**Marine Micropaleontology**
October 2018, Volume 144, Pages 14-24
http://dx.doi.org/10.1016/j.marmicro.2018.10.001
http://archimer.ifremer.fr/doc/00461/57224/




# Disconnection between genetic and morphological diversity in the planktonic foraminifer *Neogloboquadrina pachyderma* from the Indian sector of the Southern Ocean

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**Abstract :**

Eight SSU rDNA genetic types have been described in the planktonic foraminifera *Neogloboquadrina pachyderma*, but the level of correlation between genetic diversity and morphological variation remains unknown in this morphospecies. In this study, we combine molecular and morphometric analyses of specimens of *N. pachyderma* sampled during two consecutive years across a latitudinal gradient in the Indian sector of the Southern Ocean. We observe that three genetic types of *N. pachyderma* inhabit the (sub-)polar waters of the southern Indian Ocean where they have equivalent regional distributions to those previously observed in the South Atlantic. The geographic ranges of these genetic types are largely overlapping. Our morphometric data show that contrary to other planktonic foraminiferal morphospecies, there is no relationship between genetic diversity and morphological differentiation in at least two of the austral representatives of *N. pachyderma* (Type III and Type IV) despite a high morphological variability and large genetic distance between these types. These genetic types of *N. pachyderma* in the southern Indian Ocean thus constitute true cryptic species of planktonic foraminifera.

**Highlights**

► Type III and Type IV of *N. pachyderma* constitute true cryptic species. ► New protocol for rapid identification of genetic types of *N. pachyderma*.

**Keywords** : morphospecies, genetic types, polar waters, biogeography, morphometric analyses, ribosomal DNA.

## 1. Introduction

Molecular investigations during the last two decades have shed light on the nature of diversity among extant planktonic foraminifera (e.g., Huber et al., 1997; de Vargas et al., 1999; 2001; 2002; Darling et al., 1999; 2004; 2007; Stewart et al., 2001; Morard et al., 2009; 2011; 2016a; Aurahs et al., 2011; Weiner et al., 2012; 2014; 2015; Ujiié et al., 2012; Quillévéré et al., 2013; André et al., 2013). Most modern morphospecies of planktonic foraminifera appear to be composed of several genetic types, also referred to as cryptic species or genotypes, which display various levels of geographical and environmental disconnection, ranging from partly overlapping spatial distributions (e.g., de Vargas et al., 2001) to allopatric (e.g., Morard et al., 2011) and even depth-parapatric distributions (e.g., Weiner et al., 2012).

The degree of genetic and morphological divergences across these genetic types seems to be uncorrelated. The three genetic types of *Orbulina universa* diverged between 12.1 Ma and 6.1-7 Ma (de Vargas et al., 1999) and possess largely overlapping morphologies (Morard et al., 2009). In stark contrast, the genetic Type Ia of *Globigerinella siphonifera*, which diverged at 1.48 Ma from its sister type (Weiner et al., 2014), displays enough morphological characteristics to receive its own formal morphospecies name, *Globigerinella radians* (Weiner et al., 2015). These two examples indicate that the connection between genetic and morphological variations in planktonic foraminifera may be complex, and requires detailed description for sound systematic understanding. Such morphological characterization of genetic types facilitates reconstruction of ecological properties in the fossil record (Quillévéré et al., 2013; Morard et al., 2016b) and thus increases the amount of information extractable from these archives (Kucera and Darling, 2002; Morard et al., 2013; Marshall et al., 2015; Sadekov et al., 2016).

The earliest morphometric assessments of morphological variation of morphospecies across environmental gradients identified morphological clines correlated to ecological parameters (e.g., Ericson, 1959; Banner and Blow, 1960). Morphogenetic studies later showed that some of these clines mirror the genetic diversity of the studied morphospecies. One of the most prominent examples is the highly conical *Truncorotalia truncatulinoides* in subtropical waters, whilst biconvex forms occur in colder waters in the Indian Ocean (Healy-Williams and Williams, 1981). These two forms correspond to complexes of genetic types with morphological separations that can be used to track the migration of the Subtropical Front in the Southern Ocean (de Vargas et al., 2001; Renaud and Schmidt, 2003; Quillévéré et al., 2013). Relationships between a morphological cline and distribution of genetic types across environmental gradients were also demonstrated in the cases of *Globoconella inflata* (Morard et al., 2011; 2016b) and *Orbulina universa* (de Vargas et al., 1999; Morard et al., 2009), suggesting that morphological clines in planktonic foraminifera more likely correspond to the geographic distribution pattern of different genetic types and not, as previously assumed, to ecophenotypic variation.

Here, we investigate the morphospecies *Neogloboquadrina pachyderma*, which inhabits polar and subpolar waters of both hemispheres. *Neogloboquadrina pachyderma* is one of the foraminiferal morphospecies that received much attention regarding the characterization of its cryptic diversity (Darling et al., 2004; 2006; 2007; 2017) because of its importance for paleoceanography (e.g., Kennett, 1968; Brummer and Kroon, 1988) and because it constitutes 75% to 100% of the planktonic foraminiferal assemblages in high latitudes (Kucera et al., 2005).

*Neogloboquadrina pachyderma* is a non-spinose, macro-perforate trochospiral morphospecies that exhibits a high level of morphological variation. The number of chambers on the last whorl varies from 4 to 5, and the aperture ranges from a narrow slit to a high

arched opening (Bandy, 1972; Kennett and Srinivasan, 1983). The final chamber is variable in shape and often of reduced size. The morphotype is called "kummerform" when smaller than the penultimate chamber (Olsson, 1973). The initial description of *N. pachyderma* (Ehrenberg, 1861) includes both left and right-coiling morphotypes. Ericson (1959) observed that the shift in the coiling direction is associated with the April 7.2°C isotherm, which has been of great importance to Quaternary paleotemperature reconstruction (e.g., Bauch et al., 2003; Ellison et al., 2006; Eynaud et al., 2009). Darling et al. (2006) demonstrated that the coiling direction of morphotypes is congruent with two highly divergent genetic lineages, and consequently confirmed the right-coiling variant being *Neogloboquadrina incompta* as proposed by Cifelli (1961), and retained the name *N. pachyderma* for the left-coiling variant.

To date, eight genetic types of *N. pachyderma* have been described in the world ocean. Types I and IV occur in the most polar habitats of the morphospecies, in the North Atlantic and Southern Ocean, respectively. Types II and III occur in subpolar waters of the Southern Ocean. Type VII occurs in subpolar waters of the North Pacific. Types V and VI have been found only in the Benguela upwelling system (Darling et al., 2008) and Type VIII has recently been identified in the Arabian Sea (Darling et al., 2017). Types I and IV appear to be the most basal in phylogenetic inferences whilst the relationships between the recently diverging Types II, III, V, VI, and VII are partly unresolved (André et al., 2014).

Here, we study the morpho-genetic diversity of *N. pachyderma* in the Southern Ocean (i.e., Types II, III and IV). Darling et al. (2004) suggested that the diversification of Types II, III and IV might be linked to Quaternary climate dynamics and hypothesized that the distribution of the three types along the subpolar/polar oceanic fronts could be congruent with early observations of the latitudinal morphological variations occurring in the Southern Ocean (Kennett, 1968; Malmgren and Kennett, 1972). André et al. (2014) proposed a species rank for Type IV but concluded that the status of Types II and III is still unclear. They may

constitute sub-specific variants or closely related young species. To study the morpho-genetic diversity within and among these genetic types, we collected living specimens of *N. pachyderma* across a latitudinal transect sampled in the Indian sector of the Southern Ocean in the years 2011 and 2012. Based on the use of a non-destructive DNA extraction method and the development of a new genotyping protocol for rapid and cost-effective identification of the genetic types occurring in this region, we combine molecular and morphometric analyses on these specimens and then assess the level of congruence between genetic and morphological diversity in *N. pachyderma* from the Southern Ocean.

## 2. Material and Methods

### *2.1. Sample collection and hydrographic data*

The studied specimens of *Neogloboquadrina pachyderma* were collected in the southern Indian Ocean onboard the research vessel *Marion-Dufresne* during cruises OISO-19 (MD185) and OISO-21 (MD189) in 2011 and 2012, respectively, organized in the framework of the OISO program (Océan Indien Service d'Observation; CNRS-INSU observation system; Metzl, 2000). Samples were obtained from seven sites distributed along a 30° latitudinal transect from the subtropical Front to polar waters (Fig. 1, Table 1). Four sites (Stations 7, 9, 10, and 11) were investigated during two consecutive austral summers (January 16 to 30, 2011, and January 28 to February 9, 2012), and three more sites were sampled only once (Station 5 in 2011; Stations 6 and 8 in 2012). Live specimens were collected using a Multinet plankton sampler (HYDRO-BIOS® MultiNet type MiDi) equipped with five 100-µm nets hauled between 500 m water depth and the sea surface during cruise OISO-19 and between 100 m water depth and the sea surface during cruise OISO-21 (Table 1). Immediately after sampling, all living specimens of *N. pachyderma* were picked. Individuals with cytoplasm were carefully cleaned with a fine brush in filtered sea water. During cruise OISO-19,

specimens were individually transferred into 50 µl of GITC* DNA extraction buffer (Weiner et al., 2016) and stored at -20°C. During cruise OISO-21, specimens were dried for 24 hours at 4°C, and then stored on cardboard slides at -20°C. The air-dried specimens were isolated into the GITC* in the laboratory.

