



Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*

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Abstract

Symbiotic dinoflagellates belonging to the genus *Symbiodinium* are found in association with a wide variety of shallow-water invertebrates and protists dwelling in tropical and subtropical coral-reef ecosystems. Molecular phylogeny of *Symbiodinium*, initially inferred using nuclear ribosomal genes, was recently confirmed by studies of chloroplastic and mitochondrial genes, but with limited taxon sampling and low resolution. Here, we present the first complete view of *Symbiodinium* phylogeny based on concatenated partial sequences of chloroplast 23S-rDNA (cp23S) and nuclear 28S-rDNA (nr28S) genes, including all known *Symbiodinium* lineages. Our data produced a well resolved phylogenetic tree and provide a strong statistical support for the eight distinctive clades (A–H) that form the major taxa of *Symbiodinium*. The relative-rate tests did not show particularly high differences between lineages and both analysed markers. However, maximum likelihood ratio tests rejected a global molecular clock. Therefore, we applied a relaxed molecular clock method to infer the divergence times of all extant lineages of *Symbiodinium*, calibrating its phylogenetic tree with the fossil record of soritid foraminifera. Our analysis suggests that *Symbiodinium* originated in early Eocene, and that the majority of extant lineages diversified since mid-Miocene, about 15 million years ago.

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1. Introduction

Dinoflagellates of the genus *Symbiodinium* form mutualistic symbiosis with a wide range of marine invertebrates including sponges, cnidarians, and molluscs, as well as with some protistan hosts. These photosynthetic symbionts, commonly referred to as “zooxanthellae,” are crucial in the uptake of energy and enhancement of host calcification in oligotrophic tropical and subtropical environments (Muscatine, 1990). Due to the simple and almost featureless morphology of both motile and vegetative stages of *Symbiodinium*, the authors of early morphological studies claimed the occurrence of a single

pandemic species, *Symbiodinium microadriaticum* (Freudenthal, 1962; Taylor, 1974).

We know now that the genus *Symbiodinium* is exceptionally diverse as evidenced by numerous studies based on ultramorphology (Schoenberg and Trench, 1980; Trench and Blank, 1987), physiology (Banaszak and Trench, 1995; Iglesias-Prieto and Trench, 1994), biochemistry (Bishop and Kenrick, 1981; Govind et al., 1990; Withers et al., 1982), and molecules (reviewed in Baker, 2003). Until recently, molecular phylogenetic trees of *Symbiodinium* have been inferred exclusively from nuclear (nr) genes encoding ribosomal RNA. The resulting phylogenetic trees of *Symbiodinium* obtained from many cnidarian and molluscan hosts, have consistently revealed numerous divergent lineages usually referred to as clades A, B, C, and D. Similar investigations on the *Symbiodinium* harboured by foraminifera of

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the subfamily Soritinae brought to light a remarkable diversity of phylotypes, most of which (clades F, G, and H) were divergent from those found in cnidarians (Pawlowski et al., 2001; Pochon et al., 2001, 2004). These results were unexpected because the first appearance of the Soritinae in the fossil record is a recent event, dating back from about 25 to 30 million years ago (MYA) (Haynes, 1981). Furthermore, it has been shown that the acquisition of *Symbiodinium* by the ancestor of Soritinae foraminifera was a key innovation that promoted important morphological and ecological adaptations within this group of protists (Richardson, 2001).

The first *Symbiodinium* phylogeny based on a different marker than nuclear rDNA genes was inferred using the chloroplast (cp) gene coding for the ribosomal large subunit 23S Domain V (Santos et al., 2002a,b). The cp23S phylogeny was not statistically different from that generated from nuclear rDNA, providing the first independent evidence for the existence of *Symbiodinium* clades A to F. Some of these clades were also confirmed by analyses of plastid-encoded *psbA* (Takishita et al., 2003) and mitochondrial-encoded *cox1* (Takabayashi et al., 2004) genes, corroborating Santos et al. (2002a) observations. All of these studies, however, did not include the *Symbiodinium* types found in soritid foraminifera, and thus, have restricted the analyses to a portion of the currently known diversity of the genus.

Here, we have constructed *Symbiodinium* phylogenetic trees based on nr28S and cp23S, including all known phylotypes and we have statistically analysed the topological congruencies between both genes. We also tested for variation of evolutionary rates between genes and lineages. Moreover, the highly derived and diversified nature of *Symbiodinium* spp. hosted by soritid foraminifera suggests that these hosts possess some fundamental properties in driving *Symbiodinium* evolution. Here, we also ask whether faster evolutionary rates may explain the high diversity of *Symbiodinium* observed in foraminiferan hosts. Finally, we inferred the divergence timing of the different *Symbiodinium* lineages by applying an internal fossil calibration of the molecular tree and a Bayesian relaxed molecular clock approach.

2. Materials and methods

2.1. Sample collections, PCR amplification, and sequencing

Foraminiferan and cnidarian samples were collected between July 1997 and September 2003, from a total of nine localities around the world. DNA extractions were processed as described in Pochon et al. (2001). A fragment of *Symbiodinium* cp23S (Domain V, according to Harris et al., 1994) was PCR-amplified from 29

soritid and 7 cnidarian isolates by using the primers 23S4F (5'-GACGGCTGTAACATAACGG-3') and 23S7R (5'-CCATCGTATTGAACCCAGC-3'). In some cases, PCR products were recovered by excision on a 1.5% agarose gel and purified by using the MinElute gel extraction kit (Qiagen). Additionally, three *Symbiodinium* samples isolated from soritids were PCR-amplified for the nuclear large subunit 28S (Domain D1–D3), following Pochon et al. (2001).

