Universität Bremen

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Genetic diversity, biogeography and the morpho-genetic relationship in extant planktonic foraminifera

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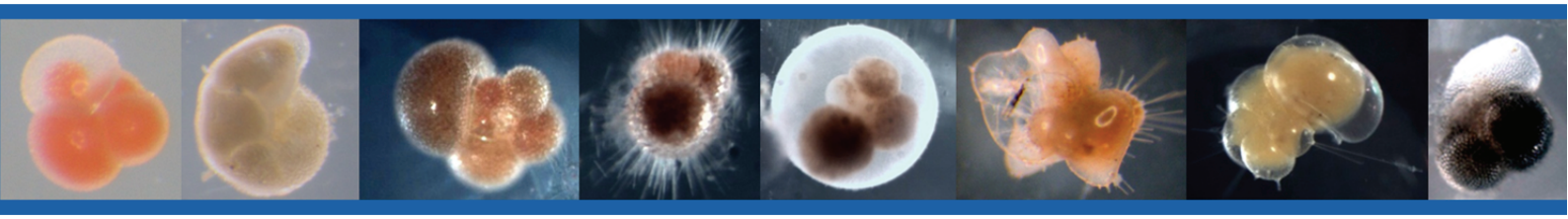

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Preface

The present PhD thesis was prepared at the Department of Geosciences at the University of Tübingen and the MARUM – Center for Marine Environmental Sciences at the University of Bremen, Germany. The work was financed by the "Landesgraduiertenförderung Baden-Württemberg" and by grant KU 2259/19 from the Deutsche Forschungsgemeinschaft (DFG) as part of the OPOCA project.

Planktonic foraminifera samples for genetic analysis were collected and processed during five research expeditions to the Mediterranean Sea, Atlantic, South China Sea and the Southern Pacific on the research vessels R/V MARIA S. MERIAN (cruise MSM15/5), R/V POSEIDON (cruises P411 and P413) and R/V SONNE (cruises SO221 and SO226/3). In addition, four research stays were carried out at the marine laboratories at Villefranche sur Mer, France, the Interuniversity Institute in Eilat, Israel, the Estación Costera de Investigaciones Marinas in Las Cruces, Chile and the Isla Magueyes Laboratories in La Parguera, Puerto Rico. The visits to the marine stations in Chile and Israel were financed by the EU FP7 research infrastructure initiative ASSEMBLE and the field trip to Puerto Rico was part of the OPOCA DFG project. The obtained planktonic foraminifera dataset was enlarged with samples from the collections of the working group and from various coauthors, as indicated for each study separately.

The eight chapters of this thesis comprise four research papers dealing with the genetic diversity, biogeography and a potential correlation of morphology and genetics in living planktonic foraminifera. Chapter 1 introduces the model organisms from a biological as well as paleontological perspective. It describes the marker gene that is used in the genetic analysis of planktonic foraminifera and elaborates the problem of cryptic diversity within the traditional morphospecies. The following section presents different modes of speciation and biogeographical distribution patterns that can be found in plankton organisms. Chapter 2 outlines the motivation and major research questions of this thesis and Chapter 3 describes the methods applied in the molecular analysis of planktonic foraminifera. Chapters 4–7 include the actual case studies that were carried out in the framework of this thesis, in the form of published or submitted research papers and Chapter 8 presents a general conclusion on the results of the studies and an outlook for future work.

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Abstract

The fossil record of planktonic foraminifera grants this group an exceptional position among marine microplankton. Foraminifera have a long tradition as proxies for the reconstruction of past ocean and climate conditions and therefore, the fossilized shells are very well studied, including evolutionary processes and phylogenetic relationships since their origin in the Jurassic. For their classification, the morphological species concept has been applied, which distinguishes about 50 different species among the modern representatives, based on the ultrastructure of their calcite shells. With the application of molecular genetic approaches on living planktonic foraminifera, the classical species concept has been challenged by the discovery of a large number of cryptic species "hidden" within the morphospecies. The total amount of cryptic diversity and its distribution between the different morphospecies, however, are still not completely resolved. In contrast to the mainly cosmopolitan occurrences of the morphospecies, many of the cryptic species exhibit differentiated distribution patterns in the ocean, marked by local adaptations to environmental factors. A morphological separation of the sibling species, however, proved very difficult and was so far only possible after detailed morphometric studies.

The present thesis aims at contributing to the ongoing research on living planktonic foraminifera, by studying the genetic diversity within three selected morphospecies, the biogeographical distribution patterns of their cryptic species and the relationship between genetic and morphological variability.

The study on *Hastigerina pelagica* examined its genetic diversity and biogeographical distribution in the Mediterranean Sea, Atlantic, Caribbean Sea and the Western Pacific. Only three already known cryptic species were discovered and they were shown to exhibit a global distribution in the ocean, but vertical segregation in the water column. For the analysis of *Globigerinoides sacculifer*, a high number of samples from around the world was amassed, including all different morphotypes of this highly variable plexus, that were, however, revealed to be genetically completely homogenous. The third species that was chosen for analysis was *Globigerinella siphonifera* that is marked by a high genetic as well as morphological variability. The examination of a large number of samples allowed the complete resolution of its cryptic diversity and a separation of the plexus into three species.

Despite an extensive sampling effort, the number of newly detected cryptic species from these studies was unexpectedly low. This indicates that for the well-studied morphospecies most cryptic species might by now be detected and that genetic variability is not even prevalent in all morphospecies. The results presented in this thesis further imply that the amount of genetic diversity cannot be predicted from the characteristics of a morphospecies. The correlation of morphological traits with genetic variability appeared to be possible to some extent in the studied morphospecies, however, generally speaking, morphology and genetics of planktonic foraminifera appear to evolve rather independently of each other. The biogeographical distribution patterns of the cryptic species of the studied morphospecies speak for a prevalence of large scale dispersal and gene flow in planktonic foraminifera, while at the same time possibilities are given for the establishment of

reproductive isolation, such as in the vertical dimension in the water column or by local adaptations to different ecological parameters.

Zusammenfassung

Der fossile Befund planktonischer Foraminiferen verleiht dieser Organismengruppe eine außergewöhnliche Position innerhalb des marinen Mikroplanktons. Ihre Nutzung als Proxies für die Rekonstruktion vergangener Ozean- und Klimabedingungen hat eine lange Tradition, was zur Folge hat, dass ihre fossilisierten Schalen sehr gut untersucht und ihre Evolution und phylogenetischen Verhältnisse seit ihres ersten Auftretens im Jura genau bekannt sind. Für die Klassifikation der Arten wurde das morphologische Artkonzept zu Grunde gelegt, das die modernen Vertreter basierend auf der Struktur ihrer kalzitischen Schale in ca. 50 Arten unterteilt. Die Anwendung molekulargenetischer Methoden an planktonischen Foraminiferen stellte das klassische Artkonzept allerdings in Frage, da eine große Zahl kryptischer Arten innerhalb der morphologischen Arten entdeckt wurde. Das komplette Ausmaß dieser kryptischen Diversität sowie seine Verteilung zwischen den morphologischen Arten konnten bisher nicht im Detail geklärt werden. Im Gegensatz zu den morphologischen Arten, die sich größtenteils durch eine globale Verbreitung auszeichnen, weisen die kryptischen Arten eher differenzierte Verbreitungsmuster mit lokalen Anpassungen an Umweltfaktoren im Ozean auf. Eine morphologische Unterscheidung dieser nahverwandten Arten stellte sich jedoch als äußerst schwierig heraus und war bisher nur nach detaillierten morphometrischen Studien möglich.

Die vorliegende Arbeit hat das Ziel, durch Studien zur genetischen Diversität von drei ausgewählten morphologischen Arten, zu den biogeographischen Verbreitungsmustern ihrer kryptischen Arten und zum Zusammenhang zwischen genetischer und morphologischer Variabilität, einen Beitrag zur aktuellen Forschung an lebenden planktonischen Foraminiferen zu leisten.

Die Studie an *Hastigerina pelagica* untersuchte deren genetische Diversität und biogeographische Verbreitungsmuster im Mittelmeer, Atlantik, der Karibik und dem westlichen Pazifik. Dabei wurden nur drei bereits bekannte kryptische Arten entdeckt, für die eine globale Verbreitung im Ozean, aber eine vertikale Trennung in der Wassersäule nachgewiesen werden konnte. Für die Analyse von *Globigerinoides sacculifer* wurde eine große Zahl an Proben aus aller Welt zusammengetragen, die alle morphologischen Typen, die innerhalb dieser Gruppe beschrieben wurden, beinhalteten. Auf genetischer Ebene konnten allerdings keinerlei Unterschiede zwischen diesen Morphotypen festgestellt werden. *Globigerinella siphonifera*, deren hohe genetische und morphologische Variabilität bereits bekannt ist, wurde als dritte Art für die Studien dieser Arbeit ausgewählt. Die Untersuchung einer großen Zahl an Proben erlaubte eine Aufgliederung der kompletten kryptischen Diversität und eine Spaltung der Gruppe in insgesamt drei Arten.

Generell betrachtet war, trotz einer außergewöhnlich hohen Zahl an Foraminiferen-Proben, die Anzahl neuentdeckter kryptischer Arten in den Studien dieser Arbeit überraschend gering. Dies impliziert, dass für die meisten gut untersuchten morphologischen Arten bereits alle kryptischen Arten identifiziert zu sein scheinen, und dass genetische Variabilität nicht in allen morphologischen Arten vorherrschend ist. Des Weiteren zeigen die Ergebnisse dieser Arbeit, dass das Ausmaß an genetischer Diversität einer morphologischen Art nicht anhand deren Charakteristika vorhergesagt werden kann. Die

Verbindung morphologischer Merkmale mit genetischer Variabilität war zumindest teilweise erfolgreich für die analysierten Arten, generell jedoch scheinen Morphologie und Genetik planktonischer Foraminiferen unabhängig voneinander zu evolvieren. Die biogeographischen Verbreitungsmuster der hier untersuchten kryptischen Arten im Ozean deuten darauf hin, dass großflächige Ausbreitung und Genfluss in planktonischen Foraminiferen überwiegen. Gleichzeitig jedoch sind Möglichkeiten für die Entstehung reproduktiver Isolation gegeben, zum Beispiel in vertikaler Richtung in der Wassersäule oder durch lokale Anpassungen an unterschiedliche Umweltparameter.

1. Introduction

1.1. The biology of extant planktonic foraminifera

Planktonic foraminifera are exclusively marine eukaryotic microbes with a global occurrence in the world's ocean (e.g. Hemleben *et al.* 1989). Despite their wide distribution throughout all climatic zones, these single celled holoplanktonic organisms exhibit relatively low abundances with on average about 10 individuals per m^3 of the water column. The most prominent feature of the group is the construction of calcite shells around their cell, granting them an extraordinary position in the plankton as important carbonate producers with an average of 3 Gt $CaCO_3$ in the global ocean per year (Schiebel 2002). These shells sink to the seafloor once the organism dies, where they accumulate in great numbers in the sediment and form the so called *Globigerina* ooze (e.g. Vincent & Berger 1981). Consequently, planktonic foraminifera are marked by an excellent fossil record, which can be traced back to their origin in the Jurassic about 180 Ma (e.g. Cifelli 1969), and they experience wide applications in micropaleontological and oceanographic studies as tools for the reconstruction of past ocean surface properties (e.g. Kucera & Schönfeld 2007).

The exact position of foraminifera in the tree of life was for a long time ambiguous, due to the lack of common morphological features with other groups of protists (Pawlowski 2000). Traditionally, they were grouped in the phylum Granuloreticulosea, because of their granular anastomosing pseudopodia (Lee *et al.* 1985). Later they were shifted to the phylum Rhizopoda (Corliss 1994). In the first studies based on molecular data of the ribosomal RNA gene (rDNA), which were published in the early 1990s, they were placed close to *Dictyostelium* and *Entamoeba* in the eukaryotic tree (Pawlowski *et al.* 1994; Darling *et al.* 1996a). Today, based on multi-gene evidence of benthic foraminifera, the phylum Foraminifera is considered to be part of the eukaryotic supergroup Rhizaria, which comprises amoeboid and skeleton-building protists (Figure 1.1; Caron *et al.* 2012; Sierra *et al.* 2013). More specifically, they form the monophyletic group Retaria together with Radiozoa (Polycystinea and Acantharea), but branch within the Radiozoa, which consequently are left as a paraphyletic group (Figure 1.1; Sierra *et al.* 2013). This fact clearly reveals the still high level of uncertainty in the systematics of protists. Planktonic foraminifera, in particular, belong to the foraminifera class Globothalamea, the order Rotaliida and the suborder Globigerinina (Hayward 2013; Pawlowski *et al.* 2013).

Owing to their relatively low abundances in the ocean, living planktonic foraminifera, in contrast to their fossilized counterparts, have not been in the focus of intense studies for a long time. As a consequence, knowledge on the biology and physiology of these organisms accumulated rather slowly. The best-studied feature of the group, though, is the calcite shell including the processes of its construction (Bé *et al.* 1979; Hemleben *et al.* 1986). The multi-chambered tests consist of a mono- or bilamellar calcite wall, which is secreted from an organic membrane (e.g. Hemleben *et al.* 1989). The wall contains integra-

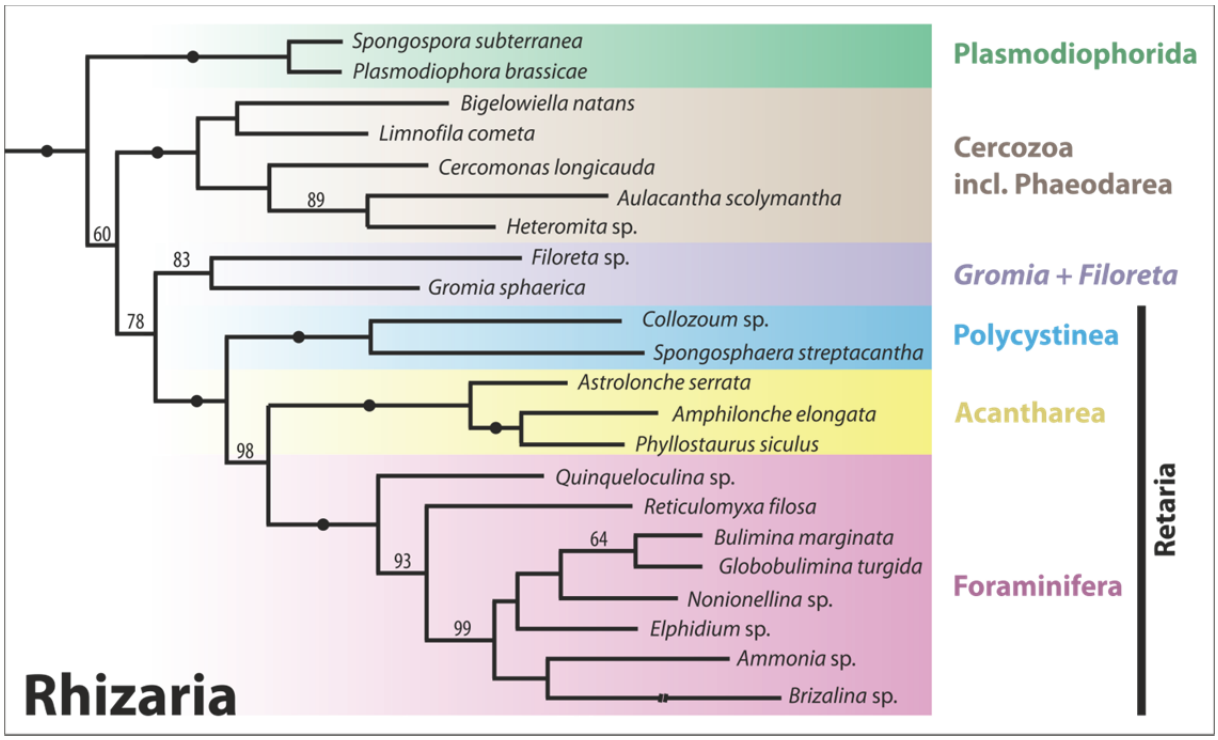

Figure 1.1: Maximum likelihood phylogenetic tree of the eukaryotic supergroup Rhizaria based on 36 genes, showing the position of the foraminifera within the Retaria. Numbers at branches indicate the topological support by bootstrap replicates, circles indicate maximum support (redrawn after: Sierra *et al.* 2013).

ted pores and ornaments like spines or pustules. Throughout their ontogeny, planktonic foraminifera grow chamber by chamber and experience severe morphological changes (Brummer *et al.* 1987) until they reach their adult size, which can measure up to ~1000 µm in the largest species. The shell is constructed as a protection for the cell, however, for the function of cellular processes, exchange with the environment is indispensable. This is realized via the aperture or the pores (e.g. Bé *et al.* 1980), where the rhizopodia extrude to the outside of the shell and uptake of oxygen as well as food takes place (Schiebel & Hemleben 2005). Planktonic foraminifera were observed to either exhibit a herbivorous, carnivorous or omnivorous feeding strategy, feeding on different types of algae (e.g. diatoms, dinoflagellates and thecate algae) or copepods (Figure 1.2A; Anderson *et al.* 1979; Spindler *et al.* 1984). For the ingestion of food, the rhizopodia wrap the prey, secrete adhesive substances and transport tissue particles via rhizopodial streaming into the shell, where digestion takes place in vacuoles (Hemleben *et al.* 1989). This process allows planktonic foraminifera to consume organisms larger than themselves (Caron *et al.* 2012).

Although planktonic foraminifera generally follow a heterotrophic living strategy, some species possess algal symbionts, which enable them to use energy from photosynthesis in exchange for metabolites and a stable microenvironment in the calcite shell (e.g. Faber *et al.* 1988). The predominant symbionts in spinose foraminifera are certain species of dinoflagellates or chrysophycophytes (e.g. Anderson & Be 1976; Faber *et al.* 1988; Gast & Caron 1996), which are taken up from the open water during the early living stages of the foraminifera with a high species specificity (Hemleben *et al.* 1989). The symbionts are usually enclosed within host vacuoles and are transported to the peripheral cytoplasm on

a diel cycle (Figure 1.2B; Anderson & Be 1976). Laboratory experiments by Bé *et al*. (1982) revealed a strong link between host and symbionts, with the observation of premature gametogenesis or suppression of calcification when the symbionts were artificially repressed. In contrast to these close associations, commensals (mostly dinoflagellates) can frequently be observed on the surface of the host, seemingly taking advantage of the favorable microcosm, acquiring nutrients around the foraminifera shell without providing carbon products to the host in return (Alldredge & Jones 1973). In addition, parasites are known to be present on or in the shells feeding on the cytoplasm of the foraminifera. Mostly, these are small free-swimming dinoflagellates, sporozoans or bacteria (Figure 1.2C; Spindler & Hemleben 1980; Hemleben *et al.* 1989).

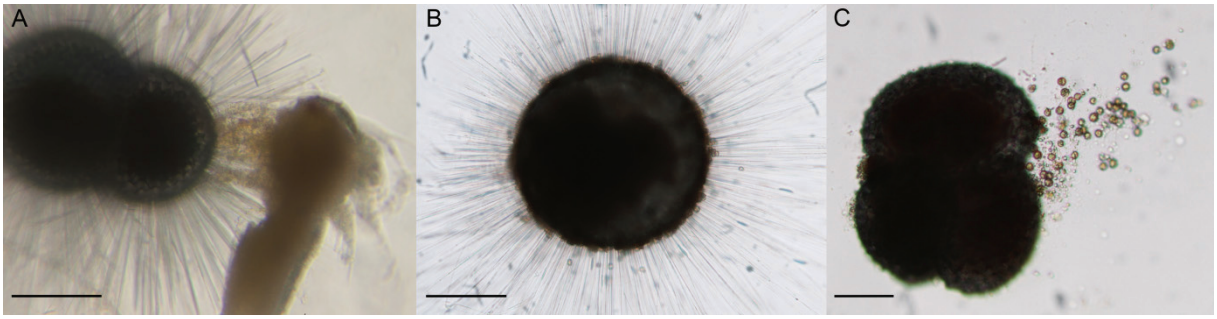

Figure 1.2: Images of living planktonic foraminifera kept in laboratory cultures. **A)** *Globigerinoides sacculifer* feeding on an *Artemia salina* nauplius via rhizopodial streaming. Scale bar 150 µm. **B)** *Orbulina universa* with algal symbionts on the surface of the shell and in the peripheral cytoplasm. Scale bar 100 µm. **C)** *Globigerinoides ruber* with parasites on and in the shell feeding on the cytoplasm of the foraminifera. Scale bar 50 µm. (Photos: A. Weiner)

If a species is associated with symbionts, its distribution in the water column is restricted to the euphotic zone, since it is dependent on light. The abundance of symbiont-free species varies largely with depth, with the deepest observations of living individuals at about 1500-2000 m water depth (e.g. Hull *et al.* 2011). Although the number of individuals is usually expected to be higher in the depth layers of the chlorophyll maximum (Schiebel & Hemleben 2005), a correlation between primary productivity and foraminifera abundance cannot always be confirmed (Kucera *et al.* 2013). Regardless of the facts that these single celled organisms are not capable of active movement and that until now no cellular mechanisms for the control of dwelling-depth have been observed, some species were reported to be restricted to certain depth layers (Kuroyanagi & Kawahata 2004; Schiebel & Hemleben 2005) or to even migrate in the water column throughout their life cycle (Figure 1.3; e.g. Emiliani 1971).

The life cycle of planktonic foraminifera seems to be marked by sexual reproduction as sole reproductive strategy, as far as known today (e.g. Hemleben *et al.* 1989). Although gamete fusion has never been observed in laboratory cultures, it is assumed that planktonic foraminifera are dioceous and only gametes of two different parents can fuse to form a zygote (e.g. Schiebel & Hemleben 2005). In contrast to benthic foraminifera that often exhibit an asexual reproducing stage, planktonic foraminifera appear to only possess a gamontic generation. However, the stages occurring after zygote formation are not yet known, preventing conclusions on the timing of meiosis as well as on the existence of

resting stages (Hemleben *et al.* 1989). In order to increase the likelihood of gamete fusion, which can be problematic in non-motile widely dispersed organisms, planktonic foraminifera produce high numbers of free-swimming biflagellated gametes and eventually as gametogenic adults sink down and accumulate near the thermocline to assure spatial proximity (Hemleben *et al.* 1989). The empty parent shells sink to the seafloor after gametogenesis, whereas the juveniles grow by adding chambers to their shells and rise again to surface waters (Erez *et al.* 1991). A further process to enhance chances for successful fertilization was reported for a couple of surface dwelling species, which seem to synchronize their gamete release with the moon phases, exhibiting either a lunar- or semilunar reproduction cycle (Figure 1.3; Spindler *et al.* 1979; Almogi-Labin 1984; Loncaric *et al.* 2005). Whereas e.g. in *Hastigerina pelagica* this reproduction cycle seems to be intrinsically triggered by an endogenous factor (Spindler *et al.* 1979), the cycle of other species might rather be influenced by external environmental stimuli (Bijma *et al.* 1990; Erez *et al.* 1991), which makes it more susceptible to natural disturbing factors (Jentzen *et al.* 2014).

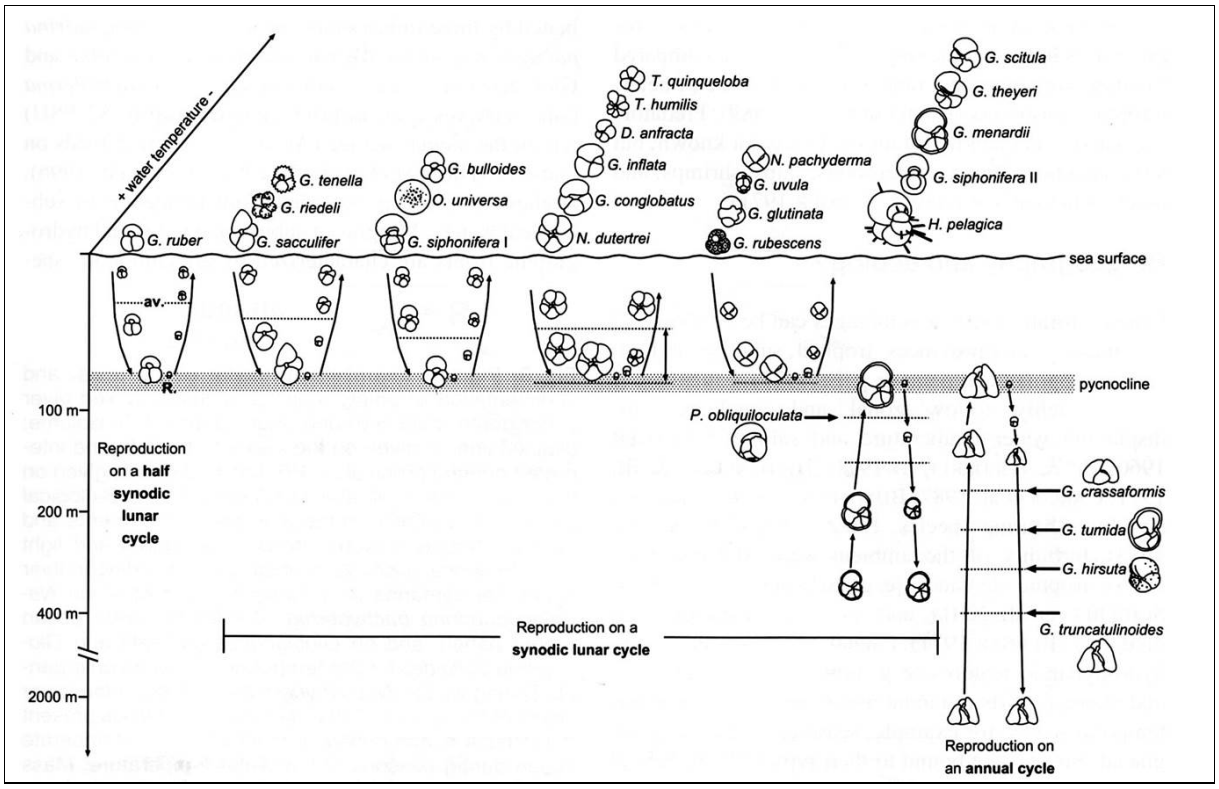

Figure 1.3: Potential distribution of planktonic foraminifera morphospecies in the water column, separated according to their preferred water temperature and dwelling depth. Some species were reported to migrate in the water column throughout their ontogeny, accumulating as gametogenic adults at the thermocline. Especially the surface dwelling species were described to reproduce according to the lunar cycle (from: Schiebel & Hemleben 2005).

1.2. The fossil record of planktonic foraminifera

The high numbers of shells in the sediments of the global ocean, their outstanding preservation as well as the exceptional continuity of the fossil record allow tracking of evolutionary and speciation events since the first appearance of planktonic foraminifera in the Jurassic. The fossil record provides insights into species origination and duration as well as ancestor-descendant relationships on high temporal and spatial scales (e.g. Benton & Pearson 2001). The origin of the entire group of planktonic foraminifera, however, is still uncertain, yet it is assumed that they descended from a benthic ancestor, which changed to a meroplanktonic and subsequently to a holoplanktonic living form (Simmons *et al.* 1997). Hart *et al*. (2003) argue that the first representatives of planktonic foraminifera evolved in the early Jurassic, during a time of severe environmental disruption, which may have been the trigger for aragonitic foraminifera of the genus *Oberhauserella* to adopt a meroplanktonic mode of life. Thereafter, radiations in the plankton are thought to have occurred following extinction events in the Jurassic and Cretaceous, by evolution from surviving planktonic species, without a second benthos-plankton transition (e.g. Tappan & Loeblich 1988; Norris 1991). This monophyletic status of planktonic foraminifera, however, was questioned by molecular data based on the rDNA of planktonic as well as benthic foraminifera, which discovered a polyphyletic origin from the benthos, from at least two ancestral benthic lineages (Darling *et al.* 1997; Ujiié *et al.* 2008). Furthermore, Darling *et al*. (2009) reported the existence of species with a tychopelagic lifestyle that are able to live in both benthos and plankton, and therefore have an ecological advantage, which might allow them rapid recolonization of the plankton after major extinction events, such as the K/T-Event. This discovery further complicates the elucidation of early planktonic foraminifera evolution, which still is not entirely resolved. The origin of the modern planktonic foraminifera fauna, though, can be traced back to an adaptive radiation in the Miocene among survivors of a severe reduction in diversity at the Eocene/Oligocene boundary, during which all forms except for the globigerines became extinct (Cifelli 1969; Kucera & Schönfeld 2007; Aze *et al.* 2011).

Besides tracking the evolutionary history of planktonic foraminifera, the fossil record also provides excellent opportunities for paleo-ecological investigations, such as the dating of marine sediments using planktonic foraminifera as stratigraphic markers (e.g. Bolli *et al.* 1989). Since the chemical and isotopic composition of the shells records the properties of the ambient seawater from the time when they were constructed, planktonic foraminifera are also widely used in paleoclimatology for the reconstruction of chemical and physical properties of surface ocean water in the past (e.g. Kucera & Schönfeld 2007). In addition, the assemblages of planktonic foraminifera shells in the sediment can be used to reconstruct past sea-surface temperatures (Kucera *et al.* 2005), upwelling intensities (Conan *et al.* 2002), and primary productivity (Ivanova *et al.* 2003). The application of foraminifera as proxies in micropaleontological studies requires an exact species identification as well as knowledge of their ecological preferences, given that physiology and habitat vary among species. Since such studies are based solely on fossilized shells, the only way to classify species is by means of the morphological species concept. Consequently, species

classification has traditionally been conducted on easily recognizable and stable morphological characteristics of the calcite shells (e.g. Kennett 1976) and morphologic similarities between fossil and living forms have been used as indicators of similar ecological preferences (Kucera & Schönfeld 2007). Parker (1962) considered the surface ultrastructure of the shell, like the existence of spines and pores, as conservative morphological features for the differentiation of taxa. Srinivasan and Kennett (1976) realized that the ultrastructure can be subject to phenotypic variations, which they, however, considered as ecophenotypes that should be combined in one taxon. In addition to this high phenotypic plasticity, some shell features have evolved in parallel in only distantly related lineages (Norris 1991; Coxall *et al.* 2007), what poses a further challenge for the correct taxonomy and systematics of extant planktonic foraminifera on the basis of morphology alone (Aze *et al.* 2011). Due to these problems, the history of foraminifera classification is marked by countless species emendations as well as the discrepancy between those authors who conceptualized narrowly following the slightest morphological variation (e.g. Saito *et al.* 1981), and those who rather hold on to the concept of phenotypic plasticity and lump morphological variants into one morphospecies (e.g. Parker 1962).

Table 1.1: The 46 planktonic foraminifera species currently considered as valid, plus the two species with serially arranged chambers, which occupy an uncertain position, separated into five different morphogroups. Species classification follows the species list of Hemleben *et al*. (1989), which is based mainly on morphologic characteristics of the calcite shell. In addition, the present list is updated by taxonomic revisions that were possible following the morpho-genetic analysis of three species. *Globigerinoides elongatus* (marked by *) and *Globigerinoides* sp. (ruber white) are based on a taxonomic revision of *Globigerinoides ruber* by Aurahs *et al*. (2011), *Neogloboquadrina incompta* (marked by **) was separated from *Neogloboquadrina pachyderma* by Darling *et al*. (2006), and the former species *Streptochilus globigerus* (Hemleben *et al.* 1989) was considered identical to *Bolivina variabilis* (marked by ***) by Darling *et al*. (2009) based on genetic evidence.

Macroperforate spinose	**Macroperforate nonspinose**	**Microperforate nonspinose**
Globigerina bulloides	*Globorotalia anfracta*	*Candeina nitida*
Globigerina falconensis	*Globorotalia cavernula*	*Globigerinita glutinata*
Globigerinoides conglobatus	*Globorotalia crassaformis*	*Globigerinita uvula*
*Globigerinoides elongatus**	*Globorotalia hirsuta*	*Globigerinita minuta*
Globigerinoides ruber	*Globorotalia menardii*	*Tenuitella iota*
Globigerinoides sp. (ruber white)*	*Globorotalia scitula*	*Tenuitella fleisheri*
Globigerinoides sacculifer	*Globorotalia truncatulinoides*	*Tenuitella parkerae*
Orbulina universa	*Globorotalia tumida*	
Beella digitata	*Globorotalia ungulata*	**Monolamellar**
Globigerinella siphonifera	*Globorotalia theyeri*	
Globigerinella calida	*Globorotalia inflata*	*Hastigerina pelagica*
Globigerinella adamsi	*Neogloboquadrina dutertrei*	*Hastigerinella digitata*
Orcadia riedeli	*Neogloboquadrina incompta***	
Turborotalita quinqueloba	*Neogloboquadrina pachyderma*	**Serially arranged chambers**
Turborotalita clarkei	*Pulleniatina obliquiloculata*	
Turborotalita humilis	*Globoquadrina conglomerata*	*Bolivina variabilis****
Globoturborotalita rubescens	*Globorotaloides hexagonus*	*Gallitellia vivans*
Globoturborotalita tenella	*Berggrenia pumilio*	
Sphaeroidinella dehiscens		

Still relying on shell ultrastructure as the sole basis for taxonomic classification, in the 1980s authors came to a consensus about the number of modern planktonic foraminifera species, which they limited to about 50 morphospecies (Kennett & Srinivasan 1983; Hemleben *et al.* 1989). The combination of morphological and genetic data in the last years led to a taxonomic revision of three morphospecies (Darling *et al.* 2006, 2009; Aurahs *et al.* 2011), resulting in 46 valid species of foraminifera with a purely planktonic lifestyle and two species found in both plankton and benthos (Table 1.1).

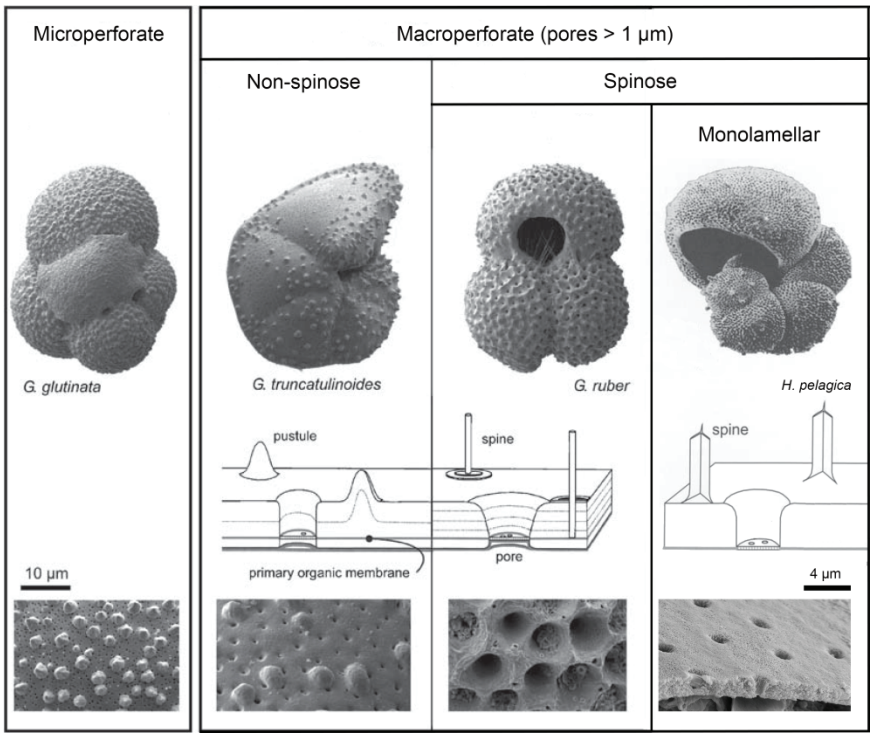

Figure 1.4: Separation of the four morphogroups of strictly planktonic foraminifera morphospecies: the microperforate nonspinose, macroperforate nonspinose, macroperforate spinose and the monolamellar Hastigerinidae. The separation of the groups is based on the shell features pore size, spines and shell thickness. For each group a typical representative is shown (modified from: Kucera (2007) and Schiebel and Hemleben (2005)).

The strictly planktonic morphospecies can be separated into four general morphogroups based on characteristic shell features (Figure 1.4; Hemleben *et al.* 1989). The macroperforate spinose, the macroperforate nonspinose and the microperforate nonspinose species possess bilamellar calcite shells, separating from them the family Hastigerinidae (with the species *Hastigerina pelagica* and *Hastigerinella digitata*). The latter can be considered an own morphogroup due to the monolamellar shell wall in addition to several further unique characteristics, like the possession of a cytoplasmatic bubble capsule (Alldredge & Jones 1973; Hemleben *et al.* 1989). The monolamellar fragile shells of the Hastigerinidae are hardly preserved in the sediments and consequently the first appearance date and the origin of the group are not entirely clear (Aurahs *et al.* 2009a). A further separation criterion between the main morphogroups is the formation of spines, which are an integrated part of the shell wall of spinose species (e.g. Lipps 1966). According to the fossil record, the lineages leading to the modern macroperforate spinose and nonspinose species split up about 70 million years ago in the Late Cretaceous with both groups evolving from a common ancestor of the genus *Hedbergella* (Liu & Olsson 1994). The spines appeared in the Early Cenozoic, most likely as an adaptation to the newly acquired carnivorous feeding habit and the first associations with symbionts (Olsson *et al.* 1999). The pore size further is used as a characteristic to separate off the microperforate group with

pore sizes smaller than 1 µm (Steineck & Fleisher 1978). The origin of this group can also be dated back to the Late Cretaceous, however, it is reported to have evolved from the genus *Guembelitria* (Liu & Olsson 1992). In addition to these four groups with spiral shells, two species with a serial chamber arrangement are often found in plankton nets and therefore they were for a long time considered to be planktonic species (e.g. Smart & Thomas 2006). However, Darling et al. (2009) could show that one of these species found in the plankton is genetically identical to the benthic species *Bolivina variabilis* and that the planktonic form is just one stage of its tychopelagic life cycle. Therefore, although they are by some authors still considered as planktonic species (Ujiié *et al.* 2008; Kimoto *et al.* 2009), their real status remains unresolved.

1.3. Genetic diversity of planktonic foraminifera

The ribosomal RNA gene as molecular marker

The genetic analysis of foraminifera, both planktonic and benthic, had a fairly late start compared to other groups of organisms mainly due to the problem that pure foraminifera DNA is difficult to obtain (Pawlowski 2000). As mentioned before, foraminifera are associated with symbionts living on or in the shell, parasites and also food particles. Therefore, it is nearly impossible to keep them under axenic conditions and consequently, DNA extracts of foraminifera very often contain contaminant DNA, which outnumbers and therefore masks the foraminifera signal (Langer *et al.* 1993; Wray *et al.* 1993). A further problem of initial molecular analysis on foraminifera was the fact that universal PCR primers did not align to foraminifera DNA (Pawlowski 2000). The contamination problem was overcome by working on total foraminifera RNA extracts, which contain a large number of gene transcripts (Pawlowski *et al.* 1994) or by using foraminifera gametes as a DNA source, which are known to be largely free of symbionts and food particles (Darling *et* al. 1996b). Both techniques delivered sequences that grouped together in the phylogenetic tree, but were substantially different from any other known sequences (Pawlowski 2000).

Once the first rDNA sequences of both benthic and planktonic foraminifera had been obtained (e.g. Merle *et al.* 1994; Pawlowski *et al.* 1994; Darling *et al.* 1996a), foraminifera specific primers could be designed, allowing a more rapid and specific amplification of foraminifera DNA. The focus thereby lay on the ribosomal DNA, since this gene complex is found in all domains of life and it occurs in several copies in the genome, making it a useful marker for phylogenetic studies (Pawlowski *et al.* 2012). Although the general structure of the foraminifera rRNA gene is the same as in all other eukaryotes, comprising the large subunit (LSU) and the small subunit (SSU) separated by an internal transcribed spacer (ITS) region (Figure 1.5; Pawlowski 2000), its high length is peculiar within eukaryotes and explains the trouble at the beginning of foraminifera molecular analysis. The complete SSU fragment of some planktonic foraminifera species already measures more than 4 kb in sequence length, which is about twice as much as in other eukaryotes (de Vargas *et al.* 1997). The extraordinary length can be attributed to highly variable expansion segments integrated as loops in the helices of the SSU rDNA. This part of the rDNA typically consists

of a mosaic of conserved and variable regions in all eukaryotic organisms. The variable regions lie at the periphery of the assembled ribosome after their transcription into rRNA, without being involved in the translation machinery and therefore they can be subject to expansions and modifications, which are, however, especially severe in foraminifera (Habura *et al.* 2004). The planktonic foraminifera SSU rDNA contains three unique variable regions (37/e1, 41/e1 and 46/e1) with high genetic variability that differs strongly between the different groups of foraminifera, complicating automated sequence alignments (de Vargas *et al.* 1997). Since these variable regions are not involved in the translation process of the assembled ribosome, it was assumed that the insertions in the SSU rDNA are functionally and phylogenetically insignificant (Wuyts *et al.* 2001). Yet, this mosaic of alternating variable and conserved regions with different diversification rates allows combined phylogenetic reconstruction at various taxonomic levels (Pawlowski *et al.* 2012).

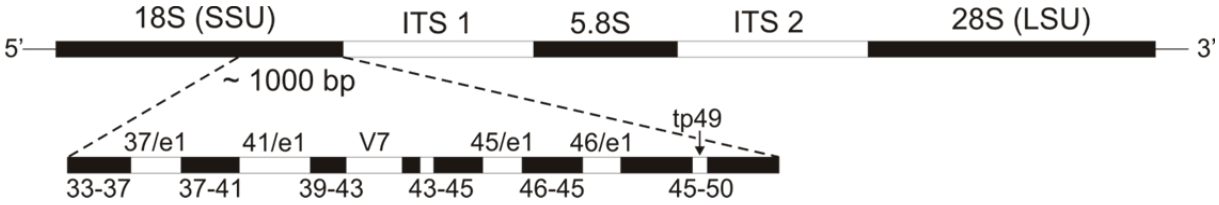

Figure 1.5: Schematic representation of the structure of the ribosomal DNA. Black areas indicate more conservative regions and white areas variable regions. The enlarged 1000 basepair (bp) fragment of the 3' end of the SSU rDNA represents the fragment that is commonly used in foraminifera molecular analysis with its mosaic of conservative regions and integrated variable expansion segments (Redrawn and modified from: Grimm *et al.* 2007).

A further peculiarity of the foraminifera rDNA is the high number of gene copies in the genome. Since high amounts of rRNA are required in each cell, its gene occurs in the genome in tandem repeats, which in eukaryotic cells usually add up to several hundred copies (Long & Dawid 1980). A study conducted on three benthic species though showed that in foraminifera between 10,000-30,000 copies of the gene can be found (Weber & Pawlowski 2013), marked by intraindividual sequence divergence (Pawlowski 2000). Although only few groups with intraindividual variability have been found so far in planktonic foraminifera, it is very likely that they also possess a high number of gene copies. The peculiar structure found in the rDNA of planktonic foraminifera, marked by high numbers of gene copies and large expansion segments, might also be reflected in the rest of the genome, which could be a reason that so far only a small number of genes (SSU and LSU rDNA, actin, tubulin and polyubiquitin genes) could be amplified for some species.

By using sequence information of the SSU rDNA to examine phylogenies reconstructed on the basis of the fossil record, the major conclusions drawn from morphological similarities were supported by the genetic evaluation (Darling *et al.* 1997; de Vargas *et al.* 1997). Planktonic foraminifera were confirmed as a sister group to benthic foraminifera (e.g. Wade *et al.* 1996), although the rate of rDNA evolution in planktonic species was calculated to be about 50-100 times faster than in their benthic relatives (Pawlowski *et al.* 1997).

This fact was explained to be due to high reproduction rates in the plankton and potential changes in DNA repair and replication mechanisms and is reflected in the unusually long branches in molecular phylogenetic trees, which are especially pronounced in the spinose group (Ujiié *et al.* 2008). Another observation from the fossil record that was supported by the molecular analysis is the general separation of species into four morphogroups (Figure 1.6; e.g. Aurahs *et al.* 2009a). Nevertheless, although a high congruence between morphology and genetics was found on first sight, the discovery of high levels of "hidden" genetic diversity within the morphospecies finally changed this perception.

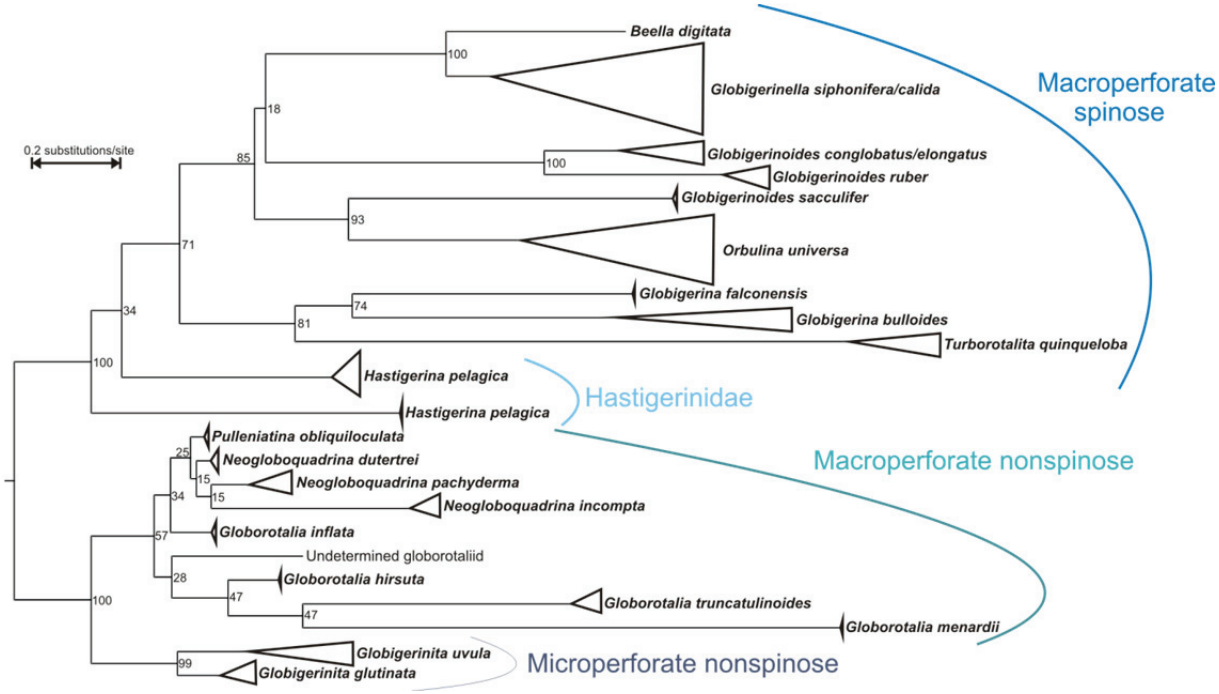

Figure 1.6: Maximum likelihood tree for planktonic foraminifera on the basis of a MAFFT alignment of a 600 bp fragment of the SSU rDNA, reflecting the separation of the four morphogroups (macroperforate spinose, Hastigerinidae, macroperforate nonspinose and microperforate nonspinose). Numbers at nodes indicate bootstrapped confidence values for the nodes. Tree inference and calculation of bootstrap values was conducted in RAxML in the CIPRES gateway and the tree was arbitrarily rooted for better visibility. Intra-morphospecies genetic variability is collapsed.

Cryptic diversity in planktonic foraminifera

The focus on the fossil record, which for a long time was predominant in foraminifera research, had the obvious consequence that the morphological species concept (e.g. Simpson 1951) was the sole basis for species classification. As a result, the status of biological species was largely neglected, as was the question about a potential agreement between the two concepts. The biological species concept regards a group of organisms as a species that interbreed, but are reproductively isolated from other such groups (Mayr 1963). This species concept is limited to sexually reproducing organisms and is considered "non-dimensional" since it does not include the time and space of occurrence of a species in its definition (Mayr 1992).