During both cruises OISO-19 and OISO-21, vertical profiles of temperature and chlorophyll-*a* fluorescence were measured at identical stations with Conductivity-Temperature-Depth (CTD Sea-bird 911 plus) casts.

*2.2. Sequencing and genotyping*

2.2.1. DNA extraction, amplification, and sequencing

DNA extractions of 502 specimens from cruise OISO-19 (2011) and of 482 specimens from cruise OISO-21 (2012) were performed using the GITC* buffer as described in Weiner et al. (2016). After DNA extraction, shells were retained for morphometric investigation.

Amplification of a fragment of 310 to 341 bp localized at the 3' end of the SSU and encompassing the variable regions 37/f and 41/f (Fig. 2 and Supplementary Fig. A.1) was carried out using newly designed *N. pachyderma*-specific primers NP1fb (5'-CCGGACACACTGAGGATTGACAGGCAATATC-3'), and NP5r (5'-CTGTTGGGCTCTGAAAGCAACGAACGTG-3'). The PCR mix per reaction was composed of 23.26 µl MilliQ water, 3 µl standard Taq-reaction buffer X10 (New England Biolabs), 0.96 µl of each primer at 10 µM, 0.30 µl of BSA 100X (New-England Biolabs), 0.26 µl of dNTP 20mM, 0.26 µl of Taq-polymerase 5U/µl (*Thermus acquaticus* YT-1 polymerase with 5' flap endonuclease activity by New England Biolabs) and 1 µl of DNA template. The cycling conditions include initial denaturation at 95°C for 10 min, amplification

at 94°C for 30s, at 54°C for 30s, and at 72°C for 30s, repeated during 44 cycles, and final extension at 72°C for 10 min.

We first screened the diversity present in the samples of cruise OISO-19 by directly sequencing between 6 and 60 PCR products per station as *N. pachyderma* abundance is quite variable. We additionally sequenced a few individuals (n = 26) from cruise OISO-21 originating from four stations (Table 2). The PCR products were purified using EZNA Cycle Pure kits (Omega bio-tek), and sequenced by an external provider (Biofidal Vaulx-en-Velin, France) on an ABI prism 3730XL sequencer (Applied Biosystems).

Direct sequencing of the PCR products generated sequences with ambiguous bases reflecting intra-genomic variability between SSU-rDNA copies, a widespread phenomenon in non-spinose planktonic foraminifera (Darling et al., 2004; André et al., 2014). Among the 152 obtained sequences, 49 included only a few (5 or less) ambiguous bases and were deposited on NCBI under the accession numbers KU365032 to KU365128. The remaining 103 sequences allowed genetic type identification but included a larger number of ambiguous bases. They are provided as Supplementary Table A.1.

2.2.2. Protocol for rapid genetic identification

Sequencing revealed only Types II, III, and IV in our sample set (see results), and only these genetic types may occur in our samples. To achieve faster and more cost-effective identification of these genetic types, we developed a two-step genotyping approach.

First, we observed that PCR products using NP1fb/NP5r primers were consistently shorter in Type IV individuals (Fig. 2). This observation was confirmed by a manual screening of the published sequences of Types II, III, and IV available on the *Planktonic*

*Foraminifera Ribosomal Reference database* (PFR[2], Morard et al., 2015) that includes all published sequences of planktonic foraminifera prior to 2015, including those of Darling et al. (2000; 2004). On the 111 available sequences covering the region amplified in our study, 19 belonged to Type II and had a length of 335 to 328 bp (mean value 332.5 bp), 32 belonged to Type III and had a length of 342 to 330 bp (mean value 338 bp), and 60 belonged to Type IV and had a length of 314 to 308 bp (mean value 310.5 bp).

Second, we developed a Restriction Fragment Length Polymorphism protocol (RFLP) to discriminate between Type II and Type III. We selected the endonuclease enzyme Taqα1 (New England Biolabs), cutting at sequence 5' TCGA 3' which occurs once in sequences of Type II and twice in sequences of Type III (Fig. 2). Five µL of the PCR product, identified as Type II or III after the initial migration, were mixed with 3.4 µl of distilled water, 1 µl of buffer solution (NEB buffer 3.1 by New England Biolabs), 0.1 µl of BSA 100X (New-England Biolabs), and 0.5 µl of enzyme. The mix was then incubated for two hours at 65 °C. The obtained digested products were then migrated for 15-20 minutes at 100 V on a 3% agarose gel with a 100-1000 bp ladder.

The protocol was tested on 14 products for which sequences were available (9 of Type II, and 5 of Type III) prior to application of the entire sample set. As expected, Type II displayed a two-band pattern (one of the two bands being characteristically located at ~250 pb; Fig. 2). However, Type III displayed a four-band pattern (one of the four bands being characteristically located at ~150 pb; Fig. 2) instead of the expected three-banded pattern. The four-banded pattern of Type III corresponds to a mix of partially or totally digested sequences as well as possible intra-genomic variants lacking one restriction site. Close examination of our sequences together with those available on the PFR² database (Morard et al., 2015) revealed that variation at restriction sites is rare (~5 % for the first restriction site, and ~1% for the second). The results obtained for the 14 products were consistent with the sequences,

validating our protocol which was subsequently applied to all the PCR products identified as Type II or III following the initial migration.

*2.3. Morphological analyses*

Preserved shells after DNA extraction were first observed under the dissecting stereo microscope to determine the number of chambers in the last whorl, and to distinguish normal from kummerform specimens. To ascertain the morphological variability within *N. pachyderma*, some selected shells were photomicrographed using a Hitachi S-570 SEM (Lyon I University). In addition, images of shells of Type III and Type IV from cruises OISO-19 and OISO-21 were taken in umbilical view, using an automated incident light microscope driven by analySIS® software at the University of Angers. The standard edge view (i.e., lateral view showing the aperture) was not analyzed because this position was highly unstable in the case of *N. pachyderma*, impeding any homologous positioning of specimens. Images of two aberrant right coiling specimens were subjected to a horizontal mirror transformation. Shell size was measured using analySIS® software, computing 21 parameters including size and shape-related data (Bollmann et al., 2004; Clayton et al., 2009).

Morphological variation among genetic types is characterized through two-dimensional shell outline analyses in umbilical view. SHAPE v. 1.3 (Iwata and Ukai, 2002) and the integrated ChainCoder program was used to extract shell outlines and record them as a chain code. Outlines were analyzed using elliptical Fourier analysis (Kuhl and Giardina, 1982) which describes the outline as a sum of trigonometric functions of decreasing wave-length, i.e., harmonics (Healy-Williams, 1984; Foote, 1989) and produces a data set of normalized Fourier descriptors suitable for statistical comparison (Kuhl and Giardina, 1982). The Chc2Nef program of SHAPE was used to calculate the elliptical Fourier descriptors using

the chain code, normalizing for the first harmonic. We selected the required number of harmonics needed for analysis using the method developed by Crampton (1995) (Supplementary Table A.2.). We applied Principal Component Analysis and a one-way MANOVA, and associated pairwise tests of significance to select coefficients using the PAST software v.2.17 (Hammer et al., 2001).

## 3. Results

### *3.1. Hydrographic data and distribution of* Neogloboquadrina pachyderma

According to temperature and fluorescence data acquired during the OISO cruises in 2011 and 2012, temperate (12.3°C) to polar waters (1.6°C) were sampled (Fig. 3). With the exception of Station 5 during cruise OISO-19 (2011), thermal stratification in the upper 100m remained very weak throughout both latitudinal transects (Fig. 3), and polar waters south of the Polar Front showed little vertical temperature variation. The thermal structure of the water column was similar in both years. Lower primary productivity occurred in 2012.

As expected for sub-polar to polar morphospecies, the abundance of *N. pachyderma* increased southwards (Table 1). Meilland et al. (2017) showed that during cruise OISO-21 (2012), the average abundance of *N. pachyderma* ranged from 14 individuals per $m^3$ near the Sub-antarctic Front in the north (Station 6) to between 100 and 200 individuals per $m^3$ in the upper 100 meters in the southern part of the sampling area close to and south of the Polar Front (Stations 9-10-11). The depth stratified casts performed during cruise OISO-19 (2011) revealed that living specimens could be found down to 500 meters depth, with the exception of Station 5, where standing stocks were generally low (Table 1). The majority, i.e. 91.2 %

(Station 10) to 96.7 % (Station 7) of the collected individuals occurred in the surface 100 m of the water column (see Meilland et al., 2017).

*3.2. Geographic distribution of genetic types*

A total of 242 specimens (49%) from cruise OISO-19, and 411 specimens (85%) from cruise OISO-21 were successfully amplified (Table 2). We identified 16 specimens of Type II, 535 specimens of Type III and 101 specimens of Type IV.

