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Phylogeny and Rates of Molecular Evolution of Planktonic Foraminifera: SSU rDNA Sequences Compared to the Fossil Record

Colomban de Vargas,[1] Louisette Zaninetti,[1] Heinz Hilbrecht,[2] Jan Pawlowski[1]

[1] Département de Zoologie et Biologie Animale, Université de Genève, CH-1224 Chêne-Bougeries, Switzerland
[2] Geological Institute, Eidgenössische Technische Hochschule Zürich, Sonneggstrasse 5, CH-8092 Zürich, Switzerland

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Abstract. Planktonic foraminifera are marine protists, whose calcareous shells form oceanic sediments and are widely used for stratigraphic and paleoenvironmental analyses. The fossil record of planktonic foraminifera is compared here to their molecular phylogeny inferred from ribosomal DNA sequences. Eighteen partial SSU rDNA sequences from species representing all modern planktonic families (Globigerinidae, Hastigerinidae, Globorotaliidae, Candeinidae) were obtained and compared to seven sequences representing the major groups of benthic foraminifera. The phylogenetic analyses indicate a polyphyletic origin for the planktonic foraminifera. The Candeinidae, the Globorotaliidae, and the clade Globigerinidae + Hastigerinidae seem to have originated independently, at different epochs in the evolution of foraminifera. Inference of their relationships, however, is limited by substitution rates of heterogeneity. Rates of SSU rDNA evolution vary from 4.0×10^{-9} substitutions/site/year in the Globigerinidae to less than 1.0×10^{-9} substitutions/site/year in the Globorotaliidae. These variations may be related to different levels of adaptation to the planktonic mode of life. A clock-like evolution is observed among the Globigerinidae, for which molecular and paleontological data are congruent. Phylogeny of the Globorotaliidae is clearly biased by rapid rates of substitution in two species (*G. truncatulinoides* and *G. menardii*). Our study reveals differences in absolute rates of evolution at all taxonomic levels in planktonic foraminifera and demonstrates their effect on phylogenetic reconstructions.

Key words: Planktonic foraminifera — Molecular phylogenetics — Rates of substitution — Ribosomal DNA

Introduction

Planktonic foraminifera (Globigerinida) are an important group of protists ubiquitous among the marine zooplankton. Their calcareous, perforate tests (shells) accumulate on the ocean floor and create one of the most widespread marine sediment types, the ‘‘Globigerina ooze.’’ Fossil globigerinids are widely used in micropaleontology for stratigraphic analysis of ancient sediments and for paleoecological and paleogeographic reconstructions (Berggren et al. 1995). The evolution and phylogenetic framework of the Neogene globigerinids (Miocene-Recent, 24 Mya) is much better known than for any other group of fossils in this epoch (Kennett and Srinivasan 1983).

In paleontology, planktonic foraminifera are discriminated from benthic ones by larger perforations of the test and, to some minor extent, by the presence of more or less globular chambers. The classification of planktonic foraminifera is exclusively based on morphologic characters of the test (Loeblich and Tappan 1988). Most of these characters present a high level of plasticity and are often subject to iterative evolution in different lineages

Correspondence to: C. de Vargas

(Kennett and Srinivasan 1983). Scanning electron microscopy (SEM) studies have shown that ultrastructure of the wall is the only conservative character in a given lineage of globigerinids.

Although detailed data are available on stratigraphic ranges of the various planktonic foraminiferal lineages, their origin and phylogenetic relationships are uncertain. Little is known about the origin of first ''globigerinids,'' which appeared in the Middle Jurassic about 180 million years ago (J.E. Whittaker, personal communication). They may have originated from small benthic foraminifera, the Oberhauserellidae (Tappan and Loeblich 1988). Sporadic blooms of small globular forms observed in the fossil record during the Middle and Late Jurassic may indicate repeated phases of adaptation to the planktonic (neritoplanktonic) mode of life (Wernli 1988). It is unknown how many transitions from the benthic to the planktonic mode of life occurred in the history of foraminifera.

The evolution of planktonic foraminifera is characterized by alternating periods of radiations and extinctions (Banner and Lowry 1985). Planktonic foraminifera, unlike benthics, are considered as extremely sensitive to oceanic changes (Wei and Kennett 1986; Malmgren and Berggren 1987). Several times during their history, large-scale environmental changes in pelagic habitat have provoked the extinction of the specialized carinated, flattened forms allowing the survival of only some minute globular species. The major break occurred at the Cretaceous-Tertiary (K/T) boundary, when globigerinids experienced the highest and most abrupt rate of extinction among all marine fossil groups (Brinkhuis and Zachariasse 1988). The second important extinction occurred at the Eocene/Oligocene boundary (34 Mya ago), due to a drastic change in sea level and a drop in water temperature (Bolli 1986).

Recent planktonic foraminifera include more than 40 species that are classified in 15 genera (Kennett and Srinivasan 1983; Saito et al. 1981; Hemleben et al. 1989). Based on wall ultrastructure and stratigraphic occurrences, three main groups can be distinguished: the spinose Globigerinidae and Hastigerinidae (20 species), characterized by large perforations and a honeycomb-textured wall, the nonspinose Globorotaliidae (14 species), having more or less flattened tests (sometimes carinate) and smooth walls, and the nonspinose Candeinidae (six to seven species), whose tiny tests have smooth microperforate wall. Within each of these groups the evolutionary relationships between lineages leading to the living species are relatively well established. The origin of these groups, however, remains unclear. One theory is that all Cenozoic globigerinids derived from two Cretaceous genera (*Guembelitria* and *Hedbergella*) that survived K/T extinction (Olsson et al. 1992; Culver 1993), and that living species form a monophyletic group (Kennett and Srinivasan 1983).