When the first rDNA sequences had become available, the biological perspective could be added to the classification of planktonic foraminifera. An unexpectedly high sequence

diversity was encountered within single morphospecies, suggesting that the biodiversity of planktonic foraminifera had been severely underestimated (e.g. Huber *et al.* 1997; Darling *et al.* 1999; de Vargas *et al.* 1999). The existence of a high "hidden" genetic diversity was in fact already known for many groups of organisms (e.g. Knowlton 1993) and seemed to be especially dominant in open ocean organisms, such as dinoflagellates, copepods or fishes (Scholin *et al.* 1995; Bucklin *et al.* 1996; Miya & Nishida 1997), a fact that was attributed to strong environmental pressures that shape the pelagic biosphere (de Vargas *et al.* 2004). Nevertheless, due to the existence of the character-rich calcite shells of planktonic foraminifera that were used for species classification, the encountered high sequence diversity came unexpected.

As long as no conclusive evidence for a morphological separation of these "genetic types" exists, they are considered cryptic species. Their rDNA sequences usually show no sign of hybridization and some were observed to possess different ecological preferences. Consequently, they very likely represent the level of biological species (e.g. de Vargas *et al.* 2001). One reason for the existence of a "hidden" genetic diversity might be a potentially too low resolution of the morphospecies concept, which does not sufficiently resolve the level of biological species. Although intraspecies morphological variability has always been an obvious problem in planktonic foraminifera research, due to the lack of genetic information it was mostly dealt with as variation from the general scheme or attributed to ecophenotypic differences, but not considered as having species level significance (e.g. Malmgren & Kennett 1972; de Vargas *et al.* 2004). A second reason for the appearance of cryptic diversity could be a high rate of molecular evolution (as it was found in planktonic foraminifera; e.g. Pawlowski *et al.* 1997), which is not followed by morphological evolution at the same pace; or that morphology is subject to stabilizing selection whereas molecular variation accumulates (Bickford *et al.* 2007). The discovery of cryptic species, though, implies that a morphospecies describes a higher taxonomic level, which integrates several sibling species. De Vargas *et al*. (2004) suggested the concept of "super-species" to describe these morphological entities.

Following up on those new perceptions, search for a potential correlation between the morphological or ecophenotypic variability and the genetic background of the morpho-species was initiated. Huber *et al*. (1997) discovered that the two physiological types of *Globigerinella siphonifera* that were distinguished by the possession of two different types of symbionts (Faber *et al.* 1988, 1989), can also be separated on the genetic level as well as by different shell ultrastructures, and they proposed the acknowledgement of two biological species. De Vargas *et al*. (1999) and Morard *et al*. (2009) could show that the three sibling species of *Orbulina universa* are marked by differences in shell porosity. A combined morpho-genetic analysis on *Globorotalia truncatulinoides* revealed not only the existence of five cryptic species in the morphospecies, but also related variances in shell-conicalness to the genetic background instead to ecological influences (Quillévéré *et al.* 2013). Although the latter studies were able to present slight morphological differences between the now called pseudo-cryptic species, the differences were not sufficiently precise to have an impact on the taxonomy of the group. Only few studies so far detected a correlation

between morphology and genetics that was strong enough to allow for a taxonomic revision of the studied morphospecies: *Neogloboquadrina incompta* could be separated from *Neogloboquadrina pachyderma* based on the observation that the genetic separation corresponds to the different coiling directions of the shells (Darling *et al.* 2006) and Aurahs *et al*. (2011) could show that *Globigerinoides elongates*, which was synonymized with *Globigerinoides ruber*, is genetically as well as morphologically distinct, allowing for the status of a separate acknowledged species (Table 1.1).

Due to an intense screening of planktonic foraminifera morphospecies for genetic diversity so far 26 morphospecies have been analyzed genetically, and overall 66 cryptic species were discovered in 16 of these sequenced species (for review see: Darling & Wade 2008; and Chapter 8). This suggests that cryptic diversity is a prevalent pattern in planktonic foraminifera morphospecies and its discovery just requires the analysis of an adequate number of individuals from different geographic locations. However, already now it is obvious that the amount of genetic diversity is not distributed homogeneously between the different morphospecies. Whereas in *Neogloboquadrina pachyderma* seven cryptic species were discovered (Darling *et al.* 2004; Darling *et al.* 2007), sequences of both *Neogloboquadrina dutertrei* and *Globorotalia inflata* revealed the existence of only two (Darling *et al.* 2003; Morard *et al.* 2011). This uneven distribution of diversity might be due to the fact that the rate of molecular evolution varies between morphospecies, within morphospecies and even between different regions of the SSU rDNA (Pawlowski & Lecroq 2010). As a consequence, the genetic distances between cryptic species differ substantially in the different morphospecies (Table 1.2) and the establishment of a universal threshold of genetic divergence for planktonic foraminifera to objectively separate the taxonomic levels of genus, species and populations is prevented (Göker *et al.* 2010), leaving the classification of biological species to be a subjective procedure.

Table 1.2: Differences in the amount of cryptic diversity "hidden" within selected morphospecies and the wide range and high values of sequence divergence within each morphospecies that inhibit the establishment of a universal threshold for species delineation. Sequence divergence was calculated as pairwise distances between 600 bp fragments of the 3' end of the SSU rDNA of the cryptic species within each morphospecies using MEGA (Tamura *et al.* 2011). Sequences for the distance calculations were taken from GenBank.

Morphospecies	Cryptic species	Sequence divergence [%]
Hastigerina pelagica	3	4 – 42
Neogloboquadrina pachyderma	7	0.7 – 7.5
Globigerina bulloides	12	0.4 – 27
Globorotalia inflata	2	1.7

The discovery of "hidden" genetic diversity in planktonic foraminifera morphospecies has severe implications on their application as paleo-proxies, since these rely on the assumption of genetic continuity and ecological homogeneity of each morphospecies (Kucera & Darling 2002). The fact that ecological differences were found between the cryptic species thus implies that paleoceanographic reconstructions contain significant noise due to the lumping of physiologically and ecologically distinct biological species (Darling *et al.* 2000), that naturally also exhibit different geochemical signatures in their shells. Nevertheless, the knowledge on the existence of genetic diversity within planktonic foraminifera morpho-

species can be used to improve the accuracy and reliability of such studies (Kucera & Darling 2002), by attributing so far unexplained geochemical variability in morphospecies to the presence of different genetic types. Therefore, it is not only important to further screen morphospecies for genetic variability, but to also consider the biogeographical distribution patterns of the cryptic species and their adaptations to different ecological parameters.

A striking advantage in foraminifera research compared to other plankton groups is the possibility for a combination of the fossil record and molecular data in order to date evolutionary events. Molecular clock analysis can be used to impose time on the molecular phylogeny if gradual evolutionary change of the SSU rDNA is assumed (Rutschmann 2006). However, since the evolutionary rates vary extensively between the different foraminifera lineages, calculating a global molecular clock for the whole group is not possible. On the other hand, evolutionary rates within individual groups (e.g. the spinose planktonic foraminifera) can be considered fairly constant and therefore, molecular divergence and speciation events can at least be dated within such a limited group by calibrating against first appearance dates known from the fossil record (Darling *et al.* 1999). By estimating the ages

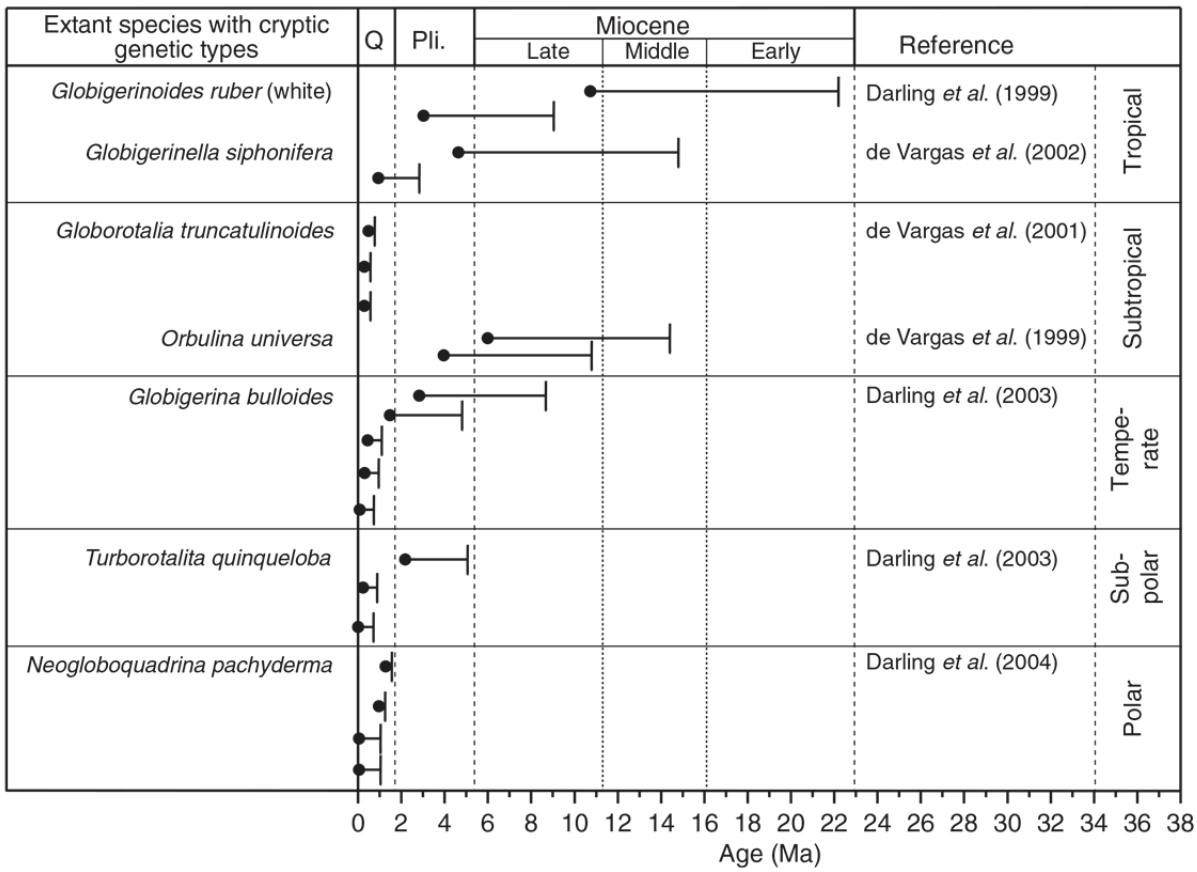

Figure 1.7: Molecular clock estimates for the divergence of cryptic species of various morphospecies, separated by their different distribution ranges. In many cases, the cryptic species already diverged several million years ago, whereas others show high diversification rates in the Quaternary. Indicated are the maximum ages of the genetic types as they are estimated on the phylogenetic tree, associated with their level of uncertainty. (from: Kucera & Schönfeld 2007)

of the cryptic species, it was demonstrated that in many cases they already diverged several million years ago, although these calculations are associated with a high uncertainty (Figure 1.7; e.g. de Vargas *et al.* 2002; Kucera & Schönfeld 2007). Nevertheless, they can be used as a further support for the status of cryptic species as separate biological species that have been constant throughout a long time range.

1.4. Speciation and biogeographic distribution patterns in plankton

The discovery of high genetic diversity in planktonic foraminifera inevitably raises questions on the possible modes of speciation in plankton that can cause such a high diversification. Traditionally, it has been assumed that microplankton species exhibit a cosmopolitan distribution throughout the world ocean (e.g. Miya & Nishida 1997; Finlay & Esteban 2001). The ocean on first sight appears as a homogenous continuum without any obvious barriers for the dispersal of small plankton organisms. The fact that they lack any means of active propulsion makes them subject to passive transport by ocean currents and the only potential limit to their global distribution would therefore be restricted connectivity between ocean basins. Furthermore, microbial species tend to have large absolute population sizes, allowing for rapid passive dispersal, large scale distribution and global gene flow (e.g. Norris 2000). Consequently, it seemed impossible for non-motile microbes to establish differentiated distribution patterns and it was postulated that theoretically, every species occurs everywhere, and the species assemblage at a certain habitat is the result of solely the ecological properties of the habitat (e.g. Finlay 2002; Fenchel & Finlay 2004). Furthermore, Finlay (1998) concluded from this ubiquitous occurrence and the resulting low endemism that the global species richness of protists might be relatively low and is well presented by the local species richness. In general, this suggests that reproductive isolation and speciation in plankton must be reduced. Yet, this is contrasted by the observation of a high species diversity in many groups of plankton either on the morphological or genetic level (e.g. Sáez *et al.* 2003; Irigoien *et al.* 2004; Amato *et al.* 2007; Goetze 2011) and also by evidence from the fossil record for high rates of species origin and extinction (e.g. Lazarus 1983).

The origin of new species in the pelagic environment is described by various theoretical speciation concepts (Figure 1.8; e.g. Norris 2000), which include spatial isolation or distinct adaptations as answer to divergent natural selection (Pierrot-Bults & van der Spoel 1979). The most apparent ones of these speciation models are the allopatric and vicariance models (Figure 1.8A and C), which describe the scenario of a separation of populations of one species by an impenetrable barrier that can either be of hydrographic or geographical origin, respectively (Butlin *et al.* 2008). The populations on either side of the barrier are reproductively isolated from each other and consequently over time diverge into separate species through accumulation of different genetic mutations. The continents can be considered to represent such geographic barriers, inhibiting circumglobal distribution of tropical species (e.g. Arnold & Parker 2002; Goetze 2003). Many temperature tolerant plankton organisms, however, were shown to be transported around the southern tip of

Africa, maintaining gene flow between the Indopacific and the Atlantic (e.g. Peeters *et al.* 2004). For species with a cosmopolitan distribution that co-occur in the same geographical region, the sympatric speciation model appears more likely, since it describes the possibility for reproductive isolation without the need for spatial isolation (Norris 2000).

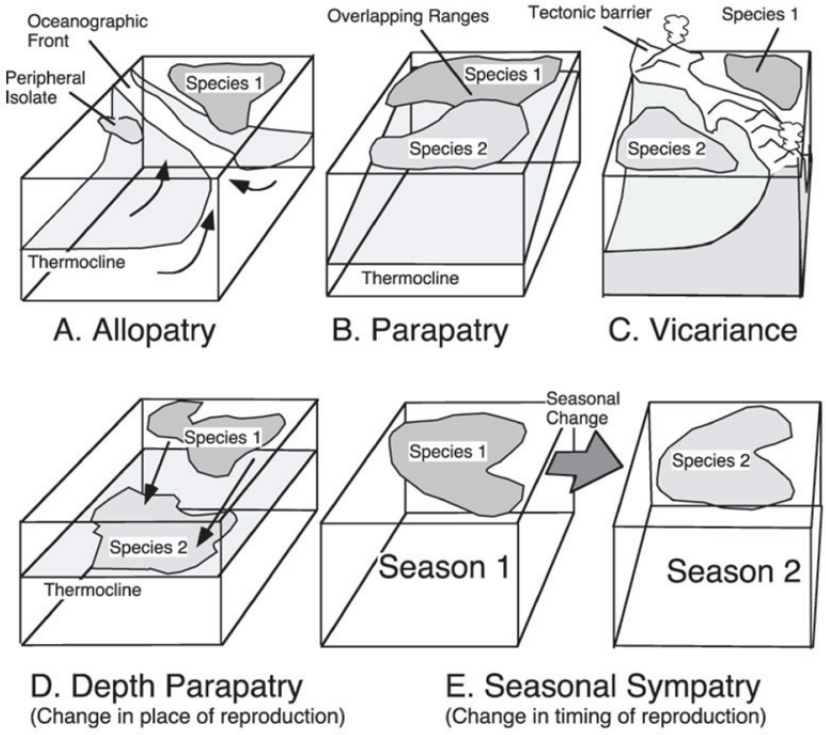

Figure 1.8: Theoretical models for speciation processes in the pelagic environment. **A)** Allopatric speciation by divergence on both sides of a hydrographical barrier, **B)** parapatric speciation in the same geographical range along a hydrographic gradient due to different selection pressures, **C)** vicariance describes speciation due to separation by a geographical barrier, **D)** depth parapatric speciation, which relies on a change in reproduction depth of two populations and **E)** seasonal sympatry, which is the consequence of a shift in the timing of reproduction (from: Norris 2000).

A detailed understanding of speciation processes in plankton requires, after all, the downscaling of observations from a global to a more local scale to disclose the inhomogeneous nature of the pelagic habitat with strong environmental gradients not only on a horizontal but also on a vertical perspective. As a consequence the high potential for speciation in plankton becomes apparent. A study focusing on the biogeography of protists revealed that the area and temporal dimension covered by active cells (described as their "home range") are much smaller than the area and time period that could potentially be covered by passive dispersal of resting stages (Weisse 2008). Further, a high dispersal rate does not necessarily provoke a large scale distribution, since this still requires successful establishment of the immigrants (Weisse 2008). The actual distribution of a species thus largely depends on physico-chemical variables, as temperature, salinity and pH, and biological factors such as food availability, predation pressure and competition (e.g. Arnold & Parker 2002; Weisse 2008). Such a dependence on abiotic factors can also be observed in the occurrence of planktonic foraminifera morphospecies in the ocean, that are distributed according to large scale biogeographies, following surface temperature gradients. The result is a distinction of five major planktonic foraminifera provinces in the ocean (Tropics, Subtropics, Transitional, Subpolar and Polar Regions), which exhibit a latitudinal diversity gradient with a high species diversity in the Tropics and Subtropics and only few species that can survive in the Polar Regions (e.g. Berger 1969; Bé & Hutchinson 1977). This large

scale distribution could be the consequence of the existence of a sharp thermocline in the Tropics and Subtropics, which creates a large number of niches in the water column compared to the homogenous water mass of the Polar Regions (Al-Sabouni *et al.* 2007). Although each morphospecies seems to have a cosmopolitan distribution within its preferred temperature range, the abundance of individuals is fairly low, provoking a patchy distribution of populations (e.g. Tolderlund & Bé 1971). This patchiness depends on the environmental preferences of the species, whereby the populations are separated by areas of unfavorable habitat (Pierrot-Bults & van der Spoel 1979). If the geographical distance between the populations is high enough to prevent gene flow, the potential for allopatric speciation is severely elevated. Yet, a spatial separation of populations not only occurs on a horizontal perspective, but also in a vertical dimension in the water column (Sommer 1982), where large environmental gradients (like temperature and salinity) can exist over small distances. If two species diverge along such a gradient, due to differences in selective pressure, they adapt to different water depths and shift their reproduction range apart. This mode of speciation is described as parapatry or depth parapatric speciation (Figure 1.8B and D; e.g. Pierrot-Bults & van der Spoel 1979; Briggs 1999; Schluter 2001). Since planktonic foraminifera morphospecies were observed to occupy different depth levels in the water column (Figure 1.3; Schiebel & Hemleben 2005), the vertical dimension clearly has to be considered when trying to explain the origin of the high genetic diversity within planktonic foraminifera morphospecies.

A biological mode of speciation, common between entirely sympatric populations, is a shift in the timing of reproduction (e.g. Palumbi 1994). Since for some planktonic foraminifera species, a dependence of reproduction on the lunar or semilunar cycle was suggested (Bijma *et al.* 1990), population divergence might happen through a temporal shift of the reproduction peak around this lunar periodicity (Figure 1.8E; Norris 2000). This would reduce the probability for gene flow, leading to reproductive isolation. On an even smaller scale, reproductive isolation can be enforced by changes in mate recognition or gamete incompatibility (Palumbi 1994).

Considering the high genetic diversity within planktonic foraminifera morphospecies, which is assumed to represent the level of biological species, the prevalence of speciation in plankton becomes obvious. The differentiated biogeographic distribution patterns of these cryptic species can be used to draw conclusions on the mode of speciation and the historical processes that shaped the now observable patterns (Arnold & Parker 2002; Kocher 2005).

Observations of differentiated distribution patterns were reported for the three sibling species of *Orbulina universa* (de Vargas *et al.* 1999; Morard *et al.* 2009). They clearly show a non-random distribution, occurring at different hydrographic conditions, depending on the primary productivity of the surface water. Whereas one of them was present in areas of high chlorophyll concentration, the second was restricted to oligotrophic and the third to extremely oligotrophic conditions. This distribution pattern very likely is the result of a sympatric or parapatric speciation event between the populations of a highly abundant

species that adapted to different environmental pressures. Conversely, a horizontal segregation was found between the cryptic species of *Globigerinoides ruber*. Aurahs *et al*. (2009b) could show that the two most closely related cryptic species occur in different basins of the homogenous Mediterranean Sea, whereas they co-occur in the more mixed region of the eastern Atlantic. This present day distribution pattern was explained to be the result of vicariant separation of Atlantic and Mediterranean populations during a glacial maximum. After reproductive isolation on either side of the Strait of Gibraltar, reinvasion of the Atlantic species into the entire Mediterranean Sea was unsuccessful due to competitive exclusion, whereas the mixed waters of the Atlantic allow a co-existence of both types. The two sibling species constituent in the morphospecies *Globorotalia inflata* were shown to exhibit a strictly allopatric distribution pattern (Morard *et al.* 2011). They are separated along the Antarctic Subpolar Front, which restricts one of them to transitional and subtropical and the second one to Antarctic water masses, clearly indicating a previous allopatric speciation event. Notwithstanding the fact that highly differentiated distribution patterns have been shown to be maintained, the possibility for global gene flow in planktonic foraminifera cannot be entirely excluded, since many cryptic species or genetic types exhibit complete genetic homogeneity independent of their geographic location (Norris 2000). The genetic homogeneity within the bipolar genetic types of the subpolar/polar morphospecies *Turborotalita quinqueloba*, *Neogloboquadrina incompta* and *Globigerina bulloides* is a striking example and suggest the existence of continuous trans-tropical gene flow (Darling *et al.* 2000; Darling & Wade 2008). Furthermore, each of the sibling species of *O. universa* presents genetic identity throughout its global range (de Vargas *et al.* 1999) and the same holds for several other species (Darling & Wade 2008). This pattern suggests that the distribution of plankton organisms is influenced by a mixture of high dispersal and gene flow and local restrictions and adaptations.

Although the divergence of two populations into separate species can occur rapidly, it usually is a continuous process extending over many generations and it can take even millions of years for species to form (Coyne & Orr 2004). The application of molecular clocks allows dating of the speciation events of planktonic foraminifera and eventually a relation to geologic events in the past (Knowlton 2000; Darling *et al.* 2004). Since speciation takes place over such enormous time scales, it is possible that geographic and biological elements of speciation alternate over time until complete genetic isolation is achieved (Norris & Hull 2011), a pattern that cannot be revealed by the distribution of living organisms.

2. Motivation and Objectives

Owing to their excellent fossil record, planktonic foraminifera play an important role as proxies for the reconstruction of past oceanic conditions (e.g. Kucera *et al.* 2005). The chemical signature of their shells records the properties of the ambient seawater, to which a specimen was exposed during the time of biomineralisation of its shell (e.g. Kucera & Schönfeld 2007). Given that every species possesses its distinct ecological adaptations and physiological characteristics, the geochemical composition of the shell consequently varies between separate species (e.g. Hemleben *et al.* 1989). In order to receive high resolution paleoceanographic reconstructions, a precise taxonomy and an exact knowledge of the level of biological species and their ecological requirements is thus of essential importance. As a consequence, the biological perspective of this enigmatic group of microplankton still requires close attention. Especially the number of extant morphospecies that are analyzed in regard to their extent of hidden genetic diversity has to be further increased, to be able to estimate the biological diversity of planktonic foraminifera. So far, cryptic diversity seemed to be prevalent in all morphospecies studied, partly reaching surprisingly high numbers of cryptic species per morphospecies (for a review see: Darling & Wade 2008). However, a morphological differentiation of the cryptic species was so far rarely achieved (Darling *et al.* 2006; Aurahs *et al.* 2011), but is indispensable in order to recognize biological species in the sediment assemblages. In addition, the biogeographical distribution patterns of cryptic species require further examination, in order to discover small scale adaptations that would have consequences for the application of foraminifera in micropaleontological studies. Regional endemism of cryptic species has been discovered before (e.g. Aurahs *et al.* 2009b), however, it is not yet known if restricted distribution patterns are an exception or the rule in planktonic foraminifera. The biogeographic distribution patterns of cryptic species in the present day ocean further are of great interest, because they represent an indicator of the modes of speciation prevalent in plankton that are responsible for the origin of the unexpectedly high biodiversity encountered in many groups of marine protists. Large scale sampling of planktonic foraminifera throughout the world ocean is therefore now required, covering as much of the distribution range of a morphospecies as possible to enhance chances to detect its entire constituent cryptic diversity and to characterize the distribution patterns of the cryptic species.

With the aim to contribute to the ongoing research on the genetic diversity of planktonic foraminifera, the relationship between their morphology and genetics as well as their biogeographic distribution patterns in the world ocean, the following three research questions were tested in the present PhD thesis:

I) Is cryptic diversity a pervasive phenomenon in planktonic foraminifera morphospecies and can its extent be predicted from the characteristics of a morphospecies, such as its morphological variability, abundance and distribution in the ocean?

II) Does a potential morphological distinction of cryptic species depend on the phylogenetic distance between them?

III) Is the biogeographical distribution of cryptic species of planktonic foraminifera marked by high endemism or rather global dispersal and gene flow?

In order to address these research questions, three different planktonic foraminifera morphospecies, *Hastigerina pelagica*, *Globigerinoides sacculifer* and *Globigerinella siphonifera*, were selected as ideal candidates for the studies. They all occur globally throughout the low latitude regions of the ocean (e.g. Hemleben *et al.* 1989) and can, therefore, be compared in regard to the prevalence of either circumglobal dispersal and gene flow across the colder waters of the Temperate Regions or local adaptations and high potential for the establishment of genetic isolation. Extensive sampling in the Tropics, Subtropics and Temperate regions allowed a detailed analysis of the genetic diversity, morphometry and biogeography of these species throughout their distribution ranges.

Hastigerina pelagica exhibits rather low abundances in the ocean, what generally grants a species high potential for genetic isolation, because of large geographical distances between the populations (e.g. Pierrot-Bults & van der Spoel 1979). Only three cryptic species had been discovered before within the morphospecies, but this number was based only on few samples (Aurahs *et al.* 2009a). The now available collection of samples permitted further screening of the morphospecies for cryptic diversity. Extended sampling in the Mediterranean Sea and Atlantic and as new locations in the Caribbean Sea and Western Pacific was carried out to check for restricted distribution patterns of the cryptic species. The genetic divergence between the known cryptic species had been observed to be unusually high (compare Table 1.2) and therefore, they seemed promising for the detection of morphological variability. Chapter 4 of the present thesis comprises the studies carried out on this morphospecies.

Globigerinoides sacculifer constitutes a highly abundant and cosmopolitan plexus of four different morphotypes. Because of the high morphological variability it seemed likely that a comparable amount of genetic diversity might be detected, now that a large enough dataset was available, covering wide areas of the morphospecies' distribution range. Furthermore, with knowledge on the genetic background of the morphospecies, the taxonomic validation of the morphotypes could be examined. Chapter 5 describes the findings on the genetic diversity and biogeography of *G. sacculifer*.

The *Globigerinella siphonifera* plexus had long been in the focus of genetic and morphometric analyses. The morphospecies is known to contain an unusually high genetic diversity (de Vargas *et al.* 2002; Darling & Wade 2008), which due to its partly only slight genetic divergence had not been entirely resolved. Furthermore, the status of its sister species *G. calida* as a separate species still remained questionable. The accumulation of a large dataset allowed further examination of the extent of cryptic diversity within this morphospecies plexus and the biogeographical distribution patterns of the cryptic species. The cryptic species are marked by highly different genetic distances between each other, and are therefore a promising example to study the relationship between genetic and morphological evolutionary rates. The genetic, biogeographic and morphometric studies carried out on the *G. siphonifera* plexus are represented in Chapters 6 and 7 of this thesis.

3. Methods applied in single-cell foraminifera genetics

3.1. Sampling of planktonic foraminifera

Sampling of planktonic foraminifera for genetic analysis was either conducted during sea-going expeditions or during visits to marine research stations. In both cases, sampling took place at locations with a water depth greater than 100 m and preferably even off the shelf, since planktonic foraminifera occur in open ocean habitats and are consequently not found in great numbers close to the coast (Hemleben *et al.* 1989). The exact locations sampled by the working group and by coauthors are indicated in Figure 3.1. Depending on the equipment of the vessel, sampling was carried out either with a multi-closing net (MCN, 1 m^2 opening, 100 µm mesh size) or with a small handheld plankton net (0.5 m diameter opening, 100 µm mesh size). The use of a MCN allows stratified sampling of the water column in five different depth intervals. We routinely towed vertically from a water depth of 700 m, below which hardly any living individuals are found (e.g. Arnold & Parker 2002), and divided the water column in the intervals 700-500 m, 500-300 m, 300-200 m, 200-100 m and 100-0 m in order to retain consistency between all sampling stations. In most cases a second net was taken at the same station separating the upper 100 m in five equal intervals. Stratified sampling of the water column allows a detailed analysis of the vertical distribution of different foraminifera species. When sampling from small boats the plankton net was used that can be towed by hand. This net was applied in two different ways, by sampling vertically from a water depth of 100 m to the surface or by sampling horizontally by pulling the net behind the boat at a water depth of ~5 m for 5 minutes.

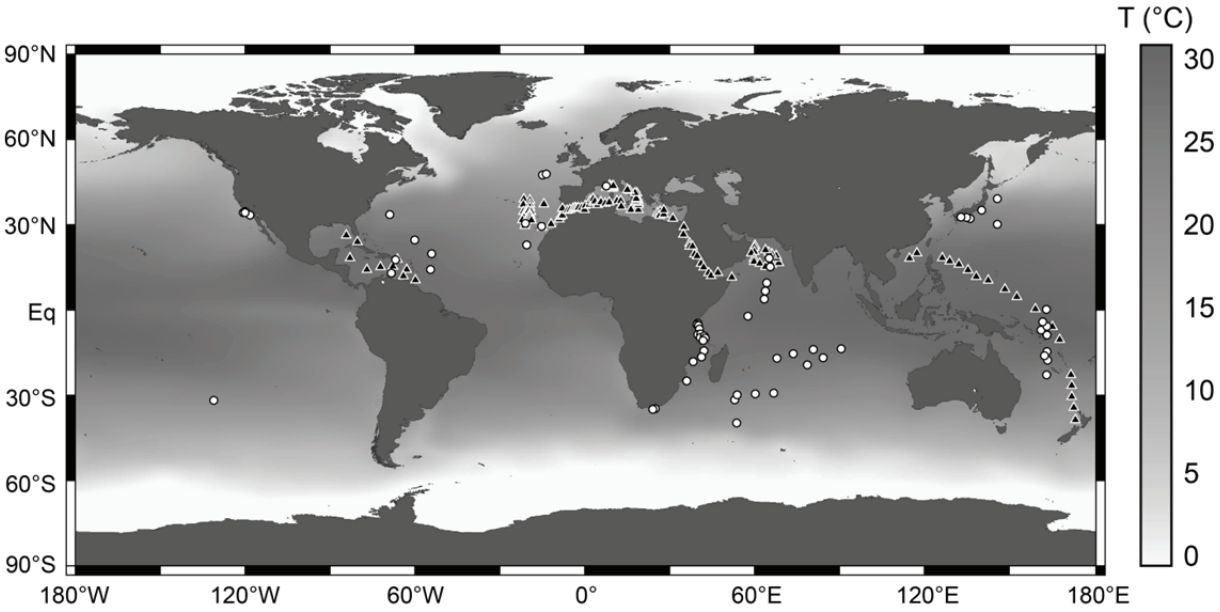

Figure 3.1: World map showing all sampling sites from which planktonic foraminifera specimens were collected for studies included in the present thesis, combining expeditions from the working group (triangles) and from coauthors (circles; exact descriptions of the cruises are found in the publications). Grey shading in the background of the map indicates sea surface temperature on an annual average (Ocean Data View).

In order to obtain untouched, undisturbed individuals, in Puerto Rico planktonic foraminifera were also sampled by diving in water depth up to 5 m. Using this approach, specimens are taken right out of their natural environment by capturing them in plastic jars, avoiding the damage of the organisms caused by entangling in plankton nets. At most open ocean stations sampled onboard the research vessels, CTD (conductivity, temperature, depth) data were collected and chlorophyll concentrations were measured using a fluorescence probe in order to be able to characterize the environmental conditions of the water column.

Immediately after resurfacing the net, plankton samples were washed out of the collection cups of the nets with filtered sea water (filtered over 63 µm gauze). Picking of planktonic foraminifera started as soon as possible, however, depending on the number of samples obtained, they sometimes had to be stowed in the fridge until the next day to finish picking. All foraminifera present in the plankton sample, including empty shells, were isolated and cleaned under stereomicroscopes using a fine brush and immediately transferred to cardboard slides for preservation (Figure 3.2A-C). After cleaning the specimens again to prevent attachment of contaminants and arranging them individually on the slide, the slides were air dried and frozen at -80 °C until they were transported to Germany on dry ice.

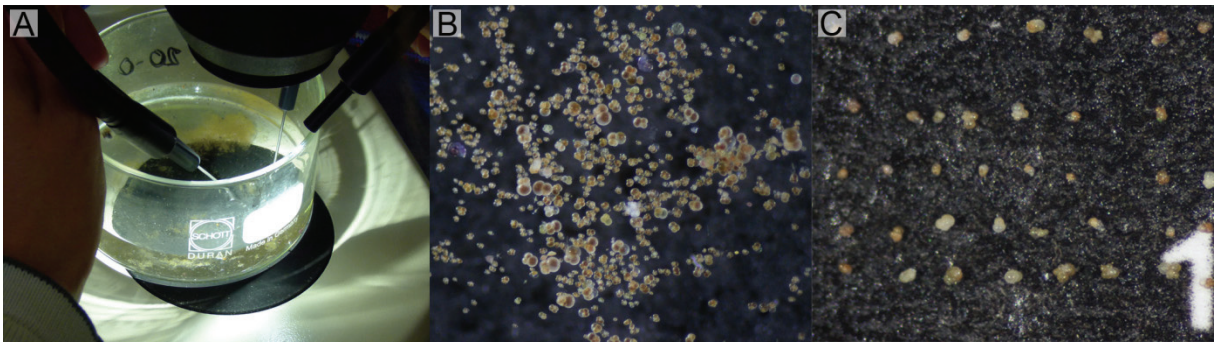

Figure 3.2: A) Picking of planktonic foraminifera specimens out of the plankton sample under a stereomicroscope (Photo: M. Kucera). **B)** Accumulation of planktonic foraminifera specimens in a plankton sample (Photo: A. Weiner). **C)** Cleaned individuals arranged and air dried on a cardboard slide for preservation (Photo: WG Kucera).

3.2. Culturing of planktonic foraminifera

All specimens collected by diving as well as healthy looking individuals from plankton tows were kept in culture until they were observed to undergo gametogenesis. These culturing experiments were carried out onshore during visits to the marine stations in Eilat, Las Cruces and Isla Magueyes with the aim to increase the DNA yield in molecular analysis by working with gametogenic foraminifera, which comprise multiple genomes with only little contamination by symbionts and food particles (compare Darling *et al.* 1996b). Therefore, individual spinose foraminifera were cleaned of obvious contaminants and transferred to 50 ml cell culture jars with an air permeable lid (Figure 3.3A). These jars contained ~30 ml filtered sea water, filtered over 0.25 µm pore size Millipore filter to prevent further con-

tamination. All culturing jars were kept at a constant temperature corresponding to the water temperature at the sampling stations and under daylight lamps in a day-night cycle. Each foraminifera was fed an *Artemia salina* nauplius every other day by sticking the brine-shrimp into its spines and pseudopodia network. Usually, within one day after sampling the foraminifera had recovered from sampling stress and rebuild their spines (Figure 3.3B). As soon as the specimens were observed to shed their spines again and sink to the bottom of the jar, which are indicators for approaching gametogenesis (Bé *et al.* 1983), they were transferred to smaller vials containing 5 ml of freshly filtered sea water and from now on were observed even more frequently. At the time of formation of granular cytoplasm close to the aperture followed by the appearance of a mass of fast moving flagellated gametes (Figure 3.3C), the shell containing the gametes was placed into a 1.5 ml Eppendorf tube containing 50 µl of ethanol and frozen at -20 °C.

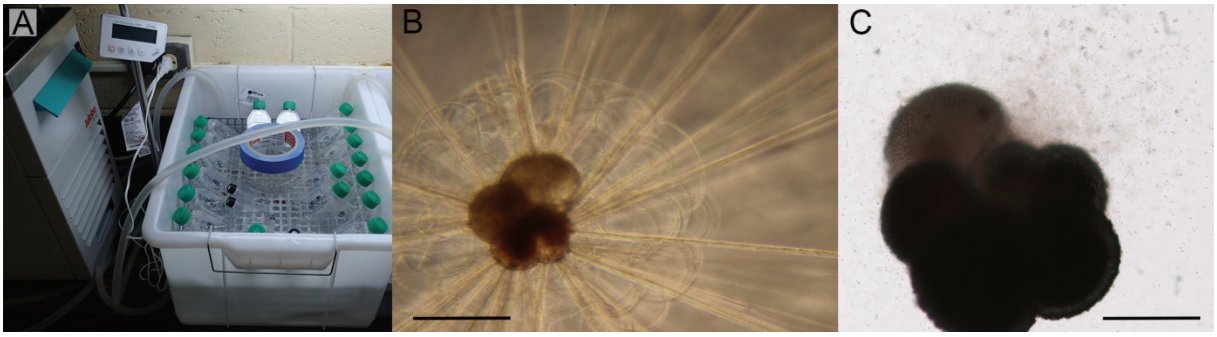

Figure 3.3: A) Culturing jars containing individual foraminifera specimens kept at constant temperature in a water bath under a daylight lamp in day-night cycle until gametogenesis can be observed (Photo: A. Weiner). **B)** *Hastigerina pelagica* in culture with regrown spines and an extended cytoplasmatic bubble capsule, scale bar 200 µm (Photo: WG Kucera). **C)** *Globigerinella siphonifera* in culture producing thousands of highly motile gametes that escape from the aperture, scale bar 100 µm (Photo: A. Weiner).

3.3. DNA extraction and molecular analysis

Back home in the laboratory, frozen specimens were individually picked off the cardboard slide, photographed under a stereomicroscope, taxonomically identified and catalogued. DNA extraction of the preserved individuals was then carried out by applying either the DOC (Holzmann & Pawlowski 1996) or GITC protocol (e.g. Morard *et al.* 2009), the latter allowing preservation of the calcite shell, which subsequently can be used for morpho-metric analysis.

1) *DOC buffer:* Each individual was transferred with a brush to a 1.5 ml Eppendorf tube containing 25 µl DOC buffer. 100 ml DOC buffer consist of 10 ml 1 M Tris- HCl pH 8.5, 0.8 ml 0.5 M EDTA, 10 ml 10% DOC (Sodium deoxycholate), 2 ml 10% Triton-X-100 and 77.2 ml H_2O_{bidest}, stored at room temperature. The tubes were then incubated under gentle shaking at 60 °C for one hour, during which the calcite shell is dissolved. Afterwards the extractions were stored in the fridge at 4 °C until further analysis.

2) *GITC buffer:* Each foraminifera was placed into a 1.5 ml Eppendorf tube containing 50 µl GITC buffer. 100 ml GITC buffer contain 50 g GITC, 10.6 ml 1 M Tris- HCl pH 8.5, 10.6 ml 20% Sarcosyl, 1.05 ml Mercaptoethanol, filled up to 100 ml with H_2O_{bidest}. The tubes

were then heated for one hour at 70 °C under shaking. After separating the empty shell from the DNA extract, 50 µl Isopropanol were added to the extract and the tubes were kept at -20 °C over night. The next day they were centrifuged at 18,000 rpm for 15 minutes and the Isopropanol was removed. The resulting pellet was washed with 50 µl Ethanol (96 %), which was taken off again after another centrifugation step (18,000 rpm, 15 minutes). The pellet was then resuspended in 20 µl H_2O_{bidest} and stored at -20 °C.

The 3' fragment of the SSU rDNA (see Figure 1.5) was amplified by Polymerase Chain Reaction (PCR), which was conducted under the following settings. The reaction mix was prepared with 8.26 µl H_2O, 3 µl reaction buffer, 1.5 µl $MgCl_2$ (25 mM), 0.6 µl dNTPs (10 µM each), 0.15 µl Primer 1 and 2 (10 µM) and 0.15 µl Taq polymerase, adding 1 µl sample DNA. The PCR reaction was carried out at 95 °C for 2 min, followed by 35 repetitions of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec and the terminating step at 72 °C for 10 min. In cases where no PCR product was obtained after the first PCR run, nested PCR was conducted as a second step. Therefore, after the first run with foraminifera group specific primers, the second run was conducted with species specific primers that anneal within the already covered fragment. A further optimization strategy that was applied for some samples was the dilution of the DNA extract with 20 µl of H_2O_{bidest} prior to the PCR reaction. This leads to a simultaneous dilution of PCR inhibiting substances resulting in enhanced DNA yield. In order to screen specimens for intra-individual genetic variability due to the possession of different copies of the rDNA, it was mandatory to clone the gene of a couple of individuals of each morphospecies. Therefore a ~500 bp fragment of the SSU rDNA was inserted into a plasmid vector of the Zero Blunt® Topo® PCR Cloning Kit (Invitrogen) and multiplied in chemically competent *E. coli* cells. All steps were carried out according to the manufacturer's protocol and the plasmids were afterwards purified using the PureLink® HQ Mini Plasmid Purification Kit (Invitrogen). Sequencing of all PCR products and clones was done by Sanger sequencing by an external service provider (LGC Genomics, Berlin, Germany), and followed by a bioinformatic evaluation of the sequences in order to screen for genetic variability.

4. First case study

Vertical niche partitioning between cryptic sibling species of a cosmopolitan marine planktonic protist

Agnes Weiner, Ralf Aurahs, Atsushi Kurasawa, Hiroshi Kitazato and Michal Kucera

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Idea/Hypothesis: 50%

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Interpretation: 60%

Writing of manuscript: 80%

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Vertical niche partitioning between cryptic sibling species of a cosmopolitan marine planktonic protist

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Abstract

A large portion of the surface-ocean biomass is represented by microscopic unicellular plankton. These organisms are functionally and morphologically diverse, but it remains unclear how their diversity is generated. Species of marine microplankton are widely distributed because of passive transport and lack of barriers in the ocean. How does speciation occur in a system with a seemingly unlimited dispersal potential? Recent studies using planktonic foraminifera as a model showed that even among the cryptic genetic diversity within morphological species, many genetic types are cosmopolitan, lending limited support for speciation by geographical isolation. Here we show that the current two-dimensional view on the biogeography and potential speciation mechanisms in the microplankton may be misleading. By depth-stratified sampling, we present evidence that sibling genetic types in a cosmopolitan species of marine microplankton, the planktonic foraminifer *Hastigerina pelagica*, are consistently separated by depth throughout their global range. Such strong separation between genetically closely related and morphologically inseparable genetic types indicates that niche partitioning in marine heterotrophic microplankton can be maintained in the vertical dimension on a global scale. These observations indicate that speciation along depth (depth-parapatric speciation) can occur in vertically structured microplankton populations, facilitating diversification without the need for spatial isolation.

Keywords: biogeography, depth segregation, foraminifera, niche partitioning, plankton, speciation

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Introduction

The surface of the world oceans is inhabited by enormous populations of microscopic free-floating phyto- and zooplankton. The small size of these organisms limits their ability for active dispersal on a global or even regional scale. Instead, marine plankton is passively carried by currents throughout the world oceans. In such groups where individuals or propagules can endure exposure to suboptimal environmental conditions during transport, the potential for dispersal is thus only limited by the degree of connectivity between oceanic basins. The dominance of such passive dispersal and the inability to counteract it by active movement has led to the idea that the pelagic environment is conducive for a cosmopolitan species distribution (e.g. Finlay & Esteban 2001) and initiated hypotheses questioning the potential for geographic isolation in marine microplankton (e.g. Finlay 2002; Fenchel & Finlay 2004). Considering the high potential for dispersal and the huge population sizes of the plankton, one might expect that the probability of speciation in these organisms should be reduced. Yet, speciation must be a common phenomenon in marine plankton because of abundant evidence for rapid species turnover in the fossil record (e.g. Lazarus 1983; Norris 2000; Benton & Pearson 2001) and the high global diversity in many groups of modern marine

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plankton, which is manifested morphologically (Irigoien *et al.* 2004), or genetically (e.g. Sáez *et al.* 2003; Amato *et al.* 2007).

Studies attempting to unravel speciation processes in the plankton are confronted with two challenges: the missing temporal dimension on an evolutionary scale (Norris & Hull 2011) and the difficulty to delineate evolutionarily significant units (Crandall *et al.* 2000). The latter issue boils down to the need to understand the spatial and temporal dimension of gene flow in planktonic organisms. Until now, this phenomenon has been mainly considered from a two-dimensional perspective of geographical isolation in a homogenous surface layer. However, next to the horizontal (geographical) dimension, the pelagic environment can also be structured vertically. In this dimension, large environmental gradients occur over short distances, and the scaling between active movement (buoyancy) and passive dispersal is reversed. This is particularly relevant for species whose habitat stretches across vertical structures in the water column such as the thermocline. In such species, gene flow in the vertical dimension could be significantly reduced, or a gradient could develop such that gene flow between adjacent populations is substantially more likely than between more distant populations, providing an opportunity for population divergence (e.g. Pierrot-Bults & van der Spoel 1979; Briggs 1999; Schluter 2001).

Planktonic foraminifera have been frequently used as model system to investigate speciation patterns in the plankton (e.g. Levinton & Ginzburg 1984; Norris *et al.* 1996; Norris 2000; Allen *et al.* 2006). Today, there are about 50 morphospecies inhabiting the world ocean, the majority of which show a cosmopolitan distribution within their preferred temperature range (e.g. Hemleben *et al.* 1989). Molecular genetic analyses of the last decade have revealed a high genetic variability within almost every morphospecies of planktonic foraminifera (e.g. Darling & Wade 2008). This is significant because planktonic foraminifera are obligate sexual outbreeders (Hemleben *et al.* 1989), and the absence of evidence for hybridization in these SSU rDNA genotypes implies reproductive isolation, that is, biological species (e.g. de Vargas *et al.* 2004; Aurahs *et al.* 2009a). This indicates that the species richness and biological diversity within the group is much higher than previously thought. Further, these cryptic species often show different habitat requirements and biogeography (e.g. Aurahs *et al.* 2009a; Morard *et al.* 2009), indicating that the cosmopolitan distribution observed for a morphospecies may be a combination of several more complex distribution patterns at the level of cryptic species.

Explanations for the emergence of this high number of genotypes so far mainly focused on horizontal distribution patterns (e.g. de Vargas *et al.* 2001; Darling *et al.* 2006). A horizontal separation between closely related genotypes was found for example in the morphospecies *Globigerinoides ruber* (Kuroyanagi *et al.* 2008; Aurahs *et al.* 2009a). The most closely related genotypes seemed to avoid each other, which resulted in strict habitat segregation in the Mediterranean Sea (Aurahs *et al.* 2009a). In contrast to the habitat separation found in the Mediterranean *G. ruber*, extensive gene flow was observed in genotypes of the bipolar foraminifera species *Globigerina bulloides*, *Turborotalita quinqueloba* and *Neogloboquadrina incompta* (Darling *et al.* 2000; Stewart *et al.* 2001). Genotypes with identical SSU rDNA sequences were found in Arctic and Antarctic subpolar provinces, suggesting recent trans-tropical genetic exchange (Darling *et al.* 2000). The same pattern has been observed for a number of genotypes within tropical and subtropical species, which yield identical DNA sequences in the Atlantic and Pacific oceans (e.g. Darling & Wade 2008). Thus, the biogeography of the genetic types in planktonic foraminifera does not provide strong evidence for the prevalence of allopatric speciation in the pelagic realm.