Type III was dominant at all stations. Type II was restricted to warmer waters and never occurred at sea surface temperatures (SST) below 7.5 °C. Type IV reached its maximum near the Polar Front, and was also found close to the Subtropical Front during cruise OISO-19 (Fig. 3A). Our observations are congruent with those of Darling et al. (2004; Fig. 3B). In the South Atlantic, Type II was restricted to the northernmost part of the geographical sampling range, whilst Type III occurred from temperate to subpolar waters. Type IV was restricted to polar and subpolar waters. Therefore, the spatial distribution of Type IV appeared slightly broader in our samples (Fig. 3A).

*3.3. Differences in shell morphology*

Despite having handled the specimens with care during the steps of DNA extraction, we had a rather low success rate in shell recovery. From the 652 amplified individuals, 358 specimens were damaged and had to be excluded from morphometric analysis. In addition, 50 individuals were used for isotope analyses, and not analyzed morphometrically. Among the 16 Type II individuals identified, 15 shells were recovered after DNA extraction and have been observed under the dissecting microscope (see Material and Methods 2.3.) but only one individual was available for morphometric analyses. Consequently, our outline analyses focus

on Type III and Type IV, which constitute the most dominant and most divergent genetic types in the Southern Ocean (André et al., 2014; Darling et al., 2004). Finally, 254 shells were used for morphometric analyses, with 202 belonging to Type III and 51 to Type IV.

Size differences between Type III and Type IV between the two years 2011 and 2012 are not significant (Fig. 4A, Table 3). However, shells of Type III are slightly larger at subpolar stations (Stations 7 to 10) than in polar waters (Station 11) (Fig. 4B; Table 4).

Shells of Type III and Type IV are similar in the number of chambers in the last whorl, and in the size of the last chamber relative to the entire shell (Supplementary Table A.3). Both genetic types exhibit kummerform morphologies with 5.5% of specimens of Type IV and 12.1 % of Type III. As for Type III and IV, the rare Type II specimens available include a few kummerforms (4 individuals out of 15, Supplementary Table A.3). No significant relationship exists between the number of kummerform individuals and SST (Fig. 5A). The three genetic types included individuals with 4 to 5 chambers in the last whorl (Supplementary Table A.3), and the number of 4-chambered specimens is negatively related with ambient water temperature (Fig. 5B). This relationship confirms the observation of Kennett (1968) on specimens from surface sediments describing the same relationship between the number of 4-chambered individuals and increasing latitude (Fig. 5C).

Outline shape analyses are based on all the 254 perfectly preserved Type III and IV individuals. Results of the PCA applied to the normalized Fourier descriptors again confirm that there is no correlation between genetic types and shell shape differentiation in Types III and IV (Fig. 6). Most of the shape variations observed in umbilical view are described by the first two principal components, which account for 64% of the total morphological variance. The PCA does not clearly delineate distinct clusters and a continuum of shape variation of *N. pachyderma* includes genetic Type III and Type IV. Probably due to the lower number of Type IV individuals, the PC domain corresponding to Type IV individuals is included within

the PC domain corresponding to Type III (MANOVA performed on the PC coordinates: Wilk's $\lambda = 0.948$, df1=2, df2=249, F = 6.833, Hotelling's p (uncorrected) = p (Bonferroni corrected) = 0.021). The outline shape analyses cannot discriminate between Type III and Type IV (Fig. 6). No correlation exists between principal components of shape parameters (PC1 and PC2) and surface temperature or size (Fig. 6), suggesting that the shape of *N. pachyderma* cannot be explained by those parameters. The MANOVA performed on the PC coordinates shows that the outlines of individuals with 5 chambers in the last whorl are different from other individuals with fewer chambers in the final whorl (Wilk's $\lambda = 0.88$, df1=2, df2=249, F = 16.03, Hotelling's p [uncorrected] = p [Bonferroni corrected] = $2.8\ 10^{-7}$). On the contrary, individuals with 4 chambers or 4 to 5 chambers in the last whorl are not discriminated (MANOVA performed on the PC coordinates: Wilk's $\lambda = 0.98$, df1=2, df2=200, F = 1.56, Hotelling's p [uncorrected] = p [Bonferroni corrected] = 0.21).

SEM observations of 54 shells exemplify the remarkable morphological variability within Types III and IV (Fig. 7): the number of chambers on the last whorls ranges from 4 to 5 and some individuals display a kummerform chamber. Some of the smallest individuals may be immature. Four photomicrographs of the shell ultrastructure in Type III and IV shells exemplify their porosity (Fig. 7).

## 4. Discussion

### *4.1. Distribution of genetic types in the southern Indian Ocean and South Atlantic Ocean*

The genetic types of *Neogloboquadrina pachyderma* identified along the southern Indian Ocean transects are identical to those retrieved by Darling et al. (2004) in the South

Atlantic (Fig. 3). This was expected because the southern hemisphere genetic types are transported within the Antarctic Circumpolar Current, resulting in their widespread distribution within surface waters of the South Atlantic and southern Indian Oceans. The same pattern exists in the southern genetic types of *Globoconella inflata* (Morard et al., 2011) and *Truncorotalia truncatulinoides* (Quillévéré et al., 2013), and the distribution observed in the southern Indian Ocean therefore mirrors the distribution observed in the South Atlantic.

Both data from the southern Indian Ocean (this work) and South Atlantic Ocean (Darling et al., 2004) show that Type II is mostly restricted to transitional environments. Type III displays a broader distribution, ranging from transitional to subpolar waters (Fig. 3 and Darling et al., 2004) where it dominates over the other types. Type IV is the dominant genetic type in polar waters (Fig. 3 and Darling et al., 2004). However, we additionally find that Type IV consistently occurred at all stations along the OISO-19 transect (Fig. 3). The spatial distribution of Type IV is thus broader than previously assumed by Darling et al. (2004). As the lower temperature ranges of the sampled intervals at subpolar stations (Stations 7, 9, 10, cruise OISO-19) are close to the temperatures recorded at polar stations (Station 11, Table 2), some Type IV specimens may have been expatriated away from polar waters. Nevertheless, two specimens were collected from waters well above polar temperatures ($T \geq 7.3$ °C, Station 5, cruise OISO-19, Table 2), suggesting that Type IV can indeed survive outside the polar temperature range and thus may episodically expand to the north of polar waters.

At polar Station 11, Type III and IV were found together at depth intervals corresponding to virtually constant temperatures (Table 2), suggesting that these genetic types may indeed occur together at the margins of their ecological ranges. A more refined characterization of the ecological niches of these genetic types would be required to conclude to true sympatry. The data available from subpolar waters are not sufficient to determine

whether Type III and IV co-occurring at sub-polar stations exhibit depth-related distributions or occur together (Table 2).

### *4.2. A case of true cryptic diversity in* Neogloboquadrina pachyderma

Shells of Types II, III, and IV could not be differentiated using morphological characteristics such as size, number of chambers in the last whorl, or the occurrence of kummerform chambers. Since the three genetic types exhibit kummerform chambers, this morphology is not a diagnostic trait to discriminate the genetic types of *N. pachyderma*. There is no significant relationship between the abundance of individuals with kummerform chambers and ambient water temperature (Fig. 5), suggesting that it may not be an ecophenotypic feature either but rather an ontogenetic or reproductive feature. Olsson (1973) suggested that kummerform chambers result from a slowing of growth of chambers when individuals reach their growth limit. The mix of normalform and kummerfom individuals observed in our samples may thus result from a co-occurrence of mature individuals that have reached or not their growth limit with individuals still undergoing chamber formation.

Kennett (1968) observed variations in the number of chambers in the last whorl in *N. pachyderma* collected from core-top sediments of the South Pacific. In particular, he showed that the abundance of individuals with 4 chambers in the last whorl increased southward (Fig. 5). Möller et al. (2013) suggested that these variations may result from mutual biogeographic replacements between genetic types. In contrast, according to our data, the number of chambers in the last whorl is not a morphological feature restricted to one of the studied genetic types (Supplementary Table A.3). However, comparison of comprehensive morphological and ontogenetic stages between water-column and surface sediment

individuals would be required to demonstrate that observations on core-top samples from the southern Indian Ocean can be applied to samples of living specimens. We would also need more Type II and IV specimens to compare the ratio of individuals with 4 chambers on the last whorl between genetic types along the latitudinal transect.

In Type III, a relationship exists between SST and the abundance of individuals with 4 chambers in the last whorl ($r^2 = 0.52$, $p = 0.008$) suggesting that this morphological feature may be affected by environmental conditions (Fig. 5B). The relationship is similar to previous observations by Kennett (1968), with the number of chambers in the final whorl being related to latitude and SST (Fig. 5C). Shell size within a genetic type may also be affected by environmental conditions. In Type III specimens, slightly smaller shells occur at polar stations than at subpolar ones (Table 4, Fig.4). However, as only 9 Type IV individuals occurred outside of Station 11, our data are too scarce to statistically verify the relationship between SST and the abundance of Type IV individuals with 4 chambers in the last whorl or between SST and size.

