

Extreme Differences in Rates of Molecular Evolution of Foraminifera Revealed by Comparison of Ribosomal DNA Sequences and the Fossil Record

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Foraminifera have one of the best known fossil records among the unicellular eukaryotes. However, the origin and phylogenetic relationships of the extant foraminiferal lineages are poorly understood. To test the current paleontological hypotheses on evolution of foraminifera, we sequenced about 1,000 base pairs from the 3' end of the small subunit rRNA gene (SSU rDNA) in 22 species representing all major taxonomic groups. Phylogenies were derived using neighbor-joining, maximum-parsimony, and maximum-likelihood methods. All analyses confirm the monophyletic origin of foraminifera. Evolutionary relationships within foraminifera inferred from rDNA sequences, however, depend on the method of tree building and on the choice of analyzed sites. In particular, the position of planktonic foraminifera shows important variations. We have shown that these changes result from the extremely high rate of rDNA evolution in this group. By comparing the number of substitutions with the divergence times inferred from the fossil record, we have estimated that the rate of rDNA evolution in planktonic foraminifera is 50 to 100 times faster than in some benthic foraminifera. The use of the maximum-likelihood method and limitation of analyzed sites to the most conserved parts of the SSU rRNA molecule render molecular and paleontological data generally congruent.

Introduction

Foraminifera are among the most abundant and diverse marine protists, characterized by membranous, agglutinated, or calcareous tests (Lee 1990). Known since the Early Cambrian, fossil foraminifera are widely used for stratigraphic analysis of ancient sediments and for paleoecological and paleogeographic reconstructions (Culver 1993). Current interpretation of foraminiferal evolution is therefore largely based on the study of fossil material. According to the stratigraphic record, the appearance of the major groups of foraminifera is a relatively recent geological event. Although the oldest fossils identified as simple agglutinated foraminifera (Astrorhizida) have been found in the Early Cambrian (540 MYA) (Culver 1991), the major explosion of the agglutinated Textulariida and the extinct calcareous Fusulinida occurred in the Devonian (400 MYA). Further radiation of the calcareous foraminifera led to the appearance of the calcareous porcelaneous Miliolida in the Carboniferous (350 MYA) and the calcareous hyaline Rotaliida, in the Early Jurassic (200 MYA) (Tappan and Loeblich 1988). The oldest planktonic foraminifera (Globigerinida) have been reported also from the Early Jurassic (200 MYA) (Görög 1994). The evolutionary history of foraminifera also includes several extinction events that provide additional information concerning the origin of surviving lineages.

Abbreviations: NJ, neighbor joining; MP, maximum parsimony; ML, maximum likelihood.

Key words: foraminifera, molecular phylogenetics, rates of substitution, SSU rDNA.

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Mol. Biol. Evol. 14(5):498–505, 1997

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The distinction of the major groups of fossil and recent foraminifera relies on the composition and structure of their test wall (Loeblich and Tappan 1988). A common paleontological interpretation of foraminiferal macroevolution implies the progressive transformation of the test, from the membranous-walled primitive type, to the agglutinated, and finally to the secreted calcareous wall (Hansen 1979). However, the macroevolutionary relationships in foraminifera are not easy to trace from the fossil material. The emergence of a new test type usually involves such drastic changes that identification of ancestral or intermediate forms becomes difficult. Intermediate forms may also be so small that they remain undetected in routine paleontological studies or their tests may not be preserved in sediments.

The recent development of molecular systematics has brought an important contribution to the study of the phylogenetic relationships between and within different groups of protists (Schlegel 1991). However, application of molecular methods to the study of foraminifera was seriously delayed because pure DNA samples proved difficult to obtain, and commonly used "universal" primers failed to amplify the foraminiferal DNA (Pawlowski et al. 1994). Fortunately, these problems have now been resolved. Using the specific foraminiferal primers we have obtained the sequences of the small subunit (SSU) and large subunit (LSU) ribosomal RNA genes from several species. Phylogenetic analysis of some of these sequences, compared to those of other unicellular eukaryotes, led us to place the foraminifera in the middle part of the eukaryotic tree, close to the euglenoids, slime molds, and some amoebas (see Pawlowski et al. 1994, 1996), rather than within the clade of alveolates as was recently proposed (Wray et al. 1995). The purpose of this study was to infer a foraminiferal phylogeny based on SSU rDNA sequences and

to compare the molecular data with paleontological interpretations of the foraminiferal evolution.

