fautin 2001, Stanley 2003, Fukami et al. 2004), hinders clear understanding of flexibility, specificity, and evolution of this symbiosis.

Many of the difficulties encountered in studies of reef symbioses are absent from the poorly studied pelagic foraminifera-dinoflagellate symbiosis. In the immense and moving pelagic fields of tropical and subtropical surface waters, many fewer host species are present and both symbiotic partners are single-celled. The life cycle of symbiont-bearing planktonic foraminifera (Fig. 1) dictates that juvenile foraminifers acquire their dinoflagellate symbiont(s) from the ambient sea water shortly after gametes fusion at each generation, i.e. approximately once every 2 wk or once a month (Hemleben et al. 1989, Bijma et al. 1990). Therefore, symbiosis is perpetually reset, and partners must find each other among the billions of cells dwelling in the water column. This differs from the complex acquisition modes in coral reef systems, where both vertical and horizontal transmission modes (Loh et al. 2001) and multiple symbiont acquisition events during the long ontogeny of a coral colony (Baker 2003) introduce greater symbiotic genetic complexity.

Pelagic symbiosis holds an important position in ocean ecology and has featured in numerous oceanographic and geologic investigations (Emiliani 1954, Imbrie et al. 1973, Shackleton & Opdyke 1973, Hughen et al. 1998). The success and proliferation of many planktonic foraminifer taxa has been fueled by acquisition of symbiotic algae (Norris 1996). Over the past few years, molecular investigations demonstrated the existence of several genetic types within all classical planktonic foraminifer 'species' defined using morphological criteria (Huber et al. 1997, de Vargas et al. 1999, de Vargas et al. 2002, Wade & Darling 2002). Thus, a molecular phylogenetic approach seems appropriate to start investigation of symbiosis within the planktonic foraminifera. Until now, morphological (Spero 1987) and small subunit (SSU) rDNA-based investigations (Gast & Caron 1996) of the dinoflagellates associated with planktonic foraminifera both described a single species, *Gymnodinium beii*. Here we analyze faster evolving large subunit and internal transcribed spacer (LSU and ITS respectively) rDNA from hundreds of individual hosts collected on a global scale, and achieve a higher resolution in addressing questions of symbiosis in this pelagic realm.

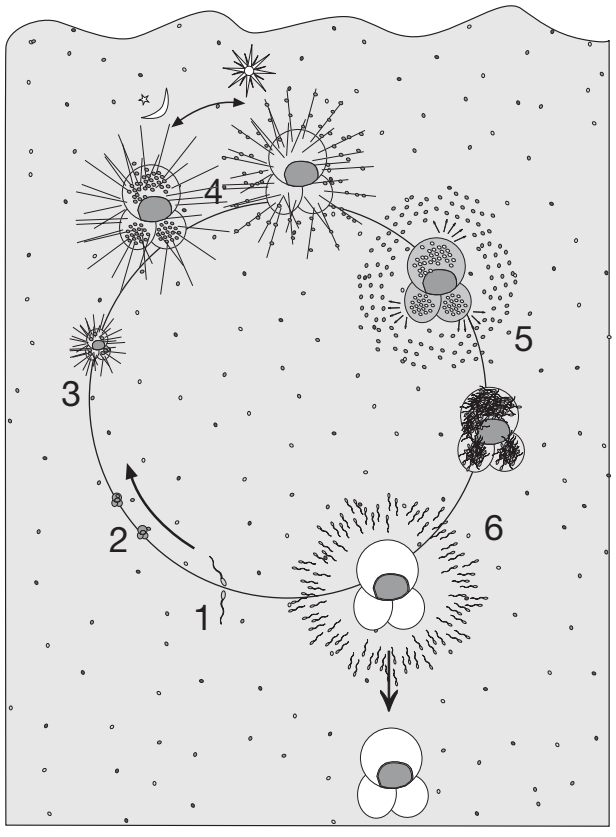


Fig. 1. Life cycle in modern symbiotic planktonic foraminifera. (1) Fusion of 2 foraminiferal gametes. (2) The juvenile foraminifer grows by secreting successive calcareous chambers, and rapidly (as early as the 3-chambered stage) acquires photosymbionts from the ambient water into specialized cytoplasmic vacuoles. (3) Endosymbionts multiply and colonize the growing foraminifer that begins to develop spines. (4) As an adult, the foraminifer spreads its symbionts along its spines to photosynthesize during the day, and brings them back into the shell at night. (5) Before gametogenesis, the foraminifer consumes some of its symbionts and expels others; all resources are focused toward gamete production. (6) The foraminifer releases its gametes into the water and dies, the carbonate shell sinking to the sea floor where it comprises much of the marine sediments in vast oceanic regions

MATERIALS AND METHODS

Sample collection and DNA extraction. Foraminifera samples were collected *in situ* using plankton nets (100 µm mesh size) from a ~200 m deep layer of surface waters during transoceanic cruises in the Atlantic, Pacific, Indian, and Southern oceans, and at some offshore stations in the Mediterranean, Caribbean, Sargasso, and Indonesian seas (Fig. 2). Planktonic foraminifera were sorted and individually picked according to morphological species using a dissecting microscope on board the vessels. Each foraminifer was carefully cleaned in filtered seawater using a microbrush, so that total DNA extraction targeted principally the genome of the host foraminifer and the hundreds of genomes of its endosymbiotic algae. Storage and DNA extraction of individual foraminiferal samples were performed as previously described in de Vargas et al. (2002).

Amplification and identification of DNA. Ribosomal DNA was amplified from hundreds of samples using standard PCR protocols and primers specific for foraminifers or dinoflagellates. Foraminiferal DNA amplification, sequencing, and genotyping methods are described in de Vargas et al. (2002). For dinoflagellates, we targeted regions of the rDNA covering the ITS-1, 5.8S, ITS-2 and the D1 and D2 regions of the LSU. We used the specific forward primers S-Dino 5′-CGCTCCCGATTGAGTGA-3′ located at the 3′ end of the SSU rDNA and L-Din6 (5′-MCCCGCTGAATTTAAGCATA-3′) at the beginning of the LSU and the reverse primer L-Din1 (5′-AACGATTTGCACGTCAGTACCGC-3′) in the LSU. Partial dinoflagellate LSU rDNA were directly sequenced from 189 individual planktonic foraminifera using an ABI 3100 Avant automatic sequencer as described in Tchernov et al. (2004). Longer fragments including the ITSs region (1.7 kb) were completed for 61 samples, priming the sequencing reaction with the internal primer 5.8S6 (5′-GCACCYDTGAAGGGCGCAGCG-3′), located in the 5.8S gene. With this primer, in addition to S-Dino and L-Din1, an overlap of more than 400 bases was achieved between each pair of consecutive strands. Shorter fragments spanning the LSU or ITS regions were sequenced using a single primer read. Based on genetic diversity obtained in our large LSU rDNA sequence alignments, we developed 2 additional methods for rapid detection of the main genetic types within the symbiotic algae.

First, an RFLP protocol was designed using the restriction endonucleases Hpy 99 I, Mfe I, and Cla I that target different sites within the region D2 of the LSU rDNA, and together allow the recognition of 4 main genetic subgroups (see 'Results'). The restrictions were performed at 37°C overnight in a total volume of 12 µl, containing 10 µl PCR product, 0.7 µl enzyme mix (Hpy 99 I: 0.5 µl; Cla I: 0.2 µl; Mfe I: 0.1 µl), and 1.2 µl of appropriate buffer. Restriction fragments patterns were visualized on 3% high-resolution agarose gels.

