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Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces

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ABSTRACT **The fossil record of planktonic foraminifers is a key source of data on the biodiversity and evolution of marine plankton. One of the most distinctive foraminiferal taxa, *Orbulina universa*, widely used as a stratigraphic and paleoclimatic index, has always been regarded as a single species. Here we present a phylogenetic analysis of *Orbulina* small subunit rDNA sequences from 25 pelagic stations covering 100° latitude in the Atlantic Ocean. The genetic data reveal the presence of three cryptic species, whose distribution is clearly correlated to hydrographic provinces, and particularly to sea-surface total chlorophyll a concentration. Our results, together with previous studies, suggest that a considerable part of the diversity among planktonic foraminifers has been overlooked in morphological taxonomies. Our data also support the idea that planktonic foraminifers, even if adapted to particular hydrographic conditions, are high-dispersal organisms whose speciation may be similar to that of other high-dispersal taxa in which reproductive mechanisms and behavior, rather than just geographic barriers to dispersal, play key roles in species formation and maintenance.**

Planktonic foraminifers are unicellular marine zooplankton whose fossil record extends back 160 million years and constitutes a fundamental archive of changes in oceanic biodiversity and paleoceanography. Despite the widespread use of foraminiferal species for paleoceanographic, stratigraphic, and evolutionary research, no large scale genetic studies have been done to develop the species concept among planktonic foraminifers; their diversity has been estimated almost exclusively on the basis of more-or-less subjective morphological classification of the tests (shells), and has never been thought to be extensive in spite of their worldwide distribution. Approximately 50 species are described in the Holocene (1). However, results based on DNA sequences suggest that oceanic biological diversity may have been seriously underestimated (2). In this study, we use molecular tools to examine the questions of planktonic foraminiferal biodiversity, distribution, and speciation in pelagic ecosystems.

As a model, we have chosen *Orbulina universa* d'Orbigny, one of the most commonly encountered planktonic foraminifers inhabiting the surface waters of the World Ocean between 60° N and 50° S. This species appeared in the fossil record 15.4 million years ago and is widely used as a stratigraphic and paleoclimatic index (3). *Orbulina* has been a focus of evolutionary studies on its origin from trochospirally coiled ancestors (4, 5) and of research on the ultrastructural variability of its last spherical chamber (6, 7). Moreover, it has been used in culture experiments of stable carbon and oxygen isotope, calcium, barium, and cadmium uptake in the test as a proxy for reconstructions of ancient sea surface water temperature and chemistry (8–12). *Orbulina universa* is considered to be the last representative of a lineage that underwent rapid anagenetic changes at the early/middle Miocene boundary, followed by morphological stasis after the appearance of the totally spherical form (13).

MATERIALS AND METHODS

Organism Collection and DNA Sequencing. Total plankton samples were collected with nets (64–500 μm mesh size) between 200 m depth and the sea surface. Most data were obtained on board the *James Clark Ross,* between the United Kingdom and the Falkland Islands during the Atlantic Meridional Transect cruise 5 (AMT-5) (14). All living *O. universa* specimens were sorted with a dissecting microscope, isolated, and transferred to Petri dishes containing filtered sea water. Total DNA extractions from individual cells were performed on the day of collection to avoid the rapid degradation of cell material that occurs when planktonic organisms are removed from the water column. Methods of extraction, PCR amplification, PCR product purification, and cloning, as well as the foraminiferal-specific primers used in this study are described in ref. 15. The 1,000-bp fragments were manually sequenced after cloning. All 425-bp fragments were directly sequenced on a ABI 377 Prism sequencer, using the internal primer *S20r*: GACGGGCGGTGTGTACAA.

