

## Molecular Identification of Algal Endosymbionts in Large Miliolid Foraminifera: 2. Dinoflagellates

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**ABSTRACT.** Large miliolid foraminifera of the subfamily Soritinae bear symbiotic dinoflagellates morphologically similar to the species of the “*Symbiodinium*” complex, commonly found in corals and other marine invertebrates. Soritid foraminifera are abundant in coral reefs and it has been proposed that they share their symbionts with other dinoflagellate-bearing reef dwellers. In order to test this hypothesis, we have analysed partial large subunit ribosomal DNA sequences from dinoflagellate symbionts obtained from 28 foraminiferal specimens, and compared them to the corresponding sequences of *Symbiodinium*-like endosymbionts from various groups of invertebrates. Phylogenetic analysis of our data shows that all soritid symbionts belong to the “*Symbiodinium*” species complex, within which they form seven different molecular types (Fr1–Fr7). Only one of these types (Fr1) branches within a group of invertebrate symbionts, previously described as type C. The remaining six types form sister groups to coral symbionts previously designated as types B, C, and D. Our data indicate a high genetic diversity and specificity of *Symbiodinium*-like symbionts in soritids. Except for type C, we have found no evidence for the transmission of symbionts between foraminifera and other symbiont-bearing invertebrates from the same localities. However, exchanges must have occurred frequently between the different species of Soritinae, as suggested by the lack of host specificity and some biogeographical patterns observed in symbiont distribution. Our data suggest that members of the subfamily Soritinae acquired their symbionts at least three times during their history, each acquisition being followed by a rapid diversification and independent radiation of symbionts within the foraminiferal hosts.

**Key Words.** Evolution, LSU, rRNA, Soritacea, *Symbiodinium*, symbiosis, zooxanthellae.

**S**ORITINAE are miliolid foraminifera common in tropical and subtropical shallow seas, with large porcellaneous discoidal tests, reaching up to 15 mm in diam. (Lehmann 1961). The family comprises three genera (*Sorites*, *Amphisorus*, and *Marginopora*) and about 6 recent species that have been described on the basis of morphological features (Gudmundsson 1994). Soritids are particularly abundant in the Indo-Pacific where they play an important role in biogeochemical mineral cycling (Murray 1991). Symbiotic association seems to have been essential for the successful adaptation of soritids to oligotrophic environments. Photosynthetic activity of symbionts provides their foraminiferal hosts with the energy necessary for survival and growth in oligotrophic environments (Hallock 1999). Several experimental studies demonstrated that algal symbiosis enhances calcification, contributing to the exceptional growth of foraminiferal tests (Lee and McEnery 1983; ter Kuile 1991).

Compared to the other extant families of large miliolid foraminifera, Archaiasinae, Peneroplidae, and Alveolinidae, which are hosts respectively to chlorophytes, rhodophytes, and diatoms (see Pawlowski et al. 2001), Soritinae bear dinoflagellates as symbionts. Morphological and ultrastructural studies of cultured isolates and in situ preparations tentatively identified soritid symbionts as belonging to the genus *Symbiodinium* (Lee and Lawrence 1990; Leutenegger 1977; McEnery and Lee 1981; Müller-Merz and Lee 1976). One study reported the isolation of dinoflagellates belonging to the genus *Amphidinium* from the soritid *Amphisorus hemprichii* (Lee et al. 1997). On the other hand, members of the dinoflagellate genus *Gymnodinium* are symbionts of spinose planktonic foraminifera belonging to the family Globigerinidae (Anderson and Bé 1976; Spero 1987).

The taxonomic identification and classification of symbiotic dinoflagellates have been hindered by the paucity of morphological characters and the limited number of cultured species. At one time, it was believed that there was a single pandemic species of symbiotic dinoflagellates described as *Symbiodinium microadriaticum* (Freudenthal 1962). The simple and almost

featureless morphology of both the motile and vegetative stages of this species gave credence to the pandemic concept. A major advance in our ability to morphologically characterize *Symbiodinium*-like zooxanthellae was the use of TEM and SEM. Loeblich and Sherley (1979) demonstrated that thecal plate patterns on *S. microadriaticum* were so different from those of other dinoflagellates that the species was transferred to a new order. Subsequent taxonomic studies, based on isoenzyme variations (Schoenberg and Trench 1980a, b), chromosome numbers (Blank and Trench 1985), and other features led to the description of several new symbiotic species belonging to seven genera classified in four orders of dinoflagellates (Banaszak, Iglesias-Prieto, and Trench 1993).

