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# A molecular approach to biodiversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny)

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**Abstract**

DNA-based studies have revealed that biodiversity has been underestimated in most groups of pelagic organisms. Recently, higher diversity has also been molecularly detected in planktonic foraminifera, which challenges the morphological concepts applied to define the species level in this group, and provides new insight in interpretation of their fossil record. Here, we present a genetic analysis based on small subunit ribosomal DNA genes from 189 individuals of the morphospecies *Globigerinella siphonifera*, collected along an Atlantic transect covering 57° of latitude. Four different, strictly homogeneous genotypes, called types I, II, III and IV, were detected. Their origin occurred in the late Miocene according to the absolute molecular clock estimation based on genetic distances calibrated within the fossil record. Biogeographic analysis shows that each *G. siphonifera* genotype is adapted to a specific environment: type I prefers oligotrophic waters, type II, although cosmopolitan, is probably adapted to mesotrophic waters, types III and IV live in highly productive waters. This emphasizes the importance of the water column stability and resulting trophic structure for planktonic foraminiferal evolution. The phenomenon of adaptive radiation of slightly different morphotypes described here in *G. siphonifera* is compared to analogous evolutionary patterns molecularly revealed in two other planktonic foraminifera, *Orbulina universa* and *Globorotalia truncatulinoides*. It implies that closely related morphotypes within classically defined planktonic foraminiferal taxa may be different species and can be markers of highly different environments. 

*Keywords:* planktonic foraminifera; ribosomal DNA; biogeography; biodiversity

## 1. Introduction

Planktonic foraminifera appeared ~160 Ma and their exceptionally complete, continuous fossil record offers one of the rare models for studying evolution and ecology at the geological timescale in a group of organisms. The great potential

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of the foraminiferal fossil archive as a stratigraphic and paleoceanographic tool rapidly resulted in a taxonomy exclusively based on morphological characters of the calcareous tests, overlooking the biological characters of the cells. The primary goal of this morphotaxonomic system was to create easily recognized, broadly distributed taxa with adequate resolution for use in stratigraphic and paleoceanographic studies. Thus, prior taxonomists have chosen to set the morphological definition of species broadly, interpreting sub-species level of morphologic variation as ecophenotypy, and thus of questionable value for biology, stratigraphy, or paleoceanography. According to the current morphotaxonomy, most planktonic foraminiferal species are cosmopolitan and occupy circum-global latitudinal climatic provinces (tropical, subtropical, temperate, subpolar or polar). The number of living morphospecies is approximately 50, according to Hemleben et al. (1989).

In recent years, DNA sequence analysis has provided new tools for further defining the boundaries of biological species (between which gene flow has been interrupted) in groups of organisms that are morphologically poorly differentiated. The molecular approach of marine biodiversity demonstrates a serious underestimation of the number of species in various groups of pelagic microorganisms (Miya and Nishida, 1997; Moore et al., 1998), including the planktonic foraminifera (Huber et al., 1997; De Vargas et al., 1999; Darling et al., 1999). For instance, De Vargas et al. (1999) analyzed ribosomal DNA sequences from 137 individuals of the morphospecies *Orbulina universa* collected over a large geographic scale in the Atlantic. They detected three cryptic, largely divergent, genetic species having a patchy distribution related to specific water-mass adaptation. This discovery demonstrates the importance of assessing the degree of cryptic genetic diversity in other morphospecies of planktonic foraminifera, and to analyze the geographic and ecologic significance of this diversity.

The species analyzed herein is *Globigerinella siphonifera*, a widely distributed, spinose planktonic foraminifer. *G. siphonifera* appears in the middle Miocene record and descends from a morphologically slightly different species, *Globigerinella praesiphonifera*, which itself originated in the early Miocene (Kennett and Srinivasan, 1983; Bolli et al., 1985). The modern biogeographic distribution of *G. siphonifera* ranges from the Southern to the Northern Hemisphere temperate waters (Lee and Anderson, 1991). It is a surface dweller, which tolerates salinity from 27 to 45‰ and temperature from 11°C to 30°C (Bijma et al., 1990a,b).

The taxonomic status of *Globigerinella siphonifera* is ambiguous. Its morphological variability is relatively high and different morphotypes have long been recognized, being described as ecophenotypes of this species (Parker, 1962). Parker (1962) was the first to suggest that *G. siphonifera* could be divided into two groups on morphological criteria. Faber et al. (1988, 1989) observed two different chrysophycophyte endosymbiotic algae in *G. siphonifera* and attributed them to two different types of hosts. Recently, Huber et al. (1997) and Bijma et al. (1998) performed a detailed morpho-genetic study on *G. siphonifera* specimens collected by SCUBA divers in Curacao and distinguished two groups presenting differences in rhizopodal arrangement, spine density, shell color, size and porosity, photosynthetic pigment content, stable isotope composition, and small subunit ribosomal DNA (SSU rDNA) sequences. These numerous physiologic, morphologic and genetic lines of evidence indicate that the two groups correspond to different species, named type I and type II (Huber et al., 1997). A third type from Southern California – named type IIb, while type II was renamed type IIa – was detected on the basis of SSU rDNA sequences by Darling et al. (1999). It appears thus from both previous morphologic and biologic research that the morphological species definition of *G. siphonifera* does not fully reflect the biological complexity of the species.

Here, we propose a new approach to study the genetic diversity of *Globigerinella siphonifera*, which consists in analyzing DNA sequence diversity from a high number of individuals collected each 3–4° latitude along an oceanic basin wide transect. This method allows rapid detection of genetic diversity within a species and gives a snapshot of its large-scale geographic structure. We also compared our data to the hydrography of the sampling sites in order to estimate which factor(s) control the distribution of *G. siphonifera* genotypes across the Atlantic Ocean.

## 2. Materials and methods

### 2.1. Organism collection and DNA extraction

Samples were collected with plankton nets (63–200-μm mesh size) between 175 m depth and the sea surface. Most data were obtained on board the *James Clark Ross*, between Ascension Island and the UK on the Atlantic Meridional Transect (Robins and Aiken, 1996) cruise 8 (AMT-8, May–June 1999) during which we took special care in isolating all specimens with a *Globigerinella siphonifera* morphology (Table 1). Specimens were also collected between the UK and the Falklands Islands during AMT-5 (September–October 1997), as well as in Puerto Rico (March 1995), Villefranche s/Mer (France, December 1995), Bermuda (April 1996), Santa Barbara (US, February–March 1998) and Eilat (Israel, May 1999). All living *G. siphonifera* were isolated from the total plankton with a dissecting microscope and transferred to Petri dishes containing filtered sea water. Specimens were then individually cleaned with a brush to eliminate spines, detritus and microorganisms at their surface. Total DNA extractions were performed on the day of collection to avoid cell degradation that occurs when planktonic organisms are removed from the water column. Organisms were individually ground in 40 μl of a guanidinium thiocyanate-based DNA extraction buffer as described in Chomczynski and Sacchi (1987). DNA extracts were preserved at −20°C.