Considering the high potential for gene flow in the marine habitat and the cosmopolitan occurrence of many planktonic foraminiferal species, the question rises on how the observed genetic diversity could have evolved. In foraminifera as well as in other planktonic organisms, alternative mechanisms for the origin of reproductive isolation have been suggested (e.g. Briggs 1999; Norris 2000). Specifically, authors have often referred to the possibility of speciation by depth parapatry (Lazarus 1983; Norris 2000). This model assumes that planktonic organisms are able to maintain a preferred vertical position in the water column. As separation takes place in the vertical direction, parapatric populations appear to occur sympatrically in the horizontal direction. Many groups of heterotrophic microplankton occur across a wide depth range, but their species are typically limited to a much narrower vertical interval (e.g. Ishitani & Takahashi 2007). Most morphospecies of planktonic foraminifera also occupy a restricted habitat in the water column, defined by their temperature tolerance or the possession of symbionts, limiting them to the mixed layer above the thermocline (e.g. Hemleben *et al.* 1989). In planktonic foraminifera, it has been hypothesized that partitioning of niches in the vertical dimension could explain changes in morphological and size disparity in the group through time (Schmidt *et al.* 2004) and in space (Al-Sabouni *et al.* 2007). The existence of a relationship between diversity and disparity and the strength of the vertical water structure is consistent with the model of depth-parapatric speciation, which could be an important mechanism

that generates diversity in response to past changes in the water column structure.

Vertical depth segregation among sister species is known from other groups of plankton, such as copepods (Mackas *et al.* 1993; Fragopoulu *et al.* 2001) and chaetognaths (Kehayias *et al.* 1994). Even though researches have shown evidence for habitat heterogeneity within the foraminiferal morphospecies *Globigerinella siphonifera* (Bijma *et al.* 1998), its correlation with genetic divergence has not been established (Huber *et al.* 1997). So far, there has been no evidence for vertical habitat separation among sibling genetic types in planktonic foraminifera. This reflects the fact that almost all studies of the distribution of these genetic types in planktonic foraminifera have been based on depth-integrated sampling (e.g. Darling *et al.* 2000; de Vargas *et al.* 2001, 2002).

Here we present the results of a global survey based on depth-stratified sampling of genetic diversity within the eurybathyal planktonic foraminifer morphospecies *Hastigerina pelagica* (d'Orbigny 1839). This species occurs in temperate to tropical waters throughout the global oceans (e.g. Parker 1960; Tolderlund & Bé 1971; Loncaric *et al.* 2005). Its vertical habitat ranges from subthermocline depths (Tolderlund & Bé 1971; Schiebel & Hemleben 2005) to the ocean surface (e.g. Hemleben *et al.* 1989). The Hastigerinidae are characterized by a number of unique morphological features. Their monolamellar wall ultrastructure, triradiate spines with lateral hooks, cytoplasmic bubble capsule (Alldredge & Jones 1973; Hemleben *et al.* 1989; Hull *et al.* 2011) and maximum shell size in excess of 1 mm separate *H. pelagica* and its sister species *Hastigerinella digitata* from all other living planktonic foraminifera. Both species lack symbionts (Hemleben *et al.* 1989). They are exclusively carnivorous and found to feed predominately on copepods (Anderson & Bé 1976; Hull *et al.* 2011).

Despite their conspicuous appearance and abundance in the plankton, limited data exist so far on the genetic variability and phylogeography of the cosmopolitan and broadly vertically occurring *H. pelagica*. Existing SSU rDNA sequences from this species form three distinct clusters (Fig. 1), with two more closely related to each other (Göker *et al.* 2010). To characterize the degree of

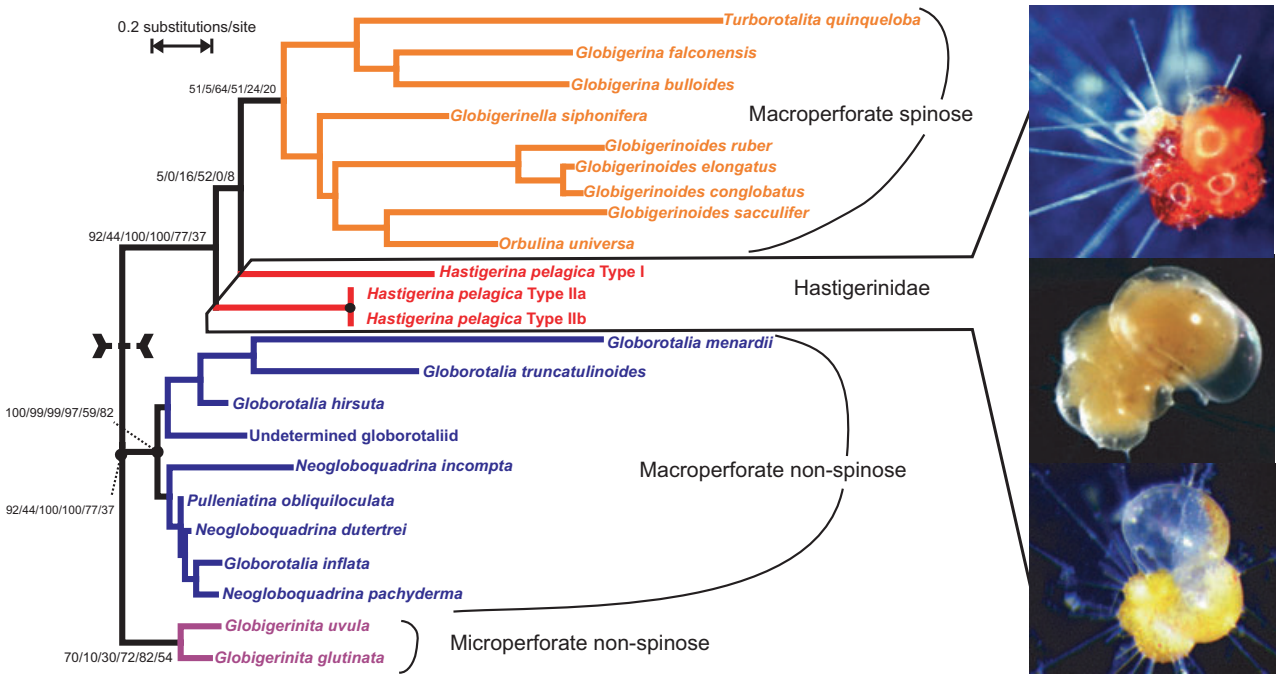

Fig. 1 Phylogenetic relationships of the four major groups of planktonic foraminifera based on a maximum likelihood reconstruction of the 3′ fragment of the SSU rDNA, modified after Aurahs *et al.* (2009b). Node supports show bootstrap values (bs) from clustalw/kalign/mafft/nralign/poa/poaglo-automated alignments (Aurahs *et al.* 2009b). Only bs values for the basal nodes of the groups of the macroperforate spinose (orange), the macroperforate nonspinose (blue) and microperforate nonspinose (purple) are shown. Sequence diversity within morphospecies has been collapsed. All node supports for the analysed *Hastigerina* sequences are shown; black circle indicates bs values of 100 for all automated alignments. As discussed in Aurahs *et al.* (2009b), the use of an automated alignment with multiple approaches allows a large coverage of the alignment space whilst avoiding the ambiguity of a manual approach. Light microscopic images of *H. pelagica* taken on board the research vessel before DNA extraction are shown to illustrate the gross morphology. Pictures show (top down) individuals of Type I, Type IIa and Type IIb. All individuals are ~0.5 mm across.

genetic and vertical differentiation within these genetic types of *H. pelagica*, we sampled this species in four oceanic regions throughout the top 700 m of the water column. Our survey reveals a vertical structuring in the occurrence and abundance of three globally distributed genotypes within *H. pelagica*, providing support for theories of depth-parapatric speciation in planktonic organisms.

Materials and methods

Sampling

Planktonic foraminifera were collected in the Mediterranean Sea and the north-eastern Atlantic Ocean during RV Poseidon cruises P321, P334, P349, P411 and P413 and during RV Meteor cruises M69/1, M71/2 and M71/3 (Table S1, Supporting information). These cruises took place throughout the years 2005–2011 and covered all seasons. Samples from the Caribbean Sea were taken onboard the RV Meteor (M78/1, Table S1, Supporting information). A multiclosing net with a mesh size of 100 μm was used for stratified sampling of the water column. Depth intervals of sampling were mainly 700–500 m, 500–300 m, 300–200 m, 200–100 m and 100–0 m. Short intervals in the upper 100 m were 100–80 m, 80–60 m, 60–40 m, 40–20 m and 20–0 m. The western Pacific was sampled during cruises MR10-06 and KT07-14 (Table S1, Supporting information) using NORPAC closing net (100 μm mesh size) with intervals of 0–25 m, 25–50 m, 50–100 m and 100–200 m and IONESS (330 μm mesh size) with intervals of 0–50 m, 50–100 m, 100–150 m, 150–200 m and 200–300 m. Planktonic foraminifera were isolated and taxonomically identified on board using a stereomicroscope. Live individuals containing cytoplasm were carefully cleaned with a brush, digitally photographed and transferred into 1.5 ml tubes for DNA isolation. Samples collected during P411 and P413, as well as during MR10-06 and KT07-14 were cleaned, then air-dried at room temperature in cardboard slides and stored at −20 °C and −80 °C until further processing.

For the biogeographic analysis, we also used data on 10 genotyped individuals of *Hastigerina pelagica* that have been previously published in Aurahs *et al.* (2009b). The specimens comprised one individual of Type I, six of Type IIa and three of Type IIb (Table S1, Supporting information). In addition to these data, sequences of only five more individuals of Type I are available in GenBank (de Vargas *et al.* 1997; Ujiie & Lipps 2009). These Type I sequences have been used in the phylogenetic analyses by Aurahs *et al.* (2009b) as shown in Fig. 1, but are not included in this study in Fig. 2, because the data on their collection depth are not available.

DNA extraction, amplification and sequencing

DNA extraction from the Atlantic specimens followed the DOC protocol of Holzmann & Pawlowski (1996). For the samples from the Pacific, the guanidine method for DNA extraction was applied (e.g. Morard *et al.* 2009). For the differentiation of genotypes of *H. pelagica* a ~450 bp large fragment of the 3′ end of the small subunit ribosomal RNA (SSU rRNA) gene was amplified by polymerase chain reaction (PCR) using the proofreading Vent® polymerase (New England Biolabs) and Ex Taq polymerase (TaKaRa Bio, Inc.). The new primers pelv3F (5′ GTGCATGGCCGTTCTTAGTTCGTG 3′) and pelv3R (5′ TATTGCCGCATCCTTCCTCTGGTT 3′) were used for amplification. PCR products were purified using the QIAquick gel extraction kit (Qiagen) and afterwards sequenced directly by an external service provider (Agowa, Berlin). The PCR products from the Pacific were purified using the Monofas DNA Purification Kit (GL Science) and directly sequenced using the Big Dye V3.1 Terminator Cycle Sequencing Kit and an ABI 3130xl Genetic Analyzer (Applied Biosystems, Inc.). Sequence chromatograms were manually scanned for ambiguous reads and corrected where possible. Sequences were then aligned manually for the recognition of the genotypes in *H. pelagica*. Sequences of 93 individuals were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/; accession nos JQ624776–JQ624868). Four sequences of low quality and three sequences shorter 200 bp allowed assignment to one of the *H. pelagica* genotypes but were not thought suitable for publication in GenBank. All Type II sequences used in this study are made available as supplement.

Phylogenetic reconstruction

The phylogenetic reconstruction of *H. pelagica* Types IIa and IIb is based on a manual alignment of 114 sequences. The alignment contains all already published sequences (direct sequences and clones) for these two genotypes (http://www.ncbi.nlm.nih.gov/) and the new sequences from this study. Based on results from Aurahs *et al.* (2009b) and Göker *et al.* (2010), all sequences were cut to the same length of 316 bp, aligned manually and then further analysed in a maximum likelihood reconstruction, using the web-based RAxML version (Stamatakis *et al.* 2008) (http://phylobench.vital-it.ch/raxml-bb/index.php) with gamma model of rate heterogeneity and maximum likelihood search. In accordance with the results from Aurahs *et al.* (2009b), we used the alignment untruncated, that is, did not check for position homology. Phylograms were constructed using the ML best tree with bootstrap values in Dendroscope 2.7.4 (Huson *et al.* 2007). In-group Kim-

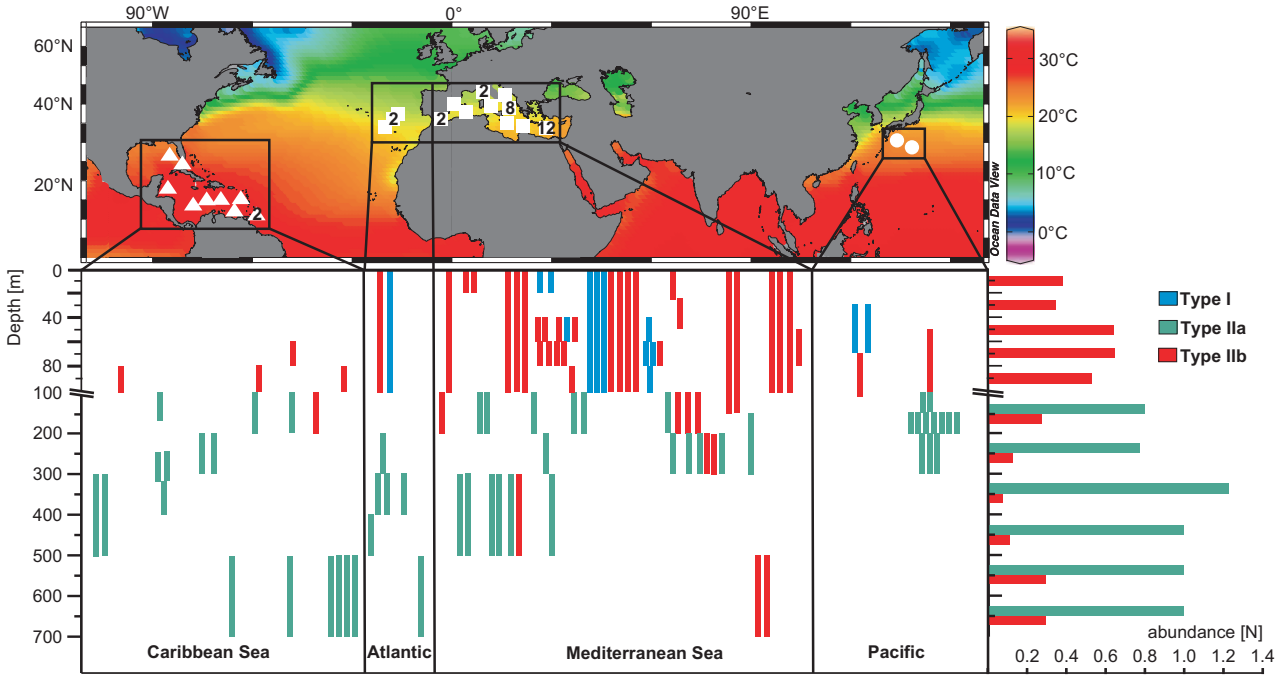

Fig. 2 Geographic and vertical distribution of the three genetic types found in *Hastigerina pelagica* in the global ocean. Colours indicate the annual mean sea surface temperature (data from the world ocean atlas, http://odv.awi.de/en/data/ocean/world_ocean_atlas_2009/), plotted using Ocean Data View 4.3.2. Symbols in the map indicate sampling locations, numbers indicate very closely located stations. The bottom panel shows the vertical distribution of individual *H. pelagica* genotypes at the respective regions along an idealized west–east transect. Bars represent depth intervals in which the respective genetic type was found. Type IIa (green bars) is only found below 100 m water depth, Type IIb (red bars) almost exclusively above 200 m. This is also the case for the few individuals of Type I (blue bars) found in our sampling. Right panel shows the number of sequenced individuals within standardized depth intervals of 20 m above and of 100 m below 100 m water depth.

ura-2 distances and nucleotide differences were calculated using the genetic distance calculation models implemented in MEGA5 (Tamura *et al.* 2011) under default setting.

Results

Sequences of a ~450 bp fragment of the 3′ end of the SSU rRNA gene were obtained from a total of 100 specimens, morphologically identified as *Hastigerina pelagica*, from 43 stations in the Caribbean Sea, eastern Atlantic Ocean, Mediterranean Sea and western Pacific Ocean. All sequences could be assigned to one of the three published genetic motives of *H. pelagica* (Aurahs *et al.* 2009b). Individuals of Type I were found at one location in the western Pacific (n = 2) and at one location in the eastern Mediterranean (n = 10; Fig. 2). The large number of specimens belonging to Type II allowed us to confirm the validity of two closely related subtypes within Type II, here named Type IIa and IIb (Fig. 3). The divergence between these two subtypes is the dominant and most strongly supported (100%) pattern in a phylogenetic analysis of all Type II sequences (Fig. 3). The two subtypes differ by a distinct nucleotide substitution pattern and short insertions/deletions in two of the variable regions of the sequenced SSU rDNA fragment. One part of the distinctive pattern is located in the middle of the expansion segment 41/e1, the other in the variable region V7 (e.g. Grimm *et al.* 2007). Individuals carrying Type IIa and IIb were found in all regions sampled in this survey, 46 individuals carried the Type IIa and 42 individuals carried the Type IIb. Types IIa and IIb co-occurred at almost all stations. In contrast to the global distribution of the genotypes, our stratified sampling revealed a remarkably consistent pattern of vertical separation of the habitat of these genetic types. For further biogeographical analyses, we supplemented our data with 10 sequences of *H. pelagica* genotypes from GenBank, where information about sampling depth was available (see Materials and Methods). An analysis of this extended data set reveals that all 13 individuals of Type I were found in the top 100 m (Fig. 2), the majority of individuals of Type IIa (65%) occurred below 200 m water depth, and no specimen was found in samples above 100 m (Fig. 2). The highest abundance of Type IIa specimens was found between

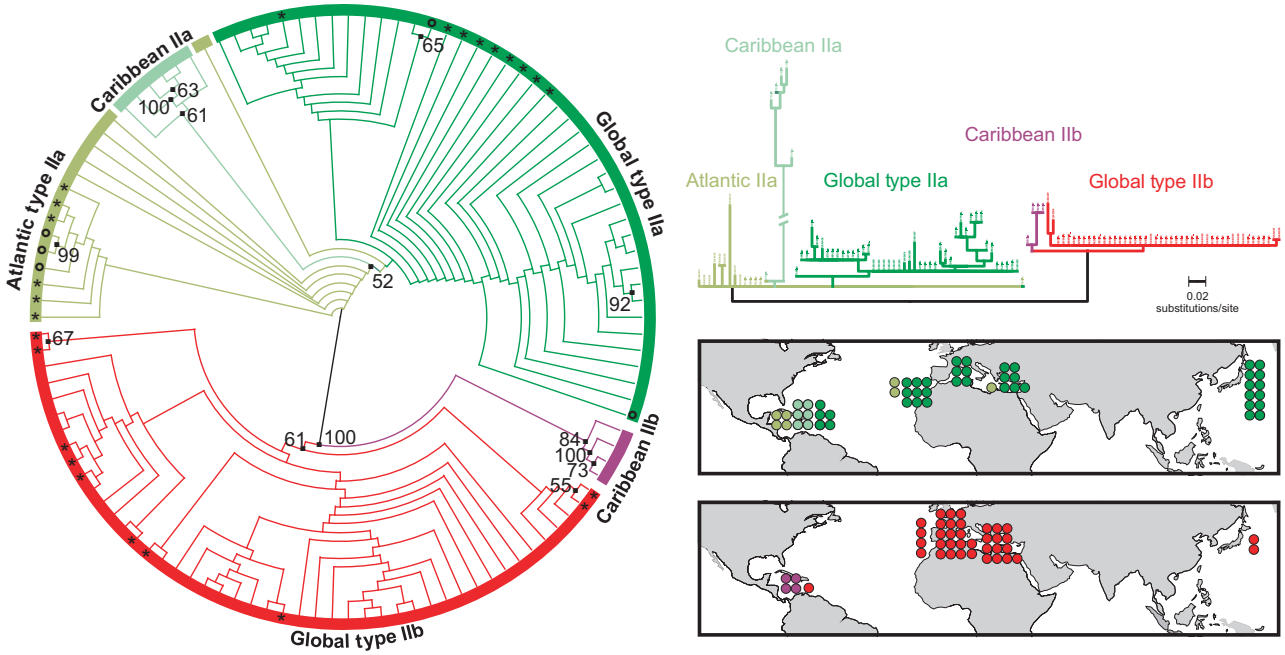

Fig. 3 Maximum likelihood based phylogenetic reconstruction of the *Hastigerina pelagica* genotypes IIa and IIb (see Materials and Methods for detail). The diagram on the left represents an unrooted circular topology, the diagram on the right shows a manually rooted phylogenetic tree. Bootstrap values higher than 50 are shown in the circular phylogram. Cloned and direct sequences taken from NCBI GenBank are highlighted by asterisks in the circular phylogram. The five clones from a single individual (accession nos FJ643402–FJ643406) belonging to the two dominant geographical motives in the genotype cluster of Type IIa are marked by small circles. The geographical distribution of individuals carrying one of the main (colour coded) genetic motives is shown in the maps. Each symbol represents one individual.

300 and 400 m water depth (Fig. 2; 1.2 individuals/interval/station; significantly ($P < 0.05$) higher than in the shallower depth intervals where the type was found). The majority of Type IIb individuals (76%) were found in the upper 100 m of the water column and only in the eastern Mediterranean Sea few Type IIb specimens (11%) occurred down to 700 m water depth (Fig. 2). Individuals of Type IIb were most abundant between 40 and 80 m (Fig. 2; 1.2 individuals/interval/station, significantly ($P < 0.05$) higher than in intervals shallower than 40 m and deeper than 100 m). Such vertical segregation of the habitats of Types IIa and IIb was consistently found in all the regions we have sampled (Fig. 2). Following a formula used in Aurahs *et al.* (2009a), with 95% level of confidence, the average abundance of Type IIa would have to have been below 0.13% in the top 100 m of the water column at all the stations sampled by us, to have remained undetected.

As our sampling took place during eleven different cruises during different years, seasons and different times of the day, we were able to discard all explanations for this pattern other than representing a genuine habitat signal. We were unable to detect any lunar periodicity-related signal (Bijma *et al.* 1990), neither in the vertical distribution of the genotypes nor in the overall abundance of *H. pelagica* (Fig. S1, Supporting information), suggesting that the observed vertical separation could not reflect a temporal offset in the reproductive cycle between the two types. Similarly, we did not find any difference in abundance or distribution among the types between seasons or daytime (Fig. S1, Supporting information), indicating that the vertical separation signal reflects neither diurnal migration nor seasonal succession. The pattern was found in samples spanning almost a decade (Fig. S1, Supporting information), suggesting that it does not represent an anomalous signal for one unusual year.

Most sequences of genotype IIb are globally identical with only a very few individuals showing nucleotide substitutions. One of the previously published sequences (GenBank accession no. FJ643397) shows two substitutions in the variable region 41/e1, which very likely represent sequencing errors. In addition, two clones from a single individual (FJ643357 and FJ643355) show a single-site mutation in the variable region V7. The only substantial and replicable deviation from the dominant global Type IIb motive can be found in three individuals from the Caribbean Sea (JQ624829–31). These sequences show four nucleotide substitutions at the beginning of variable region 41/e1 and also three substitutions and one deletion in the variable region V7. One more individual from the Caribbean Sea

(JQ624832) has two substitutions in common with these 'Caribbean sequences', but does not carry the complete pattern and could therefore indicate a hybrid sequence between the global and the Caribbean signal. The genetic variability among individual sequences of Type IIa is larger than among individual sequences of Type IIb. First, we observe a large number of seemingly random substitutions, which we interpret as intraindividual variability. This is supported by the observed level of variability among GenBank sequences that were reported to have been cloned from the same individual. These substitutions are mainly located in the variable regions 41/e1 and V7 and mostly occur in form of two basepair insertions or deletions. In addition, there are sequences that show one of two different substitution patterns that deviate significantly from the 'global' Type IIa signal and that are geographically constrained. The first variation exists in 16 individuals from multiple stations in the Atlantic as well as from the Mediterranean and Caribbean Sea (FJ643367–9, FJ643402, FJ643403, FJ643406, FJ643411–3, JQ624815, JQ624817 and JQ624819–23). This 'Atlantic' substitution pattern is characterized by a four basepair insertion and six substitutions in the variable region V7. The other substitution pattern is even more geographically restricted and was found only in five individuals (JQ624824–8) from the Caribbean Sea. These sequences show nucleotide substitutions at 20 positions and seven base insertions in the variable regions 41/e1 and V7. Among these five individuals, there is some degree of variation that is most likely an indication for intraindividual variability. The manual alignment of all Type II sequences is available as supplement (Data S1, Supporting information).

Discussion

Vertical segregation of cryptic sibling species

The consistent segregation of the vertical habitat of the genotypes IIa and IIb of *Hastigerina pelagica* is in contrast to their cosmopolitan geographical distribution (Fig. 2). As the vertical segregation is maintained throughout the sampled regions, irrespective of time (Fig. S1, Supporting information), it must reflect a genuine affinity of specimens of each type to a habitat in a different part of the water column. The complete absence of Type IIa in the surface waters is most remarkable; the chances of this pattern being due to an extremely low abundance of this genetic type in the surface waters are negligible. The occurrence of specimens of Type IIb in the eastern Mediterranean below its dominant habitat in the top 200 m of the water column is difficult to interpret. It is impossible to tell whether these specimens represent a genuine expansion of the habitat or whether they reflect natural mortality, passively falling through the water column whilst still carrying out nondegraded DNA. As the vertical separation is found among the two most closely related genetic types in *H. pelagica*, it is reminiscent of niche partitioning (e.g. Aurahs *et al.* 2009a), which is typically explained as the result of competition (Leibold 2008). On the other hand, the habitats in the photic zone and below the thermocline are fundamentally different for planktonic foraminifera and suggest that the two types may possess different adaptations (Coxall *et al.* 2007).

The exact mechanism for buoyancy control in planktonic foraminifera is not known; typical explanations involve regulations via metabolites with positive buoyancy (Hemleben *et al.* 1989). Whilst this mechanism provides a reasonable explanation for the ability to change their buoyancy, it still remains to be investigated, how (if at all) the foraminifera can detect their position in the water column and use this information to regulate the buoyancy. Despite the uncertainty in the exact mechanism, it is beyond question that species of planktonic foraminifera can be found in specific vertical intervals in the water column (Fairbanks *et al.* 1980). Interestingly, the best example for a restricted vertical position in these protists comes from the sister species of *H. pelagica*. *Hastigerinella digitata* is a rare deep-dwelling form with conspicuous digitate chambers. In situ video surveys in the Monterrey Canyon over 12 years revealed that the species occupies a narrow depth horizon of <100 m around a median depth of 300 m, immediately above the core of the regional oxygen minimum zone (Hull *et al.* 2011). Considering the morphological differentiation of its deep-dwelling sister species, it is conceivable that the observed depth segregation between the two genetic types of *H. pelagica* II will also result in morphological distinction. It is in fact possible that such morphological differentiation has already taken place but the traits are too recondite to have been identified by taxonomers. Notwithstanding the degree of morphological separation, it would appear interesting to now search for physiological or behavioural traits that are associated with the adaptation to a vertically limited habitat in this species.

The discovery of two genetic types with distinct vertical habitats has significant consequences for the interpretation of population dynamics and ecology of *H. pelagica*. The shell flux of this species is dominated by a synodic moon cycle (Spindler *et al.* 1979; Loncaric *et al.* 2005), which is thought to reflect the reproductive cycle of the species. Laboratory and in situ observations suggest a strongly synchronized reproduction peaking within a few days after full moon (Spindler *et al.* 1979). As lunar periodicity of reproduction also continued in the laboratory without the influence of the moonlight it

appears to indicate an endogenous mechanism (Bijma *et al.* 1990). Our observations did not reveal any time lag between the occurrence of mature (collected in the nets) individuals of either type (Fig. S1, Supporting information). Thus, it is likely that all three genetic types of this species follow the same endogenous 'clock'. This explanation would be consistent with the strong lunar cycle of *H. pelagica* flux observed in Atlantic sediment traps from a region (Loncaric *et al.* 2005), where all three types are expected to co-occur (Fig. 2).

Next, our discovery of distinct habitats associated with the genetic types provides an explanation for the contradictory reports on the habitat of *H. pelagica*. Whereas some authors consider the species a surface-dweller (Bé & Tolderlund 1971; Hemleben *et al.* 1989), other studies indicate its habitat as sub-thermocline (Schiebel & Hemleben 2005). Hull *et al.* (2011) reported the occurrence of *Hastigerina sp.* in the Monterrey canyon at mesopelagic depths, which seemed at odds with the lack of *H. pelagica* in plankton samples from the surface waters in the region. The observation from the Monterrey Canyon could be explained by the presence of exclusively *H. pelagica* Type IIa in that region.

Parapatric speciation in marine plankton

The genetic Types IIa and IIb of *H. pelagica* are genetically as close as many other genetic sibling types within morphospecies of planktonic foraminifera (Göker *et al.* 2010), suggesting that their divergence may represent the lowest level of relatedness associated with reproductive isolation in these pelagic protists. Therefore, it is tempting to interpret their pattern of depth segregation as a 'ghost of speciation past' (Kocher 2005). If this interpretation is correct, our observations would represent the first evidence for speciation by depth parapatry among modern planktonic foraminifera. In this model, a deep-dwelling form would have evolved from an ancestrally surface-dwelling population in *H. pelagica*, considering that the closely related relative, Type I, is also a surface-dweller (Fig. 2). Initially, in the same geographical region, one part of the surface-dwelling ancestral population would have become better adapted to greater depth. Reduced gene flow along the thus developed gradient within the population would have resulted in the emergence of two species with a depth-parapatric distribution.

Alternatively, speciation could have proceeded in allopatry and the depth-parapatric pattern would have developed upon secondary contact. Although plausible, we consider this explanation less likely, because it requires the evolution of adaptation to a deep-dwelling habitat in response to competition between two allopatric siblings, ancestrally inhabiting the same surface-ocean habitat. On the other hand, a 'retreat' from the surface habitat could have occurred in response to the tracking of a particular food resource or oceanic regime in high latitudes, which are manifested at greater depths in the lower latitudes. Such pattern of tropical submergence is known from assemblages of pelagic radiolaria, where surface-dwelling subpolar species occur at mesopelagic depth in the tropics (Ishitani & Takahashi 2007). However, we find no trace of such hypothetical high-latitude surface-dwelling ancestor of the deep-dwelling type, which would consequently have to become extinct or replaced in its habitat by the surface-dwelling type after their divergence. Similarly, there is no evidence for a residual geographic structure in the genetic diversity of the deep-dwelling type indicative for spreading away from a centre of origin.

The cosmopolitan distribution of the three genetic types has to be seen in the context of the genetic variability within each type. Indeed, sequences within each of the three genetic types show differences, which are unlikely to represent random sequencing errors. The genetic variability within the genotypes has a rudimentary geographical structure and most likely represents a population-level variability. However, unlike the segregation of Types IIa and IIb, on this level no vertical structuring in the water column could be detected within those genetic types. The sample size for Type I is arguably small, but it does not reveal a geographic signal—sequences from the eastern Mediterranean and western Pacific are all virtually identical. In fact, identical or virtually identical sequence motives have been found in the most distant sampled regions (eastern Mediterranean and western Pacific) in all three types of *H. pelagica*, suggesting the possibility of a recent or ongoing global gene flow within each type.

For the surface-dwelling Types I and IIb, this pattern is consistent with the observation of global distribution of identical SSU rDNA sequences in a large number of planktonic foraminifera (Darling & Wade 2008). *H. pelagica* has been reported from the surface waters with temperatures above 9 °C (Parker 1960), which does not exclude the possibility of passive transport between the Indian Ocean and South Atlantic via the Agulhas current. Indeed, plankton tow data from Agulhas Rings in the region SW of the Cape show that such rings carry a significant population of this species (Peeters *et al.* 2004). The presence of a weak geographical signal in Type IIb may indicate episodic isolation during Quaternary climatic cycles, although we note that the age of the signal cannot be determined owing to extreme substitution rate heterogeneity within the planktonic foraminifera (de Vargas *et al.* 1997) and the lack of a reliable fossil record of the genus. Nevertheless, the geographical structure indicates that the overall genetic

homogeneity in both types is a primary signal, not an artefact of recent anthropogenic dispersal. The existence of a global gene flow in the deep-dwelling Type IIa is more difficult to explain. At depth below 100 m, passive dispersal by currents is likely to be slower and less effective between semi-isolated basins, such as the Mediterranean and the Atlantic. On the other hand, the deeper-water habitat is more homogenous worldwide. Although Type IIa does show the highest genetic variability, suggesting the largest potential for isolation, the global distribution of sequence motives indicates that global gene flow can occur even at subthermocline depths.

The genetic variation found within the two depth-segregated genetic types allows us to address hypotheses explaining the origin of the segregation pattern. First, we note that genetic diversity is significantly higher among populations of the deep-dwelling Type IIa (Fig. 4). We attribute this observation to the fact that Type IIa inhabits a globally less connected habitat below the mixed layer. This habitat is more voluminous and more structured than the mixed-layer habitat, providing more opportunities for adaptation and isolation. The observed apparent increase in genetic diversity with depth (Fig. 4) indicates that when population range deepens, there is more potential for the evolution and maintenance of genetic structure within the species. In other words, as soon as the habitat of a species is expanded below the mixed zone and the home range remains smaller than the total habitat, vertical structuring follows with gene flow being progressively reduced vertically and horizontally with depth (Fig. 4). Our data indicate that this model applies to nonmotile microplankton, suggesting that speciation by depth parapatry does not require active means of propulsion or sensory control and could be a universal process generating diversity in the microbial plankton.

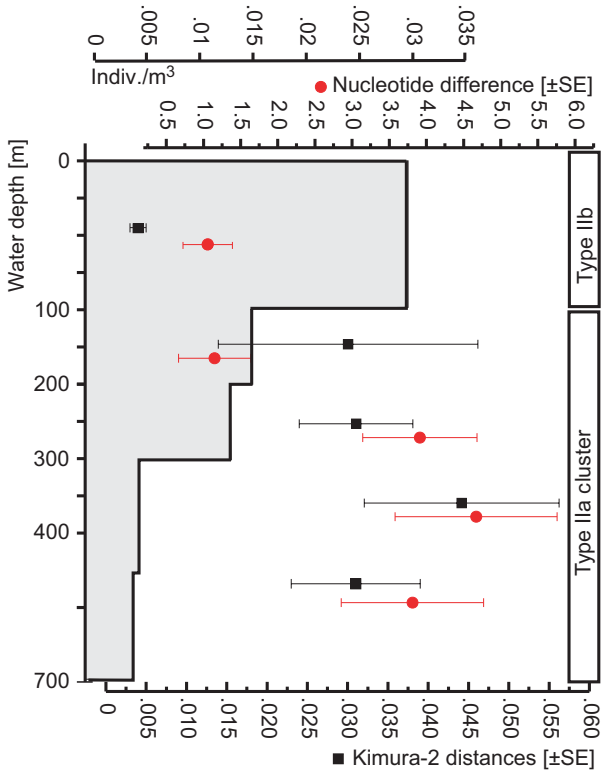

Fig. 4 Relationship between sampled water depth and genetic diversity (mean nucleotide differences and Kimura-2 distances) among *Hastigerina pelagica* individuals carrying Type IIa or Type IIb in the respective depth interval, shown together with an estimated average population density of the species in our nets (number of individuals identified as *H. pelagica* found in the respective interval, divided by total number of hauls).

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A.W., R.A., A.K. and M.K. collected the plankton samples. A.W. processed the Atlantic samples and A.K. the Pacific samples. R.A. performed the phylogenetic analysis. This research has been initiated by M.K. and H.K. A.W., R.A. and A.K. prepared the results for publication and interpreted the results. All authors drafted the manuscript and participated in writing the final version of the manuscript.

Data accessibility

DNA sequences: GenBank accession nos JQ624776–JQ624868. Sampling data (Table S1 and Fig. S1, Supporting information) and sequence alignment (Data S1, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 Manual alignment of 114 *Hastigerina pelagica* Type II sequences, both from this study and from sequences already available in GenBank.

Table S1 Supporting detail information on *Hastigerina pelagica* individuals that yielded SSU rDNA sequences.

Fig. S1 Vertical distribution of *Hastigerina pelagica* genotypes presented in this study considering different sampling attributes. Bars indicate the depth interval in which a specimen was found. Green bars represent Type IIa, red bars Type IIb, blue bars represent Type I. a) shows the distribution and abundance of the genotypes during different years of sampling, between 2005 and 2011; b) shows the distribution of the genotypes over their position in the lunar cycle during sampling; c) shows the position of the genotypes within the seasonal cycle and d) shows the abundance and distribution of the genotypes at different daytime hours. No obvious patterns in any of the considered parameters are seen, although we note that the sampling has not sufficiently covered all combinations of parameters. Thus, for example, it may appear that Type IIb is particularly abundant in December below 200 m. However, this pattern represents one sampling occasion in the eastern Mediterranean, where the deeper levels have been intensively sampled.

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5. Second case study

The cryptic and the apparent reversed: lack of genetic differentiation within the morphologically diverse plexus of the planktonic foraminifer *Globigerinoides sacculifer*

Aurore André, Agnes Weiner, Frédéric Quillévéré, Ralf Aurahs, Raphael Morard, Christophe J. Douady, Thibault de Garidel-Thoron, Gilles Escarguel, Colomban de Vargas and Michal Kucera

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Idea/Hypothesis: 20%

Data acquisition: 50%

Interpretation: 40%

Writing of manuscript: 30%

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The cryptic and the apparent reversed: lack of genetic differentiation within the morphologically diverse plexus of the planktonic foraminifer *Globigerinoides sacculifer*

Aurore André, Agnes Weiner, Frédéric Quillévéré, Ralf Aurahs, Raphaël Morard, Christophe J. Douady, Thibault de Garidel-Thoron, Gilles Escarguel, Colomban de Vargas, and Michal Kucera

Abstract.—Previous genetic studies of extant planktonic foraminifera have provided evidence that the traditional, strictly morphological definition of species in these organisms underestimates their biodiversity. Here, we report the first case where this pattern is reversed. The modern (sub)tropical species plexus *Globigerinoides sacculifer* is characterized by large morphological variability, which has led to the proliferation of taxonomic names attributed to morphological end-members within the plexus. In order to clarify the taxonomic status of its morphotypes and to investigate the genetic connectivity among its currently partly disjunct (sub)tropical populations, we carried out a global survey of two ribosomal RNA regions (SSU and ITS-1) in all recent morphotypes of the plexus collected throughout (sub)tropical surface waters of the global ocean. Unexpectedly, we find an extremely reduced genetic variation within the plexus and no correlation between genetic and morphological divergence, suggesting taxonomical overinterpretation. The genetic homogeneity within the morphospecies is unexpected, considering its partly disjunct range in the (sub)tropical Atlantic and Indo-Pacific and its old age (early Miocene). A sequence variant in the rapidly evolving ITS-1 region indicates the existence of an exclusively Atlantic haplotype, which suggests an episode of relatively recent (last glacial) isolation, followed by subsequent resumption of unidirectional gene flow from the Indo-Pacific into the Atlantic. This is the first example in planktonic foraminifera where the morphological variability in a morphospecies exceeds its rDNA genetic variability. Such evidence for inconsistent scaling of morphological and genetic diversity in planktonic foraminifera could complicate the interpretation of evolutionary patterns in their fossil record.

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Introduction

The interpretation of biogeographical and evolutionary patterns of morphologically defined species in the plankton has been challenged by the discovery of cryptic genetic diversity often linked with distinct biogeography and ecological adaptation (e.g., Saez et al. 2003; Logares et al. 2007; Kooistra et al. 2008). The interpretation of modern and fossil species distribution patterns in these groups thus hinges on the degree of congruence between their morphological and genetic divergence. As long as the morphological taxonomy consistently underestimates biodiversity, analysis of the fossil record and modern assemblages may be expected to reflect processes scaled with those at the level of biological species. This issue is particularly important for the interpretation of diversity patterns in the fossil record, because the species concept in palaeontology (and in many cases in biology as well) is based solely on phenotypic traits manifested in the shape of the classified organisms.

Planktonic foraminifera provide an excellent opportunity to test the explanatory power of

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biogeographical and evolutionary patterns derived from morphologically defined species and to assess the degree of congruence between morphological distinction and genetic divergence. Their character-rich calcite shells are abundantly preserved in marine sediments and allow direct tracking of morphospecies occurrences in time and space (e.g., Aze et al. 2011). From these occurrences, paleoceanographers derive reconstructions that are of immense importance to our understanding of past climate changes. In recent years, molecular analyses have revealed that the classical, morphological definition of species in planktonic foraminifera hides cryptic genetic and ecological differentiation (for a review, see Darling and Wade 2008). Global surveys of the Small Subunit (SSU) or Internal Transcribed Spacers (ITS) regions of ribosomal DNA (rDNA) in extant species allowed the recognition of distinct genotypes (e.g., de Vargas et al. 1999, 2001; Darling et al. 1999, 2006; Morard et al. 2009, 2011; Ujiié et al. 2010; Aurahs et al. 2011; Quillévéré et al. 2013). Considering the lack of evidence for introgression and the obligate sexual outbreeding reproductive modus in planktonic foraminifera, these genetic types have to be considered reproductively isolated and thus corresponding at least to the level of biological species. Many of these genetic types show more restricted biogeographical ranges than the morphospecies to which they have been assigned, implying that the distribution of these morphospecies does not reflect the true potential for dispersal of these organisms. Nonetheless, some of these genetic types occur throughout the range of their respective morphospecies, suggesting ongoing global gene flow. At present, the degree to which morphological divergence reflects genetic distinction in planktonic foraminifera (and potentially other plankton) remains unclear, complicating the interpretation of biogeographical and evolutionary patterns in the fossil record of this group.

Here we report on a global survey of two of the ribosomal rDNA regions (SSU and ITS-1) in the abundant and paleoceanographically important (sub)tropical species *Globigerinoides sacculifer* (Brady 1877). The survey was carried out with the aim to determine the degree of congruence between morphology and genetic divergence within this morphologically diverse species plexus, and to investigate the genetic connectivity among its partly disjunct (sub)tropical populations. *Globigerinoides sacculifer* is one of the most commonly encountered planktonic foraminifera in the (sub)tropical waters of the world oceans (e.g., Tolderlund and Bé 1971). This cosmopolitan spinose species is limited by its photosymbiotic ecology to the euphotic zone of the oceans, where it reproduces on a synodic lunar cycle (Bijma et al. 1990).

The morphology associated with the species concept of *Globigerinoides sacculifer* initially occurred during the early Miocene ~20 Myr ago, having diverged from the morphospecies *Globigerinoides trilobus* (e.g., Kennett and Srinivasan 1983; Berggren et al. 1995). The exact dating of this divergence is potentially complicated by usage of the species names *G. sacculifer* and *G. trilobus* in a way not consistent with their original species description. Similarly, the taxonomic status of *G. sacculifer* in the modern ocean is ambiguous, because of a high morphological variability among specimens of this plexus. *Globigerinoides sacculifer* sensu stricto has been taxonomically distinguished from other *Globigerinoides* morphospecies with a honeycomb shell wall texture (Kennett and Srinivasan 1983) by the presence of a sac-like final chamber (Brady 1877). Whereas the other taxonomic concepts in the plexus (Fig. 1), *Globigerinoides quadrilobatus* (d'Orbigny 1846), *G. trilobus* (Reuss 1850) and *Globigerinoides immaturus* Leroy (1939), are based on fossil material, *G. sacculifer* was originally described from subfossil sediments and the original species description clearly refers to the occurrence and habitat of this species in the plankton (Brady 1877; see also Williams et al. 2006). Numerous studies have noticed that the shells of *G. trilobus*, *G. quadrilobatus*, and *G. immaturus* are virtually identical to those of *G. sacculifer* except for the lack of the final sac-like chamber (Hofker 1959; Banner and Blow 1960; Hecht 1974; Saito et al. 1981). As a consequence, these taxa were often considered phenotypic variants of the morphospecies *G.*

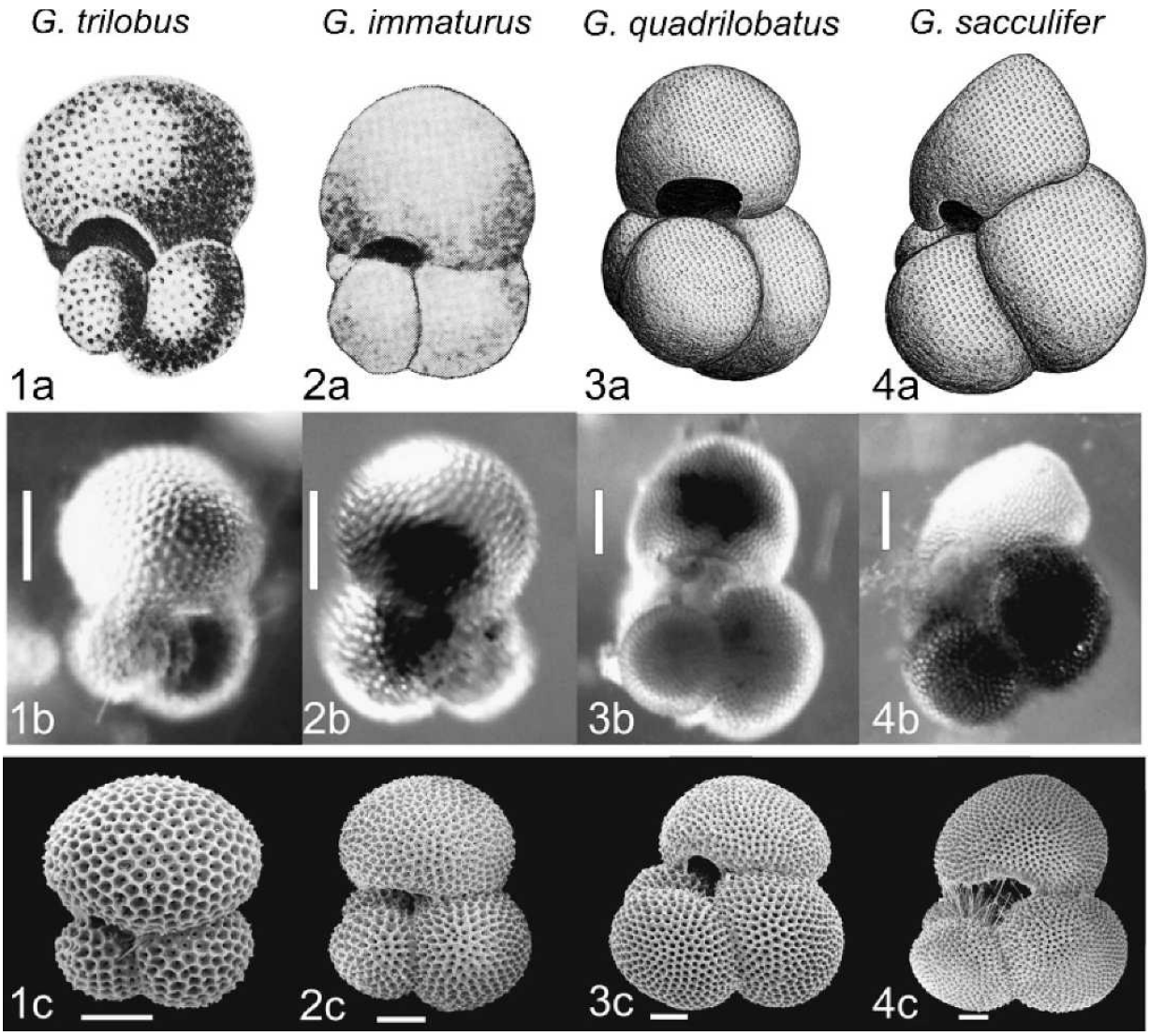

FIGURE 1. Taxonomy of the *Globigerinoides sacculifer* plexus. Row (a) shows reproductions of line drawings of (1) the holotype of *Globigerinoides trilobus* (Reuss 1850), 60×; (2), the holotype of *Globigerinoides immaturus* Leroy (1939), 60×, mirror image; (3) the lectotype selected by Banner and Blow (1960) for *Globigerinoides quadrilobatus* (d'Orbigny 1846), 100×; (4), the lectotype selected by Banner and Blow (1960) for *Globigerinoides sacculifer* (Brady 1877), 100×. Row (b) shows light microscope images and row (c) SEM pictures of genetically analyzed specimens corresponding to the four taxonomic concepts shown in (a). *Globigerinoides sacculifer* is distinguished from the other members of the plexus by its elongate sac-like final chamber. Within the members of the plexus that lack the sac-like chamber, *G. quadrilobatus* differs in having a high aperture and a tendency to possess four chambers in the last whorl, and *G. immaturus* and *G. trilobus* both exhibit lower-arched primary and supplementary apertures, but, according to the original description, in *G. trilobus* the final chamber is larger than all the earlier chambers combined. The SSU and ITS-1 sequences corresponding to the specimens in the images are labeled in Supplementary Table 1. Scale bars in (b), 0.1 mm, and in (c), 0.05 mm.

sacculifer s.l. Culture experiments have provided support for this broader taxonomic concept, suggesting that the sac-like chamber is probably a terminal event of shell growth, manifested in some but not all of the cultured specimens (Bé 1980; Bé et al. 1983; Hemleben et al. 1987; Bijma et al. 1992). Despite these culturing experiments, specimens with and without the sac-like last chamber have been continually recorded as separate taxa for paleoecological studies and transfer functions. Today, the most extensively used species names for members of the plexus are *G. trilobus* and *G. sacculifer*, but *G. quadrilobatus* and *G. immaturus* are still commonly reported, even in late Quaternary sediments (e.g., Spooner et al. 2005; Lim et al. 2006; Budillon et al. 2009; Siani et al. 2010; Wilson 2012).