Healy-William (1984) conducted the first morphological survey of *N. pachyderma* from the southern Indian Ocean, which encompasses the biogeographic ranges of the *N. pachyderma* genetic types identified between 38°S to 65 °S and described four morphotypes according to variation in shell outline observed from the studied specimens. Williams et al. (1988) suggested that these morphotypes might constitute distinct species or subspecies. Our data capture the same morphological variability but do not align with the genetic types (Fig. 6). This observation is in stark contrast with data on *Truncorotalia truncatulinoides* (Healy-Williams and Williams, 1981; Healy-Williams, 1983; Healy-Williams et al., 1985; de Vargas et al., 2001; Quillévéré et al., 2013) and *Globoconella inflata* (Morard et al., 2011; 2016b): in both morphospecies morphological variability and genetic identity are correlated. Therefore,

we conclude that the *N. pachyderma* morphotypes defined by Healy-Williams (1984) were representative of a morphological cline at the morphospecies level and not the result of morphological differences between genetic types.

The size of pores in *N. pachyderma* is around 1 µm, and is difficult to assess when concealed by organic matter (in living individuals) and crusts. Unlike other morphospecies of planktonic foraminifera such as *Globigerinella siphonifera* (Huber et al., 1997; Weiner et al., 2015) and *Orbulina universa* (Morard et al., 2009), we could not demonstrate that shell porosity in *N. pachyderma* might be of taxonomic value (Fig. 7).

## 5. Conclusions

In the southern Indian Ocean, the observed distribution patterns of Type II, Type III, and Type IV of *Neogloboquadrina pachyderma* are consistent with those previously observed by Darling et al. (2004) in the South Atlantic Ocean, but the spatial distribution of Type IV is broader than previously assumed. Whether this phenomenon is the result of hydrographic conditions or of a broader ecological niche of Type IV is unclear. Types II, III, and IV may occur together where their ecological boundaries overlap. Whether these genetic types are truly sympatric would require a more refined characterization of their ecological niches.

We conclude from our analyses that at least *N. pachyderma* Type III and Type IV constitute true cryptic species of planktonic foraminifera, since they cannot be distinguished from their shell morphology. As environmental conditions and/or geographic locations are also insufficient to predict the genetic type of a given specimen of *N. pachyderma* from the Southern Ocean, DNA analyses are the only means for unequivocal identification. As with *Trilobatus sacculifer* (André et al., 2013) and *Globigerinella siphonifera* (Weiner et al., 2015), the new data on *N. pachyderma* demonstrate that speciation in planktonic foraminifera

may not always be accompanied by morphological change. In *N. pachyderma*, the extensive morphological variability observed in the Indian Sector of the Southern Ocean does not match genetic identity, and cannot not be applied to the fossil record for paleoclimate reconstructions.

**Acknowledgements**

We thank L. Konecny for molecular biology assistance, E. Michel, L. Rossignol, M-H. Castera, and G. Isguder for assistance in sampling. We acknowledge the OISO (Océan Indien Service d'Observation) program to make the hydrographic data available to complete this study. Special thanks are given to the Institut Polaire Français Paul Emile Victor (IPEV) for the organization of the cruises during which the presented material has been collected. We also thank the Editor Richard Jordan and two anonymous reviewers for their comments on the manuscript. This work was supported by the National Research Program Tellus-INTERRVIE (FQ) of CNRS-INSU

**Appendices**

**Supplementary Table A. 1.** Sequences of *Neogloboquadrina pachyderma*

**Supplementary Table A. 2.** Elliptical Fourier descriptors and Crampton (1995) test.

**Supplementary Table A. 3.** Morphology (kummerform vs. normalform morphotypes) and number of chambers (4 to 5) on the last whorl of the sequenced and genotyped *Neogloboquadrina pachyderma* individuals.

**Supplementary Fig. A. 1**. Sequence alignment corresponding to the region of the SSU rRNA gene fragment used for the genotyping of Types II, III and IV. This region includes the foraminifera-specific variable regions 37/f and 41/f.

**Table 1.** Locations and dates of the sampling, with indications of the number of collected *Neogloboquadrina pachyderma* specimens

| Cruise | Station | Latitude (°S) | Longitude (°E) | Date | Water Depth (m) | *N. pachyderma* (collected) |
|---|---|---|---|---|---|---|
| OISO-19 | 5 | 42.5 | 52.48 | 01/21/11 | 0-20 | 0 |
| | | | | | 20-40 | 13 |
| | | | | | 40-60 | 15 |
| | | | | | 60-80 | 1 |
| | | | | | 80-100 | 1 |
| | | | | | 200-300 | 0 |
| | | | | | 300-400 | 2 |
| | | | | | 400-500 | 0 |
| | 7 | 47.7 | 58 | 01/23/11 | 0-20 | 22 |
| | | | | | 20-40 | 9 |
| | | | | | 40-60 | 66 |
| | | | | | 60-80 | 60 |
| | | | | | 80-100 | 1 |
| | | | | | 0-100 | 132 |
| | | | | | 100-200 | 2 |
| | | | | | 200-300 | 1 |
| | | | | | 300-400 | 6 |
| | | | | | 400-500 | 1 |
| | 9 | 48.5 | 65 | 01/26/11 | 0-20 | 64 |
| | | | | | 20-40 | 8 |
| | | | | | 40-60 | 23 |
| | | | | | 60-80 | 49 |
| | | | | | 80-100 | 14 |
| | | | | | 0-100 | 248 |
| | | | | | 100-200 | 11 |
| | | | | | 300-400 | 5 |
| | | | | | 400-500 | 5 |
| | 10 | 50.67 | 68.42 | 01/30/11 | 0-20 | 72 |
| | | | | | 20-40 | 21 |
| | | | | | 40-60 | 37 |
| | | | | | 60-80 | 29 |
| | | | | | 80-100 | 21 |
| | | | | | 0-100 | 398 |
| | | | | | 100-200 | 19 |
| | | | | | 200-300 | 8 |
| | | | | | 300-400 | 21 |
| | | | | | 400-500 | 8 |
| | 11 | 56.5 | 63 | 01/28/11 | 0-20 | 47 |
| | | | | | 20-40 | 71 |
| | | | | | 40-60 | 97 |
| | | | | | 60-80 | 87 |
| | | | | | 80-100 | 17 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 0-100 | 251 |
| | | | | | 100-200 | 18 |
| | | | | | 200-300 | 21 |
| | | | | | 300-400 | 7 |
| | | | | | 400-500 | 7 |
| OISO-21 | 6 | 45 | 52.1 | 01/31/12 | 0-100 | 54 |
| | 7 | 47.4 | 57.6 | 02/02/12 | 0-100 | 38 |
| | 8 | 48 | 60 | 02/02/12 | 0-100 | 125 |
| | 9 | 48.3 | 65 | 02/06/12 | 0-100 | 140 |
| | | | | | 300-500 | 6 |
| | 10 | 50.4 | 68.25 | 02/09/12 | 0-100 | 324 |
| | 11 | 56.3 | 63 | 02/07/12 | 0-100 | 189 |

**Table 2.** Number of genotyped (500) and sequenced (152) *Neogloboquadrina pachyderma*

| | | | | | Genotyped | | | Sequenced | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cruise | St. | Water depth (m) | T(°C) | Sampled | Ext. | II | III | IV | II | III | IV | Tot. id. | PCR success (%) |
| OISO-19 | 5 | 20-40 | [10.7 - 9.4] | 13 | 10 | 0 | 0 | 0 | 2 | 2 | 0 | 4 | 40 |
| | | 40-60 | [9.4 - 8.5] | 15 | 15 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 13 |
| | 7 | 0-100 | [7.5 - 3.9] | 132 | 80 | 0 | 0 | 2 | 1 | 17 | 0 | 20 | 25 |
| | 9 | 60-80 | [5.0 - 3.7] | 49 | 2 | 0 | 1 | 0 | 0 | 1 | 0 | 2 | 100† |
| | | 0-100 | [6.2 - 2.8] | 248 | 76 | 0 | 21 | 1 | 0 | 19 | 0 | 41 | 54 |
| | | 100-200 | [2.6 - 2.3] | 11 | 5 | 0 | 0 | 0 | 0 | 4 | 0 | 4 | 80† |
| | | 400-500 | [2.3 - 2.4] | 5 | 5 | 0 | 0 | 0 | 0 | 5 | 0 | 5 | 100† |
| | 10 | 0-100 | [4.8 - 3.1] | 398 | 65 | 0 | 10 | 1 | 0 | 4 | 0 | 15 | 23 |
| | | 200-300 | [2.3 - 2.2] | 8 | 8 | 0 | 0 | 0 | 0 | 2 | 1 | 3 | 37† |
| | | 300-400 | [2.3 - 2.2] | 21 | 20 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 10 |
| | | 400-500 | [2.3 - 2.2] | 8 | 8 | 0 | 0 | 0 | 0 | 4 | 0 | 4 | 50† |
| | 11 | 0-20 | [2.3 - 2.2] | 47 | 31 | 0 | 10 | 2 | 0 | 0 | 0 | 12 | 39 |
| | | 20-40 | [2.2 - 2.1] | 71 | 29 | 0 | 6 | 2 | 0 | 3 | 0 | 11 | 38 |
| | | 40-60 | [2.1 - 2.0] | 97 | 97 | 0 | 16 | 20 | 0 | 24 | 33 | 93 | 96 |
| | | 60-80 | [2.0 - 1.9] | 87 | 27 | 0 | 11 | 11 | 0 | 0 | 0 | 22 | 81 |
| | | 0-100 | [1.6 - 2.3] | 251 | 15 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 13 |
| OISO-21 | 6 | 0-100 | [8.5 - 3.7] | 54 | 53 | 1 | 17 | 0 | 1 | 10 | 0 | 29 | 55 |
| | 7 | 0-100 | [7.5 - 5.3] | 38 | 38 | 5 | 16 | 0 | 4 | 3 | 0 | 28 | 74 |
| | 8 | 0-100 | [8.2 - 4.8] | 125 | 62 | 2 | 41 | 0 | 0 | 0 | 0 | 44 | 71 |
| | 9 | 0-100 | [5.9 - 2.3] | 146 | 113 | 0 | 105 | 0 | 0 | 0 | 0 | 105 | 93 |
| | 10 | 0-100 | [4.7 - 3.5] | 324 | 113 | 0 | 109 | 0 | 0 | 2 | 0 | 111 | 98 |
| | 11 | 0-100 | [2.6 - 2.5] | 189 | 103 | 0 | 71 | 17 | 0 | 1 | 5 | 94 | 93 |

