For a better characterization of the fossil pelagic record : molecular, biogeographical and ecological diversity of planctonic foraminifers cryptic species

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Pour une meilleure caractérisation du registre fossile pélagique : Diversité morphologique, biogéographie et écologie des espèces cryptiques de foraminifères planctoniques

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La science ne sert guère qu'à nous donner une idée de l'étendue de notre ignorance.

Félicité de Lamennais

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Résumé

L'utilisation des coquilles carbonatées de foraminifères planctoniques comme marqueurs paléocéanographiques repose sur l'hypothèse fondamentale que chaque espèce morphologique correspond à une espèce biologique caractéristique d'un habitat spécifique. Cette relation empirique a été récemment remise en cause par des analyses moléculaires qui ont révélé la présence systématique de plusieurs espèces génétiques (espèces cryptiques) au sein des morpho-espèces actuelles. Il a été suggéré que ces espèces cryptiques ou génotypes, présentaient (1) des préférences biogéographiques et écologiques restreintes par rapport à leur morpho-espèce respective et (2) des différences morphologiques.

Dans ce travail, nous avons caractérisé la diversité génétique, morphologique et écologique de 4 morpho-espèces clefs de la paléocéanographie, i.e. *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* et *Globigerina bulloides.* Cette étude repose sur le développement d'un protocole d'extraction ADN non destructif pour la coquille calcaire, permettant l'analyse conjointe de la variabilité génétique et morphologique d'un même individu. Les variations de forme ou de porosité de chacun des génotypes des morpho-espèces ont été quantifiées. Il apparaît que le fort degré de plasticité morphologique largement documenté chez les foraminifères planctonique et jusqu'alors interprété comme écophénotypique, est au moins en partie la conséquence du regroupement de plusieurs génotypes présentant des morphologies et préférences écologique particulières. Sur la base des ces observations, des modèles de reconnaissance morphologique permettant d'identifier les génotypes à partir de la morphologie de la coquille, utilisables à l'échelle populationnelle, ont été développés. Afin de quantifier l'impact de l'intégration de la diversité cryptique dans les reconstitutions paléocéanographiques basées sur les assemblages de foraminifères planctoniques, les fonctions de transfert couramment utilisées ont été re-calibrées en intégrant les distributions restreintes des génotypes d'*O. universa*, *T. truncatulinoides*, *G. inflata* et *G. bulloides*. Ces re-calibrations conduisent à un degré de précision jusqu'alors jamais atteint dans les reconstructions paléocéanographiques basées sur les assemblages de foraminifères planctoniques.

Mots clefs : Foraminifères planctoniques, diversité cryptique, SSU rDNA, ITS rDNA, biogéographie, morphométrie, fonctions de transfert.

Abstract

The usefulness of calcareous shells of planktonic foraminifera as a paleoceanographic proxy relies on the key hypothesis that each morphospecies corresponds to a biological species with a specific habitat. This empirical relationship has been challenged since molecular analyses have revealed a significant level of cryptic genetic diversity among modern morphospecies of planktonic foraminifera. Previous workers have suggested that the cryptic species or genotypes (1) display narrower biogeographic and ecological ranges than their related morphospecies, and (2) exhibit shell morphological differences.

In this work, we have characterized the genetic, morphological and ecological diversity among four planktonic foraminiferal morphospecies of significance in paleoceanography, i.e. *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides*. Our study relies on the development of a new single-cell DNA extraction protocol that retains the shell, allowing direct morpho-genetic comparisons. Shape or porosity variations within each genotype have been quantified. It appears that the high degree of morphological plasticity widely documented in planktonic foraminifera and classically seen as ecophenotypy, is at least partly the spurious consequence of lumping several genotypes that display morphological and environmental preferences. Based on these observations, we developed several population-scale models, which allow recognition of the cryptic species based on their shell morphology. Finally, in order to quantify the impact of integrating cryptic diversity in assemblage-based paleoceanographic reconstructions, we have re-calibrated transfer functions based on the ecological ranges of the genotypes of *O. universa*, *T. truncatulinoides*, *G. inflata* and *G. bulloides* in the southern oceans. Such re-calibrations led to a great, previously never reached improvement in the accuracy of the assemblage-based paleoceanographic reconstructions.

Keywords: Planktonic foraminifera, cryptic diversity, SSU rDNA, ITS rDNA, biogéography, morphometry, Transfer function.

Sommaire

Introduction

Les foraminifères sont des protistes à paroi organique ou coquille carbonaté (test) mono- ou pluriloculaire. Le cytoplasme est différencié en un endoplasme inclu dans la coquille et un réseau de pseudopodes réticulés à l'extérieur (Schiebel et Hemleben, 2005). Ces organismes ont colonisé tous les milieux marins et également les eaux douces (Pawlowski et al., 1999). Cinq mille morpho-espèces ont été décrites dans l'Actuel, différenciées en 12 sous-ordres reconnus sur la base de la structure et la composition de la coquille (Armstrong et Brasier, 2005).

Les foraminifères ont attiré très tôt l'attention des naturalistes. Hérodote les observa dans les pyramides d'Egypte en 500 avant J. C. et Alcide d'Orbigny (1802-1857) décrivit 552 espèces, en grande partie dans les formations sédimentaires du bassin de Paris. Depuis ces travaux précurseurs, quelques 60 000 espèces fossiles ont été décrites faisant de ces organismes un outil de choix pour la biostratigraphie et la paléoécologie. De fait, la taxonomie en vigueur chez les foraminifères découle de la tradition paléontologique avec une définition **morphologique** de l'espèce. C'est par un ensemble de caractères typologiques observés sur un individu référent (l'holotype) qu'une espèce morphologique est définie. Cette définition s'applique aussi bien aux fossiles qu'aux représentants actuels des foraminifères.

Les foraminifères planctoniques

Les foraminifères planctoniques (Globigerinina) sont caractérisés par un test calcitique hyalin multiloculaire. Ils vivent exclusivement dans la colonne d'eau océanique et possèdent un cycle de vie sexué (Hemleben et al., 1989). Leur croissance débute par une loge initiale, appelée proloculus, et se poursuit par l'addition successive de loges constituant une coquille planispiralée, sériée (bi- ou trisériée) ou trochospiralée (Schiebel et Hemleben, 2005). Les premiers représentants des foraminifères planctoniques font leur apparition au Jurassique inférieur (Hart et al., 2003). Les foraminifères ont connu quatre périodes de diversification entrecoupées de 3 épisodes d'extinction massive : à la limite Cénomanien/Turonien (93,5 Ma), Crétacé/Paléogène (65 Ma) et Eocène/Oligocène (36,6 Ma ; Banner et Lowry, 1985).

Systématiquement, une phase de re-diversification succéda à chaque épisode d'extinction à partir de quelques espèces globuleuses de taille réduite. Les mêmes motifs architecturaux ont été exploités par les foraminifères planctoniques au cours de ces radiations (formes globuleuses et comprimées, carènes, pustules), attestant d'un fort conservatisme génétique (Norris, 1991).

Les foraminifères actuels sont regroupés en sept superfamilles distinctes dont trois principales : Globigerinoidea, Globorotaloidea, Heterohelicoidea.

Les Globigerinoidea (19 morpho-espèces actuelles ; Darling et Wade, 2008) sont des foraminifères planctoniques macroperforés **épineux** caractérisés par des formes trochospiralées ou quasi planispiralées globuleuses (Schiebel et Hemleben, 2005). Ces morpho-espèces capturent des dinoflagellés ou des chrysophycées dans la colonne d'eau au cours de leur croissance afin de réaliser la photosymbiose (Spero, 1991). Ils peuvent également être hétérotrophes en capturant des métazoaires (copépodes, ptéropodes ou ostracodes ; Caron et Bé 1984) d'autres foraminifères ou même des bactéries (Schiebel et Hemleben, 2005). Toutes les morpho-espèces épineuses peuvent recourir à ces deux modes de nutrition pour assurer leur survie à l'exception de *Globigernia bulloides,* strictement hétérotrophe (Hemleben et al., 1989). La plupart de ces morpho-espèces dépendent de la luminosité pour leur survie, et occupent donc la zone photique (0 à 100 m).

Les Globorotaloidea (18 morpho-espèces actuelles ; Darling et Wade, 2008) sont des foraminifères planctoniques macroperforés **non épineux** caractérisés par des formes trochospiralées ou dans un cas streptospiralée (*Pulleniatina obliquiloculata*). Ces foraminifères sont strictement hétérotrophes et se nourrissent principalement d'algues (diatomés, dinoflagellés, algues eucaryotes ; Anderson et al., 1979) et parfois de matière organique en suspension pour l'espèce *Globorotalia scitula*(Itou et al., 2001).

Les Heterohelicoidea sont des foraminifères planctoniques microperforés non épineux et sont représentés, dans l'Actuel, par une espèce bisériée (*Streptochilus globigerus*) et une triseriée (*Gallitellia vivans*) (Schiebel et Hemleben, 2005).

Les autres superfamilles sont représentées par 3 genres microperforés (*Globigerinita*, *Tenuitella* et *Candeina*) qui diffèrent par la structure du test et l'arrangement des loges, ainsi que par l'unique genre possédant un test monolamellaire (*Hastigerinella* ; Schiebel et Hemleben, 2005)

En fonction de leurs stratégies trophiques, ces différents groupes de foraminifères occupent des positions différentes dans la colonne d'eau, stratifiant ainsi les communautés. Les foraminifères épineux, largement dépendant de la présence de lumière occupent principalement la zone photique (0-100 m) alors que les Globorotaloidea occupent préférentiellement les couches d'eau plus profonde riches en éléments nutritifs (Ravelo et Fairbanks, 1990)

Tous les foraminifères planctoniques ont un cycle de vie sexué. Les espèce de surface ont un cycle de reproduction lunaire à semi-lunaire (Schiebel et al., 1997), alors que les espèces vivant dans les eaux plus profondes ont un cycle plus long pouvant aller jusqu'à un an par exemple pour la morpho-espèce *Truncorotalia truncatulinoides* (Hemleben et al., 1989).

Ecologie et Biogéographie

La majorité des connaissances sur la biogéographie et l'écologie des foraminifères planctoniques dérive de l'étude d'individus prélevés dans les sédiments de surface de l'Océan mondial. En effet, la difficulté de récolter des individus vivant et de les maintenir en culture ainsi que l'impossibilité de reproduire un cycle de vie complet en laboratoire ont limité les approches directes pour évaluer les facteurs biotiques ou abiotiques contrôlant leur distribution (Hemleben et al., 1989). Au regard des données issues de l'étude des sédiments, la répartition des morpho-espèces de foraminifères planctoniques semble être contrôlée par la température des eaux de surface (Bé, 1977). Cinq provinces fauniques ont été reconnues et sont caractérisées par des assemblages spécifiques : polaire, subpolaire, transition (tempéré), subtropical et tropical (Bé, 1977; Figures 1 et 2). Des expériences conduites par Bijma et al. (1990) ont démontré que les fonctions vitales des foraminifères sont inhibées quand les limites de températures naturelles des morpho-espèces sont dépassées, renforçant la thèse du contrôle de la distribution par la température. De plus, les mêmes morpho-espèces sont présentes de part et d'autre de l'équateur lorsqu'elles sont séparées par la masse d'eau tropicale. Cette distribution est qualifiée de bipolaire.

Néanmoins, il serait simpliste d'attribuer la répartition des foraminifères aux seules températures des eaux de surface (SST). Le milieu océanique est un environnement complexe en changement permanent. Les océans sont constitués d'une mosaïque de masses d'eaux aux

propriétés physico-chimiques variées qui sont en contact dans les zones frontales de convergence. La structure verticale de l'Océan évolue également selon la latitude ou au fil des saisons. Ce milieu en évolution permanente crée constamment des niches écologiques potentielles pour les foraminifères planctoniques.

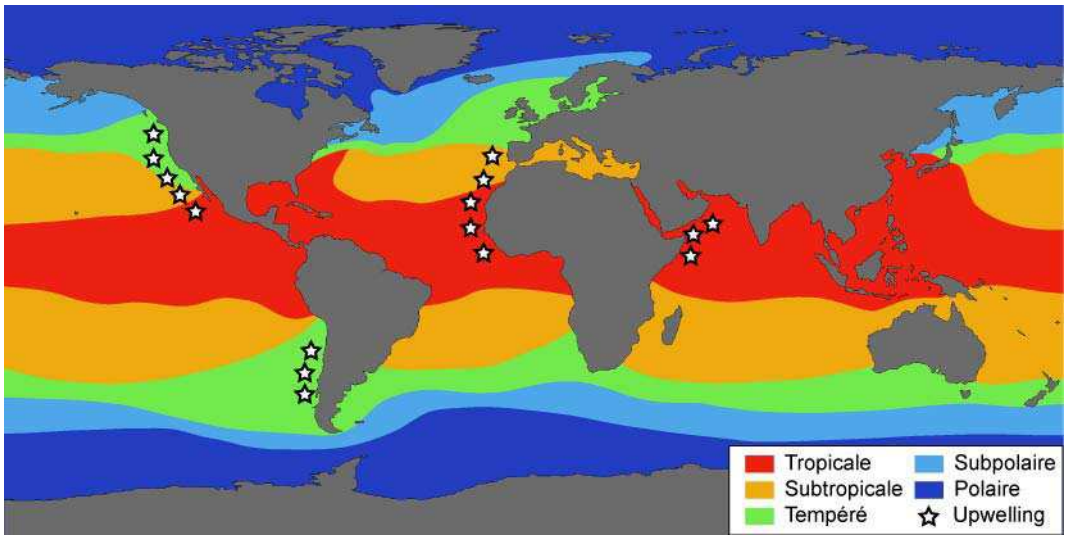

Figure 1 : Répartition géographiques des 5 provinces biogéographiques des foraminifères planctoniques (d'après Bé, 1977).

Cependant, prendre ces paramètres en compte est quasiment impossible en étudiant des spécimens en provenance des sédiments. En effet, les premiers millimètres de sédiments représentent des dizaines voir des centaines d'années de flux de tests en provenance des eaux de surface (Arnold et Parker, 1999). L'information potentielle contenue dans ces assemblages est donc condensée rendant très difficiles et aléatoires les comparaisons directes avec les conditions de surface. Pour palier à ces difficultés, il a été nécessaire de se doter de nouveaux outils pour étudier l'écologie des foraminifères. Une avancée majeure est venue de l'élaboration des filets stratifiés (MOCNESS : Multiple Opening Closing Net Environmental System ; Wiebe et al., 1985), capables de segmenter les collections de foraminifères et de rendre compte de la stratification verticale des communautés actuelles (Ravelo et Faibanks, 1990). Il a été possible d'évaluer précisément les profondeurs de vie des morpho-espèces de foraminifères et leurs préférences écologiques. De plus, la conception de pièges à sédiments, immergés en mer à des profondeurs variables pour quantifier les flux particulaires de surface, a permis d'évaluer les saisons d'occurrence des foraminifères planctoniques, ainsi que leur abondance (Northcote et Neil, 2005). Fort de ces nouveaux outils, l'écologie des

foraminifères est aujourd'hui mieux connue, fournissant des clefs de lecture du registre fossile.

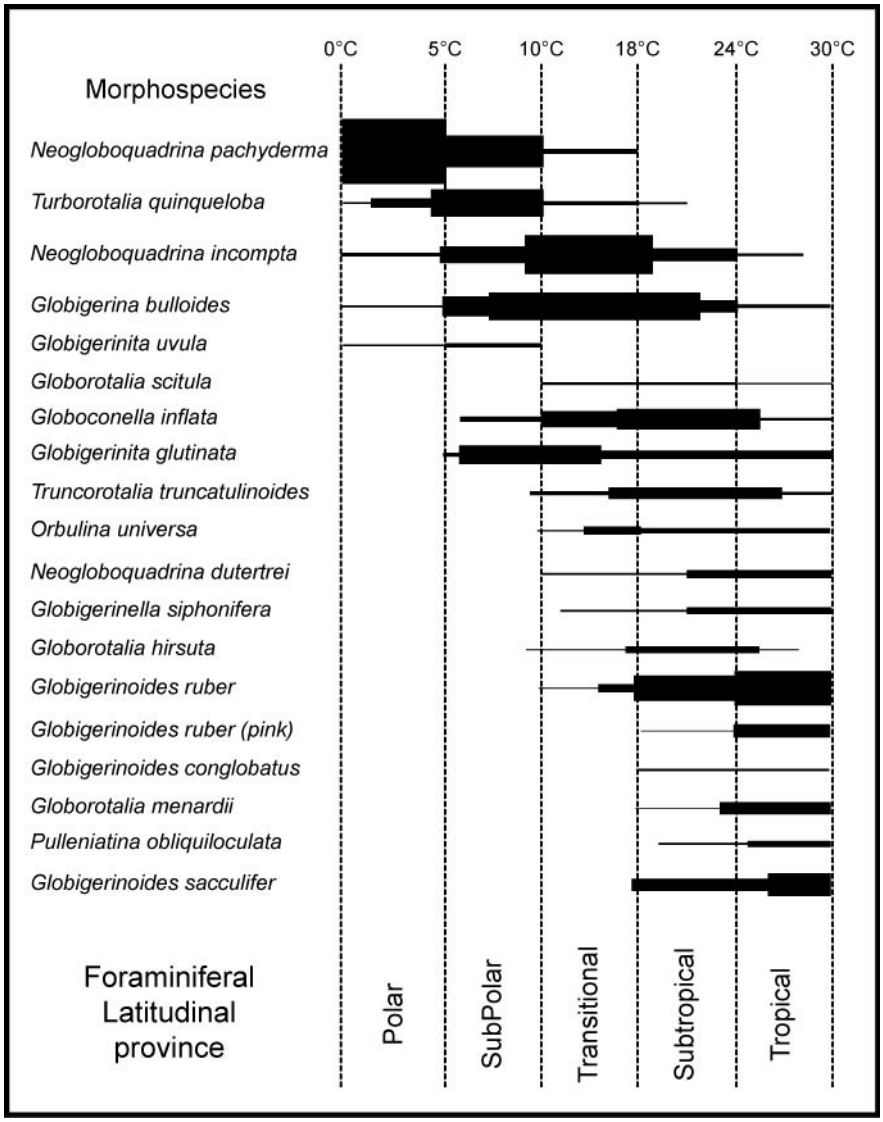

Figure 2 : Distribution des assemblages de morpho-espèces de foraminifères planctoniques dans les 5 provinces fauniques majeures (modifié de Bé et Tolderlund, 1971). Les proportions relatives des barres sont dérivées des abondances des morpho-espèces les plus communes dans les sédiments de surface de l'Atlantique (Voir Darling et al., 2008 pour les détails).

Bilan fossile et intérêt paléocéanographique

La contribution des foraminifères planctoniques à la sédimentation carbonatée pélagique est estimée de 0,36 à 0,88 Gigatonnes par an ce qui représente 32 à 80% du bilan carbonaté profond (Schiebel et Hemleben, 2005). Le bilan fossile des foraminifères planctoniques représente une source considérable d'information paléoenvironnementale grâce

à sa continuité et à la qualité de sa préservation. Ainsi, les successions fauniques de foraminifères planctoniques constituent une archive fondamentale des changements océanographiques et climatiques passés. Les reconstructions paléocéanographiques basées sur ces coquilles reposent en grande partie (1) sur l'analyse des changements d'abondances relatives des morpho-espèces dans les sédiments de surface et l'établissement de «fonctions de transfert paléo-environnementales», et (2) sur l'observation, à l'échelle d'une morpho-espèce, des variations spatiales et temporelles de compositions isotopique ou élémentaire ($\delta^{13}C$, $\delta^{18}O$, $\delta^{11}B$, Mg/Ca ; e.g. Katz et al., 2010). Ces relations empiriques établies entre les communautés actuelles de foraminifères ou composition chimique et les conditions physico-chimiques des océans (température, salinité, productivité) permettent d'inférer la dynamique et les structures thermique et trophique des océans anciens d'un point de vue géographique ou bathymétrique. Ces relations reposent sur l'hypothèse que chaque morpho-espèce de foraminifère planctonique, définie sur la base de critères typologiques, représente une espèce biologique unique avec un habitat spécifique et que cet habitat est stable au cours du temps. La validité des reconstitutions repose sur cet unique postulat. Cependant, l'obtention d'une seconde génération de foraminifères planctoniques en cultures reste, encore aujourd'hui, un écueil majeur et insoluble (Hemleben et al., 1989). En conséquence, la validité biologique (*sensu* Mayr, 1940) des morpho-espèces décrites sur la base de critères purement typologiques n'a jamais pu être testée. En effet, la capacité d'interfécondité, critère de reconnaissance des espèces biologiques, n'est pas observable dans la nature pour les groupes planctoniques. Pour palier à ce problème, il est nécessaire d'accomplir des cycles de reproduction en laboratoire pour valider les espèces biologiques (Amato et al., 2007), ce qui n'a jamais pu être fait pour les foraminifères planctoniques. La biologie moléculaire a ouvert l'accès au génome de ces organismes fournissant des clés de lectures de tous les niveaux systématiques, du régne à la population, en fonction du degré de conservation des séquences génétiques étudiées (Coleman, 2003). Ces outils récents dans l'étude des foraminifères planctoniques ont permis de tester la validité des morpho-espèces utilisées en paléocéanographie.

Apport de la biologie moléculaire

Les études moléculaires conduites sur les foraminifères planctoniques sont basées sur les gènes ribosomaux. Cette région du génome code pour la synthèse des ARN des sous-

unités ribosomales. Trois ARN sont synthétisés: la SSU (Small Sub Unit), le 5.8S et la LSU (Large Sub Unit). Il existe également deux autres fragments qui ne sont pas intégrés dans les ribosomes: les ITS 1 et 2 (Internal Transcribed Spacer ; Figure 3). Les régions qui codent pour les ribosomes (SSU, 5.8S et LSU) sont très utilisées en phylogénie (Amato et al., 2007). Les ribosomes étant indispensables à la survie de la cellule leur région codante évolue très lentement et est présente à la fois chez les eucaryotes et les procaryotes. Ces gènes servent à établir des phylogénies à l'échelle des règnes (Cavalier-Smith, 2004). Les ITS, qui ne sont pas intégrés à la synthèse de l'ARN, sont beaucoup moins stables et servent de marqueurs à l'échelle spécifique (Coleman, 2009). Ces marqueurs ont permis de reclasser les foraminifères dans le super-groupe des Rhizaria, rassemblant un assemblage hétéroclyte de protistes incluant les Radiolaires, Phytomyxea, Gromidae, Haplosporidia, Cercozoa et les foraminifères (Cavalier-Smith et Chao, 1997). Cette classification a depuis été confirmée par d'autres marqueurs moléculaires tel l'ubiquitin (Archibald et al., 2003) ou l'ARN polymérase II (Longet et al., 2003).

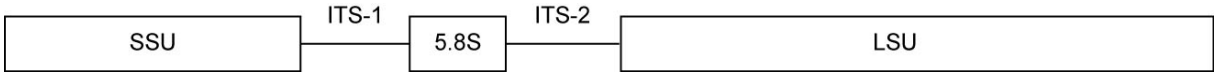

Figure 3 : Organisation fondamentale de l'ADN ribosomale nucléaire (modifié de Coleman, 2003). Les rectangles représentent les zones transcrites en ARN (SSU, 5.8S et LSU) et les traits les parties non transcrites (ITS-1 et ITS-2).

Les premières données moléculaires des foraminifères planctoniques ont été produites au milieu des années 1990 (Merle et al., 1994; Darling et al., 1996 ; Wade et al., 1996). Ces études se sont appuyées sur la petite sous-unité de l'ADN ribosomique (SSU rDNA) ou la grande sous-unité (LSU) pour reconstituer l'histoire évolutive du groupe (Pawlowski et al., 1994 ; 1997; Darling et al., 1997; de Vargas et al., 1997). Ces reconstructions ont révélé une origine polyphylétique des foraminifères planctoniques (Darling et al., 1997; Wade et Darling, 2002). En outre, un taux d'évolution de l'ADN ribosomal anormalement élevé a été observé chez les foraminifères planctoniques (de Vargas et al., 1997). Le taux de substitution moyen entre morpho-espèces est de 3×10^{-9} substitutions par site et par an (de 0,3 à 5,3 $\times 10^{-9}$ substitutions par site et par an). Ce taux est 18 fois supérieur à celui observé chez les diatomées qui est lui-même 2 à 3 fois supérieur à celui observé chez les métazoaires (de Vargas et al., 1997). Au-delà de ces taux d'évolution surprenants, ces études ont mis en évidence la présence de variations génétiques importantes au sein de la SSU de plusieurs morpho-espèces, délimitant des types génétiques ou génotypes. De Vargas et al. (1997) ont

estimé que la distance génétique séparant deux des trois génotypes observés chez la morpho-espèce *Orbulina universa* est deux fois plus élevée que la distance génétique observée entre la grenouille et l'homme. Ces types génétiques correspondent à des **espèces cryptiques** c'est-à-dire des espèces biologiques, isolées sur le plan reproductif, mais morphologiquement indiscernables (sensu Knowlton, 1993). Tous les travaux de séquençage conduits sur un nombre d'individus important, et collectés sur de larges transects trans-océaniques, ont mis en évidence de la diversité cryptique au sein des morpho-espèces analysées (Huber et al., 1997; de Vargas et al., 1997; 2001, 2002; Darling et al., 2000, 2004; Darling et Wade, 2008; Aurahs et al., 2009) à l'exception de la morpho-espèce *Globigerinoides sacculifer* (André, 2010 ; Tableau 1).

Morphoespèce	nombre d'espèces cryptiques identifiées	Marqueur moléculaire	références
Neogloboquadrina pachyderma	7	SSU rDNA	4, 6, 8
Turborotalia quinqueloba	6	SSU rDNA	4, 5, 9
Neogloboquadrina incompta	2	SSU rDNA	4, 6, 7
Globigerina bulloides	7	SSU rDNA	3, 4, 5, 8
Truncorotalia truncatulinoides	5	SSU rDNA et ITS rDNA	10, 12, 14,
Orbulina universa	3	SSU rDNA	1, 2, 10, 11
Globigerinella siphonifera	7	SSU rDNA	1, 2, 3
Globigerinoides ruber	5	SSU rDNA	1, 2, 3, 10
Globigerinoides sacculifer	1	SSU rDNA	13

Tableau 1 : Nombre de génotypes identifiés au sein de morpho-espèces séquencées sur de larges transect trans-océaniques. (D'après Darling et Wade, 2008). Références : (1) Darling et al., 1996 ; (2) Darling et al., 1997 ; (3) Darling et al., 1999 ; (4) Darling et al., 2000 ; (5) Darling et al., 2003 ; (6) Darling et al., 2004 ; (7) Darling et al., 2006 ; (8) Darling et al., 2007 ; (9) Stewart et al., 2001 ; (10) de Vargas et al., 1997 ; (11) de Vargas et al., 1999 ; (12) de Vargas et al., 2001 ; (13) André., non publié ; (14) Ujiié et Lipps, 2009

Impact de la diversité cryptique

Il apparait que la diversité spécifique des foraminifères planctoniques a été considérablement sous-estimée. C'est là conséquence du manque de descripteurs morphologiques disponibles sur le test des foraminifères, indispensable pour établir une taxonomie rigoureuse. En conséquence, les fonctions de transfert, dont le rôle est de transposer, vers le registre fossile, les préférences écologiques observées chez les morpho-espèces actuelles, se sont basées sur des agrégats d'espèces biologiques, chacune avec leurs

préférences écologiques particulières. Les reconstitutions paléocéanographiques ont donc été moyennées voir biaisées (Kucera et Darling, 2002). De plus, les expériences menées en laboratoire pour étudier la physiologie de ces organismes et en particulier établir les droites de fractionnement isotopique ou élémentaire des coquilles en relation aux variations physico-chimiques de l'eau, se sont basés sur des individus collectés sur des localités uniques, et donc appartenant à un seul génotype (Lombard et al., 2009). Ainsi, ces relations ont pu être déséquilibrées par de potentiels effets vitaux spécifiques à chaque génotype, biaisant un peu plus les applications sur le registre fossile.

Malgré ces limitations, la diversité cryptique chez les foraminifères planctoniques pourrait se révéler un avantage plutôt qu'un inconvénient. En effet, les préférences écologiques restreintes des espèces cryptiques par rapport à celles des morpho-espèces représentent une source d'informations potentielles inouïes pour les reconstitutions. Kucera et Darling (2002) ont re-calibré des fonctions de transfert en intégrant les distributions théoriques de 4 génotypes de la morphoespèce *Globigerina bulloides*. Ces auteurs on déterminé que le gain de précision potentiel lié à l'utilisation de la diversité cryptique de cet unique taxon dans les reconstitutions pouvait aller jusqu'à 34%.

Un fort degré d'écophénotypie est reconnu depuis longtemps chez les foraminifères planctoniques (e.g. Parker, 1962). Hecht et al. (1976) ont observé des variations de porosité chez la morpho-espèce *Orbulina universa* en corrélation avec les températures des eaux de surface. Ces auteurs proposèrent ce caractère comme traceur paléoenvironnemental potentiel. De Vargas et al. (1999) ont suggéré que ces variations de porosité pourraient correspondre aux différentes espèces cryptiques de cette morpho-espèce. Dès lors, si cette variabilité morphologique s'avérait être le résultat de l'agrégat des différents génotypes et non pas de l'écophénotypie comme cela avait été suggéré par Parker (1962), il serait possible de reconnaitre les espèces cryptiques sur la base de leur morphologie. Le transfert des caractéristiques écologiques restreintes des espèces cryptiques vers le registre fossile deviendrait donc possible et aboutirait à une augmentation drastique de la précision des reconstitutions paléocéanographiques basées sur les tests de foraminifères planctoniques.

Objectifs

Ce travail a pour objectif de créer les outils nécessaires pour transférer des informations liées à la diversité cryptique des foraminifères planctoniques dans les reconstitutions paléocéanographiques. Cette étude s'axe sur deux points cruciaux : (1) la détermination précise des caractéristiques biogéographiques et écologiques de ces espèces cryptiques et (2) le développement de modèles de reconnaissance morphologique des espèces cryptiques.

Cette étude s'est appuyée sur une collection unique de foraminifères planctoniques cryopréservés, réalisée depuis 1997 au cours de 8 missions océanographiques ainsi que de collectes ponctuelles réalisées à l'échelle de l'Océan mondial (Figure 4). C'est au total plus de 16.000 individus collectés dans tous les bassins océaniques des milieux tropicaux à polaire qui ont été isolés individuellement. Grâce à l'utilisation d'un tampon d'extraction d'ADN non destructif pour la coquille calcaire (voir chapitre 1), des analyses génétiques et morphométriques ont été réalisées à l'échelle individuelle pour établir une cartographie précise de la diversité cryptique de 4 morpho-espèces de foraminifères planctoniques : *Orbulina universa*, *Truncorotalia truncatulinoides*, *Glogoconella inflata* et *Globigerina bulloides*.

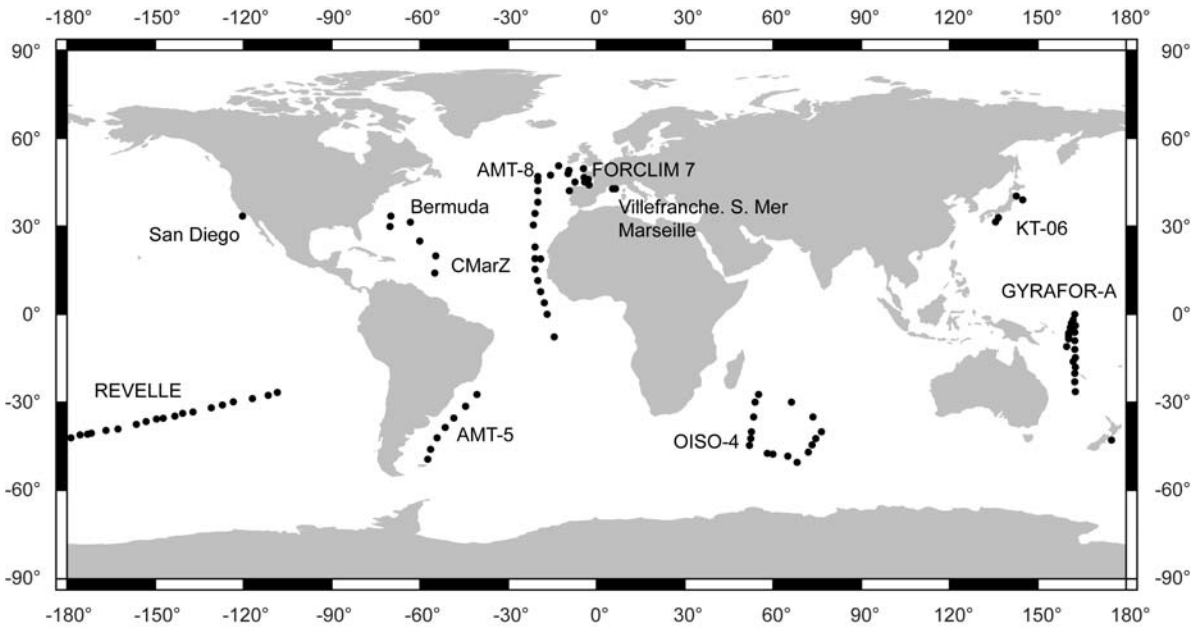

Figure 4 : Localisation géographique des stations échantillonnées et dont le matériel a servi de support à ce travail.

Les chapitres 2 et 3 sont consacrés à l'établissement de modèles de reconnaissance morphologique des espèces cryptiques d'*Orbulina universa* et *Truncorotalia truncatulinoides* respectivement. La diversité génétique de ces deux taxons a été caractérisée par des travaux antérieurs (de Vargas et al., 1999 ; 2001) conduits sur des spécimens collectés sur un transect latitudinal medio Atlantique. L'objectif du travail conduit sur ces deux morpho-espèces aura été premièrement de confirmer et valider les patterns de distribution biogéographique observés par de Vargas et al., (1999 ; 2001). Sur la base de ces collections, nous avons quantifié les variations morphologiques des espèces cryptiques de ces deux taxons à l'aide d'analyses morphométriques (porosité ou contour des tests).