Materials and Methods

Samples

The foraminiferal species included in this study were collected in the following localities: Camargue, France (*Ammonia* sp. 1, *Ammonia* sp. 2, *Quinqueloculina* sp.); St. Cyr, France (*Elphidium acuelatum*, *Trochammina* sp., *Bolivina* sp., *Massilina secans*); Golfe du Morbihan, France (*Haynesina germanica*); Antalya, Turkey (*Allogromia* sp. A); Discovery Bay, Jamaica (*Allogromia* sp. J); McMurdo Sound, Antarctica (*Astrammina rara*, *Astrorhiza triangularis*); Lake Hamana, Japan (*Trochammina hadai*); Cape Omaezaki, Japan (*Glabratella opercularis*); Gulf of Elat, Israel (*Peneroplis pertusus*, *Textularia* sp.); Isla Magueyes, Puerto Rico (*Globigerinella siphonifera*, *Orbulina universa*, *Globigerinoides conglobatus*, *Globigerinoides ruber*, *Bigenerina* sp., *Archaias angulatus*).

DNA Amplification and Sequencing

DNA extractions and PCR amplifications were performed as described elsewhere (Holzmann and Pawlowski 1996). A fragment of about 1,000 bp, localized at the 3' end of the SSU rDNA, was amplified using the specific foraminiferal primer 5'-AAGGGCACCACAA-GAACGC (Pawlowski et al. 1996) and universal primer B 5'-TGATCCTTCTGCAGGTTTCACCTAC (Sogin 1990). The amplified PCR product was purified using Spin-Bind DNA extraction units (FMC), then ligated into the pGEM-T Vector System (Promega), cloned in Supercompetent XL2-Blue cells (Stratagene), and sequenced with the *fmol* DNA Sequencing System (Promega), all according to the instructions of the manufacturers. The list of primers used for sequencing and the sequence alignment are available from pawlow@sc2a.unige.ch. The sequences reported in this paper have been deposited in the EMBL database (accession nos. Z69599–Z69618, X86093, X86095).

Sequence Analysis

All sequences were manually aligned using the Genetic Data Environment software, version 2.2 (Larsen et al. 1993). The resulting alignment was modified in reference to the universal SSU rRNA secondary structure model (Van de Peer et al. 1996). Evolutionary trees were built using: (1) the neighbor-joining (NJ) method (Saitou and Nei 1987) applied to distances corrected for multiple hits, and for unequal transition and transversion rates using Kimura's two-parameter model (Kimura 1980); (2) the maximum-parsimony (MP) method, using the heuristic search option included in PAUP 3.1.1. (Swofford 1993); and (3) the maximum-likelihood (ML) method as implemented in the fastDNAMl program (Olson et al. 1994). All analyses were based on 401 unambiguously aligned sites, including all conserved regions, excepting helices 41 and 45 (as numbered in Van de Peer et al. 1996), for which a reliable alignment was not possible. Furthermore, all gaps in individual sequences were excluded. The reliability of internal

branches in the NJ, MP, and ML trees was assessed using the bootstrap method (Felsenstein 1988) with 1,000, 500, and 100 replicates, respectively. The phylo_win program (Galtier and Gouy 1996) was used for distance computations, inference of NJ and ML trees, and bootstrapping. Phylogenetic trees were plotted using the njplot program (Perrière and Gouy 1996).

Results and Discussion

Sequence Data

Partial SSU rDNA sequences from 22 species of foraminifera representing all major taxonomic groups, including Allogromiida, Astrorhizida, Textulariida, Miliolida, Rotaliida, and Globigerinida, were obtained. The sequences correspond to the 3' region of the small subunit rDNA of *Mus musculus* (X00686) starting at position 1191 and ending at position 1854. The length of the sequences ranges from 798 to 1,460 bp, that is, up to twice as long as in most eukaryotes. This unusual length is due to the presence of several insertions; the longest one, found in *Astrammina rara*, reaches up to 260 bp. The G+C content varies depending on the group, from about 30% in Miliolida and Astrorhizida to 37% in Allogromiida and 40%–48% in other foraminifera. It ranges from 46.0% to 51.6% in the 401 sites retained for phylogenetic analysis. Among these sites, 322 were constant, 79 were variable, and 66 were parsimony-informative. The observed transitions/transversions ratio averages 3.5.