### 2.2. DNA amplification and sequencing

A ∼950-bp fragment localized at the 3′ end of the SSU rDNA was amplified by PCR using the foraminiferal specific primers S14p (De Vargas et al., 1997) or S14f3 (5′-ACGCA(AC)GTGTGAA-ACTTG-3′) coupled with SBf (5′-TGATCCATC-(AG)GCAGGTTCACCTAC-3′). The large taxo-

Table 1
Location of the sampling sites on the Atlantic Meridional Transect cruise 8, where 80% of the *Globigerinella siphonifera* were collected

| Station no. | Date | Latitude | Longitude | Types of *Globigerinella siphonifera* | Oceanic province (abbreviation) |
|---|---|---|---|---|---|
| 1 | 18.5.99 | 7.38°S | 14.31°W | II | South Equatorial Current (SEqC) 15°S–1°N |
| 2 | 19.5.99 | 4.07°S | 15.31°W | II | |
| 3 | 20.5.99 | 0.04°N | 16.42°W | II | |
| 4 | 21.5.99 | 3.53°N | 17.53°W | II, I | North Equatorial Counter Current (NECC) 1°N–8°S |
| 6 | 22.5.99 | 7.5°N | 18.59°W | II, I, IV | |
| 7 | 23.5.99 | 11.33°N | 20.09°W | II, III | Guinea Dome (GDom) 8°N–12°N |
| 8 | 24.5.99 | 15.09°N | 21°W | III, IV, II | North Equatorial Current (NEqC) 12°N–16°N |
| 9 | 25.5.99 | 18.48°N | 21°W | III, II | Canary Current Upwelling (CCUp) 16°N–20.5°N |
| 10 | 26.5.99 | 22.55°N | 21°W | III, II | Canary Current (CanC) 20.5°N–25/27°N |
| 14 | 29.5.99 | 34.22°N | 20.47°W | II | North Atlantic Subtropical Gyre-East (NASE) 26/27°N–38°N |
| 15 | 29.5.99 | 34.33°N | 20.41°W | II, I | |
| 16 | 30.5.99 | 38.1°N | 20°W | II | |
| 17 | 31.5.99 | 41.51°N | 20°W | II, III | North Atlantic Drift (NAtD) 38°N–48°N |
| 19 | 1.6.99 | 47°N | 20°W | II, III, IV | |
| 20 | 2.6.99 | 47.27°N | 19.39°W | III, II, IV | |
| 21 | 2.6.99 | 47.32°N | 14.46°W | III | |
| 22 | 3.6.99 | 48.08°N | 9.39°W | III | European Continental Shelf Water (ECSW) >48°N |

For each station, the date, the genotypes of *Globigerinella siphonifera* found, and the corresponding physical oceanic province as defined by Hooker et al. (2000) on several AMT cruises are listed.

nomic spectrum of these primers within the foraminifera (they can also amplify SSU rDNA from miliolid and allogromid foraminifera, which diverged from the rotaliid taxa several hundred million years ago) minimizes the likelihood of missing potential paralogous SSU rDNA clusters. Amplified products were then purified using the 'High Pure PCR Purification kit' (Roche) and directly sequenced on an ABI 377 Prism sequencer with the primers used for PCR amplification. In order to detect the different genetic groups, we started by sequencing ~500-bp fragments for 25 *Globigerinella siphonifera* from different water masses along the transect in the Atlantic, in the Caribbean, and Mediterranean. The whole DNA fragment (~950 bp) of four different genotypes was then sequenced, for phylogenetic analysis with other species of planktonic foraminifera, and for designing the restriction fragment length polymorphism (RFLP) protocol (see below). This fragment contains a mosaic of six variable and six conserved zones that constitute a sufficient portion of the whole gene to perform reliable phylogenetic analyses in planktonic foraminifera (De Vargas and Pawlowski, 1998; Darling et al., 1999). The amplified fragment contains the helices 32–50 of the universal SSU rRNA secondary structure model (Van de Peer et al., 1996). We checked for the presence of systematic compensatory mutations in the helices to confirm that our sequences are not non-functional pseudogenes. The clearness of the single-cell direct DNA sequences and abundant RFLP patterns, in which we never observed double or hybrid signals, strongly suggests that the different genotypes do not represent different alleles or paralogous copies within a single genome. The new sequences reported in this paper have been deposited in the GenBank database (accession numbers AJ251212, AJ251213, Z83959, Z83960 and AJ390558–AJ390580).

### 2.3. DNA sequences analyses

*Globigerinella siphonifera* SSU rDNA sequences were manually aligned to those of other related species of spinose planktonic foraminifera (GenBank accession numbers Z83961, Z83962, Z83964, Z83966, Z83967, U65634) using the Genetic Data Environment (GDE) 2.2 software (Larsen et al., 1993). Phylogenetic trees were built using 528 unambiguously aligned sites, using the maximum likelihood method (ML, Felsenstein, 1981) and the neighbor-joining method (NJ, Saitou and Nei, 1987) with distances corrected for multiple hits according to the Tajima and Nei substitution model (Tajima and Nei, 1984). ML and NJ distance analyses were performed using PAUP*, version 4.0b6 (Swofford, 2000). All sites with gaps were excluded from the analyses. For ML analysis, the MODELTEST program (Posada and Crandall, 1998) was used to compare the likelihood scores between the different nucleotide substitution models of the nested General Time Reversible (GTR) family, and find the model that best fits our data. Among-sites rate heterogeneity using the Γ-distribution (Yang, 1993), with and without the estimated proportion of invariable sites (Γ+I; Gu et al., 1995), was also examined using MODELTEST. A GTR+G model of nucleotide substitution was finally used with the Branch-and-Bound algorithm of reconstruction implemented in PAUP*. Non-parametric bootstrapping (Felsenstein, 1985) was performed with 1000 replicates for both ML and distance analyses. Distance computations were achieved with the PHYLO_WIN program (Galtier and Gouy, 1996). In order to assess if alternative phylogenetic hypotheses between the *G. siphonifera* genotypes were significantly different, the KH test (Kishino and Hasegawa, 1989) was performed to compare ML trees constrained with different topologies, using PAUP*.