The *Symbiodinium* PCR products were sequenced directly in both directions as described in Pochon et al. (2001) and compared to 59 previously published sequences (Pochon et al., 2001, 2004; Santos et al., 2002a). Additionally, nr28S and cp23S sequences from the free-living dinoflagellate *Gymnodinium simplex* (AF060900 and AJ872114, respectively) were included as outgroup. *Symbiodinium* clade designations, host species names, reference numbers, collection sites, and GenBank accession numbers of all sequences included in our analyses are given in Table 1.

2.2. Phylogenetic analyses

Three data sets (nr28S, cp23S, and both molecules concatenated) were used for phylogenetic analyses. The sequence alignment was established by using CLUSTALX (Thompson et al., 1994) and then further improved manually by using the BIOEDIT 5.0.9 sequence alignment software (Hall, 1999).

The MODELTEST v3.0b4 program (Posada and Crandall, 1998) was used to identify the best model of DNA evolution for each of our data set in maximum likelihood (ML) environment. The ML method was then performed with a heuristic search and random addition of sequences as implemented in PAUP* 4.0b10 (Swofford, 2002), with starting tree obtained via stepwise addition of taxa, and then swapped using the tree-bisection-reconnection (TBR) algorithm. The reliability of internal branches was assessed using the bootstrap method (Felsenstein, 1985) with 100 replicates by using the program PHYML (Guindon and Gascuel, 2003). Bayesian tree reconstructions with posterior probabilities were inferred using MRBAYES 2.01 (Huelsenbeck and Ronquist, 2001) and using the same model of DNA evolution as for the ML analyses. Four simultaneous Markov chains were run for 1,000,000 generations with trees sampled every 10 generations, with 50,000 initial trees discarded as “burn-in,” based on visual inspection. For the Maximum Parsimony (MP) analyses, heuristic searches were performed using PAUP*. The MP trees were constructed by using 100 repetitions of random sequence additions of taxa, starting trees obtained by stepwise addition, and branches swapped using the TBR option. Supports for branches in the MP trees were tested by bootstrap analysis with 1000 replicates.

Table 1
Sample information for *Symbiodinium* dinoflagellates included in the nr28S and the cp23S phylogenetic analyses