L'objectif du chapitre 4 était de réaliser le même exercice sur la morpho-espèce *Globoconella inflata*, abondante dans les milieux subtropicaux à subpolaires. Cependant, la diversité cryptique de ce taxon n'avait jusqu'à présent pas été étudiée. Une étape de séquençage et de développement d'un protocole d'identification rapide des génotypes a été nécessaire au préalable d'une étude biogéographique et morphométrique.

Le but du chapitre 5 a été de déterminer le gain potentiel de l'intégration de la diversité cryptique des morpho-espèces *O. universa*, *T. truncatulinoides* et *G. inflata* via l'utilisation des modèles développés dans les chapitres 2 à 4. Nous y avons également ajouté la diversité cryptique de la morphoespèce *Globigerina bulloides*. Ce taxon est le plus abondant dans les environnements de transition et nous avons identifié 4 génotypes dans nos collections dont un inédit. Le développement d'un modèle de reconnaissance morphologique des espèces cryptiques de *G. bulloides* analogue à ceux développés dans les chapitres 2 à 4 reste à être accompli. Nous avons re-calibré les fonctions de transfert utilisées pour reconstituer les températures de surface en y intégrant les tolérances thermiques restreintes des espèces cryptiques de ces 4 morpho-espèces dans les océans de l'hémisphère sud. Cette approche nous a permis d'estimer le gain de précision lié à l'intégration de la diversité cryptique dans les reconstitutions.

Chapitre 1 : Protocole d'extraction ADN

Ce premier chapitre est consacré à la méthode qui a rendu ce travail de thèse possible : l'extraction d'ADN non destructive pour la coquille calcitique des foraminifères planctoniques. Pour démontrer de façon in-équivoque que les différents espèces cryptiques présentent des morphologies différentes et ne sont des écophénotypes, il est primordial de caractériser la génétique et la morphologie d'un même individu. Cela n'a pas été possible jusqu'à présent car les tampons d'extractions ADN qui ont été utilisés pour extraire l'ADN des foraminifères sont des chélateurs de la calcite (Darling et al., 1996 ; de Vargas et al., 1997). Certains auteurs contournèrent ce problème en prélevant une partie de l'individu avant l'extraction d'ADN (Huber et al., 1997) ou en comparant la diversité moléculaire à la diversité morphologique d'une population (de Vargas et al., 2001). Malgré cela, ces approches limitaient les applications paléocéanographiques telles que l'établissement de modèles de reconnaissance morphologique *sensu stricto*.

Les objectifs de ce chapitre sont de présenter l'efficacité de ce protocole, le *GITC**, en terme de rendement (i. e. nombre de PCR positives) et en terme de conservation des tests des foraminifères planctoniques. Pour illustrer la méthode, nous avons procédé à l'extraction d'ADN de 209 spécimens de la morpho-espèces *Orbulina universa* collectés lors de la campagne GYRAFOR-A dans le Pacifique ouest équatorial en Juin 2008.

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GITC*, a new method for single cell morpho-genetic comparisons in planktonic foraminifera

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Abstract

Molecular phylogenetic studies have challenged morphospecies concept in planktonic foraminifera, revealing a significant level of cryptic (genetic) diversity in all cosmopolitan morphospecies analyzed so far. It has been suggested that the subtle morphological differences known within morphospecies and classically treated as ecophenotypic variants may differentiate cryptic species adapted to different environments. Consequently, in order to interpret the paleoecological and stratigraphic meaning of the planktonic foraminiferal fossil record, it is crucial to distinguish the morphological variability of the shell resulting from genetic isolation from that due to ecological plasticity. Here, we present a simple, rapid and efficient method, the GITC*, to extract and preserve both the shell and DNA from single living foraminiferal cells collected from the oceans. Total genomic extractions can be used for further PCR amplification, RFLP analysis or sequencing of the ribosomal nuclear DNA clusters, while the shells can be utilized for SEM or morphometric analyses. The power of this new method is illustrated through a rapid survey of the environmental features characterizing different genotypes within the morphospecies *Orbulina universa* collected through a latitudinal transect in the western equatorial Pacific. Collections at various depths along this transect show that within a complex hydrographic system as the western equatorial Pacific, the different cryptic species of *O. universa* co-occur at the same location and are apparently not stratified in the water column.

Keywords: GITC*, DNA versus morphology, planktonic foraminifera, cryptic species; biogeography

1. Introduction

1.1. Excellence and caveats of pelagic fossil records

The oceanic sediments are for the most part composed of the skeleton of different groups of planktonic protists: diatoms, coccolitophores, foraminifers, radiolarians, dinoflagellates, etc. These different taxonomic entities share a few common biological features that contribute to the exceptional quality of their fossil record: they construct extra-cellular skeletons that are preserved in deep sea sediments (Armstrong and Brasier, 2005), they live in huge populations that reproduce on a daily or weekly time scale, and they are rapidly dispersed throughout the oceans (Hemleben et al. 1989). However, they also share an important feature that limits our interpretation of this fundamental archive: they are microscopic and relatively shapeless. The lack of complex morphological features has made it difficult to classify their diversity into biological species, and thus decreases the accuracy of - if not bias- the numerous paleoclimatic, stratigraphic, or evolutionary analyses that assume a biological species concept (Kucera & Darling, 2002; Kucera et al., 2005a)

1.2. Morphological versus genetic species concepts in planktonic foraminifera

Among the skeleton-bearing pelagic taxa, the planktonic foraminifera are a unique model as they are relatively large unicellular organisms that can be rapidly manipulated with simple hand-tools under a dissecting microscope. Their fossil record is often presented as one of the few known at the “species” level (Bolli and Saunders, 1985, Norris 1991). Their calcareous shells (tests) preserved in marine sediments have built by far the most complete and continuous fossil archive on Earth, used extensively by paleoceanographers for climate reconstructions (e.g., Kennett, 1982; Lipps, 1992). In addition to the development of proxies deduced from the chemical composition of the tests (Katz et al., 2010), the use of planktonic foraminifera in paleoceanography lies on empirical correlations between the environmental parameters of the surface oceans (e.g., temperature, primary productivity) and the abundance of tests of individual species in surface sediment samples (Imbrie and Kipp, 1971; Hutson, 1977; Malmgren et al., 2001; Mix et al., 2001; Kucera et al., 2005a; 2005b). However, for historical reason mainly (and because none was able yet to establish culture of pelagic

foraminifers), taxonomy in foraminifers is almost exclusively based on morphological diagnostic characters of the calcareous tests, often even described from fossil specimens. The relatively few attempts to utilize biological characters were principally done to validate the morphologically defined "species" (Hemleben et al., 1989). In fact, the most basic biological features of planktonic foraminifera (sexuality, generation time, feeding behavior, etc) remain very speculative.

Recent application of DNA PCR amplification and sequencing methods in planktonic foraminifera have shown that the classical definition of species hides higher levels of genetic and ecological differentiation in all species analysed so far. (de Vargas et al., 1997; 1999; 2001; 2002; Huber et al., 1997; Darling et al., 1999; 2000; 2004; 2006; 2007; Stewart et al., 2001; Bauch et al., 2003; Ujiié and Lipps, 2009; see Darling and Wade, 2008 for a review). Genetically identical individuals within the SSU rDNA occur across huge geographic distances, current systems, major tectonic barriers, and can co-occur with other genotypes at the same sites (de Vargas et al., 1997; 2001; 2002; Darling et al., 2000; 2004; Darling and Wade, 2008). Genetic variations are also correlated to ecological and morphological differentiations (Huber et al., 1997; Morard et al., 2009). Together with molecular clock analyses, this strongly supports the hypothesis that the most distinct ribotypic species within classical morphospecies actually correspond to cryptic or pseudo-cryptic biological species. In addition, the diverse genotypes within a morphospecies most often display specific, generally allopatric biogeographic distributions, related to different degrees in the stability of the water mass (de Vargas et al. 2001; 2002; Morard et al., 2009). These results fundamentally alter our way of thinking about evolution and ecology in the planktonic foraminifera: they suggest that the slight morphological differences known within classical morphospecies may distinguish ancient, isolated species adapted to highly different environments. The recognition of these ecotypes within the sediments may shed new biological light onto the evolutionary reading of the pelagic fossil record, and greatly increase the accuracy of using foraminifera as paleo-climatic and stratigraphic proxies (Morard et al., 2009).

1.3. The value of direct morpho-genetic comparisons

In order to transfer the biological information acquired through genetic analysis to the interpretation of the sediment record, it is necessary to detect which morphological characters

of the shells may permit significant distinction of the eco-genotypes of planktonic foraminifera. Such morpho-genetic comparisons should ideally be conducted directly on a large number of individuals, collected over a large region in order to distinguish the morphological variability induced by environmental gradients from that due to genetic segregation and evolution. Here, we present a new, simple method of single cell DNA extraction that does not destroy the shell, where single foraminifer can be subsequently analysed for both their genetic and morphological features. The power of the protocol is illustrated through a rapid survey of success rate of DNA extraction, amplification and shell preservation of 209 specimens of *Orbulina universa* collected through a latitudinal transect in the western equatorial Pacific. The proposed method opens new avenues to investigate the environmental and functional significance of morphological variability in planktonic foraminifera. It may also be applied to other ecologically important groups of pelagic microfossils, like the diatoms or coccolithophores.

1.4. Orbulina universa d'Orbigny

Orbulina universa is a cosmopolitan, surface-dweller (Field et al., 2004) spinose morphospecies of planktonic foraminifera that is particularly abundant in the oligotrophic subtropical gyres due to its photosymbiotic ecology (Spero, 1987). The life cycle of *O. universa* consists of a juvenile stage with a trochospiral multichambered shell that is filled with cytoplasm, followed by a mature stage in which the specimen builds a final spherical chamber that entirely overlaps the juvenile chambers (Hemleben et al., 1989). Our choice to study *O. universa* to illustrate our method of single cell DNA extraction was motivated by previous molecular analyses, which make this taxon one of the best known planktonic foraminifera for its genetic diversity. Small SubUnit rDNA sequencing and genotyping of individuals from the world Ocean have revealed the existence of three cryptic specie, i.e., the Caribbean, Sargasso and Mediterranean species (de Vargas et al., 1999), whose distributions are apparently correlated to the productivity of the surface waters (de Vargas et al., 1999; Morard et al., 2009).

2. Material and methods

2.1. Sample collection

Total plankton samples were collected onboard the Alis during the cruise GYRAFOR-A (June 2008) in the Western Equatorial Pacific (Figure 1, Table 1). Collections were performed using Multi-Net tows (100 µm mesh size) which allowed completion of a continuous series of stratified hauls at various depths of the water column, along a latitudinal transect that ranges between 26°S and the Equator (see Appendix for details). The samples were distributed into plastic Petri-dishes. Using a dissecting microscope and a fine brush, all *Orbulina universa* specimens were isolated and transferred to smaller Petri-dishes with filtered sea water (0.2 µm). Each foraminifera was then individually cleaned by brushing in order to remove the organic matter, marine snow, or other organisms that may stick to the shell, and finally transferred into a 200 µl plastic tube containing 50 µl of the GITC* DNA extraction buffer (Figure 2). The brush was carefully sterilized in boiling distilled water between each individual extraction. The extraction tubes were stored at -20ºC. A total of 209 *Orbulina universa* were collected in 11 out of the 20 GYRAFOR-A stations and treated as described above.

Temperature, salinity and chlorophyll-a fluorescence vertical profiles of the upper 400 m of the water column were obtained by Conductivity, Temperature and Depth (CTD) casts at each of the 20 stations.

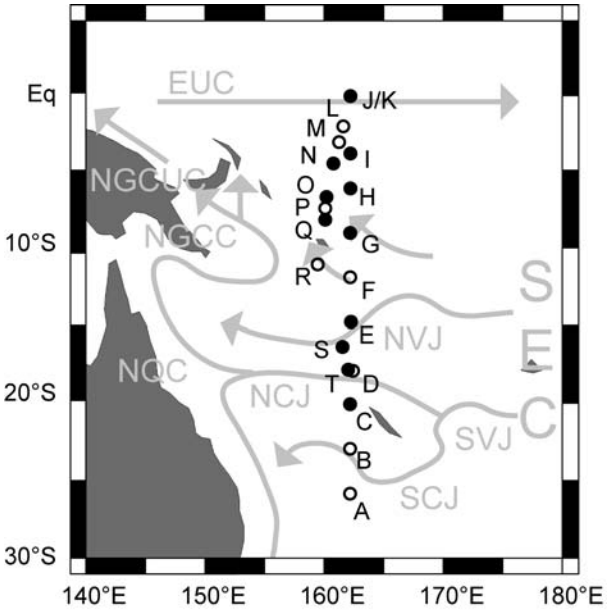

Figure 1: Geographic locations and labels of the stations sampled during the cruise GYRAFOR-A in the western equatorial Pacific. Locations include stations for which both collected specimens of *O. universa* have been genetically characterized and CTD cast performed (black circles) and stations for which only CTD casts were extracted (open circles). Location of the main current systems from Ganachaud et al. (2007). SEC = South Equatorial Current, NVJ/SVJ = North/South Vanuatu jets, NCJ/SCJ = North/South Caledonian jet, EUC = Equatorial Undercurrent, NGCUC = New Guinea Coastal under current, NGCC = New Guinea Coastal current, NQC = North Queensland Current

2.2. DNA extraction

The protocol we developed is based on the classical guanidium isothiocyanate (GITC) RNA extraction buffer first described by Chirwing et al. (1979). GITC buffer is commonly used to isolate intact RNA from animal tissues (Maniatis et al. 1982). Cellular structures disintegrate and nucleoproteins dissociate quickly from nucleic acids in the presence of guanidium isothiocyanate and reducing agent like β-mercaptoethanol. This buffer also strongly inactivates the RNAases. In order to keep the calcareous shell intact, we have modified the original composition of the buffer by removing EDTA, which is a strong calcium chelator commonly used for dissolving $CaCO_3$ skeletons (Bodine and Fernalld, 1973; Fredd and Fogler, 1998; Wade and Garcia-Pichel, 2003). The various steps of our protocol, we named GITC*, are illustrated and described in the figure 2. In total, nucleic acids of 209 specimens were extracted and PCR-amplified.

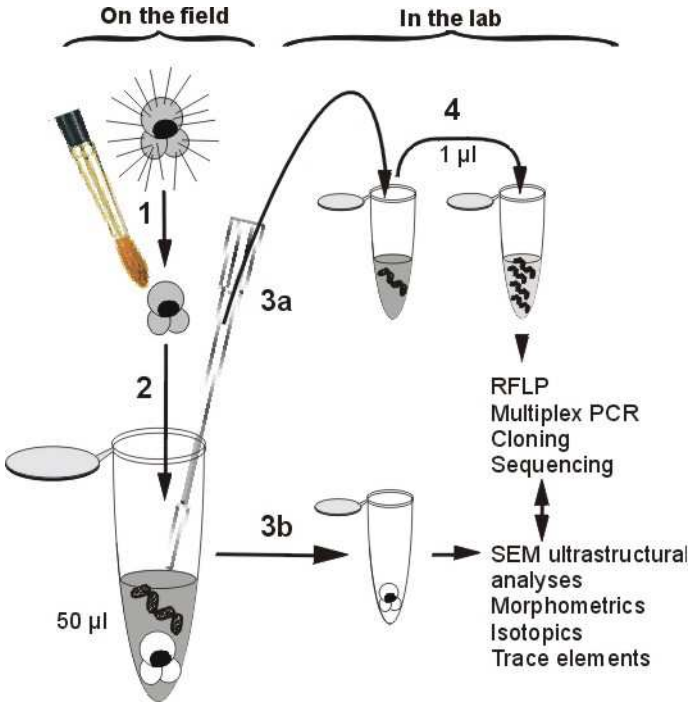

Figure 2: GITC*: a simple method for genetic and morphologic analyses on single foraminifer. 1. Isolate and clean the freshly collected foraminifer using a thin brush (see text). 2. Transfer the foraminifer into a 200 µl plastic tube containing 50 µl of the GITC* DNA extraction buffer (see note below); store at -20ºC. Carefully vortex, shortly spin, and heat 10' at 70ºC before proceeding to 3. 3a. Recover the total nucleic acids extract using a micro-tips that permit removal of the buffer without capture of the foraminiferal shell 4. DNA precipitation and PCR reaction: add to the GITC* extract 50 µl of distilled H_2O and 100 µl of 100% isopropanol. Vortex and let precipitate overnight at -20°C. Centrifuge at 15,000 rpm for 15 min. Pour off the supernatant. Wash the pellet with 100 µl of 70% EtOH, vortex, centrifuge at 15,000 rpm for 5 min. Pour off the supernatant. Leave the tube open for 2-3 h. at room temperature to evaporate the EtOH from the pellet, and dissolve in 10-40 µl of distilled H_2O. Vortex and leave 20 min at room temperature, before using 1 µl of the extraction for a standard PCR reaction (see text). Store at -20°C. 3b. Recover the empty calcareous shell in the original plastic tube for further morphologic or chemical analyses.

2.3. Molecular analyses

The internal specific foraminiferal primers S15rf (5' GTG CAT GGC CGT TCT TAG TTC 3') - S19f (5' CCC GTA CRA GGC ATT CCT AG 3') were used to PCR amplify ~600 base pair (bp) fragment, localized at the terminal 3' end of the small subunit (SSU) rDNA gene for each individual of *Orbulina universa*. A standard PCR protocol (Initial denaturation: 95°C/10"; 94°C/30'-52°C/30'-72°/30" repeated 40 times; Final extension: 72°C/10") was used to amplify the DNA extraction products.

The positive PCR-products were UV detected after slow migration on 1.5% agarose gel and ethidium bromide staining. The positive PCR products were further analyzed through

restriction fragment length polymorphism (RFLP) completed with the enzyme *Sau 96I* which cut at sequence 5'…G/GNCC… 3'. 5 µL of the PCR products were mixed with a solution containing 3.8 µL of distilled water, 1µL of NEB buffer 4 (New England Biolabs) and 0.2 µL of the *Sau*96I enzyme, and then incubated 2 hours at 37°C and inactivated at 80°C during 20 minutes. The digested products were UV detected after slow migration on 3% agarose gel and ethidium bromide staining. The Caribbean species of *O. universa* (de Vargas et al., 1999) is cut three times and displays a two bands pattern at 200 pb (indistinguishable) and a two bands pattern at ~100 bp. The Mediterranean species is cut once and displays a two bands pattern at ~400 bp and ~200 bp. Finally, the Sargasso species displays no cut.

2.4. Shell preservation

In order to estimate the effect of the GITC* extraction buffer on shell preservation, all the shells of the *Orbulina universa* specimens for which DNA was extracted were observed under binocular microscope. Fifty of the 209 GITC* processed individuals were further randomly picked, individually mounted, metalized and photographed using scanning electron microscopy (SEM). Several microstructural details (wall textute, pores, pustules) of the shells were observed using high magnification in order to qualitatively estimate the potential alteration effects of the GITC* extraction method. Because binocular microscope observations revealed the presence of buffer residues on the shells, the specimens we SEM photomicrographed were previously washed in an alcohol solution.

3. Results

*3.1. Efficacy of the GITC**

Total reaction success rate of the PCR-amplifications performed on the 209 *Orbulina universa* individuals is 77% (Table 1). The high PCR success rate we obtained among these samples indicates that the buffer efficiently penetrates the foraminiferal shell through the pores, breaks the protein matrix (Hemleben et al. 1989) at the surface of the cell, isolates the nucleic acids out of the proteins, lipids and polysaccharide structures, and extracts them out of the shell. The high success rate also reflects the small number of dead foraminifers collected in planktonic populations from the upper few hundred meters of the Ocean, the dead skeleton-bearing organisms sinking rapidly to deep-sea sediments. The relatively strong intensity of the PCR products obtained from single cell amplification is either due to the presence of a particularly large number of ribosomal clusters in foraminifera, or to the fact that the adult individuals we collected had already started their genomic multiplication for gamete production (Hemleben et al. 1989).

Station	Latitude	Longitude	Number of Specimen collected	Number of Positive PCR	Number of specimen preserved	RFLP
A	26°00'S	162°50'E	1	1	1	III
B	23°02'S	162°50'E	0			
C	20°01'S	162°50'E	67	59	60	III
D	17°66'S	162°50'E	0			
E	14°83'S	162°60'E	35	20	30	I (7), III (11)
F	11°93'S	162°51'E	0			
G	9°00'S	162°51'E	2	1	2	III
H	6°04'S	162°51'E	13	7	13	I (5), III (2)
I	3°86'S	162°55'E	0			
J	EQ	162°50'E	18	11	17	I (9), III (2)
K	EQ	162°50'E	5	5	4	I (4), III (1)
L	2°00'S	161°91'E				
M	3°00'S	161°60'E				
N	4°50'S	161°11'E	5	3	5	I
O	6°66'S	160°50'E				
P	7°40'S	160°41'E				
Q	8°15'S	160°41'E	1	1	0	III
R	10°96'S	159°80'E				
S	16°25'S	161°88'E	56	52	53	I (48), II (1), III (2)
T	17°93'S	162°66'E	6	1	5	III
			Total: 209	163	190	
			Success rate	77,03%	90,09%	

Table 1: Location of the sampling stations with the number of specimens collected, the number of positive PCR as well as the number of specimens preserved. The number of genotype identification is also given.

3.2. GITC and shell preservation*

SEM pictures show that the GITC* protocol permitted the shells to be recovered after DNA extraction. Classically, corrosion of a foraminiferal shell removes the outer wall layers thereby altering the original wall texture, and dissolution destroys parts of the shell leaving holes thereby altering the global shape of the shell (Hemleben et al., 1989). Among the specimens SEM photomicrographed, no evidence of corrosion induced by the GITC* protocol was found (Figure 3).

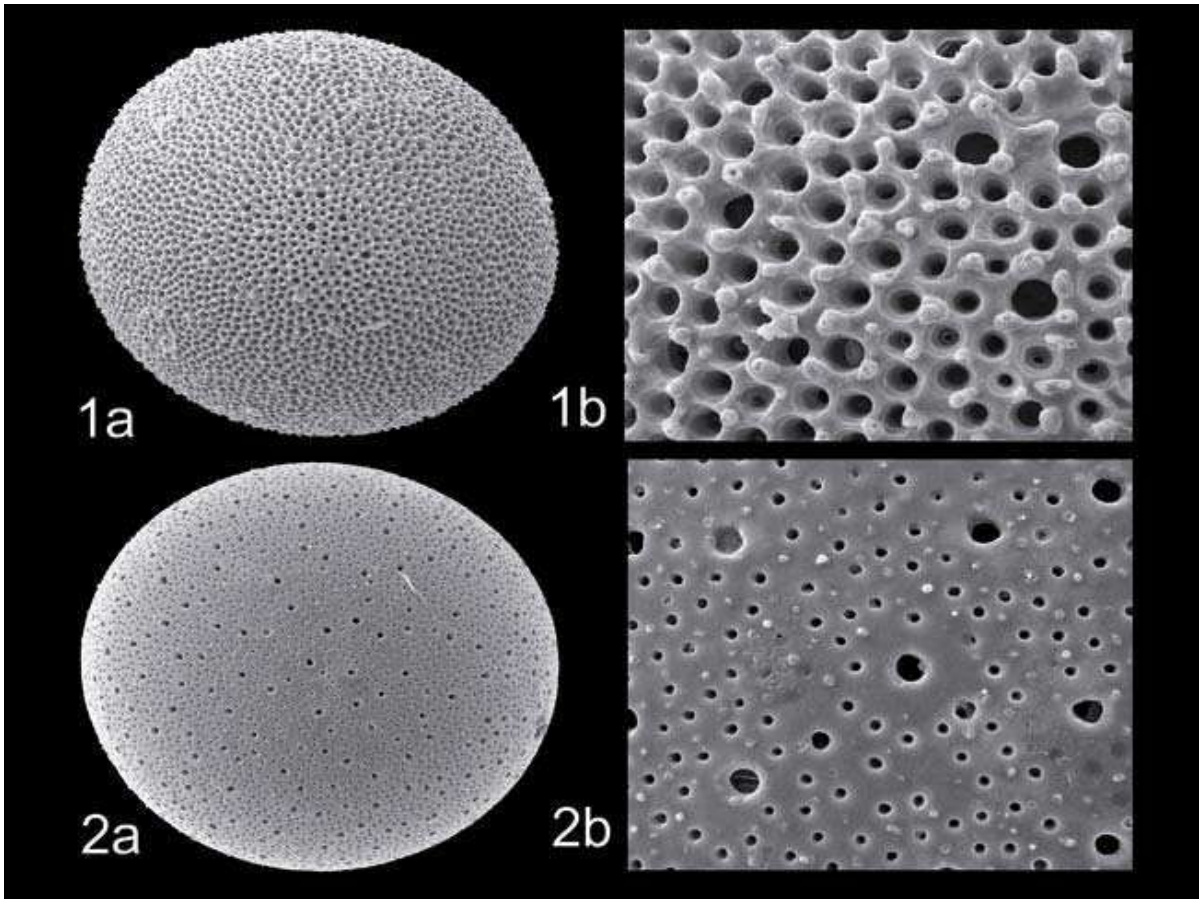

Figure 3: SEM photomicrographs of 2 previously genotyped specimens of the Caribbean (1) and the Mediterranean (2) species of Orbulina universa. Magnifications are x100 for whole specimens (a) and x500 for microstructural pictures (b). The specimens and wall-textures are perfectly preserved, even after the use of the GITC*. Porosity and pore size distribution patterns observed here are those characterizing each of the genotypes. As described by Morard et al. (2009). The Caribbean species indeed displays a globally unimodal pore size distribution and high porosity values whereas the Mediterranean species displays a bimodal pore size distribution and lower porosity values.

Among the 209 individuals for whom DNA was extracted, only 19 were found to exhibit a broken shell. The broken features probably result from the mechanical constraints (i.e. vortex homogenizations and centrifugations) underwent by the foraminifera during the DNA extraction process. The limited amount of observed alteration patterns demonstrates the power of the GITC* protocol for single-cell morpho-genetic comparisons. In the case of *O. universa*, the use of the protocol allowed us to rapidly identify the porosity patterns that characterized, in particular, the Mediterranean and Sargasso cryptic species (Morard et al., 2009; see Figure 3).

3.3. Identification of genotypes

RFLP analyses allow recognizing the three cryptic species originally described by de Vargas et al. (1999) in the Atlantic Ocean (Table 1). The specimens we collected from the GYRAFOR-A transect in the western equatorial Pacific are thus assigned to the Mediterranean (81 specimens), Caribbean (77 specimens) and Sargasso species (1 specimen) of *Orbulina universa*.

3.4. Distribution of genotypes and hydrography

In most of the collecting stations, the Mediterranean and Caribbean species of *Orbulina universa* co-occur (Table 1; Figure 4). The Sargasso species remains greatly under-represented in our dataset. Even taking into account discrepancies in the number of specimens collected at each station, the distribution pattern of the Mediterranean and Caribbean species of *O. universa* is unclear and not in agreement with previous large scale studies (de Vargas et al., 1999; Morard et al., 2009). Contrary to what we observe along the GYRAFOR-A transect (Figure 4), these studies showed that the distributions of the cryptic species were correlated to the productivity of the surface waters, with the Mediterranean species being most abundant in vertically mixed and nutrient-rich water masses, and the Caribbean and Sargasso species occurring in stratified oligotrophic water masses. In addition, the hauls collected at various depths of the water column allow no observation of depth stratification between the Mediterranean and Caribbean species (Figure 5). These results suggest that the relationships between nutrient availability and the distribution pattern of the cryptic species of *O. universa* are probably more complex than previously suspected.

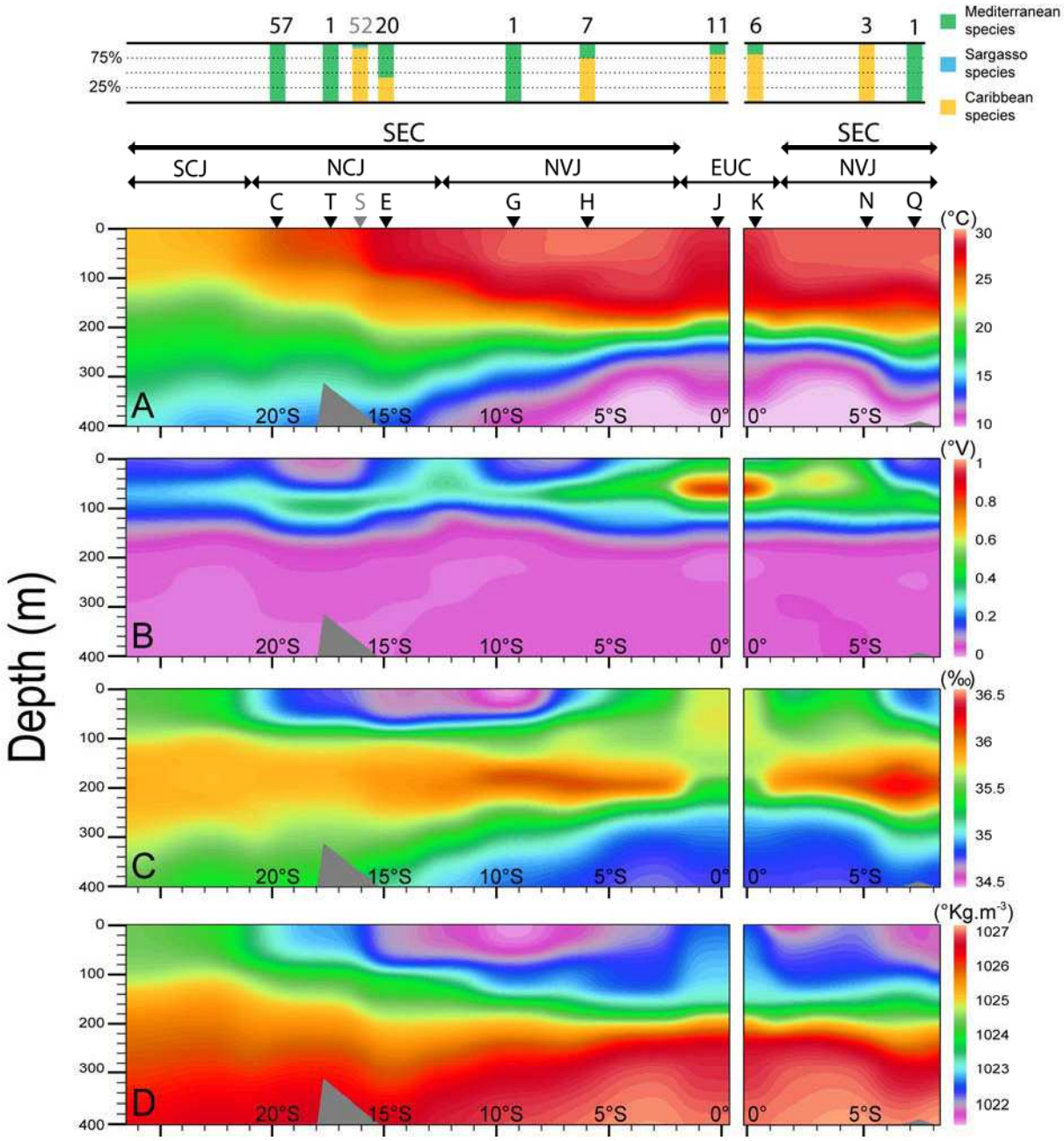

Figure 4: Latitudinal distribution of the Mediterranean, Caribbean and Sargasso (only one specimen in station S) cryptic species of *Orbulina universa* along the temperature (A), fluorescence (B) salinity (C) and density profiles (0 to 400 m depth) of the cruise GYRAFOR-A in the Western Equatorial Pacific (June 2008). The total number of genotyped *O. universa* and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. Latitudinal position of oceanic jets from Ganachaud et al. (2007); NCJ/SCJ = North/South Caledonian jet, NVJ = North Vanuatu jets, EUC = Equatorial Undercurrent.

Co-occurrences at stations between the Mediterranean and the Caribbean species may be related to the complexity of the hydrographic system occurring in the western equatorial Pacific, in peculiar because of the island ridges that are located in the area. The South Equatorial Current is divided in three main jets when it crosses the ridges of Fiji, New

Caledonia and Vanuatu islands (Figure 1; Ganachaud et al., 2007). Based on the water density and fluorescence patterns measured along the GYRAFOR-A profiles (Figure 4), it appears that the stations where we found co-occurrences of the Caribbean and Mediterranean species correspond to the frontal zones between major currents of the western equatorial Pacific. Consequently, such a complex oceanic circulation system leads to melt water masses and consequently disorganize, in our opinion, the biogeographic patterns of *O. universa* genotypes.