Heterogeneity of Substitution Rates in Foraminiferal rDNA

The alignment of foraminiferal sequences shows a large sequence divergence within planktonic species and between planktonic and benthic lineages. In order to determine whether the planktonic foraminiferal sequences could be pseudogenes, we have verified, for each species, that the secondary structure of the amplified fragment conforms to the standard model (Van de Peer et al. 1996). We have also cloned and sequenced, for each planktonic species, several amplification products, always finding the same sequence. This rules out the possibility that the sequence heterogeneities observed in planktonic foraminifera could be due to intraspecific variations.

In order to evaluate the rates of molecular evolution in different foraminiferal lineages, we have compared the number of substitutions with the approximate divergence times established according to the fossil record (table 1). For each group of foraminifera, excepting the Allogromiida, Astrorhizida, and Rotaliida, we have chosen the most closely related species or genera, with relatively well known phyletic relationships. The Allogromiida and Astrorhizida were excluded because no fossil data are available. Within the Rotaliida, we could only estimate an inferior bound to the divergence time between *Bolivina* and *Glabratella*.

Our data reveal that the different foraminiferal lineages possess extremely variable rates of substitution. The rate of rDNA evolution of planktonic Globigerinida

Table 1
Divergence Times and Rates of Evolution in Different Lineages of Foraminifera

	Divergence Time (MYA) ^a	Number of Substitutions ^b	Substitutions Per Site ^c	Rate of Substitution (per site per year) ^d
Globigerinida				
<i>G. conglobatus</i> – <i>G. ruber</i>	5–10	52	0.048	3.2×10^{-9}
<i>Orbulina</i> – <i>Globigerinoides</i> ^c	17–25	89	0.084	2.0×10^{-9}
Miliolida				
<i>Archaias</i> – <i>Peneroplis</i>	45–55	4	0.007	0.07×10^{-9}
Textulariida				
<i>Bigenerina</i> – <i>Textularia</i>	55–65	1	0.002	0.02×10^{-9}
Rotaliida				
<i>Ammonia</i> sp. 1– <i>Ammonia</i> sp. 2.	15–25	11	0.02	0.5×10^{-9}
<i>Bolivina</i> – <i>Glabratella</i>	>90	11	0.02	0.1×10^{-9}

^a Divergence times determined from the fossil record are given according to following references: Globigerinida (Cordey 1967; Bolli and Saunders 1985), Miliolida (Colom 1971), Textulariida (Loeblich and Tappan 1988), *Ammonia* spp. (Rupp 1986), *Bolivina*–*Glabratella* (Loeblich and Tappan 1988). The ages of *Bigenerina*–*Textularia* and *Bolivina*–*Glabratella* divergence may be overestimated.

^b Observed number of differences between two sequences out of 591 sites reliably aligned between all foraminifera. A larger number of sites was used here than for tree building in order to avoid very small numbers of differences.

^c Number of substitutions per site after correction for multiple hits according to Kimura's two-parameter model.

^d Times from the first fossil appearance of compared genera or species were added to obtain the divergence time ($2T = T_1 + T_2$). The rate of substitution (r), was calculated as $r = K/(2T)$, where K is the number of nucleotide substitutions per site (Li and Graur 1991).

^e *Globigerinoides ruber*.

appears to be 50 to 100 times faster than that in some other foraminiferal lineages. Within the benthic foraminifera, the evolutionary rates vary by a factor of 30; the highest values are found in the *Ammonia*–*Elphidium* group, and the lowest in the agglutinated Textulariida. The estimated rate of substitution of the Miliolida (7×10^{-11} per site per year) is close to the estimated rates of vertebrate rRNAs (Jaeger, Tong, and Denys 1986; Hedges, Moberg, and Maxson 1990). The rDNA of planktonic foraminifera, however, evolves more than 10-fold faster than the highest rate (20×10^{-11}) proposed for 18S rRNA evolution in plants (Ochman and Wilson 1987).

This is the first report of such extreme differences in the rate of rDNA evolution within a group of organisms. It is well known that the evolution of rRNA genes is accelerated in some fast-clock species (Philippe, Chenuil, and Adoutte 1994). In these cases, however, the rate differences between lineages are usually small. Within the echinoids, the comparison between 28S rRNA sequences and the fossil record shows that the evolutionary rates of different lineages vary by a factor of three (Smith, Lafay, and Christen 1992). Similar variations have been reported from sequence analysis of the β -globin gene among hominoids (Koop et al. 1989). Exceptionally high variation (up to 138% for nonsynonymous substitutions) was observed in the evolutionary rate of *rbcL* gene sequences among seed plants (Bousquet et al. 1992).

