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The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulations

Kate F. Darling,[1] Christopher M. Wade,[2] Dick Kroon,[1] Andrew J. Leigh Brown,[3] and Jelle Bijma [4]

Abstract. Molecular phylogenetic analysis of the small subunit ribosomal RNA gene of planktic spinose foraminifers shows that morphospecies may represent clusters of different and often highly divergent genotypes. In some cases the level of divergence may justify separate taxonomic status as distinct "cryptic" species. Molecular evolution rate estimates, based on fossil record evidence, suggest that the cryptic divergences may have occurred many millions of years ago. An investigation of their distribution in the Caribbean (tropical zone), Coral Sea and Mediterranean Sea (subtropical zone), and Southern California Bight (transitional zone) indicates that genotypes are transported across water mass boundaries, and it is proposed that the direction of gene flow follows the prevailing global ocean surface circulation pattern. At the present time the prevailing currents transport tropical/subtropical genotypes from the Pacific to Atlantic around the South African Cape. Cooler water transitional genotypes may transit from Pacific to Atlantic in gene corridors opened during glacial periods.

1. Introduction

The relationship between present-day planktic foraminiferal assemblages and their distribution within oceanic water masses has been used extensively for reconstructing past oceanic environments. A considerable amount of climatic information still remains buried within the sediments, however, and will remain so until a more comprehensive understanding of the foraminiferal biological system is obtained. Ocean flux studies, using sediment traps, and culture experiments are making a considerable contribution. However, these approaches cannot answer the fundamental questions of whether morphotypic variants recognized within a paleontologically defined species (morphospecies) represent natural variation within a population, phenotypic variation induced by different environmental conditions, or separate species with different ecological requirements. Using a molecular phylogenetic approach, we hope to help resolve these pivotal questions which are of considerable importance to paleoceanographers.

The isolation of sequences from the planktic foraminiferal ribosomal rRNA gene [*Darling et al.*, 1996] has created a new dimension in the investigation of foraminiferal phylogenetic relationships. Molecular phylogenetic analysis of small subunit (SSU) rDNA sequences obtained from extant species of foraminifers has shown that the foraminifera form a distinct monophyletic group within the eukaryotes and may represent one of the earliest diverging eukaryotic groups sequenced to date [*Darling et al.*, 1996; *Pawlowski et al.*, 1996; *Wade et al.*, 1996; *de Vargas et al.*, 1997], contrary to the interpretation of these results by *Conway Morris* [1998]. Comparison of SSU gene sequences within the foraminiferal group shows that the planktic foraminifera are polyphyletic and did not evolve from a single benthic ancestor [*Darling et al.*, 1997; *de Vargas et al.*, 1997]. However, the spinose species within the *Globigerinella*, *Globigerinoides*, and *Orbulina* genera do cluster together as a monophyletic group, with the divergence of the *Globigerina* lineage lying ancestral to the other spinose genera [*Darling et al.*, 1997].

Small subunit rDNA sequences show sufficient evolutionary information to allow the determination of both distant and close evolutionary relationships within the main planktic spinose group [*Darling et al.*, 1997]. The rRNA gene evolves at an unprecedented rate in planktic spinose foraminifers [*Darling et al.*, 1997], 50-100 times faster than in some benthic foraminifers [*Pawlowski et al.*, 1997]. Consequently, large differences are observed, even between closely related species, which provides high resolution in phylogenetic analyses, permitting both between and within lineage comparisons.

In this study we have adopted a molecular phylogenetic approach to investigate the spatial distribution of 19 different genotypes of five spinose planktic foraminiferal groups, collected in the Caribbean (tropical zone), Coral Sea and Mediterranean Sea (subtropical zone), and Southern California Bight (transitional zone). Such analyses will enable us to investigate whether planktic foraminiferal rDNA genotypes are genetically isolated into separate populations, partitioned by oceanographic or terrestrial barriers, or whether

[1]Department of Geology and Geophysics, Grant Institute, University of Edinburgh, Edinburgh, Scotland, United Kingdom.
[2]Department of Genetics, University of Nottingham, Nottingham, England, United Kingdom.
[3]Centre for HIV Research, University of Edinburgh, Edinburgh, Scotland, United Kingdom.
[4]Department of Geosciences, University of Bremen, Bremen, Germany

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they cross ocean fronts to form global uniform populations. An examination of the distribution of genotypes will allow us to determine whether specific water masses have characteristic genotypic assemblages and trace gene flow within the ocean circulation.

We have focused specifically on specimens of the same morphospecies in each of three biogeographical zones in order to examine the SSU rRNA genotypic diversity within the surface waters. It would be logical to assume that if genetic differences exist within a morphospecies, distinct genotypes might be associated with a specific morphotypic variant. However, it is quite possible that shell morphology may not be a true measure of planktic foraminiferal diversity. It has been demonstrated that two distinct genotypes of *Globigerinella siphonifera* cannot be discriminated morphologically without close analysis of test ultrastructure [*Huber et al.*, 1997]. Clear genetic differences can be identified between genotypes in such cryptic speciation events. We have used molecular phylogenetic analysis techniques to examine the genotypic diversity within a morphospecies and identify potential cryptic speciation events. We have also estimated within lineage evolution rates by calibration against accurate fossil record datum levels, which can be used to estimate approximate times for genotypic divergences.

2. Methods

2.1. Planktic Foraminiferal Collection

Planktic foraminiferal specimens were collected either by drift net at a depth of 5 m or individually by Scuba diving at a depth of 3-8 m. Taxonomic identification was confirmed using a stereo microscope or an inverted compound microscope. In the initial phase of our investigation, large quantities of genomic DNA was obtained by culturing specimens in the laboratory to the stage of gametogenesis [*Darling et al.*, 1996]. We are now able to amplify the SSU rDNA from single specimens.

2.1.1. Tropical zone (Caribbean). *Globigerinoides sacculifer* (Brady), *Globigerinoides ruber* (d'Orbigny) "pink" form, *Orbulina universa* (d'Orbigny), *G. siphonifera* (d'Orbigny) type I and type II, and *Neogloboquadrina dutertrei* (d'Orbigny) were collected off the west coast of the Caribbean island of Curaçao and cultured onshore at the Caribbean Marine Biological Institute, as described by *Darling et al.* [1996]. Specimens of *G. ruber* and *Globigerinoides conglobatus* (Brady) (GenBank accession numbers Z-69599 (*G. ruber*) and Z-69600 (*G. conglobatus*) were collected from Isla Magueyes, Puerto Rico, and sequenced by *Pawlowski et al.* [1997].