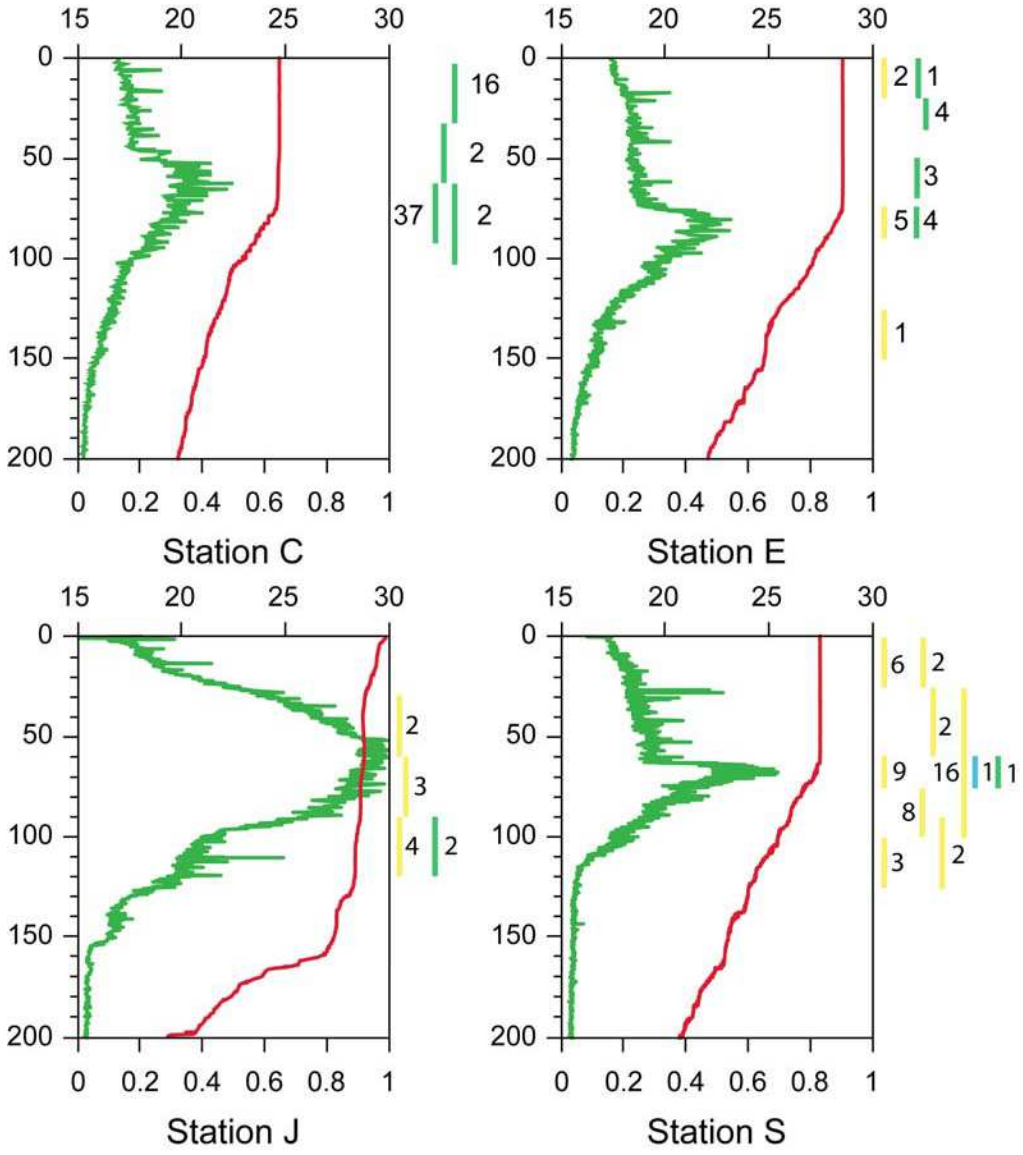

Figure 5: Depth distributions of the Mediterranean (green) and Caribbean (yellow) species of *O. universa* at stations C, E, J and S of the GYRAFOR-A transect (see Figure 1 for location). Temperature gradients in red, chlorophyll-a gradients in green. The vertical bars at the right side of the profiles represent the depths at which the genotyped specimens were collected using the Multinet system.

4. Discussion

*4.1. Advantages of the GITC**

In addition to preserving the shell (Figure 3), the GITC* protocol has several advantages. It does not require any sophisticated equipment in the field and allows rapid isolation of the specimens on board scientific vessels. Contrary to the classical phenol/chloroform extractions that are time consuming, need much attention during several steps, and require large amounts of material, all steps of DNA extraction (separation-precipitation-dissolution) can be completed in a single tube. This simplifies the handling of large numbers of samples, reduces DNA loss during manipulation, and allows us to adjust the final DNA concentration. For juvenile individuals, PCR reaction may even be carried out in the original extraction tube, although this procedure would not allow shell preservation. In comparative tests (data not shown), the GITC* protocol was also much more effective than other easy DNA extraction methods like proteinase-K digestion (Palumbi et al., 1991), or the DOC protocol (Pawlowski 2000). Finally, the cost is economical compared to the different DNA isolation kits available on the market (about 1 € for 20 samples).

4.2. Form and function in pelagic taxa

This discrepancy between morphological and genetic/ecologic differentiation may be the rule rather than an exception in the planktonic realm (Darling et al., 1999; 2004; 2006; de Vargas et al., 1999; 2001; 2002; Stewart et al., 2001), where the evolution of form and function seems to have been controlled by protection from- or interaction with- other organisms, rather than competition for resources and resource space as in most terrestrial and coastal ecosystems (Smetacek 2001). The pelagic realm itself, a three-dimensional and rather homogeneous milieu in perpetual motion, may also act as a force constraining the evolution of size and other morphological features in planktonic taxa (Tappan and Loeblich 1973). In modern foraminifera, this “pelagic selection pressure” on shell design is reflected by the paucity of species morphologically described in the plankton (~50), compared to the benthos (~ 5000) (Armstrong & Brasier, 2005)).

In any case, most common planktonic -and often considered as cosmopolitan- morpho-species analysed so far using PCR based technologies, from the pico to the micro fractions,

were found to be composed of a complex of different genetic entities (cyanobacteria and prochlorophytes -Fuhrman and Campbell, 1998-, haptophytes and diatoms -Bucklin et al., 2001; Rynearson and Armbrust, 2004; Amato et al., 2007-, dinoflagellates -Scholin et al., 1995; Bolch et al., 1999; Logares et al., 2007-, foraminifera -Kucera and Darling, 2002; de Vargas et al., 2004; Darling and Wade, 2008-, copepodes -Bucklin et al. 1996-, fishes -Miya and Nishida, 1997). Moreover, it is increasingly obvious that the distinct phylogenetic groups within highly similar morphology often have, independently of geographic distance, different horizontal or vertical biogeographic ranges (Huber et al., 1997; Toledo and Palenik, 1997; de Vargas et al., 1999; 2001; 2002; Morard et al., 2009), physiological features (Huber et al., 1997; Rynearson and Armbrust, 2004), or even radically distinctive genomic compositions (Moore et al., 1998). These results should now spawn a search for estimating the complexity of this hidden world of diversity at the genetic and/or physiological levels is now necessary in order to decipher the functional entities structuring the marine ecosystem, and to draw a valid biogeography of the global Ocean. This is particularly important for the skeleton-bearing planktonic taxa, i.e. the diatoms, coccolitophores, foraminifers, radiolarians ostracods, and pteropods, because (1) they are the principle actors of carbon export to the seafloor, and thus of great importance for the possible biological response to atmospheric changes due to human activities and (2) they have the most complete and well-preserved fossil records in oceanic sediments, a fundamental archive for the study of organismic and climate evolution.

5. Conclusion

The simple method of DNA extraction we present here has allowed us to easily extract DNA from single specimens of *Orbulina universa* without destruction of the calcareous shells, and rapidly observe shell porosity patterns that characterize the Mediterranean and Caribbean genotypes of the morphospecies. The GITC* protocol requires minimal handling, is of low cost, and permits long-term preservation of the samples. Subsequent PCR amplification of the foraminiferal rDNA had a success rate of ~77%, and the shells were adequately preserved for further morphological analyses.

Understanding how morpho-genetic diversity is distributed within the pelagic domains inevitably requires protocols that allow the study of a high number of samples collected over global geographic scale in the open ocean and if possible during different seasons. We also need analyses at the individual level, the level on which natural selection acts. Since most skeleton-bearing pelagic taxa belong to the protists, classically ranged in the category "micro-organisms" and treated like bacteria (i.e. maintained in culture and clonally amplified before further investigation), very few protocols have been developed for single-cell DNA extraction (Pawlowski 2000, Sebastian and O'Ryan, 2001), and even less for direct morpho-genetic comparison (Sherebakova et al. 2000). In fact, most pelagic protists cannot be maintained in laboratory culture, but are large enough to be manipulated under a dissecting microscope, and thus treated as individuals. The GITC* protocol presented here demonstrates that morpho-genetic study at the individual level is easily realizable in foraminifera, and may be applied, with slight modifications, to the diatoms, radiolarians, and coccolitophores. Such approach will take advantage of the accuracy of DNA sequence analyses used as a taxonomic, phylogenetic, and phylogeographic tools, to provide us with biological understanding of the extraordinarily complete fossil archives deposited at the bottom of the ocean.

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Chapitre 2 : Diversité morphologique des espèces cryptiques d'*Orbulina universa* (d'Orbigny)

Orbulina universa est un cas d'étude idéal pour le développement d'un modèle de reconnaissance morphologique des espèces cryptique. En effet, cette morpho-espèce épineuse largement distribuée dans l'Océan mondial (60°N à 50°S) attira l'attention des micropaleontologues de part sa morphologie singulière (i.e. loge terminale sphérique au stade adulte). Ainsi, la diversité morphologique de ce taxon (i.e. variation de taille et de porosité de la loge terminale) a largement été documenté (Bé et al., 1973) et même proposée comme marqueur paléo-environnemental (e.g., Hecht et al., 1977). Plus récemment, ce taxon a été choisi comme organisme modèle pour étudier la relation hôte-symbionte (Gast & Caron, 2001; Shacked & de Vargas, 2006) au sein du domaine pélagique. De plus, la diversité génétique de ce taxon, apparemment limitée à 3 espèces cryptique (de Vargas et al., 1999), limite le risque de sous-échantillonnage d'un des génotypes.

Cette étude s'appuie sur une large collection d'individus réalisée dans les principales masses d'eau tropicales à tempérées de l'Océan mondial. En plus de valider les répartitions biogéographiques des espèces cryptiques d'*Orbulina universa* observées dans l'Atlantique par de Vargas et al. (1999), et de caractériser leurs préférences environnementales respectives, cette étude représente le premier développement d'un modèle de reconnaissance morphologique des espèces cryptiques *sensu stricto*.

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Morphological recognition of cryptic species in the planktonic foraminifer *Orbulina universa*

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Abstract

One of the key hypotheses of paleoceanography is that planktonic foraminiferal morphospecies record reasonably stable and homogeneous oceanographic and climatic characteristics over their geographic and stratigraphic ranges. The discovery of numerous genetically-defined cryptic species challenges the morphospecies concept in planktonic foraminifera and paleoceanographic interpretations based on them. Here, we present a combined genetic and biometric analysis of *Orbulina universa* specimens in the Atlantic, Indian and Pacific Oceans. Our study is based on shells retained after DNA extractions. On those genotyped shells, we perform biometric analyses (shell size and thickness, inner porosity and pore surface distribution). Our genetic data confirm the presence of three cryptic species of *O. universa* in the world ocean, whose distributions are primarily correlated to the productivity of the surface waters. The Mediterranean species of *O. universa* is most abundant in the vertically mixed and nutrient-rich areas of the low to mid-latitudes, whereas the Caribbean and Sargasso species occur in stratified and oligotrophic subtropical waters. Our biometric data show no correlation between shell size and inner porosity within each cryptic species of *O. universa*. Combining Principal Component Analyses with MANOVAs performed on shell pore surface distribution, we demonstrate that the three different cryptic species are characterized by significant morphological differentiation. The Caribbean species typically exhibits large pores and higher porosity values, while the Mediterranean and Sargasso species are characterized by smaller pore areas and shell porosity. A model based upon pore surface distribution correctly assigns 60% to 90% of the specimens to their corresponding genotype. Although the inner shell surface of the Sargasso species resembles that of the Mediterranean species, our model demonstrates that the pore surface distributions of these two cryptic species can be distinguished. Finally, the Sargasso species exhibits significantly thinner shells than the two other cryptic species.

Keywords: Planktonic foraminifera; *Orbulina universa*; cryptic species; genotype; shell porosity; biometrics

1. Introduction

1.1. Morphological species and cryptic diversity in planktonic foraminifera

Planktonic foraminifera (Globigerininae) are one of the most abundant and significant fossil remains that constitute a fundamental archive of past climate and paleoceanographic changes (e.g., Kennett, 1982; Hemleben et al., 1989; Lipps, 1992). Many of the paleoceanographic reconstructions based on planktonic foraminifera rely on empirical relationships between properties of surface waters (e.g., temperature, primary productivity) and the relative abundance of morphospecies in surface sediments (e.g., Imbrie and Kipp, 1971; Hutson, 1977; see Kucera et al., 2005a for a review). These studies assume that particular morphospecies of planktonic foraminifera are characterised by stable ecological preferences that allow reconstruction of sea surface temperatures and other water mass properties (e.g., Kennett, 1968a; CLIMAP, 1976). Since the use of species specific paleoproxies requires a high degree of taxonomic consistency, the morphological definition of planktonic foraminiferal species has been set very broadly, and morphological variation has been regarded as intra-specific variability or ecophenotypy (Kennett, 1976; Hecht et al., 1976; Healy-Williams et al., 1985).

The recent discovery of cryptic species in modern planktonic foraminifera challenges the ecological significance of broadly defined morphospecies. Molecular data show that all the morphospecies analyzed so far are composed of a complex of three to six distinct genotypes (e.g., Kucera and Darling, 2002; de Vargas et al., 2003; Darling and Wade, 2008), each of them potentially exhibiting a distinct biogeography and/or ecology. The large genetic distances between many of these morphologically cryptic genotypes, the high degree of genetic identity within each genotype and their environmental/geographical separation are clearly consistent with species-level taxonomy (e.g., de Vargas et al., 1999; Kucera and Darling, 2002). Furthermore, rates of molecular evolution suggest that separation time between genotypes may have occurred up to several millions of years ago (de Vargas et al., 1999; 2001; Darling et al., 1999; 2006). It appears that paleoenvironmental reconstructions using a broad morphospecies concept are probably based on aggregates of biogeographically and ecologically distinct populations and may therefore contain significant noise (Kucera and Darling, 2002).

It has been recently suggested that the subtle morphological differences between morphospecies of planktonic foraminifera could be used to distinguish cryptic species (Huber et al., 1997; de Vargas et al., 2001). Separating cryptic species on the basis of shell morphology would make it possible to transfer this concept to paleoceanography. Here, we will test the hypothesis of a link between genetic and morphological differentiation in *Orbulina universa*. Morphological variation in *Orbulina* has been analyzed both in living conditions (e.g., Spero and Williams, 1988; Bijma et al., 1990; Caron et al., 1990; Lea et al., 1995; Spero et al., 1997; Wolf-Gladrow et al., 1999; Zeebe, 1999; Bemis et al., 2000; Köhler-Rink and Kühl, 2005) and in sediments (e.g., Bé et al., 1973; Bé and Duplessy, 1976; Hecht et al., 1976; Malmgren and Healy-Williams, 1978; Colombo and Cita, 1980; Deuser, 1987; Anand et al., 2003; Eggins et al., 2004). Small subunit rDNA sequences analyses of individuals collected from the Atlantic and a few Pacific localities have revealed the existence of three cryptic, largely divergent species having a patchy distribution that appeared to follow the water-masses (de Vargas et al., 1999).

Here, we analyze variation in pore surface distribution, porosity and wall thickness in modern *O. universa* and also present extensive new genetic data from all the subtropical oceans. De Vargas et al. (1999) noted that the three cryptic species of *Orbulina universa* are morphologically very similar, but also suggested that differences in shell porosity may allow morphological discrimination between the genotypes. Variations in wall thickness have also been observed (e.g., Deuser, 1987) and more recent studies have suggested that thickness is not purely a function of gametogenetic calcification (Billups and Spero, 1995). Our investigation combines molecular and biometric approaches based on individuals collected in the Atlantic, Pacific and Indian Oceans and seeks to compare the genetic identity of individual foraminifera with morphological data derived from their shells.

1.1. Orbulina universa d'Orbigny, 1839

Orbulina universa is one of the most commonly encountered planktonic foraminifera inhabiting the surface waters of the global ocean, between ~60°N and ~50°S (Arnold and Parker, 1999). This cosmopolitan, spinose species is particularly abundant in the oligotrophic subtropical gyres due to its photosymbiotic ecology (Spero, 1987). The life cycle of *O. universa* consists of a juvenile stage with a trochospiral multichambered shell that is filled with cytoplasm, followed by a mature stage in which the specimen builds a final spherical

chamber that entirely overlaps the juvenile chambers (Hemleben et al., 1989). Once formed, the spherical chamber is of a fixed size, and no further growth occurs except wall thickening and gametogenetic calcification that happens immediately prior to reproduction (Deuser et al., 1981; Caron et al., 1990). The final chamber typically shows two types of perforations in the shell: large (~7-10 μm) holes, representing multiple apertures (Bijma et al., 1990) through which the pseudopodia emerge and small (~2-5 μm) pores, that are blocked by a membrane for gas transport (Bé et al., 1980). Following the available literature, porosities and pore surface distributions analyzed in this paper include both types of perforations (e.g., Bé et al., 1973; Bijma et al., 1990).

Orbulina universa appeared during the middle Miocene, between 15 and 14 Ma (Lourens et al., 2005). The fossilized shells are ubiquitous in marine sediments and their mean size has been used as a paleoceanographic index (Bé and Duplessy, 1976; Malmgren and Healy-Williams, 1978). Several studies have attempted to analyze the relationship between the final chamber morphology and environmental parameters (Bé et al., 1973; Hecht et al., 1976; Colombo and Cita, 1980). In surface sediments from the Indian Ocean, Bé et al. (1973) found an inverse correlation between shell size and latitudinal occurrence. These authors also showed that shell diameters, pore diameters, and total shell porosities reach their maxima in low latitudes (see also Frerichs et al., 1972; Hetcht et al., 1976).

2. Material

We collected living *Orbulina universa* in plankton tows from all the subtropical and tropical oceans (Figure 1). In the Atlantic Ocean, specimens were collected during the cruises AMT-8 (May-June 1999; Robins and Aiken, 1996) and C-MarZ (April 2006; http://www.cmarz.org/CMarZ_RHBrown_April06/index.htm). Individuals were also obtained from the South Indian and South Pacific Oceans, respectively during the cruise-transects OISO-4 (January-February 2000; Metzl, 2000) and REVELLE (January-February 2004; http://shipsked.ucsd.edu/schedules/2004/rr_2004/constable/). The plankton samples were collected with ring net tows (100 μm mesh size) from ~200 m depth to the sea surface. During the cruise C-MarZ (NW Atlantic), zooplankton collection was also performed using 1/4-m^2 Multiple Opening/Closing Nets and Environmental Sensing Systems (MOCNESS; Wiebe et al., 1985) with 64 μm mesh size, which allowed completion of a continuous series of stratified hauls at various depths of the upper 200 m of the water column. After wet sieving net samples, all living specimens of *O. universa* were individually cleaned with a fine brush, isolated on the day of collection into a DNA extraction buffer (see below), and stored at -20ºC. In this study, we genetically analyzed 689 specimens from 42 stations (Table 1).

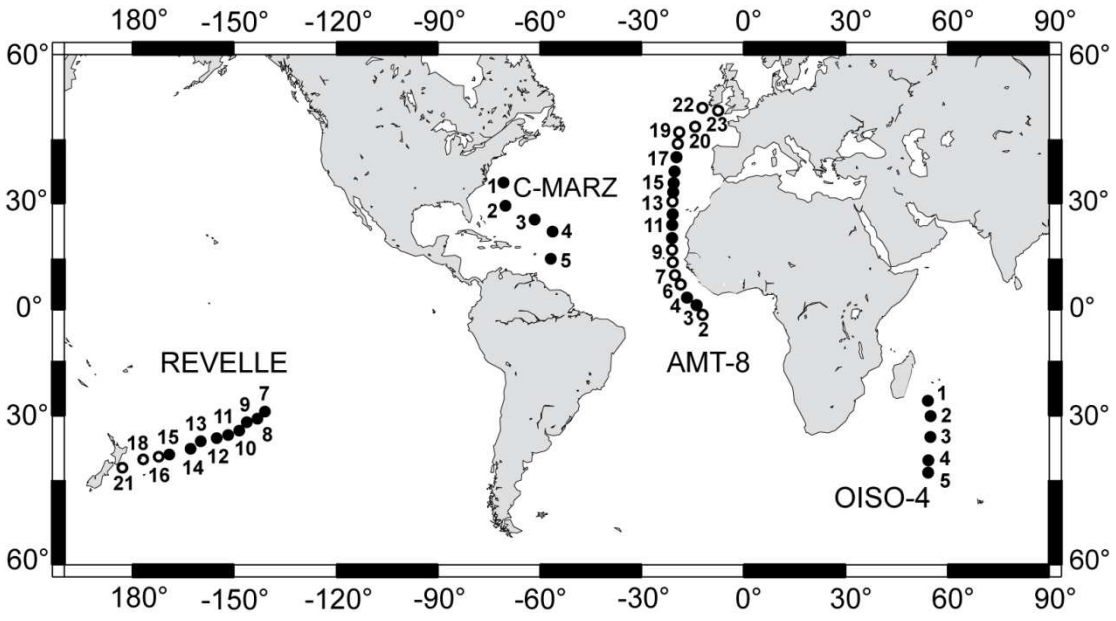

Figure 1: Geographic location and labels of the oceanic stations sampled during the cruises AMT-8, C-MarZ, OISO-4 and REVELLE. Locations include stations for which the collected specimens of *Orbulina universa* were both genotyped and morphologically analyzed (black circles) and stations for which they were only genotyped (open circles).

Temperature, salinity and chlorophyll-a fluorescence vertical profiles of the 250 m upper water column were obtained by Conductivity, Temperature and Depth (CTD) casts at most of the AMT-8, C-MarZ, OISO-4 and REVELLE stations (Table 1). These hydrographic data are used here to characterize surface water mass boundaries throughout each of the collecting transects.

Cruise	Station no.	Latitude	Longitude	Cryptic species of *Orbulina universa*	CTD Collection	Number of genotyped specimens analyzed for biometric analyses		
						Carribean sp.	Sargasso sp.	Mediterranean sp.
AMT-8	1	07°63'S	14°49'W		T, F			
AMT-8	2	04°07'S	15°31'W	Med				
AMT-8	3	00°04'S	16°42'W	Med	T, F			9
AMT-8	4	03°53'N	17°53'W	Car, Med	T, F	2		6
AMT-8	6	07°37'N	18°59'W	Med	T, F			
AMT-8	7	11°33'N	20°09'W	Med	T, F			
AMT-8	8	15°09'N	21°00'W	Med	T, F			
AMT-8	9	18°48'N	21°00'W	Med	T, F			
AMT-8	10	22°55'N	21°00'W	Car, Sar	T, F		3	8
AMT-8	11	26°41'N	21°48'W	Car, Sar			2	1
AMT-8	12	27°03'N	21°52'W	Car, Sar, Med		1	2	2
AMT-8	13	30°28'N	21°33'W	Sar	T, F			
AMT-8	14	34°22'N	20°47'W	Car, Sar	T, F	1	6	
AMT-8	15	34°33'N	20°41'W	Car, Sar, Med			9	2
AMT-8	16	38°10'N	20°00'W	Car, Sar, Med	T, F		1	4
AMT-8	17	41°51'N	20°00'W	Car, Med	T, F	1		15
AMT-8	18	45°56'N	19°58'W	Med	T, F			
AMT-8	19	46°22'N	20°00'W	Med	T, F			
AMT-8	20	47°00'N	20°00'W	Med	T, F			
AMT-8	22	48°12'N	09°24'W	Med	T, F			
AMT-8	23	48°58'N	09°15'W	Med	T, F			
AMT-8	24	49°07'N	04°44'W		T, F			
C-MarZ	1	33°54'N	69°93'W	Med	T			4
C-MarZ	2	29°85'N	70°14'W	Sar, Med	T		1	1
C-MarZ	3	24°87'N	60°13'W	Car, Med	T	11		1
C-MarZ	4	19°82'N	54°72'W	Car, Med	T	26		2
C-MarZ	5	14°01'N	54°91'W	Car, Sar, Med	T	18	1	26
OISO-4	1	27°59'S	54°59'E	Car	T, F	19		
OISO-4	2	30°00'S	53°30'E	Car	T, F	5		
OISO-4	3	35°00'S	53°30'E	Med	T, F			10
OISO-4	4	40°01'S	52°53'E	Med	T, F			11
OISO-4	5	42°31'S	52°29'E	Med	T, F			10
REVELLE	7	33°39'S	137°12'W	Car, Sar	T, F	1		
REVELLE	8	34°03'S	140°05'W	Car, Sar		4	2	
REVELLE	9	34°73'S	143°27'W	Car, Sar	T, F	1		
REVELLE	10	35°39'S	146°29'W	Car, Sar		3	2	
REVELLE	11	36°07'S	149°48'W	Car, Sar, Med	T, F	3	1	4
REVELLE	12	36°73'S	152°58'W	Car, Sar, Med		3	4	4
REVELLE	13	37°45'S	156°00'W	Car, Med	T, F	1		21
REVELLE	14	38°52'S	161°23'W	Car, Sar, Med			2	17
REVELLE	15	39°62'S	166°69'W	Med	T, F			6
REVELLE	16	39°97'S	168°41'W	Med				6
REVELLE	17	40°60'S	171°64'W		T, F			
REVELLE	18	40°87'S	173°00'W	Med	T, F			
REVELLE	20	41°97'S	178°56'W		T, F			
REVELLE	21	42°95'S	176°26'E	Med				
					Total:	100	36	170

Table 1: Location of the sampling stations, with indications of the identified cryptic species of *Orbulina universa*.

3. Methods

3.1. DNA extraction, amplification and RFLP analysis

DNA extractions of 383 specimens were performed using the classical guanidium isothiocyanate (GITC) DNA extraction buffer (for details see de Vargas et al., 1997). For the remaining 306 specimens, the original composition of the GITC extraction buffer was modified by removing EDTA, which has the property to dissolve $CaCO_3$ (Wade & Garcia-Pichel, 2003; de Vargas et al., 2003). We find that, even in the absence of EDTA, the buffer is able to efficiently penetrate the shell of planktonic foraminifera and isolate the nucleic acids. This so-called GITC* protocol makes the DNA extraction step non-destructive for the calcareous shell, and allows us to obtain, from the same individual, both the calcareous shell and the DNA for combined morphological and genetic analyses.

In this study, PCR amplification of a ~1100 base pair (bp) fragment, localized at the terminal 3' end of the small subunit (SSU) rRNA gene, was carried out for each individual of *Orbulina universa*. Methods of PCR amplification, PCR product purification and cloning, as well as the foraminifera-specific primers used in this study are described by de Vargas et al. (1997). The length of the 689 *O. universa* SSU rDNA sequences were compared to those obtained from specimens collected in the Atlantic Ocean by de Vargas et al. (1999).

The ribosomal PCR products were further analysed through restriction fragment length polymorphism (RFLP). Although the RFLP method does not allow the detection of new cryptic species of *Orbulina universa* within our sampled material (because it fails to recognize small changes characterised by a few substitutions), it allowed us to easily and quickly determine whether our 689 specimens were related to any of the RFLP genotypes described by de Vargas et al. (1999). We used the *Sau*96I restriction enzyme to cut the nucleotide sequence at a specific pattern for each genotype (for details see de Vargas et al., 1999) and to discriminate between the different genotypes. Distinct patterns for each genotype were UV-detected after migration of the digested PCR products on 1.5% agarose gel and ethidium bromide coloration.

3.2. Biometry

3.2.1. Data acquisition

The shell diameter of 160 intact specimens among 306 selected for biometric analysis was measured before each individual was mounted on a stub and gently cracked (Figure 2). A shell fragment was hand-picked and its inner surface was photographed using a scanning electron microscope (SEM) with ×500 or ×550 magnifications. Following Bé et al. (1973), all pore measurements were made on the inner shell surface in order to circumvent the problem of the radial divergence of pores due to wall thickening (see also Hemleben et al., 1989, de Garidel-Thoron, 2001). The viewing angle for SEM pictures was normal to the center of the inner shell surface in order to minimize overall pore distortion. In addition to inner surfaces, wall thicknesses of 70 shell fragments from the C-MarZ (North Western Atlantic) and REVELLE (South Pacific) cruises were also SEM photographed with ×2000 magnification and then measured.

Because the contrast between the pores and the surrounding shell varied in a wide grey scale range within each SEM photograph of the inner shells, pore areas were manually marked, and then digitally scanned using OPTIMAS v. 6.51 (see Fisher et al., 2003). For each photomicrograph, the number of pores and their individual area were extracted for statistical treatments (Figure 2).

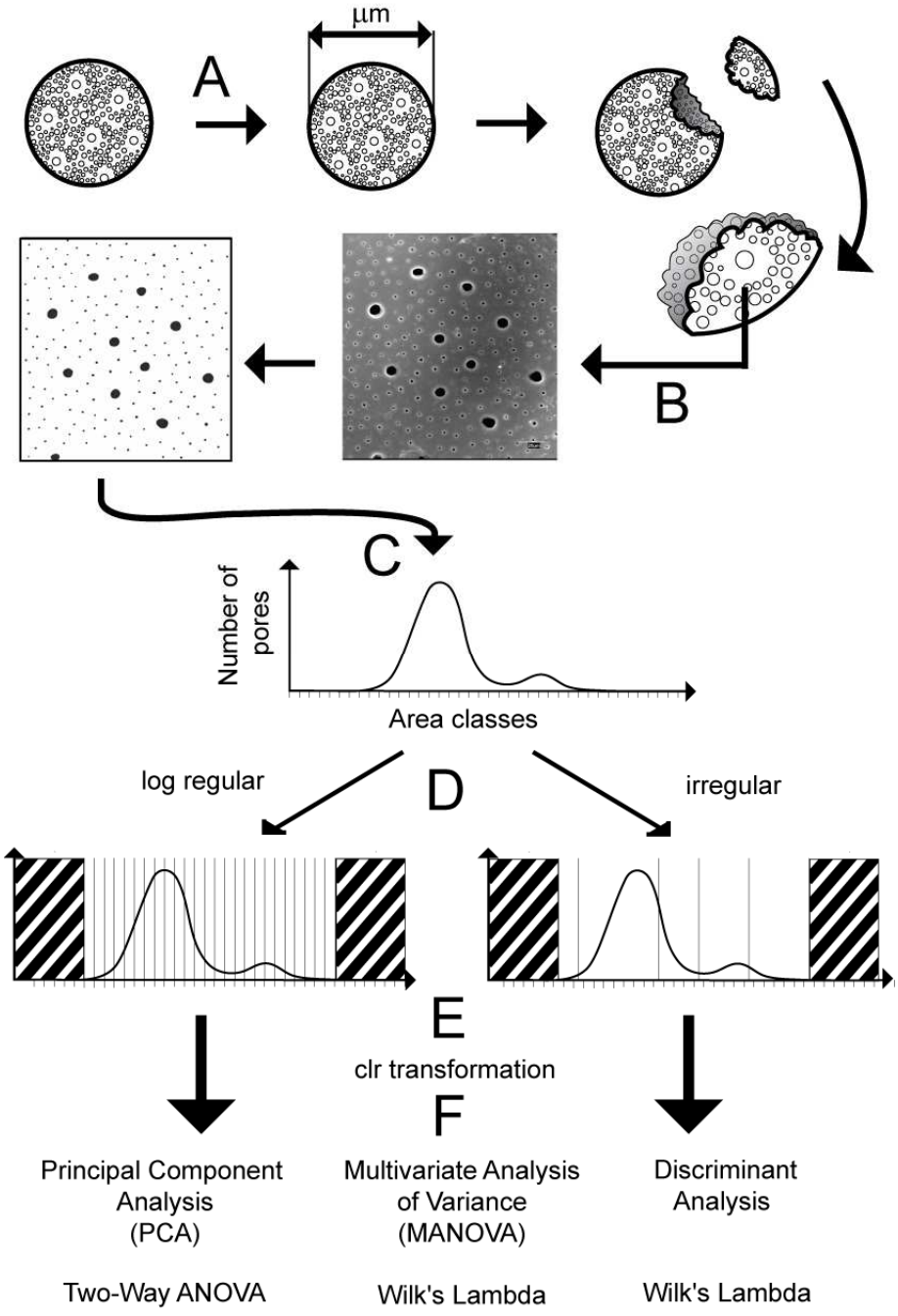

Figure 2: Procedure of data acquisition, transformation and statistical analysis. After measuring the individual diameters of intact specimens, the shells of 306 *Orbulina universa* specimens previously genotyped were cracked (A). A shell fragment was hand-picked and its inner surface was SEM photomicrographed; pore areas were then manually blackened and digitally scanned (B). Pore outlines were automatically digitized and the individual pore areas extracted and bias-corrected (C). For each individual specimen, two observational vectors of raw frequencies (pore counts) of Log_{10}-transformed corrected pore area values were computed, using 25 and 5 classes (D); then, the standardized frequency distributions of pore areas were transformed using the centered log-ratio (*clr*) technique (E). Finally, the two *clr*-transformed compositional datasets were submitted to distinct multivariate approaches (F, G) in order to quantify and partition the variation in PSD of the three cryptic species of *O. universa*.

3. 2. 2. Data correction

Preliminary comparisons between specimens indicated that the OPTIMAS v. 6.51 image analyzer systematically generates a nonlinear (allometrical) overestimation bias of the pore areas, thus requiring a correction model to be constructed. We drew a series of 40 black circles of increasing known surface areas covering the full range of observed pore areas; their measured areas were then extracted. The measured (X) and real (Y) surface area allowed the computation of a correction power model $Y = 0.479\,X^{1.14}$ (r = 0.998) with coefficients estimated by least-squares regression. The resulting relation was used to compute unbiased estimates of the observed pore areas.