Second, genotype-specific primers were designed for use in multiplex PCR reactions. The reverse primers YDG1.1 (5′-AGTRACTCCGCAGAGAAACGT-3′) within the LSU rDNA, and YDG1.2 (5′-ACACAACCRGCAGATGCACAG-3′), YDG2.1 (5′-CTGTGCAGGAGTT GGCRCAAT-3′), and YDG2.2 (5′-GGATCAAGAGATCAAGAAGAC-3′) within the ITS-2 rDNA were used in combination with the dinoflagellate forward primer 5.8S6 to produce specific products of different size and identify the same 4 main dinoflagellate genotypes.

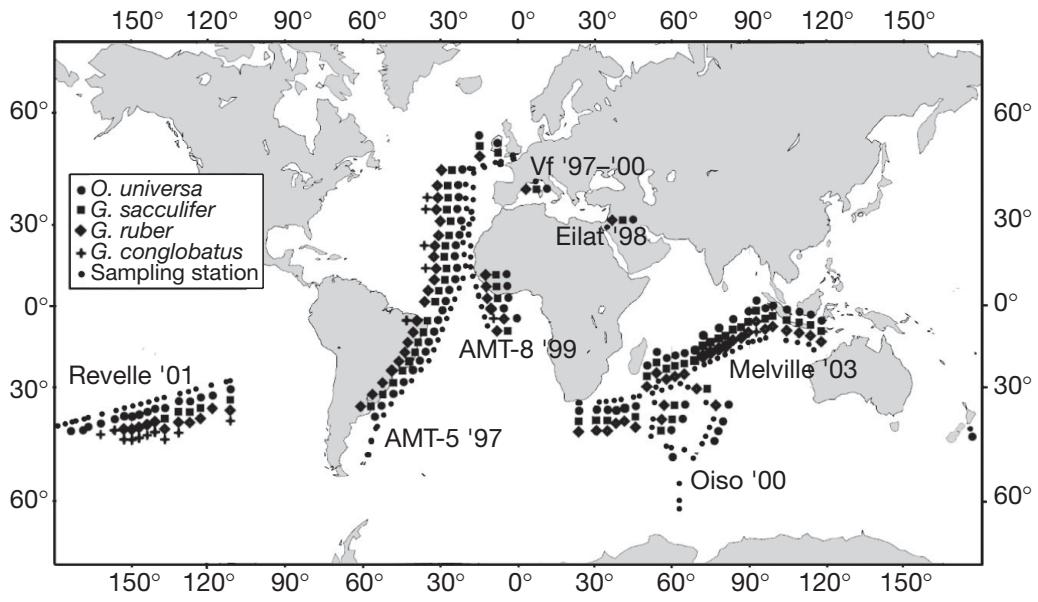


Fig. 2. Sampling locations. The occurrence of 4 morphospecies (*Orbulina universa, Globigerinoides sacculifer, G. ruber, G. conglobatus*) of planktonic foraminifer that have established symbiosis with dinoflagellates is indicated at each station. All morphospecies except *G. conglobatus* were almost universally collected at each tropical-subtropical station between ~40°N and 40°S, independent of season. Cruise names and year are indicated

Each multiplex PCR targeted 2 dinoflagellate types in a single amplification step (YDG1.1 + YDG2.1 + 5.8S6; YDG1.2 + YDG2.2 + 5.8S6). The reactions were optimized and ultimately set as follows: annealing temperature 58°C, extension temperature 68°C, addition of 0.5 ml $MgCl_2$ (25 mM) per reaction tube, common forward primer in a 2:1 concentration ratio compared to each specific primer. PCR products were visualized after migration on 2% agarose gel. A total of 592 foraminiferal extractions were genotyped using the restriction fragment length polymorphism (RFLP) and multiplex PCR approaches. Foraminifer host genotyping was performed for each DNA extraction, where dinoflagellates were identified as described in de Vargas et al. (1999). All new sequences produced in this study are available in GenBank, accession numbers DQ195278 to DQ195376 and DQ198020 to DQ198077.

Phylogenetic analysis. Sequences were automatically aligned with Clustal X software (Thompson et al. 1997), and alignments were visually examined and adjusted using the GDE program (Smith et al. 1994). Phylogenetic reconstructions were performed using the maximum likelihood (ML) and neighbor joining (NJ) approach implemented in PAUP* software (Swofford 1998). For maximum likelihood computations, the type of nucleotide substitution model and the use and shape of the gamma-distribution were estimated through likelihood ratio tests based on our data and using the Modeltest program (Posada & Crandall 1998). The same parameters were used for Bayesian statistics analyses, in which 4 Monte Carlo Markov Chains were run in parallel for 1 000 000 generations using MrBayes (Huelsenbeck & Ronquist 2001). For large data sets maximum likelihood phylogenies were generated with PHYML software (Guindon & Gascuel 2003), using the option that allows the program to estimate model parameters. Multiple phylogenetic analyses were performed for both the long and shorter rDNA fragments. Comparison of substitution rates between symbiotic dinoflagellate groups were performed with the RRTree program (Robinson-Rechavi & Huchon 2000), using *Karenia* and *Prorocentrum* spp. as outgroups. Mean genetic distances (Tajima & Nei 1984) between groups were calculated using Mega software (Kumar et al. 2004).

RESULTS

Evolutionary history of dinoflagellates symbionts of planktonic foraminifera

We first analyzed all unique dinoflagellate LSU rDNA phylotypes obtained from single planktonic foraminiferal cells of the morphospecies *Orbulina universa*, *Globigerinoides ruber*, *G. sacculifer*, and *G. conglobatus* collected worldwide. The LSU rDNA sequences were aligned with orthologous genes from free-living dinoflagellates of the *Prorocentrum* spp. and *Karenia* spp. groups (chosen as outgroups following a basic local alignment search tool [BLAST] search) and crude phylogenetic analysis of all dinoflagellate LSU rDNA available in GenBank. Closely related sequences of free-living *Gymnodinium simplex* and *G. corii*, and the only living member of the family Suessiaceae, *Polarella glacialis* (Montresor et al. 1999), were also included in the analyses. All phylogenetic analyses reveal that the pelagic symbionts form a monophyletic group, sister to the coastal-benthic *Symbiodinium* species complex (Fig. 3). Maximum likelihood and bayesian statistics suggested that the free-living