Recent molecular genetic studies have provided tools for the identification and classification of symbiotic dinoflagellates, making it possible to study the genetic diversity of natural populations. Particular attention was given to the *Symbiodinium*-like species involved in symbiosis with reef-building corals. Small subunit (SSU) rRNA gene sequences and RFLP patterns were used to assess the genetic diversity of *Symbiodinium*-like zooxanthellae cultured in vitro (Rowan and Powers 1992) or sampled in their natural environment (Rowan and Powers 1991a, b). Both RFLP and sequence data showed the presence of three distinct groups among *Symbiodinium*, called types A, B, and C (Rowan and Powers 1991a, b; 1992). Genetic identification of these types and their phylogenetic relationships with other dinoflagellate genera was demonstrated by subsequent molecular studies based on Large subunit (LSU) rDNA sequences (McNally et al. 1994; Wilcox 1998; Zardoya et al. 1995; reviewed in Rowan 1998). Intraspecific diversity and polymorphism of zooxanthellae in corals led to the identification of symbiont zonation and their possible impact on coral bleaching (Rowan and Knowlton 1995; Rowan et al. 1997).

Until recently, the only molecular data available for dinoflagellate symbionts of Soritinae consisted of three complete SSU rDNA sequences obtained from *Amphisorus hemprichii*, *Marginopora kudakajimaensis* (Lee, Wray, and Lawrence 1995) and *Sorites orbiculus* (Langer and Lipps 1995). Analysis of these sequences confirmed the taxonomic status of foraminiferal zooxanthellae as belonging to the “*Symbiodinium*” complex. However, because SSU rRNA genes evolve at a slow rate, the molecule provides only limited information about phylogenetic

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Table 1. Collection locality and date of collection of foraminiferal hosts, DNA extract number, and EMBL accession number of symbiont sequences.

Host species	Collection site	Date	DNA extract <sup>a</sup>	Accession number
<i>Amphisorus hemprichii</i>	Elat, Israel	Jan'97	378	AJ291531
<i>Amphisorus hemprichii</i>	Maldives	Oct'97	650	AJ291535
<i>Amphisorus hemprichii</i>	Taba, Israel	Apr'99	1338	AJ291533
<i>Amphisorus hemprichii</i>	Elat, Israel	Apr'99	1341	AJ291532
<i>Amphisorus hemprichii</i>	Elat, Israel	Apr'99	1360	AJ291530
<i>Amphisorus hemprichii</i>	Elat, Israel	Apr'99	1366	AJ291514
<i>Amphisorus</i> sp.	Taba, Israel	Apr'99	1275	AJ291523
<i>Amphisorus</i> sp.	Elat, Israel	Apr'99	1333	AJ291524
<i>Amphisorus</i> sp.	Guam, Micronesia	Jul'99	1635	AJ291525
<i>Marginopora vertebralis</i>	Lizard Island	Jul'97	490	AJ291515
<i>Marginopora vertebralis</i>	Guam, Micronesia	Jul'99	1582	AJ291537
<i>Marginopora vertebralis</i>	Guam, Micronesia	Jul'99	1584	AJ291539
<i>Marginopora vertebralis</i>	Guam, Micronesia	Jul'99	1643	AJ291536
<i>Marginopora vertebralis</i>	Guam, Micronesia	Jul'99	1645	AJ291538
<i>Sorites</i> sp.	Safaga, Egypt	Jun'96	207	AJ291534
<i>Sorites</i> sp.	Lizard Island	Jul'97	489	AJ291516
<i>Sorites</i> sp.	Florida Keys	Jul'98	751	AJ291513
<i>Sorites</i> sp.	Florida Keys	Jul'98	836	AJ291527
<i>Sorites</i> sp.	Elat, Israel	Apr'99	1305	AJ291512
<i>Sorites</i> sp.	Elat, Israel	Apr'99	1318	AJ291522
<i>Sorites</i> sp.	Elat, Israel	Apr'99	1334	AJ291521
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1591	AJ291519
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1593	AJ291529
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1631	AJ291528
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1650	AJ291517
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1678	AJ291520
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1679	AJ291526
<i>Sorites</i> sp.	Guam, Micronesia	Jul'99	1690	AJ291518

<sup>a</sup> Foraminiferal DNA Collection identification number (see Materials and Methods).

relationships among foraminiferal symbionts. On the basis of their findings, Lee, Wray, and Lawrence (1995) suggested that the symbionts in corals and soritid foraminifera may have shared a common evolutionary history.