2.1.2. Subtropical zone (Coral Sea and Mediterranean Sea). *Globigerina bulloides* (d'Orbigny), *G. conglobatus* (Brady), *G. sacculifer*, *G. ruber* "white" form, *O. universa*, *G. siphonifera* type II, and *Globigerinita glutinata* (Egger) were collected off the Great Barrier Reef (GBR), Australia, 0.8 nautical miles due east of Ribbon Reef 10. They were cultured onshore at Lizard Island Research Station located in the Cairns section of the GBR as described by *Darling et al.* [1997]. *O. universa* (GenBank accession number Z83961-2) was collected from the Mediterranean Sea and sequenced by *de Vargas et al.* [1997].

2.1.3. Transitional zone (Southern California Bight). *G. ruber* white form, *O. universa*, *G. bulloides*, and *G. siphonifera* type II were collected ~2 km NNE off the Catalina Marine Science Centre, Santa Catalina Island, California (33°23'N, 118°26'W). Taxonomic identification was confirmed using an inverted compound microscope and a stereo microscope.

2.2. Molecular Methods

The SSU rRNA gene used in this study is one of the three genes that encode the three RNA subunits of the ribosome, the site of protein synthesis within the cell. We have examined a region of ~1000 base pairs (1000 nucleotide sites) at the terminal end of the SSU gene. We have targeted the 1000 bp fragment of the SSU gene using the polymerase chain reaction (PCR) technique which allows the specific amplification of foraminiferal SSU rDNA. The amplified region is then sequenced, and the foraminiferal sequences generated are aligned relative to each other. Of the ~1000 bp sequenced, 540 sites were sufficiently conserved to permit alignment between all the foraminiferal taxa in the study. These 540 unambiguously aligned sites have been used to generate the genetic distances and phylogenetic trees. Genetic distances were determined by calculating the number of nucleotide differences (mutations or nucleotide substitutions) between foraminiferal taxa and correcting for unseen changes (reverse or multiple substitutions).

2.2.1. DNA extraction, amplification, and sequencing of the SSU rRNA gene. DNA extraction from Caribbean and Coral Sea specimens was as described previously [*Darling et al.*, 1996]. DNA was extracted from Southern California Bight specimens by crushing individuals in a lysis buffer and incubating them at 60°C for 1 hour [*Holzmann and Pawlowski*, 1996]. The PCR amplification and direct automated sequencing of an ~1000 bp region of the terminal 3' end of the foraminiferal SSU rRNA gene was as described by *Darling et al.* [1996, 1997]. At least two individual specimens were fully sequenced for each genotype and duplicated.

2.2.2. Sequence analysis. Partial SSU rDNA sequences of the planktic foraminiferal specimens were aligned manually within the Genetic Data Environment (GDE) package [*Smith et al.*, 1994]. Phylogenetic trees were constructed on the basis of 540 unambiguously aligned nucleotide sites. Distance-based phylogenetic analyses were performed using both the neighbor-joining [*Saitou and Nei*, 1987] (program NEIGHBOR) and Fitch-Margoliash [*Fitch and Margoliash*, 1967] (program FITCH) methods within version 3.52c of the phylogeny inference package PHYLIP [*Felsenstein*, 1993]. Nucleotide sequence distances were estimated for all pairwise sequence comparisons using the generalized two-parameter (maximum likelihood F84) model (program DNADIST) [*Felsenstein*, 1993], which incorporates unequal rates of transition and transversion substitutions and also allows for different frequencies of the four nucleotides [*Kishino and Hasegawa*, 1989]. Bootstrap resampling (2000 replicates) [*Felsenstein*, 1985] was employed to assign

support to the neighbor-joining trees (programs SEQBOOT and CONSENSE). Maximum likelihood [*Felsenstein*, 1981] phylogenetic analyses were performed using DNAML (PHYLIP, *Felsenstein*, 1993]. Maximum parsimony [*Fitch*, 1971] analyses were performed using the PAUP package [*Swofford*, 1993]. The planktic foraminiferal SSU rDNA sequences presented in this study are deposited in GenBank, accession numbers AF102227-AF102230.

3. Results

3.1. General Tree Topology

The neighbor-joining tree presented in Figure 1 represents an extension of the tree shown by *Darling et al.* [1997]. The phylogeny has been extended to include the nonspinose species *G. glutinata* from the Coral Sea and the Southern California Bight genotypes of *G. siphonifera*, *O. universa*, *G. ruber* and *G. bulloides*. In addition, we have included two Caribbean genotypes of the spinose species, *G. ruber* and *G. conglobatus* [*Pawlowski et al.*, 1997] and one Mediterranean genotype of *O. universa* [*de Vargas et al.*, 1997]. The phylogeny is rooted on *Allogromia*, a membraneous-walled benthic foraminifer which is thought to have diverged early in the history of the clade [*Tappan and Loeblich*, 1988]. All tree construction methods employed (neighbor-joining, Fitch-Margoliash, parsimony and maximum likelihood) were generally highly consistent in the foraminiferal relationships inferred (data not shown), although the phylogenetic placement of the highly divergent "long-branch" species, *G. bulloides* and *Globorotalia menardii*, were not resolved.

With the exception of the nonspinose planktic species *N. dutertrei* and *G. glutinata*, the planktic and benthic foraminiferal species cluster separately within the reconstructed phylogeny. Both *N. dutertrei* and *G. glutinata* branch off deep within the benthic group but separately from