Phylogenetic Analyses. Small subunit (SSU) rDNA sequences were manually aligned, by using the GDE 2.2 software (16). The resulting alignment followed the universal SSU rRNA secondary structure model and was improved in reference to the compensatory mutations. Phylogenetic trees were reconstructed by using 290 unambiguously aligned sites, using the maximum likelihood method with a transitions/transversions ratio of 2, as implemented in the FASTDNAML program (17). Distance computations were achieved with the PHYLO_WIN program (18). We estimated the number of expected substitutions separating the Pacific and Atlantic populations by calibrating a molecular clock on the fossil record—given 19 million years for the divergence time between *Globigerinoides sacculifer* and *Orbulina* (19)—and by using a 3.5 million-year minimum hypothetical divergence time because of the closure of the Panama Isthmus (20). A mean *Orbulina* individual lineage rate (21) of 3.3 substitutions per site per 10^9 years was inferred from a maximum likelihood reconstruction

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Abbreviations: SSU, small subunit; AMT, Atlantic meridional transect.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. Z69599, Z83961, Z83962, Z83964, Z83967, AJ229077–AJ229109, U65632, and U80791).

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using the three different *Orbulina* 1,000-bp SSU rDNA sequences added to the alignment analyzed in ref. 21, which contains 14 other planktonic foraminiferal species. This rate is a conservative estimate based on the conserved, clearly homologous parts of the gene (546 sites of a total of 1,200).

Restriction Fragment Length Polymorphism Analysis. The endonuclease *Sau*96I (Boehringer Mannheim) was used to discriminate between *Orbulina* genotypes. It cuts after the nucleotidic positions (309, 499, 669, 814, 908), (118, 597, 890), and (741) for the Caribbean type (988 bp), Mediterranean type (977 bp), and Sargasso type (973 bp) PCR products, respectively. Distinct patterns for each genotype were UV-detected after migration of the digested PCR products on 1.5% agarose gel and ethidium bromide coloration.

Chlorophyll Concentration Data. Seawater samples were collected during Cruise AMT-5 from a nontoxic supply (7 m) at 2-hour intervals while in passage. Phytoplankton were harvested by filtering 1,000- to 6,000-ml samples through 25 mm GF/F filters using positive-pressure filtration. Pigments were extracted from the filters into 90% acetone with the aid of ultrasonication and centrifugation and filtered through Teflon syringe filters to remove debris. Extracts were then analyzed for a range of chlorophyll, carotenoids, and pheopigments by reverse-phase HPLC (22).

RESULTS

We started by sequencing a 1,000-nt fragment of the gene coding for the nuclear ribosomal SSU rDNA from several specimens collected offshore from Puerto Rico, Bermuda, and the French Riviera. Comparison of observed genetic distances separating the *O. universa* from these three localities shows very important divergences: 12% between the Mediterranean and Sargasso Sea representatives, which differ by 15.1% and 17.3%, respectively, from the Caribbean specimens. Such large genetic distances are comparable to those separating morphologically recognized species of planktonic foraminifers that clearly originated several million years ago in the fossil record (15, 21) and encouraged us to further explore the extent of genetic variation within *Orbulina*. We sequenced a 425-nt fragment of the same gene for 25 additional specimens collected at 23 offshore stations at 400-km intervals along a 50° N–50° S Atlantic transect (14). As shown in Fig. 1*A*, all sequences cluster in one of the three genotypes of *Orbulina,* which we have called Mediterranean, Caribbean, and Sargasso types. Very rare mutations separate individuals within each type, even if they come from geographically remote areas (e.g., station 4 and 23 for the Mediterranean type). Surprisingly, such sequence identity is also observed in *Orbulina* from the Eastern and Western Pacific, collected in the Santa Barbara Basin (California), and off the Great Barrier Reef (Australia), representing the Mediterranean and Caribbean types, respectively. To clarify the geographic boundaries between the three *Orbulina* genotypes, we characterized 100 additional specimens across the Atlantic by using PCR-based restriction fragment length polymorphism.