3.2. Statistical analysis

3.2.1. Dataset construction

For each specimen, we first computed a D-class observational vector w of raw frequencies (pore counts) of Log_{10}-transformed corrected pore area values (full data are given in the Appendix of the online supplements of this paper; see below for details concerning the two D-values we selected in this work). Nevertheless, both the radial divergence of pores (related to shell concavity) on the SEM photomicrographs and the occasional presence of shell and cytoplasm scraps did not permit observation of the same shell area for each individual, thus precluding any direct comparison of the observational vectors. Consequently, standardization of frequency distributions of pore areas was achieved by compositional closure, i.e., by computing for each individual a compositional vector x of pore area relative frequencies. The compositional closure operation transforms the D-dimensional observational real space $\Re_+^D$ into a (D-1)-dimensional compositional space (a D-part unit *simplex* S^D), which unfortunately introduces a spurious correlation effect in the analyzed dataset – by definition, the covariance matrix of a *simplex* is singular. This makes classical methods of multivariate analysis (e.g., PCA, MANOVA, Discriminant Analysis) unreliable (Chayes, 1960; Aitchison, 2003).

In order to circumvent this problem, the centered log-ratio (*clr*) transformation was applied to each compositional vector x (Aitchison, 2003). This transformation is the application going from the compositional space S^D to the real space $\Re^D$ defined by:

$$y = clr(x) = \ln\left(\frac{x}{g(x)}\right),$$

where $g(x) = \sqrt[D]{\prod_{i=1}^{D} x_i}$ is the geometric mean associated to the compositional vector x. The *clr*-transformation can be achieved when $g(x) > 0$, then requiring the previous replacement of zeroes by values >0. Following Sandford et al. (1993), each zero value was replaced by $\varepsilon = 0.55 \times T^{-1}$ where T is the total number of pores counted for the specimen (for each specimen, α equals 55% of the smallest possible relative frequency value). Then, the D x_i-values are proportionally readjusted so that $\sum_{i=1}^{D} x_i = 1$, and the *clr*-transformation is finally performed.

3.2.2. Cutting mode

Prior to compositional closure ($w \rightarrow x$) and *clr*-transformation ($x \rightarrow y$), the raw frequencies (pore counts) of the corrected pore areas were first recorded for each specimen within an observational vector w made of $D = 40$ Log_{10}-surface classes ranging from -1 to 3 (i.e., from 0.1 to 1000 μm^2) by constant increment of 0.1 Log_{10}-unit. The weakly informative classes with null to extremely weak and exceptionally observed raw frequencies were eliminated (classes of very small or very large pore areas), thus defining a first, fine and regular cutting mode made of 25 bins ranging from -0.1 to 2.4 (0.8 to 250 μm^2).

A second cutting mode was then elaborated from the first one by computing, for each cryptic species i (including n_i specimens) and each of the 25 bins j, the arcsine-transformed proportion $\theta_{i,j} = \arcsin\left(\sqrt{\log\left(p_{i,j} + 1\right)}\right)$ of specimens where at least one pore is recorded. The use of the angular transformation unskews the distribution of a proportion estimate $p_{i,j}$ to a normal variate with expected mean $\alpha_{i,j}$ radians and an associated variance independent of $\alpha_{i,j}$ (e.g., Sokal and Rohlf, 1995). Then, we performed an UPGMA cluster analysis of the 25×25 Euclidian distance matrix of arcsine-transformed specimen proportions within each cryptic species. This analysis provided 5 well-separated clusters of 0.1-bins, defining the 5 pore area classes of this second, rough and non-regular cutting mode: [0,8-1,25 μm^2], (1,25-7,94 μm^2],

(7,94-19,95 µm²], (19,95-63,09 µm²] and (63,09-251 µm²]. This more robust cutting mode summarizes the pore area signal with a minimum loss of information and strongly decreases the noise linked to the number of null values in the *simplex*.

3.2.3. Analysis

In order to characterize and compare the 25 and 5-classes pore surface distribution (PSD) profiles for the three cryptic species of *Orbulina universa*, we submitted the dataset of 306 *clr*-transformed compositional vectors *y* to three different multivariate approaches (Figure 2). We first appraised the significance of PSD differences among cryptic species and among oceanic regions (i.e., patterns of genotype-related and geographic/ecophenotypic-related PSD differentiation). This was done by performing a principal component analysis (PCA) coupled with two-factor analyses of variance (ANOVA). The PCA allows the reduction of the total PSD variability recorded in the dataset to a few (two in this work) principal components best summarizing the overall morphological variability; then the two-factor ANOVA is used in order to test for significance of the among-group or among-regions differences observed in the space of the first two components. Second, an optimal simultaneous separation of all three genetically defined cryptic species based on the among-group (*not* total) PSD variability was performed by calculating a one-way multivariate analysis of variance (MANOVA, using Wilks' λ test statistic) coupled with a canonical variates analysis (CVA, a multigroup discriminant analysis). Last, we established the equations (linear functions) that optimally classify the specimens into their genetically assigned cryptic species based on their PSD pattern. This was done by performing discriminant analyses (also using Wilks' λ test statistic, which is computationally equivalent to Hotelling's T^2 statistic in a two-sample setting) on each of the three possible pairs of cryptic species. In addition to the computation of the percentage of correctly assigned specimens, a leave-one-out cross-evaluation procedure was applied to each pair of cryptic species in order to estimate the stability and robustness of the resulting discriminant function.

Finally, in order to appraise the significance of shell thickness variation among cryptic species of *Orbulina universa*, a one-way ANOVA was applied on wall thickness measurements. All computations were performed using the PAST software v. 1.75b (Hammer et al., 2001).

4. Results

4.1. Identification of genotypes

Different sizes of the original SSU rDNA PCR fragments allowed discrimination of three genotypes among the 689 specimens we analyzed from the Atlantic, South Indian and South Pacific Oceans. Further RFLP analyses of the ribosomal PCR products confirmed the preliminary discrimination between the genotypes. Three different RFLP patterns were observed and were all similar to those obtained from PCR-RFLP analyses of specimens collected through an Atlantic transect by de Vargas et al. (1999). The specimens studied here could thus be assigned to the previously described Mediterranean (409 specimens), Caribbean (211 specimens), and Sargasso (69 specimens) species of *Orbulina universa* (de Vargas et al., 1999).

4.2. Geographic distribution of genotypes and hydrography

Although the Sargasso species of *Orbulina universa* is yet to be recognized in the Indian Ocean (Table 1), our dataset is sufficiently detailed to identify the overall distribution of the Caribbean, Mediterranean and Sargasso species. It confirms that the geographic distribution of these cryptic species is not random (Table 1). For example, among the 42 collected stations, 19 yielded the Mediterranean species alone while the three species co-occurred at 7 stations only.

Our data confirm the observation of de Vargas et al. (1999) that the geographic distribution of the cryptic species of *Orbulina universa* is probably correlated with the primary productivity of the surface waters. The Mediterranean species is more abundant in nutrient-rich waters, where higher levels of chlorophyll-a concentration in the mixed layer are recorded. In the eastern Atlantic, the Mediterranean species is found in frontal zones in the subtropical and equatorial current systems (Figure 3). Its presence off West Africa is likely related to coastal upwelling forced by the trade winds, as pointed out by de Vargas et al. (1999; 2003). In the southern Indian (Figure 4) and Pacific (Figure 5) Oceans, the Mediterranean species is also found in areas of high levels of chlorophyll-a concentration in

the mixed layer, within or close to the frontal zones of the subtropical current systems. The Caribbean and Sargasso species of *O. universa* display similar distributions to one another, and it remains difficult to characterize productivity-related specializations between these cryptic species. Both species are systematically collected in areas of oligotrophic oceanic conditions, typically in stratified water masses of the subtropical gyres (Figs. 3, 4, 5). Co-occurrences of all three cryptic species (Table 1) in AMT-8 stations 12, 15 and 16 (Figure 3), REVELLE stations 11, 12 and 14 (Figure 5) and C-MarZ station 5 (Figure 6A) probably reflect transitional water mass mixing of frontal zones but it is also possible that the species are depth stratified.

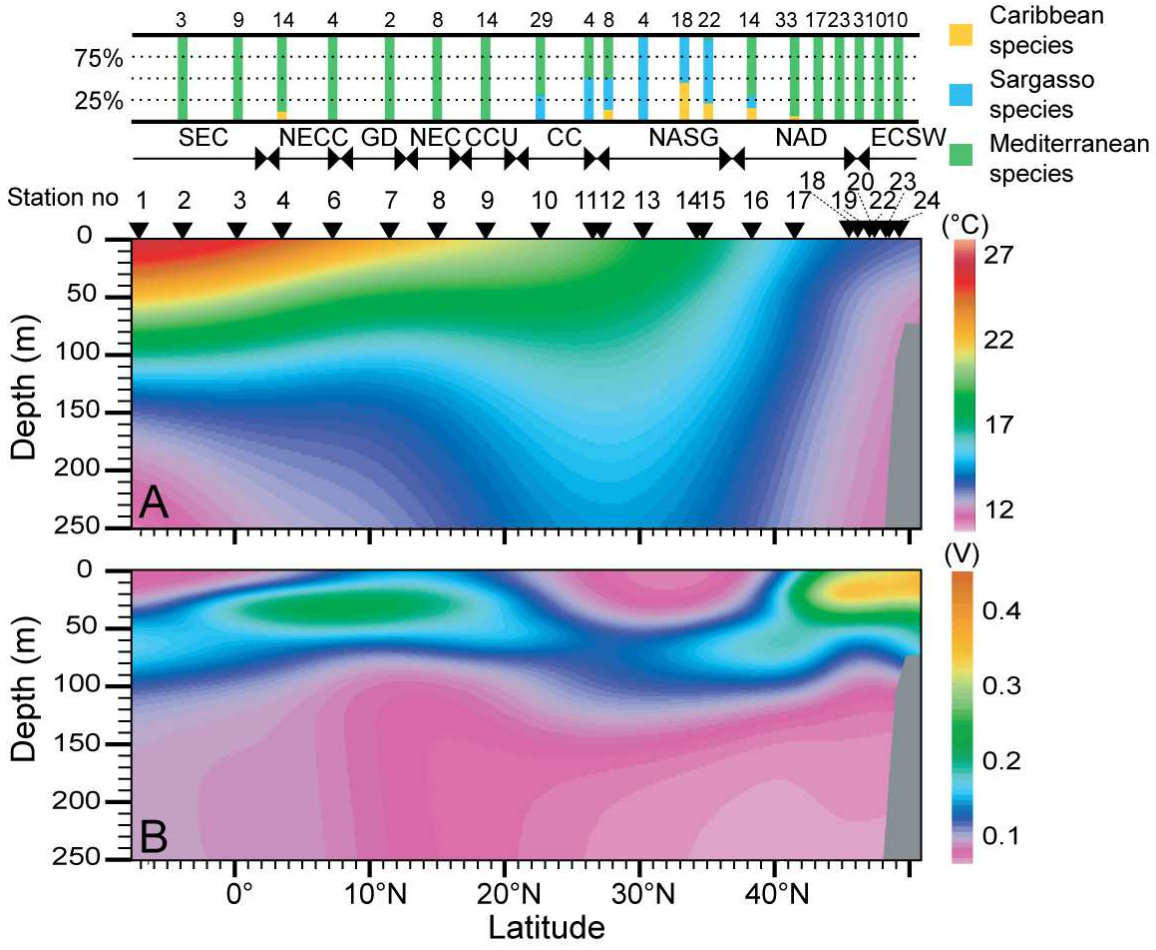

Figure 3: Latitudinal distribution of the Mediterranean, Caribbean and Sargasso cryptic species of *Orbulina universa* along the temperature (A) and fluorescence (B) profiles (0 to 250 m depth) of the cruise AMT-8 in the eastern Atlantic (May-June 1998). The total number of genotyped *O. universa* and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. Latitudinal position of oceanic fronts from Hooker et al. (2000); SEC = South Equatorial Current, NECC = North Equatorial Counter Current, GD = Guinea Dome, NEC = North Equatorial Current, CCU = Canary Current Upwelling, CC = Canary Current, NASG = North Atlantic Subtropical Gyre (East), NAD = North Atlantic Drift, ECSW = European Continental Shelf Water.

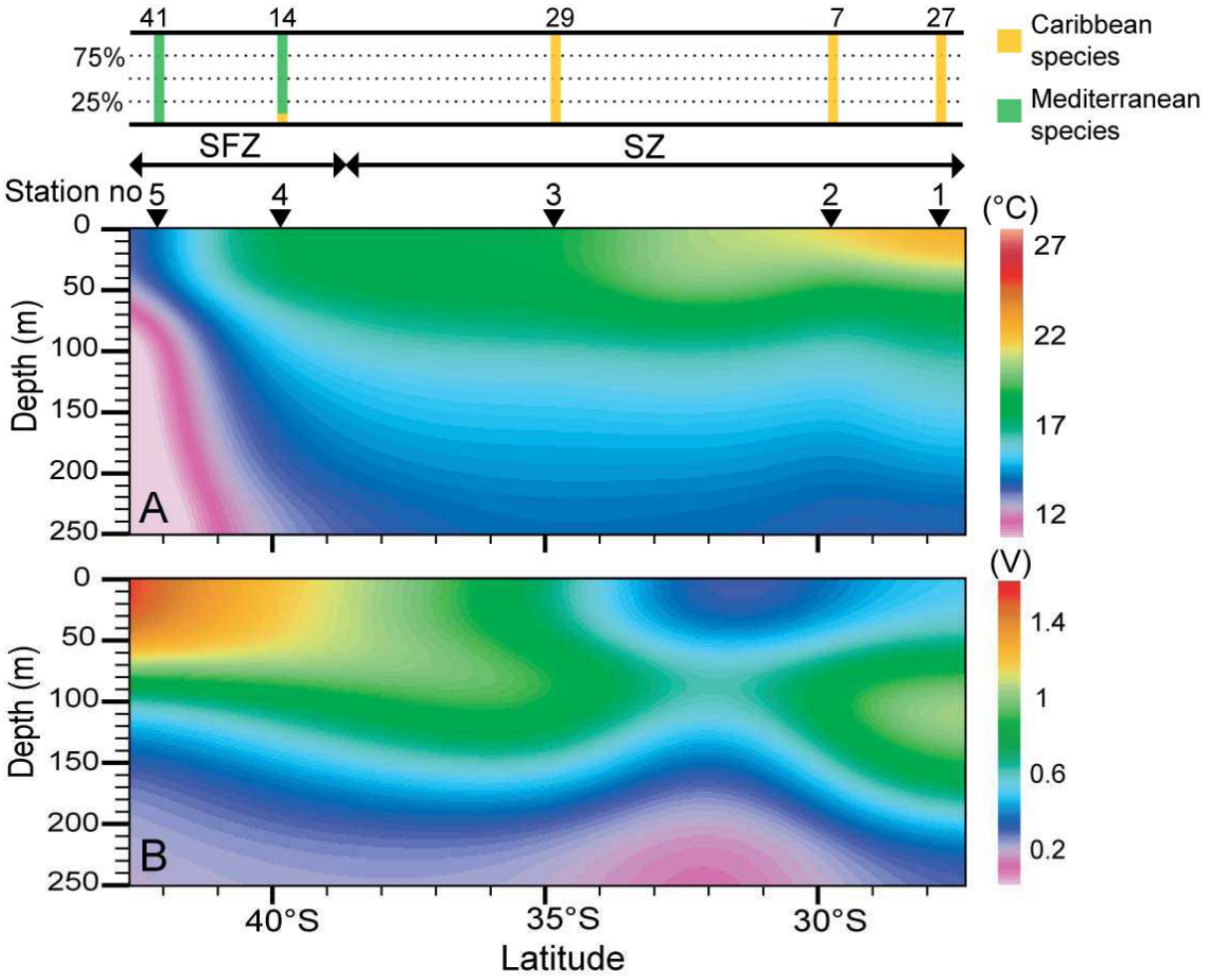

Figure 4: Latitudinal distribution of the Mediterranean and Caribbean species of *Orbulina universa* along the temperature (A) and fluorescence (B) profiles (0 to 250 m) of the cruise OISO-4 in the south Indian Ocean (January-February 2000). The total number of genotyped *O. universa* and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. Position of the subtropical front from Belkin and Gordon (1996); SZ = Subtropical Zone, SFZ = Subtropical Frontal Zone. Note that the Sargasso species has not been found during the cruise OISO-4.

Based on the available data, there is no correlation between the abundance of each cryptic species in a given sampling station and the depth of the thermocline (defined at each station as the minimum of the derivative of the function T = f(Z), where Z is the depth and T the temperature, Table 1). A one-way ANOVA indicates that no significant differences exist in the abundance of the three cryptic species with respect to the mean thermocline depth of the localities where they were collected ($F = 0.32$; d.f. = 2, 44; $p = 0.73$). This result suggests that the occurrence and abundance of each cryptic species at sampling locations do not relate to the depth of the thermocline.

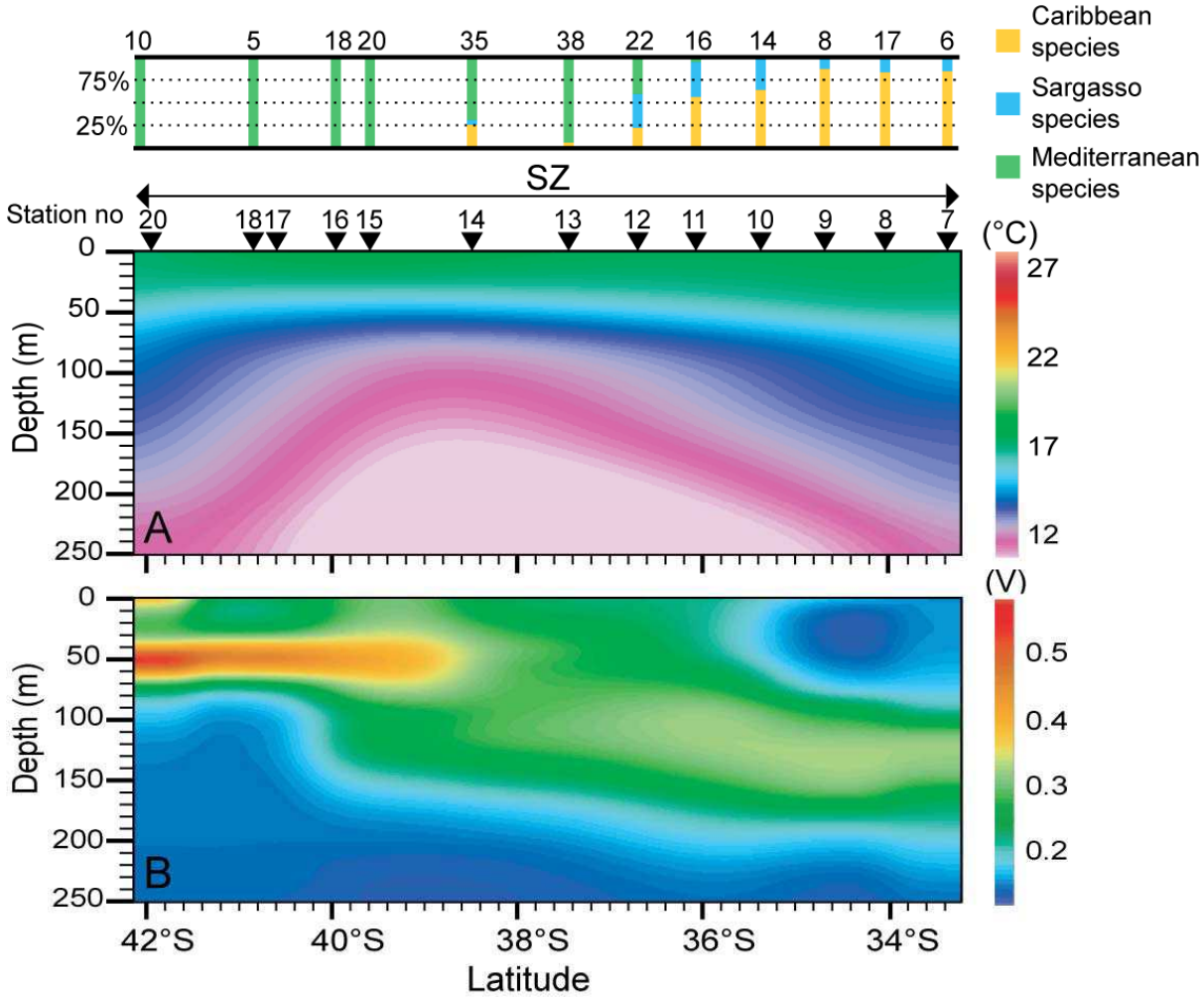

Figure 5: Latitudinal distribution of the Mediterranean, Sargasso and Caribbean species of *Orbulina universa* along the temperature (A) and fluorescence (B) profiles (0 to 250 m) of the cruise REVELLE in the south Pacific (January-February 2004). The total number of genotyped *O. universa* and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. SZ = Subtropical Zone (Tomczak and Godfrey, 2003).

Although the geographic distribution of the Mediterranean species and that of the Caribbean and Sargasso species appear to be constrained by the productivity of the surface waters, it is apparently not primary correlated with temperature (Figs. 3, 4, 5). The classically partitioned planktonic foraminiferal provinces (tropical, subtropical and transitional in the case of our dataset), which are principally related to water mass temperature (e.g., Bé, 1977), do not coincide with the geographic ranges of the cryptic species of *Orbulina universa*. As pointed out by de Vargas et al. (1999), the use of ring net tows (here at cruises AMT-8, OISO-4 and REVELLE) cannot rule out the possibility of vertical, and thus thermal segregation of the different cryptic species. However, our stratified (i.e. MOCNESS) sampling from the cruise C-MarZ, along a transect extending from the sub-tropical northern Sargasso Sea to the

equatorial waters of the northeast Brazil (Figure 1), revealed no clear depth zonation between the cryptic species of *O. universa* (Figure 6B).

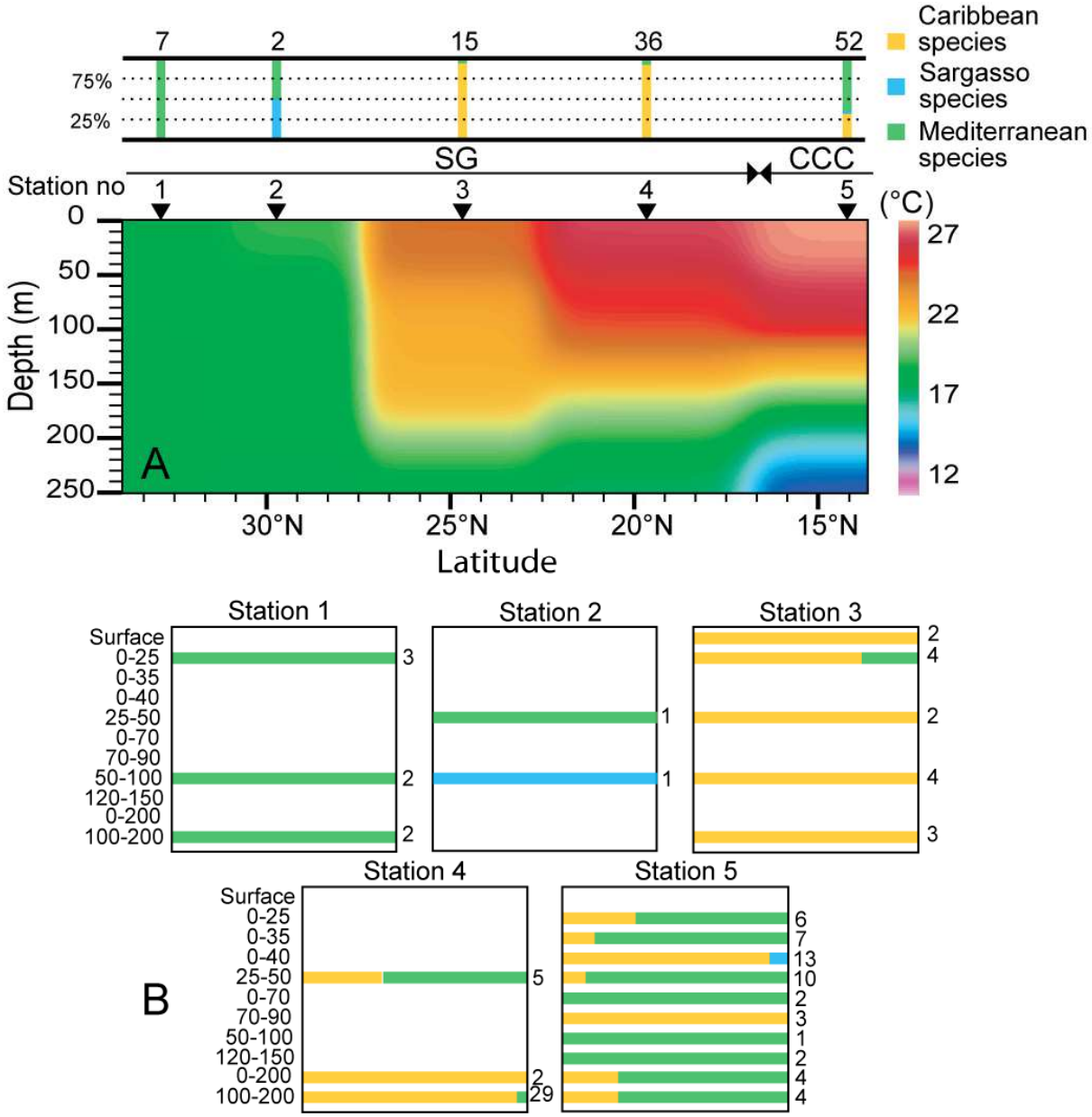

Figure 6: Latitudinal distribution of the Mediterranean, Sargasso and Caribbean species of *Orbulina universa* along the temperature (A) profile (0 to 250 m) of the cruise C-MarZ in the western north Atlantic (April 2006). The total number of genotyped *O. universa* and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. SG = Subtropical Gyre, CCC = Caribbean Counter Current (Tomczak and Godfrey, 2003). The number and ratio of the Mediterranean, Sargasso and Caribbean species collected using a MOCNESS sampling are given according to the sampling depth for each station (B).

For example, in the thermally stratified tropical waters of station 5, the abundance of collected specimens peaks at ~40-50 m, probably within the deep chlorophyll maximum, but there is no discrimination between the ratio of Mediterranean and Caribbean species above, within and below these depths (Figure 6B). These data suggest that the distribution of the

cryptic species of *O. universa*, both in horizontal and vertical dimensions of the oceans, is probably not primarily correlated with temperature. Rather, they confirm the importance of water mass stratification and nutrient availability for spatial segregation among cryptic species.

4.3. Biometry

4.3.1. Porosity and allometry

Overall porosity (i.e., % of pore surface per unit area) was estimated for all the specimens of *Orbulina universa* for which the total photomicrograph was exploited (see Appendix). Our data show that the Caribbean species exhibits more variable (ranging between 4.1% and 29.9%) and globally higher porosity values (mean value = 12.2%; 95% asymmetrical Confidence Interval: [3.6%; 26.9%]) than the Mediterranean (range: 2.0-20.9%; mean value = 6.0%; 95% CI: [2.1%; 12.0%]) and Sargasso species (range: 4.0-17.5%; mean value = 6.3%; 95% CI: [2.5%; 12.0%]). In order to test the assumption that individual size does not influence porosity, tests of correlation between *O. universa* shell diameter and the overall % of pore surface per unit area (following Bé et al., 1973) were conducted on all the specimens for which both the shell diameter was measured and the total photomicrograph area was exploited (corresponding to 31 specimens of the Mediterranean species and 22 specimens of the Caribbean species considered together, Figure 7). The correlation coefficients are: $r_{Pearson} = 0.33$ ($p_{[r=0]} = 0.015$) and $r_{Spearman} = 0.37$ ($p_{[r=0]} = 6.3\times10^{-3}$), indicating a weak, but significant positive log-linear relationship between shell size and total porosity. Such a relationship holds when considering the modal area value of small pores ($r_{Spearman} = 0.32$, $p_{[r=0]} = 0.026$) and large pores ($r_{Spearman} = 0.34$, $p_{[r=0]} = 0.017$).

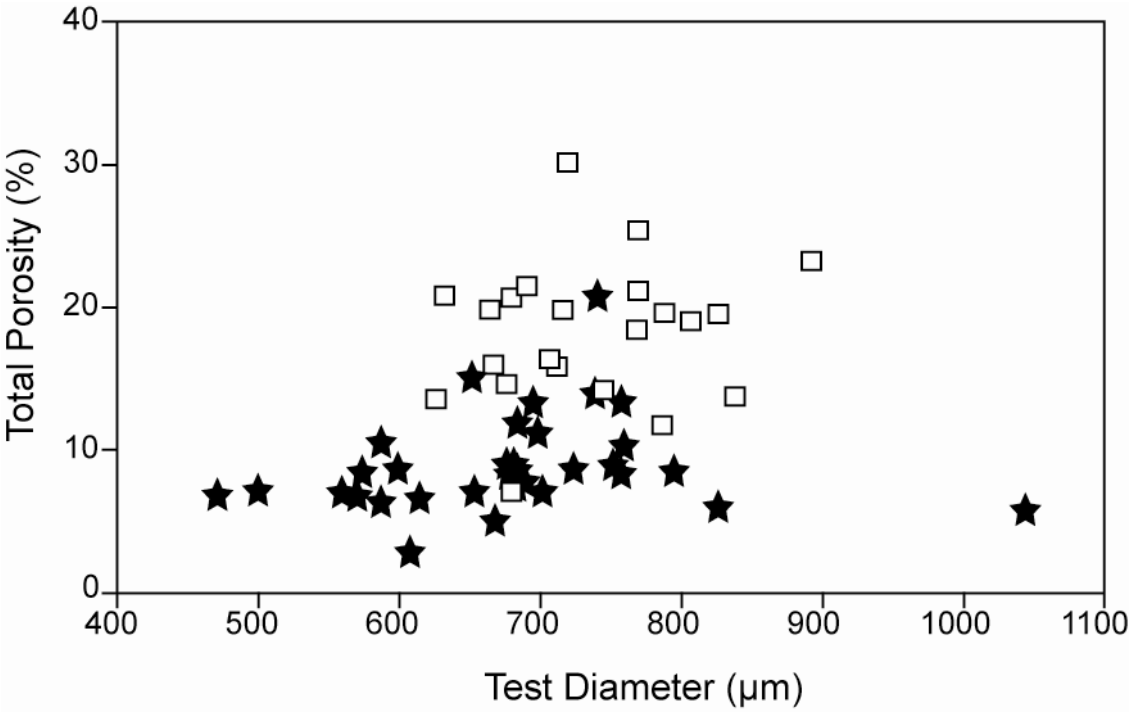

Figure 7: Relationship between individual shell porosity (% pore surface per unit area) and the size (shell diameter) of *Orbulina universa* specimens. Black stars: Mediterranean species (31 specimens); open squares: Caribbean species (22 specimens). The weak, but significant positive size-porosity relationship observed at the morphospecies level vanishes at the cryptic species level (see text for details).

The relationship between shell diameter and overall % of pore surface per unit area is noticeably weaker than that published by Bé et al. (1973) in an examination of specimens from plankton tows from the Indian Ocean. Indeed, these authors computed a highly positive allometric relationship ($r_{Pearson}$ = 0.71; $r_{Spearman}$ = 0.66) between foraminiferal shell diameter and total porosity. We believe that Bé et al.'s allometric relationship is actually the spurious consequence of sample mixing (clearly apparent in figure 13c of Bé et al., 1973) of different cryptic species of *Orbulina universa* with significantly different mean sizes and total porosities ($Size_{Caribbean\ vs.\ Mediterranean}$: Student-*t* = 2.47, df = 51, p = 0.017; $Porosity_{Caribbean\ vs.\ Mediterranean}$: Student-*t* = 8.0, df = 51, p = 2×10^{-10}). Indeed, when the cryptic species are considered independently, the correlation coefficients between shell diameter and total porosity are: $r_{Pearson}$ = 0.15 ($p_{(r=0)}$ = 0.42) and $r_{Spearman}$ = 0.26 ($p_{(r=0)}$ = 0.16) for the Mediterranean species and $r_{Pearson}$ = 0.19 ($p_{(r=0)}$ = 0.39) and $r_{Spearman}$ = 0.098 ($p_{(r=0)}$ = 0.66) for the Caribbean one. Thus, at the cryptic species-level, there is no correlation between shell diameter and porosity of the samples analyzed in our study.

4.3.2. Differentiation in pore surface distribution among cryptic species

The genetic differentiation between the Mediterranean, Caribbean and Sargasso species of *Orbulina universa* is associated with a clear PSD differentiation. The two-factor ANOVAs performed on the first principal planes coordinates (accounting for 78.9% and 69.7% of the total variance of shell PSD for the 5- and 25-class datasets, respectively; Figure 8) provide a highly significant differentiation among cryptic species for both cutting modes (Table 2).

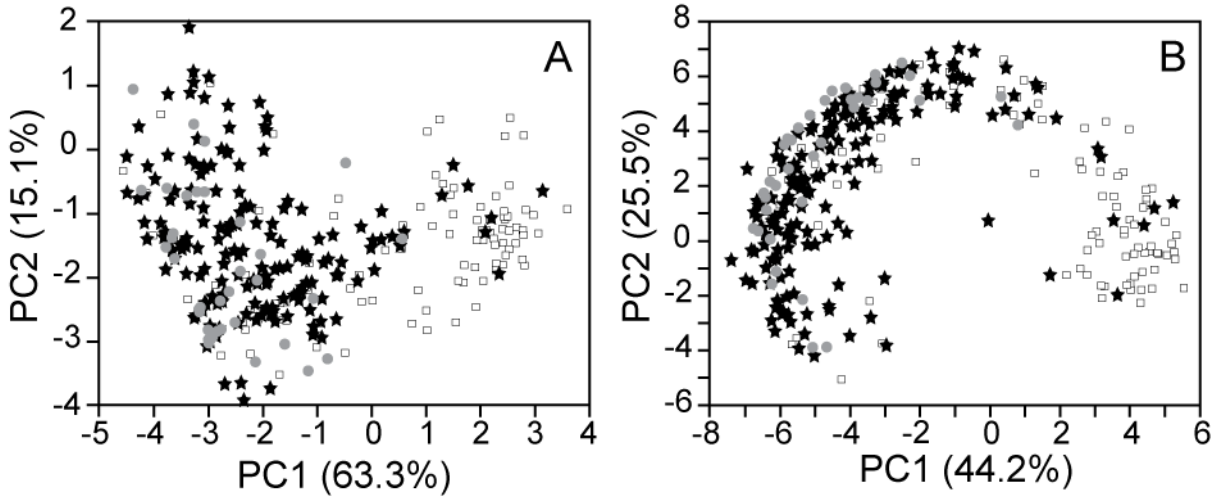

Figure 8: Principal Component Analysis performed on the 5-class (A) and 25-class (B) datasets. Black stars: Mediterranean species; grey circles: Sargasso species; open squares: Caribbean species. Two-way ANOVA results associated to these PCAs are given in Table 2.