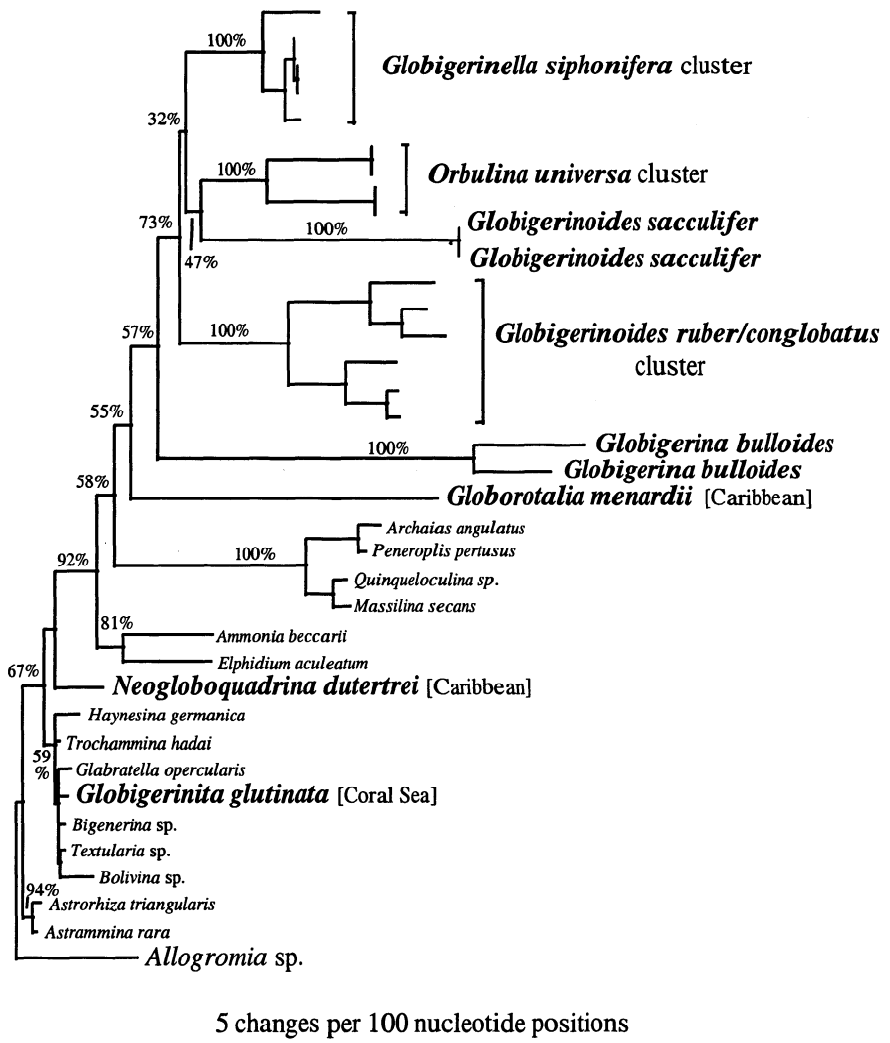


Figure 1. Neighbor-joining phylogenetic tree of planktic and benthic foraminiferal species rooted on *Allogromia*, a membraneous walled benthic foraminifer which is thought to have diverged early in the history of the clade. The planktic species sequenced in this study are highlighted in bold and placed within a background of benthic species sequenced by *Pawlowski et al.* [1997]. The phylogeny was reconstructed on the basis of 540 unambiguously aligned nucleotide sites of the terminal 3' region of the small subunit ribosomal RNA gene. Bootstrap values reflect the degree of support for a particular cluster within the tree. As bootstrapping is considered a conservative statistical test, a value of 70% is taken as indicating a robust grouping [*Hillis and Bull*, 1993].

one another, providing conclusive support for the polyphyletic origins of the planktic foraminifera from benthic taxa [*Darling et al.*, 1997; *de Vargas et al.*, 1997].

Branches within the main spinose group were strongly supported, with the *G. siphonifera*, *O. universa*, *G. sacculifer*, and *G. ruber*/*G. conglobatus* branches each supported in 100% of the bootstrap replicates. The relative order of branching of the main spinose lineages was, however, inconsistent because of the separation of the long-branch spinose lineages by relatively short internodes.

3.2. Genotype Clusters

3.2.1. *Globigerinella* cluster. The *G. siphonifera* type I and type II clades cluster within a single lineage, supported in 100% of the bootstrap replicates (Figure 2). The type I and type II specimens cluster distinctly from one another, with the type II clade supported in 98% of the bootstrap replicates. Two forms of type II *G. siphonifera* were observed. One form, type IIa, was observed in both the tropical/subtropical zones (Caribbean and Coral Sea) and the transitional zone (Southern California Bight). A second form, type IIb, has so far only been identified in the transitional zone. Despite the fact that all type II sequences are very similar, with only 1.1% nucleotide sequence distance (genetic distance), there is strong bootstrap support (87%) for the type IIa subcluster. In contrast, the distance between type I and type II is 4 times greater (4%).

3.2.2. *Orbulina* cluster. The *O. universa* cluster was supported in 100% of the bootstrap replicates within the phylogenetic tree (Figure 2). Tropical/subtropical specimens of *O. universa* collected from the Caribbean and Coral Sea showed complete sequence identity throughout the amplified 980 base pair fragment of the SSU rRNA gene. Three individual transitional zone, Southern California Bight specimens had identical sequences and were also virtually identical to the Mediterranean genotypes (three base pair differences in the whole fragment). These genotypes are highly divergent from the tropical/subtropical specimens, with a genetic distance of 9.5%.

3.2.3. *Globigerinoides ruber*/*conglobatus* cluster. Tropical *G. ruber* specimens have been obtained from two separate Caribbean locations (Figure 2). The first were of the pink form, collected offshore Curaçao [*Darling et al.*, 1996], and the second were collected offshore Puerto Rico [*Pawlowski et al.*, 1997]. Subtropical *G. ruber* specimens of the white form were collected from the Coral Sea. The