Several investigators have used LSU rRNA gene fragments to examine the phylogeny of free-living (Lenaers et al. 1991; Zardoya et al. 1995) and symbiotic dinoflagellates (Wilcox 1998). According to these studies, variable domains in the LSU rDNA are much more appropriate for studying relationships between dinoflagellate genera and species than is the case for the SSU rDNA. Therefore, in the present study, partial LSU rDNA sequences were used to examine the phylogenetic relationships of dinoflagellate symbionts in Soritinae. Our choice was also determined by the large number of comparable sequences available from the database, including yet unpublished ones (Baker 1999).

#### MATERIALS AND METHODS

**Material.** Soritids used in this study were collected from the Caribbean Sea (Florida), the Red Sea (Safaga, Eilat), western Indian Ocean (Maldives), western Pacific (Guam), and the

Great Barrier Reef (Lizard Island). Detailed data on collection localities, and date, as well as DNA sequence accession numbers in Genbank are given in Table 1.

**DNA extraction, PCR amplification, and sequencing.** Prior to the DNA extraction, the specimens were cleaned with a fine brush in order to remove any debris and associated microorganisms and washed in sterile sea water. DNA of foraminifera and their symbionts was extracted either by using DOC lysis buffer (as described in Holzmann and Pawlowski 1996) or DNeasy Plant Minikit (Qiagen, Basle, Switzerland). All specimens, except very small ones, were broken and only fragments of the tests were taken for extraction. The remaining parts of the tests were preserved for future SEM studies. Each DNA extraction received a Foraminifera DNA Collection identification number that appears in Table 1.

One  $\mu$ l of DNA extract was added to each PCR reaction that was performed in a total volume of 50  $\mu$ l with an amplification profile consisting in 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 120 s at 72 °C, followed by 5 min at 72 °C for final extension. The amplified PCR products were purified using High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), sequenced directly with ABI PRISM Big Dye Terminator Cycle Sequencing Kit, and analysed with an ABI 377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the instructions of the manufacturers.

The amplified fragment of dinoflagellate rDNA includes about 100 nucleotides (nt) of the 3'-end of the SSU rDNA, the whole ITS region (ITS1 + 5.8S + ITS2) and about 900 nt of the 5'-end of the LSU rDNA (Fig. 1). Its total length ranged from 1572–1701 nt. PCR amplifications were performed with the dinoflagellate-specific primer S-DINO (5'-CGCTCCTA-CCGATTGAGTGA) situated at the 3'-end of the SSU rDNA, and the universal primer L0 (5'-GCTATCCTGAG-

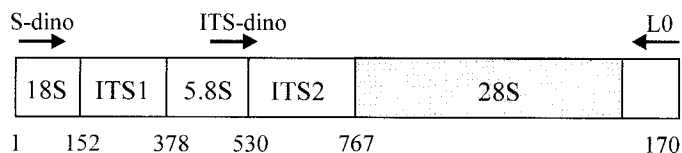


Fig. 1. Diagram of the amplified fragment of ribosomal DNA showing the position of amplification and sequencing primers. The numbers below indicate the beginning nucleotide of each region and the total length of the fragment according to accession number AJ291512. The shaded area corresponds to the region analyzed in this study.

(AG)GAAACTTCG) situated about 800 nt downstream from the 5'-end of the LSU rDNA. An additional PCR primer used for sequencing was ITS-DINO (5'-GTGAATTGCAGAACTCC), situated in the ITS region. Specific dinoflagellates primer S-DINO was designed in order to amplify broad range of dinoflagellates species, however, it cannot be ensured that it recovers all of them. All sequences were obtained by unidirectional sequencing of at least two different PCR products.