St. = Station. T = temperature range. Sampled = number of specimens in GITC* buffer. Ext. = number of specimens whose DNA has been extracted. Tot id = number of *N. pachyderma* with genetic type identification
†PCR success rate based on a very small number of DNA extractions

**Table 3**. Tests for size differences between *Neogloboquadrina pachyderma* Type III and Type IV from Station 11

| | OISO-19 | | | | | OISO-21 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Kolmogorov-Smirnov test | | | Mann-Whitney test | | Kolmogorov-Smirnov test | | | Mann-Whitney test | |
| | D | p | p (perm) | p | p (perm) | D | p | p (perm) | p | p (perm) |
| Dmax | 0.18 | 0.66 | 0.62 | 0.28 | 0.27 | 0.34 | 0.18 | 0.18 | 0.22 | 0.22 |
| Dmin | 0.24 | 0.28 | 0.27 | 0.29 | 0.30 | 0.36 | 0.13 | 0.13 | 0.26 | 0.27 |
| Dmean | 0.25 | 0.25 | 0.23 | 0.27 | 0.27 | 0.52 | 0.29 | 0.26 | 0.52 | 0.53 |

**Table 4.** Test for size differences of *Neogloboquadrina pachyderma* Type III between the subpolar (Stations 7 to 10) and the polar (Station 11) stations

| | Kolmogorov-Smirnov test | | | Mann-Whitney test | |
|---|---|---|---|---|---|
| | D | p | p (perm) | p | p (perm) |
| OISO-19 | 0.48 | $5.9\ 10^{-5}$ | 0.0002 | $3.2\ 10^{-5}$ | 0.0002 |
| OISO-21 | 0.31 | 0.005 | 0.004 | $4.2\ 10^{-4}$ | 0.0002 |

Type III and Type IV of *N. pachyderma* constitute true cryptic species.

New protocol for rapid identification of genetic types of *N. pachyderma*.

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**Figure captions**

**Fig. 1.** Geographic location of the oceanic stations sampled during the OISO cruises. Black stars correspond to stations sampled twice in 2011 and 2012, white stars correspond to stations sampled in 2011 only, and gray dots to stations sampled in 2012 only. Blue shading indicates the relative abundance of *Neogloboquadrina pachyderma* in planktonic foraminiferal assemblages from surface sediments, interpolated from the ForsCenS database (Siccha and Kucera, 2017). Black solid lines delineate the oceanic fronts (Meilland et al., 2017).

**Fig. 2.** Procedure for direct and RFLP genotyping of austral genetic types of *Neogloboquadrina pachyderma*. The region of rDNA corresponds to the last ~1000 bp of the SSU. Names of variable regions after Pawlowski and Lecrocq (2010).

**Fig. 3** A: Genetic types of *Neogloboquadrina pachyderma* recovered along latitudinal vertical profiles of temperature (°C) and fluorescence (volts) corresponding to the OISO transects. The number of sequenced/genotyped individuals and relative abundances (%) of each genetic type are given for each station, positioned along latitudes. STZ: Sub-Tropical Zone; SAZ: Sub-Antarctic Zone; PFZ: Polar Frontal Zone; AZ: Antarctic Zone (Ikeda et al., 1989). See figure 1 for details on the geographic location of the transects. The environmental diagrams were drawn using Ocean Data View V.4 (Schlitzer, 2011). B. distribution of genetic types of *N. pachyderma* in the South Atlantic Ocean according to Darling et al. (2004). The environmental diagrams were drawn using Ocean Data View V. 4. (Schiltzer, 2011) using the annual SST extracted from the World Ocean Atlas 2013 (Locarnini et al., 2013).

**Fig. 4.** A: Size comparison of *Neogloboquadrina pachyderma* Type III and Type IV (Dmax: maximum diameter; Dmin: minimum diameter; Dmea: mean diameter) as measured from Station 11 during the cruises OISO-19 (2011) and OISO-21 (2012). B: size comparison of the maximum diameter (Dmax) of *N. pachyderma* Type III between subpolar (Stations 7, 9, 10) and polar stations (Station 11) during the cruises OISO-19 and OISO-21. Box plots (Tuckey, 1977) show median values (central bar), upper and lower quartiles, and extremes of the data (whiskers). Data points are shown by markers.

**Fig. 5.** A: Abundance of kummerform specimens against SST. B: abundance of specimens with 4 chambers in the last whorl according to SST. C: abundance of specimens with 4

chambers in the last whorl according to latitude among *Neogloboquadrina pachyderma* from core-top samples of the South Pacific Ocean (data from Kennett [1968]) and among *N. pachyderma* sampled with plankton nets during cruises OISO-19 and OISO-21.

**Fig. 6.** Principal Component Analysis performed on the normalized Fourier descriptors data set extracted from the shell outlines of *Neogloboquadrina pachyderma* (umbilical views, TIII = Type III, TIV = Type IV). The outlines of some selected individuals are shown in gray on the graph showing the 1$^{st}$ principal component (PC1) against the 2$^{nd}$ principal component (PC2). The values of PC1 and PC2 are plotted against sea surface temperature (SST) and maximum diameter of the individuals (Max Diameter). Linear regressions are shown in dashed lines.

**Fig. 7.** SEM photomicrographs of representatives of Types II, III and IV of *Neogloboquadrina pachyderma* collected during the cruise OISO-19 (2011). The Type II specimen may be a juvenile. St = sampling station from which the specimens were collected; III a-b: photomicrographs of the shell ultrastructure of Type III specimens. IV a-b: photomicrographs of the shell ultrastructure of Type IV specimens.