The relative congruence between our molecular tree and the phylogenetic hypotheses based on strato-phenetic analyses of the worldwide foraminiferal fossil record (Kennett and Srinivasan, 1983) allowed us to calibrate the deep nodes of the tree in geological time. Divergence times of the four *Globigerinella siphonifera* genotypes were first estimated by calculating individual lineage absolute rates of nucleotide substitution (or local molecular clocks). Rates were measured by simply dividing, on the best ML tree, the length of the branches (in number of substitution per nucleotide) leading to the modern species by their duration (in million years) based on stratigraphic time. Alternatively, the null hypothesis of clock-like behavior among sequences was statistically tested. Relative rate tests (RRT) were performed using the RRTree program version 1.1 (Robinson-Rechavi and Huchon, 2000) in order to detect (1) if one or several of the phylogenetic clusters in the tree evolved significantly faster than other(s) and (2) which individual sequence may have an abnormal rate of substitution. Likelihood ratio tests (LRT) were also conducted using PAUP* to compare the scores of ML trees conducted with and without a molecular clock assumption, and excluding or not the 'fast-clock' sequences. Mean substitution rates were then calculated from ultrametric ML trees constructed with a molecular clock assumption, and used to estimate the divergence times of the *G. siphonifera* genotypes.

### 2.4. RFLP analysis

After DNA sequence analyses, two restriction enzymes that cut the nucleotide sequence at a specific pattern were selected in order to rapidly discriminate between the different *Globigerinella siphonifera* genotypes. PCR products digestions were performed using the endonucleases *Sau*96I and *Hae*III (Roche), according to the following protocol: 12.5 μl of the ∼950-bp SSU rDNA PCR products were directly digested for 5 h at 37°C in a total volume of 25 μl containing 2.5 μl of the diluted enzyme (1.25 U), 2.5 μl of 10× buffer (Roche) and 7.5 μl of distilled $H_2O$. Distinct patterns for each genotype were UV-detected after migration of the digested PCR products on 2% agarose gel, and ethidium bromide coloration.

### 2.5. Conductivity, temperature and depth (CTD) data and chlorophyll concentration

During the AMT-8 cruise, sea water samples were continuously collected at a depth of 7 m from a non-toxic underway supply. Mixed layer temperature and salinity data were obtained from a CTD connected to the water supply. In addition, phytoplankton was harvested every 2 h by filtering 2000- to 5000-ml samples through 25-mm GF/F filters using positive-pressure filtration. Pigments were extracted from the filters into 90% acetone by ultrasonication and centrifugation and filtered through Teflon syringe filters to remove debris. Extracts were then analyzed for a range of photosynthetic and photoprotectant pigments by reverse-phase high performance liquid chromatography (HPLC) as described in Barlow et al. (1997).

## 3. Results

### 3.1. Genetic diversity in Globigerinella siphonifera

We first sequenced partial SSU rDNA (∼500 bp) from 25 *Globigerinella siphonifera* specimens collected in the Caribbean (Puerto Rico), Mediterranean (Villefranche s/Mer) and Atlantic (AMT-5 and Bermuda). The sequences clustered into four distinct types with almost no detectable variation within each type (see below), even in the most variable region of the gene (sequences alignment available upon request to the senior author). The comparison of our DNA sequences to those available in the GenBank indicates that three of our genetic types are strictly identical to the *G. siphonifera* genotypes I, IIa (Huber et al., 1997) and IIb (Darling et al., 1999). The fourth type corresponds to a still undiscovered and distantly related genotype. We adopted a simple, one-level taxonomic nomenclature (I, II, III, IV) to name the different *G. siphonifera* genotypes. This system, where we renamed the type IIb of Darling et al. (1999) 'type III', and named our new genotype 'type IV', has the advantages to preserve the classical and abundantly described *G. siphonifera* types I and II, and to be more adaptable in case new *G. siphonifera* genotypes are discovered at different seasons or areas in the global Ocean.

SSU rDNA sequences from one representative of each genotype were then elongated to ∼950 bp, aligned to their homologues from other species of spinose planktonic foraminifera, and phylogenetically analyzed (Fig. 1). The four genotypes cluster in a monophyletic group supported by 95/100% bootstrap values (ML/NJ methods).

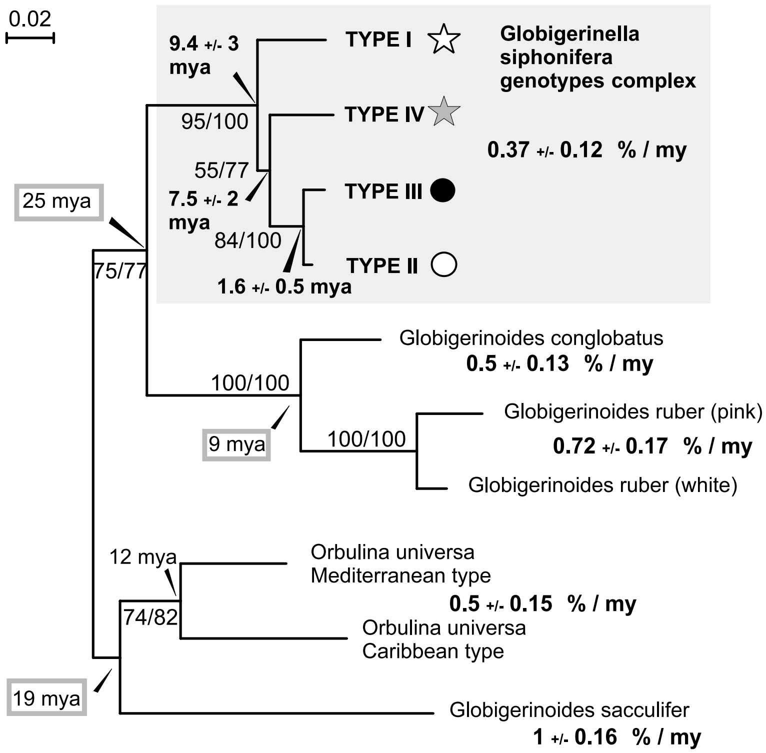


Fig. 1. SSU rDNA phylogenetic relationships (ML tree) between the four *Globigerinella siphonifera* genotypes (symbolized by circles and stars, in reference to Fig. 3) and six other species of spinose planktonic foraminifera. The boxed divergence times are based on the fossil record. Bold numbers on the right of the tree give the mean rates of DNA substitution with their standard errors for each lineage. The divergence times of the four *G. siphonifera* genotypes, estimated using lineage-specific molecular clocks, are indicated in italic. Bootstrap percentages (1000 replicates) for ML and NJ methods are given next to each internal branch of the tree. Scale is in substitutions per site. Note: the sequences from the *G. siphonifera* types I, II, III and IV come from individuals respectively collected in Puerto Rico, Villefranche s/Mer, AMT-5 station 2, and AMT-8 station 8. The two different genotypes of *Orbulina universa* were described elsewhere (De Vargas et al., 1999).