This differentiation is higher when comparing the Caribbean and Mediterranean species than for the two other possible pairs of cryptic species (Caribbean vs. Sargasso and Sargasso vs. Mediterranean species; Table 2). The first canonical planes of the MANOVAs (Figure 9), which account for ~99% of the variance of shell PSD for both cutting modes, also reveal a clear and highly significant morphological differentiation among cryptic species (Table 2).

Species samples	ANOVA			Wilk's lambda			
	df1; df2	F	p-value	λ	df1; df2	F	p-value
5 classes							
Overall	2; 301	243.60	4.03×10^{-87}	0.53	10; 600	22.46	7.51×10^{-36}
Caribbean-Sargasso	1; 134	29.03	2.90×10^{-07}	0.65	5; 131	13.85	7.40×10^{-11}
Caribbean-Mediterranean	1; 268	183	4.50×10^{-37}	0.56	5; 265	41.81	1.20×10^{-31}
Sargasso-Mediterranean	1; 203	59.22	5.00×10^{-14}	0.90	5; 200	6.02	3.25×10^{-5}
25 classes							
Overall	2; 301	87.98	5.86×10^{-36}	0.43	10; 560	5.84	2.03×10^{-27}
Caribbean-Sargasso	1; 134	9.82	2.10×10^{-3}	0.58	25; 111	3.16	1.83×10^{-5}
Caribbean-Mediterranean	1; 268	94.64	5.10×10^{-21}	0.50	25; 245	9.80	6.79×10^{-25}
Sargasso-Mediterranean	1; 203	0.90	0.34	0.71	25; 180	2.91	2.22×10^{-5}

Table 2: Overall and pairwise cryptic species two-way ANOVAs and Wilk's Lambda tests associated to PCA (Figure 8) and MANOVA (Figure 9) results, testing for differences in pore surface distribution between cryptic species, based on the 5-class and 25-class datasets.

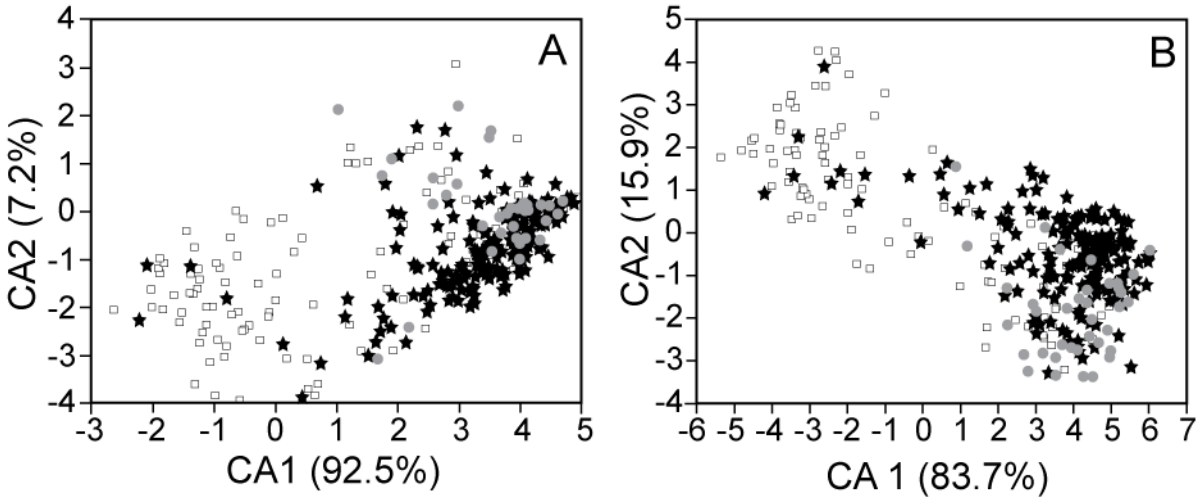

Figure 9: Multiple Analysis of Variance performed on the 5-class (A) and 25-class (B) datasets. Symbols as Figure 8. The probabilities (Wilks'λ) associated to the MANOVAs are given in Table 2.

For each pair of analyzed cryptic species, the Discriminant Analysis returns a function optimizing the correct classification of specimens (Table 3, Figure 10). The 5-class cutting mode allows the correct classification of 65% to 91% of the studied specimens (depending on the pair of cryptic species considered), whereas the 25-class cutting mode allows the correct classification of 72% to 91% of the specimens. The leave-one-out cross-evaluations return essentially the same levels of classification efficiency. In both cutting modes, the highest differentiation is observed between the Caribbean and Mediterranean species, then between

the Caribbean and Sargasso species, and then between the Sargasso and Mediterranean species (Table 2).

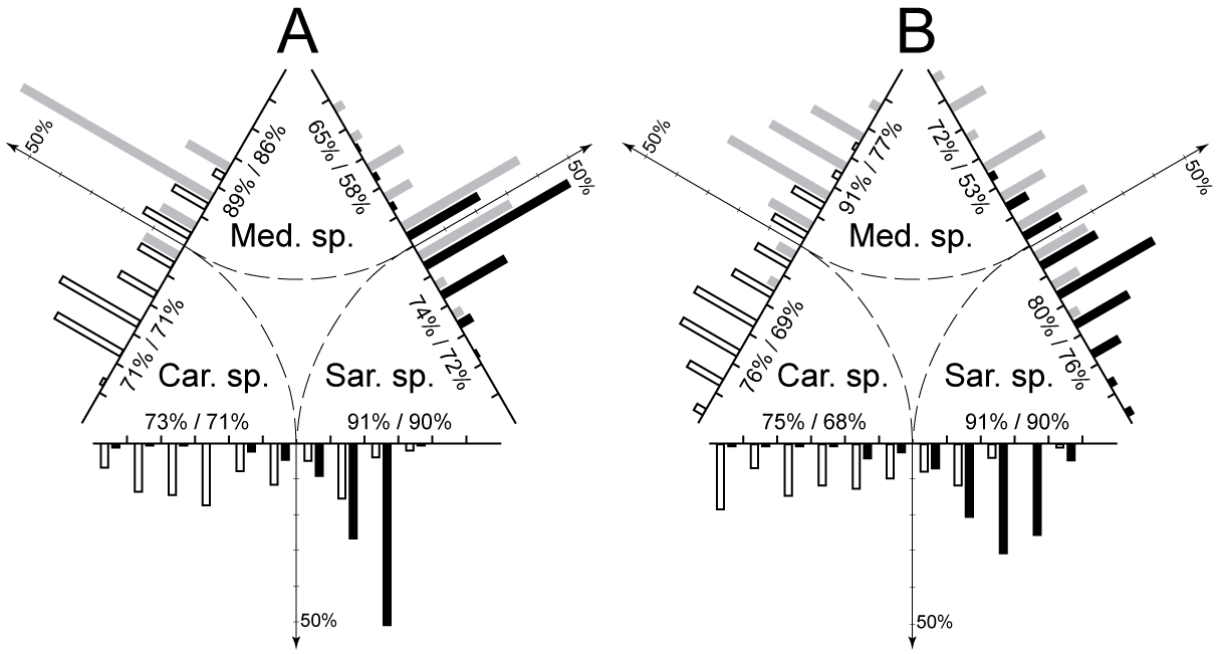

Figure 10: Results of the Discriminant Analyses performed on the three possible pairs of cryptic species of *Orbulina universa* for the 5-class (A) and 25-class (B) datasets. Each side of a "triangle" is the discriminant axis associated to a pair of cryptic species, allowing for their optimal separation based only on their pore surface distribution. Black, grey and opens bar represent the percentage of specimens of the Mediterranean, Sargasso and Caribbean species, respectively. For each cryptic species pole, the first number below each discriminant axis gives the percentage of correctly classified specimens in analyses including all specimens, whereas the second number gives the result of the leave-one-out cross-evaluation. See Table 3 for numerical details on the discriminant functions.

Pore size classes (log / µm²)		Carribean sp. *vs*. Sargasso sp.	Caribbean sp. *vs*. Mediterranean sp.	Sargasso sp. *vs*. Mediterranean sp.
25-class cutting mode				
(-0.1;0]	(0.7943; 1]	-0.1904	-0.0554	-0.1552
(0;0.1]	(1; 1.2589]	0.9286	0.3471	-0.2839
(0.1;0,2]	(1.2589; 1.5849]	-0.8672	-0.1143	0.9821
(0,2;0.3]	(1.5849; 1.9953]	0.3228	-0.1188	-1.2457
(0.3;0,4]	(1.9953; 2.5119]	0.5546	0.0913	-0.6246
(0,4;0.5]	(2.5119; 3.1623]	-0.5325	0.1624	1.1347
(0.5;0,6]	(3.1623; 3.9811]	0.3957	0.3115	-0.0237
(0,6;0.7]	(3.9811; 5.0119]	0.7392	0.132	-0.8363
(0.7;0.8]	(5.0119; 6.3096]	-0.3262	-0.3171	0.1621
(0.8;0.9]	(6.3096; 7.9433]	-0.1751	0.1143	-0.3048
(0.9;1]	(7.9433; 10]	-0.3086	0.1771	1.1103
(1;1.1]	(10; 12.5893]	-0.4125	-0.9532	-0.998
(1.1;1.2]	(12.5893; 15.8489]	0.0447	0.1921	0.3056
(1.2;1.3]	(15.8489; 19.9526]	0.5119	-0.2754	0.263
(1.3;1.4]	(19.9526; 25.1189]	-0.4651	-0.2661	0.6532
(1.4;1.5]	(25.1189; 31.6228]	-0.3347	0.0268	0.3101
(1.5;1.6]	(31.6228; 39.8107]	-0.1764	0.1552	0.6920
(1.6;1.7]	(39.8107; 50.1187]	0.1753	-0.3706	-0.287
(1.7;1.8]	(50.1187; 63.0957]	-0.5425	-0.7789	-0.7017
(1.8;1.9]	(63.0957; 79.4328]	-0.1109	-0.3374	0.0015
(1.9;2]	(79.4328; 100]	-0.5251	-0.6215	0.2762
(2;2.1]	(100; 125.8925]	-0.139	-1.3432	-2.9368
(2.1;2,2]	(125.8925; 158.4893]	0.1795	-0.3918	-0.8983
(2,2;2.3]	(158.4893; 199.5262]	-0.1019	-0,0207	1.2346
(2.3;2.4]	(199.5262; 251. 1886]	1.356	4.2545	2.1716
Offset constant		0.127	-1.9178	-1.2431
5-class cutting mode				
(-0.1;0.1]	(0.7943;1.2589]	0.235	0.1649	-0.0677
(0.1;0.9]	(1.2589; 7.9433]	0.5681	1.0296	-0.4313
(0,9;1.3]	(7.9433; 19.9526]	-0.5302	-0.3112	0.477
(1.3;1.8]	(19.9526; 63.0957]	0.0376	0.1732	0.9139
(1.8;2.4]	(63.0957;251. 1886]	-0.3105	-1.0565	-0.8919
Offset constant		1.6148	3.8363	0.4518

Table 3: Coefficients of discriminant functions established for pair couple of cryptic species in both cutting modes.

4.3.3. Differentiation in pore surface distribution within cryptic species

Samples analyzed in this study were collected in various regions of the world ocean (i.e., E Atlantic, NW Atlantic, S Indian and S Pacific Oceans) that yielded sea surface temperatures (SSTs) ranging from ~13°C to ~29°C.

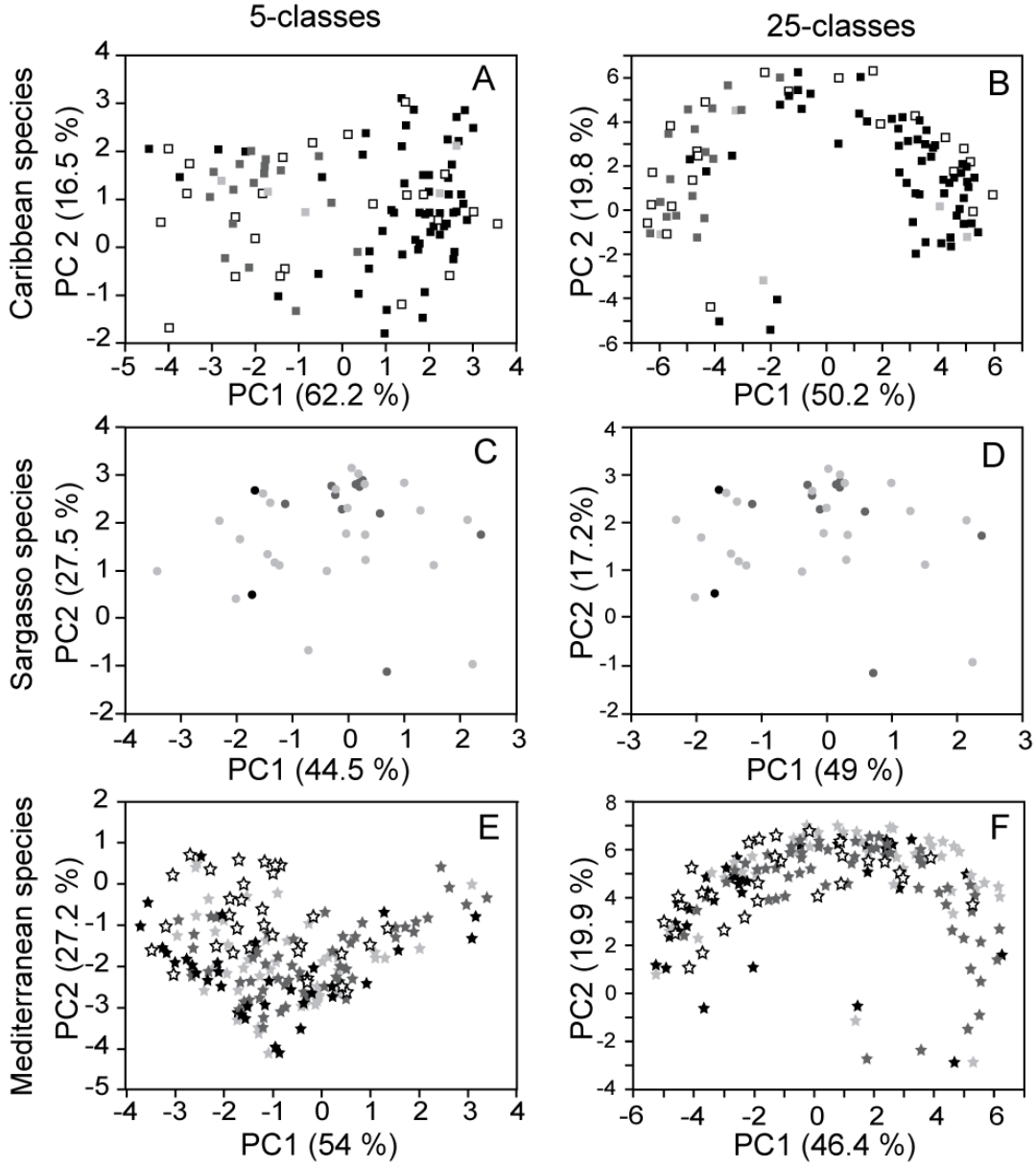

Figure 11: Principal Component Analyses performed for each cryptic species on the 5-class (A) and 25-class (B) datasets. Stars: Mediterranean species; circles: Sargasso species; squares: Caribbean species; open symbol: AMT-8 (east Atlantic); black symbol: C-MarZ (NW Atlantic); light grey symbol: OISO-4 (south Indian Ocean); dark grey symbol: REVELLE (south Pacific). Two-way ANOVA results associated to these PCAs are given in Table 4.

First, in order to depict the pattern of inter-basinal PSD differentiation, PCAs coupled with two-factor ANOVAs on the first two principal components were applied separately to each individual cryptic species (Figure 11). We thus tested for PSD differentiation within cryptic species of different regions of the world ocean. Our results show that whereas there is some morphological variability among the Caribbean species, especially between C-MarZ stations and the three other regions, both Mediterranean and Sargasso species are homogenous throughout the oceanic basins (Table 4). Shell PSDs within the Caribbean species of *O. universa* show a high overall differentiation between sampled regions. The pairwise ANOVAs realized for each pair of sampled regions indicate a very high PSD difference between Caribbean morphotypes from the cruises C-MarZ and REVELLE, whereas the Caribbean morphotypes from OISO-4 are not significantly different from those from AMT-8 and REVELLE.

5 classes	Caribbean sp.			Sargasso sp.			Mediterranean sp.		
Cruise samples	df1; df2	F	p-value	df1; df2	F	p-value	df1; df2	F	p-value
Overall	3; 94	52.98	**3.54×10^{-29}**	2; 31	0.15	0.88	3; 163	3.64	0.01
AMT-8/OISO-4	1; 26	3.69	0.06				1; 75	2.13	0.15
OISO-4/REVELLE	1; 38	1.54	0.22				1; 86	8.46	**4.00×10^{-3}**
OISO-4/C-MarZ	1; 76	34.14	**1.87×10^{-8}**				1; 62	12.53	**5.00×10^{-4}**
AMT-8/REVELLE	1;19	26.78	**2.45×10^{-6}**	1; 31	1.45	0.24	1; 102	2.13	0.15
AMT-8/C-MarZ	1; 57	21.29	**6.78×10^{-6}**	1; 22	0.12	0.75	1; 78	3.42	0.07
REVELLE/C-MarZ	1; 69	141	**2.29×10^{-25}**	1; 10	0.82	0.42	1; 89	2.69	0.11
25 classes									
Overall	3; 94	75.56	**2.64×10^{-39}**	2; 31	0.55	0.60	3; 163	5.2	1.60×10^{-3}
AMT-8/OISO-4	1; 26	2.39	0.12				1; 75	7.72	**6.30×10^{-3}**
OISO-4/REVELLE	1; 38	4.15	0.05				1; 86	13.74	**2.63×10^{-4}**
OISO-4/C-MarZ	1; 76	23.46	**2.43×10^{-6}**				1; 62	0.35	0.55
AMT-8/REVELLE	1;19	5.00	**0.03**	1; 31	0.71	0.41	1; 102	1.30	0.25
AMT-8/C-MarZ	1; 57	121.90	**9.20×10^{-23}**	1; 22	0.8	0.42	1; 78	10.76	**1.30×10^{-3}**
REVELLE/C-MarZ	1; 69	180.80	**2.39×10^{-30}**	1; 10	2.8	0.16	1; 89	6.09	0.01

Table 4: Overall and pairwise two-way ANOVAs associated to PCA results (Figure 11), testing for differences in pore surface distribution of each cryptic species between cruises, based on the 5-class and 25-class datasets.

Second, we plotted the observed morphological variation within each cryptic species as recorded by the first two principal components of PCAs with SST (measured at 10 m depth; Kucera et al., 2005a). On the one hand, these plots indicate no clear SST pattern of PSD differentiation for both the Mediterranean and Sargasso species (Figure 12, Table 5), as also shown by inter-basinal comparisons. On the other hand, the Caribbean species exhibits a marked SST-related PSD heterogeneity shown by both the 5-class and 25-class datasets, corresponding to the existence of two distinct morphological groups, one occurring only at SSTs higher than ~24°C.

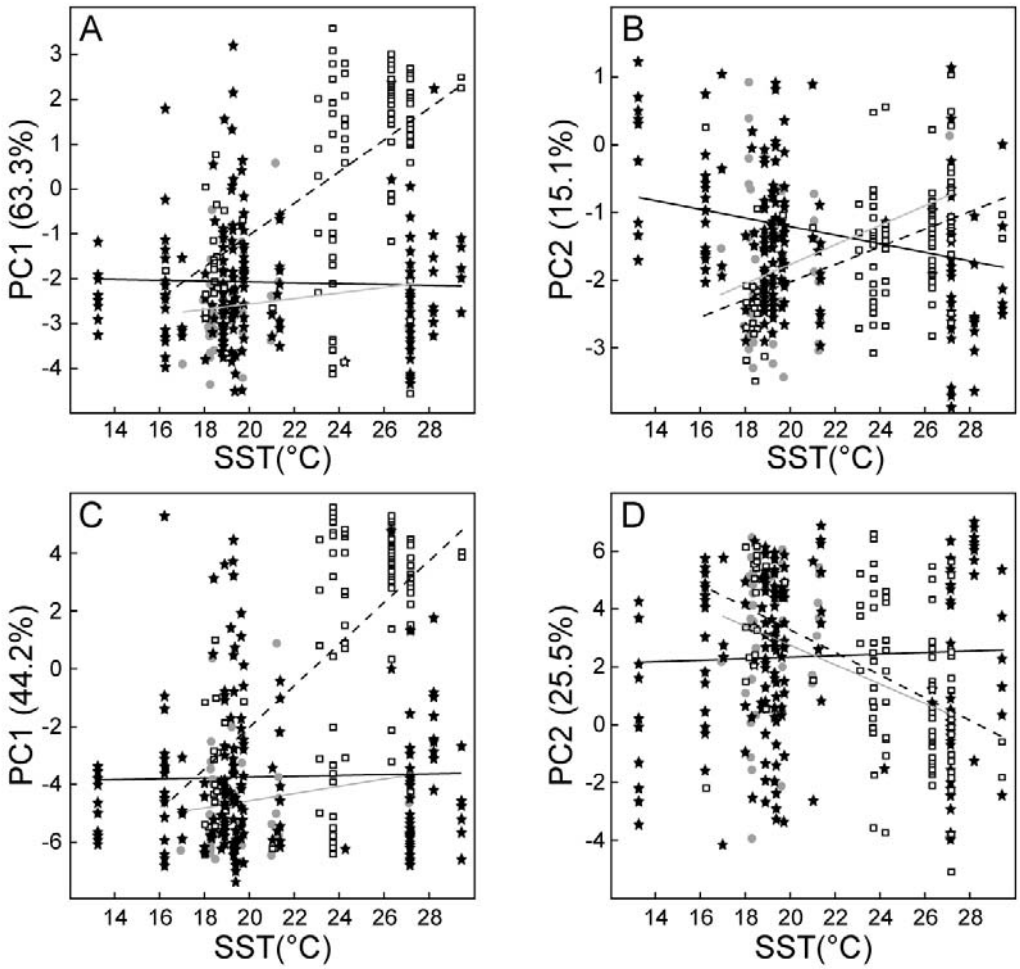

Figure 12: Biplots of SST *vs.* first two principal components calculated for the 5-class (A, B) and 25-class (C, D) datasets. SST interpolated when not available from CTD casts (error rate <0.5°C). SST-PC least-square regressions for the Mediterranean (black stars), Sargasso (grey circles), and Caribbean (open squares) species shown as solid, grey and dashed lines, respectively (see Table 5 for associated correlation coefficients).

Cryptic species	SST *vs.* PC1		SST *vs.* PC2	
	r	p-value	r	p-value
5 classes				
Caribbean sp.	0.54	**4.35×10^{-9}**	0.45	**2.3×10^{-6}**
Sargasso sp.	0.14	0.4	0.19	0.25
Mediterranean sp.	-0.02	0.74	-0.36	**1.03×10^{-6}**
25 classes				
Caribbean sp.	0.59	**6.22×10^{-11}**	-0.46	**1.12×10^{-6}**
Sargasso sp.	0.11	0.5	-0.19	0.24
Mediterranean sp.	0.008	0.9	0.03	0.61

Table 5: Pearson correlation coefficients associated with linear relations between the first two principal components and SST, estimated for the 5- class and 25-class datasets (Figure 12).

4.3.4. Shell thickness differentiation among cryptic species

The ANOVA involving the Mediterranean (n = 30; mean thickness = 11.75 ± 6.07 µm), Caribbean (n = 27; mean thickness = 14.25 ± 7.16 µm) and Sargasso (n = 12; mean thickness = 6.51 ± 4.47 µm) species indicates a significant wall thickness variation among cryptic species of *Orbulina universa* (Table 6). Furthermore, contrast analysis for each pair of cryptic species indicates that while there is no differentiation between the Mediterranean and the Caribbean species based on their wall thickness, individuals of the Sargasso species exhibit significantly thinner shells.

Species samples	df1; df2	F	*p*-value
Overall	2; 66	6.271	**3.21×10^{-3}**
Caribbean-Sargasso	1; 37	11.86	**1.40×10^{-3}**
Caribbean-Mediterranean	1; 55	2. 05	0.15
Sargasso-Mediterranean	1; 40	7.286	**0.01**

Table 6: Overall and pairwise cruise samples one-way ANOVAs associated to wall thickness measurements, testing for differences between each cryptic species.

5. Discussion

5.1. Environmental significance of shell porosity in Orbulina universa

The relationship between shell porosity in planktonic foraminifera and environmental parameters was first recognized by empirical research (Bé, 1968; Frerichs et al., 1972; Bé et al., 1973; 1976) and later confirmed in culture studies (Bijma et al., 1990). These studies have suggested that in most planktonic foraminiferal taxa, shell porosity tracks latitude and is correlated with temperature as a function of oxygen solubility in water. Planktonic foraminiferal shell porosity has been subsequently considered as climatically significant and used as a paleoceanographic index (Colombo and Cita, 1980; Haenel, 1987; Fisher, 2003; Fisher et al., 2003).

Bé et al. (1973) examined the variation in shell size and shell porosity of *Orbulina universa* specimens from plankton net tow and sediment samples from the Indian Ocean. Size and shell porosity of the final chamber were found to correlate with latitude. According to these authors, the geographic distribution of shell porosity in *O. universa* displays clinal patterns of higher porosities in the warm waters of the tropical areas, and lower porosities occurring at higher latitudes, where surface waters are cooler. Our results suggest that this relationship originates from collected samples in which Bé et al. (1973) mixed up at least two cryptic species of *O. universa* (see previous section; Figs. 11 and 12; see also Figure 13c of Bé et al., 1973).

Indeed, the shells of the Caribbean species, found in the Indian Ocean in the oligotrophic and warm waters of the subtropical zone (Figure 4), are characterized by variable but relatively high porosity values linked with larger pores (Figure 13). When coupled with the SST-PSD relationship observed for this cryptic species (Figure 12), this high PSD-variability suggests that it may be actually made of two, yet unseparated distinct cryptic species based on the analysis of rDNA 18S.

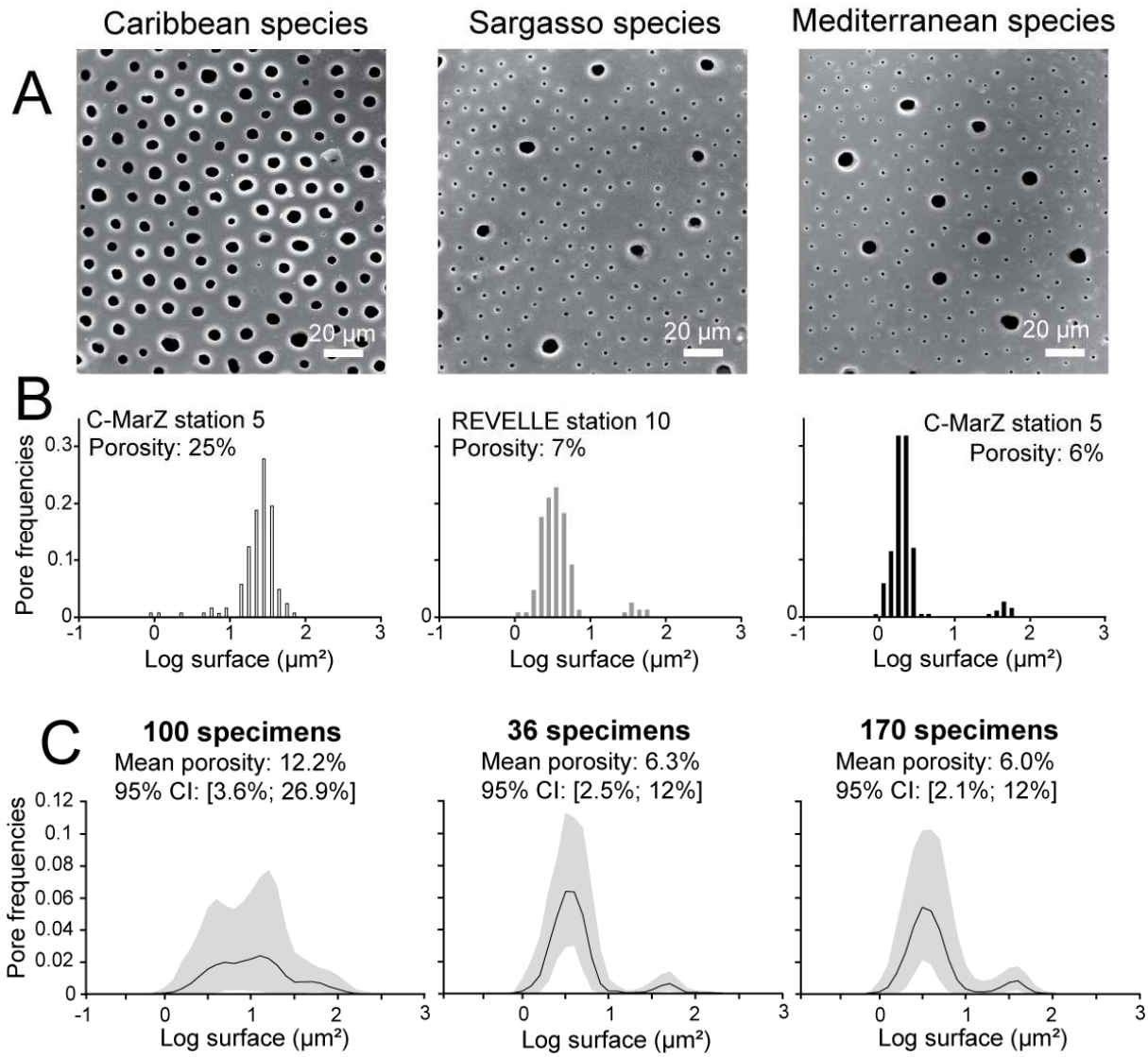

Figure 13: (A) SEM photomicrographs and (B) associated pore surface distribution diagrams of the inner shell surface of 3 specimens of the Caribbean, Sargasso and Mediterranean species of *Orbulina universa*. Note that the specimen of the Caribbean species exhibits large pores with a unimodal PSD, while the specimens of the Mediterranean and Sargasso species are characterized by lower small-pore areas (with a bimodal PSD) and shell porosities (see also Figure 7). (C) Mean pore data frequencies and overall porosity values (with associated asymmetrical 95% Confidence Intervals) for each of the three cryptic species (relative frequencies recorded within 40 Log_{10}-surface classes ranging from -1 to 3, i.e., from 0.1 to 1000 µm^2). The dark line represents the mean frequency distribution of pore surface classes and the grey area is the ± 1 standard deviation interval. Although the inner shell porosity of the Sargasso species resembles that of the Mediterranean species, the discriminant models demonstrate that the pore surface distributions of these two cryptic species can be distinguished (Figure 10).

In contrast, the Mediterranean species typically exhibits smaller porosity values and pore surfaces than the Caribbean species (Figure 13). In the Indian Ocean, Mediterranean genotypes were collected in productive and cool waters of the subtropical frontal zone (Figure 4). Considering the water masses characteristics of specimens we studied, the morphological

differences we observe among cryptic species are similar to those evoked by Bé et al. (1973). This strongly suggests that the purported relations between porosity and water temperature and salinity are the spurious consequence of a hidden biological diversity. Although laboratory experiments showed that changes in *O. universa* shell porosity do correlate with treatment variables in culture, these changes appear moderate relative to those we observed among cryptic species (compare Figure 7 with Table 8 in Bijma et al., 1990). We conclude that the latitudinal changes in shell porosity described by previous studies of *O. universa* (Bé et al., 1973; 1976; Hetcht et al., 1976) are not due to environmental factors alone but have a significant genetic component.

5.2. Cryptic diversity and ecophenotypy in planktonic foraminifera

Since first proposed by Parker (1962), numerous studies have shown that planktonic foraminiferal morphospecies exhibit morphological gradients with latitude. The significance of these observed gradients has not been clearly understood and the various morphotypes were classically considered as ecophenotypic variants (Kennett, 1968a; 1968b; Malmgren and Kennett, 1972; Hecht, 1974; Healy-Williams et al., 1985; Williams et al., 1988; Healy-Williams, 1992). We find that morphological variation in *Orbulina universa* clearly owes much to the expression of different cryptic species adapted to different environments.

Erroneous attributions of morphological clines to environmental factors may be more common than previously suspected in planktonic foraminifera. In the first genetic study of cryptic diversity in planktonic foraminifera, Huber et al. (1997) discovered genetic differences between two cryptic species of *Globigerinella siphonifera* that matched differences in their shell size, porosity, spine density and depth-related ecology (see also Bijma et al., 1998). In addition, inter-basin genetic studies of this spinose and symbiont-bearing morphospecies (Darling et al., 1999; de Vargas et al., 2002) have highlighted geographic segregation among cryptic species that is correlated with water mass stratification and productivity. These results strongly suggest that previously described ecophenotypes within *G. siphonifera* (see for example Parker, 1962; Hecht and Savin, 1972) correspond to different biological species adapted to distinct environmental conditions.