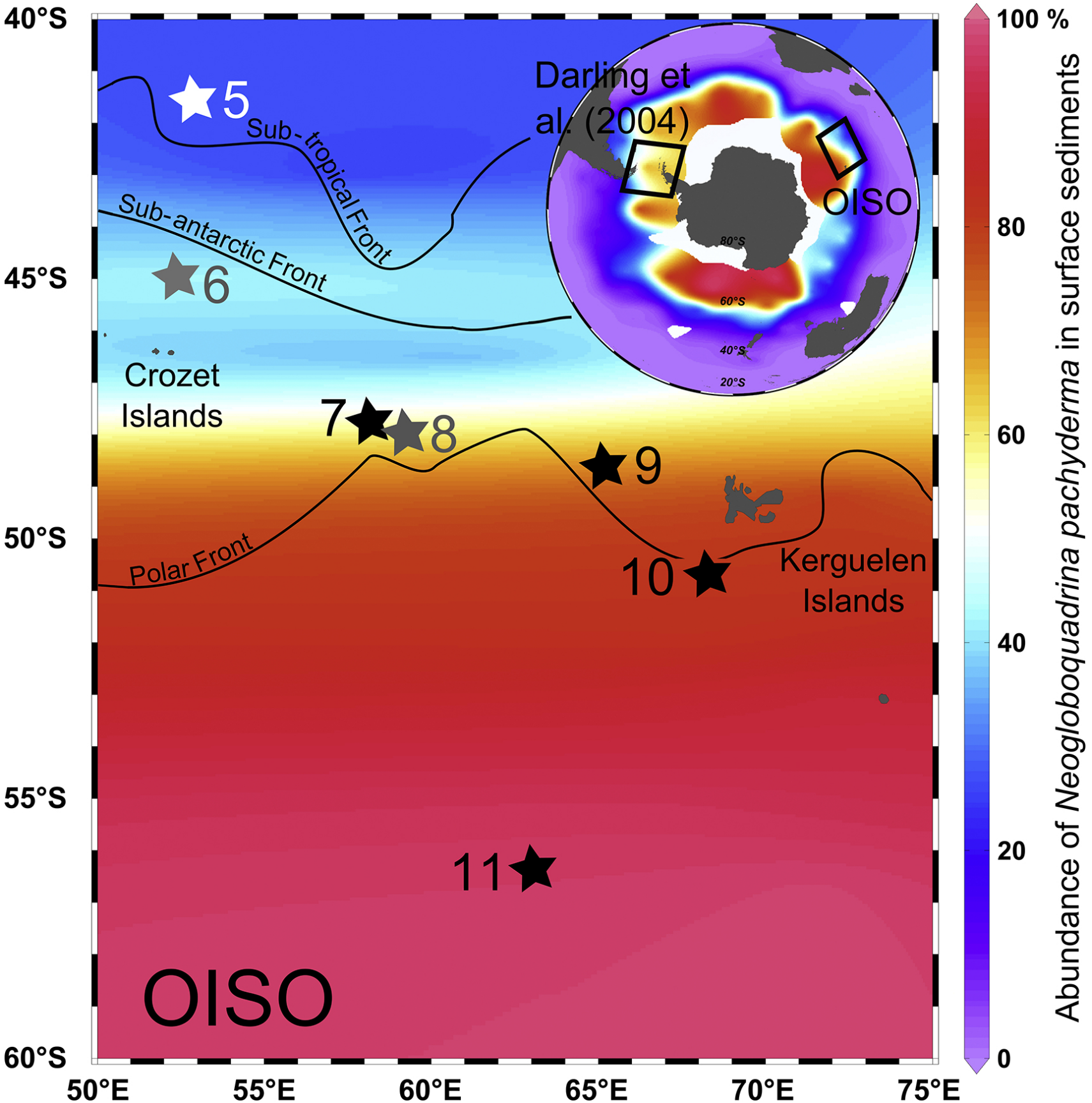
40°S
45°S
50°S
55°S
60°S
50°E
55°E
60°E
65°E
70°E
75°E
5
6
7
8
9
10
11
Sub-tropical Front
Sub-antarctic Front
Polar Front
Crozet Islands
Kerguelen Islands
OISO
Darling et al. (2004)
OISO
80°S
60°S
40°S
20°S
100 %
80
60
40
20
0
Abundance of Neogloboquadrina pachyderma in surface sediments

Figure 1

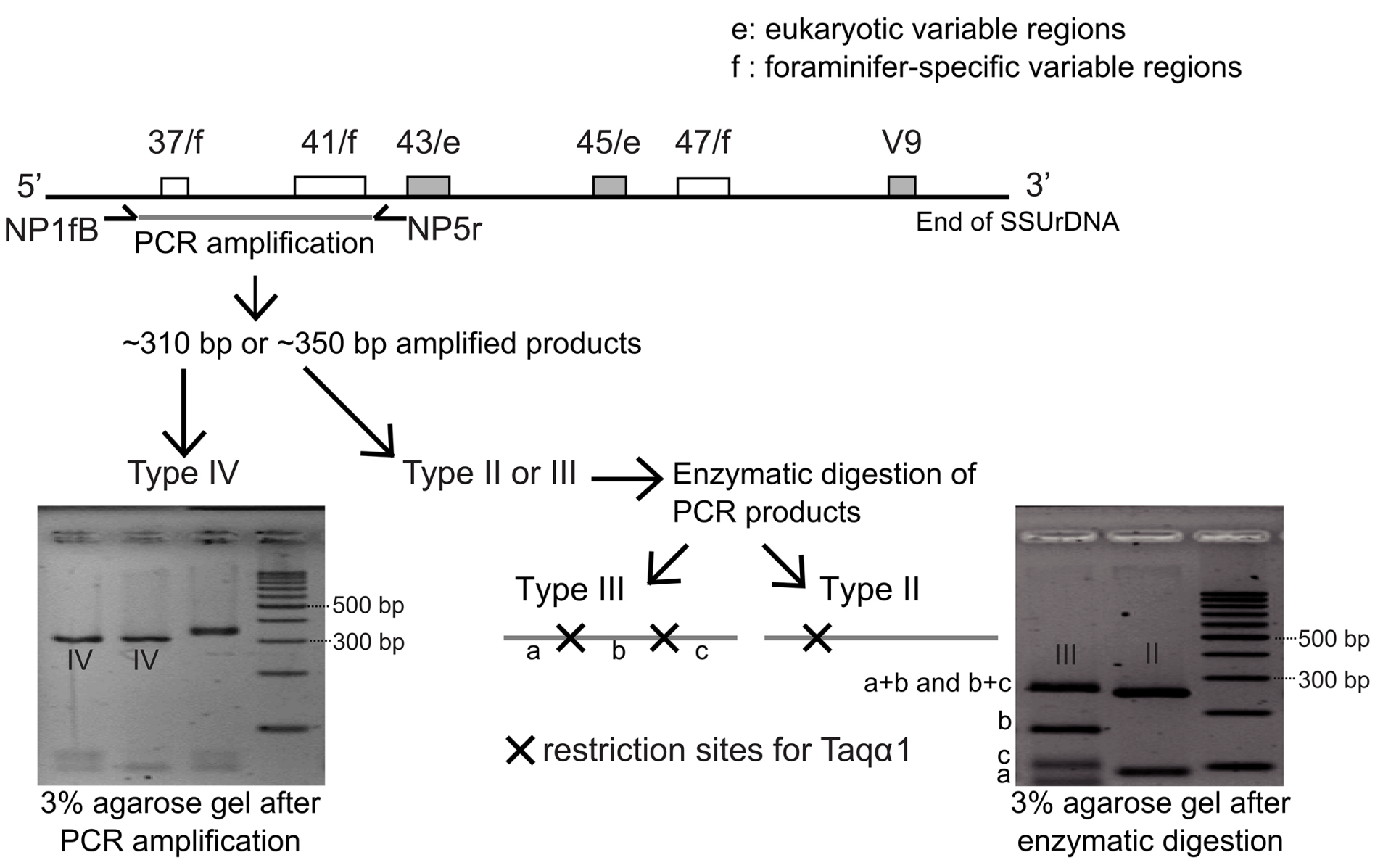
e: eukaryotic variable regions
f : foraminifer-specific variable regions
37/f
41/f
43/e
45/e
47/f
V9
5'
3'
NP1fB
PCR amplification
NP5r
End of SSUrDNA
~310 bp or ~350 bp amplified products
Type IV
Type II or III
Enzymatic digestion of PCR products
Type III
Type II
a
b
c
restriction sites for Taqα1
IV
IV
500 bp
300 bp
3% agarose gel after PCR amplification
III
II
a+b and b+c
b
c
a
500 bp
300 bp
3% agarose gel after enzymatic digestion