Here, we propose to test different hypotheses on phylogenetic relationships between and within the major groups of recent planktonic foraminifera using 18 partial SSU rDNA sequences. This is the first attempt to use molecular data to establish the phylogeny of globigerinids. Few chemo-taxonomical studies of planktonic foraminifera were limited to the amino acid composition of fossil specimens (Robbins and Healy-Williams 1991). The first rDNA sequences of planktonic foraminifera have been reported by Merle et al. (1994), Darling et al. (1996), and Wade et al. (1996). The origin and phylogeny of benthic foraminifera inferred from LSU and SSU rDNA sequences were established recently (Pawlowski et al. 1994, 1997).

Although rRNA genes are commonly used in phylogenetic reconstructions, very little is known about rDNA rates of evolution compared to the large number of works on evolutionary rates in protein coding genes (Doolittle et al. 1996). In this study, the exceptional knowledge of divergence times between fossil lineages leading to recent planktonic foraminiferal species, allowed us to report absolute SSU rDNA rates of evolution and evaluate their influence on phylogenetic reconstructions.

Materials and Methods

Samples. The planktonic foraminiferal species were collected in the following localities: Caribbean, Isla Magueyes, southwestern Puerto Rico (*Globigerinella siphonifera, Orbulina universa, Globigerinoides conglobatus, Globigerinoides ruber, Globigerinoides sacculifer, Hastigerina pelagica, Globorotalia menardii*), Mediterranean Sea, Villefranche sur Mer, France (*Globigerina bulloides, Globigerinella calida, O. universa, G. sacculifer, Globorotalia truncatulinoides, Globorotalia inflata*), and the Sargasso Sea, Bermuda (*Globigerinita glutinata, Neogloboquadrina dutertrei, Globorotalia hirsuta, G. truncatulinoides, G. ruber*). The specimens were collected with plankton nets (64–500 μm mesh size) from about 50 oblique plankton tows between 100 m depth and the sea surface. The net remained open during all immersion time and was sampled for 15–30 min.

DNA Extraction. Plankton samples were distributed in glass dishes and examined with a dissecting microscope. The foraminifera were rapidly isolated and transferred to other receptacles containing filtered sea water. Foraminiferal specimens were individually cleaned by brushing to eliminate the spines, detritus, and microorganisms at their surface. Some specimens of each species were stored on micropaleontological slides for SEM examination. DNA extractions were performed immediately after collecting to avoid damage of the fragile specimens and artifacts due to degradation of cell material. The specimens were individually (or in small groups) ground in 50 μl of the extraction buffer containing 100 mM of TRIS (pH = 8.5), 4 mM of EDTA, 1% of Na-deoxycholate, and 0.2% of Triton x-100, then incubated for 1 h at 60°C, and, finally, insoluble material was removed by centrifugation. At least 10 DNA extractions were achieved for each species.

DNA Amplification and Sequencing. A DNA fragment localized at the 3′ end of the SSU rRNA gene, of about 1100 base pairs (bp), was

amplified by PCR. For each species, at least three PCR amplifications were accomplished using different DNA extracts. To circumvent the problem of contamination by endosymbiotic genomes, we used foraminiferal specific primers S14p (5′AAGGGCACCACAAG(AC-)GCG) or S14f1 (5′AAGGGCACCACAAGAACGC) coupled with a universal primer SB (5′GTAGGTGAACCTGCAGAAGGATCA) (Sogin 1990). The amplified PCR products were then purified using Spin-Bind DNA extraction units (FMC), 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. For each species, two additional amplifications were directly sequenced in order to corroborate the cloned sequence and examine the intraspecific variations of rDNA sequences. The sequences reported in this article are deposited in the EMBL data base (accession numbers Z83957–Z83974).

Sequence Analysis. The 18 planktonic foraminiferal sequences were manually aligned, using the Genetic Data Environment 2.2 software (Larsen et al. 1993). Seven sequences of benthic foraminifera (*Bolivina* sp., *Glabratella opercularis, Trochammina hadai, Textularia* sp., *Bigenerina* sp., *Astrorhiza triangularis*, and *Allogromia* sp.—accession numbers Z69607, Z69609–Z69613, Z69616; Pawlowski et al. 1997) were added to the alignment. The resulting alignment was then modified in reference to the universal SSU rRNA secondary structure model (Van de Peer et al. 1996) and according to the frequent compensated mutations found in the conserved regions. We used the following methods and software to build the evolutionary trees: 1) the maximum likelihood method (ML), with a transitions/transversions ratio of 2, as implemented in the fastDNAml program (Olsen et al. 1994); 2) the neighbor-joining method (NJ) (Saitou and Nei 1987) applied to distances corrected for multiple hits and for unequal transition and transversion rates using Kimura's 2-parameter model (Kimura 1980); and 3) the maximum parsimony method (MP), using heuristic search option and 10 replicates for random stepwise addition of taxa, included in PAUP 3.1.1. (Swofford 1993). A total of 521 unambiguously aligned DNA sites was retained for the phylogenetic analysis. Two other sets of 577 and 619 sites were used for subgroup analyses. The reliability of internal branches in the ML, NJ, and MP trees was assessed with the bootstrap method (Felsenstein 1988) with 100, 1000, and 500 replicates. One thousand bootstrap replications were performed for the ML, MP, and NJ analyses of the subgroups. Phylo_win program (Galtier and Gouy 1996) was used for distance computations, and NJ and ML trees building and bootstrapping. Njplot program (Perrière and Gouy 1996) was used to plot phylogenetic trees.