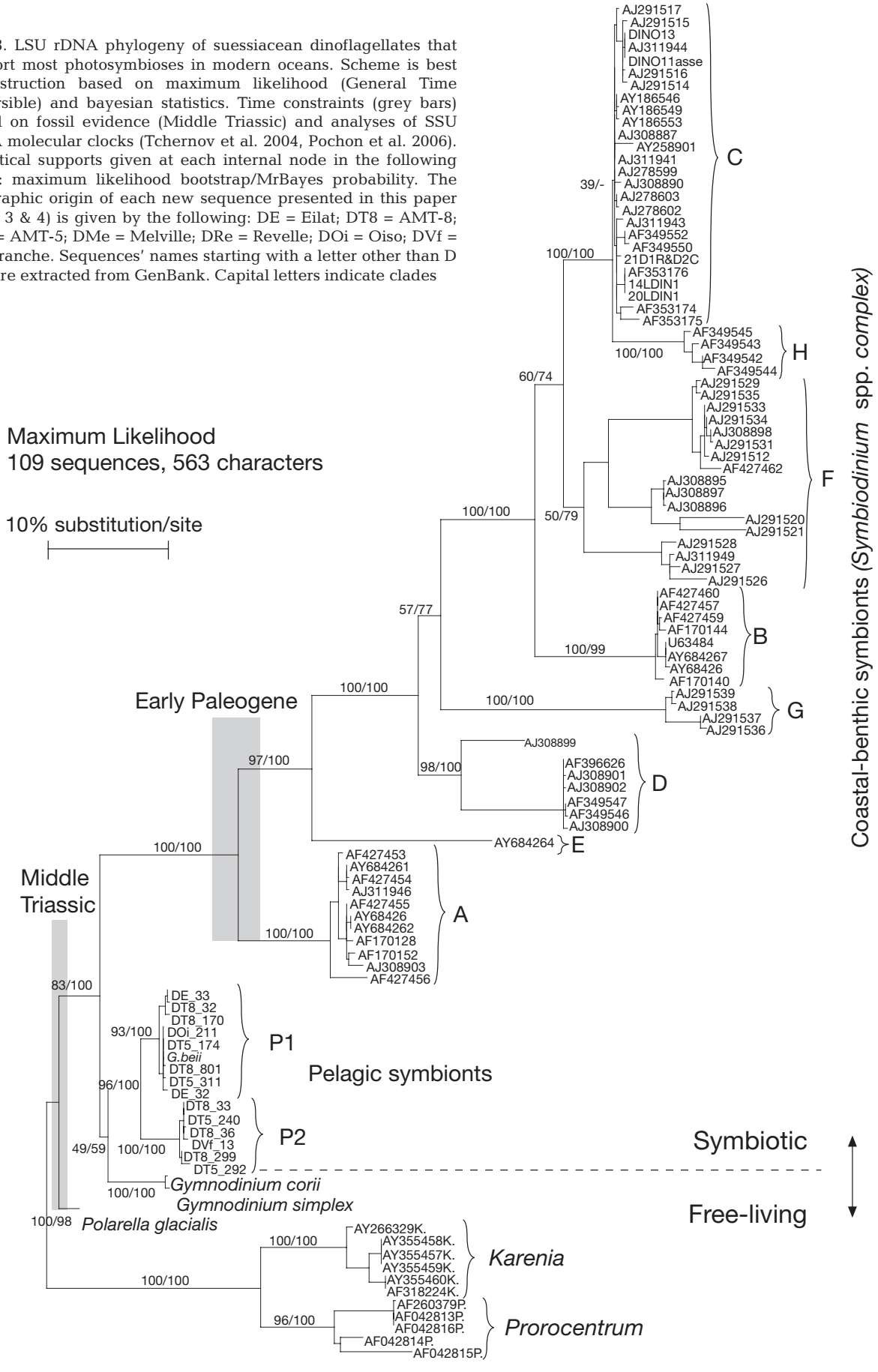


Fig. 3. LSU rDNA phylogeny of suessiacean dinoflagellates that support most photosymbioses in modern oceans. Scheme is best reconstruction based on maximum likelihood (General Time Reversible) and bayesian statistics. Time constraints (grey bars) based on fossil evidence (Middle Triassic) and analyses of SSU rDNA molecular clocks (Tchernov et al. 2004, Pochon et al. 2006). Statistical supports given at each internal node in the following order: maximum likelihood bootstrap/MrBayes probability. The geographic origin of each new sequence presented in this paper (Figs. 3 & 4) is given by the following: DE = Eilat; DT8 = AMT-8; DT5 = AMT-5; DMe = Melville; DRe = Revelle; DOi = Oiso; DVf = Villefranche. Sequences' names starting with a letter other than D were extracted from GenBank. Capital letters indicate clades

Gymnodiniales are basal to the group of pelagic symbionts, while *Polarella glacialis* appeared as an ancestor of both pelagic and coastal-benthic symbionts, confirming their taxonomic classification within the order of the Suessiales (Fensome et al. 1993, 1996, Montresor et al. 1999).

Perhaps the most striking feature of the symbiotic dinoflagellate tree is the huge differences in substitution rates between the coastal-benthic and the pelagic species. In the LSU rDNA phylogenies, the pelagic symbionts display significantly shorter branch lengths, suggesting much lower rates of molecular evolution (Fig. 3). Genetic distances from the ancestor *Polarella glacialis* are, as a mean value, 3.42 times larger for the coastal-benthic symbionts compared to the pelagic symbionts (Table 1). This calculation treats the *Symbiodinium* spp. as a single group; however, all *Symbiodinium* clades except A evolved at a much faster rate, ~6 times faster than the pelagic symbionts.

Relative rate tests were performed to confirm that the benthic-coastal *Symbiodinium* species complex evolved significantly faster than the pelagic symbionts