<i>Symbiodinium</i> designation	Host species name	Reference number	Collection site	nr28S GenBank No.	cp23S GenBank No.	Study
Clade A	<i>Aiptasia pallida</i>	FLAp#4 ^a	Florida, USA	AF427453	AY035404	Santos et al. (2002a)
	<i>Cassiopea xamachana</i>	Cx	Jamaica	AF427454	AY035406	Santos et al. (2002a)
	<i>Tridacna gigas</i>	T	Indo-Pacific	AF427455	AY035412	Santos et al. (2002a)
	<i>Zoanthus sociatus</i>	Zs ^a	Jamaica	AF427456	AY035414	Santos et al. (2002a)
Clade B	<i>Plexaura kuna</i> (1)	Pk13 ^a	Florida, USA	AF427458	AY055231	Santos et al. (2002a)
	<i>Plexaura kuna</i> (2)	Pk702	San Blas, Panama	AF427459	AY035419	Santos et al. (2002a)
	<i>Plexaura flexuosa</i>	PurPflex ^a	Florida, USA	AF427460	AY035420	Santos et al. (2002a)
	<i>Aiptasia pulchella</i>	HIAp	Hawaii	AF427457	AY035421	Santos et al. (2002a)
Clade C	<i>Ctenactis echinata</i>	458X	Guam, USA	AJ308887	AJ872078	This study
	Unknown anemone	Ua#31 ^a	Okinawa, Japan	AF427463	AY035425	Santos et al. (2002a)
	<i>Lobophyllia</i> sp.	1673J	Guam, USA	AJ311943	AJ872079	This study
	<i>Heliopora cerulea</i>	50X	Guam, USA	AJ308888	AJ872080	This study
	<i>Sorites</i> sp. (1)	1479X	Panama	AJ621128	AJ872081	This study
	<i>Sorites</i> sp. (2)	1466X	Panama	AJ620945	AJ872082	This study
	<i>Porites cylindrica</i>	8X	Guam, USA	AJ308892	AJ872083	This study
	<i>Sorites</i> sp. (3)	489J	GBR, Australia	AJ291516	AJ872084	This study
	<i>Marginopora vertebralis</i> (1)	490J ^a	GBR, Australia	AJ291515	AJ872085	This study
	<i>Amphisorus hemprichii</i> (1)	84X	Guam, USA	AJ872075	AJ872086	This study
Subclade D1	<i>Haliclona koremella</i>	PSP1-05 ^a	Palau	AF427464	AY055241	Santos et al. (2002a)
Subclade D2	<i>Acropora palifera</i>	542X	Guam, USA	AJ308902	AJ872087	This study
	<i>Acropora</i> sp.	1655J	Guam, USA	AJ311948	AJ872088	This study
	<i>Acropora bruegmanni</i>	A024	Okinawa, Japan	AF396627	AY035429	Santos et al. (2002a)
	<i>Pavona decusata</i>	63X ^a	Guam, USA	AJ308900	AJ872089	This study
Clade E	Free-living dinoflagellate	CCMP421 ^a	New-Zealand	AF060899	AY055240	Santos et al. (2002a)
Subclade F2	<i>Sorites</i> sp. (4)	1335J	Elat, Israel	AJ830912	AJ872090	This study
	<i>Sorites</i> sp. (5)	1320J	Elat, Israel	AJ830914	AJ872091	This study
	<i>Sorites</i> sp. (6)	215J	Safaga, Egypt	AJ830911	AJ872092	This study
	<i>Sorites</i> sp. (7)	206J ^a	Safaga, Egypt	AJ830908	AJ872093	This study
Subclade F3	<i>Amphisorus hemprichii</i> (2)	5243X ^a	Guam, USA	AJ872076	AJ872094	This study
	<i>Amphisorus hemprichii</i> (3)	5244X	Guam, USA	AJ830916	AJ872095	This study
	<i>Amphisorus hemprichii</i> (4)	1635J	Guam, USA	AJ291525	AJ872096	This study
	<i>Amphisorus hemprichii</i> (5)	188X	Guam, USA	AJ308895	AJ872097	This study
Subclade F4	<i>Sorites</i> sp. (8)	1334X ^a	Florida, USA	AJ621145	AJ872098	This study
	<i>Sorites</i> sp. (9)	1628X	Panama	AJ621135	AJ872101	This study
	<i>Sorites</i> sp. (10)	836J	Florida, USA	AJ291527	AJ872102	This study
	<i>Sorites</i> sp. (11)	1349X	Florida, USA	AJ621146	AJ872099	This study
	<i>Sorites</i> sp. (12)	1350X	Florida, USA	AJ621147	AJ872100	This study
Subclade F5	<i>Montipora verrucosa</i>	Mv	Hawaii	AF427462	AY035422	Santos et al. (2002a)
	<i>Sorites</i> sp. (13)	971X	Reunion Island	AJ872077	AJ872103	This study
	<i>Sorites</i> sp. (14)	1593J	Guam, USA	AJ291529	AJ872104	This study
	<i>Sorites</i> sp. (15)	650J ^a	Maldives	AJ291535	AJ872105	This study
Clade G	<i>Marginopora vertebralis</i> (2)	1645J	Guam, USA	AJ291538	AJ872106	This study
	<i>Marginopora vertebralis</i> (3)	1582J ^a	Guam, USA	AJ291537	AJ872107	This study
	<i>Marginopora vertebralis</i> (4)	1584J ^a	Guam, USA	AJ291539	AJ872108	This study
Clade H	<i>Sorites</i> sp. (16)	1653X ^a	Panama	AJ621131	AJ872113	This study
	<i>Sorites</i> sp. (17)	1286X	Florida, USA	AJ621148	AJ872111	This study
	<i>Sorites</i> sp. (18)	1382X	Panama	AJ621129	AJ872112	This study
	<i>Sorites</i> sp. (19)	751J	Florida, USA	AJ291513	AJ872109	This study
	<i>Sorites</i> sp. (20)	1678J ^a	Guam, USA	AJ291520	AJ872110	This study

^a *Symbiodinium* samples used for Shimodaira–Hasegawa congruency tests between nr28S and cp23S topologies (reduced data set in the electronic Appendix A).

2.3. Statistical analyses

Homogeneity tests of base frequencies were conducted on all alignments by using the base frequencies χ^2 test as implemented in PAUP* to estimate if the sequences have evolved with the same pattern of nucleotide substitution. A partition-homogeneity test, using a heuristic search of 100 replicates, was applied on the alignments of nr28S and cp23S to verify if the two genes can be analysed in concatenation. Additionally, we compared the best topology of each data sets obtained separately by using the Shimodaira–Hasegawa topological congruency test (SH-test; Shimodaira and Hasegawa, 1999).

To assess which one of the two markers evolves faster, the relative evolutionary rates of the two genes were estimated by plotting the pairwise corrected genetic distance (according to the model indicated by MODELTEST) of both markers. The slope of the linear regression was taken as the relative evolutionary rate value.

To test whether pairs of lineages evolve at similar rates, relative-rate tests (RRT) were performed by using the program RRTREE 1.1 (Robinson-Rechavi and Huchon, 2000). Clades were compared two by two and their sister clade was used as outgroup.

2.4. Molecular dating

Likelihood ratio tests for the molecular clock were performed as described in Swofford et al. (1996) and PAUP* was used to obtain the likelihood scores of each phylogeny. We estimated the divergence times by using the Bayesian relaxed molecular clock approach implemented in MULTIDISTRIBUTE (Kishino et al., 2001; Thorne and Kishino, 2002; Thorne et al., 1998). This program allows the incorporation of multiple time constraints, and takes into account both molecular and palaeontological uncertainties to estimate the variance of divergence times. Two calibration points issued from the fossil record were used: (1) the emergence of Soritinae, with a lower limit at 25 and an upper limit at 30 MYA (Haynes, 1981), and (2) the closure of the Isthmus of Panama, with a lower limit at 3 and an upper limit at 4 MYA, separating Indo-Pacific and Caribbean *Symbiodinium* clade H (Pochon et al., 2004).

3. Results

3.1. Sequence data

The alignment of nr28S sequences was 801 base pairs (bp) long. The model of evolution estimated with MODELTEST corresponded to the SYM+G model (Zharkikh, 1994).