Until now, only 16 SSU sequences of the *Globigerinoides sacculifer* plexus have been published, 11 originating from a single location in the NW Pacific and the five remaining

TABLE 1. Location of the sampling stations for *Globigerinoides sacculifer* with hydrographic data, collection and DNA extraction methods, numbers of sequenced individuals and, in the case of stations with cloned specimens or replicates, numbers of sequences (in italics). Data from the literature are also included. Numbers in brackets correspond to *Orbulina universa* ITS-1 sequences.

Cruise	Ocean	Station	Longitude	Latitude	Date
AMT-8	Atlantic	10	21 W	22.55 N	June 1999
	Atlantic	13	21.34 W	30.28 N	June 1999
C-Marz	Atlantic	1	69.2 W	33.33 N	April 2006
	Atlantic	3	60.3 W	24.5 N	April 2007
	Atlantic	4	54.3 W	19.5 N	April 2008
	Atlantic	5	54.7 W	14 N	April 2009
Eilat	Red Sea	N/A	34.6 E	29.3 N	May 1999
Gyrafor A	Pacific	B	162.5 E	23.02 S	June 2008
	Pacific	E	162.6 E	14.83 S	June 2008
	Pacific	G	162.51 E	9 S	June 2008
	Pacific	H	162.5 E	6.04 S	June 2008
	Pacific	K	162.5 E	0	June 2008
	Pacific	N	161.11 E	4.5 S	June 2008
	Pacific	P	160.4 E	7.4 S	June 2008
	Pacific	S	161.88 E	16.25 S	June 2008
	Pacific	T	162.66 E	17.93 S	June 2008
Gyrafor B	Indian	F	80.216 E	14.213 S	June 2007
	Indian	I	73.16 E	15.57 S	June 2007
	Indian	L	67.38 E	17.21 S	June 2007
KT06	Pacific	N/A	N/A	N/A	May 2006
Melville	Indian	1	23.7 E	35.1 S	June 2003
	Indian	2	24.5 E	35 S	June 2003
	Indian	10	52.6 E	31.8 S	June 2003
	Indian	11	59.8 E	29.8 S	June 2003
	Indian	12	59.8 E	29.8 S	June 2003
	Indian	16	78 E	19.7 S	June 2003
	Indian	17	78 E	19.7 S	June 2003
	Indian	18	83.7 E	17.2 S	June 2003
	Indian	20	89.9 E	14 S	June 2003
OISO-4	Indian	2	53.3 E	30 S	Febr 2000
	Indian	4	53.23 E	40 S	Febr 2000
	Indian	17	66.24 E	29.59 S	Febr 2000
Revelle	Pacific	6	130.98 W	32.04 S	Febr 2000
Meteor 69/1	Atlantic	N/A	5.46 W	35.58 N	August 2006
	Atlantic	323	5.51 W	35.58 N	August 2006
	Atlantic	324	5.39 W	35.57 N	August 2006
	Atlantic	366	0.31 W	35.55 N	August 2006
	Atlantic	N/A	2.45 E	39.6 N	August 2006
	Atlantic	N/A	2.44 E	39.14 N	August 2006
	Atlantic	395	2.31 E	38.57 N	August 2006
	Atlantic	N/A	2.43 E	39.16 N	August 2006
Meteor74/1	Indian	955	67.6 E	19.6 N	Sept 2007
	Indian	957	64.4 E	20.33 N	Sept 2007
Meteor78/1	Atlantic	164	83.38 W	18.30 N	March 2009
	Atlantic	222	64.28 W	12.1 N	March 2009
	Atlantic	238	60.14 W	10.56 N	March 2009
Merian15/5	Atlantic	N/A	4.13 E	37.11 N	July 2010
Poseidon321	Atlantic	175	21.27 W	30.36 N	May 2005
	Atlantic	179	22.29 W	31.59 N	May 2005
	Atlantic	181	22 W	33 N	May 2005
	Atlantic	185	20.14 W	35.50 N	May 2005
Poseidon334	Atlantic	N/A	19.30 W	31.36 N	March 2006
	Atlantic	67	20 W	33 N	March 2006
	Atlantic	N/A	20 W	34.20 N	March 2006
Curacao	Atlantic	N/A	68.56 W	12.7 N	1996
Great Barrier Reef	Pacific	N/A	N/A	N/A	1996
Puerto Rico	Atlantic	N/A	67 W	17.49 N	March 1995
Villefranche	Atlantic	N/A	7.18 E	43.42 N	Dec 1995
KT02-15	Pacific	Okinawa	145 E	39 N	May 2002

TABLE 1. Extended.

Gear	Mesh size	CTD	DNA buffer	No. of SSU sequences	No. of ITS-1 sequences	Published in
net	100 μm	yes	GUAN	3	0	this study
net	100 μm	yes	GUAN	1, *3*	0	this study
MOC net	64 μm	yes	GITC*	1	2 (1)	this study
ring net	64 μm	yes	GITC*	0	2	this study
ring net	64 μm	yes	GITC*	1	1	this study
MOC net	64 μm	yes	GITC*	2	3	this study
net	63-200 μm	no	GUAN	8	0	this study
multinet	100 μm	yes	GITC*	1	1	this study
multinet	100 μm	yes	GITC*	2	2	this study
multinet	100 μm	yes	GITC*	2, *8*	1, *5*	this study
multinet	100 μm	yes	GITC*	1	1, 5	this study
multinet	100 μm	yes	GITC*	2	2	this study
multinet	100 μm	yes	GITC*	1, *3*	1	this study
multinet	100 μm	yes	GITC*	1	1	this study
multinet	100 μm	yes	GITC*	0	1	this study
multinet	100 μm	yes	GITC*	0	0 (1)	this study
multinet	100 μm	yes	GITC*	3	4, *6*	this study
multinet	100 μm	yes	GITC*	2	3	this study
multinet	100 μm	yes	GITC*	8, *14*	10, *18*	this study
ORI net	330 μm	yes	GITC*	2	4, *8*	this study
plankton nets	65 - 200 μm	yes	GITC*	2	0	this study
plankton nets	65 - 200 μm	yes	GITC*	1	0	this study
plankton nets	65 - 200 μm	yes	GITC*	2	0	this study
plankton nets	65 - 200 μm	yes	GITC*	3	0	this study
plankton nets	65 - 200 μm	yes	GITC*	4	0	this study
plankton nets	65 - 200 μm	yes	GITC*	3	0	this study
plankton nets	65 - 200 μm	yes	GITC*	1	0	this study
plankton nets	65 - 200 μm	yes	GITC*	2	0	this study
plankton nets	65 - 200 μm	yes	GITC*	2	0	this study
net	100 μm	yes	magic GUAN	4	0	this study
net	100 μm	yes	magic GUAN	2	0	this study
net	100 μm	yes	magic GUAN	3	0	this study
net	100 μm	yes	GITC*	1, *3*	1, *5*	this study
surface pump	63 μm	no	DOC	2	1, *3*	this study
multinet	100 μm	no	DOC	1	1, *5*	this study
multinet	100 μm	no	DOC	1	0	this study
multinet	100 μm	no	DOC	1	1, *3*	this study
surface pump	63 μm	no	DOC	1	1, *3*	this study
surface pump	63 μm	no	DOC	2	2, *6*	this study
multinet	100 μm	no	DOC	3	1, *2*	this study
surface pump	63 μm	no	DOC	2	0	this study
multinet	100 μm	no	DOC	1	0	this study
multinet	100 μm	no	DOC	1	0	this study
multinet	100 μm	no	DOC	1	0	this study
multinet	100 μm	no	DOC	3	0	this study
multinet	100 μm	no	DOC	3	3, *5*	this study
multinet	100 μm	no	DOC	4	5, *11*	this study
multinet	100 μm	no	DOC	4	0	this study
multinet	100 μm	no	DOC	2	0	this study
multinet	100 μm	no	DOC	1	0	this study
multinet	100 μm	no	DOC	4	0	this study
surface pump	63 μm	no	DOC	3	0	this study
multinet	100 μm	no	DOC	8	0	this study
surface pump	63 μm	no	DOC	3	0	this study
net	63-200 μm	no	tris-EDTA	1	0	Darling et al. (1996)
scuba diver	-	no	tris-EDTA	1	0	Darling et al. (1997)
net	64-500 μm	no	tris-EDTA	2	0	de Vargas et al. (1997)
net	64-500 μm	no	tris-EDTA	1	0	de Vargas et al. (1997)
NORPAC net	63 μm	no	GITC*	11	0	Ujiie and Lipps (2009)

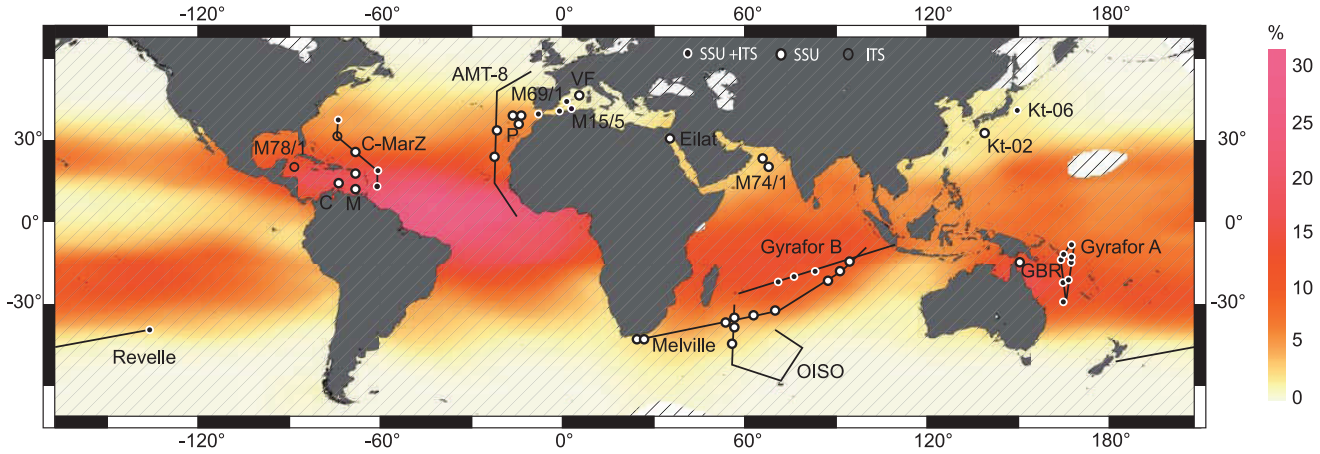

FIGURE 2. Location of ship tracks, names of the various cruises, and sampling stations for individuals of the *Globigerinoides sacculifer* plexus. Black circles represent stations where both SSU and ITS-1 sequences have been obtained; white circles represent stations where only the SSU has been sequenced; open circles mark stations were only the ITS-1 has been sequenced. Details of all localities are given in Table 1. Colors indicate the relative abundance of the *G. sacculifer* plexus in planktonic foraminiferal assemblages from surface sediments, interpolated from data in the MARGO database (Kucera et al. 2005; MARGO Project Members 2009) and Siccha et al. (2009).

originating from the South Pacific (Coral Sea), Atlantic (Caribbean Sea) and Mediterranean (Wade et al. 1996; Darling et al. 1997; de Vargas et al. 1997; Pawlowski et al. 1997; Ujiié and Lipps 2009). All these sequences were attributed to *G. sacculifer*. These sequences are virtually identical, but the available data are not sufficient to address the global diversification or to resolve the correlation between morphological and genetic divergence in the plexus. Here, we assess the rDNA genetic diversity of the SSU and ITS regions from the *G. sacculifer* plexus throughout the world oceans. Single-cell DNA analyses are performed on 148 individuals representative of all major morphotypes, i.e., *Globigerinoides trilobus*, *G. immaturus*, *G. quadrilobatus*, and *G. sacculifer* s.s. (Fig. 1). Such analyses allow us to determine whether or not there is cryptic diversity in *G. sacculifer* s.l. and to investigate the congruence between genetic and morphological diversity within the plexus.

Material and Methods

Sampling.—Specimens of *Globigerinoides sacculifer*, *G. immaturus*, *G. trilobus*, and *G. quadrilobatus* were collected from ring and stratified plankton tows (64-µm to 200-µm mesh sizes) and by pumping surface water through a sieve with 64-µm mesh size (see Table 1 for details). The sampling cruises took place from 1995 to 2010 and covered almost the entire geographic range reported for *G. sacculifer* (Fig. 2). Right after sampling, live specimens were taxonomically identified, in most cases photographed and carefully picked from the plankton, cleaned with a fine brush, and transferred individually into a DNA extraction buffer. Specimens were then stored at –20°C until further processing in the laboratory. Hydrographic vertical profiles of the water column were obtained at most stations of collection by using temperature and fluorescence sensors (Table 1).

DNA Extraction, Amplification, and Sequencing.—DNA extractions were performed using DOC (Pawlowski 2000) and guanidinium isothiocyanate (GITC*) DNA extraction buffers (e.g., Morard et al. 2009). The GITC* method kept the calcareous shell intact after DNA extraction for further morpho-genetic comparisons. Polymerase Chain Reaction (PCR) for both SSU and ITS-1 was performed using proofreading VENT® polymerase (New England Biolabs) and Thermus aquaticus YT-1 polymerase with 5′ flap endonuclease activity (New England Biolabs). A combination of universal and foraminifera-specific primers was used for the amplification of a fragment of the 3′ end of the SSU rDNA and for the complete ITS-1 region (Table 2, Fig. 3). The use of multiple primer pairs that were developed in the course of the project helped us to improve the amplification success rates (e.g.,

TABLE 2. PCR primers used in this study for the amplification of the SSU and ITS-1 regions. The primers S19F_Orb and 5.8S_R1_For were used for the amplification of the ITS-1 of *Orbulina universa* (see Methods).

Primer name	Pair	Target region	Amplicon length (bp)	Published by	Sequence 5′–3′
S19f	S15rf	SSU	~ 700	New	CCCGTACTAGGCATTCCTAG
S15rf	S19f	SSU	~ 700	New	GTGCATGGCCGTTCTTAGTTC
S14p	SBf	SSU	~ 700	Ujiié and Lipps 2009	AAGGGCACCACAAGAGCG
SBf	S14p	SSU	~ 700	Ujiié and Lipps 2009	TGATCCATCAGCAGGTTCACCTAC
saccv2F	saccv2R	SSU	~ 540	New	ACCACAAGCGCGTGGAGCAT
saccv2R	saccv2F	SSU	~ 540	New	GCACGTGTGCAGCCCAGGAC
S98f	5.8S7fp	ITS-1	~ 1200	New	CCTCCGGAAAAAGGCTTATGCAGGCA
S96f	5.8S7fp	ITS-1	~ 1200	New	TGCAGGCATTTCACGTATGCTCCTATA
5.8S7fp	S96f/ S98f	ITS-1	~ 1200	New	GTIAGTAAGGTGTTCCRCAGCC
saccITSF	saccITSR	ITS-1	~ 530	New	CGCCCGTCGCTCTTACCAAT
saccITSR	saccITSF	ITS-1	~ 530	New	ACCCGCCCATGGACCAATGT
S19F_Orb	5.8S_R1_For	ITS-1	~ 770	New	CTAACTAGGAATGCCTYGTACGG
5.8S_R1_For	S19F_Orb	ITS-1	~ 770	New	GGTAAGTAAGGTGTTCCRCAGCC

from 44% to 70% for the SSU). The reason for obtaining sequences of the ITS-1 region was the observation from species of non-spinose planktonic foraminifera in which this region showed higher rate of substitution and thus allowed finer differentiation (de Vargas et al. 2001; Morard et al. 2011). The ITS-1 sequences obtained here are the first from a species of spinose planktonic foraminifera. Therefore, in order to verify their attribution to the analyzed species, we have also generated ITS-1 sequences from two mature specimens of *Orbulina universa* (Table 1; NCBI accession numbers JQ004254 and JQ004255), which is consistently placed in SSU rDNA phylogenies as the sister species to *Globigerinoides sacculifer* (Aurahs et al. 2009b). These two specimens from the NE Atlantic and SE Pacific Oceans were identified as the Mediterranean genotype (de Vargas et al. 1999) on the basis of RFLP analyses. Their ITS-1 sequences were obtained using two new specific primer pairs shown in Table 2. For *G. sacculifer*, after preliminary analysis of the first ITS sequences, we created a primer set for a short fragment within the ITS, covering the region where we had located the positions with the highest variability (Table 2). These shorter fragments were cloned using the blunt end TOPO® PCR cloning kit (Invitrogen) and sequenced with

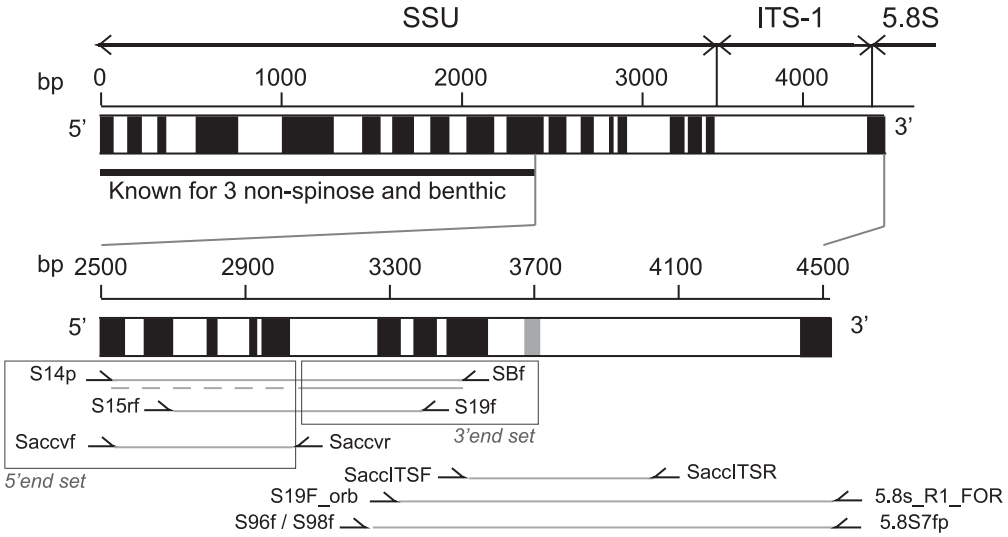

FIGURE 3. Scheme of the SSU, ITS-1, and 5.8S regions of the planktonic foraminiferal rRNA genes showing the positions of the amplified regions with appropriate primer pairs (gray lines) for *Globigerinoides sacculifer*. Variable and conserved regions are shown in white and black, respectively. The gray region shows the position of the insert found in specimens of *G. sacculifer* s.l. from the Atlantic Ocean. *Neogloboquadrina dutertrei* (NCBI EU199449) is taken as reference for SSU 5′ end length (in pair base). SSU 3′ end, ITS-1, and 5.8S lengths are based on sequences of *G. sacculifer*.

the M13F/M13R standard primers. In addition to the SSU and ITS-1 PCR products that were sequenced directly with the primers used for amplification, we cloned the whole ITS-1 from seven individuals and the partial SSU from two individuals from the Indian and Pacific Ocean. All original sequence chromatograms were checked by eye for ambiguous reads and sections of low quality of resolution. Our detailed information about the sequence coverage and length for each specimen can be drawn from the online supplement (Supplementary Table 1). Additionally, sequences of 16 specimens were compiled from the literature for comparison (Table 1).

Phylogenetic and Phylogeographic Analyses.—In a first manual alignment of the SSU and ITS sequences, we noticed that direct sequences of both regions contained a few ambiguous reads or unique base changes, which were not present in sequences obtained by cloning. The overall incidence of the ambiguous reads and unique base changes is extremely low and close to the level that is expected from the proofreading efficiency of the polymerases. We have nevertheless kept this variability for all subsequent phylogenetic analyses (Supplementary Table 1).

Both SSU and ITS-1 sequences were aligned using MUSCLE v. 3.7 (Edgar 2004) and ClustalW v. 2 (Larkin et al. 2007). The Modeltest 0.1.1 software (Posada and Crandall 1998) was used to select the best-fit nucleotide substitution model for each alignment according to the Akaike Information Criterion (AIC) (Akaike 1974). Phylogenetic trees were computed using PhyML, version 3.0 (Guindon and Gascuel 2003) and a Median-Joining network (Bandelt et al. 1999) was obtained using Splitstree v. 4.11.3 (Huson and Bryant 2006). Matrices of patristic distances for the ITS-1 region (sum of tree-branch lengths on a path between a pair of sequences) were then generated with R, version 2.12.2 (R Development Core Team 2008) using the APE package (Paradis et al. 2004) and expressed as percent of nucleotide differences.

CHRONOS Database.—We searched the NEPTUNE Database (www.Chronos.org, search generated by R.A. using Chronos XML on 18 October 2011) for reports of fossil individuals of *Globigerinoides sacculifer*, *G. trilobus*, *G. immaturus*, and *G. quadrilobatus* from global marine sediments. The resulting table listed all original taxonomic assignments, which comprise a combination of various binomial and trinomial species names (e.g., *G. quadrilobatus immaturus*, *G. quadrilobatus* s.l., *G. trilobus sacculifera*). Therefore, as far as possible, we filtered the names according to the taxonomic concept followed in this study.

Results

Altogether, we obtained DNA sequences from 148 specimens of the *Globigerinoides sacculifer* plexus from 54 stations (Supplementary Table 1). Of the specimens that were taxonomically unambiguously assigned upon collection, we obtained SSU rDNA and ITS-1 sequences corresponding to all four common morphospecies of the plexus: *G. sacculifer* ($n = 37$), *G. trilobus* ($n = 29$), *G. immaturus* ($n = 9$), and *G. quadrilobatus* ($n = 11$) (Fig. 1; Supplementary Table 1). These new sequences are deposited in Genbank with accession numbers JQ004100 to JQ004175 and JQ995373 to JQ995390 for the SSU region, and JQ004176 to JQ004253 and JQ973709 to JQ973734 for the ITS-1 region.

Within the plexus, the new and previously published SSU rDNA sequences (Supplementary Table 1) were virtually identical. Given that multiple primer pairs were used for PCR amplifications and sequencing (Table 2), three partly overlapping subsets of sequences were considered within our alignment (Fig. 3). The longest subset covers the whole fragment (Fig. 3) and consists of 107 sequences, 49 of which being completely identical. The second subset covers the front part of the fragment and is made of 138 sequences, 70 of which are identical to the base. Finally, the third subset covers the rear end of the fragment and is made of 129 sequences, 105 of which are identical to the base. In total, 65 sequences differed by up to 19 nucleotide changes from the consensus. In all cases except one, the observed small differences showed no reproducible patterns. For five randomly selected individuals that exhibited such differences, three independent PCR and sequencing replicates were carried out. These confirmed the

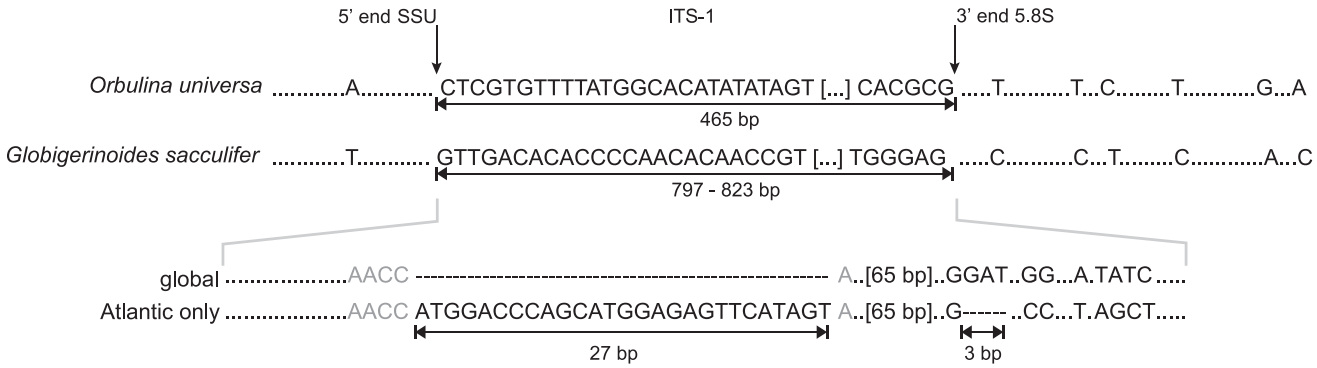

FIGURE 4. Comparison of the degree of divergence in the SSU, 5.8S, and ITS-1 sequences between the two sister species *Globigerinoides sacculifer* (NCBI JQ004220) and *Orbulina universa* (NCBI JQ004254). Dots mark identical bases in aligned regions; also shown are the lengths of the ITS-1 in base pairs (bp). The ITS-1 of *O. universa* and *G. sacculifer* are so divergent from one another that they cannot be aligned. The inset below shows the difference in the sequence motive of the ITS-1 between the dominant global haplotype and the Atlantic haplotype; dashes mark nucleotides that are missing in the other sequence.

absence of reproducible nucleotide changes within the replicated sequences (NCBI JQ995381 to JQ995390) and indicated that these changes, all located close to the 5′ or 3′ end of the sequences, represent amplification or direct sequencing artifacts. The only repeatable pattern was found in one specimen from the Caribbean Sea (NCBI JQ004126), which showed a substitution pattern (4 bp) in the SSU region identical to a sequence from the same region published by de Vargas et al. (1997). However, this individual carried an ITS-1 sequence identical to those found throughout the world oceans, and the significance of the substitution pattern in the SSU region thus remains unclear.

The 107 ITS-1 sequences obtained from 71 different individuals showed a higher degree of differentiation than the virtually identical SSU sequences. In contrast to the genetic homogeneity of the SSU rDNA, the ITS sequences revealed an interesting geographical signal and confirmed the presence of intra-individual variability in the analyzed gene complex. The clones of eight individuals assigned upon collection to *G. sacculifer* and *G. trilobus* from the Atlantic Ocean showed a unique substitution pattern in the ITS-1 (Fig. 4). This exclusively Atlantic haplotype was associated with clones from the same specimens that yielded the dominant globally distributed ITS haplotype, suggesting intra-individual variability in these specimens. In the direct sequences and clones of the "global" ITS-1 haplotype, only small variation at nine positions has been found (Fig. 5). In these sequences, the variable sites are located at the same positions, but show various permutations of character states. These permutations, also found among clones of the same individuals, are consequently consistent with the existence of intra-individual variability.

None of the observed small differences in the SSU and in the ITS-1 are linked with morphological differentiation between individuals that were attributed to one of the *Globigerinoides trilobus*, *G. quadrilobatus*, *G. immaturus* or *G. sacculifer* s.s. morphotypes (Fig. 6). An ANOSIM nonparametric test (Clarke 1993) indicates that there is no correlation between genetic distance and morphology ($R = -0.010$; $p_{(R = 0)} = 0.565$ [10,000 permutations]).

Discussion

Genetic Diversity in Globigerinoides sacculifer.—The results of our survey of the *G. sacculifer* plexus are in stark contrast with the expectation based on other species of planktonic foraminifera. All extensively genetically studied morphospecies of modern planktonic foraminifera have so far revealed the presence of more than one distinct genetic type (see review in Darling and Wade 2008). The absence of genetic variability in the SSU rDNA region within the global population of the plexus contrasts with the large differences among genetic types in all other intensively

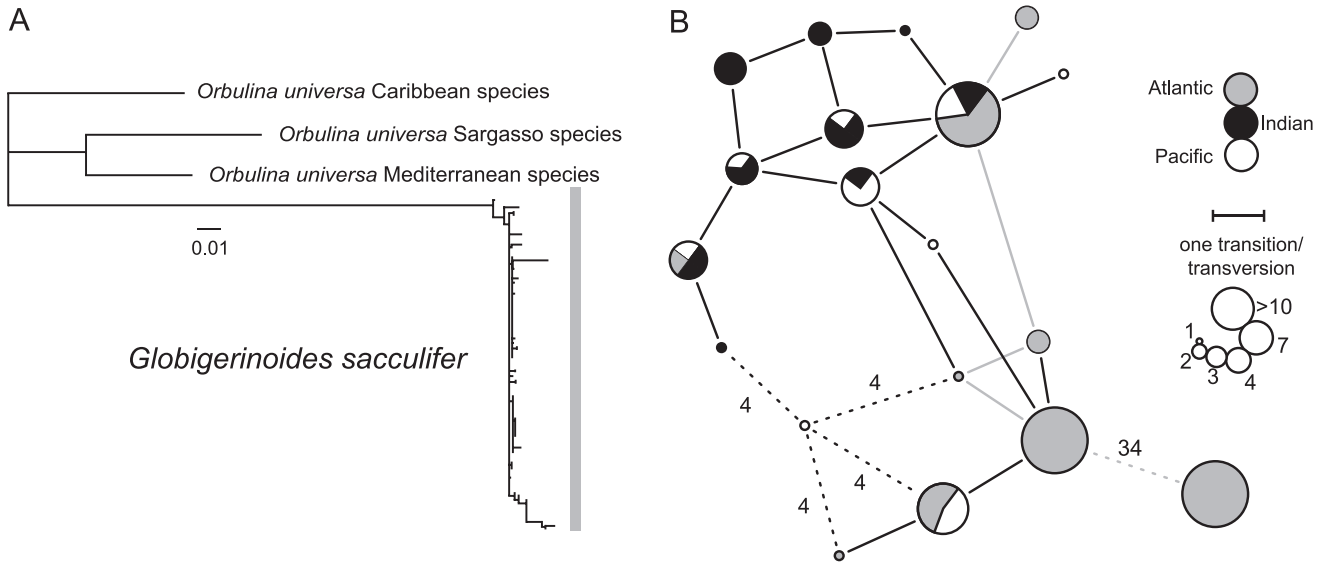

FIGURE 5. A, SSU-based phylogenetic tree for all available sequences (Supplementary Table 1) of the *Globigerinoides sacculifer* plexus using the three genotypes of *Orbulina universa* as outgroup. For details see *Material and Methods*. B, Median-joining network based on a manual alignment of 594 bp in the ITS-1 region of 78 sequences of the *G. sacculifer* plexus. Calculation of this haplotype network excludes all parsimony-uninformative sites. The original output was manually modified for legibility. Lines represent substitutions between haplotypes; gray lines connect haplotypes that may be encountered in the same individual; dotted lines, not to scale, indicate more than three substitutions (number given close to the lines). Size of the circles is proportionate to the number of sequences per haplotype. The largest haplotype difference (34 substitutions) is a result of two inserts and one deletion, as shown in Figure 4.

studied spinose planktonic foraminiferal species (Göker et al. 2010). However, this alone cannot be taken as unambiguous evidence for the absence of genetic types. In the non-spinose planktonic foraminiferal species *Truncorotalia truncatulinoides* (de Vargas et al. 2001) and *Globoconella inflata* (Morard et al. 2011), inter-genotype variability consistently appears at the level of the faster-evolving ITS-1 region.

To exclude the possibility that genetic differentiation in the *Globigerinoides sacculifer* plexus is also first manifested in this region, we have supplemented the SSU rDNA survey with a large data set of partial and complete ITS-1 sequences. These ITS-1 sequences show a higher degree of variability than the SSU data, which appears in two different ways. First, one consistent sequence pattern (haplotype) was found only in the Atlantic, and second, a number of minor variations (SNPs) were found throughout the world ocean (Fig. 5). This degree of variation is consistent with a population-level signal, and the number of substitutions separating individuals is more than 15 times lower than among ITS-1 genotypes of both *Truncorotalia truncatulinoides* and *Globoconella inflata* (Table 3, Fig. 6), which constitute the only reference points for this rDNA array. The available ITS-1 data on the *G. sacculifer* plexus thus do not indicate the presence of distinct genetic types and the observed variability is not correlated with morphological taxonomy within the plexus.

Morphological Taxonomy versus Genetic Divergence.—Similar lack of rDNA diversity has been documented in several deep-sea benthic foraminifera (e.g., Tsuchiya et al. 2009; Majewski and Pawlowski 2010), but until now, all detailed morphogenetic comparisons in planktonic foraminifera indicated a higher degree of genetic differentiation than that suggested by morphological taxonomy (Huber et al. 1997; Darling et al. 2006; Morard et al. 2009, 2011; Aurahs et al. 2011; Quillévéré et al. 2013). This would suggest that morphological taxonomy consistently underestimates the biological diversity in planktonic foraminifera, a pattern reported from many other groups of organisms (e.g., Bickford et al. 2007). If this pattern could be extrapolated into the fossil record and if the relationship between morphological taxonomy and genetic divergence remained similar, then the fossil record of planktonic foraminifera could indeed be used

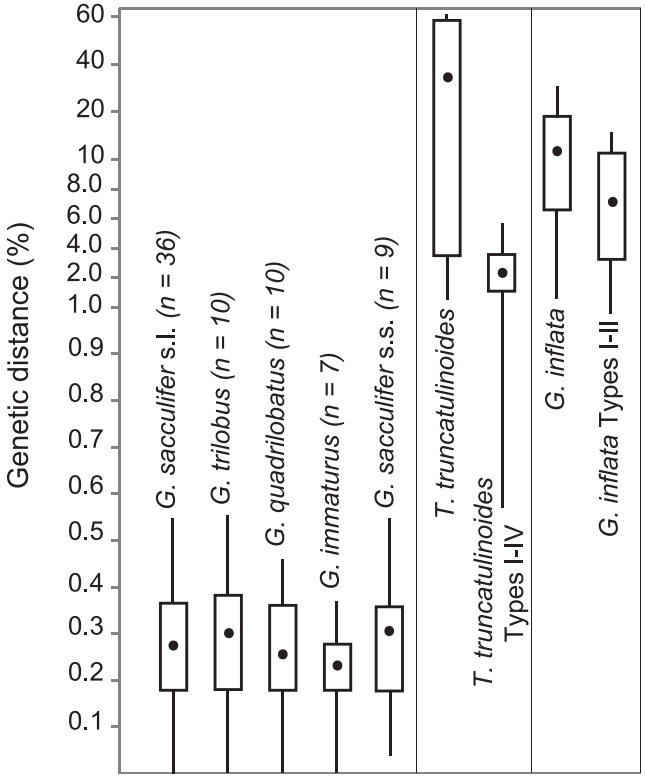

FIGURE 6. MUSCLE alignments-based ITS genetic diversity within the *Globigerinoides sacculifer* plexus and within the morphospecies *Truncorotalia truncatulinoides* and *Globoconella inflata* (data from de Vargas et al. 2001 and Morard et al. 2011, respectively). Box plots give the mean (black circles), 1st through 3rd quartiles (open rectangles), and 95% confidence interval (lines) patristic distance values expressed as percent of nucleotide changes within the entire plexus (*G. sacculifer* s.l.) and within each of the four morphotypes of the plexus. For *T. truncatulinoides* and *G. inflata*, the patristic genetic distances within genotypes (Types I to IV of *T. truncatulinoides*, Types I and II of *G. inflata*) are also given. Note the changes in the left-hand scale of patristic distances.

to infer processes at the level of biological species. Our results challenge this assumption by showing that at least in one case in the modern planktonic foraminifera, the degree of genetic differentiation is lower than that suggested by morphological taxonomy. The existence of at least four morphological species (Fig. 1), of which at least two have been extensively used for modern members of the *Globigerinoides sacculifer* plexus (i.e., *G. sacculifer* [s.s] and *G. trilobus*), contrasts with the lack of genetic differentiation in the studied gene regions. This implies that the morphological variability within the plexus has been, so far uniquely among planktonic foraminifera, taxonomically over-interpreted.

To underline the anomalous lack of genetic differentiation within the *Globigerinoides sacculifer* plexus, we have compiled data on the number of genetic types, number of synonyms, ages, and abundances of seven morphospecies of modern spinose planktonic foraminifera for which extensive genetic data are available (Fig. 7). Whereas there does not appear to be any relationship between the number of genetic types and abundance (expressed as mean number of CHRONOS reports of the species per million years), we observe positive correlation between the number of genetic types and age of the morphospecies and the number of synonyms that exist for the morphospecies. In both cases, *G. sacculifer* deviates from the general relationship. Interestingly, next to *G. sacculifer*, the largest deviation from the relationship is shown by *Orbulina universa*, the sister species to *G. sacculifer*. When both species are removed, the coefficients of determination increase from 0.09 to 0.73 for species age and from 0.27 to 0.87 for the number of synonyms (Fig. 7). We note that the estimate of the

TABLE 3. Patristic genetic distances (in percent) among morphospecies (and among cryptic species for *Truncorotalia truncatulinoides* and *Globoconella inflata*) derived from Muscle and Clustal W automatic alignments. Clustalw + G-block represents Clustal alignments cured using the G-block software. Regular and bold characters for mean and maximum distances, respectively. Distances above 100% are underlined.

ITS	MUSCLE	Clustal W	Clustal W + G-block
Globigerinoides sacculifer	0.77 / **4.93**	0.77 / **4.34**	1.10 / **3.49**
Truncorotalia truncatulinoides	34.1 / **63.6**	61.5 / **126**	41.8 / **82.7**
Type I	1.53 / **2.20**	1.54 / **2.10**	1.20 / **1.92**
Type II	2.58 / **6.36**	2.32 / **5.40**	1.82 / **4.35**
Type III	2.86 / **5.96**	2.77 / **6.85**	1.85 / **4.35**
Type IV	1.65 / **2.35**	1.46 / **2.10**	0.92 / **1. 65**
Globoconella inflata	11.8 / **37.3**	44.3 / **167**	18.8 / **87.2**
Type I	6.62 / **25.3**	8.53 / **36.8**	4.20 / **16.7**
Type II	7.6 / **23.4**	43.1 / **149**	16.90 / **81.5**

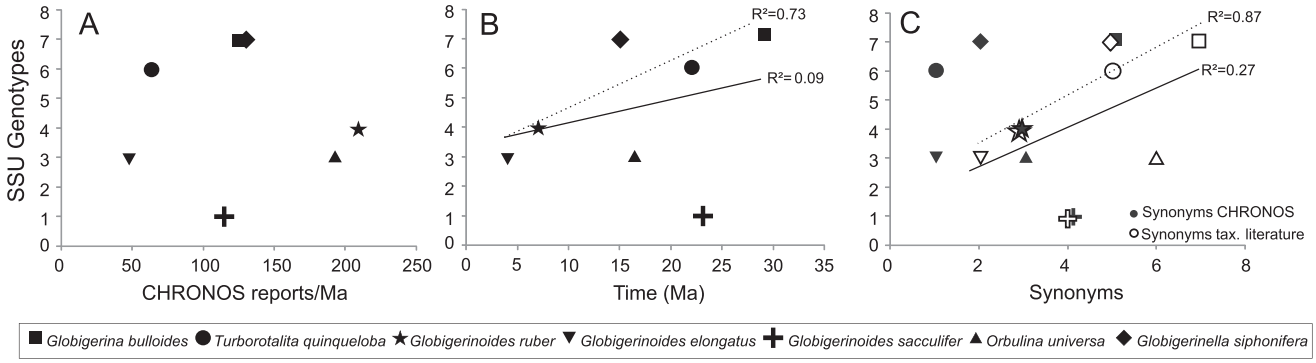

FIGURE 7. Relationships between the number of reported SSU rDNA genotypes in the seven extensively studied spinose planktonic foraminiferal morphospecies and the average number of the morphospecies reports per million years from the CHRONOS database (A), the age of the morphospecies (after Kucera and Schönfeld 2007; Aurahs et al. 2011) (B), and the number of junior synonyms for the morphospecies collated from the literature (open symbols: Saito et al. 1981; Kennett and Srinivasan 1983) and from the CHRONOS database (black symbols) (C). Dotted lines in B and C indicate linear regressions for all species (in C for literature synonyms); solid lines indicate linear regressions without *G. sacculifer* and *O. universa* (see text). Coefficients of determination for regression without those two species are significant at the 5% level (*t*-test for $r = 0$), but we note that the sample size in this case is too small for a robust statistical assessment.

number of synonyms for each of the analyzed species is fraught with a considerable level of subjectivity. For example, when only synonyms that appear in the CHRONOS database are used, the deviation of *O. universa* and *G. sacculifer* from the rest of the test group remains, but the correlation within the rest of the test group is much weaker.

Although observations are not phylogenetically independent, and although the sample size is small for a robust statistical analysis, these results suggest that the number of genetic types in planktonic foraminiferal morphospecies is generally proportionate to the age of the morphospecies (thus opportunity for speciation) and the number of synonyms (thus indication for morphological divergence), but that this relationship does not apply (or is offset) for the members of the *Orbulina universa–Globigerinoides sacculifer* clade. Either some types of morphological variability in planktonic foraminifera are more prone to taxonomic treatment or there exists an objective difference in the rate of biological speciation among clades of planktonic foraminifera. Either way, the fact that the relationship between morphological and genetic diversity in planktonic foraminifera is not consistently scaled implies that the fossil record of this group does not necessarily reflect species-level processes, contrary to the assumption of many paleobiological studies.

Significance of Morphological Variation in the Globigerinoides sacculifer Plexus.—The lack of correlation between morphological features of the shell used in taxonomic concepts within the *G. sacculifer* plexus and actual genetic differentiation requires an alternative explanation for the observed morphological variability. One clue to the meaning of the morphological variation within the plexus could potentially come from the fossil record. To this end, we have compiled data on the occurrence of the four main members of the plexus (Fig. 1) from the CHRONOS database of species occurrence records in deep-sea sediments (Fig. 8). We realize that these data reflect a combination of objective morphological observations and subjective usage of the four taxonomic concepts. Seen from this perspective, the data indicate that the four taxonomic concepts have been applied to fossil specimens throughout the stratigraphic range of the plexus, beginning almost immediately after the oldest records of the plexus. The most commonly used labels for members of the plexus have been *G. trilobus* and *G. sacculifer*, with *G. immaturus* consistently being rarely used and *G. quadrilobatus* being used preferentially in the Miocene. The latter two names have apparently never been used in the Atlantic Ocean (Fig. 8). If the taxonomic concepts of the four members of the plexus were used consistently by the workers whose biostratigraphic data are compiled in the

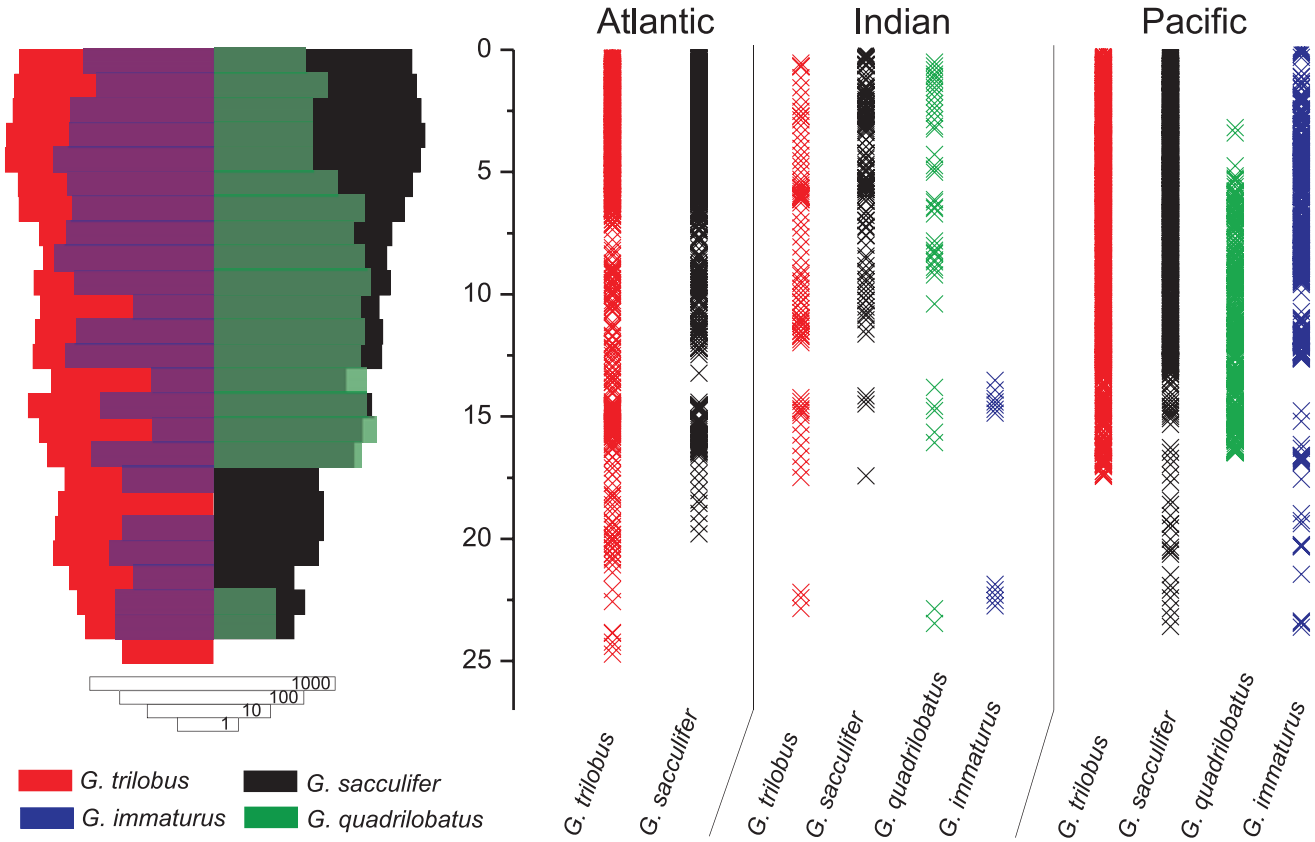

FIGURE 8. Analysis of stratigraphic occurrences of the four main members of the *Globigerinoides sacculifer* plexus as recorded in the CHRONOS database (see *Material and Methods*). The left-hand diagram shows total occurrences averaged per 1-Myr intervals on a logarithmic scale. The right-hand diagram plots the actual dated record separately for the three main ocean basins.