It is evident that the latitudinal distribution of the three genetic types of *Orbulina* is not random (Fig. 1*C*). Ten stations contain the Mediterranean type only, seven others are Caribbean type-specific, and both types co-occur in four stations. The Sargasso type is only dominant at station 18. Furthermore, it seems that each genotype is adapted to different hydrographic conditions: (*i*) The Mediterranean type is present in areas of upwelling and high chlorophyll concentrations in the

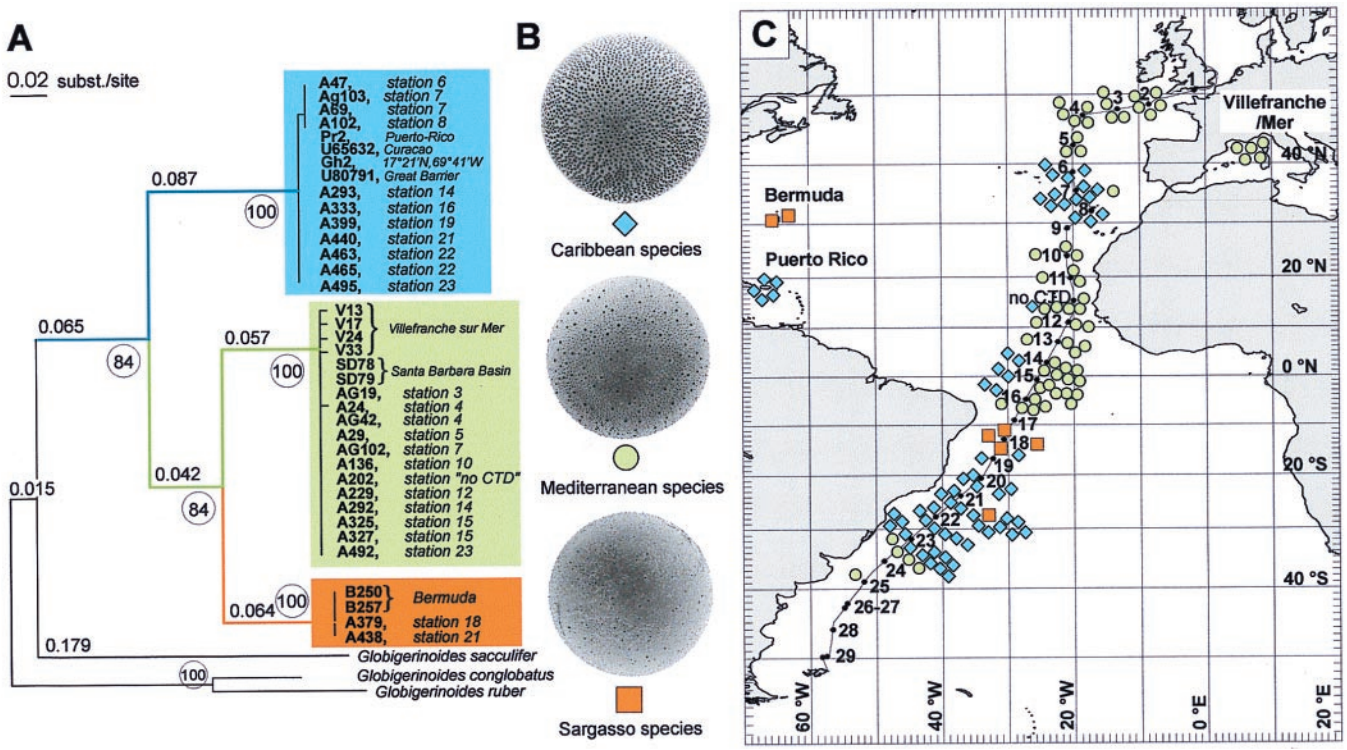

FIG. 1. Cryptic speciation in *Orbulina universa.* (*A*) SSU rDNA-based phylogenetic relationships between 37 *Orbulina* from Atlantic and Pacific pelagic stations and three representatives of the genus *Globigerinoides,* chosen as outgroup. DNA and fossil analyses (15) have shown that *G. sacculifer* is an ancestor of *Orbulina*, both lineages splitting about 19 million years ago. Blue, green, and orange frames, respectively highlight the Caribbean, Mediterranean, and Sargasso *Orbulina* genotypes. Scale and branch lengths are given in substitutions per site, bootstrap proportions (1,000 replicates) are encircled next to each internal branch. (*B*) Scanning electron microscope pictures of the tests from the three *Orbulina* species; note the differences in perforation size and density. Diameters of the tests are, from top to bottom: 600, 540, and 648 m. (*C*) Distribution of the three *Orbulina* species across the Atlantic Ocean. Colors and symbols for the different genotypes are the same as in *A* and *B*. Specimens from Puerto Rico, Villefranche-sur-Mer, Bermuda, and AMT-5 (14) were collected in March 1995, December 1995, April 1996, and September–October 1997, respectively.

mixed layer. The South Atlantic records correspond exactly to the frontal system between the southward flowing Brazil Current and the northward flowing Falkland Current. Their presence off West Africa is clearly correlated with upwelling forced by the trade winds, with a northern limit that matches the sharp decrease in surface-water chlorophyll just at the boundary of the eastern limb of the subtropical gyre. This shift in chlorophyll occurs close to the northern subtropical convergence where the Azores Current heads south along the west coast of Africa. Records of this genotype north of 40° N stop at the southern edge of the North Atlantic Current and the northern edge of the subtropical gyre. Their presence in the Mediterranean (Villefranche-sur-Mer) and California Borderlands is concurrent with local upwelling systems. (*ii*) The Caribbean type is associated with oligotrophic oceanic conditions. Its distribution in both the North and the South Atlantic corresponds