The length of the complete cp23S Domain V alignment reached 925 bp, but 306 characters were excluded

from all analyses due to variable areas with multiple indels that made alignment unreliable. The model of evolution corresponded to the TrN+G model (Tamura and Nei, 1993).

χ^2 homogeneity test of base frequencies indicated no significant deviations for both alignments (nr28S: $\chi^2=21.95$; $df=141$; $P=1.0$ and cp23S: $\chi^2=56.91$; $df=147$; $P=1.0$). The partition-homogeneity test on the concatenated sequences was performed and showed no significant incongruence between the two data sets ($P=0.68$). The concatenated alignment was 1420 bp long. The model of evolution corresponds to the TrN+G. Alignment data are available upon request.

3.2. Phylogenetic analysis

The nr28S and cp23S phylogenetic trees constructed under ML, MP, and Bayesian criteria produce very similar topologies (Fig. 1). In most analyses, the branching order of clades was extremely well conserved between both genes, the main difference being the position of clade E, which was found either branching as the sister group to the assemblage comprising clades B, C, F, and H (cp23S tree), or next to the clade A as sister to other clades (nr28S tree). In general, the plastid-based phylogenetic analyses were characterized by lower bootstrap support (BS) than the nuclear set (Fig. 1), especially at the C, F, and H root. This was due to the unstable position of clade H, which clustered within clade F in the Bayesian tree (data not shown). However, SH-tests revealed significant differences in chloroplastic topologies constraining clade H within clade F ($L_1-L_2=-27.8259$; $P=0.003$) or within clade C ($L_1-L_2=-27.7966$; $P=0.005$), indicating that under the ML criterion, the position of clade H as sister group to clade C seems to be stable. Another feature that characterized the cp23S data set was the extremely long branch of *Symbiodinium* D1. The exclusion of this sequence did not change the topologies but increased the BS in general (data not shown). In spite of the overall similarity of both nuclear and chloroplastic trees, their topologies appeared as statistically different according to SH-tests (electronic Appendix A) even when clades D1 and E were excluded from the analyses (data not shown). To test whether this unexpected incongruence was due to topological differences within or between clades, we have reduced our trees to keep only the two most divergent sequences per clade and one sequence per subclade F (see Table 1). The SH-test performed with the reduced data sets showed no significant differences between topologies, meaning that the unexpected incongruence outlined above was due to the sum of small differences within each clades, especially within the divergent clade F.

In the concatenated analysis, all nodes, except the one at the root of clades D and G, display much higher BS (electronic Appendix B; Fig. 2). The clade