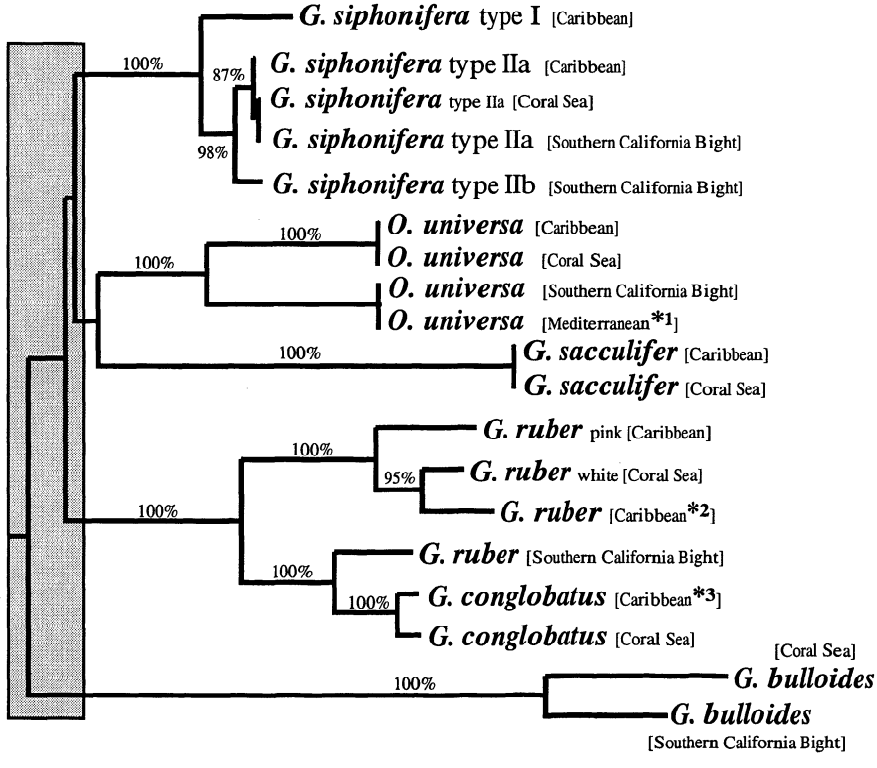


Figure 2. Neighbor-joining phylogenetic tree showing the relationships between the planktic spinose foraminiferal genotypes of morphospecies found in both the transitional and tropical/subtropical zones. The phylogeny represents a subtree of that shown in Figure 1. The shaded area denotes the radiation of the spinose species in the late Oligocene, dated at between 30 and 27 Ma from the fossil record. Bootstrap values are expressed as a percentage and reflect the degree of support for a particular branch within the tree. Three additional planktic spinose sequences incorporated in the phylogeny are denoted *[1] [*de Vargas et al.*, 1997] and *[2] *[3] [*Pawlowski et al.*, 1997]. Biogeographic divisions are (Caribbean) tropical zone, (Coral Sea) and (Mediterranean) subtropical zone and (Southern California Bight) transitional zone.

Caribbean and Coral Sea specimens cluster within a single group supported in 100% of bootstrap replicates. Within this group, the specimens collected offshore Puerto Rico cluster with the white form collected from the Coral Sea (95% bootstrap support). The pink specimens of *G. ruber*, collected from Curaçao, are divergent from this cluster with an average genetic distance of 5.5%.

G. ruber specimens were also collected from the transitional zone. Two specimens were obtained, which showed complete sequence identity in the amplified region of the SSU gene. The transitional zone specimens were highly divergent from the tropical/subtropical specimens, with a mean genetic distance of 10.8% and were strongly associated with *G. conglobatus* (100% bootstrap support).

G. conglobatus and tropical/subtropical specimens of *G. ruber* were separated by a mean genetic distance of 11.6%. In contrast, a mean genetic distance of 4.4% was observed between *G. conglobatus* and transitional *G. ruber*. The Caribbean and Coral Sea forms of *G. conglobatus* were closely related with a genetic distance of 1.1%.

3.2.4. *Globigerina bulloides* cluster. Subtropical and transitional zone specimens of *G. bulloides* clustered together within the evolutionary tree, with this association supported in 100% of the bootstrap replicates (Figure 2). Three individuals obtained from the Southern California Bight showed complete sequence identity. The genetic distance between the transitional zone specimens and the subtropical specimen was 8.3%.

4. Discussion

The molecular phylogenetic analysis of rRNA gene sequences derived from planktic foraminiferal species has proved to be highly informative in the determination of the evolutionary relationships amongst extant foraminiferal species. Complementing the morphotypic phylogenies based on the fossil record, the molecular data have provided additional evidence to help resolve the evolutionary relationships between planktic foraminiferal species and has demonstrated the polyphyletic origins of the planktic foraminifera from benthic taxa [*Darling et al.*, 1997]. As the molecular phylogeny has expanded with the addition of new taxa, the evolutionary relationships between taxa have become clearer, allowing direct comparison with phylogenies generated from the fossil record. For the first time we present evidence to demonstrate the existence of a high degree of genetic diversity within planktic spinose foraminiferal morphospecies. The examination of the distribution of foraminiferal genotypes presented within this study also provides a novel direction of approach for the study of oceanographic circulation in both the present and the past.

4.1. Comparison of the Planktic Spinose Molecular Phylogeny With the Fossil Record

The spinose planktic species form a distinct clade within the phylogenetic tree, with the spinose lineages characterized by long branch lengths separated by relatively short internodes which renders their relative placement within the phylogeny difficult to determine. This is consistent with their divergence occurring over a relatively short period of time, as suggested by the fossil record.

The first member of the genus *Globigerina* is thought to be *Globigerina officinalis* [*Blow*, 1979; *Spezzaferri and Primoli Silva*, 1991], which appeared in the late Eocene. *Globigerina praebulloides* diverged at ~30 Ma [*Pearson*, 1993; *Berggren et al.*, 1995], giving rise to *G. bulloides* in the early middle Miocene [*Kennett and Srinivasan*, 1983]. The fossil record indicates a similar divergence time for the *Globigerinella* lineage from *Globigerina*, with the appearance of *Globigerinella obesa* [*Spezzaferri and Premoli Silva*, 1991]. *Globigerinoides* is clearly a polyphyletic genus [*Kennett and Srinivasan*, 1983; this study]. *G. ruber* and *G. conglobatus* share a common ancestor in the molecular phylogeny, and the most likely candidate for the ancestral divergence of this lineage from *G. praebulloides* is thought to be *Globigerina primordius*, at ~29Ma [*Spezzaferri and Premoli Silva*, 1991]. The *G. sacculifer*/*O. universa* lineage is also thought to have diverged in the late Oligocene, with *Globigerina woodi* as a possible early ancestral form (27 Ma) [*Kennett and Srinivasan*, 1983]. The molecular phylogeny is therefore consistent with data from the fossil record and corroborates fossil evidence that the radiation of the spinose species was within a relatively short time period, dated from the fossil record at between 30 and 27 Ma (Figure 2).

4.2. The Spinose rDNA Genotype Clusters

4.2.1. *Globigerinella* genotype cluster. The divergences within the *Globigerinella* cluster indicate that there are at least two *siphonifera* branches (Figure 2). The earliest divergence is observed between *G. siphonifera* type I and type II, and there is now a great deal of biological evidence to suggest that they are at least separate species [*Darling et al.*, 1997; *Huber et al.*, 1997; *Bijma et al.*, 1998]. The molecular phylogeny also indicates a degree of genetic variation within the *G. siphonifera* type II cluster, in particular, between Caribbean/Coral Sea/Southern California Bight type IIa and Southern California Bight type IIb. The divergence between the *G. siphonifera* type I and type II branches may be quite ancient. The *Globigerinella* cluster has relatively short branch lengths compared with the other spinose clusters, which is indicative of a lower rate of evolution (Figure 2). Consequently, relatively few mutations in the *Globigerinella* lineage may reflect a comparably greater period of time than in other spinose groups.