precisely with the boundaries of the subtropical gyres. The two Caribbean-specific areas (boreal stations 6–8 and austral 19–23) match the physical oceanic provinces North Atlantic Subtropical Gyre and South Atlantic Tropical Gyre determined by the new density first-derivative methodology applied during earlier cruises on the same transect (23). The Caribbean genotype is also present in oligotrophic waters off the Great Barrier Reef (24). (*iii*) Preferences of the Sargasso type are more difficult to establish, as the genotype was found in two AMT stations only. It could be an extreme oligotrophic taxon, as the main areas of its range are among the most saline and unproductive of the Atlantic. Bermuda is exactly in the center of the western North Atlantic gyre. Likewise, station 18 had the lowest chlorophyll a concentration of any station from which we obtained genetic data from *Orbulina*.

The co-occurrence of Mediterranean and Caribbean types in the tropical Atlantic between 4° S and 2° N may reflect watermass mixing or watermass heterogeneity within the complex system of frontal zones and currents near the equator including the North Equatorial Counter-Current, upwelling along the South Equatorial Current, and the Brazil Current.

Thus, the distribution of the different genotypes of *Orbulina* appears to be correlated with primary phytoplankton production. This is confirmed by a clear latitudinal correlation between the presence of a particular genotype and the concentration of total chlorophyll a in the surface water (Fig. 2). A shift from the Mediterranean to the Caribbean genotype occurs at the boundary value of 100 ng/liter chlorophyll a. Physical hydrographic factors such as temperature and salinity are not obviously correlated with genotype distribution.

The morphology of the three *Orbulina* genotypes is nearly identical. However, scanning electron microscopy reveals that the Caribbean type has relatively large pores and a thick test, whereas the Mediterranean type is microperforate and thin-shelled. The pores of the Sargasso type are even smaller and less numerous (Fig. 1*B*). Similar differences in porosity of *Orbulina* tests have been observed across the whole Indian Ocean by Bé *et al.* (6) but have been interpreted as ecophenotypic variations of a single species. However, Hecht *et al.* (7) used Bé's data set to demonstrate abrupt, discontinuous change in shell porosity between *Orbulina* inhabiting the equatorial and central water masses of the Indian Ocean. This morphological bimodal distribution separated by a hydrographic front is in agreement with our genetic data and confirms that some morphological features allow for discrimination between the genotypes.