Rates of Substitution. We compared the number of substitutions in the 521 sites of alignment with the divergence times inferred from the fossil record to evaluate absolute rates of molecular evolution in different lineages. Rates were calculated only for pairs of species whose phylogenetic relationships based on the fossil record were confirmed by molecular data. Stratigraphic ranges and phylogenetic relationships for each species were established from broad reviews of micropaleontological data (Kennett and Srinivasan 1983; Bolli et al. 1985). Discrepancies between authors were accommodated by appropriate intervals of divergence times. Calculation of sequence divergences was based on distances corrected for multiple hits and for unequal transition and transversion rates following Kimura's 2-parameter model (Kimura 1980). We evaluated rates of evolution based on genetic distances rather than character state changes to specific branches of a MP tree (Smith et al. 1992) to avoid problems related to uncertainty in the branching order (Sorhannus 1996). The procedure adopted here is independent from the reconstruction method and, therefore, less sensitive to artifactual branching due to possible unequal rates of evolution.

Results and Discussion

Sequence Data

We obtained 18 partial SSU rDNA sequences of 14 planktonic foraminiferal species, four of them were represented by two geographic varieties. These sequences represent the four existing families of globigerinids (Globigerinidae, Hastigerinidae, Globorotaliidae, Candeinidae), including eight out of 15 living genera. They correspond to the 3′ terminal region of the SSU rRNA gene of *Mus musculus* (X00686) starting at position 1191 and ending at position 1854. The region includes the universal helices 32 to 50 (Van de Peer et al. 1996).

The most distinctive character of foraminiferal sequences is their unusual length. The examined fragment counts from 977 bp in *O. universa* to 1178 bp in *G. menardii*, which is about twice as much as in most other eukaryotes. The almost complete SSU rRNA gene sequence of *O. universa* consists of more than 4000 bp, compared to 2000 bp for typical eukaryotic sequences of this gene (unpublished data). The total alignment of our sequences contains 1736 sites. The examined fragment can be divided into seven constant and six variable regions (Fig. 1). Foraminifera are unique eukaryotes to have variable regions I, II, and V in the loops of helices 37, 41, and 46. Region I corresponds to the universal variable region V6 of the prokaryote structure model (Neefs et al. 1990). It is important to notice that sequence variability is unequal between the different groups of foraminifera. The alignment of spinose Globigerinidae + Hastigerinidae is possible in the constant regions only. The nonspinose Globorotaliidae and Candeinidae can be aligned with the benthic foraminifera also in the variable regions II, III, and V (Fig. 1). In order to include a maximum number of sites we applied our phylogenetic analyses separately to all foraminifera (planktonic and benthic), to the spinose Globigerinidae + Hastigerinidae and to the nonspinose Globorotaliidae + Candeinidae. The obtained DNA matrices contain respectively 521/577/619 sites, of which 308/355/486 are constant, 151/147/66 are parsimony-informative, and 62/75/67 are uninformative.

The G+C content averages 49% in all sets of sequences. The transitions/transversions ratio among and between the different groups of foraminifera averages 2.06. Species belonging to the Rotaliida-Textulariida group are separated mainly (sometimes only) by transitions. 113 pairs of compensatory mutations were detected in planktonic foraminiferal sequences. They are particularly abundant in the stems of the helices 42, 47, 48, and 49, and in the fast evolving species (*G. bulloides, G. sacculifer, H. pelagica, G. menardii*, and *G. truncatulinoides*). The presence of such large numbers of compensatory mutations confirms that our planktonic foraminiferal sequences are true rRNA genes, rather than

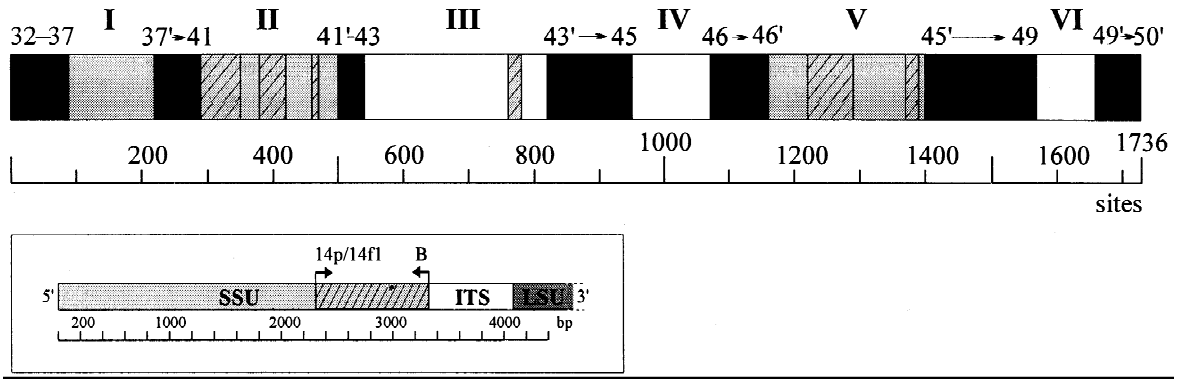

Fig. 1. Diagram of the alignment of the 18 analyzed planktonic foraminiferal sequences. Black rectangles represent the unambiguously aligned sites, used for all-species phylogenetic analysis and for calculating molecular evolutionary rates. Universal helices number are indicated above each conserved region. Rectangles numbered I–VI are the variable regions. Shaded rectangles I, II, and V constitute unique structures among eucaryotes. Hatched rectangles depict sectors where alignment is only possible between the planktonic Globorotaliidae, Candeinidae, and the benthic Textulariida-Rotaliida groups. The location of the SSU rRNA gene's fragment used in this study, with the position of the amplification primers, is shown in the frame. Scale is given according to the complete SSU rRNA gene sequence of *Trochammina* sp. (accession number X86095).