It is premature to speculate about cryptic diversity and ecophenotypy relationships in other morphospecies of spinose planktonic foraminifera because we lack large scale inter-basin genetic analyses and/or direct morphologic characterization of the cryptic diversity

within these taxa. However, some of them may constitute good candidates for better understanding the significance of morphological clines in planktonic foraminifera. For example, *Globigerinoides ruber* and *Globigerina bulloides* - apparently composed of four (Darling et al., 1997; 1999; Kucera and Darling, 2002) and six (Darling et al., 1999; 2000; Stewart et al., 2001; Kucera and Darling, 2002) distinct cryptic species, respectively - show ambiguous taxonomic status with latitudinally divergent morphotypes (see Parker, 1962; Orr, 1969; Hetcht, 1974, Robbins and Healy-Williams, 1991 for *G. ruber*; Bandy, 1972; Malmgren and Kennett, 1976; 1978 for *G. bulloides*). The modest number of specimens surveyed to date for both *G. ruber* and *G. bulloides* suggests that there may well be additional undiscovered genotypes in both clades.

Further evidence for relationships between cryptic diversity and ecophenotypy can be found in the non-spinose planktonic foraminifera. Healy-Williams et al. (1985) showed that the population structure of *Globorotalia truncatulinoides* in the southern Ocean consists of a complex of three sub-populations with distinct morphologies and depth-related specializations whose abundance varies with latitude. Single-cell DNA and morphometric analyses performed on plankton tow specimens from the South Atlantic have revealed that *G. truncatulinoides* actually corresponds to a complex of at least four cryptic species adapted to particular hydrographic conditions (de Vargas et al., 2001). However, they showed that two of these four cryptic species are characterized by similar morphologies and geographic occurrences. Accordingly, as for *Orbulina universa*, the morphological cline evidenced by Healy-Williams et al. (1985; see also Kennett, 1968a) is very likely to be the expression of different biological species adapted to different environments (de Vargas et al., 2001).

Another convincing example is given by *Neogloboquadrina pachyderma*, which yields some genetic differences (Bauch et al., 2003; Darling et al., 2004; 2007) that correlate with differences both in shell coiling direction and biogeographic distribution (Darling et al., 2006). The relationship between ratio of coiling direction in *N. pachyderma* and water mass temperature has long been established and many paleoceanographic studies have assumed that it reflects ecophenotypic variation (e.g., Ericson, 1959). Darling et al. (2006) have demonstrated that the coiling direction in *N. pachyderma* is a genetic trait, not a morphological character reflecting ecophenotypic variation, and then suggested the adoption of the name *Neogloboquadrina incompta* for the right coiling forms. Furthermore, numerous additional morphological characters in *N. pachyderma*, including shell outlines, number of chambers, wall thickness and ultra-structure have been correlated with latitude in the southern hemisphere (Kennett, 1968b; Malmgren and Kennett, 1972b). Healy-Williams (1992) showed

that the latitudinal population structure of *N. pachyderma* in the Indian and Atlantic Oceans is composed of several discontinuous morphological sub-populations, easily discernible by eye. Significant stable isotope differences, which cannot be explained by seasonal temperature variations, have also been found between morphological variants (Healy-Williams, 1992).

5.3. Implications for paleoceanography

Contrary to classical views, ubiquitous species of planktonic foraminifera may be less common than previously invoked. This changes our understanding of planktonic foraminiferal ecology, evolution and the paleoceanographic inferences that can be drawn from the classical morphospecies concept.

In addition to the development of paleo-proxies deduced from the chemical composition of calcite in planktonic foraminiferal shells (e.g., Emiliani, 1954; Spero and Williams, 1988; Fischer and Wefer, 1999), the use of these organisms in paleoceanography expanded rapidly since Imbrie and Kipp (1971) developed quantitative empirical calibrations between the relative abundance of a morphospecies in a given surface sediment sample and the sea surface temperature. Although temperature may not be the only factor affecting the taxonomic richness and relative abundance of planktonic foraminifera (e.g., Lipps, 1970; Hallock, 1987; but see Rutherford et al., 1999; Brayard et al., 2005; Morey et al., 2005; Escarguel et al., 2008), this empirical relationship has been extensively used to develop "transfer functions" that allow the prediction of ancient environmental parameters from the abundance and/or occurrence of species in sediment samples (e.g., Imbrie and Kipp, 1971; CLIMAP, 1976; Hutson, 1977; Malmgren et al., 2001; Mix et al., 2001; Kucera et al., 2005a; 2005b).

In this context, the morphological recognition of cryptic species of planktonic foraminifera may significantly increase the resolving power of such paleoceanographic reconstructions (Darling et al., 2000; Kucera and Darling, 2002). Indeed, if cryptic species have different restricted environmental preferences, then their recognition in the fossil record may increase the accuracy of the environmental predictions. For example, Kucera and Darling (2002) modelled the effect of splitting *Globigerina bulloides* into three cryptic species in the Atlantic Ocean. These authors showed that the incorporation of such cryptic species resulted in a substantial (up to 34%) decrease in error rate of the transfer functions. They also

suggested that the total decrease in prediction error rate might be tremendous if all morphospecies could be split into cryptic species.

In their new approach for assessing reliability of environmental estimates in assemblages of planktonic foraminifera, Kucera et al. (2005a; 2005b) have attempted to limit bias associated with the presence of cryptic species by geographically constraining the foraminiferal calibration datasets. Individual genetic types among planktonic foraminifera display narrower ecological requirement ranges, and thus potentially greater degree of endemicity than their related morphospecies (Darling et al., 1999; 2004; 2006; de Vargas et al., 1999; 2001; 2002; Stewart et al., 2001; this study). Thus, reducing the geographical coverage of calibration data set logically contributes to increase the statistical accuracy of transfer functions. Our results suggest that the approach developed by Kucera et al. (2005a; 2005b) is only the first step in the application of cryptic diversity in paleoceanographic reconstructions. In order to transfer the biological information acquired through genetic analysis to the interpretation of the sediment record, it is necessary to characterize the morphological differences among the planktonic foraminiferal eco-genotypes.

In the case of *Orbulina universa*, we need to be able to recognize distinguishing characters in the fossil record. Several circumstantial arguments appear to be favourable for such recognition. First, there are only three, yet recognized cryptic species in *O. universa*, making it feasible to transfer the biological information to the interpretation of the fossil record. Second, our PSD-based model correctly predicts the genotype of 65% to 91% of modern specimens, depending upon the pair of cryptic species considered (Figure 10). The potential to attribute a single fossil specimen to a genotype remains limited with such a model, especially in the case of the Mediterranean and Sargasso species identification (Figure 13), but fortunately there are significant differences in shell thickness between these two genotypes (Table 6). Our results suggest that, although discrimination is not perfect, it is possible to distinguish between populations of shells from the plankton and this approach should work equally in the fossil record. It is possible that sediment-hosted specimens may be biased against thin-walled forms, which include the majority of the Sargasso genotype, thus limiting much of the record to the more abundant genotypes. Third, according to molecular evolutionary rates, separation between genotypes occurred during the Miocene: the Mediterranean-Sargasso stem lineage diverged from the Caribbean species ~12 myr ago, and the splitting of Mediterranean and Sargasso species occurred 6 to 7 myr ago (de Vargas et al., 1999). If the PSD or shell thickness characteristics evidenced here are stable through time (see Figure 4 in de Vargas et al., 2003), then consequences for paleoceanography may be

extremely significant. We caution, however, that it is possible that there are other short-lived genotypes that have not yet been recognized in the fossil record. Indeed, the abundance of living genotypes certainly raises the question of how "bushy" the foraminiferal phylogenetic tree may be even within a seemingly fairly conservative group like *Orbulina*.

Of course, consequences for paleoceanography rely on the evolutionary connection between morphological, genetic and environmental differentiations in the fossils. For instance, as already pointed out by de Vargas et al. (2003), we can only speculate for those times that the (possible) fossil cryptic species of *Orbulina universa* evolved in unique morphologies. Further research is needed to evaluate, back into the fossil record, the temporal and spatial stabilities of the PSD and shell thickness characters we identify among extant cryptic species to characterize the past window of application of our model.

6. Conclusion

Genetic analyses of unicellular organisms are usually destructive and do not allow combined DNA and morphological analyses. In our study, the application of the GITC* protocol on a large number of *Orbulina universa* specimens collected worldwide in the open ocean allowed us to extract DNA from single specimens without destruction of the calcareous shell, and thus to investigate the environmental significance of shell pore surface distribution and wall thickness variability within this morphospecies. We recognize three cryptic species of *O. universa* which have distinct geographic distributions that are correlated with the productivity of the surface waters. The phylogeography of *O. universa* is also reflected in small, but recondite differences in shell morphology. Hence, the various genotypes of *Orbulina universa* are not strictly cryptic taxa (e.g., Knowlton, 1993), since they can be distinguished, at least on a population level from one another. Our results suggest that even seemingly simple morphologies, like the spherical tests of *Orbulina*, may yield information on species diversity if studied with sufficient care.

Theoretically, integration of further morphological characters, such as the pre-adult trochospiral shell morphology (e.g., proloculus size, coiling, and chamber growth rates) could also reveal valuable new criteria for distinguishing genotypes of *O. universa* and then contribute to further refine paleoceanographic reconstructions. Nevertheless, study of such small and delicate parts of the shell (< 1 μm wall thickness for the pre-adult trochospiral stage; Bé et al., 1973) remains difficult due to mechanical constraints (i.e., vortex homogenization and centrifugation) during the DNA extraction process and then SEM specimen preparation. Moreover, study of such morphological features for paleoceanography will remain highly limited due to their scarce preservation in the fossils.

There is still a long way to go from our methodology based on SEM pictures of the inner pore surface distribution of *Orbulina universa* specimens collected in the open ocean, to the development of a routine recognition of cryptic species in sediments. Estimations of dissolution effects on shell microstructure are for example needed. Understanding of the time-window of applicability of our model will require an accurate evaluation of the temporal stability of cryptic species morphologies back into the fossil record. We believe that a finer characterization of ecological preferences among cryptic species of *O. universa* is necessary in order to increase the power and accuracy of the transfer functions based on planktonic

foraminifera. Finally, the development of semi-automatic protocols for morphological recognition of cryptic species will enable palaeoceanographers to safely and quickly extract higher-precision paleoclimatic data from sediments.

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Chapitre 3 : Diversité morphologique des espèces cryptiques de *Truncorotalia truncatulinoides* d'Orbigny

Ce troisième chapitre s'inscrit dans la même logique que le second : la caractérisation des espèces cryptiques d'un taxon déjà largement étudié : *Truncorotalia truncatulninoides*. C'est un foraminifère non-épineux, caréné, strictement hétérotrophe et pouvant vivre de 0 à 2000 m de profondeur (Schiebel and Hemleben, 2005). Diversifiée à hauteur de 5 génotypes (de Vargas et al., 2001 ; Ujiée & Lipps 2009), cette morpho-espèce a fait l'objet de nombreux travaux de morphométrie depuis les années 1980 (e.g., Healy Williams & Williams, 1981). Un fort degré d'écophénotypie a été observé chez ce taxon amenant Healy-Williams et al. (1985) à suspecter la présence de sous-populations dans l'Océan mondial.

Ce travail a débuté par la quantification morphologique de spécimens génotypés de la campagne REVELLE (Pacifique sud) et OISO-4 (Océan Indien). Grâce à la caractérisation génétique, morphologique et écologique de ~600 spécimens, nous avons fortement amélioré la connaissance de ces espèces cryptiques. Il en résulte un modèle de reconnaissance morphologique robuste discriminant les espèces à affinités subtropicales (types I, II et V) de celles occupant les zones de transition et subpolaires (types III et IV). Ce modèle permet de reconstituer les migrations du front nord subtropical de l'hémisphère sud durant le Pléistocène récent.

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Truncorotalia truncatulinoides and the value of global scale single-cell morphogenetic analyses in planktonic foraminifera

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Abstract

Genetic analyses of planktonic foraminifera have unveiled significant levels of cryptic diversity, thus challenging the usefulness of morphospecies concept for paleoceanographic reconstructions. Here, we present single-cell combined genetic and morphological analyses performed on living *Truncorotalia truncatulinoides* collected across the world oceans. Our morphogenetic comparisons allow us to (1) detect five different genetic types (Types I to V) within the morphospecies *T. truncatulinoides*, (2) statistically analyse shape variations among these genotypes, and (3) re-assess the biogeographic patterns and hydrographic distribution of morphogenetic diversity within *T. truncatulinoides*. Types I inhabit the warm (sub)tropical waters of the south hemisphere. Types II and V are found in the warm (sub)tropical waters of the Atlantic and NW Pacific, respectively. Types III and IV are restricted to the productive subtropical and the cold subpolar frontal zones of the southern ocean, respectively. Single-cell morphogenetic comparisons reveal significant differences in test morphology among most of the genotypes of *T. truncatulinoides*. These differences are especially obvious when comparing clusters of genotypes across the subtropical fronts in the southern Ocean, with large, highly conical left (Indian Ocean) or right-coiled (Pacific Ocean) specimens north of the North subtropical front (Type I), and small, axially compressed and biconvex left-coiled specimens south of this front (Types III and IV). Our morphogenetic data bring additional evidence for a late Pleistocene invasion of the southern Ocean by novel *T. truncatulinoides* genotypes, and show that this eco-evolutionary pattern corresponds to a global latitudinal biogeographic event. We finally build a model based upon test outline analyses, which correctly assigns up to 75% of the specimens to their corresponding cluster of genotypes. Application of this model to sediment samples may contribute to monitor migrations of the subtropical front during the late Pleistocene.

1. Introduction

Calcareous shells (tests) of planktonic foraminifera (Globigerininae) are commonly used in paleoceanographic studies to reconstruct sea surface environmental conditions and upper ocean structure (e.g., Mulitza et al., 1997). These studies lie on the ground hypothesis that each individual morphospecies has its own, stable through time ecological preferences that can be used for reconstruction of past water mass properties (e.g., Kennett, 1968; Malmgren et al., 2001). The use of species-specific paleoproxies requires a high degree of taxonomic consistency. Since the CLIMAP global reconstruction of glacial oceanic conditions (CLIMAP, 1976), the morphological definition of planktonic foraminiferal species has been set very broadly, and morphological variation has been classically regarded as intra-specific variability or ecophenotypy (e.g., Kennett, 1976; Hecht et al., 1976; Healy-Williams and Williams, 1981; Healy-Williams et al., 1985).

A growing body of molecular studies have revealed that the classical, morphological definition of species in planktonic foraminifera hides higher levels of genetic and ecological differentiation (de Vargas et al., 1997; 1999; 2001; 2002; Huber et al., 1997; Darling et al., 1999; 2000; 2004; 2006; 2007; Stewart et al., 2001; Bauch et al., 2003; Kuroyanagi et al., 2008; Aurahs et al., 2009; Ujiié and Lipps, 2009; see Darling and Wade, 2008 for a review). Based on the molecular data currently available, phylogenetic analyses of the SSU (Small SubUnit) or ITS (Internal Transcribed Spacers) regions of ribosomal DNA (rDNA) on both spatially and environmentally well-sampled extant morphospecies, allowed the recognition of two to seven distinct genotypes per morphospecies (Darling and Wade, 2008). The large genetic distances between many of these genotypes, the high degree of genetic similarity within each genotype, the oldness of their separation times and their environmental and geographical separations strongly suggest that they correspond to cryptic biological species. Paleoceanographic reconstructions using a broad morphospecies concept are consequently based on aggregates of biogeographically and ecologically distinct species; they contain significant noise and the usual assumption of homogeneity made in these reconstructions is seriously challenged (Kucera and Darling, 2002; Kucera et al., 2005a).

Nevertheless, several subtle morphological differences known within morphospecies and classically treated as ecophenotypic variants may distinguish cryptic species adapted to highly different water masses (Huber et al., 1997; Morard et al., 2009), then making possible to transfer the biological information acquired through genetic analysis to paleoceanography. For such an achievement, it is necessary to detect which characters of the test may permit

significant distinction of the planktonic foraminiferal cryptic species. Direct morphogenetic comparisons should ideally be conducted on a large number of single individuals collected from large geographic ranges in order to distinguish the morphological variation induced by environmental triggers from that due to intraspecific genetic isolation and drift. Such comparisons are now feasible with the development of the GITC* buffer (de Vargas et al., pers. comm.) that makes the DNA extraction step non-destructive for the foraminiferal calcareous test. In this paper, the tests of ~600 genetically-characterized *Truncorotalia truncatulinoides* specimens collected in different water masses of the world oceans are morphometrically analyzed.

Truncorotalia truncatulinoides is a non-spinose, non-symbiotic trochospiral planktonic foraminifera that originated 2.8 myrs ago in the subtropical southwest Pacific (Lazarus et al., 1995), later spreading into the Indian and Atlantic Oceans ~2.0 myrs ago (Spencer-Cervato and Thierstein, 1997; but see Sexton and Norris, 2008). Today, *T. truncatulinoides* inhabits a wide depth range, from the surface mixed layer to depths well below the thermocline (Hemleben et al., 1985; Mulitza et al., 1997; Cléroux et al., 2007). Restricted to tropical and subtropical waters in the northern hemisphere, it exhibits a wider temperature tolerance in the southern hemisphere, where its distribution extends to subpolar waters (e.g., Kennett, 1968; Malmgren, 1983). Morphological changes (large and highly conical to small and axially compressed tests), then considered as ecophenotypic, were correlated to latitudes from analyses of subtropical to subpolar surface sediments in the southern hemisphere (Kennett, 1968; Healy-Williams and Williams, 1981; Lohmann and Malmgren, 1983; Healy-Williams et al., 1985; Pharr and Williams, 1987; Lohmann, 1992).

Our choice to study *T. truncatulinoides* was motivated by previous molecular analyses along a latitudinal gradient in the southwest Atlantic, which revealed the existence of four distinct genotypes based on their specific ITS rDNA sequences (de Vargas et al., 2001). Recently, a novel genetic type (Type V) was described based on SSU rDNA variations (Ujiié and Lipps, 2009). Morphometric and genetic analyses of plankton samples, linked the morphological latitudinal cline observed from surface sediment samples to the stepwise distribution pattern of four different genetic types which adapted to specific hydrographic provinces southward (de Vargas et al. 2001). However, most of these genetic and morphometric analyses were not performed on the same specimens, then failing to *unequivocally* demonstrate that the cryptic species of *T. truncatulinoides* indeed exhibit distinct morphologies. Here, single-cell morphogenetic analyses on a larger number of individuals from the world oceans allow us to investigate the significance of the relationships

between genetic and morphological differentiation within this morphospecies. Based on these analyses, we further attempt to build a model that allows recognition of the cryptic species of *T. truncatulinoides*.

2. Material

Specimens were collected using plankton tows from the subtropical to the subpolar oceans (Figure 1). In the Indian and Pacific Oceans, they were collected onboard the *Marion-Dufresne* during the cruise OISO-4 (January-February 2000; Metzl, 2000; 177 specimens) and onboard the *R/V Roger Revelle* during the cruise REVELLE (January-February 2004; http://shipsked.ucsd.edu/schedules/2004/rr_2004/constable/; 281 specimens), respectively. For these two cruises, genetic data by de Vargas et al. (2004) were completed by doubling the number of genotyped specimens. Additional specimens were collected in the Atlantic Ocean onboard the *R/V Ronald H. Brown* as part of the CMarZ project (April 2006; http://www.cmarz.org/CMarZ_RHBrown_April06/index.htm; 242 specimens), in the Mediterranean Sea offshore Marseille onboard the *R/V Antédon II* (February 2008; 96 specimens), and in the northwest Pacific during the cruise KT06-11 (June 2006; 17 specimens). At OISO-4, REVELLE and KT-06 stations, plankton samples were collected using vertical ring net tows (100 µm mesh size) from ~200 m depth to the sea surface. At CMarZ and Marseille stations, 1/4-m^2 Multiple Opening/Closing Nets and Environmental Sensing Systems (MOCNESS; Wiebe et al., 1985) and 1-m^2 MULTINETs with 100 µm mesh sizes were used, respectively, to sample specific layers of the water-column.

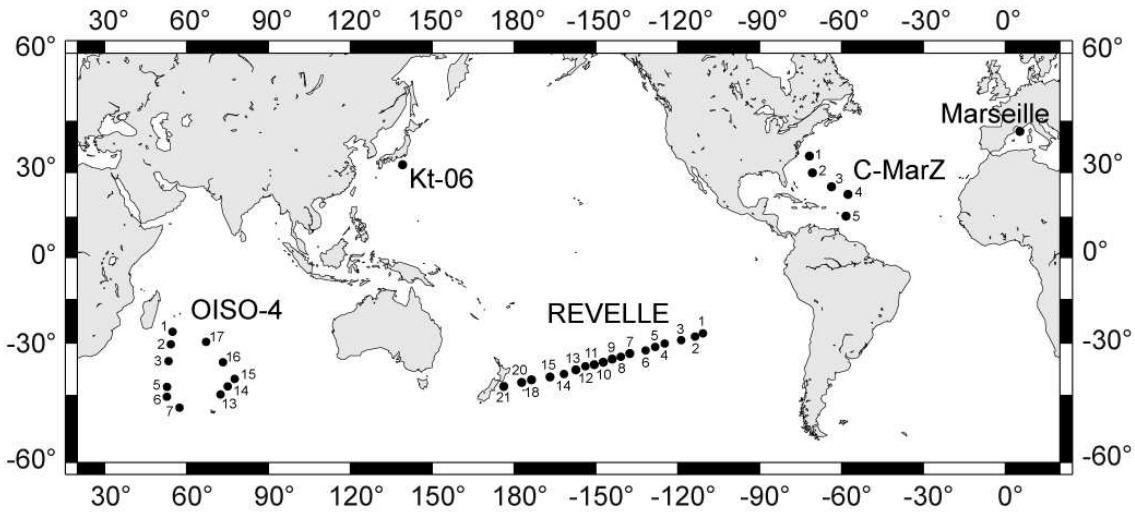

Figure 1: Geographic location and labels of the oceanic stations sampled during the cruises OISO-4, REVELLE, C-MarZ, KT-06 and offshore Marseille.

Our study is based on the analysis of 813 living specimens of *T. truncatulinoides* from 36 stations (Table 1). Each of these specimens was isolated on the day of collection into the

GITC* buffer, and stored at -20ºC. Other specimens collected at 28 of the sampling stations were directly dried after filtration.

Hydrographic data are used to characterize surface water mass features and boundaries. At OISO-4 stations, Conductivity Temperature and Depth (CTD) casts provided water temperature and chlorophyll-a fluorescence profiles of the 250 m upper water column. At all sampled stations, temperature (SST) measurements were also recorded between 7 and 10 m depth.

Cruise	Station	Latitude	Longitude	Nb of specimens within each genotype	% of left-coiled specimens for each genotype	Oceanic provinces
Marseille	N/A	43°40'N	07°15'E	II (96)	93	Mediterranean
C-MarZ	1	33°54'N	69°93'W	II (69)	91	Subtropical Gyre
C-MarZ	2	29°85'N	70°14'W	II (36)	83	Subtropical Gyre
C-MarZ	3	24°87'N	60°13'W	II (97)	29	NEC
C-MarZ	4	19°82'N	54°72'W	II (31)	13	NEC
C-MarZ	5	14°01'N	54°91'W	II (5)	0	NEC
OISO-4	1	27°59'S	54°59'E	I (16)	100	SZ
OISO-4	2	30°00'S	53°30'E	I (19); III (2)	100; 100	SZ
OISO-4	3	35°00'S	53°30'E	I (2); III (6)	100; 100	SZ
OISO-4	5	42°31'S	52°29'E	III (3); IV (2)	100; 100	SFZ
OISO-4	6	45°00'S	52°05'E	IV (10)	100	PFZ
OISO-4	7	47°40'S	58°00E	IV (14)	100	PFZ
OISO-4	13	44°58'S	73°21'E	III (3)	100	SFZ
OISO-4	14	42°30'S	74°53'E	III (12)	100	SFZ
OISO-4	15	40°00'S	76°24'E	III (6)	100	SFZ
OISO-4	16	34°59'E	73°28'E	I (1); III (45)	100; 96	SFZ
OISO-4	17	29°59'S	66°24'E	I (32); III (2)	100; 100	SZ
KT-06	E	34°00'N	140°00'E	V (17)	6	SZ
REVELLE	1	27°67"S	111°55"W	I (39)	5	SZ
REVELLE	2	27°99"S	113°08"W	I (39)	0	SZ
REVELLE	3	29°33'S	118°95'W	I (20)	0	SZ
REVELLE	4	30°66'S	124°77'W	I (5)	0	SZ
REVELLE	5	31°35'S	127°83'W	I (4)	0	SZ
REVELLE	6	32°04'S	130°98'W	I (3)	66	SZ
REVELLE	7	33°39'S	137°12'W	I (2); III (17)	100; 89	SZ or SFZ
REVELLE	8	34°03'S	140°05'W	I (1); III (4)	0; 100	SZ or SFZ
REVELLE	9	34°73'S	143°27'W	III (6)	83	SFZ
REVELLE	10	35°39'S	146°29'W	III (2)	100	SFZ
REVELLE	11	36°07'S	149°48'W	III (19)	100	SFZ
REVELLE	12	36°73'S	152°58'W	III (33)	97	SFZ
REVELLE	13	37°45'S	156°00'W	III (26)	93	SFZ
REVELLE	14	38°52'S	161°23'W	III (24)	92	SFZ
REVELLE	15	39°62'S	166°69'W	III (18)	100	SFZ
REVELLE	18	40°87'S	173°00'W	III (6)	100	SFZ
REVELLE	20	41°97'S	178°56'W	III (5)	80	SFZ
REVELLE	21	42°95'S	176°26'E	III (6)	100	SFZ
				Total = 805		

Genetic data at OISO-4 and REVELLE stations from de Vargas et al. (2004) and this work, at C-MarZ and Marseille from this work.

Table 1: Location of the sampling stations, with indications of the identified cryptic species of *Truncorotalia truncatulinoides* and their number, and coiling mode of specimens that have been studied here. EC = North Equatorial Current, SZ = Subtropical Zone, SFZ = Subtropical Frontal Zone, PFZ = Polar Frontal Zone.

3. Methods

3.1. DNA extraction, PCR amplification and RFLP analysis

We used the GITC* buffer procedure to extract DNA and retain calcareous tests of the specimens (de Vargas et al., 2004; Morard et al., 2009). Following de Vargas et al. (2001), for each individual, the ITS-2 region was amplified using the couple of foraminiferal specific primers 5.8S12f and L2TAigta. All positive PCR products were digested using the endonuclease *Sau 96 I* (New England Biolabs). Distinct RFLP patterns were UV-detected after slow migration of the digested PCR-products on 3% agarose gel, and ethidium bromide staining, then allowing distinction between Types I+V, II, III and IV as described by de Vargas et al. (2001). Since the band patterns of Type V (Ujiié and Lipps, 2009) were identical to those of Type I, we developed an additional RFLP procedure to discriminate between these two genotypes, using the enzyme *BbVI* (New England Biolabs), which cuts the sequence 5'…GCAGC$(N)_8$…3'. 5µl of the PCR products were mixed with a solution containing 3.5µl of distilled water, 1µl of NEB buffer 2 solution (New England Biolabs) and 0.5 µl of the *BbVI* enzyme, and then incubated at 37°C during 2 hours. In this method, Type I of *T. truncatulinoides* displayed a two bands pattern at 400 and 200 bp, while Type V displayed no cut.

3.2. Shape Descriptors

597 perfectly preserved tests out of the 813 previously genotyped specimens were retained for further morphometric analyses. The remaining specimens were excluded from our analyses because of slight alteration patterns likely resulting from the mechanical constraints (i.e. vortex homogenizations and centrifugations) occurring during the DNA extraction process.

The coiling direction of each specimen was recorded (Table 1), and all individual tests were subjected to morphometric analysis. Shape variation among specimens was appraised by extracting test outlines in two dimensions, with two perpendicular (edge and umbilical) views of oriented specimens in order to approximate the three-dimensional test morphology. The

tests were mounted on glass cover slips with double-sided tape. At first, they were oriented on their edge view, the apertural side ahead. Edge views of *T. truncatulinoides* have already been shown to represent most of the latitudinal shape variation in specimens from both modern and Pleistocene Oceans (Healy-Williams and Williams, 1981; Lohmann and Malmgren, 1983; Healy-Williams, 1983; Healy-Williams et al., 1985; Pharr and Williams, 1987). The cover slips were mounted on a universal stage allowing identical orientation of the tests, and digitized under the microscope using an optical image analyzer (OPTIMAS v. 6.51). Each outline was then manually traced in the anti-clockwise direction starting from the acute margin of the last chamber. From this origin, the x- and y-coordinates of 64 equally spaced points were extracted for each individual outline. Variations in the coiling direction were taken into account by subjecting right-coiled specimens to a horizontal mirror transformation. Then they were measured as left-coiled tests, in order to pool both right- and left-coiled directions into the shape analysis. The procedure of shape capture was repeated with the umbilical views. It was also applied, for both views, to a set of non-genotyped specimens from the same collecting stations.

Test sizes were extracted as umbilical shape areas. Any outline can be described in a simple way using a Fourier transform method (Foote, 1989), which allows the generation of a set of shape-representative variables that are suitable for statistical comparisons (Crampton, 1995). It has been successfully applied to the study of planktonic foraminifera (Quillévéré et al., 2000; 2002; de Vargas et al., 2001; Renaud and Schmidt, 2003). To appraise shape changes alone, size of the specimens was standardized by dividing the initial coordinates of the outlines by the area. An elliptic Fourier transform (Kuhl and Giardina, 1982) was then performed on the data points with the program PAST 2.0 (Hammer et al., 2001). Any single outline was thus expressed as a sum of trigonometric functions of decreasing wave-length, i.e., the harmonics.

Much of the shape information can be summarized by the first few harmonics – the higher the rank of the harmonic, the more details of the outline it describes. The content of morphological information added by each harmonic was estimated here by using the cumulative power (Crampton, 1995) as a function of the harmonic rank. For each specimen data set, the first 6 harmonics reached 97.1% and 98.5% of the average total power for edge and umbilical views, respectively. Based on multiple measurements performed on a single specimen and on both views, we found that the measurement error rate for these 6 harmonics remain <10%. Additional harmonics, accounting for less than 2.9% (edge view) and 1.5% (umbilical view) of the total power, were seen as adding high-frequency noise. Thus, the

Fourier coefficients from the 7th harmonic and higher were not considered for further analyses. In addition, following Renaud et al. (1996), (1) the zero harmonic is not relevant to the shape analysis because it represents the starting point of the outlines, and (2) the first harmonic, which fits the general outline to a single ellipse, is extremely sensitive to measurement error. Consequently, the Fourier coefficients corresponding to these harmonics were deleted. Each harmonic being characterized by four Fourier coefficients, a set of 20 coefficients corresponding to 5 harmonics was then extracted for each individual outline.

3.3. Shape Analyses

Based on these individual outlines, we first appraised the extent of shape differentiation among cryptic species of *Truncorotalia truncatulinoides* by applying a principal component analysis (PCA) to the Fourier coefficient data set. PCA allows reduction of the total inter-individual shape variability to a few principal components that best summarize the overall morphological variation within the data set. An optimal separation of all five genetically-assigned cryptic species based on the among-group shape variability was further performed by calculating a one-way multivariate analysis of variance (MANOVA) and associated pairwise tests of significance (using Hotelling's T^2 statistics). This procedure was applied, for both edge and umbilical views, to all genetically characterized specimens. Finally, Discriminant Analyses (also using Hotelling's T^2 statistics) were performed on the Fourier coefficients of pairs of genotype clusters, each cluster including genotypes that exhibit, for both views, similar morphologies based on previous multivariate analyses. This was done in order to establish the linear functions that optimally classify the specimens into their genetically assigned genotype, or cluster of genotypes of similar morphology, based on their test outlines. In addition to the computation of the percentage of correctly assigned specimens, a leave-one-out cross-evaluation procedure was applied for both edge and umbilical views, in order to estimate the stability and robustness of the resulting discriminant functions. These linear functions were then applied to 341 specimens that were not genetically characterized, in order to illustrate the usefulness of the resulting model for morphological recognition of cryptic species in *T. truncatulinoides*. All statistic analyses were performed using the PAST software 1.94 (Hammer et al., 2001).

4. Results

4.1. Identification of genotypes

First PCR-based RFLP analysis, using the endonuclease *Sau 96 I*, allowed discrimination between four different patterns. These patterns are similar to those obtained from PCR-RFLP analyses of *Truncorotalia truncatulinoides* samples collected in the Atlantic Ocean (Figure 8 in de Vargas et al. [2001]), and correspond to genetic Types I/V (201 specimens), II (338 specimens), III (248 specimens) and IV (26 specimens). Second, RFLP analysis based on the use of the enzyme *BbVI*, discriminates two different patterns between Types I (184 specimens) and V (17 specimens).

4.2. Geographic distribution of genotypes

Different genotypes co-occurred in only 7 out the 35 sampled oceanic stations. Our data confirm previous observations that Types I, II and V of *Truncorotalia truncatulinoides* are restricted to warm (sub)tropical waters (de Vargas et al., 2001; 2004; Ujiié and Lipps, 2009). As shown in Table 1, Type I is restricted to the southern hemisphere, north of the North subtropical front, Type II was only found in the Atlantic and Mediterranean, and Type V remains to date restricted to the Northwest Pacific. Additional collections from the North Pacific are clearly needed for further defining the biogeographic distribution of Type V. In the southern hemisphere, the OISO-4 and REVELLE transects cross 4 and 2 oceanic fronts, respectively (Figure 2). The *T. truncatulinoides* Types I, III and IV are segregating in a similar way along the environmental gradients associated to these transects. While Type I specimens are found in subtropical regions, Type III specimens inhabit the complex, highly productive zone south of the North subtropical front, and Type IV specimens were only collected within cold and highly productive waters of the polar frontal zone, south of the sub-antarctic front. This latitudinal distribution of genotypes in the southern Ocean is particularly obvious when observing variations in the abundances of Types I, III and IV along the temperature and chlorophyll-a gradients (0-250 m depth) crossed during the OISO-4 cruise (Figure 3). Remarkably, genotypes co-occur only at boundary stations located in water masses

in-between the subtropical and subtropical frontal zones (Types I and III in stations Oi-2, -3, -16, -17 and Re-7, -8) and in-between the subtropical and polar frontal zones (Types III and IV in station Oi-5). No *T. truncatulinoides* were found south of the polar front.