Figure 2

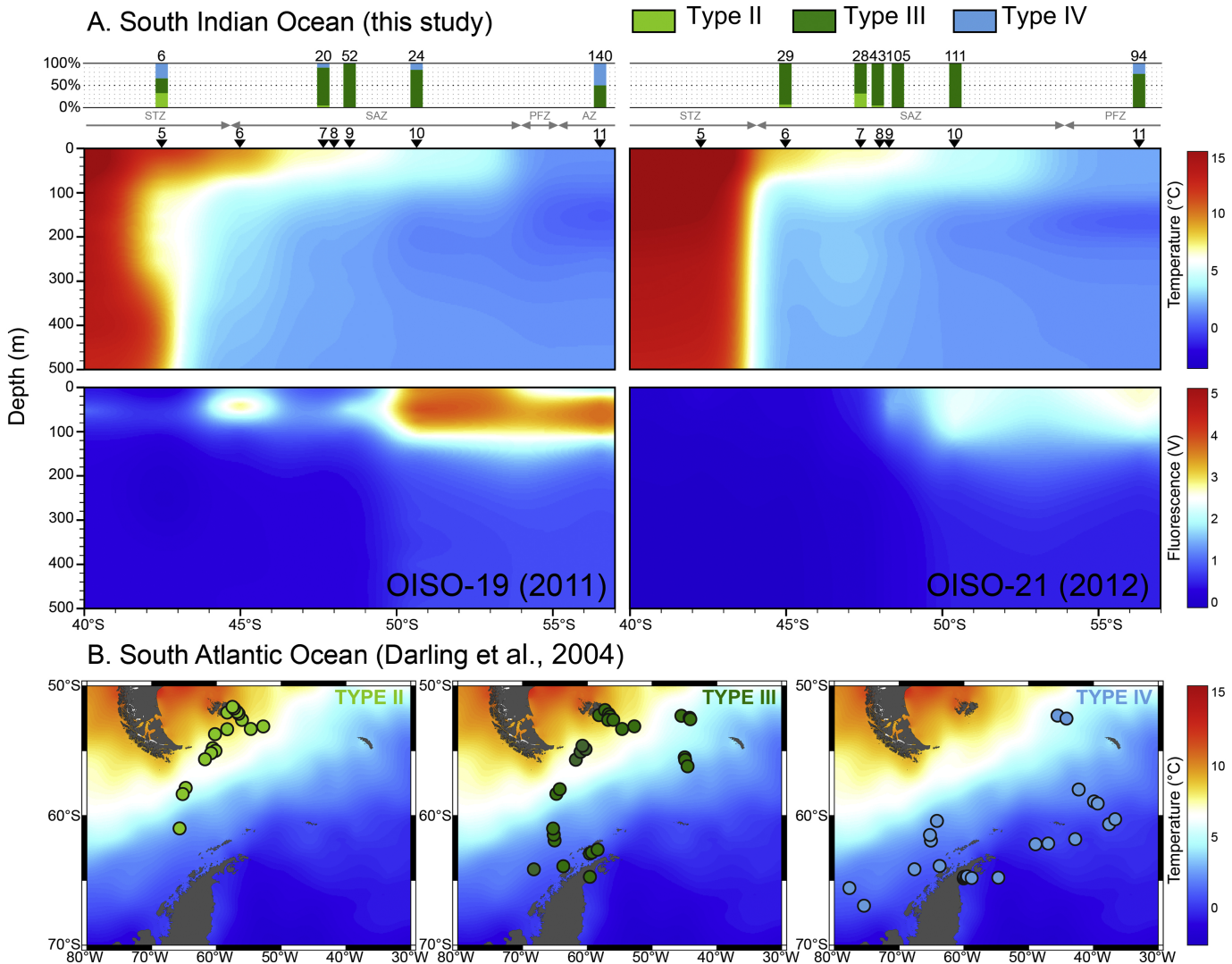
A. South Indian Ocean (this study)
Type II
Type III
Type IV
100%
50%
0%
6
20
52
24
140
29
28
43
105
111
94
STZ
SAZ
PFZ
AZ
5
6
7
8
9
10
11
Depth (m)
0
100
200
300
400
500
Temperature (°C)
15
10
5
0
Fluorescence (V)
5
4
3
2
1
0
OISO-19 (2011)
OISO-21 (2012)
40°S
45°S
50°S
55°S
B. South Atlantic Ocean (Darling et al., 2004)
TYPE II
TYPE III
TYPE IV
50°S
60°S
70°S
80°W
70°W
60°W
50°W
40°W
30°W

Figure 3

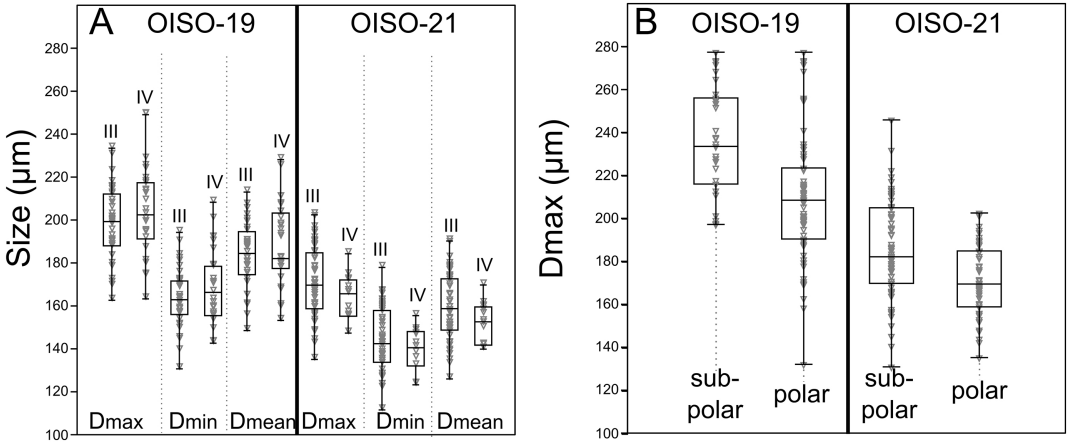
A
OISO-19
OISO-21
Size (µm)
280
260
240
220
200
180
160
140
120
100
III
IV
Dmax
Dmin
Dmean
B
OISO-19
OISO-21
Dmax (µm)
sub-polar
polar

Figure 4

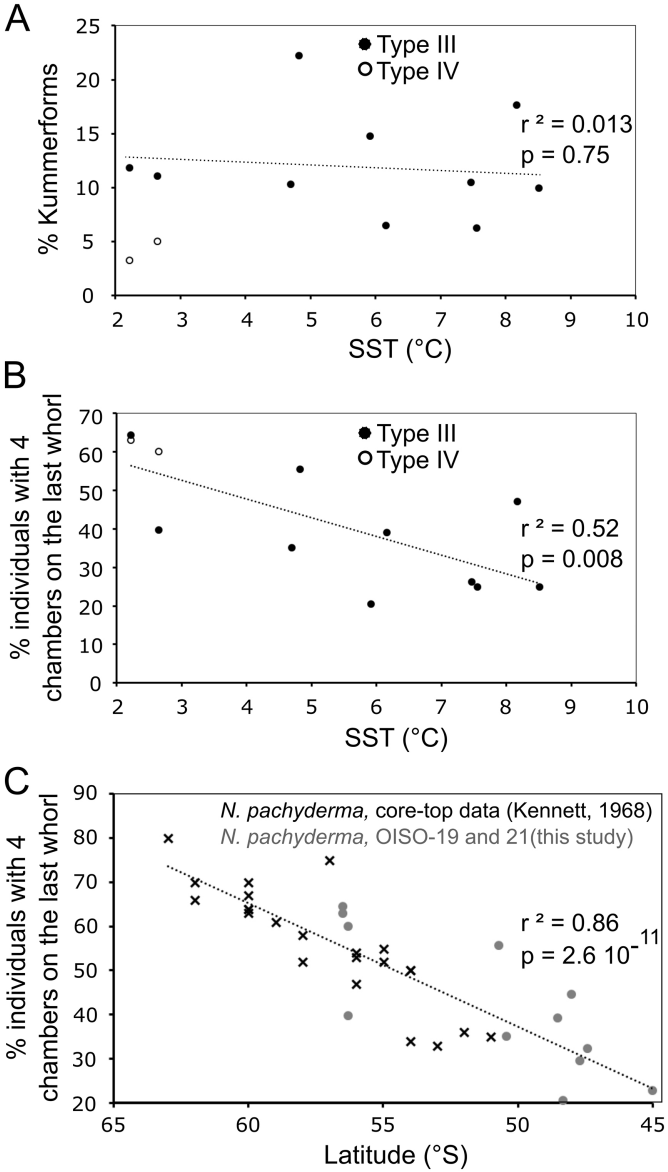
A
% Kummerforms
SST (°C)
Type III
Type IV
r ² = 0.013
p = 0.75
B
% individuals with 4 chambers on the last whorl
SST (°C)
Type III
Type IV
r ² = 0.52
p = 0.008
C
% individuals with 4 chambers on the last whorl
Latitude (°S)
N. pachyderma, core-top data (Kennett, 1968)
N. pachyderma, OISO-19 and 21(this study)
r ² = 0.86
p = 2.6 10-11