**Sequence analysis.** The sequences were aligned by using Clustal X (Thompson, Higgins, and Gibson 1994) and further improved manually by using GDE 2.2 software (Larsen et al. 1993). Two methods were used for sequence analysis: the neighbor-joining (NJ) method (Saitou and Nei 1987) applied to distances corrected for multiple hits and for unequal transition and transversion rates, and using Kimura's two-parameter model (Kimura 1980); and the maximum likelihood (ML) method as implemented in the fast DNAm1 program (Olsen et al. 1994). The reliability of internal branches in the NJ and ML trees was assessed by 1,000 and 100 bootstrap replicates, respectively. The PHYLO-WIN program (Galtier and Gouy 1996) was used for distance computations, tree building, and bootstrapping.

## RESULTS

**Phylogenetic analyses.** Phylogenetic analyses were performed on a fragment of 622 sites located in the LSU rDNA (Fig. 1), for which a large number of sequences of *Symbiodinium*-like dinoflagellates from corals and other hosts were available from GenBank (Baker 1999). Twenty-eight sequences of foraminiferal symbionts have been compared to 32 sequences of *Symbiodinium*-like dinoflagellates representing 6 cultured species and 28 isolates from corals and other marine invertebrates. The sequences of *Gymnodinium simplex* and *Gymnodinium beii*, the latter one isolated from the planktonic foraminifer *Orbulina universa* (Spero 1987), were used as an outgroup following Wilcox (1998). Sequences representing *Symbiodinium* types A, B, and C (Rowan and Powers 1991a) were used to indicate the different genetic groups in our phylogenetic tree.

The sequences of the *Symbiodinium* species complex form 9 distinct types (Fig. 2). Five types are composed exclusively of foraminiferal symbionts and three types contain solely coelenterate symbionts and cultured species of *Symbiodinium*. Only one type includes a mixture of symbionts from foraminiferans and other hosts. Sequence divergence between the clades ranges from 5% to 25%, while divergence within the clades averages 1%. Because of important genetic distances separating the different types, each of them can be considered most probably as a separate species.

The phylogenetic relationships within the *Symbiodinium* species complex are similar in neighbor joining and maximum likelihood (data not shown) analyses. In both analyses, type A branches next to the outgroup in the basal part of the tree, followed by a radiation of the other types. Type E, together with type D (data not shown), appear as a sister group to the type Fr6. Type B clusters with foraminiferal types Fr2+Fr3+Fr4+Fr5 forming a clade which branches as a sister group to the types Fr1 and C. However, the relationships between type B and types Fr2+Fr3+Fr4+Fr5 are not stable. Changing the species selection (data not shown) may result in type B moving to the position basal to clade Fr2+Fr3+Fr4+Fr5 and types Fr1 and C. In ML analysis, type Fr2 changes its phylogenetic position, by branching basal to clades B+Fr2+Fr3+Fr4+Fr5, and to the types Fr1 and C.

## DISCUSSION

**Phylogenetic position of soritid symbionts.** All DNA sequences presented herein belong to the "*Symbiodinium*" species complex. This term describes an assemblage of free-living and symbiotic species of *Symbiodinium*, and some species of *Gymnodinium*, that group together according to their ribosomal DNA sequences (Wilcox 1998). This group is well separated from the rest of the dinoflagellates, including other symbiotic genera, such as *Amphidinium*, *Glenodinium*, and *Scrippsiella* (Gast and Caron 1996; Rowan 1998).

Although our PCR primers are designed to amplify a broad range of dinoflagellate lineages, we have not found evidence for the presence of any other type of symbiotic dinoflagellates in Soritinae. Other endosymbionts, such as *Amphidinium*-like dinoflagellates reported by Lee et al. (1997), are very rarely isolated (JLL., pers. observ.), which renders them difficult to detect by the direct sequencing-based molecular approach used in this study.

Our molecular identification of soritid symbionts as belonging to the "*Symbiodinium*" species complex is in agreement with morphological studies describing them as typical *Symbiodinium*-like zooxanthellae (Leutenegger 1977, 1984; McEnery and Lee 1981; Müller-Merz and Lee 1976). The present findings confirm previous SSU rDNA-based molecular analyses that indicated close relationships between *Symbiodinium* and foraminiferal symbionts (Langer and Lipps 1995) and earlier speculations suggesting that the zooxanthellae of foraminifera and marine invertebrates shared some common evolutionary history (Lee, Wray, and Lawrence 1995).