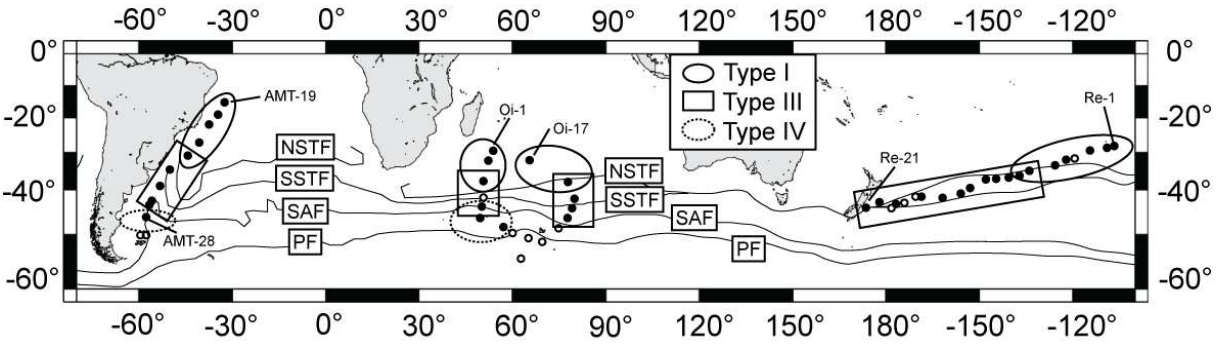

Figure 2: Location of the oceanic stations where *Truncorotalia truncatulinoides* specimens have been genotyped in the southern Ocean, and biogeographic distribution of the genotypes. Data from the south Atlantic (cruise AMT-5) from de Vargas et al. (2001). Data from the Indian and Pacific Oceans from de Vargas et al. (2004) and this work. The positions of the main oceanic fronts are from Carter et al. (2008). NSTF and SSTF = north and south subtropical fronts; SAF = sub-antarctic front; PF = polar front.

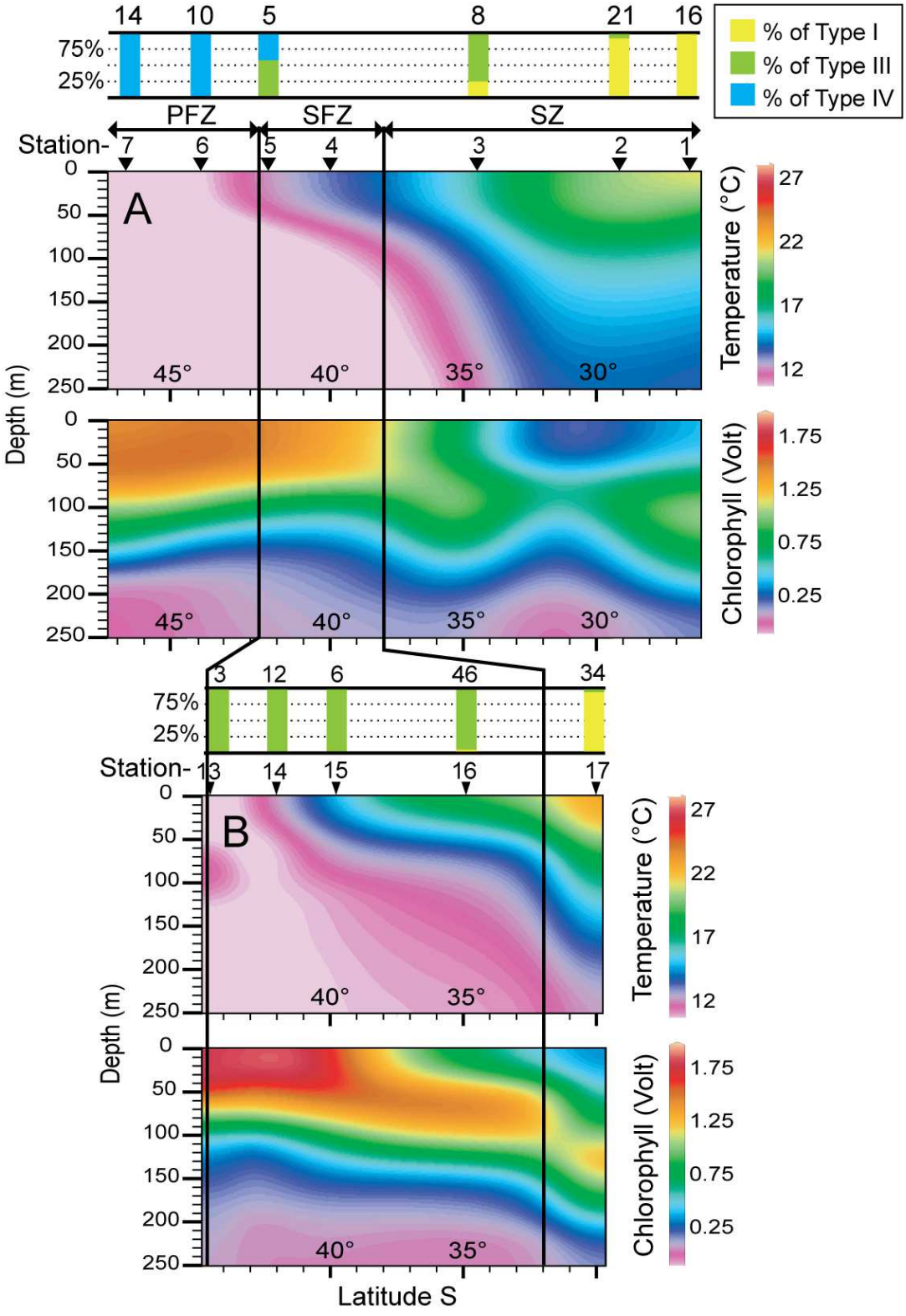

Figure 3: Latitudinal distribution of the genetic Types I, III, and IV of *Truncorotalia truncatulinoides* along temperature and chlorophyll-a profiles (0 to 250 m depth) crossed during the cruise OISO-4 in the South Indian Ocean (A = western leg; B = eastern leg). The total number of genotyped *T. truncatulinoides* and % of each genotype is given for each station, positioned along the latitudes with a black triangle. Although the sampling effort was not strictly the same at each station, the number of specimens is still indicative of the abundance of the species at each station. SZ = subtropical zone, SFZ = subtropical frontal zone, PFZ = polar frontal zone.

4.3. Morphological differentiation among cryptic species

We confirm previous observations that Types III and IV of *Truncorotalia truncatulinoides* contain exclusively left-coiling forms, Type II exhibits mixed modes of coiling directions (de Vargas et al., 2001), and Type V is right-coiled (Ujiié and Lipps, 2009). We find, however, that Type I specimens exhibit inter-basinal differences in their coiling mode, since the population sampled in the south Pacific is composed of right-coiling forms, while Indian and Atlantic Oceans populations are left-coiled (Table 1).

Results of the PCAs applied to the Fourier coefficients show that the genetic differentiation among cryptic species correlates with a morphological differentiation. Most of the shape variations are described by the first two principal components which account for 65% and 69% of the total morphological variance for edge and umbilical views, respectively. For edge views (Figure 4A), PC2 axis records a dimension of shape changes from conical, axially extended tests in Types I, II and V to relatively biconvex and elongate, axially-compressed tests in Types III and IV. For umbilical views (Figure 4B), PC1 axis records a dimension of shape changes from globally circular tests in Types III and IV to more elongate tests in Types I, II and V. The morphological space defined by the combination of PC1 scores of umbilical views and PC2 scores of edge views (Figure 4C) allows visualization of the three-dimensional shape variations that characterize the cryptic species of *T. truncatulinoides*.

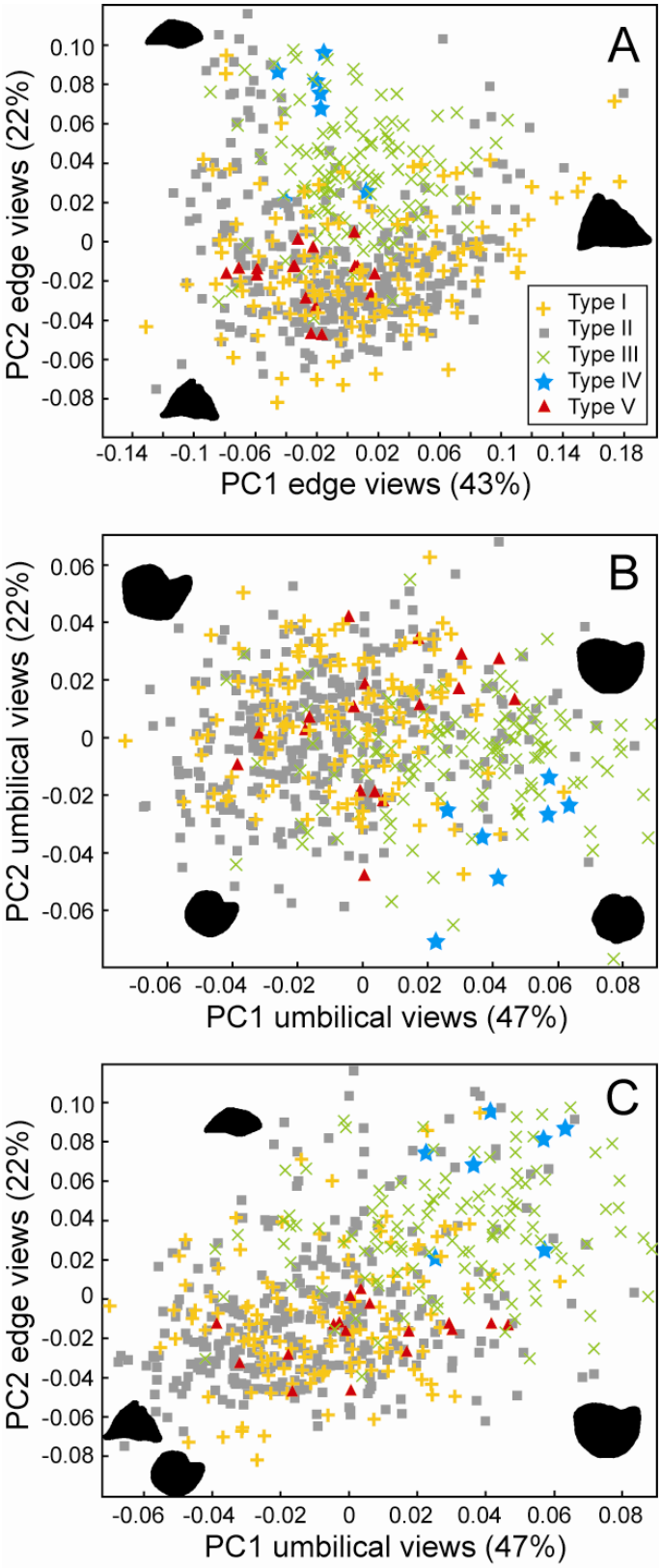

Figure 4: Principal Component Analysis performed on the Fourier coefficient data set extracted from the edge (A) and umbilical (B) views of specimens of *Truncorotalia truncatulinoides* Type I, II, III, IV and V. (C) Biplot of PC1 scores of umbilical views and PC2 scores of edge views. Outlines of several specimens are shown in order to visualize morphological changes within the planes of principal components.

MANOVAs performed on the Fourier coefficients indicate an important variation among cryptic species for both edge (Wilks'λ = 0.410; df = 80, 2259; F = 7.17; p = 1.2×10^{-65}) and umbilical views (Wilks'λ = 0.376; df = 80, 2259; F = 7.94; p = 7×10^{-75}). Post hoc pairwise comparisons among couples of cryptic species (Table 2) indicate a highly significant morphological differentiation between Types I, II, III and IV. Type V is apparently similar to Type I based on the test outlines in egde and umbilical views. Nevertheless, low pairwise values associated with Types IV and V may result from the weak number of specimens collected for these two genotypes.

Edge views					
	Type I	Type II	Type III	Type IV	Type V
Type I		**1.84E-07**	**1.07E-32**	**1.32E-06**	**2.37E-02**
Type II	**1.84E-06**		**6.69E-58**	**7.02E-09**	**2.30E-04**
Type III	**1.07E-31**	**6.69E-57**		**4.85E-03**	**6.82E-14**
Type IV	**1.32E-05**	**7.02E-08**	**4.85E-03**		**8.60E-02**
Type V	2.73E-01	**2.30E-03**	**6.82E-13**	8.61E-02	
Umbilical views					
Type I		**5.08E-07**	**1.23E-32**	**1.87E-16**	4.02E-01
Type II	**5.08E-06**		**1.40E-58**	**5.88E-19**	**4.08E-02**
Type III	**1.23E-31**	**1.40E-57**		**1.21E-06**	**4.74E-05**
Type IV	**1.87E-15**	**5.88E-18**	**1.21E-05**		**1.43E-02**
Type V	1	4.07E-01	**4.74E-04**	1.43E-01	

Table 2: Hotelling's pairwise comparisons with sequential Bonferroni corrected (bottom-left values) and uncorrected (top-right) values (Holm, 1979), testing for shape differences among cryptic species of *Truncorotalia truncatulinoides*. Significant values in bold.

4.4. A model for morphological recognition of cryptic species

The combination of two Discriminant Analyses showed in figure 5 points out two opposite clusters of specimens that exhibit conspicuous, easily identifiable morphological differences. The first cluster holds Types I, II and V that exhibit large and highly trochospiral tests. The second cluster is composed of Types III and IV that exhibit smaller, axially-compressed tests. For these two clusters of cryptic species, the Discriminant Analysis returns a function that optimizes the correct assignment of specimens based on test outlines in edge and umbilical views. The resulting "three-dimensional" model allows the correct classification of 81% and 76% of the analyzed specimens from clusters 1 and 2, respectively. In addition, 16% (cluster 1) and 21% (cluster 2) of the specimens fall into a partial (one over two) matching zone. Finally, it turns out that only 2.6% (cluster 1) and 3.1% (cluster 2) of the

sampled specimens yield incorrect classifications. The leave-one-out cross-evaluations return essentially the same levels of classification efficiency (Table 3).

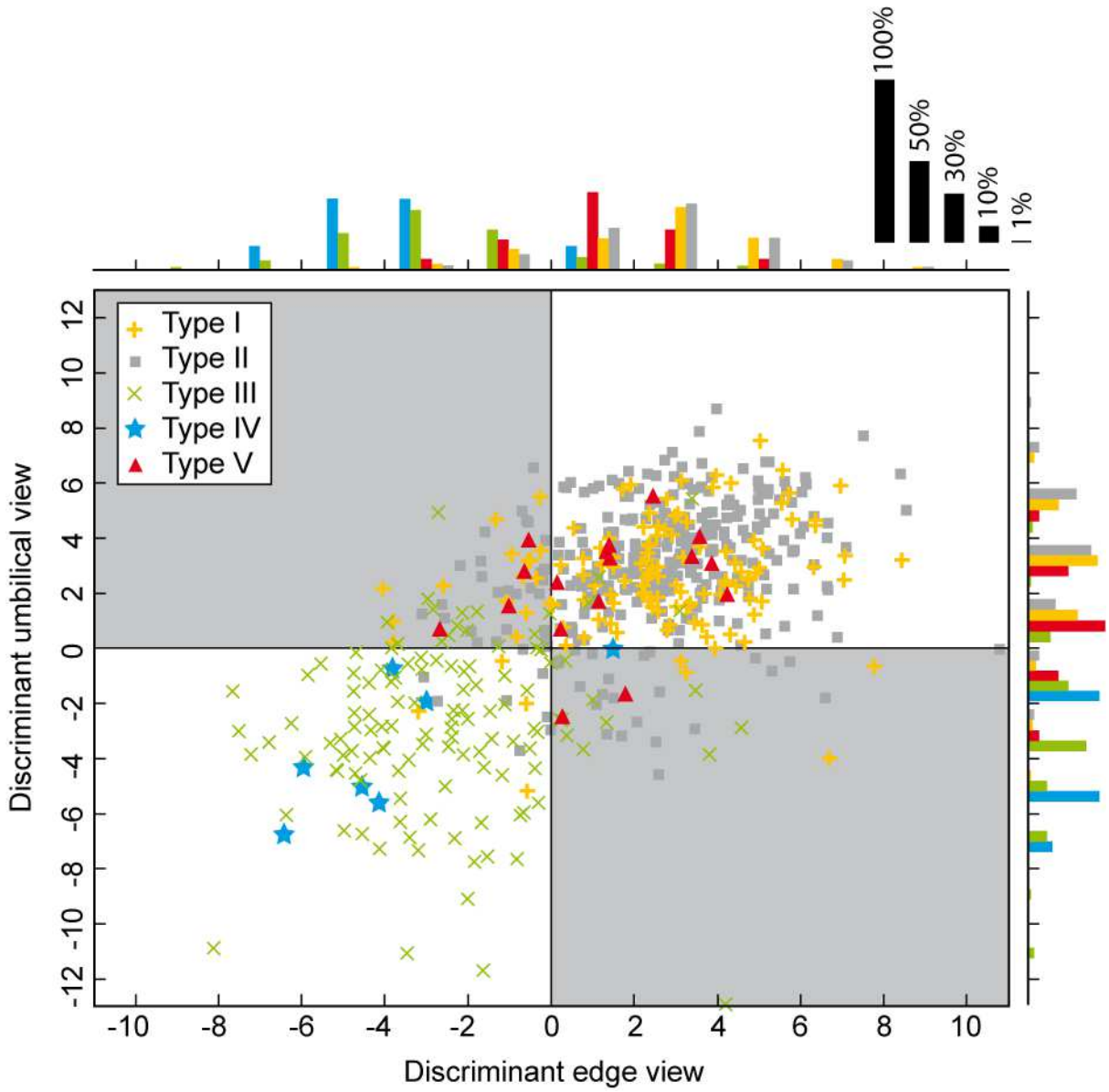

Figure 5: Graphical representation of the Discriminant Analysis-based "three-dimensional" model for the morphological recognition of *Truncorotalia truncatulinoides* genotype clusters 1 (warm water Types I, II and V) and 2 (cold water Types III and IV) based on test outlines in edge and umbilical views. The graph is divided into 4 zones, two corresponding to a "match zone" (in white) where specimens classify in the same cluster for both views, and a "partial match zone" (in gray) where classification remains ambiguous. Score frequencies for each genotype and both views are indicated by histograms. Yellow, grey, green, blue and red bars represent specimens of Type I, II, III, IV and V, respectively.

	A - Model			B - Leave-one-out cross evaluation			C - Non-genotyped specimens		
	Overall	Cluster 1	Cluster2	Overall	Cluster1	Cluster2	Overall	Expected cluster1	Expected cluster2
Number of specimens	597	467	130	597	467	130	341	199	142
Correct assignments based on edge views	87.4%	87.3%	87.7%	86.9%	87.1%	86.1%	84.2%	81.9%	87.3%
Correct assignments based on umbilical views	89.9%	91.2%	85.4%	88.2%	90.3%	80.8%	84.4%	87.9%	79.6%
Correct assignations based on both views	80.0%	81.1%	76.1%	78.3%	80.2%	71.5%	75.1%	74.4%	76.1%
Partially correct assignations based on both views	17.3%	16.3%	20.8%	18.5%	16.9%	23.8%	18.5%	21.1%	14.8%
Incorrect assignations based on both views	2.9%	2.6%	3.1%	3.2%	2.8%	4.6%	6.4%	4.5%	9.18%

Table 3: Results of our model for morphological recognition of clusters of *T. truncatulinoides* genotypes. The % of specimens assigned to each of the two clusters is given for both discriminant functions (edge and umbilical views) and for a resulting "three-dimensional" assignation (A). Success rates of the model are indicated based on the % of correctly, partialy correctly and incorrectly assigned specimens. (B) Results of the leave-one-out cross-evaluation procedure. (C) Results of expected assignations into one of the clusters after the linear functions of the discriminant analyses were applied to 341 non-genotyped specimens.

5. Discussion

Our results strongly support a non-random biogeographic distribution of the cryptic species within the morphospecies *Truncorotalia truncatulinoides*. Co-occurrences of genotypes are scarce, and only occur at water mass boundaries (Table 1, Figure 2). The morphological characters originally assigned to the *T. truncatulinoides* genotypes in the Atlantic Ocean (de Vargas et al., 2001) are unequivocally confirmed in other oceanic basins, and can thus be applied to planktonic foraminifera from the world oceans. The single-cell morphogenetic analyses used herein show that warm and oligotrophic water Types I (southern Ocean), II (Atlantic) and V (NW Pacific) exhibit large, highly conical tests with flat or concave spiral side in edge view. Cryptic species from colder, nutrient-rich water in the southern Ocean (Types III and IV) are characterized by smaller, axially compressed tests with flat or even convex spiral side in edge view. In umbilical view, the tests from Types III and IV appear more elongated than those from lower latitude cryptic species.

Analyses of *T. truncatulinoides* tests from surface sediments of the Southern Ocean have revealed morphologically distinct populations associated to particular water mass (e.g., Kennet, 1968; Lohmann and Malmgren, 1983; Heally-Williams et al., 1985). For example, Lohmann and Malmgren (1983) documented some general shape changes with latitude, grading in edge view from highly conical in tropical areas to axially compressed in cold water areas. These changes in conicalness were interpreted as ecophenotypic, although no transitional morphotypes were detected. The lack of transitional morphotypes was thought to result from either an inadequate sample distribution or a mathematical bias associated with the procedure of shape analysis. Our direct morphogenetic comparisons indicate that the lack of transitional morphotypes rather originates from the presence of conical tests from Type I and axially compressed tests from Types III and IV in the samples analyzed by Lohmann and Malmgren (1983), collected respectively north and south of the North subtropical front.

Our data show that when cryptic species are considered together, shape and size are indeed significantly correlated with environmental parameters, here documented as SST changes (Figure 6). Such correlation has been classically interpreted as ecophenotypy in *T. truncatulinoides* (e.g., Kennett, 1968; Lohmann, 1992) as well as other morphospecies of planktonic foraminifera (e.g., Hecht and Savin, 1972; Hecht, 1976; Malmgren and Kennett, 1978). However, one-way MANOVA and ANOVAs based on PC2 scores of test edge views (Figure 4) and shell areas indicate highly significant overall and pairwise morphological

differences between the five identified genotypes (Table 4). These results indicate that the identified relations between SST and shell size or shape, as observed in surface sediments from the southern hemisphere along tropical to subpolar gradients, cannot be solely attributed to ecophenotypy. Rather, they are at least partly the spurious consequence of ecologically and/or biogeographically distinct inter-genotype differences.

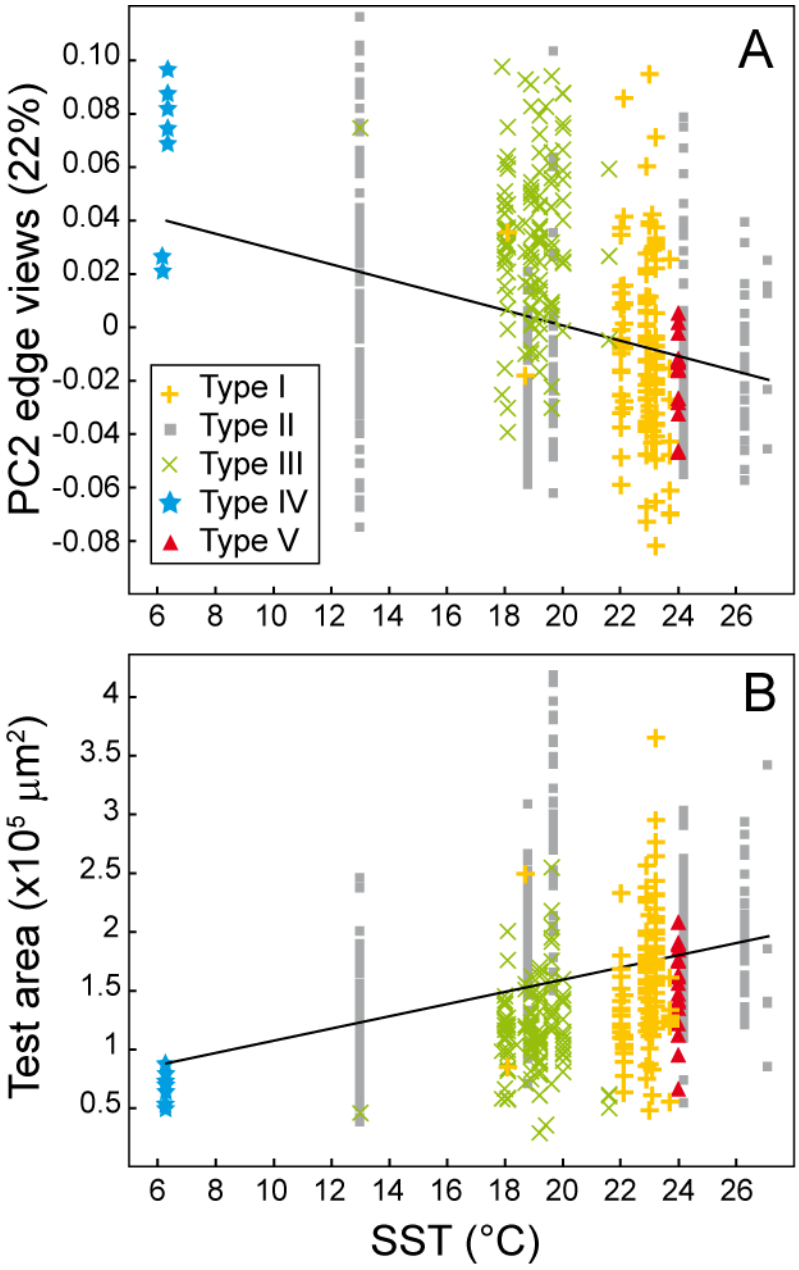

Figure 6: Biplots of SST vs. (A) PC2 calculated for edge views Fourier coefficient data sets and (B) size measured as test area in umbilical view, with least square regressions shown as solid lines in both cases. Associated Pearson correlation coefficients: SST-PC2: r = -0.32, p = 2.32E-15; SST-size: r = 0.38, p = 2.34E-22. SST measured at 10 m depth following Kucera et al. (2005b).

A - PC2 and areas					
Overall: Wilks' λ = 0.33; df = 8. 1180 ; F = 108.8 ; p = **7E-136**					
p cor. \ p uncor.	I	II	III	IV	V
I		**2.03E-03**	**6.23E-10**	**1.34E-04**	0.93
II	**2.03E-02**		**1.44E-18**	**2.34E-04**	0.23
III	**6.23E-09**	**1.44E-17**		**5.15E-03**	**2.62E-03**
IV	**1.34E-03**	**2.34E-03**	5.15E-02		**9.30E-05**
V	1	1	**2.62E-02**	**9.23E-04**	
B - PC2					
Overall: F = 48.11; df = 4. 591; p = **5E-05**					
Q \ p	I	II	III	IV	V
I		0.99	**9.67E-05**	**1.72E-05**	0.95
II	0.24		**1.89E-04**	**1.72E-05**	0.91
III	6.30	6.06		**9.99E-03**	**1.94E-05**
IV	10.90	10.67	4.60		**1.72E-03**
V	0.99	1.23	7.29	11.90	
C - area					
Overall: F = 31.98; df = 4. 591; p = **4E-24**					
Q \ p	I	II	III	IV	V
I		0.64	0.18	**1.72E-05**	0.99
II	1.95		**3.35E-03**	**1.72E-05**	0.46
III	3.09	5.04		**5.69E-04**	0.31
IV	8.78	10.73	5.68		**1.73E-05**
V	0.39	2.34	2.70	8.38	

Table 4: (A) MANOVA and associated post hoc Hotelling's pairwise comparisons (with [lower triangle] and without [upper triangle] Bonferroni corrections), and one-way ANOVA and associated contrast analyses (using Tukey's Q statistics) associated to (B) PCA results (PC2 scores in edge view; Figs. 4, 7) and (C) areas (Figure 6), testing for overall and pairwise differences among cryptic species of *Truncorotalia truncatulinoides*. Significant values are in bold.

De Vargas et al. (2001) suggested that evolutionary differentiation leading to the modern cryptic species of *T. truncatulinoides* originated in the Late Pleistocene during stepwise colonisations of southern oceanic provinces. This hypothesis was based on a correlation between molecular clock inferences (de Vargas et al., 2001) and the abundant sediment data that makes *T. truncatulinoides* one of the best known fossil morphospecies of planktonic foraminifera in time and space (Kennett, 1968; Herman, 1972; Healy-Williams, 1983; Lohmann and Malmgren, 1983; Pharr and Williams, 1987; Jenkins and Gamson, 1993; Lazarus et al., 1995; Spencer-Cervato and Thierstein, 1997; Sexton and Norris, 2008). Restricted to the tropical and subtropical environments during the first two million years of its evolution, *T. truncatulinoides* recently colonized the frontal zones in the southern hemisphere. A first ephemeral range expansion to northern subantarctic waters occurred at ~500 ka during marine isotopic stage 13 (Pharr and Williams, 1987). Since ~300 ka, the morphospecies has been shown to occur there pervasively and it further expanded to southern subantarctic waters at ~200 ka during marine isotopic stage 7 (Kennett, 1970). Typically, specimens that invaded

southern high latitudes during the late Pleistocene were smaller than lower latitude specimens, and exhibited elongate, axially compressed tests (Kennett, 1970; Healy-Williams, 1983; Pharr and Williams, 1987). Heterochronic speciation processes were hypothesized based on the resemblance between the adult morphotypes of high-latitude cryptic species and the juveniles from lower latitudes types (de Vargas et al., 2001). The morphologic features observed in sediments from southern latitudinal gradients are identical to those we have highlighted from *T. truncatulinoides* Types I and III-IV collected using plankton-net (Figure 7). Our morphogenetic data from the South Indian and Pacific Oceans suggest that the steps of adaptive speciation linked to southern migration of the *T. truncatulinoides* species complex, initially recognized in the South Atlantic Ocean, correspond to global latitudinal biogeographic events.

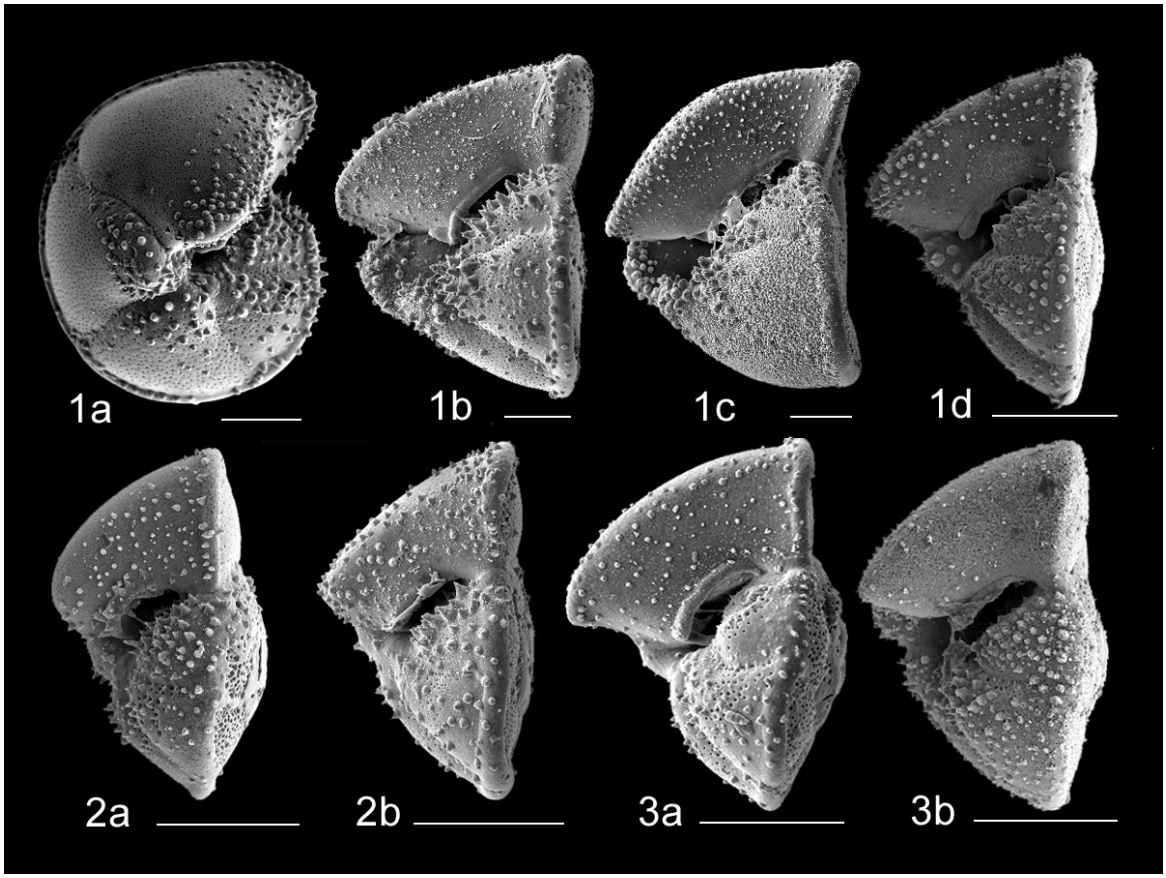

Figure 7: Scanning Electron Microscope pictures of southern Ocean *Truncorotalia truncatulinoides* specimens from which DNA has been extracted and PCR-amplified (scale bars represent 100 µm for all pictures). All specimens from the cruise OISO-4 (Indian Ocean). Labels 1, 2, 3 for Types I, III and IV, respectively; umbilical view for specimen 1a, edge view for all others. Types I, III and IV specimens from stations Oi-17, Oi-16 and Oi-7, respectively. *Truncorotalia truncatulinoides* grades from highly conical mature forms with flat or even concave spiral side in the subtropical zone (Type I, 1b, c), to smaller, rather biconvex forms in colder water areas of the subtropical frontal zone (Type III, 2a, b) and the polar frontal zone (Type IV, 3a, b). These latter forms resemble juveniles of the subtropical zone (Type I, 1d).