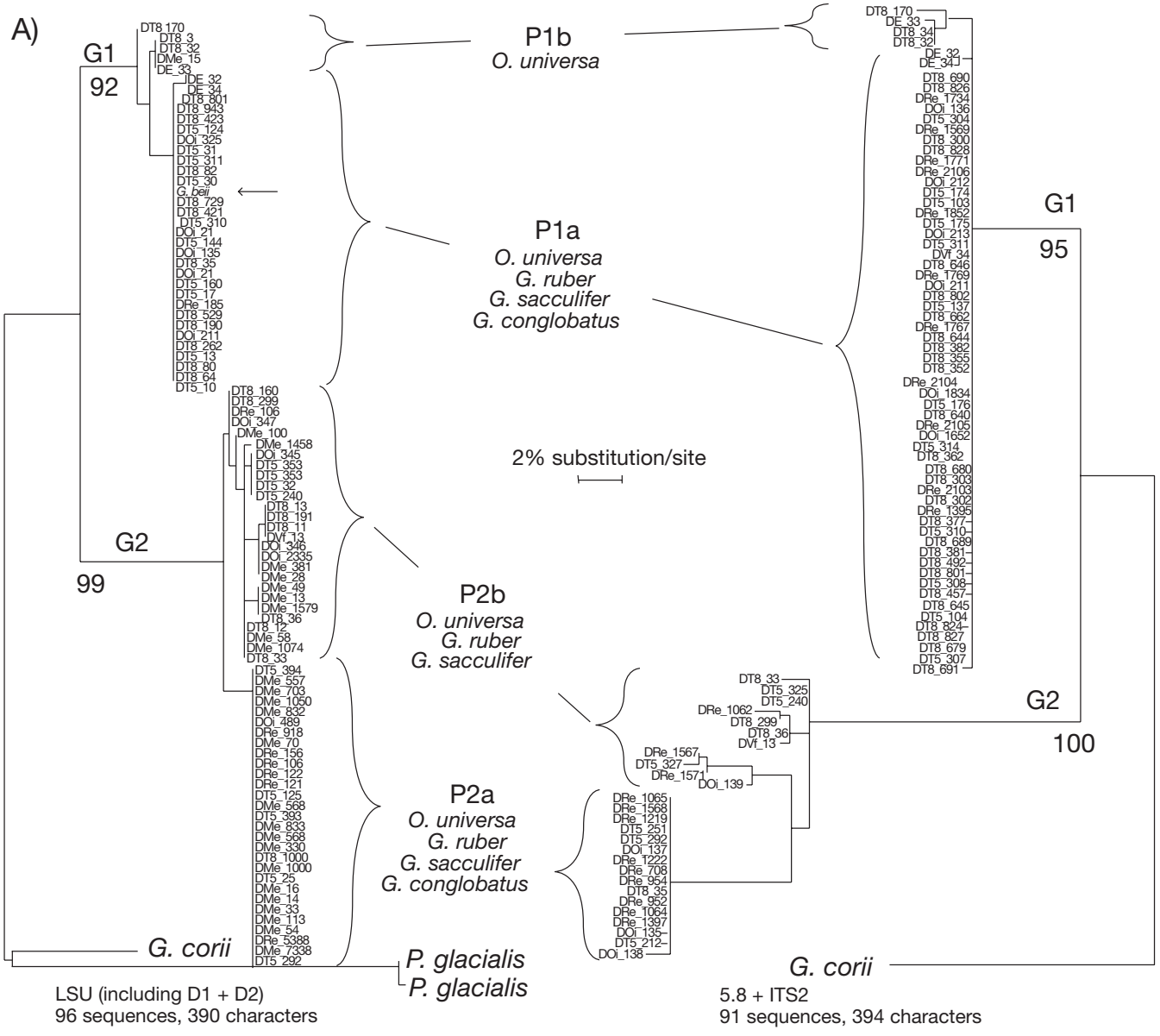
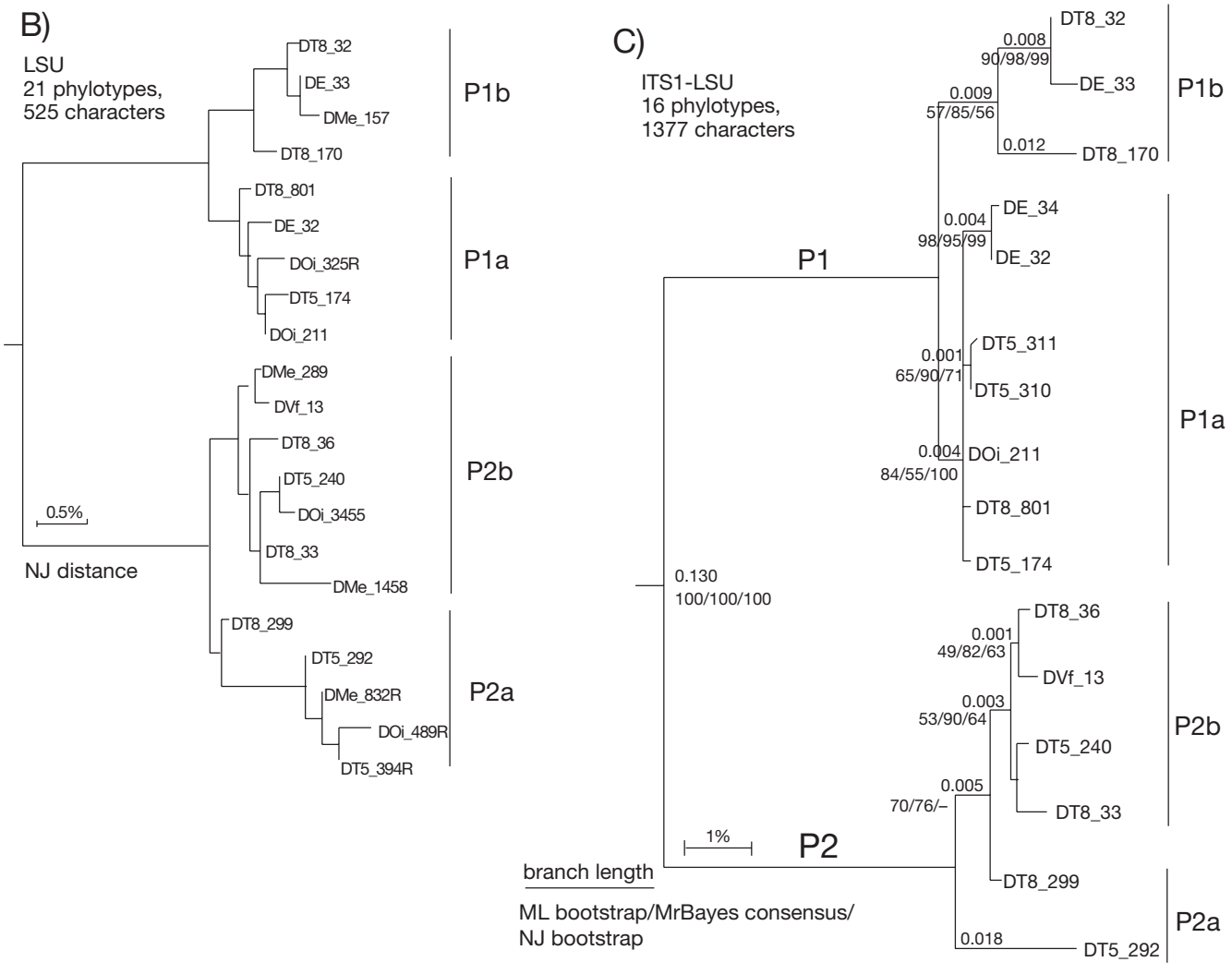


Fig. 4. (A) Comparison of maximum likelihood reconstructions using D1-D2 fragment of the LSU rDNA and ITS-2 gene. Trees demonstrate great redundancy in phylotypes sequenced from different hosts species and geographic locations. Note: data sets do not fully overlap. Arrow marks previously sequenced LSU rDNA of *Gymnodinium beii* (Wilcox 1998). (continued opposite)

(Table 2). Most differences in rates of substitutions between the defined phylogenetic groups were statistically significant ($p < 0.005$), ruling out the use of a simple absolute molecular clock to calibrate in time the evolutionary history of marine dinoflagellate-based photosymbiosis.

Genetic diversity and phylogeny within the pelagic symbiotic dinoflagellates

The genetic diversity of the pelagic symbiotic dinoflagellates is greater than anticipated from previous morphological and genetic studies, in which a single species *Gymnodinium beii* was described (Spero 1987, Gast & Caron 1996). In total, 21 dinoflagellate LSU ribotypes were obtained from 189 individual foraminifer hosts analyzed in this study. In all analyses, which used LSU and ITS regions either independently or concatenated, the rDNA diversity was split into 2 main clusters that we termed P1 and P2 (Figs. 3 & 4A). This main division may be comparable to the 'clade' level of separation within the *Symbiodinium* species complex. Each of the main clusters P1 and P2 can be further divided into 2 subgroups termed P1a-P1b and P2a-P2b (Fig. 4B). These subgroups are apparent when examining the D1-D2 region of the LSU and are supported by analyses of our long rDNA fragments from the ITS1 to the LSU (Fig. 4C). Note that our RFLP and multiplex PCR protocols were based on the LSU rDNA D1-D2 region and discriminated between these 4 genetic groups (as presented in Fig. 4B,C).



(Fig. 4 continued) (B) Details of partial LSU phylogeny using 21 unique phylotypes recognized in this study. (C) Phylogeny of 16 of the 21 unique symbiont phylotypes (all from *Orbulina universa* hosts) based on a long (1377 bp) fragment of rDNA spanning ITS-1 to LSU. *Globigerinoides* species include *G. sacculifer*, *G. ruber* and *G. conglobalus*

Table 1. Mean genetic distances (Tajima & Nei 1984) between free-living *Gymnodiniales* (*corii-simplex*), pelagic symbionts, coastal-benthic *Symbiodinium* spp., *Polarella glacialis*, and the *Karenia-Prorocentrum* spp. outgroup. Distances were calculated from the alignment used to generate the phylogeny of Fig. 3 using Mega software (Kumar et al. 2004). Genetic distances of pelagic and coastal-benthic clusters from their ancestor *P. glacialis* in **bold**. SD provided for each genetic distance (in parentheses)