CHRONOS database, then one could conclude that the morphological variability encompassed in these four taxonomic concepts (Fig. 1) has been a persistent feature of the plexus throughout its existence. Combined with the observed lack of correlation between morphology and genetic distinction, the fossil data thus appear to support the conclusion that the morphological traits used to define the four main taxonomic concepts within the plexus are not evolutionarily relevant.

Instead, the morphological variability could reflect marked differences in morphology during the individual growth, which becomes differentially expressed in individuals of different sizes. This argument is supported by the apparent differences in shell size among the species holotypes, where the honeycomb wall structure in relation to shell size is considerably less prominent in *Globigerinoides sacculifer* and *G. quadrilobatus* than in the other two species. This difference is also reflected in the individuals from our collection displayed in Figure 1. The specimens in our collection that we identified as *G. trilobus* and *G. immaturus* were significantly smaller in size than the *G. quadrilobatus* and *G. sacculifer* specimens. Indeed, culturing experiments and observations in the plankton from the Red Sea have been interpreted by Hemleben et al. (1987) as evidence that the sac-like terminal chamber that characterizes the morphotype *G. sacculifer* s.s. is an ontogenetic feature, not developed on all individuals that reach reproductive maturity.

Alternatively, the morphological variability within the plexus could represent an ecophenotypic signal, with different morphologies being expressed under different environmental conditions. The ecophenotypic plasticity is a typical feature of species of planktonic foraminifera (e.g., Kennett 1976). For the *Globigerinoides sacculifer* plexus, Hecht (1974) studied the distribution of specimens with the sac-like final chamber as well as the morphology of specimens without the sac-like cham-

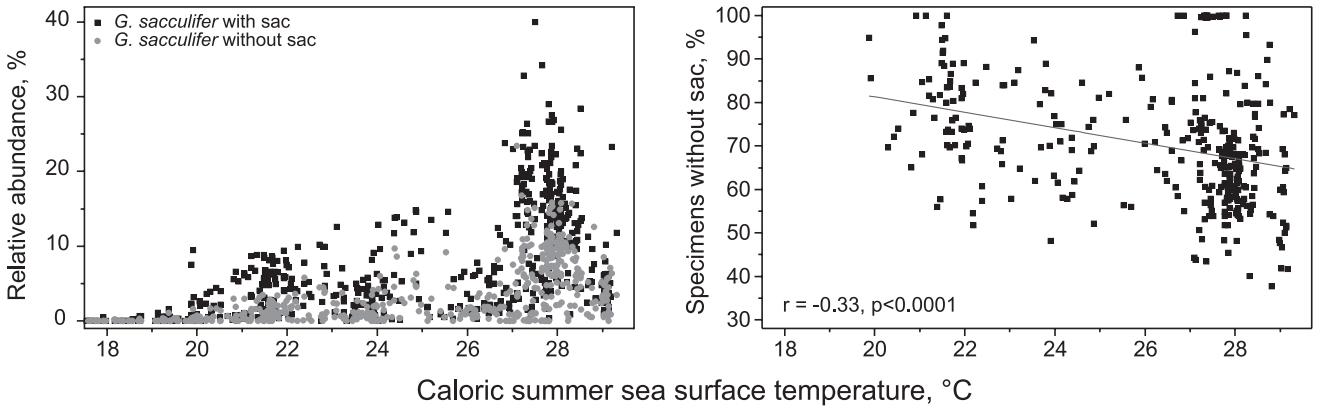

FIGURE 9. The left-hand panel shows the abundance of *Globigerinoides sacculifer* with and without the sac-like terminal chamber in surface sediments of the North Atlantic plotted against caloric summer SST at 10 m depth (abundance and SST data from Kucera et al. 2005). The right-hand panel shows the proportion of specimens without the sac-like chamber in all samples where the abundance of both forms together was higher than 5% in the assemblages; the line shows linear regression; its statistical significance is shown in the figure.

ber. He concluded that the abundance of the sac-like chambers within the plexus increased toward the tropics. This observation is clearly supported by an analysis of the MARGO North Atlantic data set of coretop abundances (Fig. 9). Specimens with and without the final sac-like chamber occur within the same range of temperatures, but the abundance of the *G. sacculifer* s.s. morphotype increases with temperature. It is therefore likely that the manifestation of some or all of the morphological types within the plexus reflects a combination of the existence of different ontogenetic growth stages, which are differentially reached under different environmental conditions. As a consequence, our results imply that all modern representatives of the plexus should be treated as members of a single biological species. Of the four main members of the plexus, *G. sacculifer* is the only one whose original description (Brady 1877) refers to it as occurring in the modern plankton, all other names referring exclusively to fossil material. By the principle of priority, the name *G. quadrilobatus* (d'Orbigny 1846) should be considered the senior synonym for the plexus. However, because we cannot entirely exclude the possibility that the fossil representatives of the plexus with different morphologies could represent different species, we propose that the name *G. sacculifer* be used for all modern specimens of the plexus. The use of "with sac" or "without sac" (i.e., presence or absence of a sac-like final chamber) should be used solely as the description of morphotypes within this species.

Global Dispersal and Gene Flow in Globigerinoides sacculifer.—The fact that same haplotypes on the SSU and ITS-1 sequences in *G. sacculifer* are found globally, throughout the subtropical and tropical habitat of the species, implies either ongoing and effective gene flows or an extremely reduced substitution rate (see also Darling et al. 1999). The latter hypothesis is not consistent with the observed existence of insertion and deletion and polymorphic sites in the ITS-1 region in the species (Fig. 5), which indicates ongoing intra-population differentiation. In addition, *G. sacculifer* and *Orbulina universa* consistently cluster as sister species in SSU rDNA phylogenies (Darling et al. 1997; de Vargas and Pawlowski 1998; Aurahs et al. 2009b), but their ITS-1 sequences are so derived that they cannot be aligned (Fig. 5). As a consequence, we conclude that the lack of geographic differentiation in *G. sacculifer* probably does not result from a slowdown in evolutionary rate. Instead, the data appear more consistent with the existence of an ongoing (or very recent) effective gene flow among the partly disjunct warm-water populations of the species between the Indo-Pacific and the Atlantic. Because planktonic foraminifera are passively dispersed by surface currents, this conjecture implies that the transport of specimens or propagules of this species by the Agulhas Current and Agulhas Rings from the Indian

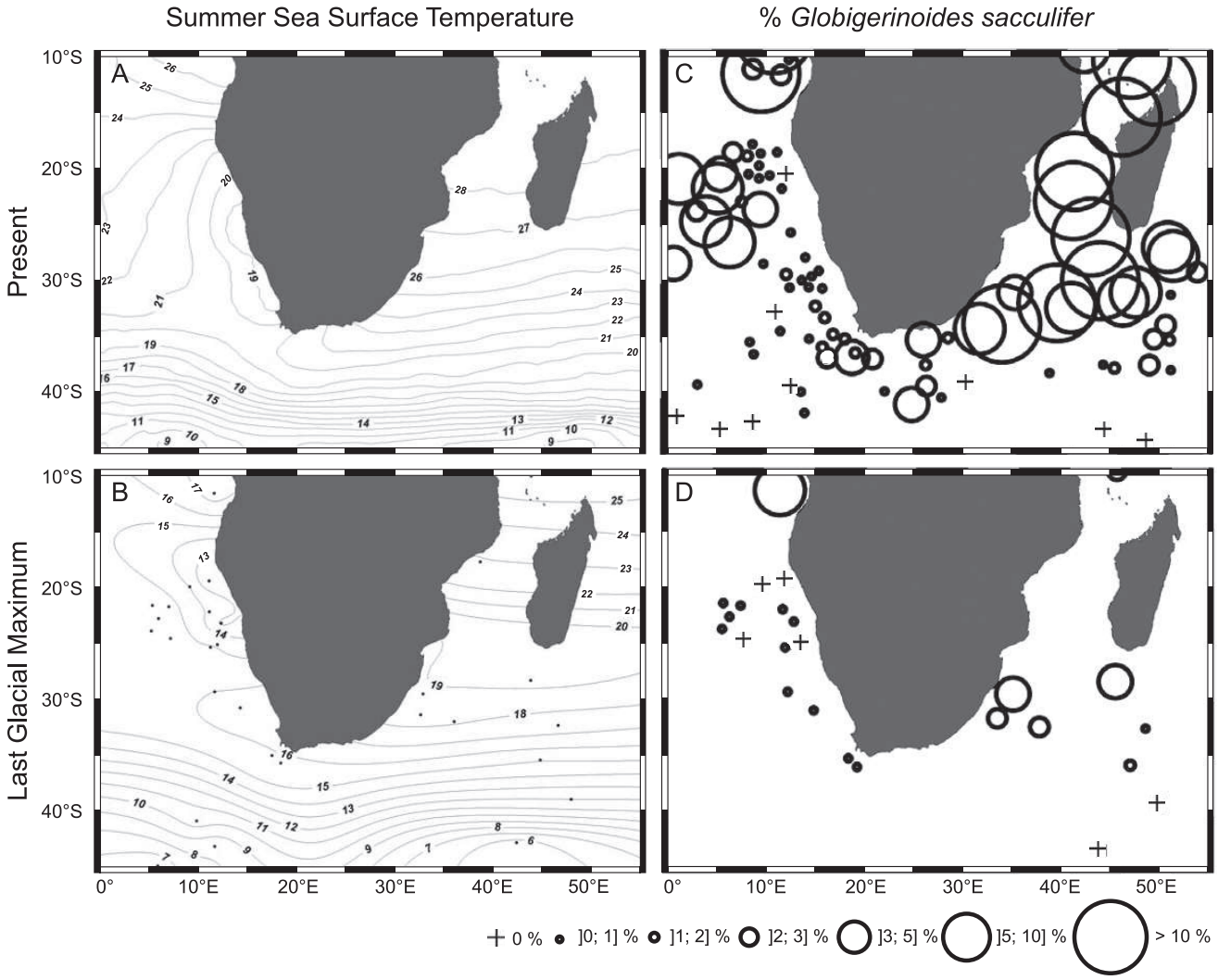

FIGURE 10. Modern (A) and last glacial maximum (B) summer sea-surface temperatures (data from the MARGO Project Members [2009]) in the Atlantic and Indian Oceans around southern Africa. The right-hand maps show relative abundances of the *Globigerinoides sacculifer* plexus (i.e., including specimens with and without sac-like final chamber) in planktonic foraminiferal assemblages from surface sediments (C) and last glacial maximum sediments (D). Each symbol represents one locality. The abundance data are from Kucera et al. (2005) and Barrows and Juggins (2005).

Ocean into the South Atlantic is effective enough to saturate the Atlantic population with specimens carrying Indo-Pacific haplotypes.

Indeed, Peeters et al. (2004) report large standing stocks of *Globigerinoides sacculifer* from plankton nets samples retrieved in isolated Agulhas Rings in the Southern Atlantic. The passive transport of this species into the Atlantic is clearly visible in the pattern of its abundance in surface sediment samples (Fig. 10). Considering the pattern of surface current flow between the two oceans (Beal et al. 2011), the advection of *G. sacculifer* between the Indo-Pacific and the Atlantic is at present mainly unidirectional. In fact, the transport is likely to have remained unidirectional since the closure of the Panama Isthmus in the Pliocene (Groeneveld et al. 2006).

The unidirectionality of the gene flow in *Globigerinoides sacculifer* from the Indo-Pacific into the Atlantic is consistent with the occurrence of an exclusively Atlantic ITS-1 haplotype (Fig. 5). We hypothesize that this haplotype originated in allopatry, during an intermittent isolation of the Atlantic Ocean, but its evolution was not associated with the development of intrinsic reproductive isolation mechanisms, such that inbreeding was not prevented upon secondary contact with the Indo-Pacific population. This is evidenced by the fact that the Atlantic haplotype occurs

together with the dominant global haplotype in the same specimens. Considering that it is only found in the faster-evolving ITS-1 and does not correspond to a differentiation signal in the SSU, and that there is no evidence for genetic recombination between the haplotypes, we conclude that its occurrence reflects a recent isolation event. The most recent opportunity for isolation between the Indo-Pacific and the Atlantic (sub)tropical water masses occurred during the last glacial maximum, when the circum-Antarctic currents were displaced toward the north and constricted the Agulhas exchange (Flores et al. 1999; Peeters et al. 2004; Bard and Rickaby 2009; Beal et al. 2011). The chance for westward advection of individuals of *G. sacculifer* around the Cape was thus considerably reduced, as the environment that had to be crossed to reach the Atlantic became too cold (Fig. 10), close to the temperature tolerance of the taxon (14–32°C [Bijma et al. 1990]). The resulting restriction of the passive transport of *G. sacculifer* around the Cape is reflected in the abundance of this species in deep-sea sediments from the region that have been dated to the last glacial maximum (Fig. 10).

In this model, the intermittent restriction of the gene flow between Indo-Pacific and Atlantic populations of *Globigerinoides sacculifer* on glacial/interglacial time scales would have been too short to result in the evolution of distinct SSU genetic lineages. If the propensity of *G. sacculifer* to maintain an effective gene flow between the two basins during interglacials has been a persistent attribute of the species throughout its existence, then these two mechanisms combined could explain why only a single global SSU genetic type is found today within the *G. sacculifer* plexus. The only other plausible explanation for the lack of genetic differentiation would be the existence of a recent population bottleneck. Here, any genetic variability that had developed over time in the *G. sacculifer* plexus would have been reset by an extinction event, sparing only a small, genetically homogeneous population from which all the modern representatives would have descended. Low levels of genetic diversity have been shown in planktonic foraminifera thought to have undergone such recent bottlenecks (e.g., Aurahs et al. 2009a). However, the fossil distribution of the *G. sacculifer* plexus does not indicate any evidence for a population bottleneck (Figs. 8, 10). To our knowledge, in the literature, such an event has never been reported for *G. sacculifer* at any time throughout the existence of the species.

Conclusions

Our survey of the genetic variability in the modern representatives of the *Globigerinoides sacculifer* plexus reveals the existence of a single genetic type. The lack of cryptic genetic diversity is in stark contrast to the morphological variability and the usage of multiple taxonomic concepts for members of this plexus. Our results imply that at least in the modern plankton, the morphospecies *G. sacculifer*, *G. immaturus*, *G. trilobus*, and G. *quadrilobatus* all correspond to a single biological species with a cosmopolitan distribution. In paleoceanographic reconstructions based on morphological and/or chemical signals in the fossil shells of planktonic foraminifera, the assumption is indirectly made that each morphospecies corresponds to a biological species adapted to a unique habitat and possessing unique biomineralization physiology. Over the last decade, this assumption has been called into question by extensive genetic surveys, which revealed the existence of multiple distinct and differentially adapted genetic types within individual morphospecies (de Vargas et al. 1999, 2002; Darling and Wade 2008; Morard et al. 2009; Quillévéré et al. 2013). In the case of *G. sacculifer*, the assumption of the congruence between morphological and biological species is violated in the opposite direction: multiple morphotypes correspond to a single species. This observation is significant because it shows not only that the morphological taxonomy does not reflect genetic differentiation, but also that the scaling between the two may be inconsistent.

The lack of geographic structuring among the surveyed specimens is interpreted as evidence for an effective gene flow from the Indo-Pacific into the Atlantic tropical and subtropical habitats of the species. The exis-

tence of an exclusively Atlantic ITS-1 haplotype indicates that this gene flow can be intermittently reduced, most likely during glacial intervals, but the latest disruption apparently has not been sufficient to result in speciation. If the propensity for "genetic homogenization" following short periods of isolation was a persistent feature in the evolutionary history of the species, then the fossil record of the *G. sacculifer* plexus could be interpreted as the occurrence of a long-ranging single, ecologically successful, morphologically variable lineage.

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6. Third case study

Phylogeography of the tropical planktonic foraminifera lineage *Globigerinella* reveals isolation inconsistent with passive dispersal by ocean currents

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Phylogeography of the Tropical Planktonic Foraminifera Lineage *Globigerinella* Reveals Isolation Inconsistent with Passive Dispersal by Ocean Currents

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Abstract

Morphologically defined species of marine plankton often harbor a considerable level of cryptic diversity. Since many morphospecies show cosmopolitan distribution, an understanding of biogeographic and evolutionary processes at the level of genetic diversity requires global sampling. We use a database of 387 single-specimen sequences of the SSU rDNA of the planktonic foraminifera *Globigerinella* as a model to assess the biogeographic and phylogenetic distributions of cryptic diversity in marine microplankton on a global scale. Our data confirm the existence of multiple, well isolated genetic lineages. An analysis of their abundance and distribution indicates that our sampling is likely to approximate the actual total diversity. Unexpectedly, we observe an uneven allocation of cryptic diversity among the phylogenetic lineages. We show that this pattern is neither an artifact of sampling intensity nor a function of lineage age. Instead, we argue that it reflects an ongoing speciation process in one of the three major lineages. Surprisingly, four of the six genetic types in the hyperdiverse lineage are biogeographically restricted to the Indopacific. Their mutual co-occurrence and their hierarchical phylogenetic structure provide no evidence for an origin through sudden habitat fragmentation and their limitation to the Indopacific challenges the view of a global gene flow within the warm-water provinces. This phenomenon shows that passive dispersal is not sufficient to describe the distribution of plankton diversity. Rather, these organisms show differentiated distribution patterns shaped by species interactions and reflecting phylogenetic contingency with unique histories of diversification rates.

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Introduction

In many groups of marine microplankton, morphologically defined species tend to underestimate diversity [1,2]. Cryptic speciation is prevalent in these groups, manifested in genetic differences that are not accompanied by the development of morphologically divergent traits [3]. In consequence, diversity patterns and species biogeography derived from observations of morphospecies may not reflect processes at the level of biological species.

This observation has consequences for the interpretation of biogeographic patterns of marine microplankton. At the morphological level, species often appear globally distributed, but their constituent cryptic lineages may show more differentiated patterns [4]. In theory, such spatially structured distribution may reflect either dispersal limitation, differential adaptation or niche incumbency [5,6]. The fundamental difference among these scenarios lies in the ubiquity of gene flow and in the importance of species interactions. Under dispersal limitation, genetic drift associated with the establishment of abiotic barriers may lead to the differentiation of allopatric sister lineages. If dispersal is not the primary restriction and species interaction is of subdued importance, then distribution of species should reflect the spatial realization of suitable niches. If, however, species interactions are important then the occupancy of the realized niches will be influenced by competitive exclusion, leading to a pattern of niche incumbency. Because of the manifest differences among the predictions of these three scenarios, an observed species biogeography could in theory be used to draw conclusions about the importance of dispersal and species interactions for the distribution and diversity of marine plankton.

Because of the prevalence of cryptic speciation and the often cosmopolitan distribution of morphospecies in plankton, an assessment of these three end-member scenarios for biogeographic patterns requires an extensive global sampling of genetic diversity, covering the entire range of the studied lineage. Here we use the genetically most diverse morphospecies of planktonic foraminifera as a model to assess global biogeography of DNA-delineated cryptic species in view of these scenarios. Most morphospecies of

planktonic foraminifera have a cosmopolitan distribution within their preferred temperature range [7] and evidence exists that gene flow in these obligate sexual outbreeders occurs on a global scale [8,9]. On the other hand, there is abundant evidence that morphospecies of planktonic foraminifera represent complexes of reproductively isolated but morphologically indistinguishable cryptic species [4]. In most cases such cryptic species reveal restricted distribution patterns, indicating that biogeographies of morphospecies in this group are not representative for processes at the level of biological species [10–12].

Earlier studies of the phylogeography of planktonic foraminifera attempted to identify the pattern of speciation that has led to the observed distribution or the environmental factors influencing it, but the importance of biological interactions has been largely overlooked [4,13,14]. Aurahs *et al.* [10] first noted that the distribution of genetic lineages of *Globigerinoides ruber* could be best explained by competitive exclusion and the concept was then used by Seears *et al.* [15] to explain the occurrence of genetic types of planktonic foraminifera in the Arabian Sea.

In this study we present the results of a global survey on the foraminifera lineage *Globigerinella* [16], which is abundant in the surface waters in tropical and subtropical provinces throughout the world ocean (**Fig. 1**). The dominant morphospecies in this lineage, *G. siphonifera* tolerates a temperature range from 11°C to 30°C and a salinity range from 27–45‰ [17] and it is limited vertically to the euphotic zone due to its association with symbionts. In the modern ocean, *Globigerinella calida* [18] has been described as its sister species, but it is morphologically similar and its status as a separate species remains unclear. This study includes specimens that have been assigned to that species name as well. Within the typical *G. siphonifera* morphology, two divergent types were distinguished by different cellular morphology and symbionts [19,20], and potentially also by morphological, physiological, chemical and genetic differences [21,22]. The high degree of variability in the *G. siphonifera* lineage is reflected in its genetic diversity. Analyses of the small ribosomal subunit RNA gene (SSU rDNA), which is part of the only gene complex known so far in planktonic foraminifera, identified a large number of genetic lineages, which show no evidence for introgression and are typically considered as cryptic species [4,21–24]. Based on these data, the *G. siphonifera* group appears to be the most genetically diverse lineage of modern planktonic foraminifera [4].

Although the existing sampling has been far from exhaustive, it seemed to indicate that individual cryptic genetic lineages within *G. siphonifera* are cosmopolitan [4], but their proportions vary with surface ocean properties [23]. Such distribution could be explained by a combination of unlimited dispersal and differential adaptation, but it remains uncertain whether it stands the test of global sampling. Here we analyze SSU rDNA sequence data from a global survey that covers the distribution range of *G. siphonifera* both latitudinally, across the tropical and subtropical oceans and their satellite semi-isolated marginal seas (**Fig. 1**) in order to study its biogeography and draw conclusions on the emergence of the observed high genetic diversity.

Materials and Methods

Ethics statement

The field collections carried out for the purpose of this paper did not involve endangered or protected species. Locations of all sampling stations are given in **Table S1**. The sampling was carried out in open ocean and followed the regulations for the exclusive economic zones (EEZ) of the coastal countries, provided for each expedition by the respective authority. No specific permission was required to collect the analyzed plankton.

Sampling

Specimens of *Globigerinella siphonifera* were collected during 26 expeditions between 1996 and 2012 covering all seasons and water depths from the surface to 700 m (**Table S1**). The sampling represents a combination of plankton hauls during ship cruises, including stratified sampling, with nearshore collections by small nets and scuba diving. Mesh size varied from 100 to 200 μm. In all cases, individual foraminifera were separated from the rest of the plankton and taxonomically identified using stereomicroscopes. Living specimens still containing cytoplasm were cleaned using a brush and either transferred to 1.5 ml tubes for direct DNA extraction or air-dried on cardboard slides and stored at −20 or −80°C until further processing. In addition, the dataset was enhanced by inclusion of 45 sequences of *G. siphonifera* available in GenBank (**Table S1**). In order to resolve the phylogeny of the *G. siphonifera* sequences, to root the tree, and to estimate divergence times among the main lineages, we have attempted to obtain SSU rDNA sequences of the sister species *Beella digitata*. Eight specimens of that species have been collected from plankton nets in the Western Mediterranean (**Table S1**).

DNA extraction, amplification and sequencing

DNA extraction followed either the DOC protocol of Holzmann & Pawlowski [25], during which the shell is dissolved, the guanidine method [26] or an urea method where the DNA is extracted in a mixture of 100 mM Tris (pH 8), 100 mM NaCl, 1% Sarcosyl, 8 M Urea and 2 mM TCEP, kept at room temperature. The latter two methods allow preservation of the shell. Polymerase chain reaction (PCR) was used to amplify a ~350 to 1000 bp fragment of the 3′ end of the SSU rDNA either using the proofreading Vent® polymerase (New England Biolabs) or Taq DNA Polymerase (Qiagen). The amplified fragments include all sequence sites necessary to differentiate between the genetic lineages of *G. siphonifera*. Details on extraction, amplification and primers for all individuals are given in **Table S1**. PCR products were purified using the QIAquick gel extraction kit (Qiagen), Wizard® PCR clean up (Promega) or DNA Gel Extraction Kit (Millipore). Products were sequenced directly by external service providers (Agowa, Berlin and University of Edinburgh Gene Pool). In order to constrain intra-individual variability, eight individuals from different regions were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) with TOP10 chemically competent cells. Sequence chromatograms were checked manually for ambiguous reads and corrected where possible. All new sequences longer than 200 bp were submitted to GenBank (http://www.ncbi.nlm.nih.gov/; accession nos.: KF769560-KF769948).

Delineation of genetic lineages

The primary sequence alignment was carried out in MAFFT v. 6.935b [27] using the option -auto, which allows the program to decide on the optimal alignment algorithm (**Alignment S2 in File S1**). Aurahs et al. [28] have shown that MAFFT handled best the particular sequence structure of foraminiferal SSU rDNA out of six alignment programs tested. The alignment was used to define the main genetic lineages and to group identical sequences (here referred to as 'ribotypes' (RT)), which present the same combination of certain sequence motifs within the amplified fragment of the SSU rDNA. This analysis identified the presence of three main lineages, which further split into up to seven clades. The initial automated alignment was split into three subalignments corresponding to the three main genetic lineages (**Alignments S4–6 in File S1**). For each subalignment sequence chromatograms were checked by eye for sequencing errors, sequence ends

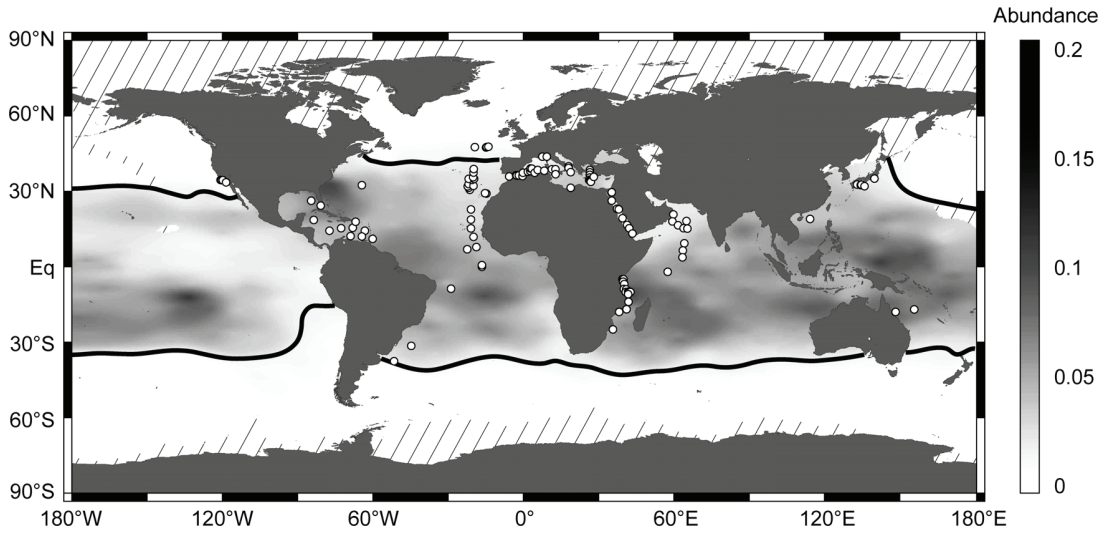

Figure 1. World map indicating the distribution of the target species and sampling sites for this study. Gray shading indicates the relative abundance of *Globigerinella siphonifera* as it is found in planktonic foraminiferal assemblages from surface sediments interpolated from data in the MARGO database [64] by Ocean Data View [65] in default projection. Black lines show the borders of occurrence with a threshold of 1%. White circles indicate the sampling stations of all samples included in this study. Diagonal lines indicate areas where no data are available.
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were trimmed and length-polymorphic regions were left-aligned by default in MESQUITE v. 2.75 [29]. The SSU rDNA of foraminifera is characterized by the occurrence of highly length-polymorphic regions (LPR) in the core structure, which hinder the computation of straightforward alignments with consistent homology of individual base pairs [28]. The number of inferred parsimonious changes in these regions would be highly depending on the alignment, the hypothetical homology of individual sites. Therefore, we opted for treating each LPR as a single, complex character (an oligonucleotide motif) in the ribotype analysis instead.

Due to the different length of the individual accessions, and the particular nature of foraminifer expansions segments, the direct application of median-joining networks [30] to establish relationships between ribotypes of each major genetic lineage was not feasible. Instead ribotypes were analyzed based on the variable positions in each subalignment. Differing sequence patterns (point mutations and LPR motifs) were coded as a binary matrix, in which characters with more than two states were represented by a corresponding number of half-weighted binary characters. A point mutational pattern involving the nucleotides A, C, and G would be coded as 1 0 0, 0 1 0, and 0 0 1 using three characters with a weight of 5 instead of the standard weight of 10. LPR motifs were coded accordingly at this step. Mutation patterns that were only present in a single sequence were not considered separately, but merged with the nearest ribotype for abundance analysis. The resultant binary matrices comprising up to 19 ribotypes were then analyzed using NETWORK v. 4.5 (Fluxus Technologies Inc.) to compute median-joining (MJ) networks [30].

The recognition of ribotypes allowed us to structure the genetic diversity within *G. siphonifera* between the level of the three main lineages and the ribotypes into discrete and objectively defined genetic types, using a threshold of three mutational events. Ribotypes separated by three or fewer mutational events were considered to belong to the same genetic type. Earlier studies reported the existence of different ribotypes within the genome of one single individual in some but not all species of foraminifera [31]. Consistent with earlier investigations of intraindividual variability within the spinose planktonic clade [8], in our study, only one ribotype per individual was found, which was apparent by the lack of ambiguous sequence reads and was verified by cloning, which revealed identical sequences within single individuals. The apparent lack of hybridization among the ribotypes would suggest that they may represent genetically isolated units. However, we cannot entirely exclude the existence of hybrids with the present data because of insufficient cloning depth. Therefore, to avoid an over-interpretation of the genetic diversity and arrive at a number of distinguishable lineages, we reserve the (cryptic) species rank for genetic types.

ML tree inference and bootstrapping

To resolve the phylogenetic relationships of the *G. siphonifera* lineages and *B. digitata* to the rest of the planktonic foraminifera, the MAFFT sequence alignment from Aurahs *et al.* [28], including sequences of 23 planktonic foraminifera morphospecies, was used as a basis to which the new sequences were aligned by the sequence adding function in MAFFT v. 7 [32] (**Alignment S1 in File S1**). Settings were left to default. This enlarged alignment was then used for tree inference under the maximum likelihood (ML) criterion with RAxML-HPC2 v. 7.6.3 [33] via the CIPRES Gateway [34]. The alignment was used without further manipulation or filtering. Branch support for the ML tree of the general foraminifera MAFFT alignment was established with the fast implementation (option –x) [35] of nonparametric bootstrapping (BS) [36]. The number of necessary replicates was determined by automatic bootstopping with the majority-rule tree based criterion (option -#autoMRE). The per-site rate approximation model [33] was used for the fast BS phase followed by a slow final model optimization under the general time reversible model allowing for between-site variation modeled via a gamma distribution (GTR + Γ; option -m GTRCAT). Run parameters were set to infer in one run the best-known ML tree and perform a full BS analysis (option –f a).

To resolve further the relationships among the genetic types of *G. siphonifera*, a set of analyses has been carried out including only sequences of *G. siphonifera* and *B. digitata*. Following Aurahs *et al.*

[28], the stability of the topology has been evaluated by a multiple alignment approach. To this end, automated alignments have been used, based on the default settings of the online-available, up-to-date versions of MAFFT v. 7, MUSCLE v. 3.7 [37] and KALIGN v. 2 [38]. Tree inference was conducted under the same settings as described above and without prior manual modification of the alignments.

Molecular clock and speciation rates

In order to estimate the divergence time among the genetic lineages within *G. siphonifera*, a molecular clock approach was applied, using the *G. siphonifera*/*B. digitata* MAFFT alignment. *B. digitata* was used as an outgroup to define the *Globigerinella* root. Molecular clock analysis was performed using Bayesian methods implemented in BEAST v. 1.7.5. [39] via the CIPRES Gateway. The alignment was tested under various clock models (strict, uncorrelated lognormal and uncorrelated exponential). The split between *G. siphonifera* and *B. digitata* is marked in the fossil record by the first appearance of the species *Beella praedigitata* [40,41]. This event is dated to 10.2 Ma in Aze *et al.* [41]; the age of the oldest reported occurrence of this species in deep-sea sediments is listed in the CHRONOS database as 11.96 Ma (http://chronos.org) [42]. Here we used the mean of the two ages (11.08 Ma) and associate this date with an uncertainty of 0.88 Ma. Detailed settings were the same for all three clock models tested. The distribution of the fixed node age prior was considered normal. The GTR+Γ+I (adding a parameter for the proportion of invariant sites) was used as a substitution model, to allow for different evolutionary rates between variable and conserved regions of the SSU rDNA. Speciation rate was considered constant under the Yule-Process and a UPGMA tree was calculated as a starting tree. Markov-Chain-Monte Carlo (MCMC) analyses were conducted for 10,000,000 generations, with a burn-in of 1000 generations and saving each 1000th generation. The maximum clade credibility tree with median node heights was calculated in TREEAnnotator from the BEAST package, with a burn-in of 100 trees and a posterior probability limit of 0.0. The resulting tree was then analyzed in FigTree v. 1.3.1 [43].

To test for trait dependency of changes in birth-only speciation rates among different clades, we applied a covariates generalized linear model (GLM) approach [44] on the trees produced by the lognormal and exponential uncorrelated clocks. This method allows to test, whether or not the presence of a certain trait had a significant effect on the speciation rate within given clades in a phylogenetic tree, taking branch-lengths into account. If reliable phylogenetic trees exist, it is considerably more powerful than traditional tests for changes in speciation rate, that only compare the number of lineages within adelphotaxa [45]. The test was performed in R v. 3.0.1 [46], using the package 'ape' v. 3.0.8 [47].

Assessment of sampling intensity

For the global dataset and for the separate regions, first-order-Jackknifing [48,49] was performed in R v. 3.0.1 to estimate the number of genetic types expected to occur in each region, given their occurrence in the sampling sites. Such test provides a first assessment whether or not the sampling was sufficient to detect all genetic lineages present in each region. For that, each station was treated as a separate sample, independent of the other stations, and it was assumed that the samples are sufficiently random and well distributed to allow such an approach, and cover the world ocean area to an extent that allows them to be assumed homogenous. The jackknifing is insofar most useful for this dataset, as it is fully independent of any possible interaction of different genetic types within the same quadrat, and offers a very good bias-correction for low densities per sample [49].

Results

In addition to the 45 sequences from GenBank, in this study we obtained 370 partial sequences of the 3′ end of the SSU rDNA representing 338 individuals of *Globigerinella siphonifera* from 108 stations of 25 expeditions in seven regions of the world ocean (**Table S1**). The 3′ end of the SSU rDNA, routinely used in foraminifera molecular studies, includes the helices 32 to 49 [50] and additional foraminifera specific expansion segments of variable length. Most sequence divergence was found in the expansion segments 37/e1, 41/e1, 45/e1 and 46/e1, the variable region V7 consisting of several helices and the terminal part of helix 49 (Tp49) [51]. Furthermore, point mutations were also found in the sequentially and structurally conserved regions (helices 32–49) of foraminifera SSU rDNA (**Table S2**). All sequences obtained either by direct sequencing or cloning showed a clear signal and could be attributed without doubt to one of the main genetic lineages. We did not observe any intraindividual variability neither by seeing ambiguous reads at consistent positions or by observing variability among sequences from cloned specimens, which would be the case if individuals contained different ribotypes in the multiple copies of the SSU rDNA. Additionally, we obtained 25 sequences of eight individuals of *Beella digitata* covering the entire fragment of the SSU rDNA used for phylogenetic inference in planktonic foraminifera by Aurahs *et al.* [28].

All sequences could be assigned to one of the three main lineages, which, applying a distance threshold of 0.1028, correspond to objectively definable taxonomic units [24]. These lineages are robust to increased taxonomic coverage, especially to the inclusion of *B. digitata* (**Fig. 2a**) and remain supported to >89% in maximum-likelihood inference. The sister relationship of *B. digitata* has been confirmed (**Fig. 2a**), supporting observations from the fossil record [40].

Following the strict definition excluding singletons, the variability of the analyzed gene fragment of *G. siphonifera* reveals the existence of 30 SSU rDNA sequence variants (ribotypes; **Table S2**). This confirms the exceptional level of diversity noted in earlier studies [4]. Within lineage I, the six separated ribotypes can be organized into two basic genetic lineages, namely Ia (RT 1+2) and Ib (RT 3–6), that differ by up to eight characters (all of them point mutations; **Fig. 3a**). Mutations occur to equal parts in the variable regions (41/e1, 46/e1 and Tp49) and in the more conserved regions (helices 33, 36, 37, 43). The five ribotypes within lineage III are only little more divergent than those in lineage I, with two (RT 4+5) being separated by up to 13 point mutations from the remaining three (RT 1–3), which differ by 3–4 characters from each other (**Fig. 3b**). Consequently, these ribotypes can be classified into three different genetic lineages, IIIa, IIIb and IIIc. Mutations separating these lineages are exclusively point mutations and are mostly found in the variable regions (37/e1, 41/e1 and V7) and only in two conserved regions (helices 37 and 38). Highest divergence is found in lineage II, where sequence variation sums up to 19 ribotypes that can be grouped into seven genetic lineages (IIa1–6 and IIb; **Fig. 4**). RT 18 and 19 are with more than 40 mutational events most distinct and assigned to lineage IIb. Mutations in lineage II are homogeneously distributed between all variable and all conserved regions.

Subsequently, the phylogenetic relationships among the 30 ribotypes organized in 12 genetic lineages within *G. siphonifera* were tested using three different alignments (**Fig. 2b**). This analysis

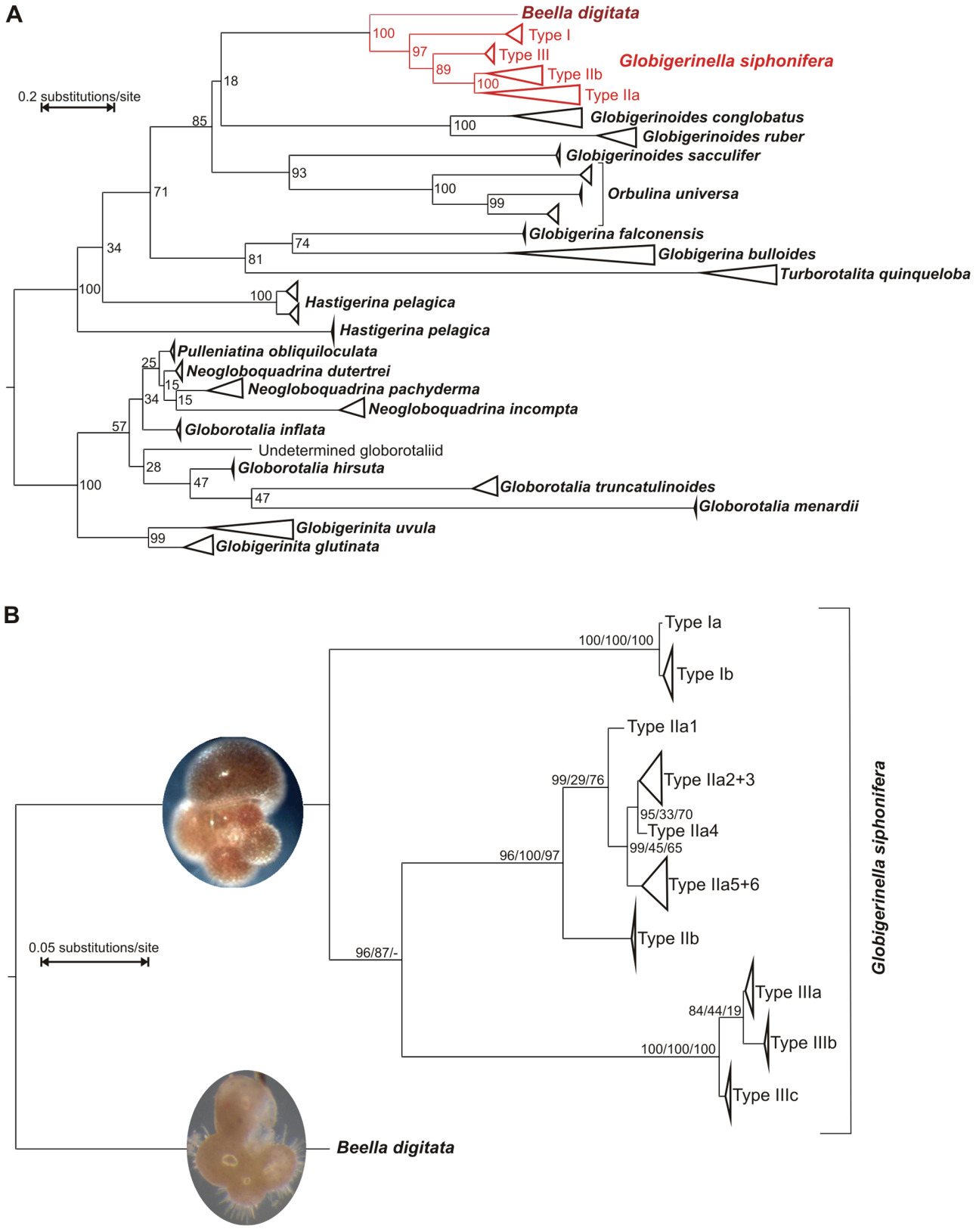

Figure 2. Phylogenetic relationships within planktonic foraminifera. A) Phylogenetic relationships of planktonic foraminifera including *Globigerinella siphonifera* and *Beella digitata*. The tree is based on the MAFFT alignment of Aurahs *et al.* [28] to which SSU rDNA sequences of *G. siphonifera* and *B. digitata* were added. Tree inference and calculation of bootstrap values was conducted in RAxML in the CIPRES gateway. Sequence

diversity within morphospecies has been collapsed, except for *G. siphonifera* where only terminal branches were collapsed. **B**) Phylogenetic tree of *G. siphonifera* with *B. digitata* as an outgroup. The tree is based on a MAFFT alignment and was calculated in RAxML on the CIPRES gateway. Bootstrap values are shown based on MAFFT/MUSCLE/KALIGN alignments. Light microscopic images of *G. siphonifera* and *B. digitata* illustrate the gross morphology. Both individuals measure ~250 μm across.
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reveals that 10 out of the 12 genetic lineages, defined as differing by more than three characters, are supported in the majority of the alignments. A resolution down to the separate ribotypes as seen in the networks, however, is not possible in the tree, and therefore the terminal branches are collapsed. The topology of the phylogram, including the inferred allocation of mutation events to branches, indicates a nested, hierarchical pattern of divergence, suggesting an ongoing process of sequential differentiation.

It is remarkable that despite the seven-fold increase in sequencing effort compared to existing data, no new major lineages within *G. siphonifera* were discovered. A similar picture appears when individual genetic lineages are considered. Here, our data complement earlier studies [4,23,24] by discovering two new genetic lineages (lineages IIIb and c; **Fig. 2b**), which is again highly disproportionate to sequencing effort. At the lowest level of divergence considered, the proportion of newly discovered sequence motifs is the highest: 16 out of 30 ribotypes are reported here for the first time. Even here, the amount of ribotype discovery is disproportionate to sequencing effort and the higher number of new motifs simply reflects the hierarchical scaling within the clade.

The geographical distribution of specimens assigned to the twelve genetic lineages reveals the existence of cosmopolitanism as well as provincialism within cryptic genetic types of *G. siphonifera* (**Fig. 5a**). Type IIIc shows the most restricted occurrence; it was

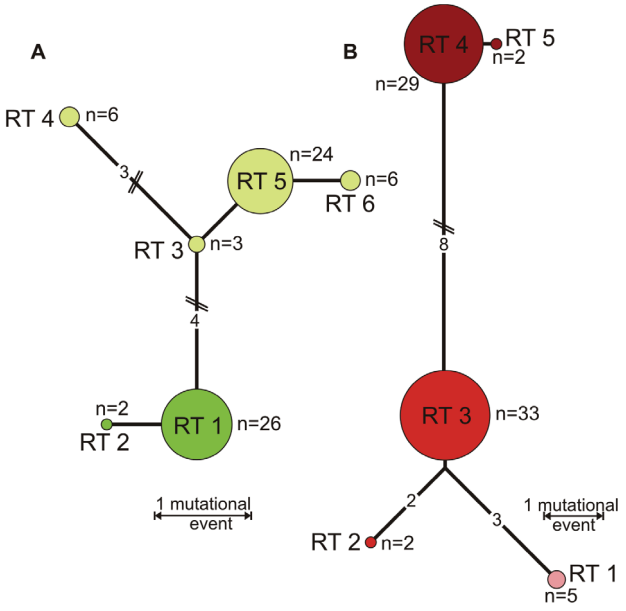

Figure 3. Ribotype networks for delineation of genetic types. **A**) Median-joining network of *Globigerinella siphonifera* lineage I showing genetic distances and relationships between ribotypes (RT) and their grouping into two basic genetic lineages, Ia (RT 1+2, bright green) and Ib (RT 3–6, light green). Numbers on links indicate amount of mutational events between two ribotypes if they are larger than one. n indicates number of individuals representing one ribotype. **B**) Ribotype network of lineage III distinguishing three basic lineages, IIIa (RT 1), IIIb (RT 2+3) and IIIc (RT 4+5), addressed by different shades of red.
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only found in the Gulf of Aquaba, where it has the highest abundance of all occurring types. Type IIIa was only found in low abundances and exclusively in the Eastern Atlantic. Type Ia seems to have a cosmopolitan occurrence since it was found in the majority of regions sampled. Types Ib, IIb and IIIb can also be considered cosmopolitan, although they are less evenly distributed. Type Ib has its highest abundances in the Western Indian Ocean and the Red Sea and very low abundances in the Atlantic, where only one individual was found. Type IIb was sampled in high numbers in the Atlantic, but only few individuals in the Eastern Pacific. Type IIIb was found in the marginal seas of the Atlantic and in the Western Indian Ocean.

The group of genetic types IIa is highly abundant globally and shows a truly cosmopolitan distribution. However, its constituent types show highly differentiated distribution patterns, characterized by a surprising difference in diversity between the Atlantic and the Pacific (**Fig. 5b**). The Indian Ocean contains the highest diversity with five different types of this lineage. Type IIa1 was found in very low abundances mainly in the Indian Ocean and one individual in the Coral Sea. Type IIa4 seems to be restricted to the Red Sea and the Western Indian Ocean. Type IIa5 is most abundant in the Arabian Sea, but also present in low numbers in the Northwestern Pacific. Type IIa6 was mainly found close to Japan, but apparently also occurs in the Indian Ocean as indicated by one individual sampled in the Arabian Sea. In contrast to the high diversity of lineage IIa in the Pacific and Indian Ocean, the diversity in the Atlantic is considerably more limited. There we only encountered two different types: Type IIa2, which except for two individuals off California seems to be restricted to the Atlantic Ocean and Type IIa3, which has a cosmopolitan distribution and occurs in every region sampled.