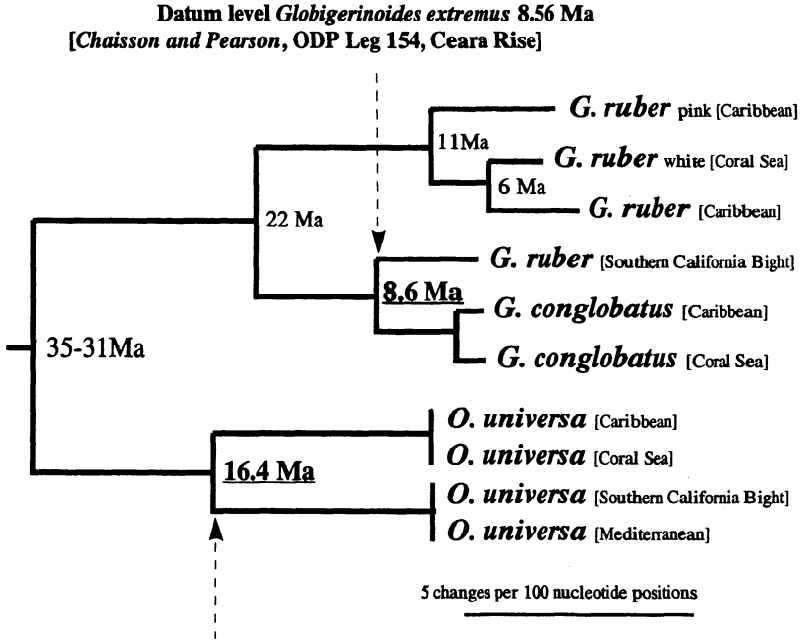
4.2.2. *Orbulina* genotype cluster. *Orbulina* made its appearance in the form of *Praeorbulina sicanus* possibly from the ancestral line of *Globigerinoides triloba* at the beginning of the middle Miocene (16.4 Ma) [*Berggren et al.*, 1995]. The molecular phylogeny clearly shows that there are at least two extant *O. universa* genotypes (Figure 2). We propose that the divergence occurred during the early radiation of the orbulines, ~16 Ma ago. At this time, *O. universa* radiated into both the tropical and temperate zones, which is suggestive of such early genetic divergence. However, as our sampling density and geographic distribution is limited, we cannot discount the possibility that earlier diverging genotypes will be found.

4.2.3. *Globigerinoides ruber/conglobatus* genotype cluster. The evolutionary relationships within the *G. ruber*

lineage have remained unresolved up to the present time. *Kennett and Srinivasan* [1983] suggest that *G. ruber* evolved from *Globigerinoides subquadratus* during the middle Miocene zone N15. Alternatively, *Blow* [1969] suggested a later appearance from *Globigerinoides bollii* within zone 16 in the late Miocene. Confusion is compounded by *Cordey* [1967], who, following extensive examination of the ontogeny of both *G. subquadratus* and *G. ruber*, concluded that two lineages are represented by the morphospecies *G. ruber*: one *G. subquadratus* and the other *Globigerinoides obliquus*. We believe that the molecular phylogenetic divergence within these lineages may help explain the reason for the differing views.

The molecular phylogeny indicates that there are two extant *G. ruber* lineages. Their genetic distances suggest that they diverged many millions of years ago (see evolution rates; Figure 3). We propose that the two *G. ruber* lineages diverged from their common ancestor, possibly *Globigerinoides primordius* or *Globigerinoides altiapertura*, within the radiations of the early Miocene (Figure 2). One lineage is represented by *G. obliquus* and the other by *G. subquadratus*. *G. obliquus* is the common ancestor of the *G. ruber* extant in the Southern California Bight at the present time and also of *G. conglobatus*. It is as yet unknown whether this genotype occurs elsewhere. We propose that the *G. subquadratus* lineage did not become extinct but remained in a refugium to reradiate in the middle Miocene and continued to the present day as suggested by *Bolli and Saunders* [1985].

4.2.4. *Globigerina* genotype cluster. *G. praebulloides* gave rise to *G. bulloides* in the early middle Miocene during the same period as the orbuline radiation [*Kennett and Srinivasan*, 1983]. Few clues to the timing of the divergence of the transitional and subtropical *G. bulloides* are available. The addition of *Globigerina falconensis* would make a



Rate calculation (r)	T(Ma)	K Sub/site	K/2T	r = substitutions x10^{9} /site/year
Caribbean + Coral Sea *G. conglobatus* / Southern California Bight *G. ruber*	8.6	0.04415	0.0026	**2.6**
Caribbean + Coral Sea *O. universa* / Southern California Bight + Mediterranean *O. universa*	16.4	0.0951	0.0029	**2.9**

Figure 3. Calibration and estimated divergence times for the *G. ruber*/*G. conglobatus* and *O. universa* rRNA genotype clusters. Datum levels used for calibration are underlined in bold. Evolution rate calculations are tabulated and estimated dates are shown for the lineage divergences resolved in the tree. Nucleotide sequence distances were estimated for all pairwise sequence comparisons using the generalized two-parameter (maximum likelihood F84) model (program DNADIST) [*Felsenstein*, 1993].

considerable contribution, as it diverged slightly earlier from *G. praebulloides*.

4.3. Chronology of the Divergences Within the Foraminiferal Phylogenetic Tree

4.3.1. Evolution rates in the foraminiferal SSU rRNA gene. The SSU rRNA gene undergoes gradual evolutionary change in the DNA sequence through time, leading to an overall increase in genetic distance between isolated populations. Consequently, it may be possible through the assumption of a "molecular clock" to impose time on the foraminiferal molecular phylogeny. Under this assumption, evolution rates would have to be constant between taxonomic groups. However, the analyses of foraminiferal SSU rDNA sequences have revealed considerable differences in evolutionary rates between foraminiferal lineages [*Darling et al.*, 1997; *Pawlowski et al.*, 1997], which are most pronounced between the planktic and benthic genera. Rate differences are also observed between planktic taxa, although there does appear to be a degree of similarity in evolutionary rates within the spinose cluster [*Darling et al.*, 1997; *de Vargas et al.*, 1997]. If individual spinose lineages are found to evolve at a constant rate, evolutionary rate could be calculated by calibration against well-dated first appearances of fossil taxa. The molecular data could then be used to date molecular divergence points and cryptic speciation events within the evolutionary tree.

Observed differences in evolutionary rates between taxa have been explained by differences in organismal generation time, differences in DNA replication in germ line cells, metabolic rate, nucleotide generation times, DNA repair efficiency, and exposure to mutagens (e.g., ultraviolet exposure) [*Hillis et al.*, 1996]. Possible differences in evolution rates between planktic foraminiferal lineages may be largely due to differences in generation time and genome turnover. High gamete numbers increase the chance of DNA replication-associated mutation. However, the factors that create evolution rate differences between planktic spinose foraminiferal lineages are likely to be very similar within the closely related genotype clusters, and consequently, there remains the possibility of a "local clock" within these clusters.