pseudogenes, as could be suggested by their unusually high substitution rates and length.

Rates of SSU rDNA Evolution

The rates of rDNA evolution vary between and within the different families of planktonic foraminifera (Table 1). In the Globigerinidae, rates are very high but relatively stable, ranging from 3.2 to 4.7, with a mean value of 4.0×10^{-9} substitutions/site/year, if we exclude the dubious high value of 7.2 obtained for *G. ruber/G. conglobatus,* that may result from uncertainty about the divergence time of both species (Cordey 1967). Rates of rDNA evolution vary in the Globorotaliidae. In *G. inflata, G. hirsuta,* and *N. dutertrei,* the SSU rDNA evolves at rates of $0.3–1.2 \times 10^{-9}$ substitutions/site/year, whereas *G. truncatulinoides* and *G. menardii* are characterized by much more rapid rates, averaging 3.5×10^{-9} substitutions/site/year. There is no global molecular clock in the SSU rDNA of planktonic foraminifera but we observe a clock-like evolution among the spinose species.

Planktonic foraminiferal rates by far surpass all rates reported until now for SSU rDNA sequences. The mean value of all computed globigerinids rates (3×10^{-9} substitutions/site/year) is about 17 times superior to the mean substitution rate proposed for 18S rRNA genes of diatoms, which evolve two to three times faster than Metazoa (Sorhannus 1996). The average distance of 0.067 substitutions/site separating the sequences of Caribbean and Mediterranean *O. universa* is about two times higher than the distance separating the frog *Xenopus* from *Homo* (0.035 substitutions/site) in the same SSU rDNA fragment (521 sites). Furthermore, our rates, particularly those of spinose species, can be considered as conservative estimates, because they are based on only 30% of the alignment, the rest of which belongs to the six variable regions that are lost for global comparison due to important differences in evolutionary speed between the groups of planktonic foraminifera.

As shown by previous data, the substitution rates in planktonic foraminifera are up to 100 times higher than in benthic foraminifera (Pawlowski et al. 1997). This is the first case of such extreme differences in rDNA evolutionary rates within a group of organisms. In echinoids, rates of rRNA evolution differ by a factor of three between irregular deep-sea infaunal and shallow water epifaunal sea urchins (Smith et al. 1992). Their divergence happened 200 Mya ago, at about the same time when first globigerinids appeared in the fossil record. The accelerated molecular evolution in globigerinids compared to the benthic foraminifera may suggest that the planktonic mode of life strongly influences the rate of rDNA substitution. This could be explained by different mode and tempo of reproduction in both groups. The benthic foraminifera reproduce slowly, occasionally undergoing alternations of asexual and sexual generations. In planktonic foraminifera only sexual reproduction is known and involves several 10^5 biflagellate gametes released by one individual. Reproduction phases range between biweekly to annual (Bé and Anderson 1976; Hemleben 1989). This interpretation draws on known relations between generation times and rates of molecular evolution in other organisms (Li 1993; Li and Tanimura 1987; Martin and Palumbi 1993). The adaptation to the planktonic mode of life could also be the factor explaining the difference of evolutionary rates between spinose and nonspinose planktonic foraminifera.

Polyphyletism of Planktonic Foraminifera

The sequences of planktonic foraminifera were compared to those of seven benthic species of the orders

Table 1. Divergence times and rates of SSU rDNA evolution between different lineages and species of planktonic and benthic foraminifera

Pairwise compared species	Divergence time (T) in million years	Number of substitutions per site[a] (K)	Rate of substitution[b] ($\times 10^{-9}$/site/years)
Globigerinidae			
Globigerinoides sacculifer - *Orbulina universa* (c, m)	16.4–21.7	0.153	3.5–4.7
Globigerinella siphonifera (c) - *Globigerinella calida* (m)	5	0.038	3.8
Globigerinella siphonifera (c) - *Globigerina bulloides* (m)	27–29	0.185	3.2–3.4
Globigerinoides ruber (c) - *Globigerinoides conglobatus* (c)	5–10	0.072	3.6–7.2
Globigerinoides ruber - *Globigerinoides sacculifer* (c, m, s)	25–28.5	0.192	3.4–3.8
Globorotaliidae			
Globorotalia hirsuta (s) - *Globorotalia inflata* (m)	17–18.5	0.034	0.92–1
Neogloboquadrina dutertrei (s) - *Globorotalia inflata* (m)	11–23.8	0.014	0.3–0.64
Neogloboquadrina dutertrei (s) - *Globorotalia hirsuta* (s)	11–23.8	0.027	0.57–1.22
Globorotalia menardii (c) - *Globorotalia inflata* (m)	17–19	0.108	2.8–3.2
Globorotalia menardii (c) - *Globorotalia hirsuta* (s)	15.8–19	0.115	3–3.6
Globorotalia menardii (c) - *Globorotalia truncatulinoides* (m)	15.8–19	0.140	3.7–4.4
Globorotalia truncatulinoides (m) - *Globorotalia hirsuta* (s)	7.4–10.5	0.078	3.7–5.3
Globorotalia truncatulinoides (m) - *Globorotalia inflata* (m)	17–18.5	0.088	2.4–2.6
Benthic species: rotaliida			
Bolivina sp. - *Glabratella opercularis*	>90	0.014	<0.08