According to an absolute molecular clock calibrated on the fossil record (21), the three *Orbulina* genotypes might have appeared in the Miocene: the Mediterranean–Sargasso stem lineage diverged from the Caribbean type 12.1 million years ago, while the splitting of Mediterranean and Sargasso genotypes occurred 6.1–7 million years ago.

DISCUSSION

The large genetic differences between the three types of *Orbulina*, the high degree of genetic identity within each genotype, their hydrographic separation, and their morphological distinction demonstrate the existence of at least three "cryptic" species in the genus *Orbulina.*

The patchy and alternating distribution of the different *Orbulina* species across the Atlantic sheds a first light on the global biogeography of biological—and not morphological—species of pelagic foraminifera. It challenges the general tendency in evolutionary interpretations of living and fossil planktonic foraminifers to interpret the morphological variations through time and space as clinal and continuous gradations. The geographic ranges of *Orbulina* species do not correspond to the classical foraminiferal provinces (tropical, subtropical, and transitional, in the case of our data), which are principally related to water mass temperature, although we cannot eliminate the possibility that the different genotypes of *Orbulina* species may live at different depths from place to place. Still, the clear correlation between genotype distribution and chlorophyll concentration from the surface waters suggests that species distribution is controlled by variations in

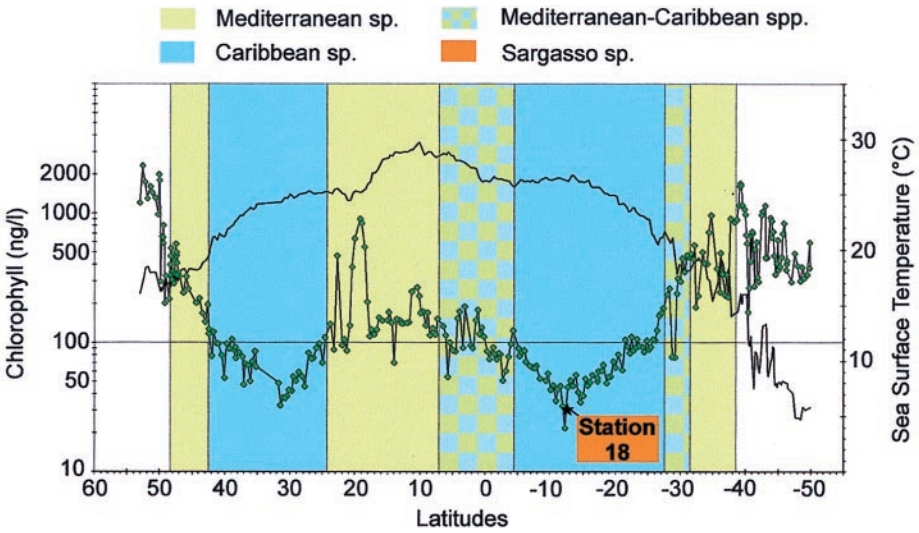

FIG. 2. Total chlorophyll a concentration (◆) and sea surface temperature (solid line) on the AMT-5 transect. Color frames on the graph represent the distribution of *Orbulina* genotypes along the geographic latitudes. Note that the chlorophyll scale (left) is logarithmic.

surface ocean productivity. Such association could be explained by specific differences between *Orbulina* genotypes in feeding behavior or symbiotic association. This latter hypothesis has been proposed to explain the faunal provincialism known from the fossil record of *Globigerinoides ruber* (25).