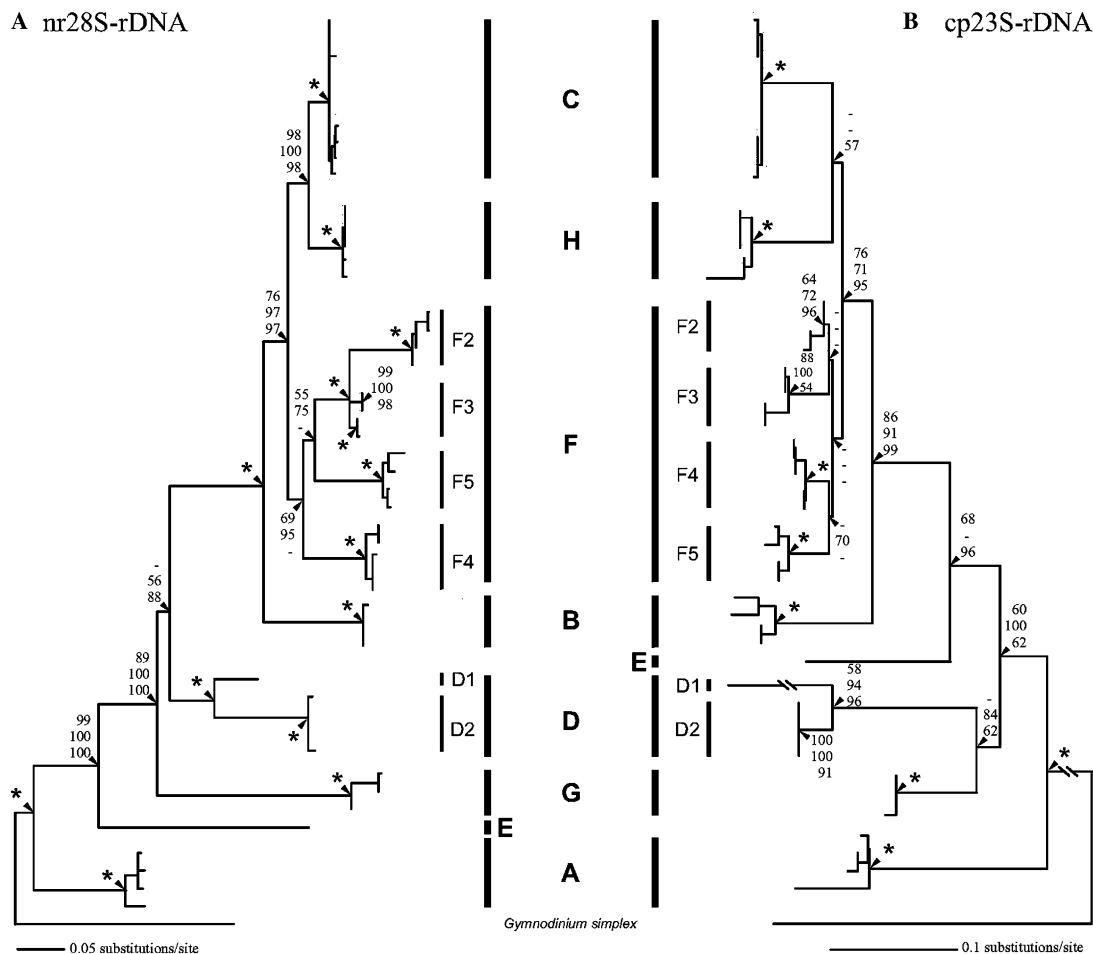


Fig. 1. Maximum likelihood phylogenetic trees of the genus *Symbiodinium* based on (A) the nr28S data set and (B) the cp23S data set. Numbers at nodes are, from top, the bootstrap values obtained with ML, the Bayesian posterior probabilities (in percentage), and the bootstrap values obtained with MP, respectively. Only values above 50 are indicated. Nodes with 100% BS in all analyses are symbolized with *. The phylogenetic trees are rooted using the dinoflagellate *Gymnodinium simplex*. The *Symbiodinium* clades and subclades are indicated with letters A to H.

interrelationships are congruent with the nr28S phylogeny with the exception of the monophyly of clades D and G. Finally, there is a good support for placing subclade F5 at the base of clade F.

3.3. Evolutionary rates

The effective relative evolutionary rate between the two markers estimated across all lineages are presented in the [electronic Appendix C](#). Only the sites included in the phylogenetic analyses were considered. The slope ($Y=0.5283$) of the linear regression ($R^2=0.7079$) was taken as the effective evolutionary rate value, excluding clade D1. The analysed nuclear region evolves twice as fast as the chloroplastic counterpart. Clade D1 is composed of a single lineage which displays an opposite trend with the chloroplastic gene evolving faster ($Y=1.77$).

The differences in evolutionary rates between *Symbiodinium* lineages were estimated by RRT tests for both markers (Table 2). In the nuclear set, clade A exhibited significantly slower evolutionary rates than any

other clade. In contrast, clades B and F displayed the highest evolutionary rates with most of the P values near the 0.05 significance level. In the chloroplastic set, clades A and G showed slightly slower evolutionary rates than the other clades with P values sometimes lower than the 0.05 significance level. Finally, clade D was found to evolve faster than all other clades due to the extremely long branch of lineage D1.

3.4. Molecular dating

Since the likelihood ratio test rejected a global molecular clock ($P<0.05$), estimates of divergence times were obtained with the Bayesian relaxed molecular clock approach (Fig. 2). The relaxed clock analyses were run on the three data sets separately. We assumed that the calibration point corresponding to the emergence of soritid foraminiferans is located at the root of clades F, H, and C (Fig. 2), considering the clade G symbionts as an independent or secondary acquisition in foraminifera (see Section 4). To ascertain the time separating the