Abbreviations: c, m, and s = species collected respectively in the Caribbean, Mediterranean, and Sargasso seas

[a] Number of substitutions per site between two sequences out of 521 sites reliably aligned between all foraminifera, after correction for multiple hits according to Kimura's two-parameter model

[b] The rate of substitution was calculated as r = K/(2T)

Rotaliida, Textulariida, Astrorhizida, and Allogromiida. *Allogromia* is a representative of the membraneous walled foraminiferal group that is considered as the most ancestral lineage (Tappan and Loeblich, 1988); it was thus chosen as the outgroup. *Allogromia* clusters invariably with *Astrorhiza*, an agglutinated species whose ancestors originated at least 540 Mya ago in the fossil record. The phylogenetic tree obtained with ML, NJ, and MP methods presents a large radiation of planktonic and benthic foraminifera (Fig. 2). The sequences of planktonic species cluster in three groups: Globigerinidae + Hastigerinidae, Globorotaliidae, and Candeinidae. The position of the Candeinidae within the radiation of benthic foraminifera is stable in all analyses. The branching order of the other groups depends on the method of analysis. In the ML tree, the Globorotaliidae branch as a sister group of *T. hadai*, within the radiation of benthic foraminifera. In the NJ and MP analyses they branch as a sister group of the clade Globigerinidae + Hastigerinidae. The clades Globigerinidae + Hastigerinidae and Globorotaliidae are supported by relatively low bootstrap percentages values, respectively by 39/88/91 and 46/41/73 in the ML/NJ/MP analyses. When only transversions are used for NJ analysis, the general structure of the tree and branching order are similar to the ML tree.

Molecular data confirm the morphotaxonomic and paleontological separation of the analyzed species in three main groups. They contrast, however, with the common view concerning the origin of these groups. Planktonic foraminifera are usually considered as a monophyletic group (Loeblich and Tappan 1988). Our data suggest independent benthic origins for the Candeinidae, the Globorotaliidae, and the clade Globigerinidae + Hastigerinidae.

There is a molecular evidence of close relationships between the microperforate nonspinose Candeinidae, represented by *G. glutinata*, and benthic Rotaliida and Textulariida. Use of transversions-only evolutionary distances (NJ) shows null dissimilarity between *G. glutinata* (Candeinidae) and most of the species of the benthic Rotaliida-Textulariida clade. This indicates that *G. glutinata* lineage comes from a relatively recent benthic → planktonic transition. In agreement with micropaleontological data, such a transition could have occurred during the Eocene-Oligocene crisis (34 Mya ago), when the first Candeinidae cryptogenically appeared in the fossil record. This could explain the atypical morphological features of the Candeinidae among planktonic species, mainly the lack of large perforations. According to micropaleontological data, similar benthic-planktonic transition already occurred in the history of foraminifera during the early Paleogene, when some minute microperforate species, originated from benthic or neritoplanktonic ancestors, invaded the pelagic domain after the K/T extinction (Brinkhuis and Zachariasse 1987; Li and Radford 1991; Liu and Olsson 1992).

An independent benthic origin is also possible for the nonspinose Globorotaliidae, as proposed by ML topology. We privilege this topology because ML method is less sensitive to the ''artifactual long branch attraction'' phenomenon, than are the NJ and MP methods (Felsenstein 1978, 1988). In fact, close relationships between Globorotaliidae and Rotaliida-Textulariida are strongly suggested by sequence similarities in the variable regions