Their phylogenetic relationships are as follows: type I branches in a basic ancestral position, followed by type IV, which gives rise to the two closely related genotypes II and III (Fig. 1). This topology is sustained by relatively high bootstrap values, and is stable in both ML and NJ analyses. However, the relative position of the genotype IV is not evident, as alternative branching hypotheses – i.e. (1) type IV in an ancestral position, and (2) type IV in a monophyletic group with type I – are not significantly rejected using a Kishino–Hasegawa test ($P = 0.2801$ for both hy-

Table 2
Pairwise genetic distances (Tajima and Nei, 1984) in % of nucleotide difference and number of observed substitutions between the ~950-bp SSU rDNA fragments of the four *Globigerinella siphonifera* genotypes

| *Globigerinella siphonifera* | Type I | Type II | Type III | Type IV |
|---|---|---|---|---|
| Type I | 0 | 1.7 | 1.6 | 1.6 |
| Type II | 20.7/180 | 0 | 0.8 | 1.5 |
| Type III | 19.4/170 | 5.4/52 | 0 | 1.4 |
| Type IV | 18.7/163 | 15.8/141 | 14.2/128 | 0 |

Above the diagonal are the standard errors of the genetic distances.

potheses 1 and 2). The genetic distances between genotypes are given in Table 2. While type I is more or less equally distant (∼19%) from all other types, there is a ∼15% nucleotide divergence separating type IV from types III and II, and a 5.4% divergence between type II and type III.

In contrast to these high inter-genotypes genetic distances, almost no differences were observed between the 500-bp DNA sequences from individuals within a same genotype, despite the huge geographic distances and even tectonic barriers separating the sites where the individuals were collected. For instance, we obtained SSU rDNA from type I individuals collected at AMT-5 station 20, AMT-8 station 6 (Table 1) and Santa Barbara, and from type II individuals sampled in Bermuda, Eilat (Red Sea), Villefranche s/Mer (Mediterranean sea), AMT-5 stations 7 and 25, and AMT-8 station 3. Despite this global sampling, the intra-genotype variability came down to only one mutation between two type II specimens. This difference may be due to allelic polymorphism or Taq polymerase errors, rather than reflect the evolutionary history of orthologous gene sequences.

### 3.2. *Rate of DNA substitution and divergence times of the Globigerinella siphonifera genotypes*

The topology of the molecular tree (Fig. 1) is congruent with the stratigraphic and morphologic phylogenetic interpretations of the planktonic foraminiferal fossil record in several points: (1) the close relationship between the *Orbulina* lineage and *Globigerinoides sacculifer* (Bolli et al., 1985), (2) the close relationship between *Globigerinoides conglobatus* and *Globigerinoides ruber* (Cordey, 1967), (3) the relatively equal distances (genetic or geologic) between the main spinose genera (here *Globigerinella*, *Globigerinoides* and *Orbulina*/*G. sacculifer*) and (4) the relatively recent separation between the white and pink varieties of *G. ruber*, that exists since at least the middle Pleistocene (700 000 yr, Thompson et al., 1979). These several homologous phylogenetic points between fossil and molecular data sets allowed us to calibrate the SSU rDNA tree with stratigraphic dates and estimate lineage-specific rates of substitution

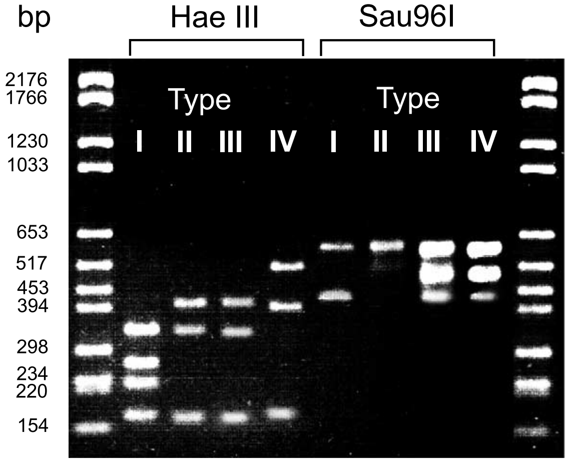


Fig. 2. Distinction of the *Globigerinella siphonifera* genotypes by using PCR-based RFLP patterns. Digested PCR products are colored after migration on a 2% agarose gel. The restriction enzyme *Hae*III distinguishes types I, II+III, and IV, while *Sau*96I distinguishes types I, II, and III+IV.

(Fig. 1). We first used the *Globigerinella siphonifera* lineage-specific rate of 0.37 ± 0.12% of substitution per million years to infer the ages of the four genotypes under the hypothesis of a constant lineage substitution rate (Fig. 1).

In case DNA sequences show a clock-like behavior, an alternative approach for estimating the divergence times is to calculate an overall molecular clock on an ultrametric tree built with a clock assumption and using one calibration date based on fossil data. An RRT using the *Globigerinoides sacculifer* sequence as outgroup and comparing the substitution rates between the main phylogenetic groups, i.e. the *Orbulina* cluster, the *Globigerinoides ruber*–*Globigerinoides conglobatus* group, and the *Globigerinella siphonifera* complex, indicated that none of the lineages evolved significantly faster, the lowest *P*-value being 0.076 (*Orbulina* versus *G. ruber*–*conglobatus*). An RRT comparing all sequences individually showed that *G. sacculifer* and *G. ruber* pink may evolve out of clock, only if a relatively high risk threshold is used ($P < 0.05$). The lowest *P*-values were 0.01, 0.023, and 0.029, respectively, for the *G. siphonifera* II/*G. ruber* pink, *G. siphonifera* III/*G. ruber* pink, and *G. siphonifera* II/*G. sacculifer* comparisons. The LRT to compare ML trees with