Discussion

A surprisingly high SSU rDNA sequence divergence is found in most morphospecies of planktonic foraminifera [4]. This sequence divergence is typically organized into a small number of lineages, which show no evidence for hybridization, their divergences appear ancient and their distribution follows a geographical structure [10,12]. For these reasons, such lineages, also referred to as "Types" or "Genetic types", are considered to represent reproductively isolated taxonomic units akin to biological species. Although this interpretation appears most likely, it is fair to state that unambiguous evidence for the status of these lineages as biological species is lacking. This is because planktonic foraminifera do not reproduce in culture, so that cross-mating experiments such as those carried out for cryptic species of diatoms by Amato *et al.* [2] are at present impossible. Because of large differences in substitution rates, it is difficult to devise a universal threshold distance for DNA-based species delineation in the group [24]. However, evidence from existing surveys suggests that most divergences in the analyzed SSU rDNA fragment are not associated with hybridization. The lack of hybridization could be shown particularly well in cases where divergent multiple copies are found in sequences of SSU rDNA, or where additionally also the associated ITS region had been sequenced [12,52]. On the other hand, an exhaustive survey of *Globigerinoides sacculifer*, a closely related species to *G. siphonifera*, revealed the existence of one

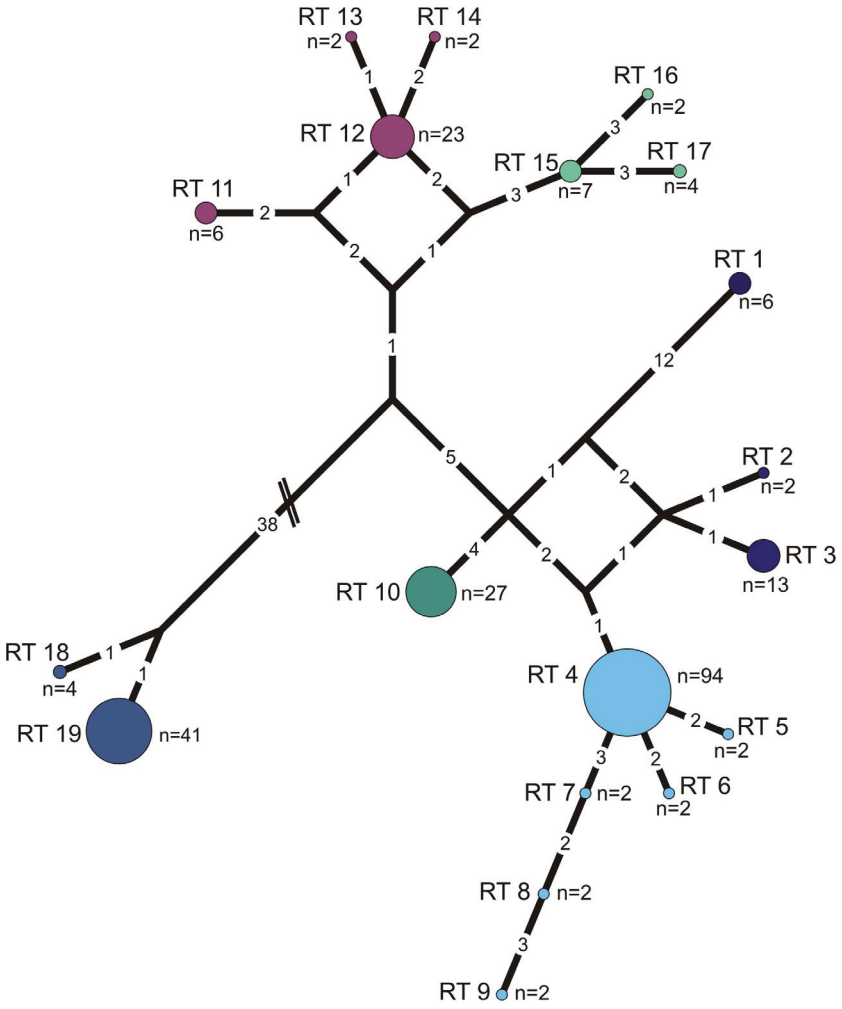

Figure 4. Ribotype network for delineation of genetic types. Ribotype network for *Globigerinella siphonifera* lineage II showing genetic distances and relationships between the 19 ribotypes (RT) and their assignment to different genetic lineages: IIa1 (RT 1), IIa2 (RT 2+3), IIa3 (RT 4–9), IIa4 (RT 10), IIa5 (RT 11–14), IIa6 (RT 15–17) and IIb (RT 18+19), addressed by different colors. Numbers at links indicate the number of mutational events between two ribotypes. n indicates number of individuals representing one ribotype.
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rare divergent SSU sequence motif, which differed by three characters, but was associated with the same ITS sequence as specimens without the SSU motif [8]. Because of this observation and the divergence structure observed in our data (**Figs. 3**, **4**), we assume that the lowest level of genetic variability in *G. siphonifera*, manifested by the 30 SSU ribotypes, may not be associated with reproductive isolation, but represents divergence and rDNA variation within species. Because of the uncertainty in the interpretation of the evolutionary status of the 30 ribotypes, when analyzing the distribution of the 12 genetic lineages, which we consider cryptic species, we cannot be entirely sure that we are not underestimating the number of reproductively isolated lineages. However, since the difference in the distribution and allocation of cryptic diversity is manifested already at the level of the 12 genetic lineages, the conclusions drawn from the lineage-level data must also apply to any unit below these.

Notwithstanding the exact status of the 12 genetic lineages, the first step before analyzing their distribution and allocation is to ask how representative the sampling has been. To this end, the first-order-Jackknifing approach (**Table 1**), which serves as an objective estimate of lineage richness that is to be expected both globally and regionally, shows that the number of lineages in our collection appears to approach the expected total number of lineages, given the assumptions of the test. Similarly, the number of sampled lineages in almost every region falls within the 95% confidence interval of the Jackknifing estimate, implying that further lineages are unlikely to have been discovered in each region by more intensive sampling. Only for the Red Sea does the test indicate the existence of at least one lineage that has not been sampled yet. This analysis confirms the empirical observation that a seven-fold increase in sampling intensity led to a disproportionately low rate of discovery of new variants and that the distribution of the proportion of new variants is scaled with their hierarchical position. Despite the higher lineage diversity than among other planktonic foraminifera species (12 in *G. siphonifera*, compared to 7 in *Neogloboquadrina pachyderma* and *Globigerina bulloides* [4]), the global survey in the "hyperdiverse" *G. siphonifera* confirms, that the total number of cryptic genetic types within morphospecies of planktonic foraminifera is limited and that the biological diversity

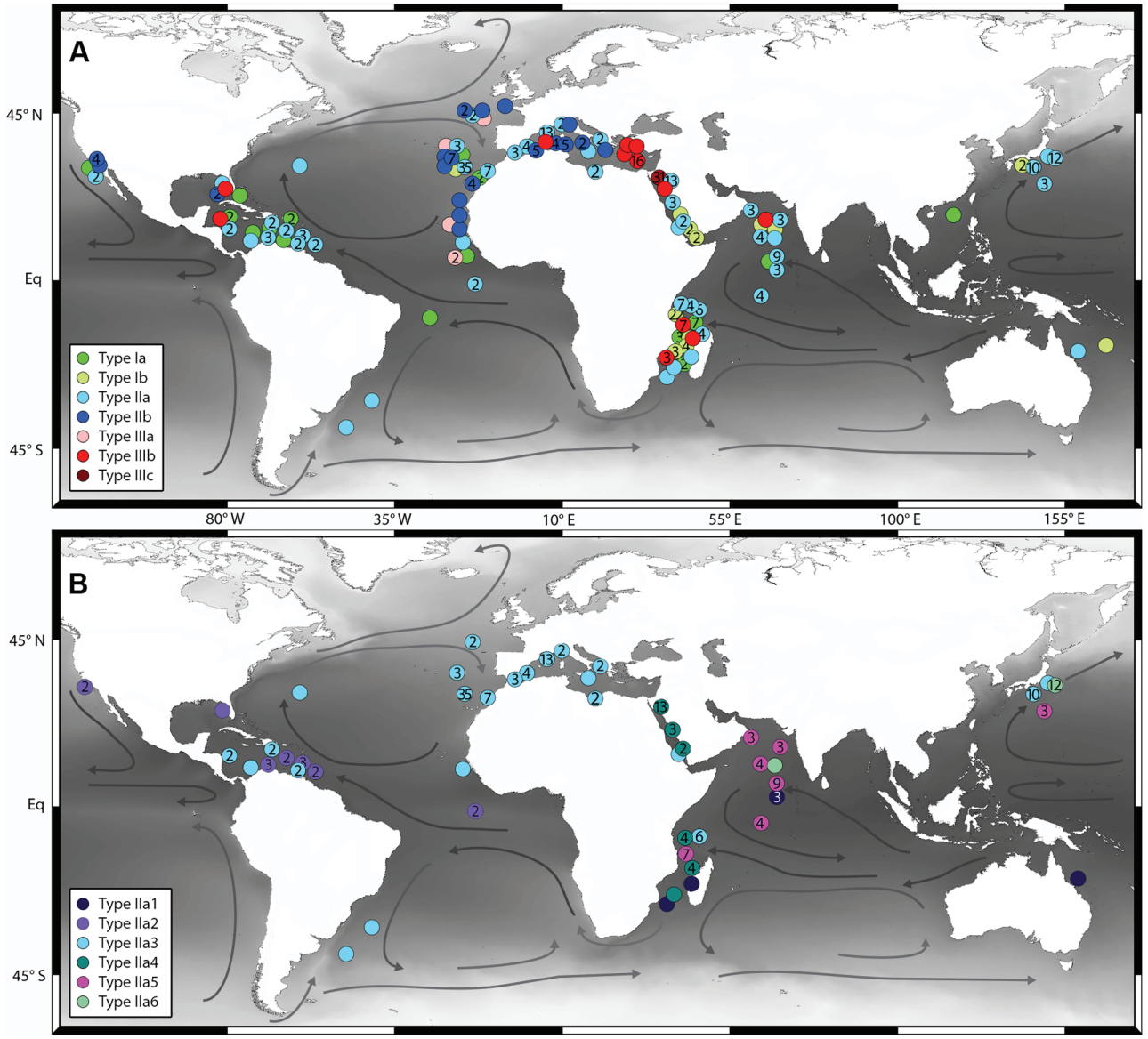

Figure 5. Biogeographic distribution of the genetic types of *Globigerinella siphonifera*. **A**) Geographic distribution of the *G. siphonifera* lineages plotted at their exact sampling locations on a map in Mercator projection. Numbers indicate the amount of individuals of one genetic type found at one station. One year mean sea surface temperature is indicated by gray shading. Arrows indicate main ocean currents. **B**) Geographic distribution of the genetic types of *G. siphonifera* lineage IIa.
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in the group may be underestimated by a factor of about 10, but not significantly more.

Observed (S_o) and estimated (S_e, first-order-Jackknifing) number of genetic types of *Globigerinella siphonifera* for the global and regional data sets. Only in the Red Sea the observed number of types does not fall within the 95% confidence interval (CI_{95}) of the estimate, suggesting the existence of at least one more genetic type in that region.

Having established that the sampling intensity, both globally and regionally, can reasonably be considered sufficient to capture the occurrence pattern of the *G. siphonifera* lineages, we first consider the relationships of these lineages within the phylogenetic tree. Here, a major finding is the uneven distribution of diversification between the three main lineages; with seven types in lineage II and only two and three types in lineage I and III respectively. Since the Jackknifing analysis suggests that our sampling approaches the real diversity in each region, the uneven distribution of types between the lineages is unlikely to be due to systematic undersampling.

The second obvious explanation for uneven allocation of diversity to lineages is their age, with older lineages having more time to accumulate species [53]. To test this hypothesis, we calculated molecular clocks for the diversification of genetic lineages within *G. siphonifera* based on the dating of the split from its sister species *B. digitata* (**Fig. 6**) [40,41]. The ages resulting from both relaxed clock models showed a more realistic distribution than the results of a strict clock model and agree remarkably well with earlier calculations based on entirely independent calibrations

Table 1. Comparison between observed and estimated number of genetic types.

Region	S_o	S_e	CI_{95}	$S_o \in S_e \pm CI_{95}$
Global	12	12.99	1.95	true
Atlantic Ocean	6	6.97	1.90	true
Mediterranean Sea	3	3	0	true
Caribbean Sea	5	5.93	1.83	true
Red Sea	5	7.67	2.61	false
Arabian Sea	6	8.73	2.76	true
Western Indian Ocean	7	7	0	true
Pacific Ocean	8	8.96	1.89	true

Observed (S_o) and estimated (S_e, first-order-Jackknifing) number of genetic types of *Globigerinella siphonifera* for the global and regional data sets. Only in the Red Sea the observed number of types does not fall within the 95% confidence interval (CI_{95}) of the estimate, suggesting the existence of at least one more genetic type in that region.
doi:10.1371/journal.pone.0092148.t001

[23]. The age for the split of the hyperdiverse lineage IIa from lineage IIb is calculated to have taken place ~5 Ma in the early Pliocene. The split between lineage II and III dates to ~7 Ma and the split of lineage I from the rest of the lineages took place ~9 Ma. Thus, as the branching order of the phylogeny alone indicates (**Fig. 2**), the highest number of genetic types is found in the youngest lineage. Based on the molecular clock estimates (**Fig. 6**), this lineage had a two to three times shorter duration than the other lineages. In consequence, lineage longevity is not feasible as an explanation for unbalanced distribution of diversity.

Thus, since the high diversity in lineage II is unlikely to be a result of undersampling and is not correlated with lineage age, we may consider the possibility of it resulting from uneven rates of diversification among the lineages [54]. We test this hypothesis by using a covariates GLM approach that analyzes trait dependency of changes in birth-only speciation rates. The results reveal that speciation rates in lineage IIa must have been significantly higher than in all other lineages within *G. siphonifera*. This result is consistent for the uncorrelated lognormal ($\chi^2 = 4.258$, df = 1, $p = .039$) as well as the exponential ($\chi^2 = 8.232$, df = 1, $p = .004$) molecular clock analysis. Thus, we conclude that increased speciation rate seems most likely to be the cause for the disproportionate accumulation of diversity that occurred in lineage IIa.

The exact factor causing an increase in speciation rate in the hyperdiverse lineage IIa is difficult to reconstruct from the phylogeny alone. However the topology of the median joining network of lineage II (**Fig. 4**) reveals a centripetal distribution of ribotypes, with missing ancestral motifs. Such distribution implies that lineage II diversified by sequential fragmentation of a population of ancestral ribotypes, which was entirely transformed during the fragmentation process. This is interesting because it speaks against speciation by peripheral isolation.

The second clue to the unique status of the hyperdiverse lineage IIa comes from its biogeography. The striking pattern of (Indo-)Pacific isolation within this lineage (Types IIa1, 4–6; **Fig. 5**) has not only consequences for the interpretation of its elevated diversity, but it offers critical evidence to evaluate the biogeography of the cryptic genetic diversity of the constituent morphological species. To this end, we consider the three end-member scenarios explaining restricted distribution in turn (dispersal limitation, differential adaptation or niche incumbency).

First, we argue that the biogeographic distribution of the genetic lineages of *G. siphonifera* (**Fig. 5**) shows that a dispersal limitation does not seem to be the likely factor causing divergence in this taxon. In every one of the three lineages we find at least one type with a cosmopolitan distribution. Even the hyperdiverse lineage IIa contains one type (IIa3) with a global occurrence. If dispersal limitation would be the prevailing factor for speciation, we should expect an accumulation of endemic types in the Atlantic. The connection between the tropical-subtropical Atlantic and Indopacific habitats of *G. siphonifera* (**Fig. 1**) is mediated by the Agulhas current, which transports warm saline water from the Indopacific to the Atlantic [55] and was shown to carry live populations of planktonic foraminifera with it [56]. Therefore, in theory, lineages originating in the Atlantic should be much less likely to be able to escape from there, whereas lineages originating in the Indopacific should be constantly passively transported to the Atlantic due to the absence of a dispersal barrier. Indeed, for some species of marine copepods genetic differentiation and isolation of Atlantic populations due to limited dispersal between ocean basins were shown [57], whereas other species revealed a cosmopolitan distribution with a lack of barriers to gene flow and also showed a connection between the Indian Ocean and the Southern Atlantic [58]. These studies revealed no evidence for a population isolated in the Pacific Ocean and the observed biogeography thus could be considered consistent with passive dispersal.

The similarity of relative abundances of genetic lineages in *Globigerinella* between the different ocean basins analyzed by non-metric multidimensional scaling (**Figure S1**) reveals a close relationship between the Atlantic Ocean with its marginal seas, the Mediterranean and the Caribbean Sea. Also the Arabian Sea and its neighboring region, the Western Indian Ocean, show a high similarity in genetic type occurrence as well as the Red Sea which is affected by inflowing water from the Arabian Sea. The analysis shows the Pacific community to be related similarly to the Atlantic as well as to the Indian Ocean, however there is no close similarity between the Indian Ocean and the Atlantic. This observation is completely contrary to what would be expected if the occurrence of genetic lineages reflected passive dispersal by currents between the Atlantic and the Indian Ocean. Our conclusion that dispersal limitation is unlikely the cause of the observed pattern is in line with widespread evidence for global mixing in tropical populations of other species of planktonic foraminifera [8,11] as well as evidence based on observations in the fossil record [59].

Second, we consider ubiquitous dispersal and differential adaptation. The accumulation of genetic types in the Indopacific could be indicative for differential adaptation of these genetic types to ecological or hydrographical conditions which are only realized in this region. We consider this explanation unlikely, because all of the endemic genetic types co-occurred upon collection in the same samples with genetic types that are cosmopolitan and there was no systematic offset in living depth among any of the genetic types, as evidenced by their occurrence in stratified plankton hauls. Further, types IIa2 and IIa3, which show a wider distribution or even are cosmopolitan, are nested within the clade comprising the endemic types. If there was a specific adaptation associated within the hyperdiverse lineage that limits its occurrence to the Indopacific then two independent evolutionary events are required to have occurred: the character had to evolve at the base of the IIa clade and then be reversed at the base of the IIa2 + IIa3 clade.

Therefore the most likely scenario to explain the distribution of the genetic types in the hyperdiverse lineage is the concept of niche incumbency [5,60]. In this scenario, we assume that the diversification of lineage IIa has taken place in the (Indo-)Pacific by sequential fragmentation of the parent population. Until the

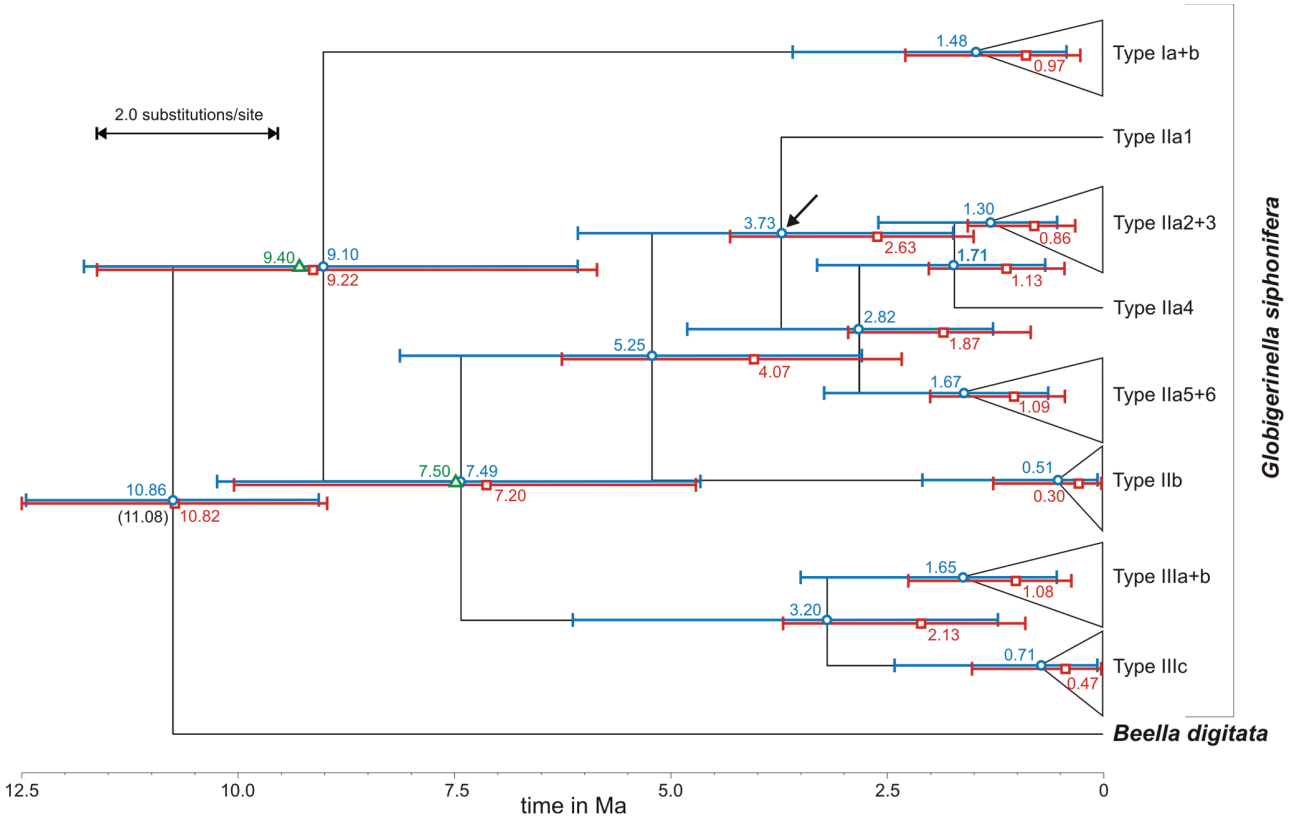

Figure 6. Molecular clock estimates for the evolution of the *Globigerinella siphonifera* lineages. Molecular phylogeny of *G. siphonifera* and *Beella digitata* based on a MAFFT alignment with time estimate ranges from the uncorrelated lognormal (blue) and exponential (red) molecular clocks. Numbers at nodes indicate the divergence ages shown with their 95% confidence intervals. Number in brackets indicates fixed age for the split of *G. siphonifera* and *B. digitata*. Green triangles and numbers show ages calculated in de Vargas *et al.* [23], except for one terminal node which seems too young. Black arrow indicates the starting point from where the presence of a certain trait had a significant effect on the speciation rate, based on a covariates generalized linear model approach.
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divergence of the IIa2 + IIa3 clade, all lineages either remained restricted to the Indopacific or their invasion efforts into the Atlantic ended in extinction. The reason for the failure of most of the genetic types in this lineage to spread into the Atlantic would be incumbency – the niche that these genetic types possess is strongly overlapping with that of an Atlantic incumbent (whichever it may be), preventing the Pacific invaders, carried with the Agulhas current, to establish a viable population in the Atlantic. On a smaller scale, an exclusion pattern may in fact be expressed in the Atlantic between the invasive types IIa2 and IIa3 which represent two closely related sister lineages. The majority of individuals of Type IIa3 were found in the Eastern Atlantic and the Mediterranean Sea, whereas type IIa2 is the dominant type in the western part of the North Atlantic and the Caribbean. Requiring only one evolutionary event (the ability of the IIa2 + IIa3 lineage to invade the Atlantic), the niche incumbency or competitive exclusion thus seems to be a more parsimonious explanation of the distribution pattern of the genetic lineages of *G. siphonifera*.

The unexpectedly high genetic diversity as well as the differentiated distribution of the genetic types in the studied planktonic foraminifera show that occurrence patterns based on morphological species are too coarse to elucidate biogeographic patterns. In agreement with previous studies [11,12], we show that the differentiated pattern of lineage distribution is unlikely to reflect dispersal limitation, but that it also does not simply reflect passive dispersal by ocean currents. Instead, these results confirm that even in marine microplankton high diversification is possible [61] and that interactions and competition between lineages together with historical contingency shape their present-day occurrence and distribution in the world ocean.

Supporting Information

Figure S1 Rendition of similarity of relative abundances of all genetic types of *G. siphonifera* in the sampling regions. In order to statistically assess the geographical structure in the occurrence of genetic lineages of *G. siphonifera*, the sampling sites were separated into seven regions of the world ocean. The similarity of relative abundances of genetic lineages among these regions was visualized using non-metric multidimensional scaling based on the Morisita similarity index [62], as implemented in the PAST software v. 2. 17c [63]. Arrows indicate the direction of surface ocean currents connecting neighboring regions.
(TIF)

Table S1 Information on individual samples and handling procedures. Detailed information on each *G. siphonifera* individual

used in the study (Sheet 1), GenBank samples added to the dataset (Sheet 2) and primer table with all different primers used (Sheet 3). (XLSX)

Table S2 Sequence differences between *G. siphonifera* ribotypes. Table showing the sequence differences and their location in the secondary structure of the SSU rDNA used for differentiation of ribotypes within the three main lineages.
(XLSX)

File S1 Sequence alignments used for phylogenetic reconstructions and delineation of genetic types. MAFFT alignment of sequences of 23 planktonic foraminifera morphospecies including representative sequences of every ribotype of *G. siphonifera* and *B. digitata* from this study (Alignment S1); MAFFT alignment of all *G. siphonifera* sequences used in this study including GenBank sequences (Alignment S2); MAFFT alignment of representative sequences of every ribotype of *G. siphonifera* and *B. digitata* (Alignment S3) and *G. siphonifera* subalignments for each of the three major lineages (Alignment S4-S6).
(ZIP)

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Author Contributions

Conceived and designed the experiments: AKMW MK. Performed the experiments: AKMW AK KFD. Analyzed the data: AKMW MFGW GWG. Contributed reagents/materials/analysis tools: AKMW AK KFD MK. Wrote the paper: AKMW MFGW AK KFD MK GWG.

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7. Fourth case study

Genetic and morphometric evidence for parallel evolution of the *Globigerinella calida* morphotypes

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Own contributions:

Idea/Hypothesis: 70%

Data acquisition: 60%

Interpretation: 60%

Writing of manuscript: 80%

Genetic and morphometric evidence for parallel evolution of the *Globigerinella calida* morphotypes

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Abstract

Molecular genetic investigations of the highly abundant extant planktonic foraminifera plexus *Globigerinella siphonifera*/*Globigerinella calida* have recently shown this group to be the genetically most diverse within planktonic foraminifera, separating it into 12 distinct genetic types. Independently, several morphological or physiological variants have been described within the group, but the correlation between the high genetic diversity and the phenotypic variability remains unclear. In this study we combine genetic data with morphometric analyses of shell shape and porosity of genotyped individuals belonging to the different genetic lineages. Our morphometric measurements suggest a differentiation of three morphotypes within the plexus, two of which possess the elongated chambers described as a typical trait of *G. calida*. These two morphotypes with elongated chambers are associated with two distinct genetic lineages. The *G. calida* morphology therefore appears to have evolved twice in parallel. Unexpectedly, we show that the two morphotypes with elongated chambers can be separated from each other by characters seen in the lateral view of their shells. This implies that the taxonomy of the extant members of the genus *Globigerinella* should be revised. A comparison with the original descriptions of members of the genus shows that two genetic types of one major lineage correspond to *G. calida*. The second group with elongated chambers is associated with one recently diverged genetic type and we propose to reinstate the name *Globigerinella radians* for this distinct form. The remaining nine of the 12 genetic types correspond to the *G. siphonifera* morphology, and in the absence of evidence for morphological differentiation, they form a paraphyletic morpho-taxon. Our results highlight the prevalence of parallelism in the evolution of shell morphology in planktonic foraminifera even at the lowest level of relatedness represented by genetic types.

Keywords: planktonic foraminifera, *Globigerinella*, shell morphology, porosity, taxonomy, evolution

Introduction

Molecular genetic studies of extant planktonic foraminifera continue to challenge our perception on the diversity within the group (e.g. Darling et al., 1999; de Vargas et al., 1999; de Vargas et al., 2002; Aurahs et al., 2009; Seears et al., 2012; Quillévéré et al., 2013). The relatively low number of accepted morphospecies (e.g. Hemleben et al., 1989) is significantly exceeded by the number of their constituent genetic types (e.g. Darling and Wade, 2008). Since most of these genetic types cannot be differentiated morphologically, they are often referred to as "cryptic species" and their discovery usually had no impact on the taxonomy of the morphospecies. Exceptions hereto are *Neogloboquadrina incompta*, which could be separated from *Neogloboquadrina pachyderma* based on genetic data confirming the observation that the two species are associated with different coiling directions (Darling et al., 2006) as well as *Globigerinoides elongatus* that was synonymized with *Globigerinoides ruber*, but recently shown to be genetically as well as morphologically distinct (Aurahs et al., 2011). Morphometric studies on *Orbulina univera*, *Globoconella inflata* and *Globorotalia truncatulinoides* revealed only slight morphological differences between the genetic types that were statistically significant, but did not allow sufficiently precise discrimination of individuals to warrant a taxonomic revision (Morard et al., 2009; Morard et al., 2011; Quillévéré et al., 2013). A study on the morphospecies complex *Globigerinoides sacculifer* surprisingly revealed that also the opposite scenario can exist: a worldwide screening of all morphotypes associated with this taxon showed that this morphospecies is genetically homogenous despite high morphological variability (André et al., 2013). In this case an over-interpretation of morphological characteristics had taken place, which lead to the usage of multiple morphospecies concepts that do not appear justified in the light of the genetic evidence. These examples underline that the connection between genetic and morphologic variability in planktonic foraminifera is complex and the resolution of species delineation requires a detailed combined genetic and morphometric analysis.

The genus *Globigerinella* was first described by Cushman (1927) to include individuals with near-planispirally coiled shells, globular to ovate chambers and fine round spines (Kennett and Srinivasan, 1983). Three extant species can be attributed to this highly diverse and abundant genus. The most abundant species is *Globigerinella siphonifera*, described as *Globigerina siphonifera* by d'Orbigny (1839), with spherical to ovate chambers and a rather tight coiling. *Globigerina aequilateralis* as it was described by Brady (1879) was later declared a junior synonym for the exact same morphology (Banner and Blow, 1960). The second most abundant morphospecies, *Globigerinella calida*, was described as *Globigerina calida* by Parker (1962). It was characterized as having trochospirally coiled evolute shells with radially elongated chambers, the final chamber separated from the previous ones and being perforated by large circular pores (Parker, 1962; Saito et al., 1981). The third morphospecies is *Globigerinella adamsi*, which was originally described as *Hastigerina adamsi* (Banner and Blow, 1959), and is characterized by its elongated digitate chambers with pointed tips. This

species is exceedingly rare. It inhabits mesopelagic waters of the Indopacific low latitude realm (Bé and Tolderlund, 1971) and was never collected for genetic analysis. All three species show a considerable level of intraspecific variability. Parker (1962) was the first to describe a potential separation of *G. siphonifera* into two or even more groups based on shell size and the degree of deviation from the planispiral coiling. She assumed though that these forms represent ecophenotypic plasticity and therefore did not treat her morphotypes taxonomically. Interestingly, the existence of two groups within *G. siphonifera* was later indicated on the basis of biological differences, especially the possession of different endosymbiotic Chrysophycophyte species (Faber et al., 1988; Faber et al., 1989). Later studies suggested a correlation between these groups and the then known two genetic types (Huber et al., 1997), including a potential differentiation between the two types based on shell porosity. In a subsequent study, Bijma et al. (1998) further linked the two groups to differences in cell physiology and shell chemistry However, none of these discoveries had an impact on the taxonomy of the genus.

Genetic studies conducted on the small subunit ribosomal RNA gene (SSU rDNA) of *G. siphonifera* subsequently demonstrated that the high diversity in this morphospecies is not limited to the morphology, but is also represented at the genetic level (Huber et al., 1997; de Vargas et al., 2002; Darling and Wade, 2008; Göker et al., 2010). Most recently, Weiner et al. (2014) showed that the high sequence diversity in the group could be assigned to three major genetic lineages, which further split into 12 distinct genetic types (**Figure 1**). Since no signs of hybridization are found between these genetic types, they may be considered to represent biological species. In these genetic studies, the exact status of *G. calida* remained unclear. The distinction of this morphospecies from *G. siphonifera* is difficult and in many cases the two species were lumped together for studies on fossils from the sediment (e.g. Siccha et al., 2009). The distinction is especially difficult among pre-adult individuals that are often encountered in the plankton. As a result, only a preliminary identification has been presented by genetic studies published to date, in which *G. calida* was suggested to represent one of the genotypes of the *Globigerinella* plexus (Type IV of de Vargas et al., 2002, and *G. calida* in Darling and Wade, 2008).

In order to resolve the relationship between genetic and morphologic variability in the genus, we have taken advantage of the recently developed methods for extraction of DNA from planktonic foraminifera that leave the shells intact for morphometric analysis (Morard et al., 2009; Weiner et al., 2014). Using these methods in combination with the imaging of genotyped specimens prior to DNA extraction, we have amassed a dataset of morphological measurements from 181 individual specimens identified by several researchers as *G. siphonifera* and *G. calida*, sampled within various regions of the world ocean. All of the specimens were genetically analyzed and could be assigned to one of the delineated genetic types. We combined measurements of shell morphology based on scanning electron microscopic as well as light microscopic images with measurements of porosity and pore size. As a result, we were able to resolve the identity of

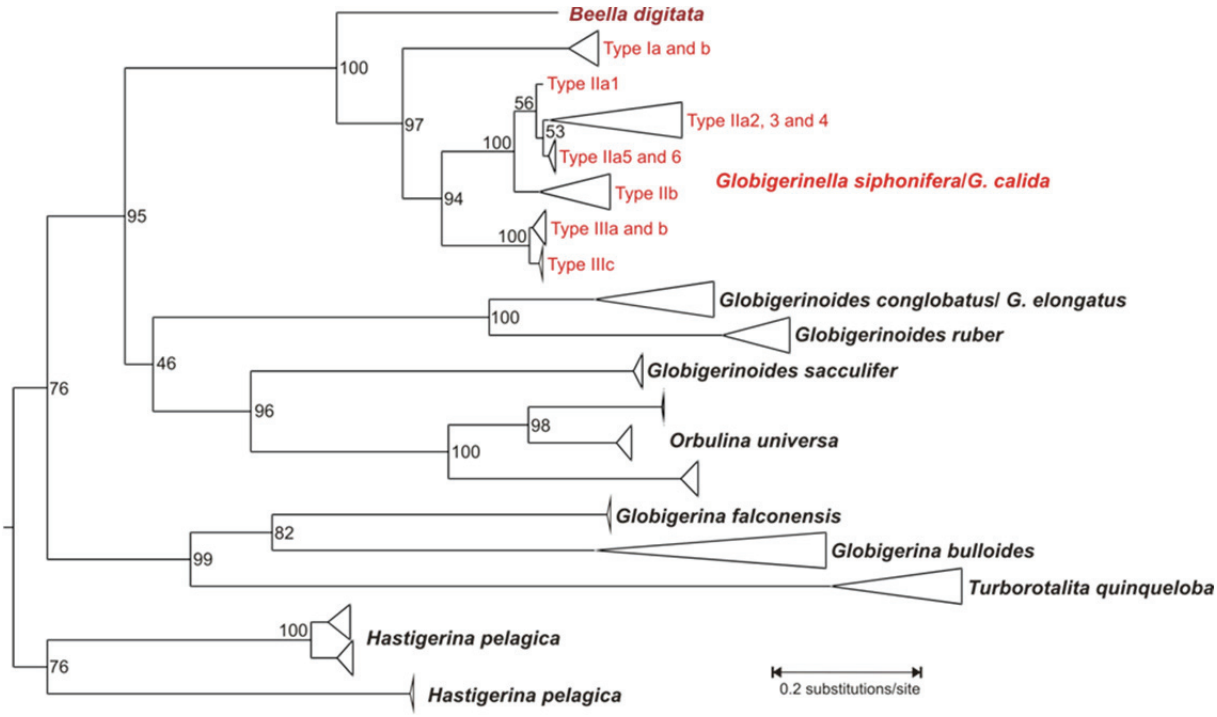


Figure 1: Maximum likelihood phylogenetic tree of the spinose planktonic foraminifera
Phylogenetic relationships among the spinose planktonic foraminifera showing the monophyletic status of *Globigerinella* and its affinity with the sister taxon *Beella digitata* and highlighting its high genetic diversity. The tree as well as the bootstrap values are based on a MAFFT alignment of SSU rDNA sequences and were calculated using RAxML via the CIPRES gateway. Sequence diversity within morphospecies has been collapsed, except for *G. siphonifera*/*G. calida* where only terminal branches are collapsed.

G. calida and revise the taxonomic concept of the *G. siphonifera*/*G. calida* plexus.

Material and Methods

Sampling, imaging and genetic analysis

In this study, images of 181 *Globigerinella siphonifera* and *Globigerinella calida* individuals were analyzed for comparisons of shell morphology with genetic identity. All of the individuals included yielded DNA sequences that could be used to assign them to one of the 12 lineages described by Weiner et al. (2014). The specimens were collected by stratified plankton tows during 13 expeditions between 2006 and 2013 (**Figure 2**, **Table S1**). The foraminifera were separated from the rest of the plankton, taxonomically identified using stereomicroscopes and in most cases digitally photographed directly on board. Living specimens still containing cytoplasm were prepared for DNA extraction. Methods for genetic analysis and the sequence data of most individuals were presented in Weiner et al. (2014). Specifically for this study we genetically characterized 44 additional specimens from a transit through the South Pacific on board RV SONNE (SO226-3, Kucera and Cruise Participants, 2013). These new samples represent topotypic material for the species concept of *G. calida* as developed by Parker (1962). They were obtained by stratified tows using a multiple closing net with a mesh size of 100 µm. Foraminifera were isolated from the plankton residues, cleaned, dried and frozen on cardboard slides until further processing in the lab. The guanidine method,

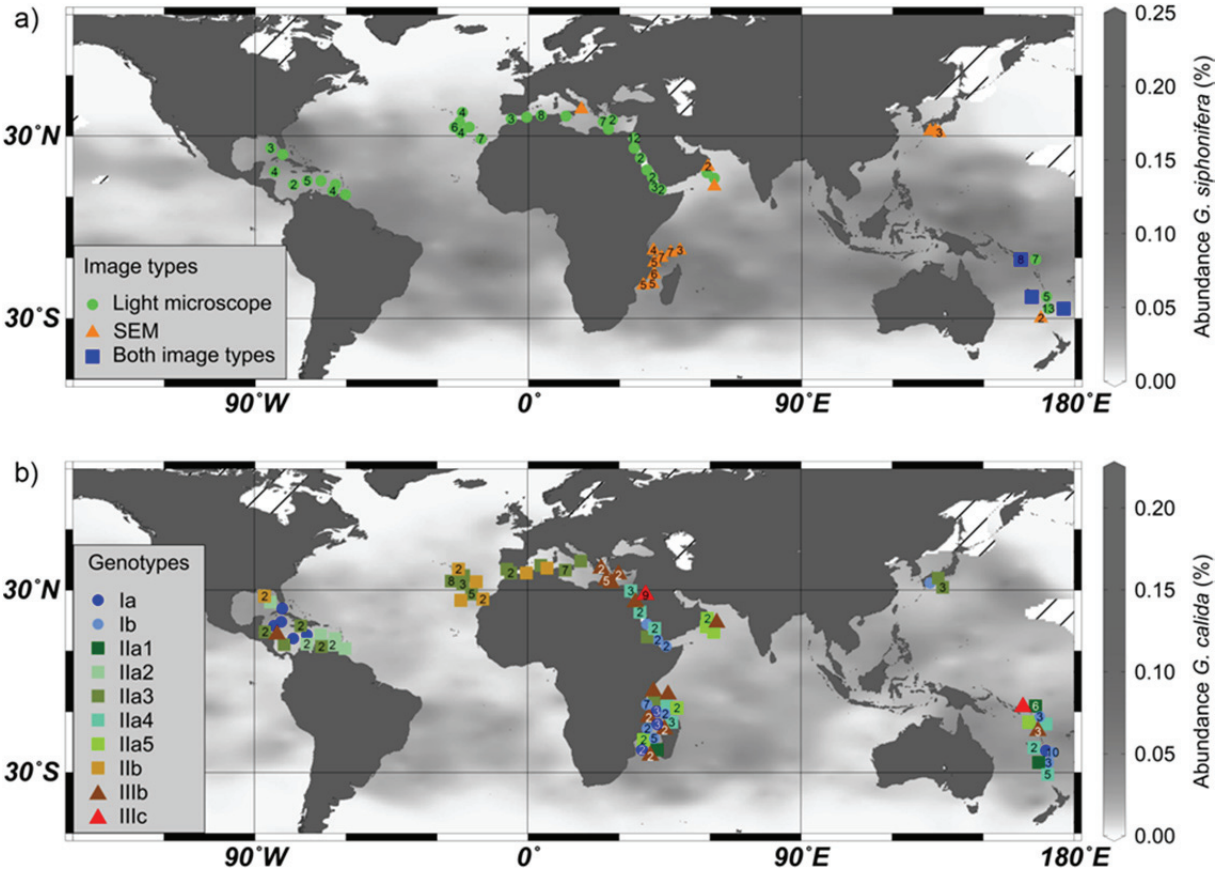


Figure 2: Geographic locations of sampled individuals
a) Sampling locations of all individuals used in the morpho-genetic comparison in this study. Different symbols indicate where only light microscopic images, only SEM images or both were available. Numbers within the symbols denote number of individuals from one sampling location. Gray shading indicates the relative abundance of *Globigerinella siphonifera* as it is found in planktonic foraminiferal assemblages from surface sediments interpolated from data in the MARGO database by Ocean Data View (Schlitzer, 2011). Diagonal lines indicate areas where no data are available.
b) The genetic identity of the analysed individuals. Symbols indicate different genetic types, following the classification by Weiner et al. (2014). Numbers within the symbols denote number of individuals from one sampling location belonging to the same genetic type. Gray shading indicates the relative abundance of *Globigerinella calida* as it is found in planktonic foraminiferal assemblages from surface sediments interpolated from data in the MARGO database by Ocean Data View. Diagonal lines indicate areas where no data are available.

which allows preservation of the shell, was used for DNA extraction (e.g. Morard et al., 2009). Light microscopic images in the standard taxonomic umbilical view were taken in the lab prior to DNA extraction. Polymerase chain reaction (PCR) was used to amplify a ~600 bp large fragment of the 3´end of the small subunit ribosomal RNA gene (SSU rDNA) using the GoTaq® G2 Hot Start polymerase (Promega) and two different primer pairs as indicated in Table S1. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and afterwards sequenced directly by an external service provider (Agowa, Berlin). Sequence chromatograms were checked manually for ambiguous reads and corrected where possible. Sequences of all 44 individuals were submitted to GenBank (http://www.ncbi.nlm.nih.gov/; accession nos: KJ202213 – KJ202256). Shells that could be recovered after DNA extraction

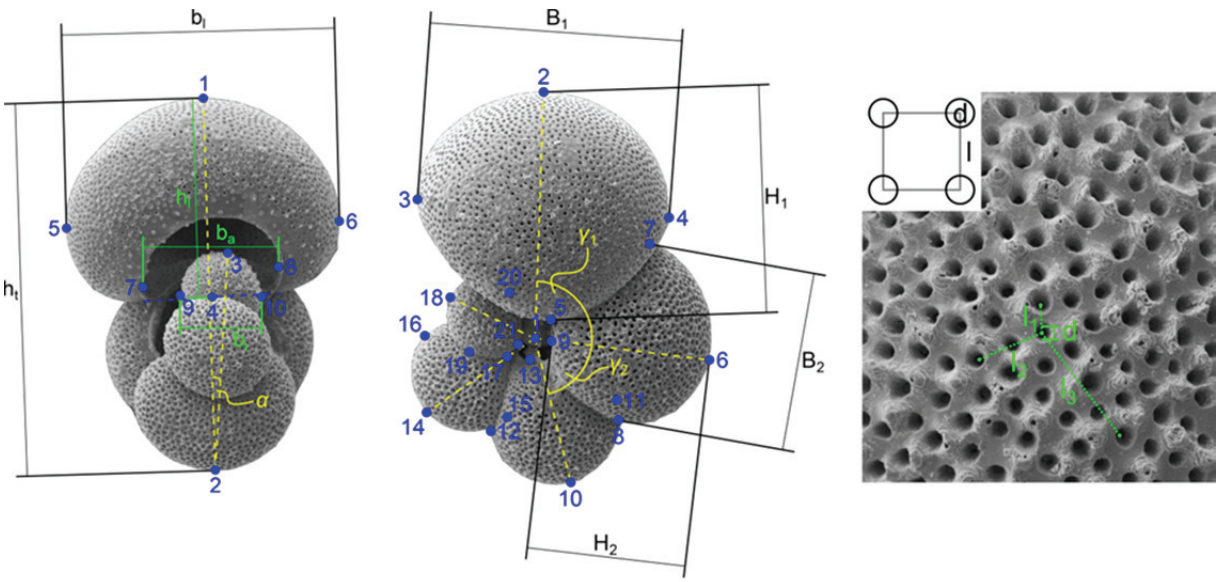
were imaged by scanning electron microscopy (SEM) from spiral/umbilical and lateral view and higher magnification close-ups of chamber wall surface were taken. In total, 37 individuals from the South Pacific yielded images that could be used for morphometric analysis.

ML tree inference and bootstrapping

In order to represent the phylogenetic position of *G. siphonifera*/*G. calida* in relation to the rest of the spinose planktonic foraminifera, sequences of 11 morphospecies were included in an automated alignment using the online version of MAFFT v. 7 (File S1, Katoh and Standley, 2013) as it is available on the CIPRES gateway (Miller et al., 2010), under default settings. This alignment was then used without further manipulation or filtering for tree inference under the maximum likelihood (ML) criterion with RAxML-HPC2 v. 7.6.3 (Stamatakis, 2006) via the CIPRES Gateway. Branch support was established with the fast implementation (Stamatakis et al., 2008, option -x) of nonparametric bootstrapping (BS; Felsenstein, 1985). The number of necessary replicates was determined by automatic bootstopping with the majority-rule tree based criterion (option -#autoMRE). The per-site rate approximation model (Stamatakis, 2006) was used for the fast BS phase followed by a slow final model optimization under the general time reversible model allowing for between-site variation modeled via a gamma distribution (GTR + Γ; option -m GTRCAT). Run parameters were set to infer in one run the best-known ML tree and perform a full BS analysis (option –f a).

Measurements of shell morphology and porosity

SEM images suitable for morphometric analysis were obtained from a total of 63 specimens of the *G. siphonifera*/*G. calida* plexus in lateral and umbilical/spiral view to quantify the main morphological features of the shell which have been used to differentiate species in the plexus. The traits have been quantified as distances and landmark positions (**Figure 3**) extracted from the images in R v. 3.0.1 (R Development Core Team, 2011). In lateral view those measurements include the height h_{total} of the specimen, the elongation of the last chamber (E_l), the deviation of the last whorl from the planispiral plane (expressed as angle α), and the extent to which the first chamber of the last whorl covers the aperture (*PS*). In umbilical/spiral view values comprise the elongation of the last chamber (E_L), the mean elongation of all chambers in the last whorl (E) and the number of chambers in the last whorl expressed as mean angle γ between successive chamber axes. To avoid the effect of unusual terminal morphologies, in Kummerform-specimens, the penultimate chamber was treated as the last chamber. Damaged specimens with fewer than three consecutive chambers in the last whorl preserved were excluded from the analysis. The data acquisition and parameter calculation was replicated, and the values used in the following represent the mean of the two replications to minimize subjectivity during data extraction.



$$h_{total} = \mathrm{h_t}\ [\mu\mathrm{m}] \qquad E = \frac{\sum_{i=1}^{n} \frac{\mathrm{H}_i}{\mathrm{B}_i}}{n} \qquad \gamma = \frac{\sum_{i=1}^{n-1} \gamma_i}{n-1} \qquad \bar{d} = \frac{\sum_{i=1}^{n} d}{n} \qquad P = \frac{\frac{\pi}{4} \times \bar{d}^2}{\bar{l}^2}$$

$$\alpha = \angle 123 \qquad E_L = \frac{\mathrm{H_1}}{\mathrm{B_1}} \qquad \bar{l} = \frac{\sum_{i=1}^{n} l_{\min}}{n}$$

$$E_l = \frac{\mathrm{h_l}}{\mathrm{b_l}}$$

$$PS = \mathrm{b_i} \in \mathrm{b_a}\ [\%]$$

Figure 3: Morphometric measurements conducted on images of *Globigerinella siphonifera*/*G. calida* individuals

Schematic representation of the measurements of shell characteristics and pore size and porosity derived from SEM images, including equations for the calculation of the derived, size-invariant parameters. Blue points represent landmarks, whose coordinates were extracted from the images. Black and green lines show distances used for the calculation of morphological parameters, which were calculated on the basis of the landmarks. Yellow dashed lines are auxiliary lines for the visualization of calculated angles. Distances H_i and B_i, and angles γ_i are only shown exemplarily on the last two chambers in umbilical view. Points 1–13 in umbilical view were also extracted from light microscopic images.