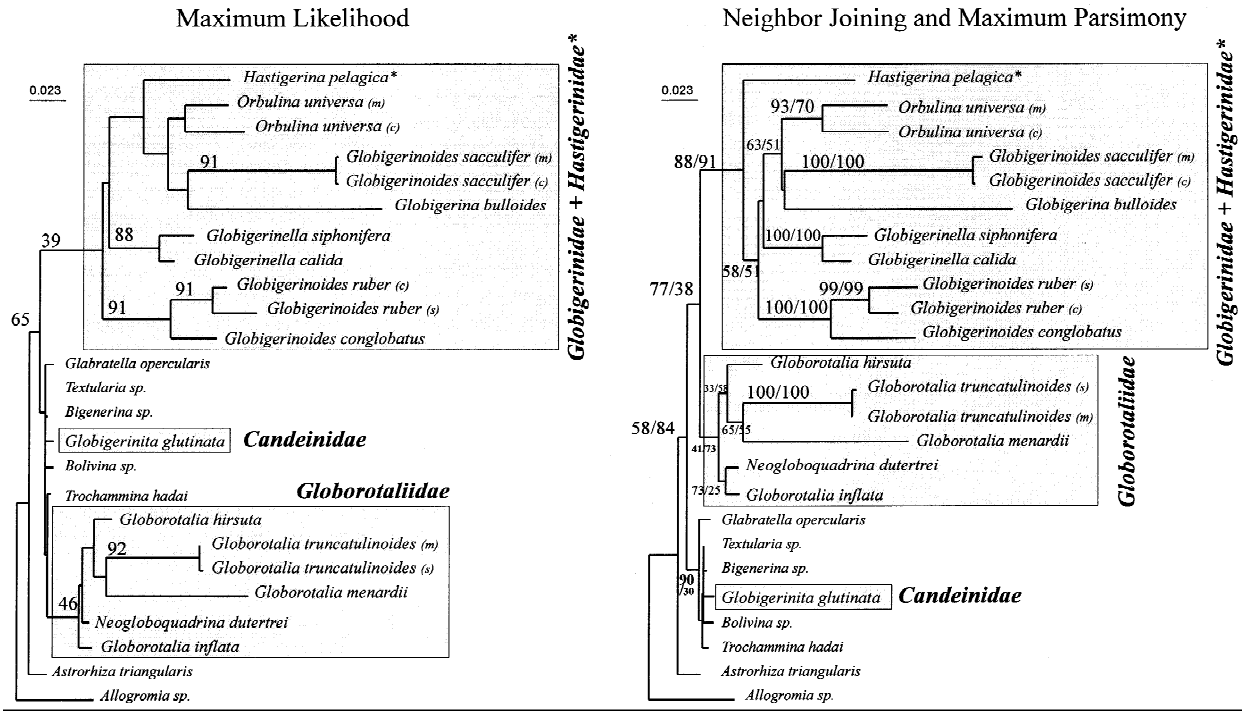

Fig. 2. Evolutionary relationships between the 14 planktonic foraminifera (shaded rectangles) and seven benthic foraminifera. The ML and NJ-MP trees were obtained using 521 unambiguously aligned sites. Bootstrap proportions greater than 50% are given next to each internal branch indicating repeatability of predicted clades. Scale is given in substitution/site. Abbreviations c, m, s are used for species collected in the Caribbean, Mediterranean, and Sargasso seas.

that were not included in our analysis because of impossibility of aligning the spinose species. In the whole alignment (1736 sites), we have counted 479 constant sites shared by the group Globorotaliidae-Rotaliida-Textulariida, of which 158 are unique to this group and thus define it among all foraminifera. In comparison, we have found only 30 constant sites common for the group Globigerinidae-Hastigerinidae-Rotaliida-Textulariida. Furthermore, 75% of the 360 constant sites of the Globorotaliidae clade are common with the Rotaliida-Textulariida, whereas only 2% are shared with the group Globigerinidae-Hastigerinidae.

Our data contrast with the paleontological view on a common origin for the Globigerinidae and Globorotaliidae. Classical models of evolutionary pathways in planktonic foraminifera assume globular, Globigerinidae-like species as ancestors and flattened, carinate, Globorotaliidae-like forms as evolutionary endmembers in planktonic specialisation (Hart 1980; Caron 1983; Bolli 1986). In the Neogene, the Globorotaliidae are supposed to diverge from some Globigerinidae ancestor (Cifelli 1982; Pearson 1993). However, our data clearly demonstrate higher DNA homology between the Globorotaliidae and the benthic species. Three hypotheses can explain our results: 1) the Globigerinidae originated from Globorotaliidae ancestors; 2) the Globigerinidae have extremely accelerated their rDNA evolutionary rates after the divergence of Globorotaliidae; 3) the Globorotaliidae have diverged independently and later than Globigerinidae. The first assumption can be clearly rejected on the basis of the fossil record, given that the spinose ancestors of Globigerinidae appeared 65 Mya ago, after the K/T crisis (Hemleben 1991), while the first Globorotaliidae emerged about 22 Mya ago (Kennett and Srinivasan 1983). The second hypothesis would implicate improbable lack of changes in evolutionary speed of Globigerinidae during at least 27 Mya and then a sudden acceleration in all spinose lineages after the divergence of the Globorotaliidae ancestors, 38 Mya ago.

In view of our data, the third hypothesis of independent origin of the Globorotaliidae is the most plausible, and also the most congruent with paleontological and biological data. Referring to the fossil record, there were few survivors of the Eocene-Oligocene planktonic foraminiferal extinction when the Globorotaliidae appeared. The only possible ancestor for the Globorotaliidae is the *G. opima–G. nana* plexus, but this group is characterized by spinose globigerinoid wall ultrastructure (Cifelli 1982). Given the weak fossil evidence for such a passage from a spinose, honeycomb to a nonspinose smooth wall, it is not surprising that the question of origin of Globorotaliidae is left open by most authors (Kennett and Srinivasan 1983; Bolli et al. 1985). From the biological point of view, similarities are observed between living Globorotaliidae and benthic foraminifera. Globorotaliidae show benthic behavior in culture and are herbivo-