4.3.2. Calibration of evolution rate against the fossil record. The calibration of evolutionary rate against the fossil record should be based on more than a single datum level, and calibration points should be evolutionarily independent [*Hillis et al.*, 1996]. At the present time the scarcity of the molecular data limits the number of datum points that can be used for calibration. Further, whilst the fossil record may provide relatively accurate datums for the first appearance of specific foraminiferal morphotypes, genetic divergence may be cryptic. Taking into account all of these considerations, rate estimates calculated from foraminiferal fossil record datum levels will be subject to a wide range of error. However, this should not deter a tentative attempt to calculate evolution rates from lineages where divergences are relatively well dated in the fossil record.

We have calculated evolutionary rates for two lineage divergences from different genotype clusters for which reliable dates are available from the fossil record and in which branch lengths are relatively similar. The first divergence chosen lies within the genotype cluster of *G. ruber/conglobatus* (Figure 3). The *G. conglobatus* line diverged from its ancestral lineage of *G. obliquus* [*Kennett and Srinivasan*, 1983] with the first appearance of *Globigerinoides extremus* at 8.56 Ma [*Chaisson and Pearson*, 1997]. There is a morphological gradation between *G. obliquus* and *G. extremus*, and it is possible that the genetic divergence may be earlier. The Southern California Bight *G. ruber* also diverged from the same ancestral branch. This datum level can therefore be used to estimate evolution rate based on the genetic distance between the Southern California Bight *G. ruber* and *G. conglobatus*. The second divergence used for calibration is the first appearance of the orbuline form of *P. sicanus* in the early middle Miocene at 16.4 Ma [*Berggren et al.*, 1995] (Figure 3). This will have to be revised if an earlier diverging *O. universa* genotype is found. The estimates of evolution rate from these two independent calibration points are close, with an estimate of 2.6×10^{-9} substitutions/site/year for the *G. ruber/G. conglobatus* divergence and 2.9×10^{-9} substitutions/site/year for the *Orbulina* genus.

The evolution rate estimates can be used to provide an estimated time for other lineage divergences within the same genotype cluster. The estimated divergence time for the Southern California Bight *G. ruber* from the other *G. ruber* lineage is 22 Ma (Figure 3). This date can be tested against the fossil record. We have proposed that the two *G. ruber* lineages diverged from a common ancestor, possibly *G. primordius* or *G. altiapertura*, within the radiations of the early Miocene. The molecular clock estimate supports this suggestion, as the first appearance of *G. primordius* is at 26.7 Ma and *G. altiapertura* at 20.5 Ma [*Berggren et al.*, 1995]. A further test may be made against the fossil record by calculating the divergence time of the *G. ruber/G. conglobatus* cluster from the *O. universa* cluster. Taking the evolution rate range between the two lineages at between 2.6 (calculated from the *G. ruber/G. conglobatus* cluster) and 2.9 (calculated from the *Orbulina* cluster), the estimated time of divergence is 35-31 Ma. This estimate, in view of the inherently wide confidence limits, is remarkably close to the 30 Ma approximate divergence date, obtained from the fossil record, for these lineages in the late Oligocene.

As an example of applying molecular clock data to estimating dates for genetic divergences not dated in the fossil record, we have attempted to estimate dates for the genetic divergences within the *G. ruber* lineage (Figure 3). The clock estimate indicates that the divergence of *G. ruber* pink from white is apparently quite ancient (11 Ma, Figure 3). If this is correct, there are two possible explanations why this early divergence has remained unseen in the fossil record. First, *G. ruber* pink may not have been pink when the divergence occurred. Second, the pink pigment may have faded in the sediment over such a long period of time [*Thompson et al.*, 1979]. It is not intended to imply that these dates are absolute, but they do hopefully provide a rough guide to the time of lineage divergences within the genotype cluster.

4.4. Planktic Foraminiferal Gene Flow in the Oceans

4.4.1. The distribution and circulation of foraminiferal genotypes. Mixing of surface waters occurs on a global scale over a relatively short time (~1000 years) interval [*Broecker*, 1974]. However, the distribution of planktic foraminiferal morphospecies is not ubiquitous, as they are distributed in faunal provinces which largely correspond to the major hydrographic regions of the worlds oceans [*Bé*, 1977]. The biogeographic distribution of individual morphospecies, however, indicates that the majority are not restricted to a single province, suggesting adaptation to a wide range of environmental conditions. We have examined the genotypic distribution of foraminiferal morphospecies from three regions lying within the tropical (Caribbean), subtropical (Coral Sea), and transitional (Southern California Bight) faunal provinces to investigate whether multiprovincialism reflects genetic isolation. Although a preliminary study, these data provide an opportunity to examine the genetic relationships within and between provinces. However, a more extensive examination of population size, seasonal succession, and depth distribution will be required in the future to determine whether they are representative genotypes.

4.4.2. Gene flow within the tropical/subtropical zone. The circumglobal continuity of warm water morphospecies of the tropical/subtropical provinces are relatively unrestricted between the Pacific, Indian, and Atlantic Oceans. However, it is unknown whether the genotypes of each morphospecies are identical throughout the tropical/subtropical province. During the interglacial periods, tropical/subtropical morphospecies from the Indian Ocean can pass around the South African Cape, allowing faunal exchange with the populations resident offshore, west of the Benguela cold water current. During glacial periods, transit of tropical/subtropical morphospecies into the Benguela system may be intermittent.

Our molecular data indicate that there is gene flow between the Pacific and Atlantic tropical/subtropical genotypes of *G. sacculifer* and *O. universa*. Complete sequence identity is observed between the genotypes, not only in the conserved region (Figure 2) but also throughout variable regions of their SSU rDNA fragment. The variable regions are shown by *Darling et al.* [1997, Figure 3]. This suggests that their genomes have not become isolated and are continually mixed. As the prevailing global surface currents are in a westerly direction throughout the tropical subtropical zones, we propose that gene mixing occurs from east to west at the present time and that the corridor off the South African Cape is not a barrier to these two tropical/subtropical genotypes.

The tropical/subtropical *G. siphonifera* type IIa also shows complete sequence identity in the conserved region, with the exception of a single polymorphic site [*Darling et al.*, 1996], between Australia and the Caribbean (Figure 2). However, *G. siphonifera* type II does have a limited number of base changes in the variable region of the gene fragment between the Coral Sea and Caribbean specimens as shown by *Darling et al.* [1997, Figure 3]. This may indicate that the *G. siphonifera* type II rate of gene transit, from east to west, is slower than in *G. sacculifer* and *O. universa*. This may be episodic in form because of environmental barriers preventing this species from passage at times as tolerance levels to different temperatures and salinities vary between species [*Bijma et al.*, 1990].