	Outgroup	*P. glacialis*	*G. corii-simplex*	Pelagic symbionts	*Symbiodinium* spp.
Outgroup		(0.022)	(0.025)	(0.025)	(0.036)
P. glacialis	0.234		(0.016)	(0.015)	(0.030)
G. corii-simplex	0.265	0.099		(0.012)	(0.027)
Pelagic symbionts	0.269	**0.097**	0.096		(0.027)
Symbiodinium spp.	0.447	**0.332**	0.310	0.314	

Abundance of pelagic dinoflagellate subgroups and phylotypes

We surveyed the relative abundance of pelagic symbiont subgroups using rDNA sequencing, as well as RFLP and multiplex PCR. A total of 592 symbiotic dinoflagellates were thus assigned to 1 of the 4 genetic subgroups shown in Figs. 4B,C. Out of the 21 unique phylotypes, 2 were vastly dominant and comprised most of the P1a and P2a subgroups (Fig. 4A). The remaining 19 phylotypes had a significantly lower abundance, most of them having been found only once. Dinoflagellates belonging to the genetic subgroups P1a and P2a were overwhelmingly abundant, representing 51.3% and 28.5% respectively of our total planktonic foraminifer samples collected worldwide (Fig. 5A). Both P1a and P2a subgroups had a small internal diversity (10 and 4 phylotypes, respectively), with the great abundance attributed to a single dominant phylotype. The genetic subgroup P1b is rare: we only recognized 23 ind. of this type (5.4%), and only 3 unique phylotypes were sequenced. Subgroup P2b comprised 16.2% of our samples (96 ind.) and is as diverse as P1a, with up to 10 phylotypes of similar abundance (Fig. 5A).

Table 2. Relative rate test (Robinson-Rechavi & Huchon 2000) of rDNA substitution (Kimura model) between groups included in the phylogeny of Fig. 3, using *Karenia* and *Prorocentrum* spp. as outgroups. *Symbiodinium* clade A was treated as a separate group: it displays much shorter branches than other *Symbiodinium* spp. clades. R: ratio between substitution rates within a group compared to substitution rates within another group relative to the outgroup; p: the exact probability associated with the test; $^{\star}p < 0.005$; $^{\star\star}p < 5 \times 10^{-6}$. Smaller p values associated with larger differences in substitution rates between lineages. Comparisons between pelagic and coastal-benthic symbionts are highlighted in bold

Lineage1	Lineage2	R	p
G. corii-simplex	*Pelagodinium*	−0.44783	0.65428
G. corii-simplex	*Symbiodinium* spp. A	−3.15401	0.00162*
G. corii-simplex	*Symbiodinium* spp. B-H	−5.78816	1×10^{-7}**
Pelagic symbionts	***Symbiodinium* spp. A**	−2.89003	**0.00386***
Pelagic symbionts	***Symbiodinium* spp. B-H**	−5.65599	**1** $\times 10^{-7}$**
Symbiodinium spp. A	*Symbiodinium* spp. B-H	−3.16338	0.00156*

Host specificity

Symbiosis in planktonic foraminifera is clearly not species-specific (Fig. 4A & 5B). Our study encompasses the full diversity of modern planktonic foraminifera that have established symbiosis with dinoflagellates, with special emphasis on the morphological species *Orbulina universa*, where 3 genetically and ecologically distinct types were previously detected worldwide (de Vargas et al. 1999, de Vargas et al. 2004). Symbiont types P1b were only found in association with *O. universa*, and were predominantly from genetic type III (Mediterranean-type in de Vargas et al. 1999) (Fig. 5B). However, the genetic subgroup P1b is rare, and its absence from the *Globigerinoides* species may simply be due to the relatively small sample size of these host species (n = 37). Members of the 3 more abundant subgroups were retrieved from all genetic and morphological species of planktonic foraminifera, proving their ability to colonize divergent hosts. In addition, multiplex PCR allowed us to detect the presence of different dinoflagellate ribotypes within a single host. Although the large majority of individual foraminifers contained a single ribotype, 37 occurrences (11%) of multiple genetic subgroups within a single foraminifer were observed. Most of these samples contained 2 different symbiotic dinoflagellate types and a few foraminifera enclosed even 3 genetic subgroups. The rare occurrence of multiple symbiont-types within a single host clearly reveals its ability to associate with different dinoflagellate phylotypes and further confirms the flexibility of this symbiosis between 2 pelagic unicellular organisms.

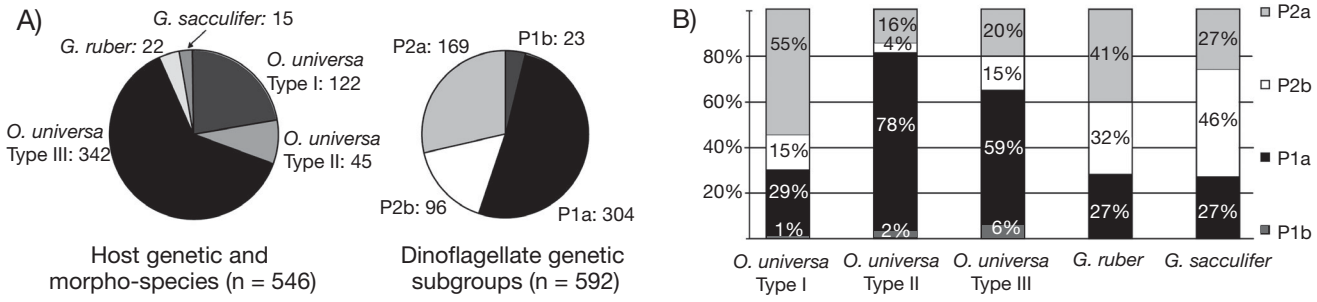


Fig. 5. (A) Proportion of host foraminiferal species and symbiotic dinoflagellate genetic subgroups among symbioses analyzed in this study (*Orbulina universa, Globigerinoides sacculifer, G. ruber*). Larger number of characterized dinoflagellate symbionts compared with hosts is due to presence of multiple symbiotic ribotypes in 11% of hosts. Symbionts of *G. conglobatus* were not included due to very low occurrence. (B) Proportion of symbiotic dinoflagellate genetic subgroups in the planktonic foraminiferal genetic and morphological species

DISCUSSION

Our sampling most likely does not exhaust the genetic diversity of dinoflagellate symbionts of planktonic foraminifers. The 21 unique LSU types identified here are a lower bound for the true diversity, and continued investigation into more rapidly evolving regions of the genus will likely expand and refine this phylogenetic scheme. Nevertheless, the widespread sampling effort over different oceanic basins and seasons, together with the large number of redundant genotypes from ~600 individual foraminifers analyzed, suggests that we unveiled most of the major phylogenetic features of this group of organisms. Note that the biogeographic and seasonal distributions of both host and symbiont genotypes will be discussed in another study.

Evolutionary history of oceanic dinoflagellate-based symbiosis

The dinoflagellates that established photosymbiotic relationships with foraminifera in the planktonic realm form a monophyletic group directly related to the dinoflagellates of the *Symbiodinium* species complex that are involved in benthic-coastal (mainly coral reef) symbioses. Thus, a family of genetically related dinoflagellates acquired the ability to form symbiosis with a wide range of protistan and metazoan hosts in subtropical and tropical oceans. These dinoflagellates have invaded both benthic and pelagic marine domains, and sustained the survival of the majority of modern marine calcifiers through photosymbiosis. Based on an absence of morphological characters, the first dinoflagellate symbiont of planktonic foraminifer was classified into the highly polyphyletic genus *Gymnodinium* (Spero 1987). Here we labeled all phylotypes of dinoflagellate symbionts of planktonic foraminifers with the letter 'P', for 'pelagic symbionts'. However, a formal description of this new genus is in progress.