Differences in the DNA substitution rates have been best analyzed among vertebrates, where two main possible causes have been put forward: the generation time effect hypothesis (Li et al. 1996) and the metabolic rate hypothesis (Martin and Palumbi 1993). The validity

of these hypotheses for foraminifera can hardly be tested because of the lack of detailed knowledge of their biology. We can only speculate about factors that could be responsible for the acceleration of evolutionary rates in planktonic foraminifera. Some Globigerinida, particularly those whose reproduction follows a lunar or semilunar periodic cycle, seem to reproduce more frequently than do larger benthic foraminifera (Hemleben, Spindler, and Anderson 1989). They probably produce many more swimmers than the benthic species. It is doubtful, however, whether these differences of generation time and productivity are sufficiently large to explain the measured 100-fold acceleration of evolutionary rates. Another factor which might contribute to the higher mutation rate of planktonic foraminifera is their increased exposure to solar UV radiations compared to benthic species. This exposure may not be higher than for some tropical shallow-water foraminifera, but the thick calcareous tests of the latter certainly provide a much better protection against UV radiation than do the delicate planktonic tests. According to Smith, Lafay, and Christen (1992), shallow-water echinoids exposed to UV radiation evolve three times faster than infaunal or deep-sea species.

Finally, we cannot exclude that the increase of mutation rates in planktonic foraminifera could result from some drastic changes in the DNA replication or repair mechanisms in these organisms. Changes in repair mechanisms are considered to be the most likely source of the differences in mutation rates that occurred during primate evolution (Britten 1986). The planktonic foraminifera have experienced strong environmental stresses, as shown by the extinction record and by the strict dependence of their taxonomic rates of evolution on the

paleotemperature curve (Berggren 1969). Whatever the mechanisms, these environmental constraints may be correlated with an exceptionally high rate of rDNA variability.

Phylogeny of Foraminifera

The phylogenetic trees inferred using NJ, MP, and ML methods indicate that foraminifera form a monophyletic group that consists of five major clades. Four clades correspond to the morphotaxonomic orders Miliolida, Astrorhizida, Allogromiida, and Globigerinida; the fifth clade includes the representatives of two orders, namely the Rotaliida and Textulariida. The monophyly of Miliolida, Allogromiida and Astrorhizida is supported in most trees by high bootstrap values; however, each of two latter orders is represented by only two closely related species. The relationships between the Rotaliida, Textulariida, and Globigerinida are weakly supported; only the Globigerinida and the *Ammonia-Elphidium* group, classically belonging to the order Rotaliida, are well-defined groups supported respectively by 69/67/60 and 94/73/82 percent bootstrap values in NJ/ML/MP analyses.

The higher-level relationships inferred from our sequence data vary depending on the method of analysis, the choice of outgroup and the length of analyzed sequences. According to the ML method (fig. 1A), the Miliolida branch first, followed by the Allogromiida and the Astrorhizida. The Textulariida, the Rotaliida, including the *Ammonia-Elphidium* group, and the Globigerinida branch within the crown of the tree. The root of the ML tree is located either on the Miliolida or the Globigerinida, depending on the choice of outgroup sequence. An ML analysis without the Globigerinida consistently locates the root of the foraminiferal radiation between Miliolida and other taxa, whatever the outgroup. When regions that can be aligned only between foraminifera are included, the Globigerinida and the *Ammonia-Elphidium* group (all characterized by very long peripheral branches) tend to be located in the lower parts of the trees.

The phylogenetic tree obtained with the NJ method differs from the ML tree in placing the Globigerinida either next to the Miliolida, with the *Naegleria* sequence as outgroup (fig. 1B), or as the earliest branch of foraminifera depending on the choice of outgroup sequences. The location of the *Ammonia-Elphidium* group in NJ analyses varies with the choice of eukaryotic outgroup sequences between late- and early-emerging positions. Use of transversions-only evolutionary distances does not significantly change the position of Globigerinida and the *Ammonia-Elphidium* group in NJ trees. The topology of the MP tree, using *Naegleria* as an outgroup, is similar to that of the ML tree (data not shown). Other outgroup sequences, however, locate the root of the foraminiferal radiation between Globigerinida, Miliolida, and other taxa as in the branching order given by the NJ method. Moreover, the MP analysis consistently places Allogromiida and Astrorhizida in one clade, supported, however, by only 52% bootstrap value.