Tropical/subtropical genotypes of *G. ruber* white show a high degree of genetic divergence between the Coral Sea and the Caribbean populations (Figure 2). This indicates that they have been isolated over a considerable period of time. Clock estimates (Figure 3) suggest that this may be as long ago as 6 Ma, although this estimate will be subject to a wide error. The *G. ruber*, Coral Sea genotype may not transit east to west as far as the Caribbean. It may be unable to tolerate exposure to the range of environmental conditions experienced while negotiating the South African Cape and so remains retained within the Indian Ocean circulation. However, extensive population studies will be required before the absence of any genotype can be established.

4.4.3. Gene flow between the tropical/subtropical zone and the transitional Southern California Bight. In the North Pacific the western tropical/subtropical population is swept into cooler, higher latitudes within the Kuroshio Current, preventing gene flow into the eastern Pacific population. The California Current flowing south joins the Northern Equatorial Current, drawing surface waters in a westerly direction, effectively restricting gene flow from the south. It is clear from the large genetic distances observed between Californian and Australian *O. universa*, *G. ruber*, and *G. bulloides* that they are genetically isolated populations. However, the presence of *G. siphonifera* type IIa also indicates that there is some degree of gene flow northward between the two zones in more cold tolerant genotypes.

The transitional populations of the Southern California Bight are not, however, restricted to the northeastern sector of the Pacific. The *O. universa* genotype found in the Southern California Bight is very closely related to the genotype found in the Mediterranean Sea (Figure 2), having only three base differences in the whole gene fragment (data not shown). This indicates relatively recent gene mixing between these populations. Preliminary data from *de Vargas et al* [1998] indicates that the geographic distribution of this population is closely related to primary production and not to temperature, as it has been identified within the Atlantic equatorial upwelling system. Two morphologically different populations of *O. universa* have been observed in the Indian Ocean which vary in test size and porosity. Their geographic distribution indicates a close correlation with water mass [*Hecht et al.*, 1976], and it is highly probable that they may represent genetically distinct populations. Morphometric analysis in future studies will determine whether the orbuline genotypes are morphologically distinct cryptic species.

The Southern California Bight *G. bulloides* is much more closely related to the Atlantic subarctic and subantarctic cooler water populations [*Darling et al.*, 1998] than to the subtropical Coral Sea population. Gene transit across the tropical zone in these populations most likely occurs during cooling cycles, when changing ocean circulation patterns may open up transient gene corridors.

5. Conclusions

The molecular evidence indicates that planktic foraminiferal diversity is much greater than ever imagined. In some cases the level of divergence may justify separate taxonomic status as distinct cryptic species. It is most probable that by increasing sample size, sampling throughout the water column, and serial sampling over seasonal succession, an even greater number of genotypes associated with each morphospecies will be identified. It remains to be determined whether genotypes can be discriminated morphotypically.

Considering the wide range of error inherent in estimating evolution rates, the estimates of divergence times made in the present study are in relatively good agreement with the fossil record. Currently, the scarcity of molecular data limits the number of datum points that can be used for calibration within individual clusters. As the foraminiferal SSU rDNA database of genotypes builds, the calibration of evolution rate should improve. Further analysis of genetic distance estimates, taking into account between-site rate variation, may also enhance the accuracy. If the genetic divergences which remain cryptic in current fossil record phylogenies can be dated, such genetic events may then be correlated with oceanographic changes in the past. The evolutionary rate within the conserved regions of the SSU rRNA gene is too slow to provide information of sufficient resolution to examine the Quaternary period. However, arrayed between the conserved regions are variable regions [*Darling et al.*, 1996] which may be used to examine closer relationships within the genotype populations. It may also be possible to isolate other foraminiferal genes for corroborative studies which may also be more suitable for population-based analysis.

The molecular phylogeny provides good evidence that the southern California Bight populations are genetically isolated from the populations in the oligotrophic tropical/subtropical zone. However, it is clear that the genotypes from the Southern California Bight gene pool enter the general ocean circulation. A more complete survey of the geographic distribution of transitional genotypes will be required to determine their global distribution. We propose that gene mixing occurs from Pacific to Atlantic at the present time, following the ocean surface circulation patterns. The dispersal of such genotypes around the oceans and the level of their genetic exchange could provide considerable information toward past ocean circulation patterns. Only when the global picture is more complete will it become possible to examine speciation processes within the marine environment.

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References

Bé, A. W. H., An ecological, zoogeographic and taxonomic review of recent planktonic foraminifera, in *Oceanic Micropaleontology*, vol. 1, edited by A. T. S Ramsay, pp. 1-100, Academic Press, San Diego, Calif., 1977.

Berggren, W. A., D. V. Kent, C. C. Swisher, and M. P. Aubry, A revised Cenozoic geochronology and chronostratigraphy, in *Geochronology: Time Scales and Global Stratigraphic Correlations*, edited by W. A. Berggren, D. V. Kent, and J. Hardenbol, *Spec. Publ. Soc. Econ. Paleontol. Mineral., 54*, 129-212, 1995.

Bijma, J., W. W. Faber, and C. Hemleben, Temperature and salinity limits for growth and survival of some planktonic foraminifers in laboratory cultures, *J. Foraminiferal Res., 20*, 95-116, 1990.

Bijma, J., C. Hemleben, B. T. Huber, H. Erlenkeuser, and D. Kroon, Experimental determination of the ontogenetic stable isotope variability in two morphotypes of *Globigerinella siphonifera* (d'Orbigny), *Mar. Micropaleontol., 35*, 141-160, 1998.

Blow, W. H., Late middle Eocene to Recent planktonic foraminiferal biostratigraphy, in *Proceedings of the First International Conference on Planktonic Microfossils*, vol. 1, edited by P. Bronnimann and H. H. Renz, pp. 199-422, , E. J. Brill, Leiden, Netherlands, 1969.

Blow, W. H., The Cainozoic Globigerinida: A study of the Morphology, Taxonomy and Evolutionary Relationships and the Stratigraphical Distribution of Some Globigerinida, 1413 pp., E. J. Brill, Leiden, Netherlands, 1979.

Bolli, H. M., and J. B. Saunders, Oligocene to Holocene low latitude planktic foraminifera, in *Plankton Stratigraphy*, vol. 1, edited by H. M. Bolli, J. B. Saunders, and K. Perch-Nielsen, pp. 155-262, Cambridge Univ. Press, New York, 1985.

Broecker, W. S., *Chemical Oceanography*, Harcourt Brace Jovanovitch, New York, 1974.