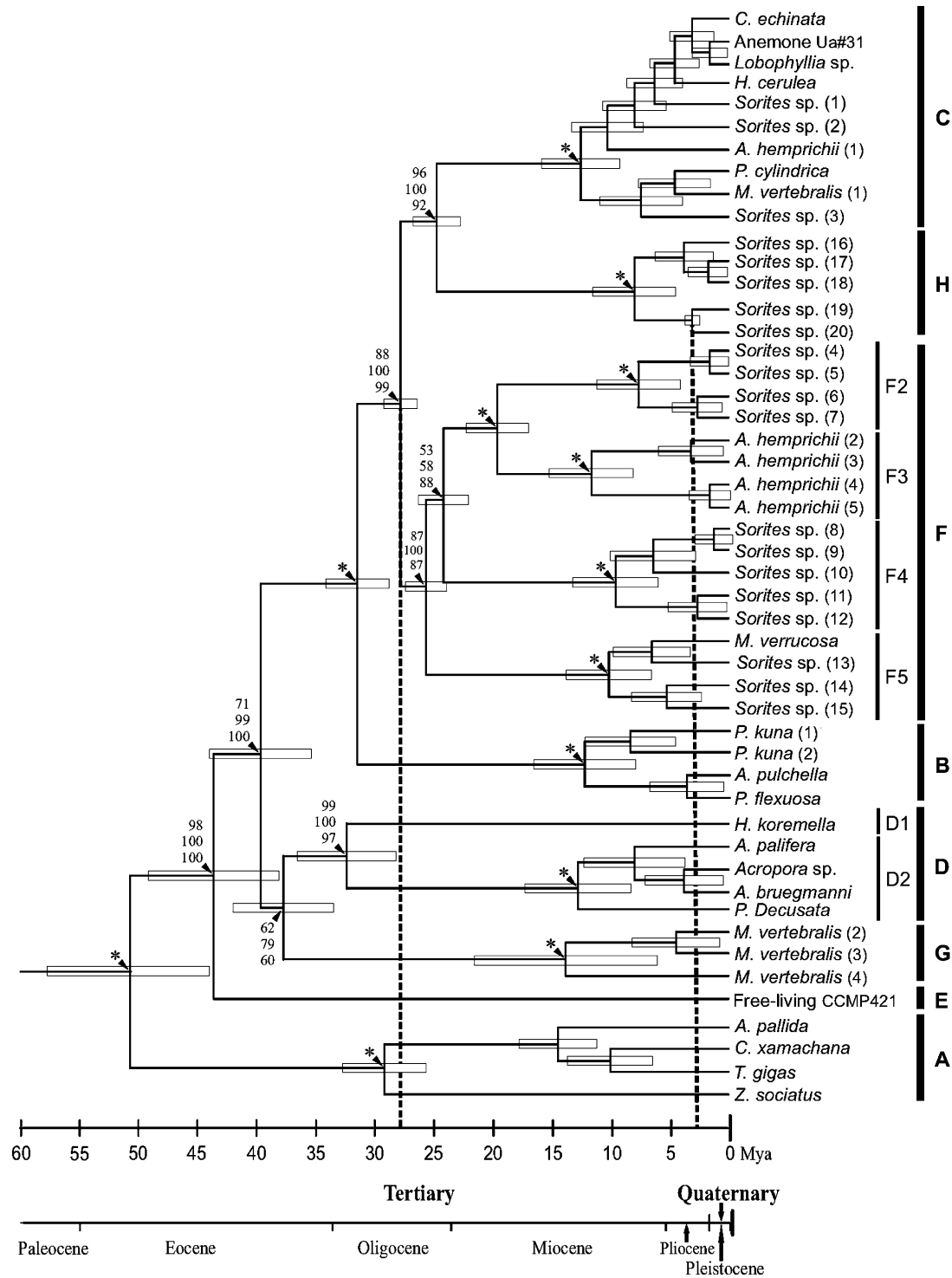


Fig. 2. Chronogram obtained from the concatenated nr28S and cp23S data sets for the *Symbiodinium* ingroup, with ages inferred from the Bayesian rate autocorrelation method using two nodes under palaeontological constraints (dotted lines). Names at leaves correspond to the *Symbiodinium* hosts shown in Table 1. Horizontal boxes stand for \pm one standard deviation around divergence ages. Numbers at nodes are, from top, the bootstrap values obtained with ML, the Bayesian posterior probabilities (in percentage), and the bootstrap values obtained with MP, respectively (see also electronic Appendix A). Nodes with 100% BS in all analyses are symbolized with *. The *Symbiodinium* clades and subclades are indicated with letters A to H.

ingroup root from the present (rttm in MULTIDIV-TIME), this method requests the test of different priors for the ingroup age (rttm = 450, 400, 250, 100, 70, 65, 60, 55, 50, 47, and 40 MYA). For the three data sets, the

standard deviation for each node was the smallest with a root prior at 50 MYA. Nuclear and chloroplastic genes analysed separately or concatenated gave very similar estimates of divergence times. As shown on Fig. 2, the

Table 2

Relative-rate tests of *Symbiodinium* lineages between nr28S (upper half of the matrix) and cp23S (lower half) data sets

	A	B	C	D	E	F	G	H
A		0.000*	0.002*	0.000*	0.000*	0.000*	0.000*	0.002*
B	0.031*		0.068	0.023*	0.056	0.698	0.667	0.044*
C	0.15	0.827		0.414	0.492	0.047*	0.418	0.942
D	0.006*	0.137	0.015*		0.899	0.062	0.896	0.743
E	0.384	0.487	0.826	0.006*		0.098	0.06	0.577
F	0.175	0.737	0.592	0.013*	0.487		0.976	0.048*
G	0.28	0.013*	0.163	0.000*	0.449	0.102		0.342
H	0.038*	0.259	0.354	0.113	0.507	0.277	0.014*	

* $p < 0.05$.

clade A diverged about 50 MYA, followed by clades E, G, D, and B, between 50 and 25 MYA. The clades C and H as well as most of extant phylotypes within all clades diverged since 15 MYA.

4. Discussion

4.1. Trees and classification in the genus *Symbiodinium*

Our study presents the first mostly congruent view of *Symbiodinium* phylogeny based on more than one molecular marker. As illustrated in Figs. 1 and 2, the nuclear and chloroplastic genes revealed a congruent view of *Symbiodinium* phylogeny, providing a solid basis to establish the classification of this genus. This view is in general agreement with the majority of previously published *Symbiodinium* phylogenies, however, our data provide stronger statistical support for most of the clades and better resolution of their relationships. Based on our analyses, we distinguish eight clades, which can be considered as subgenera of the genus *Symbiodinium*. Phylogenetic position and support for these clades, their host range and geographic distribution are discussed below:

4.1.1. Clade A

Initially described by Rowan and Powers (1991), together with clades B and C, this clade branches always at the base of the *Symbiodinium* tree, as a sister group to all other clades. Its position is stable in all analyses, including nuclear, chloroplastic, and mitochondrial genes (Pawlowski et al., 2001; Pochon et al., 2001, 2004; Santos et al., 2002a; Takishita et al., 2003; Takabayashi et al., 2004). The clade is strongly supported (100% BS) in the present and previous studies, in spite of relatively high sequence divergence (Santos et al., 2003). These symbionts have been found in a wide range of ‘zooxanthellate’ invertebrates (cnidarians and molluscs), but are absent in foraminifera. Host assemblages in Western Atlantic regions (Baker and Rowan, 1997) and in the Red Sea (Barneah et al., 2004; Karako-Lampert et al., 2004) are associated commonly with members of this clade.