We think that the position of Globigerinida near the base of foraminiferal tree should be considered as an artifact provoked by the unusually high rates of rDNA evolution in this group. It is well known that fast-evolving sequences tend to group together at the base of the phylogenetic trees (Olsen 1987). The ML and MP methods, applied to our data, seem to be less affected by the differences in evolutionary rates than the NJ method (Kuhner and Felsenstein 1994). We favor the ML method with our strict set of sites, because it generates the same internal topology for the foraminifera in rooted as well as in unrooted trees. When other methods are used, or additional sites are included, the outgroup sequence attracts the long peripheral branches of the Globigerinida.

The most convincing evidence that the position of Globigerinida at the base of some of our trees is artificial comes perhaps from the micropaleontological data. In fact, the first fossil Globigerinida appeared only in the Jurassic, that is, much later than the earliest benthic foraminifera. It is rather unlikely that Globigerinida originated independently from some unknown planktonic or benthic protists that did not leave any fossil remains. Therefore, we conclude that the tree which most probably reflects best the evolutionary history of foraminifera is the ML tree rooted on Miliolida (fig. 1A). The topology of this tree is generally congruent with the common micropaleontological interpretation of foraminiferal evolution (fig. 2). The analysis of evolutionary relationships within foraminifera based on the present molecular data leads to following conclusions:

1. *Miliolida diverged early in the evolution of foraminifera.* In view of molecular data, the divergence of Miliolida occurred much earlier than the divergence of the earliest agglutinated lineage (Astrorhizida), that is, before 540 MYA. This contrasts with the paleontological record, which shows that the earliest Miliolida diverged from some agglutinated Ammodiscida or calcareous Spiriliniida about 350 MYA (Haynes 1981, p. 156). Our data support the idea that Miliolida, together with Spiriliniida and Lagenida, diverged from some primitive Fusulinida that originated very early in the evolution of foraminifera (Tappan and Loeblich 1988). The sequences of Spiriliniida and Lagenida, however, are not available at the moment. On the other hand, it cannot be excluded that Miliolida diverged directly from some naked, membranous-walled ancestral foraminifera; indeed, some modern naked foraminifera can develop one or more chambers similar to those of some simple miliolids (Arnold 1978). The early divergence of Miliolida agrees also with the different molecular weight of miliolid actin compared to that of other foraminifera (Fahrni and Pawlowski 1994).
2. *Astrorhizida branch separately from the other agglutinated foraminifera.* Molecular data confirm the micropaleontological view that the Astrorhizida form an ancestral group to all other agglutinated and calcareous perforate foraminifera. The relationships between the Astrorhizida and the other agglutinated