To evaluate the degree of morphological separation obtained on the basis of the exactly positioned clean SEM images, for application in the field, we subsequently tested the approach on imperfectly oriented 128 light-microscopic images in the umbilical/spiral view. In these images we extracted 13 landmark points each (**Figure 3**) to calculate the elongations E_L and E on the basis of the last three chambers, as well as the mean angle γ.

Porosity measurements were obtained using SEM images with a magnification of 4000 of the surfaces of the last chamber of 66 specimens. The images were treated for contrast enhancement and where necessary, pores were manually blackened to enable automatic measurements. The maximum Feret diameter (d) and centroid coordinates of each pore were then extracted from black and white threshold images in FIJI v. 1.47q (Schindelin et al., 2012). These values were then used to calculate the porosity of the specimen (**Figure 3**). This approach yields reliable results as long as pores can be expected not to be significantly oval in first approximation. The maximum pore diameter, in contrast to the directly measured pore area, is invariant to the orientation of the pore, so that the curvature of the shell does not influence the results by distorting the pores in areas

which are not perfectly perpendicular to the plane of view. In this study, we decided not to break the shells to measure the pores from the inside, like Huber et al. (1997). As a result, our values are likely to overestimate pore size by a small amount, especially in large and thick shells.

In order to determine shell porosity, we calculated the distance of each pore to every other pore based on the obtained centroid coordinates in R v. 3.0.1 and then identified the nearest neighbor to each pore. The mean distance *l* of all nearest-neighbor-pair-distances of the specimen was then assumed to be a good approximation of the mean pore distances in that specimen. Assuming a regular pore distribution with one pore at each corner of a square with edge length *l*, we could then approximate the mean porosity *P* of the specimen ($P = (\pi/4 \times d^2)/l^2$). Even if the real pore distribution deviates from this expectation, the fact that we treat all specimens alike, leads to mutually comparable results. In 40 specimens we have taken two SEM images from the same individual, which we could use to test the reproducibility of our results using a paired *t*-test.

Statistical analysis of morphometric measurements

All statistical analyses were performed in R v. 3.0.1. We used principal component analysis (PCA, Hotelling, 1933) to evaluate the continuity of the morphospace in the *G. siphonifera*/*G. calida* plexus on the basis of the morphological parameters (excluding porosity) obtained from the SEM images, without a priori assumption on their attribution to genetic types. During that step we excluded the parameter h_{total} from the analysis, because shell height of specimens from the plankton is a function of their age and does not represent the final size at which reproduction would occur. Next, we explored to what degree specimens of distinct genetic lineages can be distinguished from the rest of the plexus by performing linear discriminant analyses/canonical variate analyses (LDA/CVA, Fisher, 1936) in the R-package MASS v. 7.3-26 (Venables and Ripley, 2002). We then repeated the same steps on the data obtained from light microscopic images.

The porosity data were tested for the influence of genotype and sampling location and their interaction term on porosity and pore size of specimens. To that end the non-parameteric Scheirer–Ray–Hare test (Scheirer et al., 1976) was applied. For all significant factors, pairwise comparisons

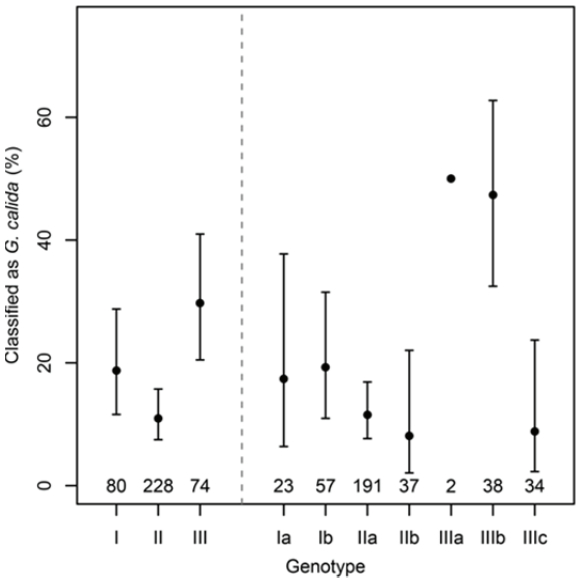


Figure 4: Percentages of individuals classified as *Globigerinella calida*

Percentages of individuals in each genetic lineage/genetic type (following the classification by Weiner et al. (2014)) of *Globigerinella* that were classified upon collection as *G. calida*. The dataset includes all 382 individuals that were genetically analyzed, independent of the existence of morphometric measurements. Vertical bars represent 95% binomial confidence intervals after (Agresti and Coull, 1998). Total number of trials *n* is given at the bottom of the graph. Most individuals classified upon collection as *G. calida* belong to either lineage I or III.

were performed using a Mann–Whitney *U* test (Mann and Whitney, 1947), during which the *p*-values were corrected after Benjamini and Hochberg (1995). To test for a relationship between pore size/porosity and shell size (approximated via shell height, h_t), we performed a Kendall–Theil robust line fitting (Kendall, 1938; Theil, 1950; Sen, 1968) implemented in R, using the equations from Helsel and Hirsch (2002) and Conover (1980). For specimens with two SEM images of the same individual, we used the one which provided a larger dataset (i.e. more pore measurements) for the analysis.

Results

Of the 382 genetically analyzed *Globigerinella* specimens (Weiner et al. (2014) and new data from South Pacific com-

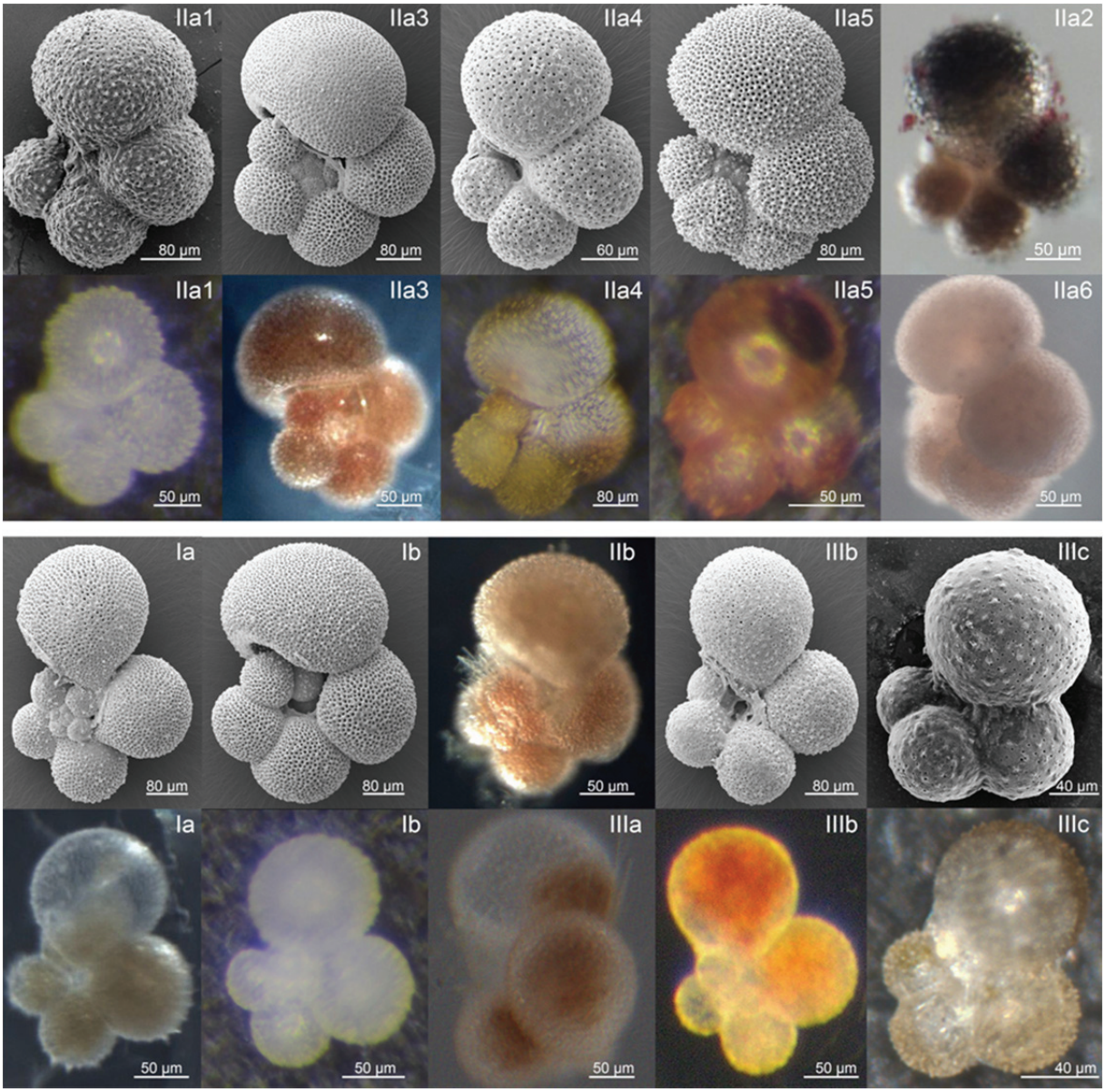


Figure 5: Images of representative specimens of the genetic types of *Globigerinella* sp.
SEM images and light microscopic images of representative individuals belonging to the different genetic types within the *G. siphonifera*/*G. calida* plexus. No SEM images are available for specimens representing types IIa2, IIa6, IIb and IIIa. The exact sampling location of each specimen is shown in Table S1.

bined), 62 were labeled upon collection as *G. calida*. In this respect, the subset used for morphometric analysis is representative, containing 42 specimens out of 181 in total originally labeled as *G. calida*. As the first step, we asked, whether or not the usage of the species name *G. calida* correlated with any of the genetic types. Here, a comparison of the taxonomic labels and genetic identification indeed reveals that the frequency of usage of *G. calida* varies significantly among the genetic lineages and genetic types (**Figure 4**). Although there is no single genetic type which is associated exclusively with specimens labeled as *G. calida*, this name has been used more frequently for specimens in lineages I and III (**Figure 4**).

Next, we ventured to resolve the correlation of genetic and morphological variability in the *G. siphonifera*/*G. calida* plexus. To this end, we first explored morphological differences among all analyzed specimens and determined how these relate to the genetic types found within this group. The high number of SEM and light-microscopic images allowed a morphometric analysis of representatives of almost every genetic type from various parts of the world ocean (**Figure 2, Figure 5, Table S2**). Most genotypes had sufficiently well preserved shells following DNA extraction to obtain representative SEM images, apart from types IIa2, IIa6, IIb and IIIa. However, it was possible to include types IIa2 and IIb in the morphometric analyses using their light-microscopic images, but those of IIa6 and IIIa proved too poor to be useful.

A PCA of the morphometric measurements carried out on SEM images (**Figure 3, Table S2**) revealed a significant size-independent variation in morphology of the individuals belonging to the *G. siphonifera*/*G. calida* plexus. The mapping of the genetic identity onto the morphospace reveals that three of the analyzed genetic types are associated with a morphology that is distinct from the rest of the plexus. The genetic types Ia and IIIb/c appear to be separated from the rest of the genetic types chiefly by higher chamber elongation (E_I, E_L, **Figure 6**). This separation is supported by the LDA, which confirms a statistically significant difference in the multivariate means between the groups ($p > 0.001$) and reveals that based on the same set of morphological measurements, 97% of the specimens can be correctly classified (**Figure 7a**). Furthermore, these three types can not only be separated from the rest, they also show morphologic differences when being compared with each other. Specimens of type Ia are characterized by the highest values for chamber elongation in spiral/umbilical view (E and E_L), while members of lineage III are marked by highest values for angle α, which describes the deviation of growth from the planispiral plane (**Figure 6**). This differentiation is also supported by the LDA ($p = 0.004$), and allows a correct classification of 95% of the specimens (**Figure 7b**).

A CVA with the remainder of genotypes (Ib, IIa1, IIa3–5) shows low correct classification rates (73%) and a general distribution of all genetic types over the whole morphospace, indicating that no distinct morphotypes can be separated within that group (**Figure S1**).

Having established the existence of three groups of genetic types that are morphologically distinct from each other, we attempted to determine whether or not these groups correspond to any of the

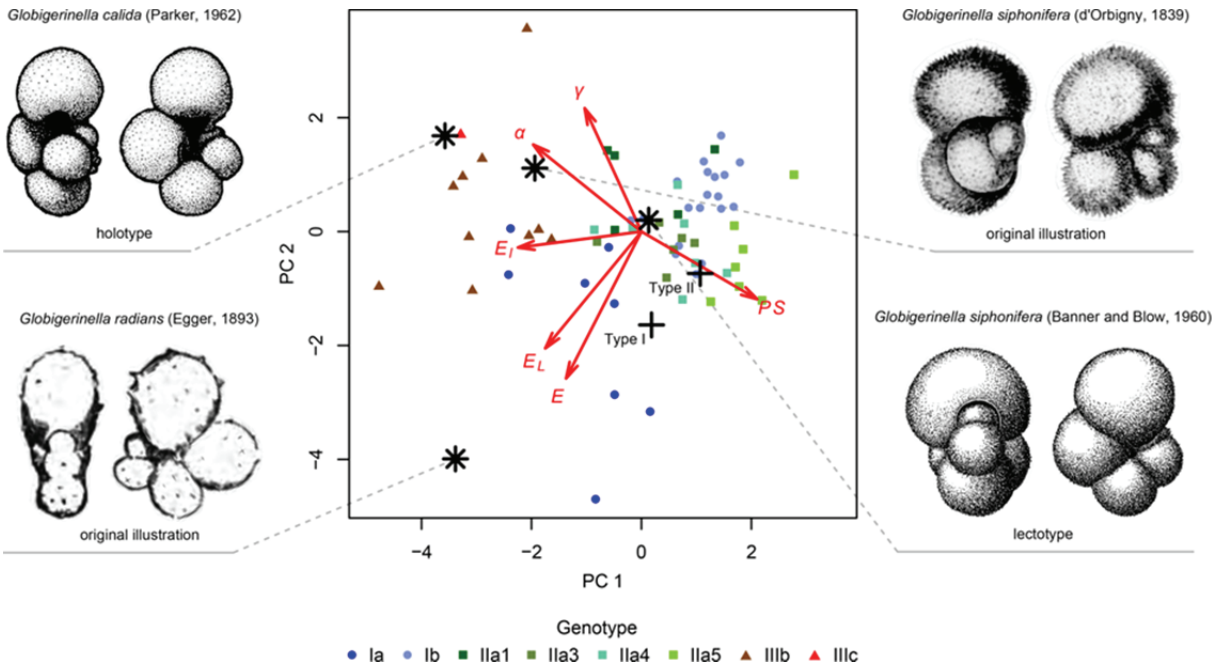


Figure 6: PCA biplot of *Globigerinella siphonifera/G. calida* individuals from SEM images with projected position of type specimens

Principal component analysis (PCA) of six size-invariant morphological characters of the *G. siphonifera/G. calida* plexus obtained from SEM images as described in **Fig. 3**. The plane of the first two principle components explains 70.9 % of the total variance. The projected position of type specimens (measurements obtained from the drawings shown on the side of the graph) are indicated as black stars. The type specimen of *Globigerinella adamsi* plots far outside the plot, because of the extreme elongation of the chambers and is thus not shown. The position of the extant types I and type II as described in Huber et al. (1997, fig. 7) are projected as black crosses. Their type I plots closer to *G. radians*, while their type II is akin to *G. siphonifera*.

existing morphological species concepts. To this end, we extracted images of type specimens from the literature, including original illustrations and designated types. This included the original illustrations of *Globigerina radians (Egger, 1893)*, *Globigerina siphonifera* (d'Orbigny, 1839), the lectotype of *Hastigerina siphonifera* (Banner and Blow, 1960) and the holotype of *Globigerina calida* (Parker, 1962). The same morphological parameters have been extracted from these images as from the genotyped individuals and based on these data the specimens were projected onto the plane of the first two principal component axes (**Figure 6**). This analysis reveals that the holotype of *G. calida* shows the highest similarity in morphology with genotypes IIIb/c. The original illustration of *G. radians* shows a specimen with highly elongated chambers and a small value of *α* as is characteristic for individuals of the genetic type Ia. The rest of the genetic types clusters around the lectotype specimen of *H. siphonifera*.

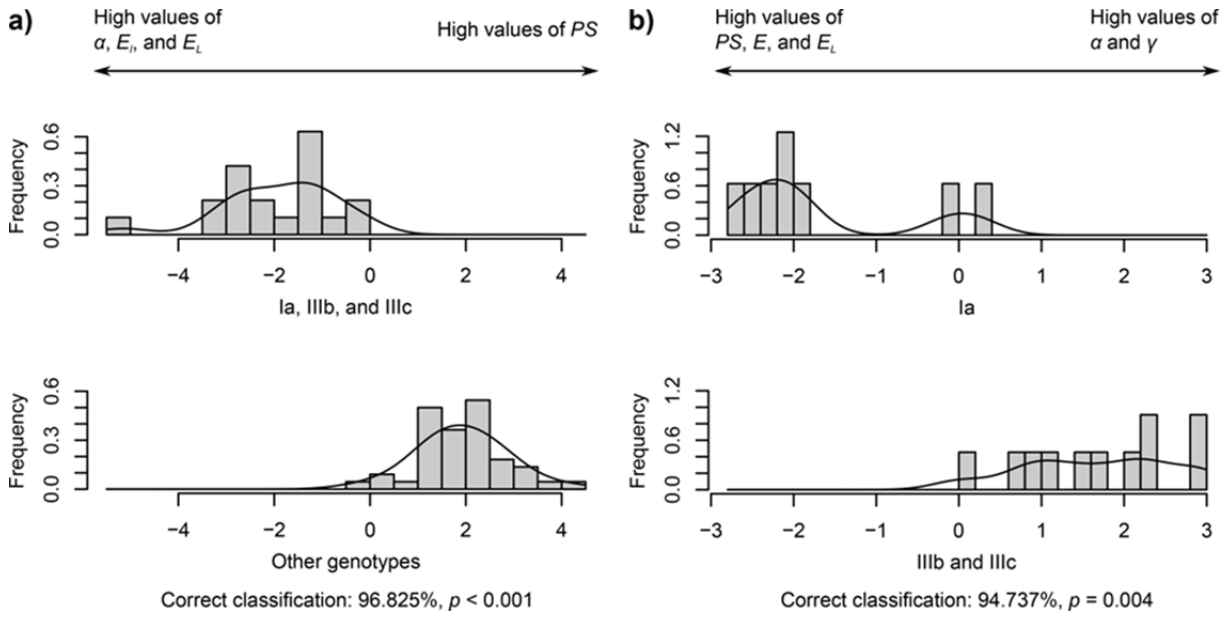


Figure 7: LDA histograms for morphological distinction between selected genotypes of the *Globigerinella siphonifera/G. calida* plexus

a) Histograms of linear discriminant analysis (LDA) between genotypes Ia+IIIb+IIIc and the other genotypes. Genotypes Ia+IIIb+IIIc are characterized by a stronger elongation of the last chamber in both lateral and umbilical/spiral view.

b) Histograms of LDA between genotypes Ia and IIIb+IIIc, showing that genotypes IIIb+IIIc are characterized by the larger value of *α* (inversely correlated with *PS*), i.e. by a more trochospiral coiling and a less equatorial aperture.

In order to determine to what degree the morphological separation is possible without the time-consuming SEM imaging, we subsequently analyzed light microscopic images of 128 genotyped individuals (**Table S2**). Since it is not possible to take images of the lateral view without fixing the specimens, only pictures from the umbilical/spiral side were available. Consequently, the number of morphological variables was limited and characters like the angle *α*, that proved important for the separation into morphological groups, could not be measured. Nevertheless, the PCA analysis of the measurements on the light microscopic images still revealed a separation, with specimens belonging to types Ia, IIIb and IIIc occupying a much smaller portion of the morphospace, albeit with strong overlap to the rest of the genetic types (**Figure 8a**). A further separation of type Ia from IIIb/c, however, is not apparent in this analysis. The morphological trait responsible for the slight separation into two groups is the elongation of the chambers (mainly the last chamber), whereas the number of chambers in the last whorl proves to be variable, but not related to a certain genetic type. This finding supports the results from the SEM analyses and confirms that chamber elongation is the most important distinction factor. In comparison to the analysis based on SEM images, a differentiation into morphological groups solely on the basis of light microscopic images proves to be difficult and the LDA only classifies 78% of individuals correctly, although the difference between the groups remains highly significant at $p < 0.001$ (**Figure 8b**). As implied by the results of the PCA, a further separation between genetic types Ia and IIIb/c by an LDA is not possible ($p = 0.738$).

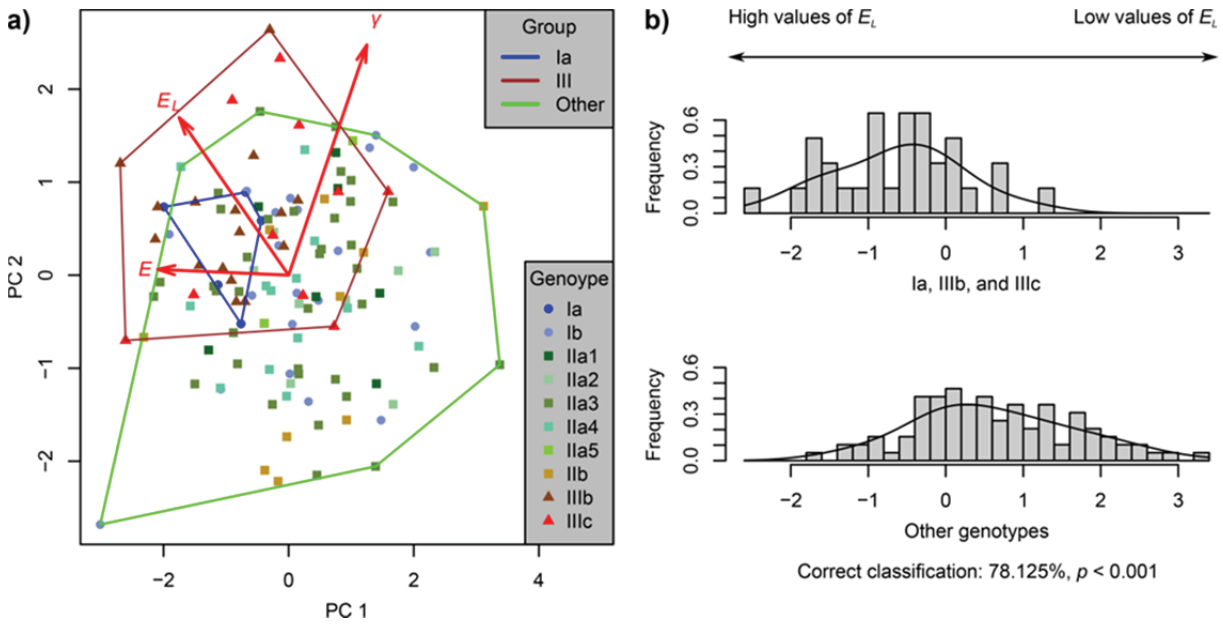


Figure 8: PCA biplot and LDA histograms of morphometric data obtained from light microscopic images of *Globigerinella siphonifera/G. calida* individuals

a) Principal component analysis (PCA) of three size-invariant characters (see Fig. 3) extracted from light microscopic images in umbilical/spiral view. Types Ia and III are situated in one sector of the plot and are mainly distinguished from the other genotypes by a stronger elongation of the last chamber, albeit showing large overlap in morphospace with the other types.

b) Linear discriminant analysis (LDA) histograms for the distinction of genetic types Ia+IIIb+IIIc from the other types on the basis of the three characters extracted from light microscope images. The correct classification rate in the LDA is larger than 78%, and the Hotelling's T2 value indicates a significant morphological difference between the groups. Nevertheless, the large overlap between the histograms confirms the PCA results indicating that distinctions between the genotypes on the basis of umbilical/spiral light microscopic images alone is less reliable than that based on exactly positioned SEM images including the lateral view.

The final characteristic of the calcite shell that might be useful for genotype differentiation is the porosity (**Table S3)**. Since differences in porosity had been detected before for individuals belonging to two different morphological types of *G. siphonifera* (Huber et al., 1997), we analyzed high magnification SEM images of shell wall surface at the last chamber of 66 specimens that had also been used for the morphometric analysis (**Figure 3**). A median of 104 pores were measured per individual. Comparing the mean pore diameter and the mean porosity with the size of the individuals we see a slight trend towards increasing pore parameters with larger shell sizes, when regarding the whole plexus as one group ($p < 0.001$, **Figure 9**). When the different genotypes are regarded as separate entities, however, this trend is only significant in genotypes IIa4 ($p_{(\text{pore size})} = p_{(\text{porosity})} = 0.028$), and IIa5 ($p_{(\text{porosity})} = 0.005$, **Table S3**). In the majority of size classes we detect the whole range of pore size and porosity values. The use of a mathematical approach to calculate the pore parameters of specimens of the *G. siphonifera/G. calida* plexus on the basis of measurements that are widely invariant in regard to viewing angle makes our results reliable, even though we could in rare cases only measure 10 pores/specimen. This is supported by the high degree of replicability of measurements on the same specimen ($n = 40$, paired *t*-test, $p_{(\text{pore size})} = 0.789$, $p_{(\text{porosity})} = 0.912$).

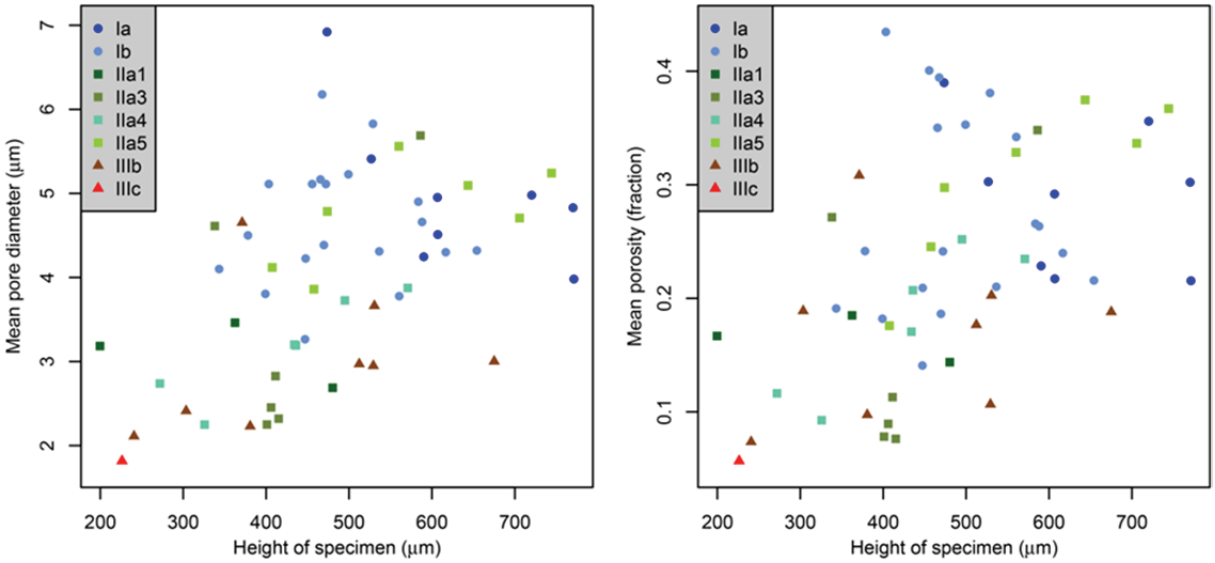


Figure 9: The relationship between pore parameters and shell size in individuals of the *Globigerinella siphonifera/G. calida* plexus

The relationship between pore size and porosity to shell size in the *G. siphonifera/G. calida* plexus. Though there is likely a relationship between shell size and pore parameters (due to the small sample sizes this is only significant in genotypes IIa4 ($p_{(\text{pore size})} = p_{(\text{porosity})} = 0.028$), and IIa5 ($p_{(\text{porosity})} = 0.005$), Kendall–Theil robust line fitting), the graph shows that in the majority of the size range the whole observed range of pore sizes and porosities is realized and the observed variation in these parameters is not merely reflecting shell size.

Testing for possible influences on the pore parameters using a Scheirer–Ray–Hare test, we detected a significant influence of the genetic background of the individuals as well as of the region in which they were sampled, but not of the interaction term of the two factors (**Table 1**). We observe large pore diameters and high porosity in individuals belonging to types Ia and Ib and small pores and low porosity values in the morphologically similar types IIIb and IIIc (**Figure 10, Table S3**). The genetic type cluster IIa is marked by a high variability in pore sizes and porosities within genetic types, with three types (IIa1, IIa3, and IIa4) showing lower values than type IIa5. Comparing the different sampling localities, we detect smaller pore sizes and porosity values in the Pacific and off Japan compared to the Indian Ocean and the Mediterranean Sea. This finding is consistent for all genetic types, which implies

Table 1 Results of a Scheirer–Ray–Hare test for the influence of genotype, sampling region, and their interaction on the pore size and porosity of specimens of the *Globigerinella siphonifera/G. calida* plexus. For a full cross-wise comparison of genotypes and sampling sites (Mann–Whitney *U* test with adjusted *p*-values) see **Table S3**.

Factor	*p*-value (pore size)	*p*-value (porosity)
Genotype	<0.001	<0.001
Region	0.008	0.008
Genotype * Region	0.100	0.053

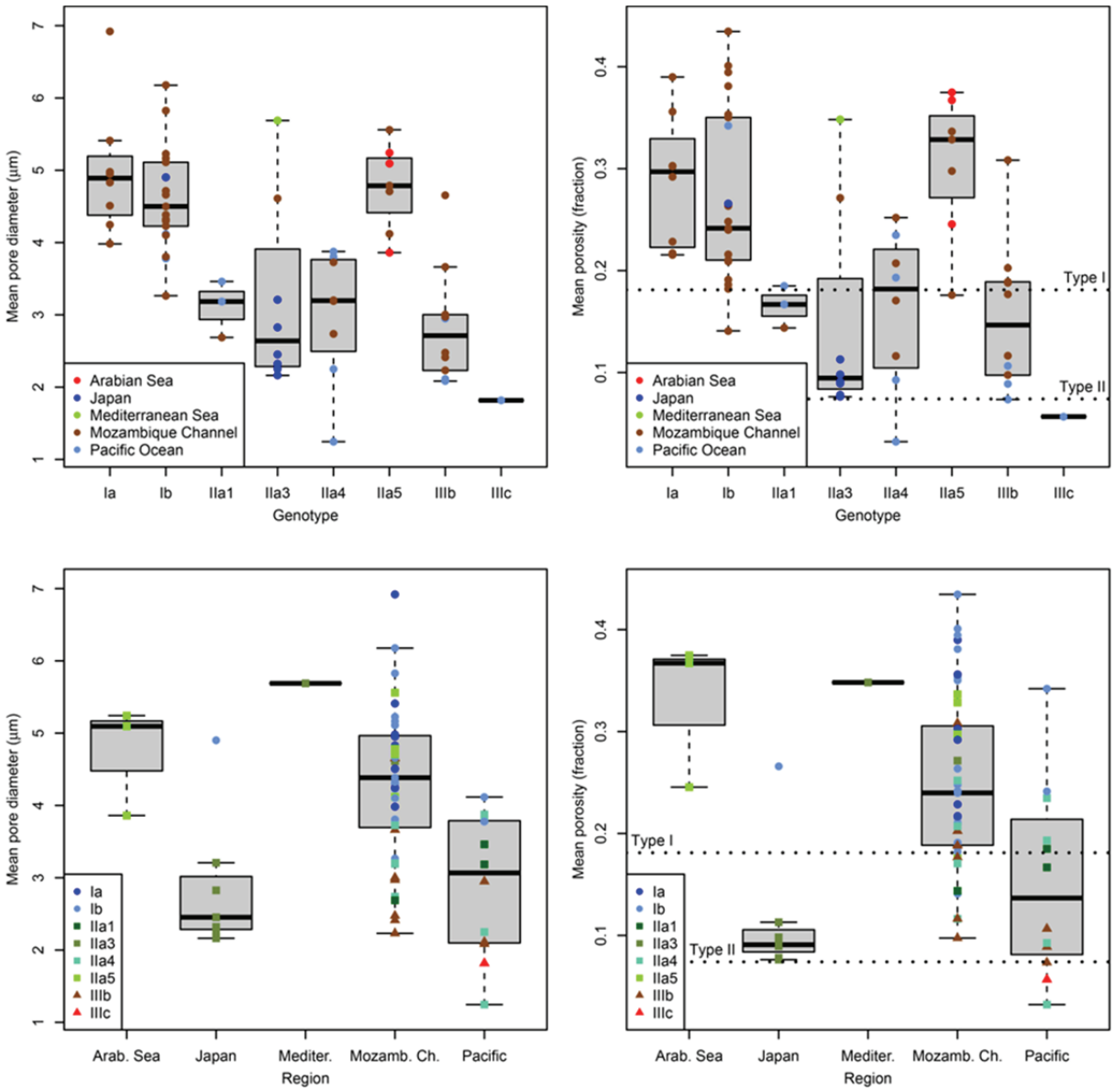


Figure 10: Boxplots for the analysis of influence of genotype and sampling location on the pore parameters in *Globigerinella siphonifera/G. calida* individuals

Boxplots and original data points showing the variability of pore size and porosity within the *G. siphonifera/G. calida* plexus by genotype and sampling location. Both genotype and sampling location have a significant influence on both parameters (**Table 1**). In the porosity plots the porosity values determined by Huber et al. (1997) as being typical for the last chamber of their types I and II are indicated by dotted lines.

that they exhibit the same direction of reaction of the pore parameters to the environmental conditions at a certain sampling locality.

Discussion

Representativeness of sampling

To date, the genus *Globigerinella* appears to be the most genetically diverse within the planktonic foraminifera (de Vargas et al., 2002; Darling and Wade, 2008) (**Figure 1, 5**). However, the amount of genetic diversity is not endless and a Jackknifing analysis presented by Weiner et al. (2014) indicated that the 12 genetic types recorded at that time were likely a comprehensive representation of the genetic diversity within the lineage. In this study, DNA se-

quences were obtained from additional 44 individuals at three stations in the southern Pacific, a region that was not sampled before. Yet, all of these sequences could be assigned to one of the genetic types of Weiner et al. (2014). This fact supports the claim by Weiner et al. (2014) that the number of sampled genetic types is close to saturation both with respect to the addition of more individuals as well as to sampling of new regions. This is important, because it allows us to assume that the image dataset we analyze is representative of the full diversity within the plexus.

Genetic identity of *Globigerinella calida* specimens

Due to the similarity between *G. calida* and *G. siphonifera*, the genetic distinction between the two species remained uncertain. However, an analysis of the original attributions given to each sampled individual included in this study indicated that the *G. calida* morphology, mainly characterized by more elongated chambers (Parker, 1962), is found in several of the delineated genet i c types (**Figures 4 and 5**). This analysis also revealed that the specimens have been labeled as *G. calida* conservatively, i.e., the majority of the specimens belonging to the genetic types associated with the *G. calida* morphology were labeled as *G. siphonifera*. This is interesting considering that the SEM-based morphometric analysis revealed a strong separation of specimens with the general *G. calida* morphology (**Figure 7a**). On the other hand, the analysis based on light microscope images (**Figure 8**) showed a higher degree of overlap between the two groups, indicating that the distinction between the two species is less obvious when working on material in the plankton.

Both, the analysis of the genetic identity of specimens labeled as *G. calida* in the field (**Figures 4**) and the morphometric analysis (**Figure 7a**) indicate that the general morphology of *G. calida* occurs independently in two unrelated lineages. Moreover, specimens belonging to these lineages can clearly be separated from each other morphologically (**Figure 7b**). Since this separation is based on a character that is only visible in the lateral view, a validation on light microscopic images was not possible. Nevertheless, specimens in the field can be observed in lateral views and the character is thus likely to be useful in field studies.

The association of two distinct "*G. calida*" morphologies with two genetically distinct lineages indicates the existence of a taxonomic confusion. Before any attempt to resolve this confusion, it has to be established that the morphological differences do not represent ecophenotypic variants. This possibility can be easily discarded on the basis of our sampling. Specimens belonging to all three morphologically recognizable groups co-occur together at the same stations and depth intervals (**Figure 2, Table S1**). If the characters associated with the broad "*G. calida*" morphology were due to ecophenotypic variability then there should have been no distinction between specimens of the *G. siphonifera* and *G. calida* morphology from the same plankton haul.

Congruence of morphotypes with existing species concepts

To clarify the relation of the three morphotypes to the originally described morphospecies we projected the morphometric values of the type specimens and illustrations onto the PCA plot, revealing a sur-

prisingly high congruence with the three morphologic groups (**Figure 6**). The *G. siphonifera* morphology appears to be most akin to the largest group of genetic types, especially when the lectotype by Banner and Blow (1960) is considered. The lectotype has been selected to represent a more typical specimen than the original drawing by d'Orbigny (1839) and a better congruence of the latter one with our samples is thus not surprising. Consequently, we conclude that most of our genetic types correspond to the current species concept of *G. siphonifera*.

The separation of the two "*G. calida*" morphologies is possible based on characters best seen in the lateral view. Individuals of the genetic types IIIb and c are marked by a higher deviation from planspirality than individuals belonging to type Ia and are therefore closer to the original description of the *G. calida* morphology, which was described as having an umbilical aperture (Parker, 1962). This is supported by the fact that the *G. calida* holotype is projected in the center of the IIIb/c group in the PCA biplot. In order to further test our assumption that the genetic lineage III corresponds to *G. calida*, we used molecular clock estimates to compare the ages of the lineages derived from molecular data to those observed for morphospecies in the fossil record (**Figure 11**, Weiner et al., 2014). The first appearance date (FAD) for *G. calida* in the fossil record lies between 3–4 Ma according to data in the CHRONOS database (http://chronos.org), the FAD for *G. praecalida* dates to 9 Ma. These ages are consistent with the *G. praecalida* morphology representing ancestral populations of

Table 2 The correspondence between genetic diversity and morphological variability within the *Globigerinella siphonifera*/*G. calida* plexus, including classification following classical taxonomy (e.g., Parker, 1962) and the revised taxonomy, based on the morphometric measurements from this study. Question marks stand for genetic types whose morphology could not be confirmed by quantitative analysis, because no suitable images were available.

Genetic type	Revised Taxonomy	Classical Taxonomy
Ia	*G. radians*	*G. calida* or *G. siphonifera*
Ib	*G. siphonifera*	*G. siphonifera*
IIa1	*G. siphonifera*	*G. siphonifera*
IIa2	*G. siphonifera*	*G. siphonifera*
IIa3	*G. siphonifera*	*G. siphonifera*
IIa4	*G. siphonifera*	*G. siphonifera*
IIa5	*G. siphonifera*	*G. siphonifera*
IIa6	*?*	*G. siphonifera*
IIb	*G. siphonifera*	*G. siphonifera*
IIIa	*?*	*G. calida*
IIIb	*G. calida*	*G. calida*
IIIc	*G. calida*	*G. calida*

lineage III, their first appearance marking the divergence between lineage III and lineage II. In this scenario, interestingly, the appearance of the *G. calida* morphology around 3 Ma corresponds to the oldest divergence among the genetic types of lineage III. Importantly, the fossil record is not compatible with the divergence age of lineages Ia and Ib, which is too young. If lineage Ia represented *G. calida*, then that morphology would have to be associated with lineage I until the divergence between genetic types Ia and Ib, when type Ib would have lost its chamber elongation. We consider this scenario less likely, because it would imply that the morphology of genetic type Ib would have to revert back to the ancestral morphology (the ancestral Miocene form of *Globigerinella*, *G. obesa*, does not possess radially elongated chambers). These observations thus further supports the assumption that extant representatives of the genetic lineage III are most affine to the species concept of *G. calida* as it has been applied in the fossil record.

Since the genetic type Ia can also be

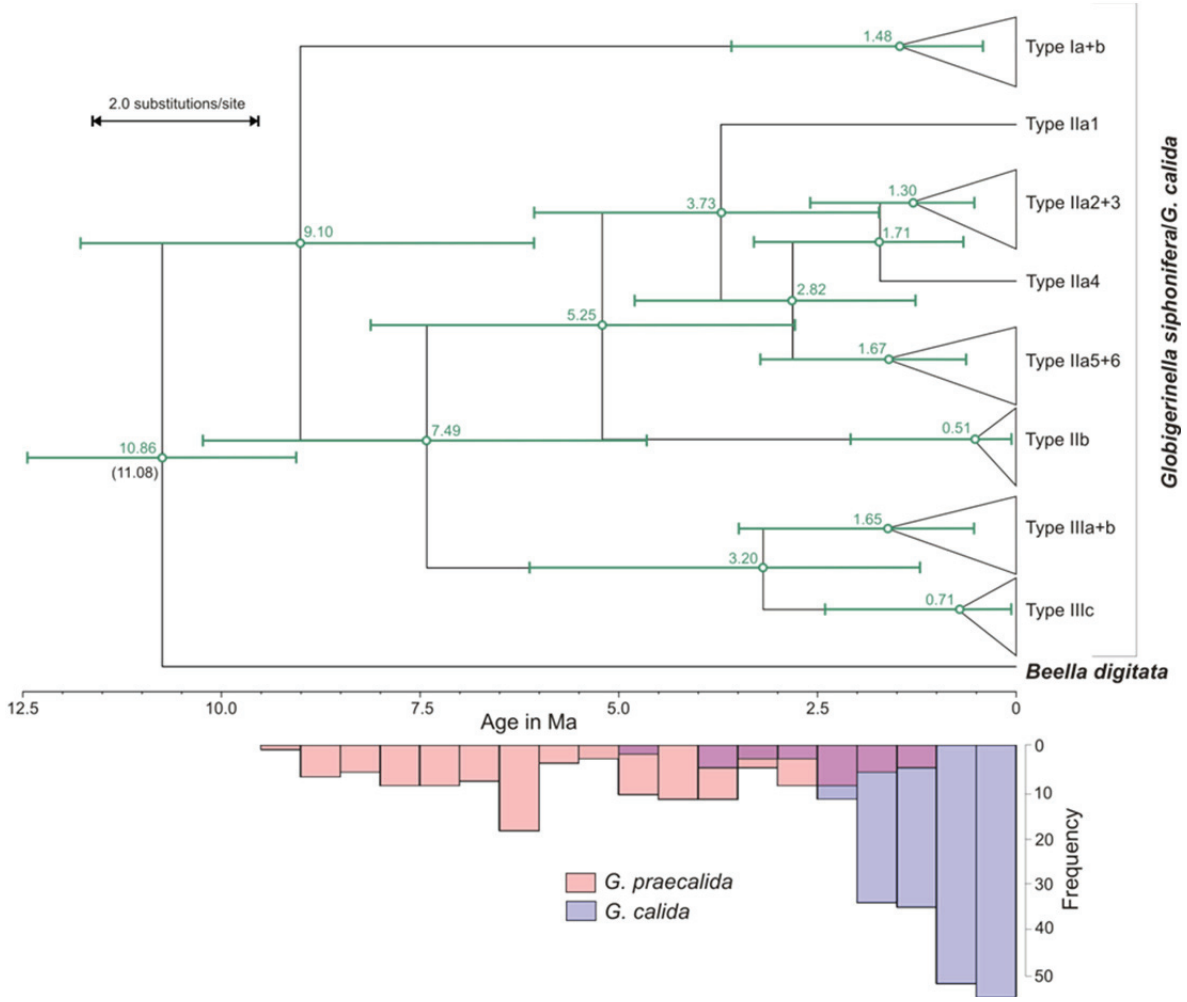


Figure 11: Molecular clock estimates for *Globigerinella siphonifera/G. calida* and their sister species *Beella digitata*

Molecular clock for *G. siphonifera*/*G. calida* and *B. digitata* based on a MAFFT alignment with time estimate ranges from the uncorrelated lognormal relaxed molecular clock, modified after Weiner et al. (2014). For details on the procedures see this previous study. Numbers at nodes indicate divergence ages with 95% confidence intervals. Number in brackets indicates fixed age for the split of *Globigerinella* and *B. digitata*. The histogram shows the occurrence of *G. calida* (blue) and *G. praecalida* (red) as it is recorded in the CHRONOS database (http://chronos.org).

separated morphologically from all other types we investigated whether it is related to some already known morphologic concept. *Globigerinella adamsi* was described as a sister to *G. siphonifera* and *G. calida* (Banner and Blow, 1959) and could be a potential candidate. However, *G. adamsi* is described as having even more elongated chambers (Banner and Blow, 1959; Parker, 1962) and so far it was exclusively found in sediments from the Pacific or Indian Ocean (Bé and Tolderlund, 1971; Hemleben et al., 1989). Since we find our type Ia also in the Caribbean Sea, *G. adamsi* is unlikely to correspond to it.

Searching the literature we discovered with *Globigerina radians* (Egger, 1893) an illustration of a specimen possessing a morphology that closely resembles that of *G. calida* but appears more planispiral. Adding the morphometric parameters of the type illustration to the PCA we see that the illustration corresponds to our specimens of type Ia, characterized by highly elongated chambers and a small value of α. We note that the original description of *G. radians* by Egger (1893) appears indistinguishable from the description of *G. calida* by Parker (1962), but the distinctly planispiral specimen illustrated by Egger (1893) differs from the holotype of *G. calida* (Fig. 6). We therefore propose to reinstate *G. radians* as a name for specimens of genetic type Ia (**Figure 12**).

To compare our morphotypes with the two types that were first described by Faber et al. (1988) and morphometrically analyzed by Huber et al. (1997), we projected the morphological traits of one specimen of type I as well as type II, figured in Huber et al. (1997, fig. 7), into the PCA morphospace of our analysis (**Figure 6**). Thereby we could show that their type II resembles our *G. siphonifera*, whereas their type I is more closely related to our *G. radians*.

The paraphyletic status of *G. siphonifera*

A taxonomic revision of the *G. siphonifera*/*G. calida* plexus is confounded by the fact that the identity of several genetic types cannot be evaluated (**Table 2**). Thus, genetic type IIIa did not yield images of a high enough quality to be included in the morphometric analyses. It can therefore not be entirely excluded that this genetic type is associated with the *G. siphonifera* morphology and not like the rest of lineage III with *G. calida*. Further difficulty arises from the fact that the majority of the genetic types appear morphologically similar. This is most troublesome for type Ib, which cannot be included into the species concept of *G. radians*, because it resembles the *G. siphonifera* morphology otherwise found in specimens of lineage II. Consequently, our taxonomic revision based on shell morphology (**Figure 6**) leads to a paraphyletic *G. siphonifera* including specimens of genetic type Ib and lineage II, which are unrelated, but cannot be separated further from each other (see **Figure S1**). It is entirely possible that traits other than those based on shell morphology will allow a separation of type Ib from *G. siphonifera* and we note that biological or physiological differences (including the possession of symbionts), were shown before to diverge between two different types of *G. siphonifera* (Faber et al., 1988; Faber et al., 1989; Huber et al., 1997).