4.1.2. Clade B

In our concatenated tree, this clade branches as sister to the clades C, H, and F with 100% BS (Fig. 2; electronic Appendix B). The same position was found in analyses of chloroplastic genes, albeit with much lower support (Santos et al., 2002a). Other studies placed it either as sister to clade F (Pawlowski et al., 2001; Pochon et al., 2001) or as sister to clade C (Baker, 2003; Takishita et al., 2003). These symbionts are particularly common in Western Atlantic regions (Baker and Rowan, 1997), where they have been found in a wide range of ‘zooxanthellate’ invertebrates, especially in gorgonian corals (Goulet and Coffroth, 1997, 2003; Lewis and Coffroth, 2004; Santos et al., 2003, 2004).

4.1.3. Clade C

This clade forms the “crown” of the *Symbiodinium* tree, branching as a sister group to clade H (92–100% BS). The relations within this clade are not well resolved, but the analysis of more variable (ITS1 and ITS2) regions demonstrated the presence of a large number of ecologically and physiologically distinct ‘types’ (LaJeunesse, 2001; LaJeunesse et al., 2004; Rodriguez-Lanetty et al., 2004; Van Oppen, 2004; Van Oppen et al., 2001). These symbionts have been found in all ‘zooxanthellate’ invertebrates groups as well as in foraminifera and ciliates (Lobban et al., 2002). They are particularly abundant in Indo-Pacific, evolving since at least 6–9 MYA (LaJeunesse, 2005) and seem to be targets of their bleaching, being more sensitive to warming episodes than most members from other *Symbiodinium* clades (Baker et al., 2004; Rowan, 2004). However, some types identified by ITS sequences are thermally tolerant (Pochon et al., 2004).

4.1.4. Clade D

This is one of few clades, which phylogenetic position is not stable. In our analyses, it branches either as a sister group to clade G, or as sister group to clades B, C, F, and H. In the previous analyses of chloroplastic genes, it was placed between clade A and other clades (Santos et al., 2002a; Takishita et al., 2003). In these studies, however, the relationship between clade D and other clades are weakly supported. The study of mitochondrial *cox1* shows even that this clade is not monophyletic, and that the two subclades D1 and D2 branch separately (Takabayashi et al., 2004). This situation is exceptional, because in all other phylogenies of *Symbiodinium*, both subclades group together and their monophyly is highly supported (97–100% BS) in our concatenated analysis (Fig. 2; electronic appendix B). Subclade D2, sometimes referred to as clade E (see Baker, 2003), groups mainly scleractinian symbionts that are thermally tolerant and which abundance is known to increase in reefs that have suffered episodes of severe bleaching (Baker et al., 2004; Fabricius et al., 2004; Rowan, 2004).

4.1.5. Clade E

This ‘clade’ branches either near the base of the tree between clade A and other clades (Figs. 1A and 2) or in the middle of the tree, as sister to clades B,C,F, and H (Fig. 1B). The latter position was also recovered in analysis of chloroplastic genes by Santos et al. (2002a). Clade E is represented here by a single sequence of a cultured strain (CCMP421), which was identified as *Gymnodinium varians* when it was originally isolated (Chang et al., 1983). As shown by several phylogenetic studies based on various rDNAs this isolate belongs clearly to the genus *Symbiodinium* (LaJeunesse, 2001; LaJeunesse and Trench, 2000; Saldarriaga et al., 2001; Wilcox, 1998). More recently, representatives of this clade have been detected in water samples from Jiaozhou Bay, PR China (Gou et al., 2003) and properly identified by Santos (2004). Because they have to date never been reported in invertebrates and protists hosts, these symbionts may represent a subgenus of *Symbiodinium* that has an exclusively free-leaving mode of life.

4.1.6. Clade F

This clade is composed of four highly divergent lineages (F2–F5) found frequently and almost exclusively in soritid foraminifera (Pawlowski et al., 2001; Pochon et al., 2001). The presence of this clade in corals was reported only twice. LaJeunesse (2001) sequenced a cultured strain isolated from *Montipora verrucosa* (now *M. capitata*) from the Hawaiian archipelago, which is now identified as belonging to subclade F5. Rodriguez-Lanetty et al. (2003) reported one sequence type belonging to clade F2 from scleractinian *Alveopora japonica* from Korean waters. All other subclades comprise only foraminiferan symbionts. The position of clade F as sister to clades C and H is stable and was found in both chloroplastic and nuclear gene analyses, however, its monophyly is only weakly supported and the relations between the four subclades are not well resolved (Fig. 1).

4.1.7. Clade G

The symbionts belonging to this clade were found initially in soritid genus *Marginopora* from Guam (Pawlowski et al., 2001; Pochon et al., 2001) but some new studies show that this clade is also represented in various invertebrates from the Great Barrier Reef, such as bioeroding sponges (Schoenberg and Loh, 2005), octocorals (Van Oppen et al., 2005a), scleractinian corals (Van Oppen et al., 2005b), and giant sea anemones (LaJeunesse, pers. comm.). The phylogenetic position of this clade either as sister group to clade D (Figs. 1B and 2, Pochon et al., 2001) or as an independent lineage (Fig. 1A) is not resolved.

4.1.8. Clade H

This foraminiferan-specific lineage was initially described as clade Fr1 (Pawlowski et al., 2001; Pochon et al., 2001). It was later considered as a separate clade

because of a high genetic distance, which separates it from clades C and F (Pochon et al., 2004). These symbionts dominate the community of Caribbean soritid foraminifera (Pochon et al., 2004). The phylogenetic position of this clade as sister to clade C is strongly supported in our nuclear and concatenated analyses (Figs. 1A and 2) but much more weakly in the plastid-based phylogenetic tree.