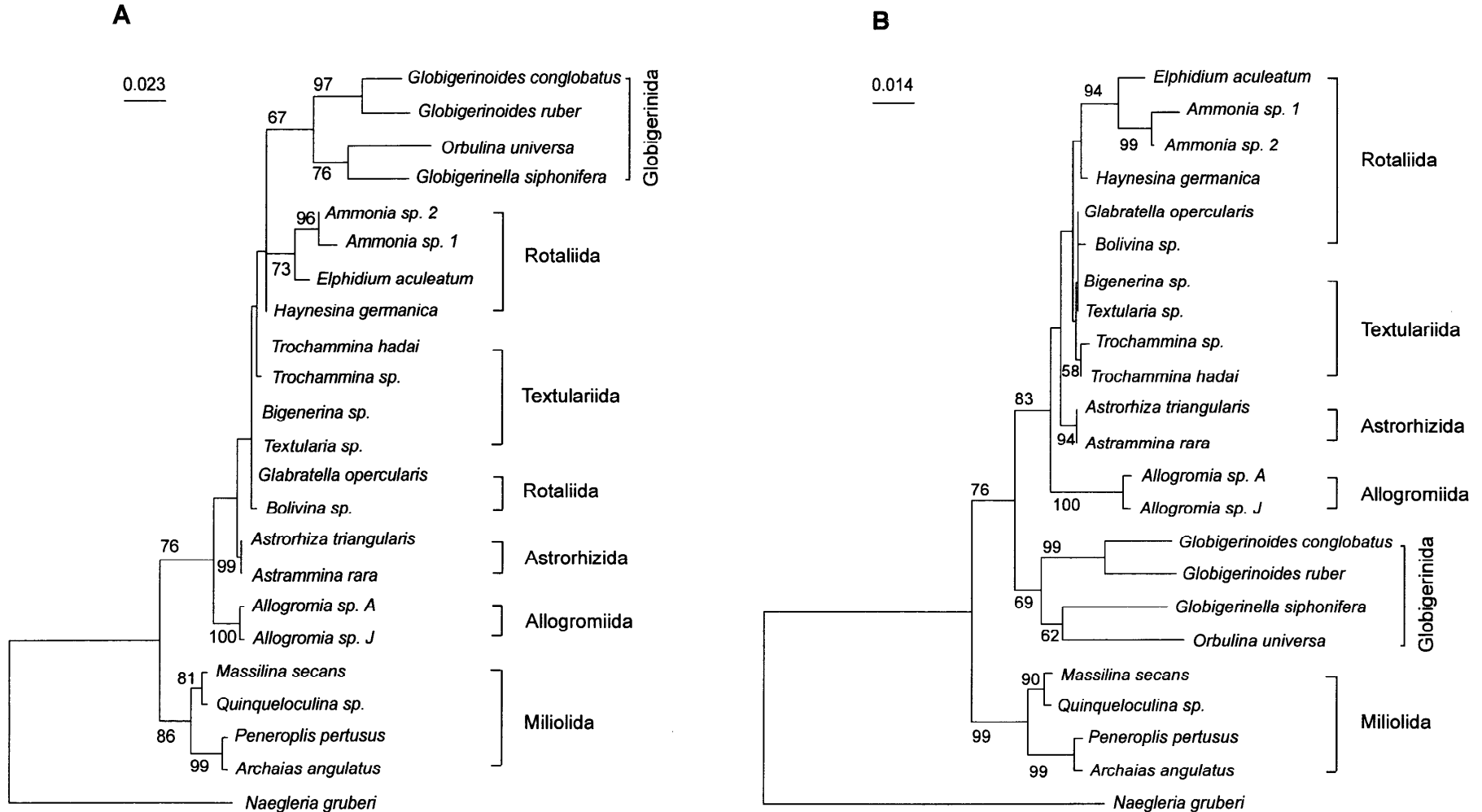


FIG. 1.—Evolutionary relationships between 22 foraminifera inferred from partial SSU rDNA sequences by (A) maximum-likelihood and (B) neighbor-joining methods. The trees are rooted using *Naegleria gruberi* as a designated outgroup. Unreliably aligned regions and all gap-containing sites were excluded, yielding 401 aligned sites. Bootstrap percentage values greater than 50% out of 100 (ML) and 1,000 (NJ) replicates are given next to each internal branch.