**Foraminiferan symbiont specificity.** Our study clearly shows that the majority of dinoflagellates living in symbiosis with Soritinae are genetically different from those found in other hosts. Among the six zooxanthella molecular types housed by soritids, five are specific for to soritids. The only exception is type Fr1, which clusters with the coral type C. Even in this case, however, the type Fr1 will form a separate clade if the number of analysed nucleotide sites is increased (data not shown).

Such host-symbiont specificity is unusual among zooxanthellate hosts. Until now, the same types of *Symbiodinium* have been found in various groups of animal hosts, including scleractinian corals, sea anemones, and molluscs (Rowan 1998). A newly discovered ciliate species also bears type C (XP., unpubl. data). To our knowledge, foraminifera appear to be the only group that possesses its own specific *Symbiodinium*-like zooxanthellae. This is particularly surprising given the well-known predisposition of foraminifera to enter endosymbiotic relationships with a wide range of diverse algal types (Lee and Anderson 1991). It has been suggested that foraminifera could easily recruit their symbionts from environmental pools (Lee, Wray, and Lawrence 1995), especially because they must renew their stock of endosymbionts after each sexual reproduction (Lee et al. 1991). Flexibility in the acceptance of different potential foraminiferal endosymbionts was considered to increase the chances of adaptation to a broader range of environmental parameters (Lee et al. 1997).

In view of our data, the exchange of symbionts between foraminifera and other hosts seems to be highly implausible. But such free symbiont exchange probably exists within the Soritinae, as different genera living in the same locality tend to share the same *Symbiodinium* genotypes. This is particularly well demonstrated for type C, which is found in all three genera of Soritinae, and for types Fr2 and Fr5, which are found in two genera.