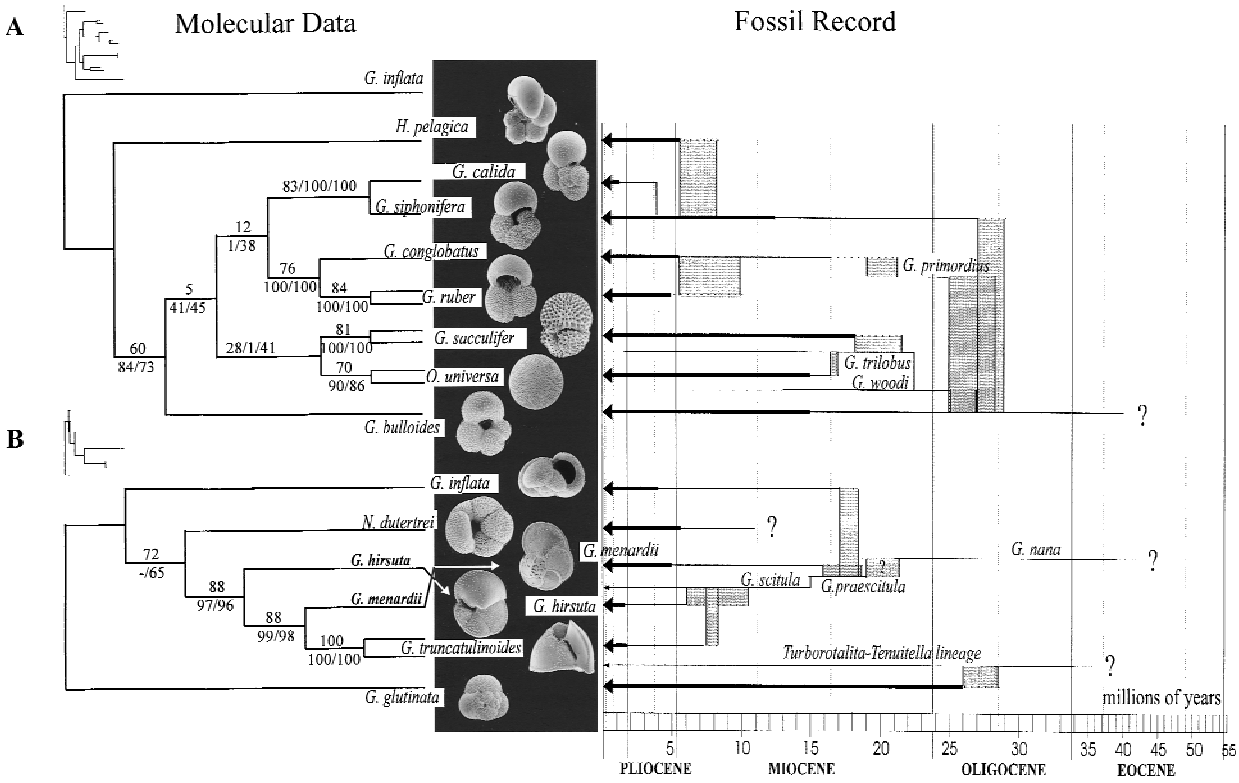

Fig. 3. Comparison of molecular and paleontological phylogeny of planktonic foraminifera. Analyses of 577 and 619 unambiguously aligned sites (SSU rDNA) of the spinose Globigerinidae-Hastigerinidae **(A)** and nonspinose Globorotaliidae **(B)** respectively, are presented on the left side. Bootstrap proportions (1000 replicates) are given for each internal branch for ML/NJ/MP analyses. Branch lengths are shown on the topology-corresponding ML trees presented next to **A** and **B** labels. Symbols in the paleontology graph: ← = stratigraphic range of the analyzed species, — = stratigraphic range of the lineage, · · · · = stratigraphic uncertainty concerning the age of lineages or species, ? = uncertain origin. Ranges of some ancestral and phylogenetically important species are indicated. Stratigraphic ages are calibrated relative to the time scale of Berggren et al. (1995). Links between the lineages and species are given with the interval of uncertainty (greyish areas).

rous rather than carnivorous as the spinose species are (Hilbrecht and Thierstein 1996; Hemleben et al. 1989). Moreover, they seem to be less adapted to the planktonic mode of life compared to the Globigerinidae which bear spines allowing them to use zooplankton as a food source in addition to phytoplankton, often possess symbionts, and thrive in highly variable surface waters.

In conclusion, we propose at least three independent episodes of benthic-planktonic transitions in the history of modern planktonic foraminifera. The spinose Globigerinidae could originate after the K/T crisis, 65 Mya ago, while the nonspinose Globorotaliidae and microperforate Candeinidae appeared much later at the Eocene-Oligocene boundary, 30 Mya ago. This interpretation agrees with general pattern in zooplankton evolution, with successive waves of invasion into the water column from the benthos, rather than by evolutionary diversification in the plankton (Rigby and Milsom 1996). In addition, planktonic stages are common in some groups of benthic foraminifera but are not well documented (Sliter 1965; Rückert-Hilbig 1980; numerous observations during our own plankton sampling in the Atlantic and Mediterranean) and morphologic similarities between some benthic and planktonic forms even present occasional problems in their distinction in the fossil record. The major extinction events at the K/T and Eocene/Oligocene boundary eliminated most species of planktonic foraminifera and opened the pelagic domain for new foraminiferal colonization.