Chaisson, W. P., and P. N. Pearson, Planktonic foraminifer biostratigraphy at Site 925: Middle Miocene-Pleistocene, *Proc. Ocean Drill. Program Sci. Results, 154*, 3-32, 1997.

Conway Morris, S., The evolution of diversity in ancient ecosystems: A review, *Philos. Trans. R. Soc. London*, Series B, *353*, 327-345, 1998.

Cordey, W. G., The development of *Globigerinoides ruber* (d'Orbigny 1839) from the Miocene to recent, *Paleontology, 10*, 647-659, 1967.

Darling, K. F., D. Kroon, C. M. Wade, and A. J. Leigh Brown, Molecular phylogeny of the planktic foraminifera, *J. Foraminiferal Res., 26*, 324-330, 1996.

Darling, K. F., D. Kroon, C. M. Wade, and A. J. Leigh Brown, Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa, *Mar. Micropaleontol., 30*, 251-266, 1997.

Darling, K. F., D. Kroon, I. Stewart, C. M. Wade, R. Dingle, T. Paramor, C. Pudsey, and J. Bijma, Planktic foraminiferal genotype distribution: Gene flow and past ocean circulation, paper presented at 6th International Conference of Paleoceanography, EXPO'98, Lisbon, Portugal, 1998.

de Vargas, C., L. Zaninetti, H. Hilbrecht, and J. Pawlowski, Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record, *J. Mol. Evol., 45*, 285-294, 1997.

de Vargas, C., R. Norris, L. Zaninetti, and J. Pawlowski, Cryptic diversity and speciation in the open ocean, *Proceedings of the International Symposium on Foraminifera: Forams' 98*, edited by J. F. Longoria and M. A. Gamper, pp. 25, Soc. Mexicana de Paleontol., Monterrey, Mexico, 1998.

Felsenstein, J., Evolutionary trees from DNA sequences: A maximum likelihood approach, *J. Mol. Evol., 17*, 368-376, 1981.

Felsenstein, J., Confidence limits on phylogenies: An approach using the bootstrap, *Evolution, 39*, 783-791, 1985.

Felsenstein, J., *PHYLIP: Manual Version 3.52c*, Berkeley Univ. Herbarium, Univ. of Calif., Berkeley, 1993.

Fitch, W. M., Toward defining the course of evolution: Minimum change for a specified tree topology, *Syst. Zool., 20*, 406-416, 1971.

Fitch, W. M., and E. Margoliash, Construction of phylogenetic trees: A method based on mutation distances as estimated from cytochrome c sequences is of general applicability, *Science, 155*, 279-284, 1967.

Hecht, A. D., A. W. H. Bé, and L. Lott, Ecological and paleoclimatic implications of morphologic variation of *Orbulina universa* in the Indian Ocean, *Science, 194*, 422-424, 1976.

Hillis, D. M., and J. J. Bull, An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis, *Syst. Biol., 42*, 182-192, 1993.

Hillis, D. M., C. Moritz, and B. K. Mable (Eds.), *Molecular Systematics*, 2nd ed., Sinauer Assoc., Inc., Sunderland, Mass, 1996.

Holzmann, M., and J. Pawlowski, Preservation of foraminifera for DNA extraction and PCR amplification, *J. Foraminiferal Res.*, *26*, 264-267, 1996.

Huber, B. T., J. Bijma, and K. F. Darling, Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny), *Paleobiology*, *23*, 33-62, 1997.

Kennett, J. P., and M.S. Srinivasan (Eds.), *Neogene Planktonic Foraminifera: A Phylogenetic Atlas*, Hutchinson and Ross, Stroudsburg, Penn., 1983.

Kishino, H., and M. Hasegawa, Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data and the branching order of the Hominoidea, *J. Mol. Evol.*, *4*, 406-425, 1989.

Pawlowski, J., I. Bolivar, J. Fahrni, T. Cavalier-Smith, and M. Gouy, Early origin of foraminifera suggested by SSU rRNA gene sequences, *Mol. Biol. Evol.*, *13*, 445-450, 1996.

Pawlowski, J., I. Bolivar, J. Fahrni, C. de Vargas, M. Gouy, and L. Zaninetti, Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record, *Mol. Biol. Evol.*, *14*, 498-505, 1997.

Pearson, P. N., A lineage phylogeny for the Paleogene planktonic foraminifera, *Micropaleontology*, *39*, 193-232, 1993.

Saitou, N., and M. Nei, The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.*, *4*, 406-425, 1987.

Smith, S. W., R. Overbeek, C. R. Woese, W. Gilbert, and P. M. Gillevet, The genetic data environment and expandable GUI for multiple sequence analysis, *Comp. Appl. Biosci.*, *10*, 671-675, 1994.

Spezzaferri, S., and I. Premoli Silva, Oligocene planktonic foraminiferal biostratigraphy and paleoclimate interpretation from hole 538A, DSDP Leg 77, Gulf of Mexico, *Palaeogeogr. Palaeoclimatol. Palaeoecol.*, *83*, 217-263, 1991.

Swofford, D. L., *PAUP 3.1: User Manual*, Smithsonian Inst., Washington, D.C., 1993.

Tappan, H., and A. R Loeblich, Foraminiferal evolution, diversification and extinction, *J. Paleontol.*, *62*, 695-714, 1988.

Thompson, P. R., A. W. H. Duplessy, and N. J. Shackleton, Disappearance of pink-pigmented *Globigerinoides ruber* at 120,000 years BP in the Indian and Pacific Oceans, *Nature*, *280*, 554-558, 1979.

Wade, C. M., K. F. Darling, D. Kroon, and A. J. Leigh Brown, Early evolutionary origin of the planktic foraminifera inferred from SSU rDNA sequence comparisons, *J. Mol. Evol.*, *43*, 672-677, 1996.

J. Bijma, Department of Geosciences, University of Bremen, P.O. Box 330 440, D-28334, Bremen, Germany. (e-mail: jbijma@uni-bremen.de)

K. F. Darling and D. Kroon, Department of Geology and Geophysics, Grant Institute, University of Edinburgh, West Mains Road, Edinburgh EH9 3JW, Scotland, U.K. (e-mail: kdarling@glg.ed.ac.uk; dkroon@glg.ed.ac.uk)

A. J. Leigh Brown, Centre for HIV Research, University of Edinburgh, Edinburgh EH9 3JN, Scotland, U.K. (e-mail: A.Leigh-Brown@ed.ac.uk)

C. M. Wade, Department of Genetics, University of Nottingham, Nottingham NG7 2UH, England, U.K. (e-mail: pdzcmw@ppdcmw.nottingham.ac.uk)

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