**Evolutionary origin of symbiosis in Soritinae.** Symbiosis

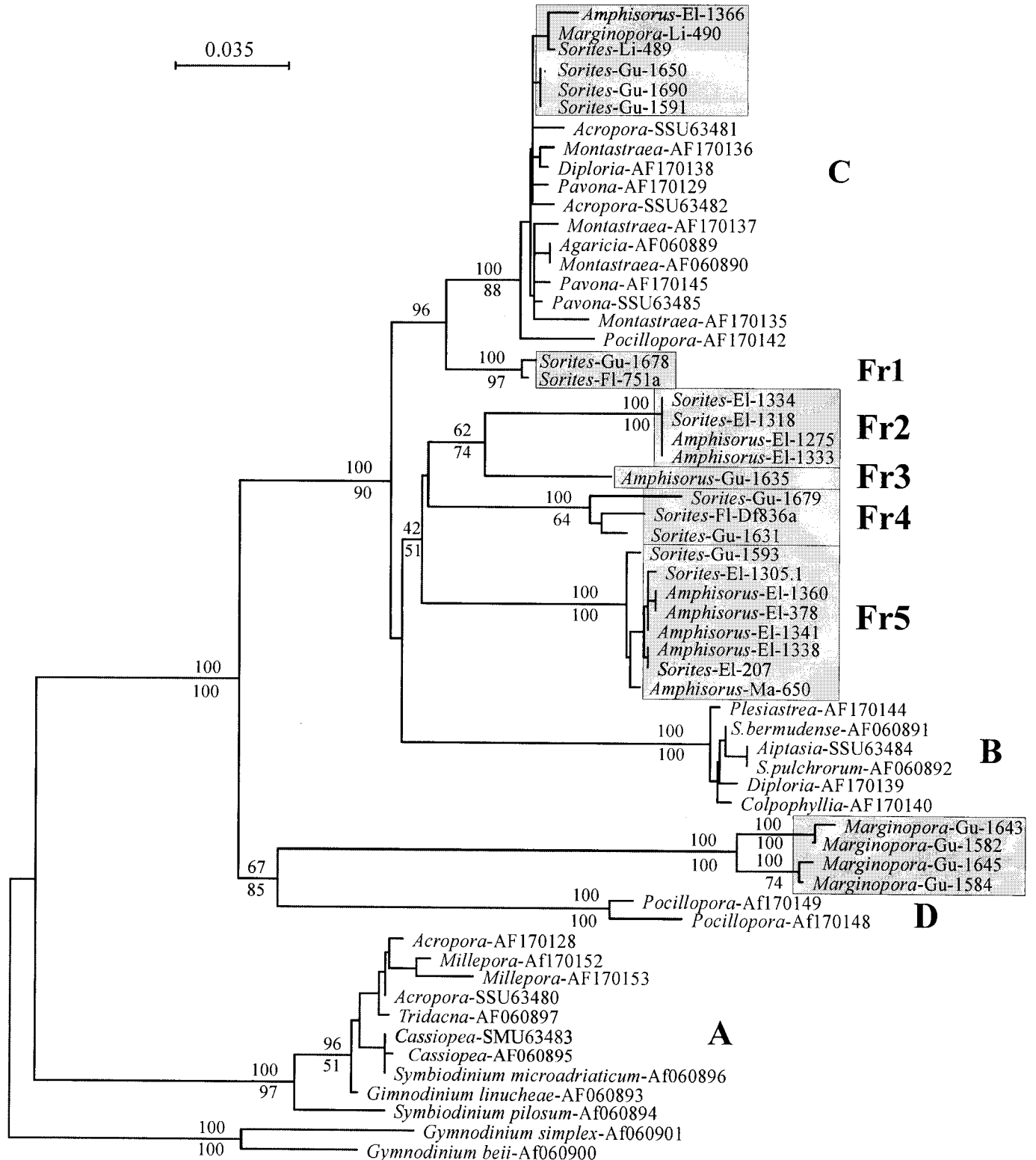


Fig. 2. The LSU rDNA neighbour joining tree of the *Symbiodinium* species complex, including 28 sequences of soritid symbionts. The clades of coral symbionts are designated by the letters A–D; the clades of foraminiferal symbionts (shaded areas) are designated by the terms Fr1–Fr7. The genus name of each foraminiferal host is followed by two letters indicating its geographic origin, and by the DNA extract number (see Table 1 for details). The numbers above and below branches correspond to bootstrap values obtained in NJ and ML analyses, respectively.

as a source of novel metabolic capabilities plays an important macroevolutionary role (Douglas 1994). Isotopic analysis of fossil foraminiferal tests shows that the acquisition of photosymbionts contributed to the radiation of Paleogene planktic foraminifera (Norris 1996). Comparison of fossil and molecular data on the phylogeny of Soritacea suggests that Soritinae evolved from some green-algae bearing Archaiasinae (MH., unpubl. data). The switch from chlorophyte to dinoflagellate symbionts seems to have acted as a trigger for the radiation in Soritinae. The origin of soritid symbionts, however, remains unclear.

Two hypotheses can be proposed to explain the origin of soritid symbionts. The first one would suggest that the earliest Soritinae acquired their symbionts from some coelenterates, such as scleractinian corals, which are assumed to have borne zooxanthellae since the Triassic (Veron 1995). The fact that all molecular types of *Symbiodinium* present in Soritinae are either very closely related to coral symbionts (C) or appear as their sister groups (C/Fr1, B/Fr2–Fr5, D/Fr6) favors this hypothesis. It is difficult to explain, however, why the genetic variability of the symbionts is much higher in soritids than in other hosts. It is puzzling that three genera of Soritinae, evolving some 25 million years ago, bear at least 6 genetically different types of *Symbiodinium*, while in the numerous coelenterate taxa that are known to possess endosymbionts for at least 240 million years, only four molecular types have been detected.

In the second hypothesis, the soritid symbionts would have originated from some free-living zooxanthellae. This is in agreement with the concept that foraminifera can easily acquire symbionts from environmental pools (Lee, Wray, and Lawrence 1995). It also fits better with the biogeographic pattern observed in some localities where more or less all soritids bear the same type of symbionts, as in the case of the types Fr3 and Fr6, present principally in Red Sea and western Indian Ocean samples. Little is known, however, about the diversity of free-living *Symbiodinium* species, and about the geographic distribution patterns of free-living and symbiotic species. Further testing of these hypotheses will demand more extensive geographic sampling of both foraminiferans and corals symbionts.

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