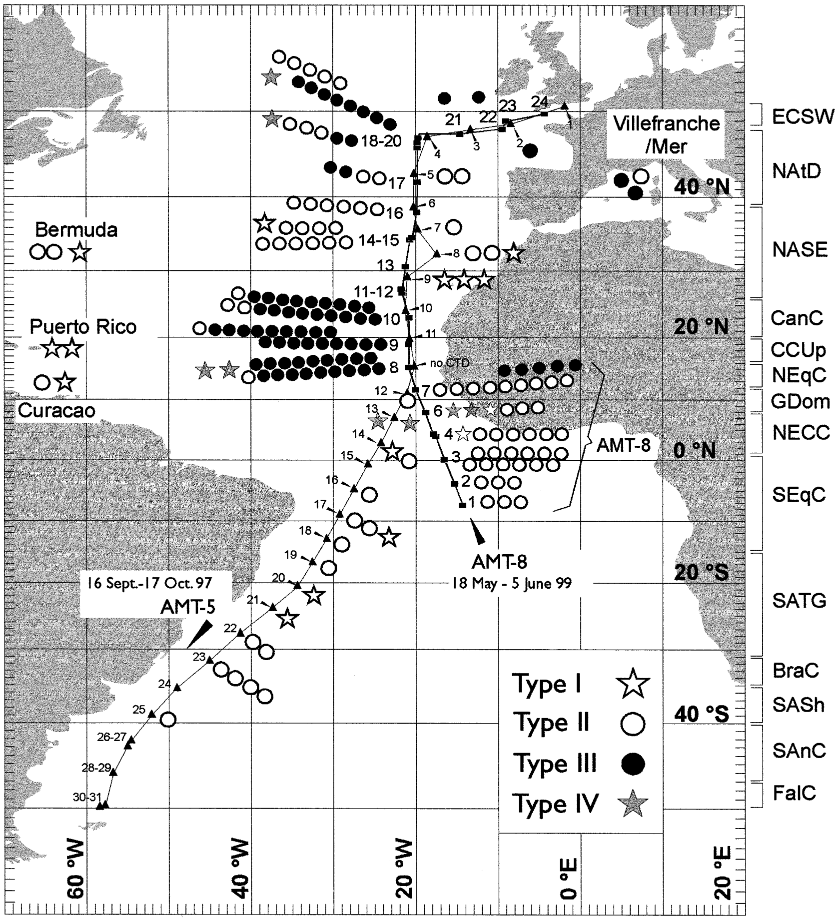


Fig. 3. Distribution map of the four genotypes of *Globigerinella siphonifera*. Each circle or star represents one individual. Types I and II from Curacao were described by Huber et al. (1997). All AMT-5 *G. siphonifera* are depicted on the right of the transect, while AMT-8 *G. siphonifera* are on the left, except for the first seven stations. AMT-8 stations (black squares) are numbered from South to North (1–24); AMT-5 stations (black triangles) are numbered in italic from North to South (1–31). On the right on the map are located the oceanic provinces defined on water-density on several AMT cruises (Hooker et al., 2000; see Table 1 caption for definitions of abbreviations).

and without clock assumption resulted in $P$-values of 0.016 (including all sequences) or 0.41 (excluding the relatively 'fast-clock' *G. sacculifer* and *G. ruber* pink). Both values do not reject the null hypothesis of substitution rate stationary, at $P < 0.01$. By dating the splitting between the *G. ruber–conglobatus* and the *G. siphonifera* clusters at 25 Ma, a global molecular clock of 0.5% per million year was inferred. This clock indicates that the ancestor of the *G. siphonifera* types II, III and IV diverged 7.5 Ma from *G. siphonifera* type I. Type IV diverged 5.6 Ma from the ancestor of types II and III, and the splitting between types II and III occurred 1.2 Ma. The clock gives also a date of 10 Ma for the separation time between *G. conglobatus* and the *G. ruber* lineages, which corresponds well to the time inferred from fossil data (Cordey, 1967) and confirms its relative accuracy.

### 3.3. Geographic and hydrographic distribution

As it covers 100° of latitude, the AMT transect crosses several major hydrographic features: tem-

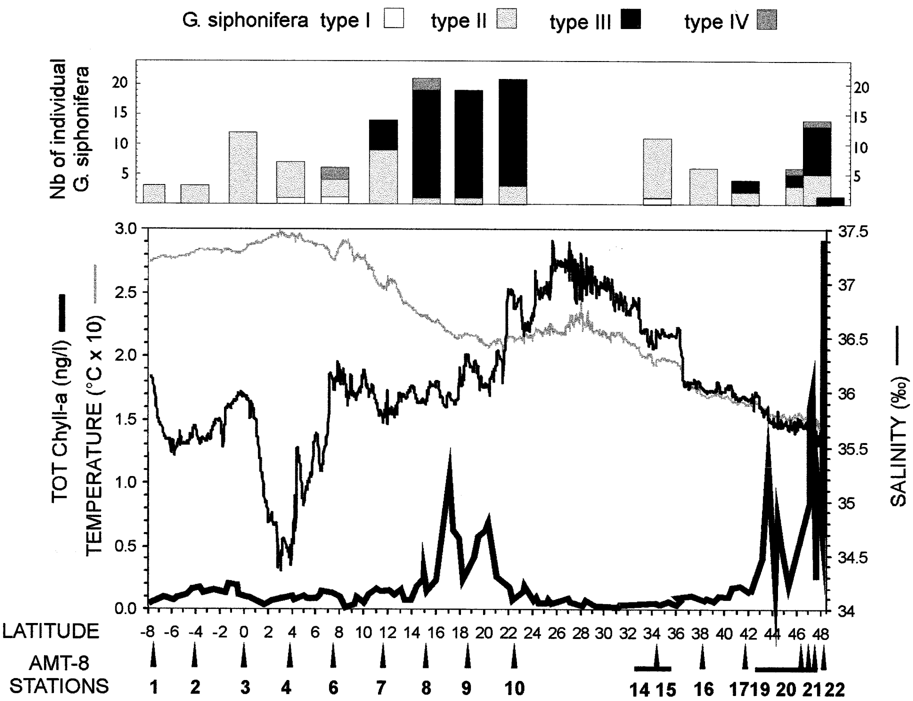


Fig. 4. Latitudinal occurrence and relative abundance of the four *Globigerinella siphonifera* genotypes, as well as sea surface total chlorophyll *a* concentration, temperature and salinity along the Atlantic Meridional Transect cruise number 8 (AMT-8). Latitudes are given from South to North. Although the sampling effort was not strictly the same at each station, the number and relative abundance of the *G. siphonifera* are still indicative of which genotype dominates at each station.

perate highly productive waters, subtropical and tropical gyres with stratified and oligotrophic waters, nutrient rich and productive upwelling waters (African coast), southern subantarctic waters, as well as several frontal zones due to currents confluence that generates mesoscale eddies as well as vertical mixing.