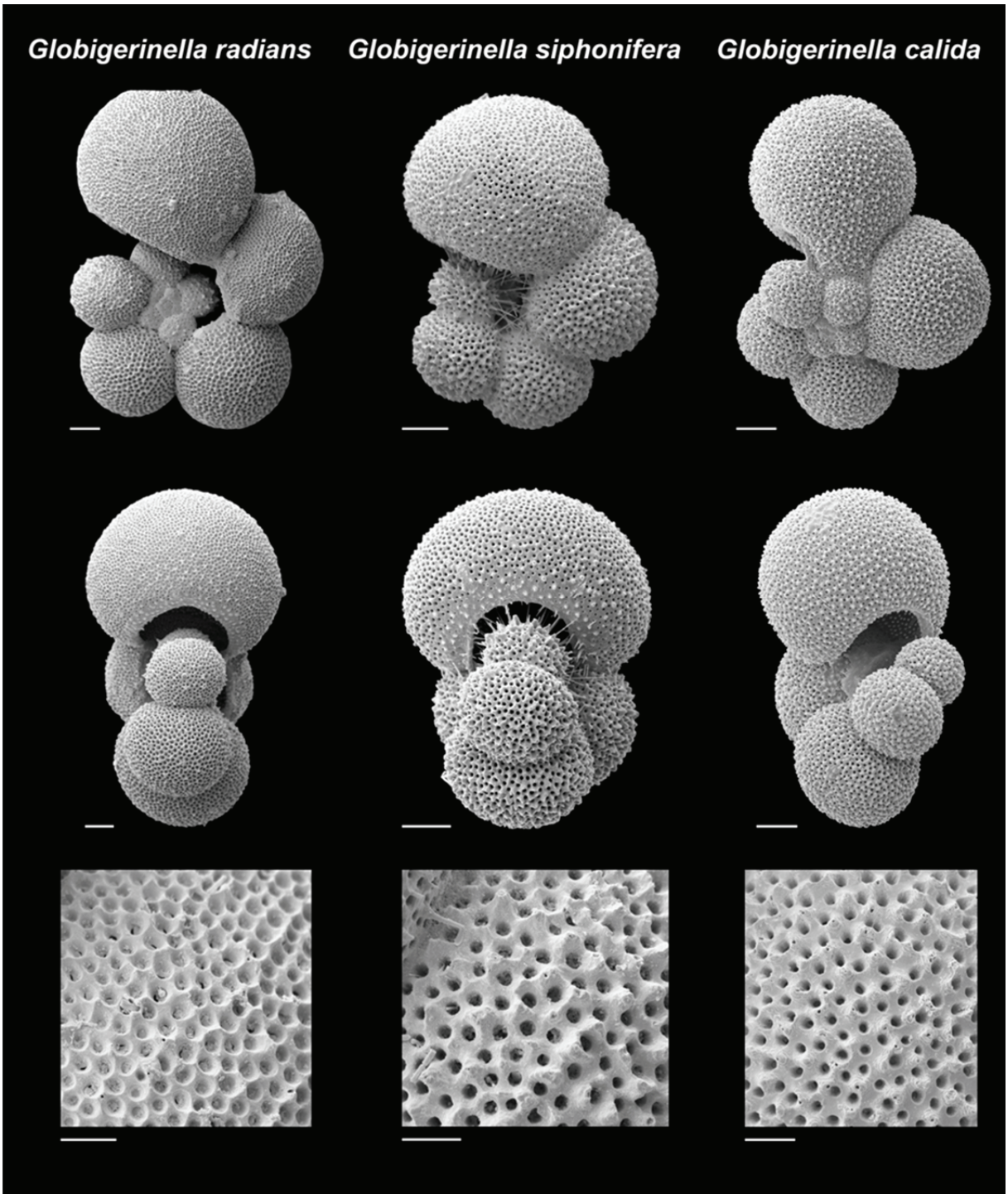


Figure 12: SEM images of the three morphotypes
SEM images of the umbilical/spiral side, apertural side and close-up view of the pores of one individual of each morphotype with their revised taxonomy. Scale bars at pictures with the whole shell are 60 µm, close-ups have a scale bar of 20 µm. The *Globigerinella siphonifera* specimen was sampled in the Arabian Sea (Station 945), *G. radians* and *G. calida* in the Mozambique Channel (stations MC-12 and MC-3 respectively).

In this context, another feature of the shell that was reported to differ between the two different types that were originally described by Faber et al. (1988) is the shell porosity (Huber et al., 1997). Differences in pore size were used to differentiate between the two types for which also a relationship to genetic divergence was suggested. In the present study we use porosity measures as a further possibility to dif-

ferentiate genetic types on a morphological basis and to assess the correlation with the types I and II as described by Faber et al. (1988). Because pores appear to facilitate gas exchange between the cytoplasm and the environment (Hemleben et al., 1989), shell porosity is primarily controlled by body size (Brummer et al., 1987). This is because cytoplasm volume increases with the cube of chamber diameter, but pore area only with the square of chamber diameter. This relationship explains the observed relationship between porosity and shell size in our data (**Figure 9**). However, within the range of shell sizes represented in our dataset, we only detected a minor influence of shell size on pore parameters, explaining a maximum of 23% of the total variation (**Figure 9, Table S3**). Therefore, we conclude that among the studied specimens porosity is not predominantly controlled by the individual ontogeny, and the observed differences require another explanation. Taking other parameters into account, both the genotype and the sampling location have a significant influence on the pore parameters (**Table 1**). The relationship is particularly strong with genotype, with five of the eight analyzed genetic types consistently showing low porosity values (**Figure 10**).

The largest pores and higher porosity is observed in specimens of lineage I. This is consistent with the results by Huber et al. (1997), suggesting that pore parameters could be used to differentiate specimens of genetic lineage I from specimens of lineage II. The values of mean porosity reported for the two genetic types in Huber et al. (1997) are slightly lower than those observed

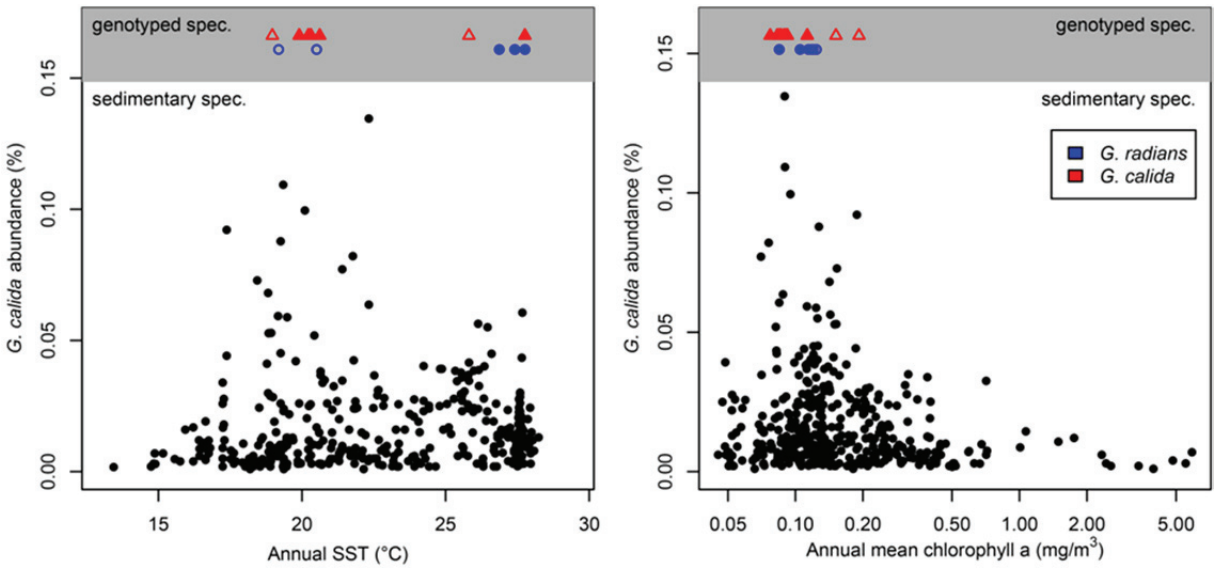


Figure 13: Comparison of environmental preferences of *Globigerinella calida* and *G. radians*
Distribution of the morphotype generally referred to as *G. calida* (Barrows and Juggins, 2005; Kucera et al., 2005) along sea surface temperature (global SST as annual mean and mean of the upper 20 m of the water column from the World Ocean Atlas, Locarnini et al., 2013) and productivity (10-year averaged annual chlorophyll a concentration from Ocean Color Web, Feldman and McClain, 2013) in the Northern Atlantic (including Caribbean) and the Mediterranean Sea. Corresponding SST and chlorophyll a values for the sampling sites of genotyped and morphologically analyzed individuals of *G. calida* and *G. radians* from this study are added at the top of the graphs (filled symbols: genotype and morphotype known, open symbols: only genotype known). Both species appear to show the same preferences for primary productivity as well as water temperature.

among the analyzed specimens (Fig. 10). This offset likely reflects the fact that we measured the pores from the outside instead of breaking the shell to measure from the inside. Unfortunately, we observe that large pores and high porosity also marks specimens of genetic type IIa5 (Fig. 10). This means that the propensity for building disproportionately large and more concentrated pores evolved at least twice in *Globigerinella* and cannot be universally used to differentiate between specimens of lineages I and II. On the other hand, the observation that there is no statistically significant interaction between genotype and sampling region suggests that the ob served differences in pore parameters are an inherent property of the genotypes and are at least partly genetically fixed. The existence of a weaker but significant relationship between pore parameters and locality implies a secondary ecophenotypic effect. This effect is consistent between genotypes (has the same sign), meaning that the pore parameters will remain offset at the same locality and may be used as a rough indicator to distinguish between genotypes I+IIa5 and rest of II+III, even though the threshold value will differ among localities.

Ecological differentiation

In many cases, cryptic species were shown to exhibit a more restricted biogeographical distribution than the morphospecies they belong to (e.g. Aurahs et al., 2009; Weiner et al., 2012). De Vargas et al. (2002) reported a non-random distribution associated with the productivity in the water column for four different genetic lineages of *G. siphonifera*, corresponding to our lineages I, IIa, IIb and III. Therefore, we also tested for a potential correlation between the distribution of the revised morphospecies and water mass characteristics. However, the fact that in many regions all three morphospecies co-occur indicates that there is no difference in their biogeographic distribution. A comparison with data from surface sediment samples as reported in the MARGO database (**Figure 2**, Barrows and Juggins, 2005; Kucera et al., 2005) also shows that *G. siphonifera* and *G. calida* share a common range of occurrence.

To test specifically for a possible affinity to different environmental settings between the two species with elongated chambers, we plotted the localities of all genotyped specimens (Weiner et al., 2014 and this study) of these morphospecies against the annual average temperature and productivity at those localities. By comparing the occurrence of *G. calida* and *G. radians* in the north Atlantic, Mediterranean Sea and Caribbean Sea with extracted sea surface temperature and chlorophyll a data we observe the exact same temperature tolerance of both *G. calida* and *G. radians* (**Figure 13**). Both morphotypes show two abundance peaks, one at a higher temperature and one in colder waters. When comparing those results with the distribution of "*G. calida*" in the sediment according to the MARGO database it appears that this pattern resonates with the occurrence of two abundance maxima in the morphospecies. Therefore, the "*G. calida*" assemblages seem to be a mixture of *G. calida* and *G. radians*.

Parallel evolution of morphological traits

By comparing the morphology of the individuals to their genetic background we were able to support our first impression that morphological divergence only maps partly onto the genetic diversity. We find

elongated chambers in individuals of lineages I and III, leaving only lineage II to completely represent the typical *G. siphonifera* morphology. Thus, unexpectedly, we are confronted with the fact that a similar chamber morphology evolved twice in *Globigerinella* and can be found in individuals belonging to different genetic lineages. The same seems to apply to the evolution of larger pores and higher porosity. This character has likely evolved early (late Miocene) in the evolutionary history of lineage I, but it also must have evolved in parallel in genetic type IIa5, most likely in the Quaternary (**Figure 11**). We suggest that the evolution of elongated chambers in two different genetic lineages is the result of parallel evolution, as it was shown before to have been the case for digitate chamber shapes in various morphospecies of planktonic foraminifera (Coxall et al., 2007). Surprisingly, in the genus *Globigerinella* we show that parallel evolution operates on the lowest taxonomic level, and that it involves not only chamber shape but also the properties of the shell wall (pore parameters).

Conclusions

The morphometric analysis of shell shape and porosity of genotyped individuals of the *Globigerinella siphonifera*/*G. calida* plexus provides evidence for the morphological differentiation of several SSU rDNA genetic types. A detailed morpho-genetic comparison allows us to use this information to revise the taxonomy of the genus. Our analyses show that the genetic types Ia, IIIb and IIIc can be separated from the rest of the altogether 12 genetic types due to their radially elongated chambers and in case of types IIIb/c also because of the deviation from planspirality. Although a separation into three morphologic groups proved to be difficult using light microscopic pictures, the differentiation conducted on SEM images is highly significant. We also discovered a difference in the porosity and pore size values between the different genetic lineages. Our data though show that the pore parameters are influenced not only by the genetic background of the individual but also by environmental factors and that like chamber shape this character also underwent parallel evolution. A comparison of the three morphologic groups with the original descriptions for members of the *Globigerinella* genus reveals that most of our genetic types correspond to the morphology of *G. siphonifera*. The genetic lineage III could be shown to most resemble the true *G. calida* morphology (Parker, 1962), which is also supported by molecular clock estimates, dating the diversification in this lineage to the same age as the FAD of *G. calida* known from the fossil record. For the third morphologic group found within the plexus, we propose the name *Globigerinella radians*, which was attributed to this morphology by Egger (1893) but virtually ignored since. We are aware of the fact that a revision of the taxonomy in *Globigerinella* for now creates a paraphyletic group with genetic types of two different lineages manifesting the *G. siphonifera* morphology, but our data do not show sufficient evidence for a separation of genetic type Ib from the rest of the *G. siphonifera* group. The fact that we observe elongated chambers as well as high porosity in different genetic types shows that in the genus *Globigerinella*, parallel evolution is highly prevalent acting on the lowest taxonomic level.

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Appendix: Systematic appendix

Genus ***Globigerinella*** Cushman 1927

The genus *Globigerinella* as described by Cushman (1927) includes species of planktonic foraminifera with nearly planispiral tests in the adult stage, globular to ovate chambers, umbilical to equatorial aperture and fine round spines (Kennett and Srinivasan, 1983). Three extant morphospecies have been assigned to this genus (e.g., Hemleben et al., 1989): *Globigerinella siphonifera* (d'Orbigny, 1839), *Globigerinella calida* (Parker, 1962) and *Globigerinella adamsi* (Banner and Blow, 1959). In the present study based on genetic and morphometric data, we further include among the extant species *Globigerinella radians* (Egger, 1893).

Globigerinella radians (Egger, 1893)

Text-figure 11

Globigerina radians Egger, 1893, p. 170, plate XIII (figs 22-24)

non *Globigerina radians* – Rhumbler, 1909, p. 148, plate XXIX (figs 2-4) – Parker, 1958, p. 278, plate 5 (fig 10) – Drooger & Kaasschieter, 1958, p. 84, plate 4 (fig 24) plate 5 (fig 6)

Type specimen: none designated; the specimen figured by Egger (1893) on plate XIII, figs 22-24; material of the Gazelle expedition was stored at the "bayerische Staatssammlung für Paläontologie und Geologie", but destroyed during world war 2; type specimen considered to be lost

Type locality: The species is originally described from sediments from the southern Indian and Pacific Ocean collected during Gazelle expedition, localities cited in Egger (1893) are west. Australia St. 87 (20°49 S, 113°46 E, depth 915 m), St. 90 (18°52 S, 116°18 E, depth 357 m); Fiji St. 130 (14°52 S, 175° 32 W, depth 1655 m)

Diagnosis: Individuals of *Globigerinella radians* possess nearly planispirally coiled highly evolute shells with typically five chambers in the last whorl. Chambers in the last whorl are radially elongated ($\overline{E_l} \approx 0.9, \overline{E_L} \approx 1.1, \overline{E} \approx 1.0$) with rounded tips. Aperture is equatorial, forming a high symmetrical arch. The surface of the shell is covered by round spines. The species differs from *Globigerinella calida* by a more planispiral coiling ($\overline{\alpha} \approx 7.9°, \overline{PS} \approx 0.9$), as well as by possessing larger pores and higher shell porosity. Unlike *Globigerinella*

adamsi, *G. radians* never shows pointed chamber tips and the degree of chamber elongation is not as extreme as in that species.

Mean shell height in lateral view: 473–771 µm (mean = 633 µm, *n* = 8)

Observed occurrences in this study (genotyped individuals): Caribbean Sea, Mozambique Channel, southwestern Pacific Ocean

Remarks: The original description of *Globigerinella radians* by Egger (1893) refers to planispirally coiled shells with a loose chamber arrangement and a significant size increase from one chamber to the next as well as a spinose surface. Subsequently, Rhumbler (1909) used the name *Globigerina radians* for a non-spinose foraminifera, although he is referring to Egger's work (1893). Rhumbler's concept was adopted by (Parker, 1958) until it was renamed as *Globigerina atlantisae* by Cifelli and Smith (1970), and later synonymized with *Tenuitella iota* (Hemleben et al., 1989). Drooger and Kaasschieter (1958) used this name for specimens from Caribbean surface sediments corresponding to a morphology that we consider to be *Globigerinella calida.*

Globigerinella calida (Parker, 1962)

Text-figure 11

Globigerina subcretacea – Drooger & Kaasschieter, 1958, p. 84, plate 4 (fig 23) plate 5 (fig 5)

Globigerina radians – Drooger & Kaasschieter, 1958, p. 84, plate 4 (fig 24) plate 5 (fig 6)

Globigerina sp. – Bradshaw,1959, p. 38, plate 6 (figs. 19, 26-28)

Globigerina calida Parker, 1962, p. 221, plate 1 (figs. 9-13, 15)

Globigerinella calida – Saito, Thompson and Breger, 1976, p. 282, plate 1 (fig. 2) plate 6 (fig. 2) plate 8 (fig. 1) – Saito, Thompson and Breger, 1981, p. 32, plate 4 (figs 2a-d) – Kennett and Srinivasan, 1983, p. 240, plate 60 (figs 7-9) – Hemleben et al, 1989, p. 18, figure 2.3 e, f

Type specimen: Holotype USNM no. 638685 (Parker, 1962)

Type locality: The species is originally described from surface sediments from the central southern Pacific Ocean, (14°44 S, 112°06 W, depth 3120 m), Downwind BG 130 (0-4 cm.)

Diagnosis: Individuals of *Globigerinella calida* possess slightly trochospirally coiled evolute shells with typically five chambers in the last whorl. The last whorl is marked by radially elongated chambers ($\overline{E_l} \approx 0.9$, $\overline{E_L} \approx 1.0$, $\overline{E} \approx 1.0$) with rounded tips. The aperture is in an interiomarginal position and cannot be seen from the spiral view. The surface of the shell is marked by round spines. The general appearance is similar to *Globigerinella radians*, however, the chambers are less elongated in side view, the species has smaller pores and less shell porosity and shows a clear deviation from planispirality ($\overline{\alpha} \approx 22.1°$, $\overline{PS} \approx 0.5$).

Mean shell height in lateral view: 226–675 µm (mean = 417 µm, *n* = 11)

Observed occurrences in this study (genotyped individuals): Caribbean Sea, Eastern Mediterranean Sea, Red Sea, Arabian Sea, Mozambique Channel, middle-western Pacific Ocean

Remarks: The original description of *Globigerinella calida* by Parker (1962) refers to trochospiral shells with rapidly enlarging chambers and the last chambers being elongated radially. Her specimens have ~5 chambers in the final whorl and the apertures are approaching an interiomarginal position. She differentiates *G. calida* from *G. siphonifera* by having less involute chambers and less spines. Parker (1962) synonymizes her description with *Globigerina* sp. described by Bradshaw (1959), however she neither refers to the *Globigerina radians* described in Drooger & Kaasschieter (1958) nor to the *Globigerina subcretacea* from the same authors, which both describe the same morphology as *G. calida*. Parker's description of the species still remains valid until today, however in Saito, Thompson and Breger (1976) the species was assigned to the genus *Globigerinella*.

Globigerinella siphonifera (d'Orbigny, 1839)

Text-figure 11

Globigerina siphonifera d'Orbigny, 1839, p. 83, plate 4 (figs 15-18)

Cassidulina globulosa – Egger, 1857, p. 296, plate 11 (fig 4)

Globigerina aequilateralis – Brady, 1879, p. 285 – Brady, 1884, p. 605, plate 80 (figs 18-21) – Egger, 1893, p. 172, plate XIII (figs 5-8)

Globigerinella aequilateralis – Cushman, 1927, p. 87 – Bradshaw, 1959, p. 38, plate 7 (figs 1, 2) – Cifelli and Smith, 1970, p. 35, plate 4 (figs 2-4) – Walker and Vilks, 1973, p. 196, plate 1 (figs 6, 7) – Saito, Thompson and Breger, 1976, p. 281, plate 3 (figs 1, 2) plate 6 (fig 7) plate 8 (figs 3, 8) – Saito, Thompson and Breger, 1981, p. 26, plate 2 (figs 2a-d) – Kennett and Srinivasan, 1983, p. 238, plate 59 (fig 1) plate 60 (figs 4-6)

Hastigerina aequilateralis – Bolli, Loeblich and Tappan, 1957, p. 29, plate 3 (fig 4)

Hastigerina siphonifera – Banner and Blow, 1960, p. 22, figures 2, 3

Globigerinella siphonifera – Parker, 1962, p. 228, plate 2 (figs 22-28) – Hemleben et al, 1989, p. 18, figure 2.3 i, k

Type specimen: Lectotype: Alcide d'Orbigny collection at the Muséum Nationale de l'Histoire Naturelle, Paris, designated by Banner and Blow (1960)

Type locality: The species is originally described from recent beach sand on Cuba (Banner and Blow, 1960)

Diagnosis: Individuals of *Globigerinella siphonifera* possess in adult stages nearly planispirally coiled involute shells with typically five chambers in the last whorl. Chambers in the last whorl are globular or ovoid ($\overline{E_l} \approx 0.7$, $\overline{E_L} \approx 0.9$, $\overline{E} \approx 0.9$). Aperture is equatorial, forming a high symmetrical arch. The surface of the shell is covered with round spines. The species differs from *G. radians* and *G. calida* by less elongated chambers especially in lateral view. In contrast to *G. calida*, *G. siphonifera* does not show a strong deviation of the growth axis from the planispiral plane ($\overline{\alpha} \approx 8.0°$, $\overline{PS} \approx 1.0$).

Mean shell height in lateral view: 200–744 µm (mean = 476 µm, *n* = 44)

Observed occurrences in this study (genotyped individuals): Caribbean Sea, middle-eastern Atlantic Ocean, Mediterranean Sea, Red Sea, Arabian Sea, Mozambique Channel, western Pacific Ocean

Remarks: The original description of *Globigerinella siphonifera* by d'Orbigny (1839) refers to planispirally coiled shells with globular chambers and many spines. His specimens have ~5 chambers in the last whorl and the aperture is elongate. The species was later renamed in *Globigerina aequilateralis* by Brady (1879), however, this name was declared a junior synonym by Banner and Blow (1960). These authors though attributed the species to the genus *Hastigerina*, which was changed again by Parker (1962), who referred to the species as *Globigerinella siphonifera*, the name which is still valid today.

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Electronic Supplements

Table S1: Information on individual samples and handling procedures.

Detailed information on each sampled individual that yielded both genetic and morphometric data, including data on handling procedures.

File S1: Sequence alignment used for phylogenetic reconstruction.

MAFFT sequence alignment used for the phylogenetic reconstruction, including sequences of 11 planktonic foraminifera morphospecies.

Table S2: Morphological parameters extracted from images

Morphology traits extracted from SEM images (averaged over replication) and light microscopic images, including genotype of the specimens used in our analyses.

Figure S1: Canonical variate analysis of *G. siphonifera*

Scatterplot of the CVA within genotypes Ib, IIa1, and IIa3–5.

Table S3: Porosity measurements and analysis

Porosity data (full dataset per individual and average per individual) used in our analyses, plus results of a pairwise comparison of pore size and porosity per genotype/sampling locality and of a Kendall–Theil robust line fitting between shell size and pore size/porosity.

File S2: R PCA functions

R script to project independently gathered data into the PCA morphospace of our analyses.

8. Concluding remarks and Perspectives

8.1. Implications of the results

The case studies presented in this thesis support the hypothesis that cryptic diversity within planktonic foraminifera morphospecies is a prevalent pattern (e.g. Darling & Wade 2008). However, they also show that it is not pervasive in the entire group of planktonic foraminifera and that it does not have unlimited dimensions. This overall assumption on the biodiversity of planktonic foraminifera is now possible, given that more morphospecies have been subject to detailed genetic analysis and by now, large collections of samples from around the world have been accumulated, which detect even geographically restricted cryptic species.

The study conducted on the enigmatic species *Hastigerina pelagica* (Chapter 4) is the first detailed investigation of a member of the Hastigerinidae. By screening the morphospecies for cryptic diversity though, the already known number of SSU rDNA genetic motifs was supported (Aurahs *et al.* 2009a). Although, sampling effort was largely enhanced (also to regions that have not been sampled before), no more than these three sibling species were discovered (Table 8.1). This result is in contrast to the high genetic diversity that might have been expected for the morphospecies from its global distribution, but low abundances in the ocean. The finding of complete genetic homogeneity of the SSU rDNA in the *Globigerinoides sacculifer* plexus was also rather unexpected (Chapter 5). Due to its high morphological variability that led to the differentiation of four morphospecies (e.g. Kennett & Srinivasan 1983), an at least equally high genetic divergence was expected. However, for the first time in planktonic foraminifera, and despite sampling a wide area of the distribution range of the morphospecies, only a unique SSU rDNA sequence motif was discovered (Table 8.1) that had already been described before (Darling *et al.* 1996a; Darling *et al.* 1997; de Vargas *et al.* 1997).

The number of genetically analyzed morphospecies was further enhanced by sequencing individuals of the morphospecies *Beella digitata* (Chapter 6). However, due to the small dataset, so far no concluding assessment of its genetic diversity could be performed. The genetic diversity of the morphospecies plexus *Globigerinella siphonifera*/*Globigerinella calida*, in contrast, could be studied in detail due to the large dataset available (Chapter 6). This group has long been known to be genetically extremely diverse (e.g. de Vargas *et al.* 2002; Darling & Wade 2008), however, due to the only slight sequence differences between some cryptic species the genetic diversity was never entirely resolved. A seven fold increase in sequencing effort and nearly global sampling now allowed elucidating the exact amount of genetic diversity existing in the group. Although the total number now sums up to 12 cryptic species in the genus *Globigerinella* (Table 8.1), most interestingly, this number was here only raised by two that had not been described before.

Table 8.1: Summary of all morphospecies of the five different morphogroups that have been analyzed genetically so far, including the number of contained cryptic species and the associated publications. Morphospecies for which too few sequences were obtained to screen for cryptic diversity are marked by "not yet determined". Bold print indicates species that were studied as part of this thesis and the newly described sequences/cryptic species. The genus *Globigerinella* is separated into the three newly declared species according to Chapter 7 of this thesis. Except for two, all of the cryptic species of this genus were known before. Publications: (1) Darling *et al.* 1997, (2) de Vargas *et al.* 1997, (3) Darling *et al.* 1999, (4) Darling *et al.* 2000, (5) Darling *et al.* 2003, (6) Darling *et al.* 2007, (7) Morard *et al.* 2013, (8) Atsushi Kurasawa, personal communication, April 2014, (9) Stewart *et al.* 2001, (10) Aurahs *et al.* 2009a, (11) Aurahs *et al.* 2011, (12) Wade *et al.* 1996, (13) Aurahs *et al.* 2009b, (14) Kuroyanagi *et al.* 2008, (15) Seears *et al.* 2012, (16) Darling *et al.* 1996a, (17) de Vargas *et al.* 1999, (18) Morard *et al.* 2009, (19) de Vargas *et al.* 2002, (20) Darling & Wade 2008, (21) Schweizer *et al.* 2008, (22) de Vargas *et al.* 2001, (23) Quillévéré *et al.* 2013, (24) Morard *et al.* 2011, (25) Darling *et al.* 2004, (26) Ujiié *et al.* 2012, (27) Darling *et al.* 2009, (28) Ujiié *et al.* 2008.

Morphospecies	Cryptic species	Reference
Globigerina bulloides	12	1-8
Globigerina falconensis	not yet determined	9, 10
Globigerinoides conglobatus	1	1, 2, 11
Globigerinoides elongatus	3	11
Globigerinoides ruber	1	2, 11-13
Globigerinoides sp. (ruber white)	4	13-15
Globigerinoides sacculifer	1	1, 2, 16, **this study (Chapter 5)**
Orbulina universa	3	1-3, 12, 17, 18
Beella digitata	**not yet determined**	**this study (Chapter 6)**
Globigerinella siphonifera	9	1-3, 10, 12, 15, 19, 20, **this study (Chapters 6 + 7)**
Globigerinella calida	**2**	**this study (Chapters 6 + 7)**
Globigerinella radians	1	2, 19, **this study (Chapter 7)**
Turborotalita quinqueloba	6	4, 5, 9, 15
Globoturborotalita rubescens	not yet determined	15
Globorotalia hirsuta	not yet determined	2, 10, 21
Globorotalia menardii	not yet determined	1, 2, 15
Globorotalia scitula	not yet determined	15
Globorotalia truncatulinoides	5	10, 22, 23
Globorotalia ungulata	not yet determined	15
Globorotalia inflata	2	2, 10, 21, 24
Neogloboquadrina dutertrei	3	2, 5, 12, 21
Neogloboquadrina incompta	2	4, 5, 10
Neogloboquadrina pachyderma	7	4, 6, 25
Pulleniatina obliquiloculata	3	5, 15, 26
Globigerinita glutinata	not yet determined	2-4, 10, 15
Globigerinita uvula	not yet determined	9, 10
Hastigerina pelagica	3	2, 10, **this study (Chapter 4)**
Bolivina variabilis	not yet determined	27
Gallietellia vivans	not yet determined	28

The disproportion between the high sequencing effort (723 newly published sequences out of the overall 1516 sequences of planktonic foraminifera that are available in GenBank to date) and the low number (2) of newly discovered cryptic species as a total of all studies of this thesis suggests that the amount of genetic diversity in planktonic foraminifera morphospecies is not as extensive as might have been concluded from previous studies

(summarized in Table 8.1). Indeed, for many well studied morphospecies the maximum number of cryptic species might already be discovered, as could be shown for *G. siphonifera* by the application of a Jackknifing approach (Chapter 6). Species of all five morphogroups by now have been subject to genetic analysis (Figure 8.1A), though due to their importance as paleo-proxies and their higher abundances in the ocean, attention has been biased towards the macroperforate spinose and nonspinose groups, of which respectively, 70% and 56% of all morphospecies have now been examined. In both groups, the number of discovered cryptic species more than doubles the number of morphospecies analyzed. However, the summary in Table 8.1 on the cryptic diversity encountered so far in planktonic foraminifera indicates that the biological diversity of the group may not be underestimated by more than a factor of ten, which is about the maximum number of cryptic species encountered in the two most diverse morphospecies.

By comparing the results of the presented as well as previous studies, it becomes obvious that the genetic diversity is distributed highly inhomogeneously between the morphospecies (Figure 8.1B) and is not even pervasive in the entire group of planktonic foraminifera. The latter fact is supported by the discovery of morphospecies that contain only one single motif in their SSU rDNA. The morphospecies studied in this thesis cover the whole range of diversity with only one sequence type in *G. sacculifer* versus nine in *G. siphonifera*, a number that is only exceeded by *Globigerina bulloides* with 12 cryptic species (see Table 8.1). The mean number consequently amounts to four cryptic species per morphospecies. This high difference in sequence diversity between morphospecies might be explained by the highly different evolutionary rates that have been described for planktonic foraminifera (Pawlowski & Lecroq 2010). Surprisingly, three of the four species with above-average cryptic diversity occur in polar to transitional regions. This is unexpected, since these regions are marked by high intermixing of the water column without providing stable niche space (Al-Sabouni *et al.* 2007), which would allow for high speciation rates. Interestingly, also in the presented studies the detected cryptic diversity does not come up to the expectations based on the characteristics of the studied morphospecies, such as low abundance in the global ocean or high morphological variability. Generally speaking, it does not seem possible to predict the exact amount of biodiversity, existent in morphospecies that have not been screened until now, based solely on the characteristics of these species.

Considering the application of planktonic foraminifera in paleontological research and the consequential requirement of an exact taxonomy and species classification, the existence of a rather limited amount of cryptic diversity would be more advantageous. A correlation with existing morphological variability could thus be facilitated and the probability for a successful differentiation of biological species in the fossil record would be raised (Kucera & Darling 2002), reducing the scatter in paleo-reconstructions arising from the application of morphospecies.

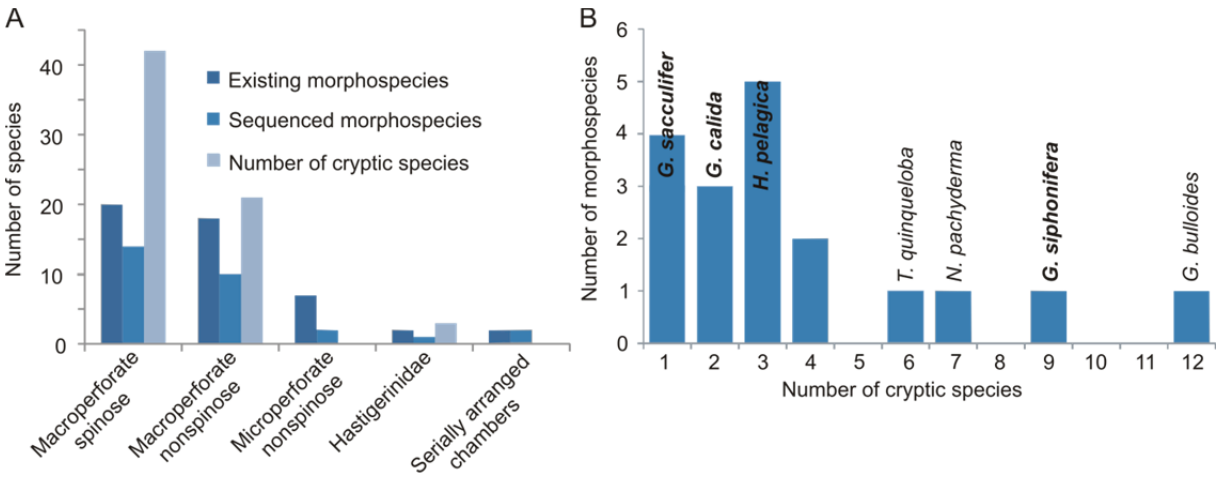

Figure 8.1: A) Comparison of the number of morphospecies that have been analyzed of each of the five morphogroups and the number of cryptic species discovered. Due to their low abundances in the ocean and their minute shell sizes, the microperforate species are still under-represented. **B)** Representation of the highly inhomogeneous distribution of cryptic diversity between the morphospecies. The average number lies at four cryptic species per morphospecies. Only the morphospecies studied in this thesis (bold print) and all morphospecies with above-average cryptic diversity are assigned to their number of cryptic species.

The objective of establishing a correlation between genetic diversity and morphological traits was thus also part of the studies presented in this thesis. Nevertheless, these studies come to the conclusion that genetics and morphology map only partly onto each other and that morphological distinction does not depend on the genetic distance between cryptic species. This statement is based on comparisons of the morphology of the cryptic species of the studied morphospecies, partly resting upon extensive morphometric analyses carried out on specimens that had been genotyped before.

The three cryptic species of *H. pelagica* could not be differentiated by a superficial inspection of their morphological traits, despite of what could have been expected from their high genetic distances between each other (Chapter 4). Surprisingly, the here conducted analysis of the *G. sacculifer* plexus revealed that the morphological variability in the group had been over-interpreted and is not genetically justified. This case is the first in planktonic foraminifera, where the morphological diversity exceeds the genetic variability and the different morphotypes therefore have to be considered either as different ontogenetic stages or ecophenotypic appearances. As a consequence, the species names that had mostly been applied in studies on the fossil record for this taxon (*G. trilobus*, *G. immaturus* and *G. quadrilobatus*) have to be replaced by *G. sacculifer* as the valid species name. The picture revealed by morphometric analysis of the *G. siphonifera*/*G. calida* plexus is marked by a high genetic as well as morphological variability, which in some parts could be brought into agreement. Not only was it possible to verify the status of *G. calida* as a separate species and sister to *G. siphonifera*, but by detecting a third morphotype within the plexus, *Globigerinella radians* could be described as a new species within the planktonic foraminifera. Surprisingly, *G. calida* and *G. radians* possess similar morphologies, characterized by elongated chambers, despite the fact that they are only distantly related to each other within the plexus. A further hint that morphological distinction of the cryptic species does not depend on their phylogenetic distances was found while analyzing their

shell porosity. Various cryptic species are marked by high porosity values, however, this morphological trait is not associated with a certain lineage, but appears in cryptic species that are only distantly related.

The examples of the present thesis show that inconsistent scaling exists between genetic and morphological diversity and that these two features evolve rather independently of each other. The study on *G. siphonifera* further indicates the presence of high rates of parallel evolution in distantly related taxa, as it was already reported from the fossil record (Coxall *et al.* 2007). In general, these observations imply that the fossil record does not reflect biological species and neither one of the two species concepts by itself mirrors the entire diversity of planktonic foraminifera. Only few examples have been shown so far in which exactly one morphology corresponds to one genetic type: in *Globigerinoides conglobatus*, *Globigerinoides ruber* (pink) and *G. radians* the morphological and biological species concepts seem to be in agreement (Table 8.1; Aurahs *et al.* 2011; Chapter 7). These cases show that once the genetic diversity of a morphospecies has been resolved, a closer look at the morphology and maybe even physiology of the cryptic species is indispensable in order to detect slight differences that correspond to the genetic background. If a correlation between these different aspects could be established, the conflict of non-matching species classifications between studies on fossil and living individuals could be minimized.

In order to obtain a comprehensive picture of the biological species of planktonic foraminifera, a consideration of their biogeographic distribution patterns and environmental adaptations are of utmost importance. The cases analyzed in the present thesis reveal a predominance of global dispersal and gene flow even for the cryptic species, but also detect geographical restrictions in the horizontal as well as vertical perspective. The dataset obtained profits from the wide spatial and temporal extension of the now available sample collections.

Every morphospecies that was screened for genetic diversity was also examined in regard to its general occurrence and the distribution patterns of its cryptic species. From the complete genetic homogeneity in *G. sacculifer* throughout its global distribution range, the only conclusion to be drawn is that this species has always been marked by global gene flow without the establishment of reproductive isolation (Chapter 5). The three sibling species of *H. pelagica* also show a global distribution in the ocean. Genetic differentiation in this case most likely took place in the vertical dimension by differential adaptation along an environmental gradient, depicting a depth parapatric speciation event, which is still reflected in today's distribution of the cryptic species (Chapter 4). Although depth parapatry was described for other groups of plankton, like copepods and chaetognaths (Mackas *et al.* 1993; Kehayias *et al.* 1994; Fragopoulu *et al.* 2001), in extant planktonic foraminifera it was observed for the first time. Most of the genetic types found within *G. siphonifera* are also able to disperse on a global scale and obtain gene flow between their populations, indicating that speciation originally must have taken place in sympatry. The only factor inhibiting global dispersal of each cryptic species of *G. siphonifera* seems to be a strong influence of species interactions, which shapes the present day distribution patterns of the sibling species, with a striking separation of diversity between the Atlantic and Pacific Oceans.

The observed high potential for global dispersal and gene flow in all of the studied species is surprising, since all three are bound to the high temperature waters of the Tropics and Subtropics and have to cross colder water masses in order to establish a circumglobal distribution. However, cosmopolitan distribution has been observed for at least some cryptic species of several morphospecies (e.g. de Vargas *et al.* 1999; Aurahs *et al.* 2009b; Quillévéré *et al.* 2013) and consequently seems to be the norm within many planktonic foraminifera, not only on the level of morphospecies, but also for the cryptic species. Nevertheless, the potential for establishment of reproductive isolation is still given. By having a look at the distribution patterns of cryptic species, in many cases it is possible to draw conclusions on the prevailing modes of speciation in planktonic foraminifera or microplankton in general. Overall, almost all different speciation mechanisms have now been detected in planktonic foraminifera (Table 8.2), a fact that points out the high potential for diversification in plankton, despite the predominance of global dispersal, and that shows that the establishment of genetic isolation is not as rare in planktonic protists as is often assumed (Benton & Pearson 2001). Furthermore, from the existence of different modes of speciation it can be concluded that the traditional view on allopatric/vicariant speciation and horizontal separation as sole diversification possibility was too limited, underestimating the complexity of microplankton. *Globorotalia inflata* was described to differentiate on either side of an oceanic current (Morard *et al.* 2011), the two most closely related genetic types of *G. ruber* probably differentiated from each other while being separated into different ocean basins during a glacial maximum (Aurahs *et al.* 2009b), but the majority of speciation events seems to occur in sympatry (Table 8.2). This fact highlights the importance of a detailed analysis of the environmental adaptations of the cryptic species. Biological speciation in planktonic foraminifera might proceed without being obviously reflected in the morphology of the new species, however, if occurring in sympatry, the newly evolved cryptic species possess different ecological preferences, since they diverged by differential adaptation along an environmental gradient. This fact is important to consider, if a morphospecies is used in paleoceanographic studies, since the different adaptations will be reflected in the isotopic compositions of the shells.

Table 8.2: Summary of the different modes of speciation that might have caused the high genetic diversity in planktonic foraminifera and might have led to the distribution patterns of the cryptic species in the present day ocean. For each speciation mode, the best described examples are shown. Bold print indicates studies from this thesis. Superscripts: (1) Morard *et al.* 2011, (2) Darling & Wade 2008, (3) Aurahs *et al.* 2009b, (4) Morard *et al.* 2009, (5) Quillévéré *et al.* 2013, (6) Ujiié *et al.* 2012.

Allopatry	**Vicariance**
Globorotalia inflata[1] *Neogloboquadrina pachyderma*[2] *Neogloboquadrina incompta*[2]	*Globigerinoides ruber*[3] (divergence between types IIa1 and IIa2)
Depth Parapatry	**Sympatry**
Hastigerina pelagica	*Orbulina universa*[4] ***Globigerinella siphonifera*** *Globorotalia truncatulinoides*[5] *Globigerina bulloides*[2] *Pulleniatina obliquiloculata*[6]

8.2. Remaining limitations

The genetic diversity within planktonic foraminifera morphospecies seems to be resolved to a wide extend, however, molecular analysis on the group still faces various limitations. The most significant surely is the low success rate in DNA amplification (e.g. 8% sequence yield from the *Globigerinoides sacculifer* samples of the working group) and additional high proportion of contamination. A low success rate might be attributed to the highly divergent and unusually long nucleotide sequences of planktonic foraminifera or to some further unknown molecular modification of the SSU rDNA. Even the application of foraminifera specific primers mostly does not enhance DNA yield and nested PCR often just intensifies the contaminant signal (own observations). The same problems very likely impede the amplification of the mitochondrial genes, which also are assumed to be highly modified (Pawlowski & Lecroq 2010). As a consequence, a high number of samples is required for the single cell approach in order to get a significant genetic signal. This might explain why so many rare and small species that are seldom sampled are still not genetically characterized.

The contamination problem was tried to overcome during the research for this thesis by working with gametogenic individuals, in which the high amount of genome copies should easily outnumber the genetic signal of the contaminant. However, despite the fact that this approach has been successfully applied before (e.g. Hemleben *et al.* 1989; Darling *et al.* 1996b) and that the individuals in culture sometimes survived several weeks during the studies for this thesis, the number of specimens performing gametogenesis was vanishingly low and they still contained contaminant DNA. This unfeasibility to obtain axenic cultures and large amounts of DNA severely limits the potential for obtaining sequence information for protein coding genes (Flakowski *et al.* 2005), which would present a necessary addition to the single gene approach applied so far. Although the SSU rDNA serves well as a marker for phylogenetic reconstructions, its high level of heterogeneity in substitution rates between and sometimes even within a morphospecies prevents the establishment of universal thresholds separating the different taxonomic levels (Göker *et al.* 2010). This fact together with the inability to observe reproduction of planktonic foraminifera in culture leaves the biospecies classification to be highly subjective and being based solely on the lack of observable hybridization in the SSU rDNA.

Regarding the biogeographical distribution of planktonic foraminifera, a constraint of patchy sampling exists due to the application of the single cell approach and the applied sampling methods. By sampling with 100 µm plankton nets, only adult specimens are obtained, but the presence of gametes or potentially resting stages cannot be detected, although these might even have wider distributions and dispersal rates compared to the sometimes restricted areas where the species finally thrives.

8.3. Future perspectives

One of the next steps of molecular genetic research on planktonic foraminifera should focus on obtaining new marker genes in order to examine the reliability of the SSU rDNA delineated genetic types and their phylogenetic relationships. Combining the information of several marker genes (e.g. SSU and LSU rDNA, mitochondrial genes or polymerase genes) might better resolve genetic information, which evolutionary potential so far could not be identified. Mitochondrial genes evolve faster than the ribosomal DNA and could therefore be used to detect present day gene flow and more recent evolutionary events (Bik *et al.* 2012). The approach of amplifying genes by primer walking (e.g. Jones & Winistorfer 1993) might be useful for obtaining the LSU gene that lies in close vicinity of the SSU rDNA and is not yet known for a large number of species.

A further exciting step in foraminifera genetics will be the elucidation of the whole genome. By now, the genome of a benthic foraminifera species has been published (Glöckner *et al.* 2014). Although the selected species is a member of the naked foraminifera and possesses several large nuclei, advantages that are not found in planktonic foraminifera, the nucleotide information obtained might still provide a solid basis for sequence alignments during attempts on planktonic foraminifera. In order to enhance the DNA yield, several specimens of the same species could be merged. As an alternative to whole genome sequencing transcriptome analysis could be carried out, as it has been successfully applied in one benthic species (Pillet & Pawlowski 2013). Since nucleotide sequences usually are heavily processed during the transcription and translation processes (e.g. Wuyts *et al.* 2002), the transcriptome can be expected to be a lot smaller than the genome, facilitating the bioinformatics data processing. Furthermore, knowledge on gene expression rates might allow conclusions on the physiology of the organism and eventually even reveal adaptations to certain environmental parameters or responses to changing conditions (Caron *et al.* 2012).

For the biogeographical analyses, the future probably lies in environmental sequencing approaches. Filtering large amounts of water and sequencing the whole filtrate (Karsenti *et al.* 2011 for methods) resolves the problem of patchy and size biased sampling. Since the number of cryptic species in planktonic foraminifera is now not expected to rise extensively anymore, this approach can be applied as a screening method to detect the real dispersal rates of microplankton in the ocean.

Investigating speciation events in plankton based on present day distribution patterns can just give a small hint about how speciation possibly can occur, since the range of a species can change throughout time, erasing the original pattern (Norris & Hull 2011). In order to obtain a more explicit picture about lineage evolution and speciation in planktonic foraminifera over large time scales, a close connection of biology and paleontology in the future will be indispensable (Benton & Pearson 2001) to combine present day observations with those from the fossil record. The present thesis aims at being one step into the direction of such a combination, by providing a comprehensive assessment of the extent of biodiversity in planktonic foraminifera and the biogeographical distribution of the

cryptic species. In addition to the general knowledge gained on diversification and distribution of microplankton in the global ocean, the present thesis eventually will enhance the applicability of planktonic foraminifera in paleontological studies, by resolving the relationship between genetic diversity and morphological variability in selected morphospecies.

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