The geographic separation of the living *Orbulina* species may suggest that speciation was controlled to some extent by hydrographic isolation. In fact, rapid allopatric speciation linked to the development of upwelling and oceanic front has been clearly shown in the fossil record of the globoconellid planktonic foraminifers in the Pliocene (26). However, an isotopic and morphological study (27) has shown that the first steps in the evolution of *Orbulina* from the *Globigerinoides–Praeorbulina* plexus was by sympatric speciation and without any discernible change in depth habitat. In our data set, the strong intraspecific genetic homogeneity indicates that a global gene flow has been maintained even between the two hemispheres and transoceanic populations. Hence, our results also challenge the assumption that oceanographic barriers are strong enough to preserve allopatry. It could be argued that the genetic identity of the ribosomal sequences results from strong adaptive selection or from an acceleration of the mutation rate followed by structural stasis of the molecule. However, we observed a clocklike behavior of the DNA-substitution process in the same SSU rDNA fragment from several species of globigerinids—the family containing *Orbulina*—by calibrating their molecular evolution with their well known fossil record (21). According to such a molecular clock, we would expect a minimum of 12 nucleotide substitutions separating the Pacific from the Atlantic populations if we hypothesize a separation since the closure of the Panama Isthmus (3.5 million years ago). But specimens from both the upwelling, chlorophyll-rich waters of the California borderlands and from the oligotrophic Great Barrier Reef system are 100% identical to our Mediterranean and Caribbean genotypes, respectively.

Apparently, even major tectonic barriers to exchange between the two oceans have not been effective in preventing gene flow between Atlantic and Pacific *Orbulina.* Thus, our data support the idea that planktonic foraminifers, even if adapted to particular hydrographic conditions, are high-dispersal organisms that can travel around tectonic barriers and can cross hydrographic fronts. We propose that, like wind-blown seeds, nonreproductive cells could cross and survive unfavorable habitats—via transport in oceanic currents, networks of upwelling systems, eddies, or water-mass submergence—and flourish where conditions are propitious. In this respect, the pattern of planktonic foraminiferal speciation may be similar to that of other high-dispersal taxa in which gamete interactions, widespread genome incompatibility, a complex mating system, or reproductive behavior play key roles in species formation and maintenance (28).

Our study raises also important questions about the biodiversity of planktonic foraminifers and its paleontological interpretation. The fossil record of planktonic foraminifers is known to contain numerous examples of intergradation between morphologically defined taxa but it has been difficult or impossible to determine which morphological features identify species-level differences and which represent within-species variability. It could be argued that the genetic speciation detected in *Orbulina* is an exception because of the lack of morphological characters observable from its spherical shell. However, morphological differences have been described in populations of *Orbulina* (7) but were erroneously attributed to environmental factors rather than genetic differences.

In fact, cryptic speciation may be a common phenomenon in planktonic foraminifera. For example, genetic work has demonstrated the existence of two cryptic species of *Globigerinella siphonifera* (29). The genotypes of *G. siphonifera* can be recognized by differences in shell porosity (29), as is also the case for the three *Orbulina* genotypes. Likewise, the morphospecies *Globigerina bulloides* and *G. ruber* have each been identified as consisting of clusters of highly variable genotypes (30) that may represent groups of related species. Our own preliminary large-scale and general genetic characterization of planktonic foraminifers has already shown that several other taxa can be split into at least two or three species, most of which can be recognized after the fact by using morphological criteria. These results for foraminifera are similar to those for other marine organisms in which genetic data help to establish the level of morphological differentiation needed to consistently identify biological species (31).

Obviously, a considerable part of planktonic foraminifer diversity has been missed or attributed to ecophenotypy by morphological taxonomies, and species concepts clearly need revision. To increase the resolving power of the Neogene fossil record, the challenge is now to combine molecular and paleontological approaches to identify which phenotypic characters correspond to differences between genotypes. Each new species is a potential new tool for micropaleontology, and can be used as a new paleoecological or stratigraphic marker. Further studies of foraminiferal genotypes will also help to define genetic provinces in the modern ocean and to understand the processes of speciation and extinction in the pelagic realm.

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