Once the four *Globigerinella siphonifera* genotypes were identified, we developed a more rapid and economical PCR-based RFLP method for their discrimination without sequencing (Fig. 2). This approach allowed us to genetically characterize 164 additional *G. siphonifera* specimens across the Atlantic, whose distribution is shown in Fig. 3. Although most sampling sites contain a mixture of two to three genotypes, the global distribution of each type is obviously not random.

Except for type II that seems cosmopolitan at a first glance (we found it in the Pacific–Santa Barbara Basin, and Red Sea–Eilat, and Darling et al. (1999) described it form the Coral Sea, Australia, and Southern California Bight), the three other types are confined to more restricted areas. Type I is typically found in tropical or subtropical regions (Puerto Rico, Curacao, Bermuda, AMT-5 station 14, AMT-8 stations 4 and 6); during AMT-8, it was mostly sampled in the northern and southern tropical gyres – NASE and SATG provinces (Hooker et al., 2000). Type III is strongly associated with highly productive waters, either in the North Atlantic transitional waters (AMT-8 stations 17–22 and AMT-5 station 2), or in areas of coastal upwelling at Villefranche s/Mer or near the African coast (AMT-8 stations 8–10), where it is clearly dominant. The occurrence of type III at AMT-8 station 7 corresponds to the record of a mid-ocean upwelling between 12 and 8°N at this site (Hooker et al., 2000). Type III was also found in the Southern California Bight by Darling et al. (1999). It is also the only type of *Globigerinella siphonifera* found north of 48°N, close the shelf break (ECSW province).

Type IV was also found in highly productive waters, in sympatry with type III (NAtD and CCUp provinces, Fig. 3) or close to them in meso-productive waters (AMT-8 station 6 and AMT-5 station 13); however, it was very rare in all samples.

To further define which parameter(s) could influence the genotypic distribution, we compared the presence (and to some extent abundance) of the different types to the quantitative surface water data obtained from the HPLC (photosynthetic and photoprotectant pigments) and the CTD (temperature and salinity) along the AMT-8 transect where most genetic data were obtained (Fig. 4). A correlation is observed between total chlorophyll *a* concentration and the presence or absence of all types. This correlation is particularly visible in case of type III, whose range and abundance are strongly coincident with the two peaks of chlorophyll *a* concentration along latitudes, and in case of type I which occurs in chlorophyll poor waters (Fig. 4). This last correlation is more obvious when the distribution of type I specimens is compared to chlorophyll concentrations along AMT-5 (De Vargas, 2000). On the other hand, the physical factors like sea surface temperature and sea surface salinity do not seem to be of direct importance for genotype distribution (Fig. 4).

As the AMT-5 and AMT-8 cruises took place during the boreal autumn and spring, respectively, we can estimate the possible seasonal effect on genotype distribution. Such information is limited by the relatively low amount of genetic data on *Globigerinella siphonifera* from AMT-5. Nevertheless, there are cases of mirror occurrences of the same genotypes at the same latitude on both cruises, as for instance the presence of type III at the northernmost stations, or the presence of type I in the NASE province (Fig. 3). Despite a very scarce sampling, type IV was also collected at AMT-8 station 6 and AMT-5 station 13, which may reflect a persisting presence of this genotype in this water mass. The only major seasonal difference between both cruises concerns the abundant presence of type III in the African upwelling in May, while no *G. siphonifera* was found in this area in September. The Mauritanian upwelling is a seasonal phenomenon related to the trade winds blowing effectively during the first part of the year and then starting to subside toward the end of the summer. The high abundance of type III individuals within the spring upwelling and its complete disappearance in September suggest that type III growth is seasonally controlled by upwelling intensity.

## 4. Discussion

### 4.1. Four species within *Globigerinella siphonifera*?

The taxonomic rank to which belong the four genotypes detected within the traditional morphospecies *Globigerinella siphonifera* is not yet clear. The systematic interpretation is limited by our single gene analysis, and by the fact that SSU rDNA does not evolve fast enough to produce variation at the population level (De Vargas et al., 2001). However, our data suggest that the modern morphospecies *G. siphonifera* may in fact be a complex of at least four different species. First, we never detected more than one genotype – or hybrid genotypes – within a single individual (using RFLP or direct sequencing), which suggests that the multiple SSU rDNA copies arranged in tandem arrays within an individual genome are highly homogeneous and evolve through a process of concerted evolution like in most organisms. Second, we observed no mutation between individuals within each genotype, even if they come from extremely remote areas separated by tectonic or hydrographic barriers, like the type II cells collected in the Pacific, Atlantic, Mediterranean and Red Sea. Mostly, this genetic and geographic homogeneity for each SSU rDNA type is maintained despite sympatric occurrence of two or more genotypes at most geographic sites (Fig. 3), which argues against gene flow between the genotypes.

In contrast, the genetic distances between the four genotypes – between 5 and 20% – are very important, considering that the SSU rDNA is one of the most conservative among all known genes. Such genetic distances distinguish supra-specific taxonomic status (genus or even higher level) in most groups of organisms. Moreover, the genetic distances between *Globigerinella siphonifera* genotypes are also of the same order of magnitude as the distances separating species of spinose planktonic foraminifera that are morphologically clearly recognized as many million years old distinct lineages in the fossil record. For instance, the genetic distances between *G. siphonifera* type I and all other types are similar to the distance between *Globigerinoides ruber* and *Globigerinoides conglobatus*; likewise, while 170 DNA fixed substitutions distinguish *G. siphonifera* type I from type II, 104 fixed substitutions separate the *G. ruber* pink and white varieties, which are known to exist at least since the middle Pleistocene (Thompson et al., 1979). In fact, the molecular clock calibrated on stratigraphic time indicates that the different genotypes are relatively old: a main radiation occurred during the late Miocene, between 8 and 5 Ma, followed by the genotypes II/III separation that happened between 1 and 2 Ma.