The eight clades described above certainly do not include a complete list of taxa in the genus *Symbiodinium*, however, these clades represent probably the major part of present *Symbiodinium* diversity. In spite of the increasing number of *Symbiodinium* sequences, the number of clades remains stable and the new ones, such as the clade H, are introduced only for the lineages identified before but not considered as distinctive taxa. We predict that in the future, some clades will be further split into numerous subclades but the general structure of the *Symbiodinium* tree as presented here will not change substantially.

4.2. The evolutionary rates

To test whether the phylogeny of *Symbiodinium* can be biased by differences in evolutionary rates, we compared the rates between lineages and between both analysed markers. Our special attention was focused on comparison between symbionts living in foraminifera and those harboured by other hosts. We considered that the high diversity of foraminiferan symbionts, illustrated by numerous clades and subclades specifically associated with only three genera of the subfamily Soritinae, are due to an acceleration of evolutionary rates. However, the RRT tests between all pairs of lineages (Table 2) did not show a consistent acceleration in evolutionary rates for all clades involving foraminiferan symbionts (C, F, G, and H). For example, in the nuclear data set, only clade F showed high evolutionary rates together with clade B, which to date has never been detected in foraminifera. The outcome of the RRT test suggests that among many others alternative factors, the mode of vertical transmission of symbionts across foraminiferan generations by multiple fission (Fujita et al., 2000) or population bottlenecks resulting from ecological and physiological isolation (LaJeunesse, 2005), may explain the specificity as well as the important diversification of foraminiferan symbionts.

A previous study suggested that in *Symbiodinium*, the cp23S evolves 1–7 times faster than the nr28S gene (Santos et al., 2002a). This can be due to the fact that Santos et al. (2002a) included in their clades analyses the highly variable regions of the cp23S. When we excluded these regions to compare both genes at the genus level, we did not observe a particularly high difference of rates between the nuclear and chloroplastic genes. Our results indicate that the nr28S evolves twice as fast as the cp23S

(electronic Appendix C). There is only one lineage, D1 represented by the *Symbiodinium* isolate PSP1-05, which shows a marked deviation of points, and which has an extremely long branch in the chloroplastic phylogenetic tree (Fig. 1B). This isolate, originally extracted from the sponge *Haliclona koremella* (Carlos et al., 1999), displays a peculiar evolutionary rate also in other genes (Takabayashi et al., 2004; Takishita et al., 2003). This acceleration could be related to the ambiguous origin of this strain, which may represent a free-living dinoflagellate (Carlos et al., 1999; Santos et al., 2002a).

4.3. Time scale of *Symbiodinium* evolution

The limitations in using the scleractinian fossil record to accurately calibrate the *Symbiodinium* tree as well as the uncertainties of its single gene phylogenies, impeded determination of the time scale of *Symbiodinium* evolution. Here, we overcame these difficulties by applying a relaxed molecular clock to our nuclear and chloroplastic rDNA phylogenetic trees and by calibrating them with the first fossil appearance of the Soritinae foraminifera about 25 MYA. Our data indicate that the first radiation event within the genus *Symbiodinium* occurred some 50 MYA, i.e., at the beginning of Eocene. This is much later than suggested by Tchernov et al. (2004), who proposed that the ancestor of the *Symbiodinium* species complex appeared at the Cretaceous-Tertiary boundary, about 65 MYA. Although, we cannot exclude that some members of clade A originated already in Palaeocene and could descend from the symbionts that passed through the K–T boundary, our study strongly suggests that the divergence of all other extant *Symbiodinium* lineages started in Eocene only. We can speculate that this divergence was related to the large Eocene radiation of scleractinian corals, during which many modern coral families appeared (Wood, 1999), and that the *Symbiodinium* lineages, which diverged at that time, subsequently replaced other symbionts present in Palaeocene corals.

Interestingly, the juxtaposition of the clocked *Symbiodinium* tree (Fig. 2) with the long-term patterns in Cenozoic global climate (Zachos et al., 2001) reveals that the major diversifications of this genus occurred during global cooling periods. The divergence of *Symbiodinium* clades A, B, D, E, and G took place during the Eocene cooling, after the late Palaeocene thermal maximum, when lower sea level and global coastlines increase promoted regional differences and biodiversity (Bice et al., 2000; Scortese, 1997). The major diversification of extant *Symbiodinium* lineages started during the mid to late Miocene, when the global temperature of oceans decreased and its circulation changed in relation to the closure of Tethys Ocean and beginning of Isthmus of Panama uplift (John et al., 2003). It is remarkable that none of extant symbionts, except those harboured by soritid foraminifera, diverged during the period between

late Oligocene warming and mid-Miocene climatic optimum (25–15 MYA). This conforms to present day observations that periods of global warming negatively affect corals symbioses (Hughes et al., 2003). Although recent studies show that corals can overcome this problem by adapting to more “thermally tolerant” symbionts (Baker et al., 2004; Rowan, 2004), the evolutionary history of *Symbiodinium* suggests that long term increase of water temperature may significantly reduce *Symbiodinium* diversity, constituting a serious threat for the survival and diversity of coral-reef ecosystems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jympev.2005.04.028](https://doi.org/10.1016/j.jympev.2005.04.028).

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