All genetic markers and analyses used herein indicate that *Polarella glacialis* belongs to the ancestral lineage that gave rise to all symbiotic dinoflagellates (Fig. 3). In addition, our reconstructions suggest that the free-living species *Gymnodinium corii* and *G. simplex* are part of the *Symbiodinium* spp.-pelagic symbionts cluster. They branch, with weak statistical support, at the base of the pelagic symbiont group (Figs. 3 & 6B). Our data can be interpreted as either 2 independent endosymbiotic transitions from free-living dinoflagellate lineages into coastal reef biota and pelagic foraminifera (Fig. 6B), or as a single symbiotic event followed by a loss of symbiotic behavior in the *G. corii* and *G. simplex* lineages (Fig. 6B). Given the impressive taxonomic range of hosts 'infected' by the Suessiaceae, a re-invention of symbiosis in the pelagic realm would not be surprising (Rowan 1998). The Suessiaceae have obviously 'learned' how to repeatedly colonize new forms of life, implying transient periods as free-living protists. Investigation of other genes and additional free-living marine dinoflagellates, yet to be collected, may clarify the early evolution of this major marine photosymbiosis.

Timing and rates of evolution of pelagic symbiotic dinoflagellates

Some constraints on the evolution of dinoflagellates and their hosts are found in the fossil record, and thus provide additional information for further interpretation of our data (Fig. 6A). First, the extant *Polarella glacialis,* which roots all symbiotic dinoflagellates in our phylogenies, is linked to the Early Jurassic fossil dinoflagellate *Umbriadinium* sp. (Palliani & Riding 2003), and more generally to the family

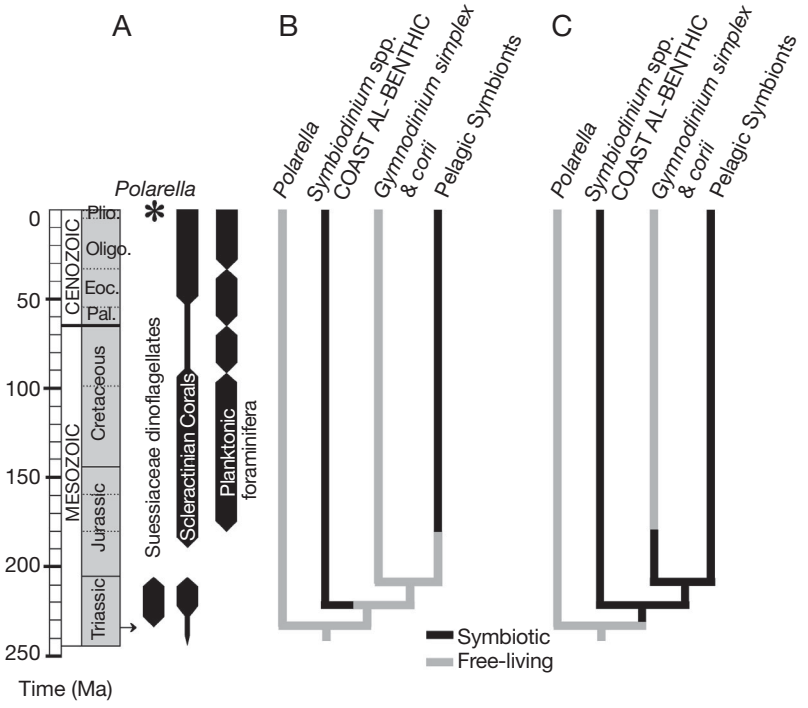


Fig. 6. Macroevolution of suessiacean dinoflagellates. (A) Phylogenetic constraints from fossil records of suessiacean dinoflagellates, scleractinian corals, and planktonic foraminifera (see text for detailed explanations and references). For each group, the origin of the fossil record, its gaps, and its presence (abundant or scarce) are schematically depicted. The asterisk indicates the presence of *Polarella glacialis* in modern plankton samples. This species does not fossilize but is firmly linked to triassic suessiacean fossils based on morphological characteristics. (B,C) Two LSU rDNA-based macro-evolutionary scenarios leading to the rise of modern dinoflagellate-based marine photosymbioses. Ma: million years ago

of the Suessiaceae. This family has been proposed to be the ancestor of the modern *Symbiodinium* spp. (Fensome et al. 1996, MacRae et al. 1996), and a great abundance of Suessiaceae cysts coincides with the first appearance of scleractinian coral reefs in Middle Triassic times, 230 to 200 million years ago (Fensome et al. 1996, Fensome et al. 1999, Palliani & Riding 2000, Stanley & Fautin 2001). In addition, studies of ancient coral reefs together with stable isotope evidence from fossil coral skeletons suggest that many of the early corals were photosymbiotic (Stanley & Swart 1995, Stanley 2003).

It appears that some suessiacean dinoflagellates established endosymbiotic relationships in coastal ecosystems soon after their origination in the Triassic. As a result, they limited or even stopped the production of diploid encysting forms, which are typically resistant stages for free-living organisms. Finding direct evidence to link the early photosymbionts with extant *Symbiodinium* spp. is thus hampered by the patchy nature of the fossil dinoflagellate record, based on cysts. However, the molecular phylogenetic position of *Polarella glacialis* confirms interpretations of the fossil record and constrains the early evolutionary steps of dinoflagellate-based photosymbiosis in the early Mesozoic (Fig. 6). Another time constraint comes from recent and independent molecular calibrations of the *Symbiodinium* species complex. The use of both absolute (Tchernov et al. 2004) and relaxed (Pochon et al. 2006) molecular clocks indicates that the origin of the modern *Symbiodinium* species complex occurred after the Cretaceous/Tertiary boundary, in the Paleocene or Early Eocene. Finally, stable isotope measurements in the shells of planktonic foraminifera indicate that some species established photosymbiosis in the late Cretaceous and that major groups successfully diversified in the Paleocene thanks to photosymbiosis (Kelly et al. 1996, Norris 1996, Berggren & Norris 1997, Houston et al. 1999, Quillevere et al. 2001). Stable isotope patterns suggest that these early photosymbioses involved dinoflagellates (D'Hondt et al. 1994). The application of a crude, lineage-specific molecular clock suggests that the origin of modern diversity in the pelagic symbiotic cluster, or the first split between the clades P1 and P2, dates back to the Early Paleogene, and thus that early symbioses in planktonic foraminifera involved dinoflagellates similar to today's pelagic symbionts.

The time constraints imposed on our phylogeny highlight the huge difference in rates of rDNA substitution between the pelagic and the coastal-benthic symbiotic dinoflagellates. In fact, it seems that there is an acceleration in evolutionary rates that starts with the slow-evolving *Polarella glacialis*, followed by the still slow pelagic symbionts, the *Symbiodinium* ancestral clade A with intermediate substitution-rates, and finally the fast-clock remainder of *Symbiodium* spp. Previously, accelerated rates of mutation in parasitic and endosymbiotic bacteria compared to related free-living strains have been noted (Lambert & Moran 1998). Two processes may contribute to this phenomenon: (1) the small population size within a host, which generates recurrent bottlenecks at each host generation, causes increased genetic drift and fixation rates, (2) the increased reliance of the endosymbiont on its host leads to a relaxation of the purifying selection and possible damage to DNA-repair genes (Dufresne et al. 2005).