The genetic arguments in favor of considering the *Globigerinella siphonifera* types as separate species are in agreement with the numerous previous studies on the biology of this species (Faber et al., 1988, 1989; Huber et al., 1997; Bijma et al., 1998). Important differences in life span, growth, photosynthetic pigments and symbionts contents, shell chemistry, porosity and coiling ratio were observed between genotypes I and II. Those two types display fundamental physiological and biological differences that reflect separate species despite only slight ultrastructural differentiation according to Huber et al. (1997). The specific distributions for each genotype (Figs. 3 and 4) also argue for distinct species adapted to different ecological niches (see below). Note that it cannot be excluded that the genotype IV we described corresponds to the morphospecies *Globigerinella calida* (Parker, 1962). This tropical to warm subtropical species may be difficult to distinguish from *G. siphonifera* in its early adult stage, using a dissecting microscope. *G. calida* evolved from *G. siphonifera* ~5 Ma (Kennett and Srinivasan, 1983), which corresponds well to the time of divergence inferred with the molecular clock for the genotype IV.

### *4.2. Environmental control on the planktonic foraminiferal biodiversity*

Based on the morphological species concept, *Globigerinella siphonifera* has a transitional–subtropical–tropical biogeographic range. In contrast the four genetic species of *G. siphonifera* have more restricted geographic distributions that likely reflect specific ecological requirements. The sea-surface total chlorophyll *a* concentration seems to be an acceptable proxy for explaining the specific biogeographic patterns of most *G. siphonifera* genotypes (Fig. 4). It suggests that types I, IV and III are adapted to oligotrophic, mesotrophic and highly productive water masses, respectively (Fig. 3). While overlapping distribution occurs between types III and IV, there is no sympatric occurrence of types III and I: they apparently occupy substantially different ecosystems in terms of water column stability and productivity.

Moreover, our sampling did not discriminate across oceanic depth, and some of the *Globigerinella siphonifera* species may also inhabit a restricted layer of the water column, particularly in tropical or subtropical environments, where stratification offers different ecological niches at various depths (in particular the oligotrophic mixed layer and the meso- to eutrophic deep chlorophyll maximum at the top the of thermocline). In fact several lines of evidence suggest this is the case for *G. siphonifera* types I and II. All experimental studies on ecology and biology of *G. siphonifera* have been conducted in stratified tropical waters, where a type I–type II assemblage is observed: Jamaica and Barbados, Faber et al., 1988, 1989; Curacao, Huber et al., 1997, Bijma et al., 1998. These field and laboratory experiments have shown that type I specimens have a lower metabolic rate, live longer and have larger shell size and porosity than type II specimens (Faber et al., 1988, 1989; Huber et al., 1997), suggesting that type I is better adapted to survive in highly oligotrophic shallow water conditions, while type II may live deeper in colder and more productive waters. This is confirmed by the horizontal hydro-geographic distribution of the genotype I which we exclusively found in subtropical or tropical stratified water masses (Fig.

3). In contrast, type II is particularly abundant in mesotrophic areas, even if it is also recorded to some extent in highly productive upwelled and cold waters in association with type III, or in oligotrophic gyres with type I (Figs. 3 and 4); the biogeographic preference of *G. siphonifera* type II for mesotrophic areas suggests it inhabits the deep chlorophyll maximum, a deep niche rich in primary producers, and a major food source exploited by several species of planktonic foraminifera (Fairbanks et al., 1982) in tropical/subtropical provinces.

This hypothesis of depth segregation between *Globigerinella siphonifera* types I and II in oligotrophic conditions is supported by comparative analyses of oxygen isotopic ratio, Mg/Ca ratio and pigment content between both types (Huber et al., 1997; Bijma et al., 1998). The higher $\delta^{18}O$ values and lower Mg/Ca ratio measured from type II specimens shells independently suggest that this type grows at a 1.8°C colder temperature than type I (Bijma et al., 1998). Such a temperature difference coincides with the start of the thermocline at the basis of the tropical mixed layer, where the deep chlorophyll maximum develops. The vertical separation of types I and II is also suggested by data from stratified tows (Fairbanks et al., 1980) displaying a bimodal distribution of *G. siphonifera* in Sargasso Sea waters (where we found types I and II – Fig. 3): a group of shallow water specimens is clearly separated from a population occurring at the thermocline.

Thus, biogeographic, hydrographic, physiological and shell chemistry lines of evidence indicate the four *Globigerinella siphonifera* genotypes are, at least in part, horizontally and/or vertically adapted to different water masses displaying different levels of chlorophyll concentrations. Chlorophyll reflects the level of productivity resulting from a specific biological assemblage of primary producers, which can be radically different (qualitatively and quantitatively) in different water masses, mainly between stratified and vertically mixed regimes. Chemotaxonomic studies of 20 different chlorophyll and carotenoid pigments along four AMT cruises (Gibb et al., 1998) have shown that areas of high total chlorophyll *a* concentration (temperate to subpolar waters, upwelling and frontal zones) are mostly inhabited by large eukaryotes and nanoflagellates, while picoplanktonic prokaryotes, mainly prochlorophytes and cyanobacteria, dominate the biomass in tropical and subtropical regions. Thus, it may be that the different *G. siphonifera* ecotypes acquired different trophic requirements or symbiotic associations during or after differentiation, constraining their biogeography to specific biological communities.

This new biogeographic concept of *Globigerinella siphonifera* distribution challenges the general view on planktonic foraminiferal biogeography, according to which species occupy huge latitudinal belt-provinces which are mainly limited by temperature variations (Rutherfold et al., 1999). The classical interpretation of large-scale, simple and monotonous foraminiferal provinces principally results from analysis of sediment deposits. It is known that such analyses are biased by current transport, dissolution and other taphonomic effects on the dead foraminiferal assemblages (Arnold and Parker, 1999), in addition to the difficulty of determining from sediment data the effective depth niche, and timing of reproduction and blooming of a living species. In fact, most studies based on plankton tows have shown that species occurrence is primarily controlled by biological components, such as life cycle, productivity and prey availability (Hemleben et al., 1989), which are secondarily linked to the physical and chemical oceanic conditions. The discrepancy between the large-scale latitudinal biogeographic patterns based on sediment observations and the complexity of the meso-scale distribution in space (latitude and depth) and time (season) revealed by plankton tows analyses may find an explanation in our data: there are four relatively old genotypes with different ecological requirements within what one thought to be one species according to the current morphological taxonomy.

### *4.3. Toward a new view of the planktonic foraminiferal present and past diversity*

Our genetic data stress the importance of species concepts on the perception of planktonic foraminiferal distribution, ecology and evolution.