Figure 5

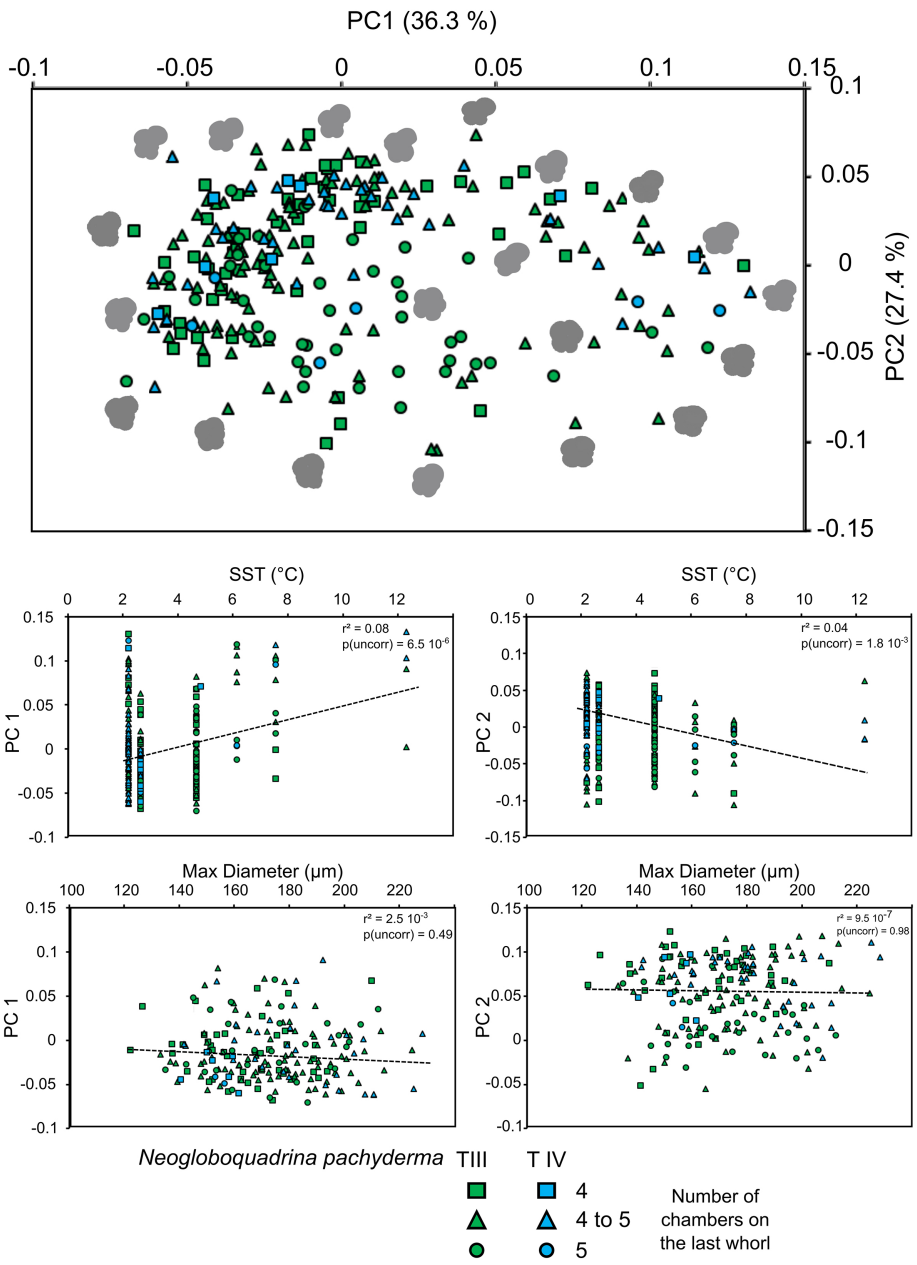
PC1 (36.3 %)
PC2 (27.4 %)
SST (°C)
r² = 0.08
p(uncorr) = 6.5 10-6
PC 1
SST (°C)
r² = 0.04
p(uncorr) = 1.8 10-3
PC 2
Max Diameter (µm)
r² = 2.5 10-3
p(uncorr) = 0.49
PC 1
Max Diameter (µm)
r² = 9.5 10-7
p(uncorr) = 0.98
PC 2
Neogloboquadrina pachyderma
TIII
T IV
4
4 to 5
5
Number of chambers on the last whorl

Figure 6

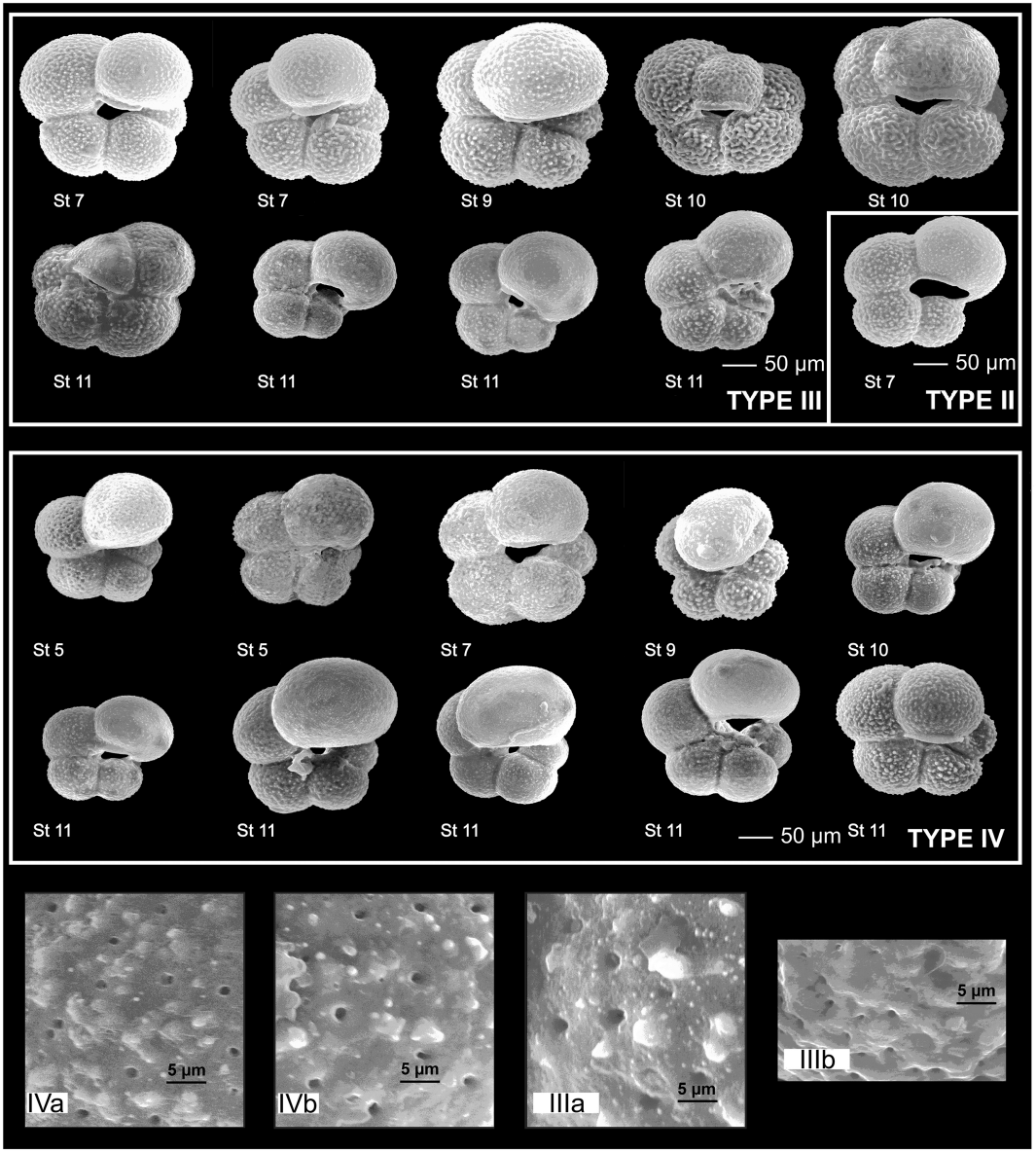
St 7
St 7
St 9
St 10
St 10
St 11
St 11
St 11
St 11
50 µm
TYPE III
St 7
50 µm
TYPE II
St 5
St 5
St 7
St 9
St 10
St 11
St 11
St 11
St 11
50 µm
St 11
TYPE IV
5 µm
IVa
5 µm
IVb
5 µm
IIIa
5 µm
IIIb

Figure 7

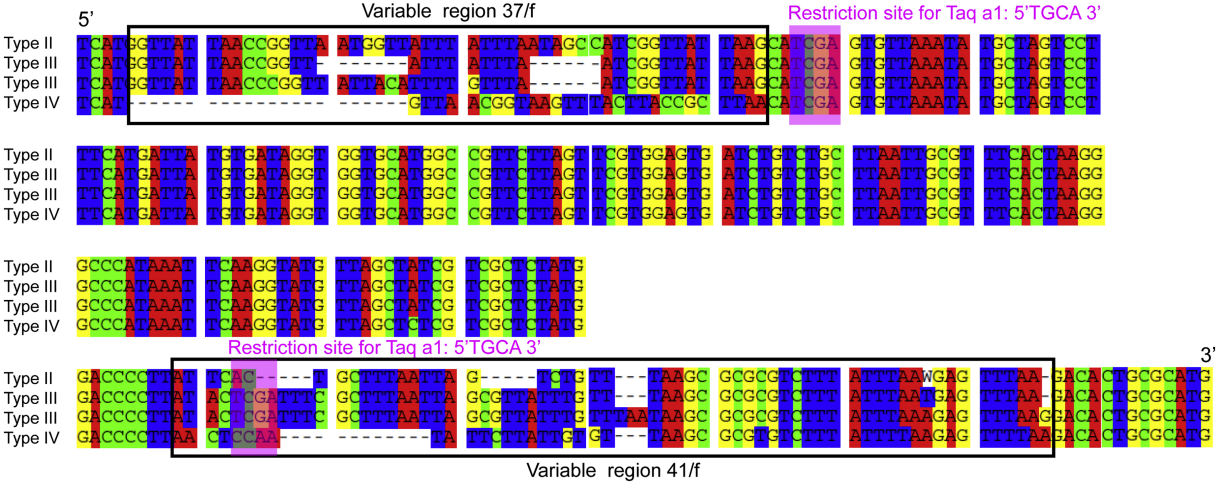
5'
Variable region 37/f
Restriction site for Taq a1: 5'TGCA 3'
Type II
Type III
Type III
Type IV
Type II
Type III
Type III
Type IV
Type II
Type III
Type III
Type IV
Restriction site for Taq a1: 5'TGCA 3'
3'
Type II
Type III
Type III
Type IV
Variable region 41/f

Figure 8