Ingroups Relationships

The relationships within the spinose and nonspinose groups have been analyzed separately. In analysis of spinose foraminifera (Globigerinidae + Hastigerinidae), *G. inflata* was arbitrarily chosen as outgroup, however, tree topology is similar using other Globorotaliidae or benthic foraminifera outgroups. The SSU rDNA-based phylogeny of the Globigerinidae is consistent with current paleontological interpretations (Fig. 3A). Phylogenetic relationships within the Globigerinidae are stable in all analyses, except for the position of the clade *G. ruber* + *G. conglobatus* that is related either to the clade of *G. siphonifera* + *G. calida* in the ML and MP trees, or to the

clade *G. sacculifer* + *O. universa* in NJ tree. The resolution of relationships between these clades seems to be as difficult for the molecular as for the paleontological data. The branches emerging from *G. bulloides* lineage in SSU rDNA tree probably represent the confused radiation from the *Globigerina* stock that is known from the late Oligocene fossil record (Chaproniere 1992; Bolli et al. 1985). The SSU rDNA-based phylogeny of the Globigerinidae corroborates paleontological hypotheses in two other points. It supports the relationship between *O. universa* and *G. sacculifer*, which is well documented in the fossil record (Bolli et al. 1985). It confirms the close and recent link between *G. ruber* and *G. conglobatus* lineages (Cordey 1967), opposed to the common idea that *G. ruber* descends from or is conspecific with the fossil *G. subquadratus* (22 Mya), an idea that is in conflict with the paleontological gap of several million years between stratigraphic ranges of both species (Bolli et al. 1985).

In our tree, the Hastigerinidae appears as a sister group to the Globigerinidae. This contrasts with paleontological interpretation suggesting that the Hastigerinidae derived from *Globigerinella*, 6–8 Mya ago (Saito et al. 1981). In view of our data, the Hastigerinidae may have evolved much earlier, probably in the Oligocene, about 30 Mya ago. Early origin of *Hastigerina* fit better with the important morphological and biological differences between this genus and other planktonic foraminifera. *H. pelagica* is unique among living spinose planktonic foraminifera with respect to its thin, delicate test, its monolamellar wall structure, triradiate spines, and the cytoplasmic bubble capsule of probably digestive function (Hemleben et al. 1989). The apparent lack of *Hastigerina* in the fossil material older than 8 Mya can be explained by both the low preservation potential of their delicate tests and their destruction during reproduction.

In analysis of the Globorotaliidae, the microperforate *G. glutinata* was chosen as an outgroup, although other foraminiferal species have given similar results. The branching order within this family is similar in all types of analysis (Fig. 3B). The molecular phylogeny of the Globorotaliidae is in disagreement with micropaleontological data concerning the position of *G. menardii.* The fossil record shows an emergence of all modern Globorotaliidae lineages from *G. praescitula* about 16–19 Mya ago, *G. menardii* lineage being considered as one of the earliest branches in this radiation (Kennett and Srinivasan 1983; Bolli et al. 1985). In the SSU rDNA tree, *G. menardii* branches with *G. truncatulinoides*, which appeared recently in the fossil record (1.8 Mya ago). DNA sequences of both species are characterized by very high—more or less equivalent after time calibration—substitution rates, about four times faster than in other Globorotaliidae. Therefore, their close relationship is much probably artifactual. In fact, the branching order within the Globorotaliidae agrees with paleontological data when either the sequence of *G. menardii* or that of *G. truncatulinoides* is removed from our analyses.

Effect of the Substitution Rates on Phylogenetic Reconstruction

It is well known that unequal rates of mutations can lead to artifactual tree topology, due to the ''long branch attraction'' phenomenon (Olsen 1987). This artifactual branching is not always easy to detect. In this study, the calibration of our DNA sequences with the excellent fossil record allowed us to evaluate absolute substitution rates and their effect on phylogenetic reconstructions.

SSU rDNA-based phylogeny of planktonic foraminifera is principally affected at the family level. There is a good evidence of independent benthic origin of the Candeinidae. However, the relationships between the spinose Globigerinidae and the nonspinose Globorotaliidae are obscured by about fivefold differences in their substitutions rates. The number of sites used for the analysis of their relationships was reduced because of the impossibility to reliably align the rapidly and slowly evolving species. Independent origin of the Globorotaliidae was mainly proposed in agreement with some biological and paleontological evidences.

In ingroup analyses, where larger numbers of sites were included, a good congruence has been found between molecular and paleontological data on evolution of the ''fast-clock'' Globigerinidae. On the other hand, the resolution of relationships within the Globorotaliidae was clearly biased by lineage-acceleration in *G. menardii* and *G. truncatulinoides.* Both species, having substitution rates four times higher than other Globorotaliidae, were consistently placed together at the top of molecular tree in all analyses, in spite of a good fossil evidence that in evolutionary history they are separated by the slow-clock lineage of *G. hirsuta.*

The rRNA genes are considered as good chronometers of molecular evolution (Woese 1987), however, absolute rates of rDNA evolution were estimated for only very few groups of organisms, including bacteria (Ochman and Wilson 1987), diatoms (Philippe et al. 1994; Sorhannus 1996), echinoids (Smith et al. 1992), and vertebrates (Hedges et al. 1990). Substitution rates vary from two to fivefold between and within these groups. The highest variations are considered as due to unreliable paleontological data (Philippe et al. 1994; Sorhannus 1996). In the case of planktonic foraminifera, whose fossil record is certainly the best among the unicellular eukaryotes, this argument can hardly be used. Therefore, our data show that variations in rDNA rates can be much higher than previously observed. This raises an important question: how accurate are the SSU rDNA-

based phylogenies in the other groups of protists? Artifactual positions for the Euglenozoa and some ‘‘rhizopods’’ due to high SSU rDNA evolutionary rate have been suggested recently (Philippe and Adoutte 1995). In view of our present data, the position of foraminifera near the base of eukaryotic tree (Pawlowski et al. 1996) may also be incorrect. It seems urgent to develop some efficient methods for detecting variations of substitutions rates in those taxonomic groups that do not have any fossil record and for evaluating the influence of these variations on phylogenetic reconstructions.

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