These mechanisms may have affected the coastal-benthic symbionts much more than the pelagic ones. Indeed, populations of the coastal-benthic *Symbiodinium* spp. can be isolated in some large coral colonies and vertically transmitted to the next host generations. They can thus genetically drift in isolation or relax their purifying selection for thousands of generations. On the contrary, pelagic symbionts are forced into a free-living stage every few weeks, at each new host generation (Fig. 1), and thus constantly re-seed the immense and worldwide genetic pool of free-floating dinoflagellates available for new symbioses.

Specificity in pelagic photosymbiosis

The absence of patterns of co-evolution in our data suggests that photosymbioses in the pelagic realm are sustained by a high flexibility in host-symbiont associations. All dinoflagellate genetic subgroups were found in association with all foraminiferal morphological and genetic species within the genus *Globigerinoides* and *Orbulina* (Fig.5). At the level of the symbionts' LSU and ITS rDNA phylotypes, strictly identical sequences were harbored by different host species (Fig. 4A). In addition, although most of the foraminifer cells analyzed herein contained a single pelagic symbiont phylotype, 11% contained symbionts of different genetic subgroups.

This high flexibility between host and symbiont species in the pelagial contrasts with the patterns of specificity frequently observed in photosymbiotic associations in coastal-benthic environments (Pochon et al. 2004). In coral reefs, the extremely long life of most hosts compared to the symbiont generation time, the dominantly asexual reproductions of the hosts, and the relative absence of *Symbiodinium* spp. free-living stages, may favor co-evolutionary processes. Patterns of symbiosis-specificity in reef ecosystems may also result from competition and adaptation to the infinity of ecological gradients and micro-niches available in such environments. However, in the pelagic world, conditions are more uniform and the biodiversity of both hosts and symbionts is relatively lower. The generation time of foraminifer hosts (2 to 4 wk) is close to that of dinoflagellate symbionts (a few days), and the critical need of the juvenile foraminifer to acquire symbionts from the open water applies a strong evolutionary pressure to maintain flexible associations. Within the wide uniform expanses of the open waters, where tiny drifting organisms are relatively diluted, and where the photosynthetic holosymbiont unit needs to be re-created at each host generation, maximum compatibility is certainly an advantage.

CONCLUSIONS

Foraminiferal symbiosis in the pelagic realm involves dinoflagellates genetically related to the coastal-benthic *Symbiodinium* species complex. However, the pace and mode of evolution of the pelagic symbiosis appears to be different. In open oceans, a lower diversity of symbionts with significantly lower rates of DNA substitution, and obligatory free-living stages that occur every few weeks, sustains a seemingly highly flexible photosymbiosis. These may be necessary conditions for survival in the plankton. The deep phylogenetic branching of the pelagic symbionts species complex, the fossil evidence that Mesozoic and Paleogene planktonic foraminifera were photosymbiotic, and the marked absence of specificity observed in modern photosymbioses, together suggest that these dinoflagellates were the major symbionts for the entire evolutionary history of pelagic foraminifera. Although the high flexibility involved in pelagic symbiosis apparently brings acclimation advantages over ecological time scales, the relative lack of biodiversity in the system may threaten pelagic symbioses over longer, geological time scales and lead to major extinction of the hosts.

Acknowledgements. We warmly thank Swati Narayan-Yadav for technical support. Many thanks also to Roger Harris, Nicolas Metzl, and Erica Goetze, who generously invited us to participate in trans-oceanic cruises in the Atlantic (AMT-5, AMT-8), Indian (Oiso), and Pacific (Revelle-2001, Melville-2003) oceans. The Captains and the crews on board research vessels 'James Clark Ross', 'Marion-Dufresne', 'Revelle', and 'Melville' allowed data collection. Thanks finally to John Dolan for his efficient editorial work, and to 3 anonymous reviewers who provided constructive critiques on the original manuscript. This work was supported by US-NSF grants OCE-0083415 (Richard Norris) and DEB-0415351 (CdV), as well as an ATIP grant awarded to CdV by the 'Centre National de la Recherche Scientifique', France.

LITERATURE CITED

Baker AC (2001) Reef corals bleach to survive change. Nature 411:765–766

Baker AC (2003) Flexibility and specificity in coral-algal symbiosis: diversity, ecology, and biogeography of *Symbiodinium*. Annu Rev Ecol Evol Syst 34:661–689

Baker AC, Starger CJ, McClanahan TR, Glynn PW (2004) Coral's adaptive response to climate change. Nature 430:741

Berggren WA, Norris RD (1997) Biostratigraphy, phylogeny and systematics of Paleocene trochospiral planktonic foraminifera. Micropaleontology 43:1–116

Bijma J, Erez J, Hemleben C (1990) Lunar and semi-lunar reproductive cycles in some spinose planktonic foraminifers. J Foraminif Res 20:117–127

Buck KR, Bolt PA, Bentham WN, Garrison DL (1992) A dinoflagellate cyst from Antarctic sea ice. J Phycol 28:15–18

Buddemeier RW, Fautin DG (1993) Coral bleaching as an adaptive mechanism: a testable hypothesis. BioScience 43:320–326

Buddemeier RW, Baker AC, Fautin DG, Jacobs JR (2004) The adaptive hypothesis of bleaching. In: Rosenberg E, Loya Y (eds) Coral health and disease. Springer Verlag, Berlin

Carlos AA, Baillie BK, Kawachi M, Maruyama T (1999) Phylogenetic position of *Symbiodinium* (Dinophyceae) isolates from tridacnids (Bivalvia), cardiids (Bivalvia), a sponge (Porifera), a soft coral (Anthozoa), and a free-living strain. J Phycol 35:1054–1062

de Vargas C, Norris R, Zaninetti L, Gibb SW, Pawlowski J (1999) Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. PNAS 96:2864–2868

de Vargas C, Bonzon M, Rees NW, Pawlowski J, Zaninetti L (2002) A molecular approach to biodiversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). Mar Micropaleontol 45:101–116

de Vargas C, Garcia-Saez A, Medlin LK, Thierstein HR (2004) Super-species in the calcareous plankton. In: Thierstein HR, Young JR (eds) Coccolithophores: from molecular processes to global impact. Springer Verlag, Berlin, p 271–298

D'Hondt S, Zachos JC, Schultz G (1994) Stable isotopic signals and photosymbiosis in Late Paleocene planktonic foraminifera. Paleobiology 20:391–406

Dufresne A, Partensky G, Partensky F (2005) Accelerated evolution associated with genome reduction in a free-living prokaryote. Genome Biol 6:R14

Emiliani C (1954) Depth habitats of some species of pelagic foraminifera as indicated by oxygen isotope ratios. Am J Sci 252:149–158

Fabricius KE, Mieog JC, Colin PL, Idip D, van Oppen JH (2004) Identity and diversity of coral endosymbionts (zooxanthellae) from three Palauan reefs with contrasting bleaching, temperature, and shading histories. Mol Ecol 13:2445–2458

Fensome RA, Taylor FJR, Norris R, Sarjeant WAS, Wharton DJ, Williams GL (1993) A classification of living and fossil dinoflagellates, Vol 7. Sheridan Press, Hanover, PA

Fensome RA, MacRae RA, Moldowan JM, Taylor FJR, Williams GL (1996) The early Mesozoic radiation of dinoflagellates. Paleobiology 22:329–338

Fensome RA, Saldarriaga JF, Taylor FJR (1999) Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies. Grana 38:66–80

Fukami H, Budd AF, Paulay G, Solé-Cava A, Chen CA, Iwao K, Knowlton N (2004) Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. Nature 427:832–835

Gast RJ, Caron DA (1996) Molecular phylogeny of symbiotic dinoflagellates from planktonic foraminifera and radiolaria. Mol Biol Evol 13:1192–1197

Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Hemleben C, Spindler M, Anderson OR (1989) Modern planktonic foraminifera. Springer Verlag, New York

Houston RM, Huber BT (1998) Evidence of photosymbiosis in fossil taxa? Ontogenetic stable isotope trends in some Late Cretaceous planktonic foraminifera. Mar Micropaleontol 34:29–46

Houston RM, Huber BT, Spero HJ (1999) Size-related isotopic trends in some Maastrichtian planktonic foraminifera: methodological comparisons, intraspecific variability, and evidence for photosymbiosis. Mar Micropaleontol 36: 169–188

Huber BT, Bijma J, Darling K (1997) Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). Paleobiology 23:33–62

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Hughen KA, Overpeck JT, Lehman SJ, Kashgarian M, Southon J, Peterson LC (1998) A new ^{14}C calibration data set for the last deglaciation. Radiocarbon 40:483–494

Imbrie J, van Donk J, Kipp NG (1973) Paleoclimatic investigation of a late Pleistocene Caribbean deep-sea core: comparison of isotopic and faunal methods. Quat Res 3:10–38

Kelly DC, Bralower TJ, Zachos JC, Premoli Silva I, Thomas E (1996) Rapid diversification of planktonic foraminifera in the tropical Pacific (ODP Site 865) during the late Paleocene thermal maximum. Geology 24:423–426

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

LaJeunesse TC (2001) Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinflagellates in the genus *Symbiodinium* using the ITS region: in search of a 'species' level marker. J Phycol 37:866–880

Lambert JD, Moran NA (1998) Deleterious mutations destabilize ribosomal RNA in endosymbiotic bacteria. PNAS 95: 4458–4462

Little AF, Oppen MJHv, Willis BL (2004) Flexibility in algal endosymbioses shapes growth in reef corals. Science 304: 1492–1494

Loh WKW, Loi T, Carter D, Hoegh-Guldberg O (2001) Genetic variability of the symbiotic dinoflagellates from the wide ranging coral species *Seriatopora hystrix* and *Acropora longicyathus* in the Indo-west Pacific. Mar Ecol Prog Ser 222:97–107

MacRae RA, Fensome RA, Williams GL (1996) Fossil dinoflagellate diversity, origins, and extinctions and their significance. Can J Bot 74:1687–1694

Montresor M, Procaccini G, Stoecker DK (1999) *Polarella glacialis*, gen. nov., sp. nov. (Dinophyceae): Suessiaceae are still alive! J Phycol 35:186–197

Norris RD (1996) Symbiosis as an evolutionary innovation in the radiation of Paleocene planktonic foraminifera. Paleobiology 22:461–480

Palliani RB, Riding JB (2000) Subdivision of the dinoflagellate cyst Family Suessiaceae and discussion of its evolution. J Micropalaeontol 19:133–137

Palliani RB, Riding JB (2003) Umbriadinium and Polarella: an example of selectivity in the dinoflagellate fossil record. Grana 42:108–111

Pawlowski J, Holzmann M, Fahrni JF, Pochon X, Lee JJ (2001) Molecular identification of algal endosymbionts in large miliolid foraminifera : 2. Dinoflagellates. J Eukaryot Microbiol 48:368–373

Pochon X, Pawlowski J, Zaninetti L, Rowan R (2001) High genetic diversity and relative specificity among *Symbiodinium*-like endosymbiotic dinoflagellates in soritid foraminiferans. Mar Biol 139:1069–1078

Pochon X, LaJeunesse TC, Pawlowski J (2004) Biogeographic partitioning and host specialization among foraminiferan dinoflagellate symbionts (*Symbiodinium*, Dinophyta). Mar Biol 146:17–27

Pochon X, Montoya-Burgos JI, Stadelmann B, Pawlowski J (2006) Molecular phylogeny, evolutionary rates and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium* (Dinophyta). Mol Phylogenet Evol 38:20–30

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Quillevere F, Norris RD, Moussa I, Berggren WA (2001) Role of photosymbiosis and biogeography in the diversification of early Paleogene acarininids (planktonic foraminifera). Paleobiology 27:311–326

Robinson-Rechavi M, Huchon D (2000) RRTree: Relative-rate tests between groups of sequences on a phylogenetic tree. Bioinformatics 16:296–297

Rodriguez-Lanetty M (2003) Evolving lineages of *Symbiodinium*-like dinoflagellates based on ITS1 rDNA. Mol Phylogenet Evol 28:152–168

Romano SL, Cairns SD (2000) Molecular phylogenetic hypotheses for the evolution of scleractinian corals. Bull Mar Sci 67:1043–1068

Romano SL, Palumbi SR (1996) Evolution of scleractinian corals inferred from molecular systematics. Science 271: 640–642

Rowan R (1998) Diversity and ecology of zooxanthellae on coral reefs. J Phycol 34:407–417

Rowan R (2004) Thermal adaptation in reef coral symbionts. Nature 430:742

Rowan R, Knowlton N, Baker AC (1997) Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature 388:265–269

Santos SR, Taylor DJ, Kinzie RA III, Hidaka M, Sakai K, Coffroth MA (2002) Molecular phylogeny of symbiotic dinofagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. Mol Phylogenet Evol 23: 97–111

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

Savage AM, Trapido-Rosenthal H, Douglas AE (2002) On the functional significance of molecular variation in *Symbiodinium*, the symbiotic algae of Cnidaria: photosynthetic response to irradiance. Mar Ecol Prog Ser 244:27–37

Shackleton NJ, Opdyke ND (1973) Oxygen isotope and paleomagnetic stratigraphy of equatorial Pacific core V28-238: oxygen isotope temperatures and ice volumes on a 10^5 year and 10^6 year scale. Quat Res 3:39–55

Smith SW, Overbeek R, Woese CR, Gilbert W, Gillevet PM (1994) The genetic data environment an expandable GUI for multiple sequence analysis. Comput Appl Biosci 10:671–675

Spero HJ (1987) Symbiosis in the planktonic foraminifer, *Orbulina universa*, and the isolation of its symbiotic dinoflagellate, *Gymnodinium Béii* sp. nov. J Phycol 23: 307–317

Stanley GDJ (2003) The evolution of modern corals and their early history. Earth-Sci Rev 60:195–225

Stanley GDJ, Fautin DG (2001) The origins of modern corals. Science 291:1913–1914

Stanley GDJ, Swart PK (1995) Evolution of the coral-zooxanthellae symbiosis during the Triassic: a geochemical approach. Paleobiology 21:179–199

Swofford DL (1998) PAUP* 4.0: phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA

Tajima F, Nei M (1984) Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol 1(3):269–285

Tchernov D, Gorbunov MY, de Vargas C, Narayan Yadav S, Milligan AJ, Häggblom M, Falkowski PG (2004) Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. PNAS 101:13531–13535

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res 24:4876–4882

Trench RK (1993) Microalgal-invertebrate symbioses: a review. Endocytobiol Cell Res 9:135–175

Wade BS (2004) Planktonic foraminiferal biostratigraphy and mechanisms in the extinction of *Morozovella* in the late middle Eocene. Mar Micropaleontol 51:23–38

Wade C, Darling K (2002) Fossilized records of past seas. Microbiol Today 29:183–185

Wilcox TP (1998) Large-subunit ribosomal RNA systematics of symbiotic dinoflagellates: morphology does not recapitulate phylogeny. Mol Phylogenet Evol 10:436–448

Editorial responsibility: John Dolan (Contributing Editor), Villefranche-sur-Mer, France

Submitted: October 25, 2005; Accepted: December 16, 2005
Proofs received from author(s): October 9, 2006