The recognition of the eco-genotypes using characters of the test may dramatically improve our understanding of the ecological and evolutionary significance of the planktonic foraminiferal fossil record. The stratigraphic and paleoecologic range of the traditional single *Globigerinella siphonifera* species is broad compared to that of each genotype. While type I may be a marker of tropical–subtropical stratified water masses for the last ~10 Myr, type III would be an excellent indicator for Pleistocene frontal and upwelling systems. In fact, the literature on planktonic foraminifera abounds in enigmatic cases of intraspecific variability in space and time at the morphologic (including coiling ratio), isotopic, or ecologic levels, which may be reconciled in a multi-genotypes – or multi-ecotypes – framework. In *G. siphonifera* for instance, Fairbanks et al. (1980) used MOCNESS tows to establish vertical (200 m depth) abundance in three different north Atlantic hydrographic regimes: slope water, Gulf Stream cold core ring and Sargasso Sea water. The data show a depth migration and decline in abundance of *G. siphonifera* flourishing in surface slope water and cold core ring, and growing slower at the base of the mixed layer (DCM) in Sargasso Sea stratified water, although displaying a separated surface water population in this last regime. The authors interpret their data in a single uniform species scenario. In the light of our results, the different abundance profiles may rather be due to specific water-mass and depth adaptations of the four *G. siphonifera* genotypes: a type III distribution profile in slope waters, a type III–II–IV one in the cold core ring, and a type I–II bimodal distribution in the stratified Sargasso Sea.

The four genotypes revealed here within a single morphospecies are not an exception. In fact, all species genetically analyzed so far, *Orbulina universa* (De Vargas et al., 1999), *Globigerinoides ruber*, *Globigerina bulloides* (Darling et al., 1999), *Turborotalita quinqueloba*, *Neogloboquadrina pachyderma* (Darling et al., 2000), and *Globorotalia truncatulinoides* (De Vargas et al., 2001), are composed of at least three to four significantly different genotypes. Apparently, there is an important difference between the present rather broad morphotaxonomic criteria applied to species description and the more narrowly circumscribed biological genotypes observed in planktonic foraminifera. However, in most cases, previous studies have already detected morphological variations within the genetically analyzed morphospecies, which were either overlooked or regarded as mere clinal variants within a single species. In addition, the first quantitative studies to test the relationship between morphologic and genetic discriminations in planktonic foraminifera have all shown that various characters of the test may display differences reflecting the genetic types. Significant variation in shell porosity was observed between the *Globigerinella siphonifera* type I and type II in Curacao (Huber et al., 1997), a character which also seems to allow distinction between the three genotypes within *O. universa* (De Vargas et al., 1999). *Globigerinella siphonifera* types I and II have also notable differences in the stable isotopic ratio of their test (Huber et al., 1997; Bijma et al., 1998). In the case of *G. truncatulinoides* (De Vargas et al., 2001), a first speciation event corresponds to the adaptation of the species to subantarctic water mass, which occurred ~300 000 yr ago according to both molecular and geologic records. Even in this relatively young genetic isolation, a significant morphometric differentiation was detected in the outlines of the tests of the southern populations using Fourier analyses (Healy Williams et al., 1985; De Vargas et al., 2001).

Moreover, in the three morphospecies – *Orbulina universa*, *Globorotalia truncatulinoides*, and *Globigerinella siphonifera* – that have been genetically examined so far using a high number of specimens from a large geographic scale (De Vargas et al., 1999, 2001, and the present study), genetic isolation events seem to have been systematically associated or followed by adaptive processes to specific hydrographic conditions. In all cases, the variations in water column stability and the resulting differences in biological communities and productivity are thought to have played a key role in adaptive radiation. In case of *Orbulina*, the biogeographic pattern displayed by the three cryptic species along AMT-5 (fig. 1 in De Vargas et al., 1999) is an alternating latitudinal occurrence between oligotrophic 'Caribbean' and 'Sargasso' species and eutrophic 'Mediterranean' species. This pattern is strikingly similar to the hydrographic distribution of the *G. siphonifera* genotypes (Fig. 3). The two planktonic foraminiferal lineages developed, independently and at different times, similar adaptive radiation patterns influenced by ocean stability and productivity.

## 5. Conclusion

Large geographic scale analysis of SSU rDNA diversity in *Globigerinella siphonifera* illustrates that the classical, morphological, definition of species – and therefore biogeography – in planktonic foraminifera may hide a more complex level of genetic and ecologic differentiation.

(1) The morphological species *Globigerinella siphonifera* is in fact a complex of four largely divergent genotypes (types I, II, III, IV), whose main divisions occurred in the late Miocene, between 8 and 5 Ma, according to the molecular clock calibrated on the fossil record.

(2) The individuals of a genotype are genetically identical within the SSU rDNA, regardless of segregation by huge geographic distances, current belts, major tectonic barriers, and in spite of the mixing with other genotypes at several stations.

(3) The different genotypes display specific latitudinal biogeographic distributions that are partly correlated to different levels of chlorophyll concentration in surface waters, reflecting different degrees in the stability of the water mass, and probably different biological communities of primary producers. The ecotypes I, IV and III are adapted to oligotrophic, mesotrophic and highly productive water masses, respectively. The cosmopolitan distribution of genotype II may reflect a vertical segregation of this type in stratified environment, i.e. its adaptation to the deep chlorophyll maximum typically found in tropical/subtropical water masses.

Emerging evidence suggests that the genetic and ecologic diversity molecularly detected in all species of planktonic foraminifera analyzed until now can be morphologically recognized using subtle morphological or chemical features of the test, like test porosity, outline, or stable isotopic and Mg/Ca ratio. In addition to the taxonomic issue, this leads to an important conclusion concerning the interpretation of the planktonic foraminiferal fossil archive: slight morphological differences within classical morphospecies may distinguish ancient, isolated genotypes adapted to highly different environments.

As DNA is definitely lost by hydrolysis in dead foraminifera at the bottom of the ocean, the only key to the past will remain the calcareous shell. The challenge is now to further detect which morphological and/or chemical characters of the tests will allow us to significantly distinguish the ecogenotypes of planktonic foraminifera in the fossil record. Solving this problem is not trivial. It requires combined genetic and morphologic studies on the same living individuals or populations of planktonic foraminifera, collected at a large geographic scale in order to distinguish the morphochemical variability of the shell induced by environmental gradients from that due to genetic segregation and evolution.

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