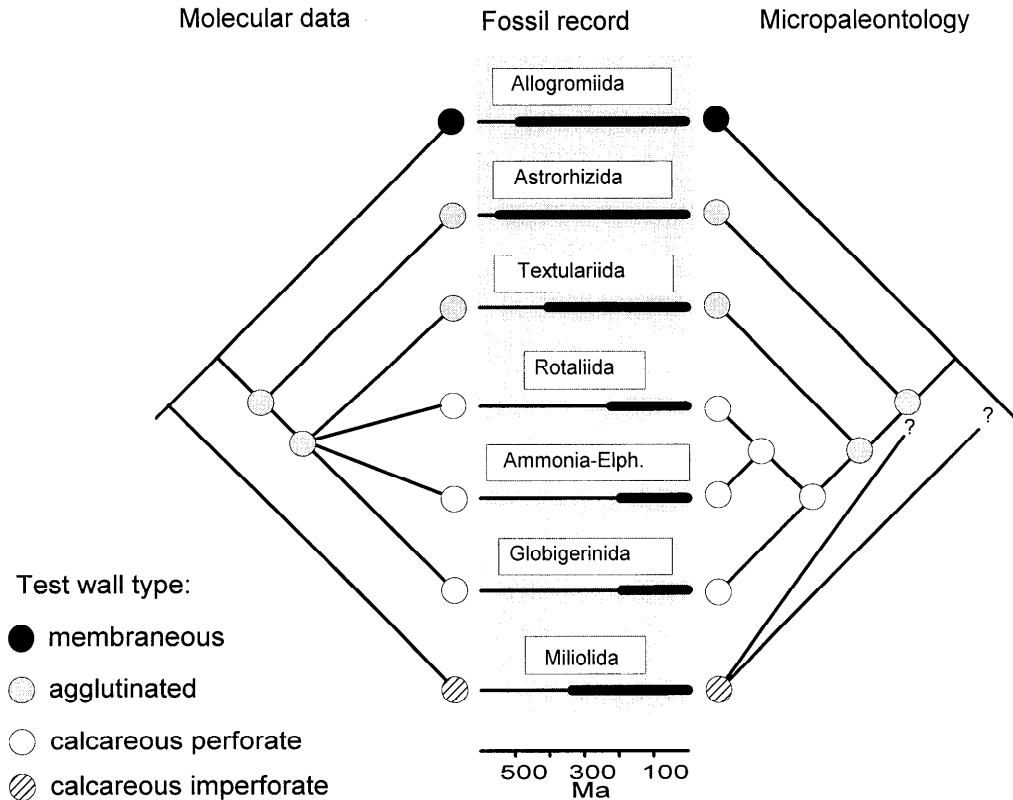


FIG. 2.—Schematic presentation of the fossil record extension (Ma = million years) of the major groups of foraminifera (Tappan and Loeblich 1988) and their relationships inferred from the molecular and micropaleontological data. The hypothetical test wall types for ancestors are indicated.

Textulariida are doubtful (Loeblich and Tappan 1989). As pointed out by some authors, the separation of the Astrorhizida from the membraneous Allogromiida may be purely artificial (Lipps 1973). In fact, in MP trees both groups are consistently placed together. Close relationships between *Astrammmina* and *Allogromia* are also supported by recent observations on their biology and ultrastructure (Bowser et al. 1995).

3. *Textulariida* and *Rotaliida* are very close relatives. In view of our data, the radiation of Textulariida and Rotaliida occurred in a relatively short time. This is in marked disagreement with the geological record, which shows that Textulariida originated about 450 MYA, while Rotaliida appeared only about 220 MYA. The Rotaliida are supposed to have derived from the aragonitic Robertiniida or the calcitic Spirilliniida (Haynes 1981, p. 238). Our data suggest rather that some lineages of Rotaliida may have derived directly from an agglutinated ancestor. This would imply an easy transformation between different types of wall structure, which is in contrast with the classical view of foraminiferal phylogeny. On the other hand, we cannot exclude that the close relationships between Textulariida and Rotaliida may result only from the slow rate of rDNA evolution in both groups. By including more sites in the analysis, we can separate both groups, but the statistical support for this separation is very weak.

This study is the first attempt to use DNA sequence data to revise the current interpretation of foraminiferal evolution. Previous chemotaxonomical studies of foraminifera were limited to the amino acid composition of fossil specimens (Robbins and Healy-Williams 1991). Foraminiferal rDNA sequences reported by other authors were used to discuss their phylogenetic position among unicellular eukaryotes (Merle et al. 1994; Darling et al. 1996). By resolving the technical problems that hindered the amplification of foraminiferal DNA, we have been able to initiate and develop the molecular systematics of foraminifera. Still, several questions concerning the phylogeny of foraminifera remain unresolved. One of the most important is perhaps the problem of the origin and phylogeny of planktonic foraminifera. To address this problem, we have obtained the SSU rDNA sequences of 15 planktonic species, which are now under analysis in our laboratory. From the data presented in this paper, however, it seems clear that the phylogenetic position of planktonic foraminifera will not be established without examining the sequences of genes with more uniform rates of substitution between the different foraminiferal lineages. Further molecular studies are also necessary to reexamine the importance of wall structure and composition in the classification of foraminifera.

Acknowledgments

We thank S. S. Bowser, J.-P. Debenay, C. Hemleben, H. Hilbrecht, R. Wernli, and J. Whittaker for help.

ful comments on an earlier version of this manuscript; J.-P. Debenay, K. Grell, H. Kitazato, A. Drod, and J. J. Lee for help in collecting some benthic species; and K. Darling for correcting taxonomic identification. Planktonic foraminifera collection was supported by a research grant from ETH Zürich (H. Hilbrecht). Specimens of Antarctic foraminifera were obtained through grants from the National Science Foundation, Office of Polar Programs (8917357 and 9220146), awarded to S. S. Bowser. This work was supported by Swiss National Science Foundation grant 31-39632.93 (J.P.)

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- DAN GRAUR, reviewing editor
- Accepted January 28, 1997