This new reading onto the biogeographic distribution patterns of *T. truncatulinoides* morpho-genotypes sheds light onto the use of this taxon as a paleoceanographic proxy. Understanding the distribution of the genotypes might significantly increase the resolving power of the reconstructions based on planktonic foraminiferal diversity. Since the five genotypes discussed in this paper have narrower geographic ranges and environmental preferences than classical *T. truncatulinoides* morphospecies, then reducing the geographical coverage of surface sediments calibration data set may limit bias associated with the cryptic diversity (Darling and Kucera, 2002; Kucera et al., 2005a). In case of *T. truncatulinoides*, splitting the ranges of Types I, III and IV in the southern hemisphere may logically contribute to increase the statistical accuracy of the so-called transfer functions, especially for monitoring past movements of the subtropical fronts. Our predictive model based on test outlines remains limited for the direct attribution of a single specimen to a genotype. Insufficient morphological differentiation occurs between Types I, II and V for such a direct attribution in subtropical waters. In addition, insufficient samples available hindered recognition of differentiation between Types III and IV in Southern Ocean waters. However, because of the narrow geographic distribution of each of the cryptic species of *T. truncatulinoides* (except Type II in the Atlantic Ocean), it is possible to distinguish between populations of tests from the plankton and from surface sediments. Application of our prediction model (Figure 5) to the non-genetically characterized samples from 28 of the sampled stations (Table 3, Figure 8) shows that the genotype cluster (Types I-II-V vs. Types III-IV) of about 75% of individuals can be predicted. Even in the absence of molecular analysis, specimens are often further attributable to one of the genotypes detected from the genetically characterized specimens, because of their peculiar geographic distributions and the scarcity of genotypes co-occurrences (at the 28 stations considered here, 26 yielded a single genotype, only 2 yielded two genotypes). As shown in Table 3 and Figure 8, 18.5% of individuals fall into the partial matching zone of the model, where genotype assignation remains ambiguous. A very low overall value of 6.5% is associated with an incorrect cluster assignation (4.52% and 9.15% for clusters 1 and 2, respectively). In other words, the prediction model completely failed for less than 10% of individuals, opening great perspectives to predict the genotypes of *T. truncatulinoides* from test samples.

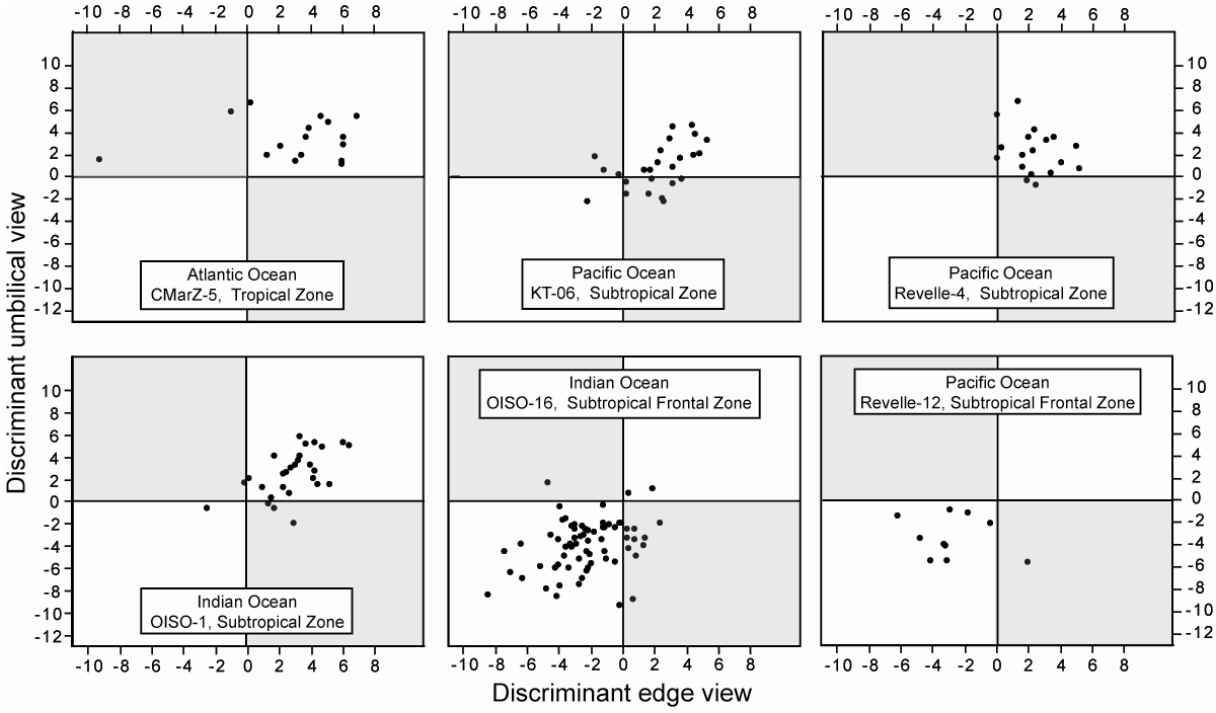

Figure 8: Three dimensional prediction model for morphological recognition of *T. truncatulinoides* cryptic species applied to non-genotyped specimens from 6 sampling stations. At each station, a great majority of the specimens (see Table 3 for details) return a correct assignation to their expected genotype (Type II at C-MarZ-5 station, Type V at KT-06 station, Type I at Revelle-4 and OISO-1 stations, Types III at OISO-16 and Revelle-12 stations). Division zones as in figure 5.

Finally, because elongate, axially compressed *T. truncatulinoides* have permanently inhabited the subantarctic waters since ~300 ka, the present-day cline in test conicalness in the Southern Ocean has probably maintained its structural sensitivity to temperature fluctuations throughout the late Pleistocene. This suggests that there is a stable connection between genetic, morphological and environmental differentiations in the fossils, making it possible to transfer the detection patterns we establish for the extent cryptic species to the interpretation of the sediment record. Thus, application of our model should work equally in the fossil record, in peculiar for monitoring past movements of the subtropical and subpolar frontal systems. Observations by Renaud and Schmidt (2003) in late Pleistocene sediments from the southern Ocean suggest that it might be possible to recognize the different cryptic species of *T. truncatulinoides* back into the fossil record (see also Lohmann and Malmgren, 1983; Lohmann, 1992). These authors have analyzed morphological changes through the last ~140 ky in *T. truncatulinoides* samples from Core PS2498, today located in the subpolar frontal zone of the southern Atlantic. Beyond size changes within left-coiled specimens, significant conicalness variations associated with changes in temperature and productivity were documented. These variations were interpreted as resulting from differences between the

genetically identified cryptic species. Our data agree with such interpretations. In the polar frontal system of the southern Ocean, inputs of highly conical specimens through time likely reflect transient invasions of tropical cryptic species (Type I or maybe Type II in the case of the Atlantic), and probably result from southward migrations of the subtropical convergence during interglacial stages of the late Pleistocene. Such transfers of genetic information to the interpretation of the fossil record may be generalized to the abundant sediment data recovered from the southern Ocean.

6. Conclusion

Because of its exceptional completeness, the planktonic foraminiferal fossil record is often considered as one of the few known at the *species* level (e.g., Bolli and Saunders, 1985). However, for historical reason mainly, and because none was able yet to obtain complete life cycles in laboratory cultures, taxonomy of modern species is almost exclusively based on morphological diagnostic characters of the tests, often even described from fossil specimens. In our study, direct morphogenetic comparisons on single-cell samples from the morphospecies *Truncorotalia truncatulinoides* collected across the world oceans, allowed us to recognize five cryptic species with distinct geographic distributions, and identify significant morphological differences in most of these cryptic species. These differences are recognizable on a population level from one another, and are particularly marked when comparing clusters of genotypes across the subtropical fronts in the southern Ocean. Comparisons with previous plankton tow data from the western South Atlantic and with sediment data-sets, suggest that the *T. truncatulinoides* genotype complex evolved through late Pleistocene speciation events associated with stepwise global latitudinal invasions of southern oceanic provinces. Our study shows that morphogenetic study at the individual level is realizable in planktonic foraminifera (see also Morard et al., 2009). Such approach will take advantage of the accuracy of DNA analyses used as a taxonomic tool, to provide a better biological understanding of the planktonic foraminiferal fossil record.

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Chapitre 4 : Diversité cryptique de *Globoconella inflata*

Ce quatrième chapitre diffère des deux précédents car la diversité cryptique de *Globoconella inflata* n'avait jusqu'alors pas été étudiée. Cette morpho-espèce est un foraminifère planctonique non-épineux, hétérotrophe abondant dans les eaux de surface lors des blooms de printemps et d'automne (Northcote, 2005; King and Howard, 2005). Ce taxon est le descendant d'une lignée ayant servi d'illustration à la théorie des équilibres ponctués, vivement débattue dans les années 1980. De fait, la littérature pléthorique consacrée à ce taxon détaille précisément les modalités de spéciation en domaine pélagique (Scott, 1983 ; Malmgren & Kennett, 1983), l'ontogénie (Wei, 1994) et la biogéographie de *Globoconella inflata* (Chapman et al., 1998). Le cadre biogéographique, écologique et stratigraphique de ce taxon est donc remarquablement contraint, facilitant l'interprétation des données moléculaires.

Mon travail a consisté tout d'abord à compléter des données moléculaires préliminaires produites par Colomban de Vargas. Les spécimens récoltés et génétiquement caractérisés du transect AMT-5 (données non publiées) ont été complétés d'une large collection de séquences issues de campagnes recoupant la quasi-totalité de la couverture environnementale de G. *inflata*. Ces analyses ont révélé la présence de deux espèces cryptiques, la première occupant les eaux tempérées à subtropicales des deux hémisphères, et la seconde les eaux subpolaires de l'hémisphère sud. La reconnaissance de ces deux génotypes sur la base de la morphologie de leur test pourrait s'avérer être un outil puissant de reconstitution des migrations du front subpolaire antarctique au cours du Pléistocène récent.

Ce manuscrit est en cours de préparation et sera soumis prochainement à *Plos Biology*.

Cryptic diversity in *Globoconella inflata* (planktonic foraminifera) and its Potential Implications for Monitoring Past Migrations of the Antarctic Subpolar Front

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Abstract

The planktonic foraminiferal morphospecies *Globoconella inflata* is widely used as a stratigraphic and paleoceanographic index. While *G. inflata* was until now regarded as a single species, we show that this morphospecies rather constitutes a complex of two cryptic species. Our study is based on sequence analyses and genotyping of 497 individuals collected from 49 oceanic stations distributed worldwide within the entire environmental range of the morphospecies. Our phylogenetic analyses of ITS rDNA sequences unveil the presence of two quite divergent genotypes. Type I inhabits the transitional and subtropical waters of both hemispheres, when Type II is restricted to the Antarctic subpolar waters. We find no co-occurrence between genotypes suggesting that they exhibit a strictly allopatric distribution on each side of the Antarctic subpolar front. Sediment data show that *G. inflata* was restricted to the transitional and subtropical environments since the early Pliocene, and finally expanded its geographic range to the southern subpolar waters ~700 kyrs ago, during marine isotopic stage 17. Our results suggest that this invading datum corresponds to a peripatric speciation event that led to the partition of an ancestral genotype into two evolutionary significant units. Finally, biometric measurements performed on *G. inflata* collected from plankton tows North and South of the Antarctic subpolar front indicate that Types I and II exhibit slight but recondite differences in shell morphology. Such differences may allow recognition of the cryptic species of *G. inflata* back into the fossil record, and contribute to monitor past movements of the Antarctic subpolar front during the middle and late Pleistocene.

1. Introduction

Planktonic foraminifera (Globigerininae) are pelagic protists whose calcareous shells constitute one of the most complete and continuous fossil archive of biodiversity changes since 180 Myrs. They are extensively used as a tool for past climate reconstructions. The biogeography of planktonic foraminifera remarkably correlates with hydrographic conditions of latitudinal oceanic provinces (Bé, 1977). Paleoceanographers derive reconstructions by utilizing empirical relations between extant environmental parameters of the surface oceans (e.g., temperature, primary productivity) and the abundance of shells of individual species in surface sediment samples (e.g., Kucera et al., 2005). These correlations lie on the key hypothesis that each species has its own, stable habitat preferences that are transferable back into the past to reconstruct changes of water mass physical properties. Individual species identity and recognition are therefore of very first importance for the use of planktonic foraminifera as a paleoceanographic proxy.

Shell morphological distinction provides the unique basis for planktonic foraminiferal taxonomy. Extant species have been partitioned and defined based on diagnostic characters of the shells (*morphospecies* concept), sometimes even primarily described from fossil specimens (Kennett and Srinivasan, 1983; Bolli and Saunders, 1985). Yet, molecular analyses applied to living specimens have challenged this concept by demonstrating that this classical taxonomy widely underestimates planktonic foraminiferal diversity. Each morphospecies analyzed so far actually comprises three to seven substantially distinct genotypes (Darling and Wade, 2008), many of which exhibiting a distinct biogeography and/or ecology (de Vargas et al., 1999; 2001; 2002), and even sometimes subtle but statistically significant differences in shell morphology (Morard et al., 2009). Ribosomal DNA genotypes (ribotypes) occur across huge geographic distances, current oceanic systems, major tectonic barriers, and can co-occur with other genotypes of the same morphospecies at the same sites of collection (de Vargas et al., 1997; 2001; 2002; Darling et al., 2000; 2004; Darling and Wade, 2008; Morard et al., 2009). However, these diverse genotypes within a single morphospecies most often display specific distributions, related to different degrees in stratification and productivity of the water column (de Vargas et al., 2001; 2002; Morard et al., 2009). Together with molecular clock analyses (de Vargas et al., 1999; 2001), these results strongly support the hypothesis that distinct ribotypes within classical morphospecies actually correspond to cryptic *biological* species. In this study, we investigate the rDNA genetic diversity and its relationship to global biogeography within the morphospecies *Globoconella inflata* to highlight its cryptic diversity.

Globoconella inflata is a macro-perforate, non-spinose morphospecies of planktonic foraminifera (Globorotaloidea) that originated 4.14 myrs ago in transitional waters of the southwest Pacific (Scott et al., 2007), later invading the global Ocean 2.09 myrs ago (Berggren et al., 1995). Although it currently exhibits a wide temperature tolerance, this thermocline-dweller (Fairbanks et al., 1982; Wilke et al., 2006) is particularly abundant in transitional and subtropical waters of both hemispheres (Bé, 1977). Our study focuses on genetic variation in SSU and the more variable ITS rDNA sequences of a large number of living specimens collected in plankton tows from the world oceans (Figure 1). The Small SubUnit (SSU) rDNA sequences have been utilised in most studies dealing with planktonic foraminiferal genetic diversity (Darling & Wade 2008). The more variable Internal Transcribed Spacer (ITS) rDNA sequences have been also used when the SSU did not provide sufficient resolution to discriminate recently evolved cryptic species (de Vargas et al., 2001). In the case of *G. inflata*, we discover two distinct ITS genotypes, which exhibit specific, strictly allopatric biogeographic distributions and subtle differences in shell morphology. Such a discovery impacts our understanding of the planktonic foraminiferal diversity and its biogeographical structuring. It may significantly contribute to increase the accuracy of paleoceanographic reconstructions based on the fossil record of these organisms.

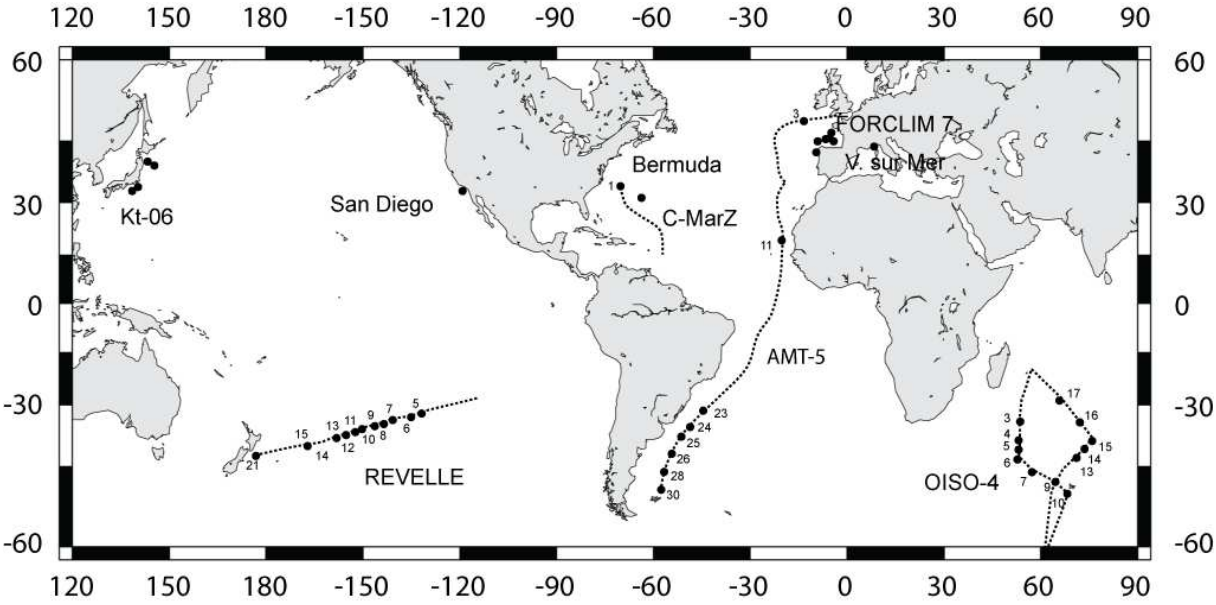

Figure 1: Geographic location and labels of the oceanic stations sampled during the cruises FORCLIM 7, AMT-5, C-MarZ, OISO-4, REVELLE, Kt-06 and offshore Villefranche-sur-mer, San Diego and Bermuda. Dashed lines represent ship routes and black circles are collecting stations from where *G. inflata* specimens have been genetically analyzed.

2. Results

2.1. Genetic Variation in Globoconella inflata

Sequencing of a fragment of 800 bp of the SSU rDNA of 16 individuals collected from 8 stations of the cruise AMT-5 (SW Atlantic; Table 1) does not illustrate any genetic variation among samples. In contrast, the complete ITS array (ITS-1, 5.8S and ITS-2) of 20 specimens from the same 8 stations shows genetic variations. We then characterized ITS sequences from 60 additional individuals collected from 33 stations that cover the entire environmental range of the morphospecies *Globoconella inflata*. 55 sequences were characterized from the same specimens in order to measure the intra-individual variations. Our dataset finally includes 135 ITS rDNA sequences from 80 individuals. The remaining 417 individuals were genetically characterized through Restriction Fragment Length Polymorphism analysis (RFLP).

Four phylogenetic analyses were successively performed. First, we only aligned unambiguous sequences from 54 specimens to bring evidence of the cryptic diversity within this morphospecies (cleITS dataset). Second, we added clones from single specimens to test the influence of non-concerted evolution on our dataset (cloITS Dataset). Third, we added sequences of lesser quality (sequences that included few ambiguous sites) in order to increase the geographic coverage of the dataset (larITS datatset). Fourth, we added three sequences from an individual (Re-1010 from REVELLE station 10) that mixes molecular traits of the two genotypes (see below) (comITS dataset). We applied a Maximum Likelihood approach according to the PhyML implementation by Guindon and Gascuel (2003) to each of these datasets. The node support was analyzed by non-parametric bootstrapping with 500 replicates in the ML analysis.

Based on those phylogenetic inferences, the sequences analyzed cluster in two largely divergent groups, that we term Type I and Type II (Figure 2). For the whole ITS region, and in all four datasets analyzed, patristic distances between the two ribotypes are systematically higher than those measured within each genotype (Table 2a). Genetic variation within individuals is greater than any differentiation among population, making these sequences uninformative at the population level (Table 2b). The node separating both genotypes shows bootstrap support values ≥98% in all but the comITS dataset (89%). No branch support up to 80% has been observed within phylotypes, with only few exceptions of terminal nodes.

A unique specimen (Re-1010) collected in the subtropical south Pacific during the cruise REVELLE, displays molecular characteristics of both genotypes, and its inclusion substantially decreases branch support of phylogenetic reconstruction. This specimen was detected based on RFLP analysis (see next section); among the 417 genotyped specimens, this was the only specimen displaying a Type II pattern among Type I population. This specimen could be representative of a third cryptic species, which remains underrepresented in our dataset.

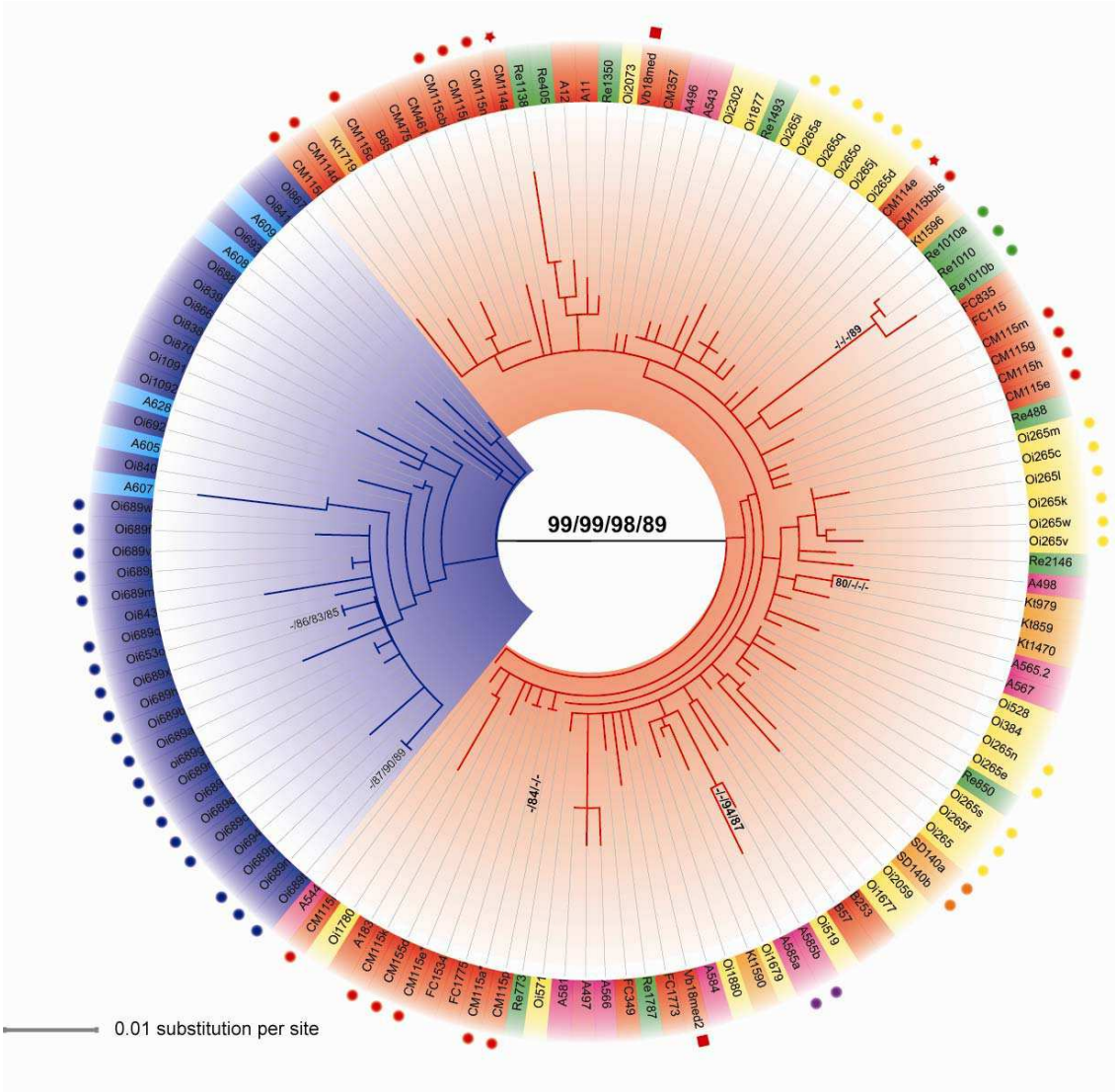

Figure 2: ITS-based evolutionary relationships between 135 clones of *Globoconella inflata* from 41 localities in the Atlantic, Pacific, Indian Ocean and Mediteranean Sea (see Table 1 and Figure 1 for station names and locations). This Maximum Likelihood inference shows the relationships between the two phylotypes (Type I in red and Type II in blue). The bootstrap scores (500 replicates) greater than 80% are given next to branches for each dataset following a CleITS/CloITS/LarITS/ComITS-dataset order. The scale and branch lengths are given in % of nucleotide substitution per site. The colors associated to leaf labels indicate geographic area of collection: blue: Indian Ocean subpolar area; light blue: South Atlantic subpolar area; Pink: South Atlantic north of the subpolar front; Yellow: Indian Ocean north of the subpolar front; red: North Atlantic; green : South Pacific; Orange: North Pacific. Circles, stars and squares associated to specific colors indicate clones sequenced from the same individuals.

Cruise	Station	Latitude	Longitude	Environmental data	Number of specimen sequenced	Number of copies from the same individual	Number of RFLP identification	TYPE
C-MarZ	1	33°54'N	69°93'W	CTD (T)	5	3; 16	79	I
OISO-4	3	35°00'S	53°30'E	CTD (T, F)	1	17	5	I
OISO-4	4	40°01'S	52°53'E	CTD (T, F)	3		9	I
OISO-4	5	42°31'S	52°29'E	CTD (T, F)	1		7	I
OISO-4	6	45°00'S	52°05'E	CTD (T, F)	5	19	0	II
OISO-4	7	47°40'S	58°00E	CTD (T, F)	8		8	II
OISO-4	9	48°31'S	64°59'E	CTD (T, F)	2		0	II
OISO-4	10	50°40'S	68°24'E	CTD (T, F)	0		2	II
OISO-4	13	44°58'S	73°21'E	CTD (T, F)	2		8	I
OISO-4	14	42°30'S	74°53'E	CTD (T, F)	2		18	I
OISO-4	15	40°00'S	76°24'E	CTD (T, F)	3		13	I
OISO-4	16	34°59'E	73°28'E	CTD (T, F)	0		3	I
OISO-4	17	29°59'S	66°24'E	CTD (T, F)	1		1	I
FORCLIM 7	I	42°37'N	10°02'W	CTD (T, F)	0		15	I
FORCLIM 7	II	44°20'N	8°45'W	CTD (T, F)	1		9	I
FORCLIM 7	III	45°35'N	7°33'W	CTD (T, F)	1		9	I
FORCLIM 7	V	45°37'N	7°33'W	CTD (T, F)	0		3	I
FORCLIM 7	VIII	45°38'N	7°36'W	CTD (T, F)	1		4	I
FORCLIM 7	XV	45°06'N	5°38'W	CTD (T, F)	3		17	I
FORCLIM 7	XXII	45°56'N	6°15'W	CTD (T, F)	0		50	I
FORCLIM 7	XXVII	46°36'N	5°49'W	CTD (T, F)	0		17	I
KT-06	C	39°00N	145°00E	CTD (T, F)	2		32	I
KT-06	E	34°00'N	140°00'E	CTD (T,F)	1		1	I
KT-06	F	33°00'N	139°00E	CTD (T,F)	3		9	I
KT-06	G	33°21'N	140°00'E	CTD (T, F)	0		1	I
REVELLE	5	31°35'S	127°83'W	SST	1		3	I
REVELLE	6	32°04'S	130°98'W	SST	1		2	I
REVELLE	7	33°39'S	137°12'W	CTD (T,F)	0		14	I
REVELLE	8	34°03'S	140°05'W	SST	1		6	I
REVELLE	9	34°73'S	143°27'W	CTD (T,F)	1		2	I
REVELLE	10	35°39'S	146°29'W	SST	1	3	1	I
REVELLE	11	36°07'S	149°48'W	CTD (T,F)	1		8	I
REVELLE	12	36°73'S	152°58'W	SST	1		5	I
REVELLE	13	37°45'S	156°00'W	CTD (T,F)	1		6	I
REVELLE	15	39°62'S	166°69'W	CTD (T,F)	1		5	I
REVELLE	21	42°95'S	176°26'E	SST	1		45	I
Villefranche-sur-Mer		43°40'N	07°15'E	SST	1	2	0	I
San Diego		32°36'N	111°11'W	SST	1	2	0	I
Bermuda	1	32°08'N	64°33'W	SST	1		0	I
Bermuda	2	32°20'N	64°33'W	SST	1		0	I
Bermuda	3	32°20'N	64°33'W	SST	1		0	I
AMT-5	3	47°98'N	13°20'W	CTD (T,F)	2		0	I
AMT-5	11	19°71'N	20°50'W	CTD (T,F)	1		0	I
AMT-5	23	31°37'S	44°52'W	CTD (T,F)	3		0	I
AMT-5	24	35°29'S	48°52'W	CTD (T,F)	2		0	I
AMT-5	25	38°50'S	51°55'W	CTD (T,F)	3		0	I
AMT-5	26	42°14'S	54°27'W	CTD (T,F)	4		0	I
AMT-5	28	46°03'S	56°42'W	CTD (T,F)	4		0	II
AMT-5	30	49°79'S	57°62'W	CTD (T,F)	1		0	II
				Total:	80		417	

Table 1: Location of the sampling stations, with indications of the the number of sequenced and genotyped specimens of *Globoconella inflata*.

			CleITS		CloITS		LarITS		ComITS	
			Min	Max	Min	Max	Min	Max	Min	Max
Inter-individuals	Type I	Median	3,84E-02		1,01E-02		1,56E-02		1,37E-02	
		95% CI	1,41E-02	8,21E-02	3,30E-03	3,76E-02	2,60E-03	3,52E-02	2,00E-03	3,18E-02
		Min-Max	1,46E-07	1,02E-01	2,00E-10	5,55E-02	2,00E-10	5,68E-02	2,00E-10	4,32E-02
	Type II	Median	5,24E-02		1,17E-02		1,83E-02		1,37E-02	
		95% CI	1,90E-02	9,06E-02	8,40E-07	2,82E-02	2,60E-03	4,33E-02	8,10E-07	3,21E-02
		Min-Max	9,60E-03	9,59E-02	2,00E-10	3,54E-02	2,00E-10	5,91E-02	2,00E-10	4,44E-02
	Type I vs. Type II	Median	1,38E-01		4,37E-02		6,70E-02		4,83E-02	
		95% CI	1,03E-01	1,80E-01	3,10E-02	5,72E-02	4,88E-02	8,86E-02	3,54E-02	6,75E-02
		Min-Max	8,26E-02	2,11E-01	5,00E-03	6,90E-02	3,82E-02	1,13E-01	2,87E-02	8,54E-02
Intra-individuals	CM115	Median			6,67E-03		1,03E-02		7,79E-03	
		95% CI			4,15E-07	1,50E-02	3,98E-10	2,34E-02	2,16E-07	1,56E-02
		Min-Max			2,00E-10	1,50E-02	2,00E-10	2,60E-02	2,00E-10	1,77E-02
	Oi265	Median			1,16E-02		1,30E-02		1,17E-02	
		95% CI			1,04E-07	1,95E-02	1,41E-07	2,11E-02	3,33E-07	1,78E-02
		Min-Max			2,00E-10	2,18E-02	2,00E-10	2,12E-02	7,06E-08	1,79E-02
	Oi689	Median			5,01E-03		7,74E-03		5,83E-03	
		95% CI			1,99E-07	1,03E-02	1,92E-07	1,82E-02	2,05E-07	1,37E-02
		Min-Max			2,00E-10	1,17E-02	2,00E-10	2,08E-02	2,00E-10	1,56E-02
	Re1010	Median							4,94E-03	
		95% CI							1,09E-03	5,79E-03
		Min-Max							8,90E-04	5,83E-03

Table 2: Inter- (A) and intra-individuals (B) patristic distances (substitutions per site) measured on phylogenetic trees obtained from the four datasets analyzed. The median, 95% nonparametric confidence interval, and minimum and maximum patristic distance values are given within and among genotyes (A), and within 4 cloned individuals (B). Specimen Re-1010 displays a Type I-like sequence with a Type II RFLP pattern.

2.2. Geographic Distribution of Genotypes

Our RFLP data set is large and detailed enough to identify the main patterns of distribution of *Globoconella inflata* Types I and II. The 49 sampled stations range through the entire biogeographic spectrum known for the morphospecies, except the northern subpolar province. Among the stations, none yielded co-occurrences of Types I and II, illustrating a strictly allopatric distribution of these two genotypes. Type I is worldwide distributed within the subtropical and transitional water masses whereas Type II is restricted to the cold, nutrient-rich water masses of the subpolar southern ocean (Figure 3A). This distribution pattern is apparently not primarily controlled by the productivity of the surface waters. Indeed, environments with the highest levels of nutrient richness (Fig 3B, C) yielded a different genotype in the south Atlantic (Type I in stations 25 and 26 of AMT-5) and the south

Indian Oceans (Type II in stations 7, 9 and 10 of OISO-4). Rather, the distribution of Types I and II is correlated, in the Southern Ocean, with the temperature of the surface waters. In both Atlantic and Indian Ocean basins, the boundary between the two types corresponds to the location of the North subpolar front, where Sea Surface Temperatures (SSTs) range between 8°C and 12°C (Figure 3B, C).

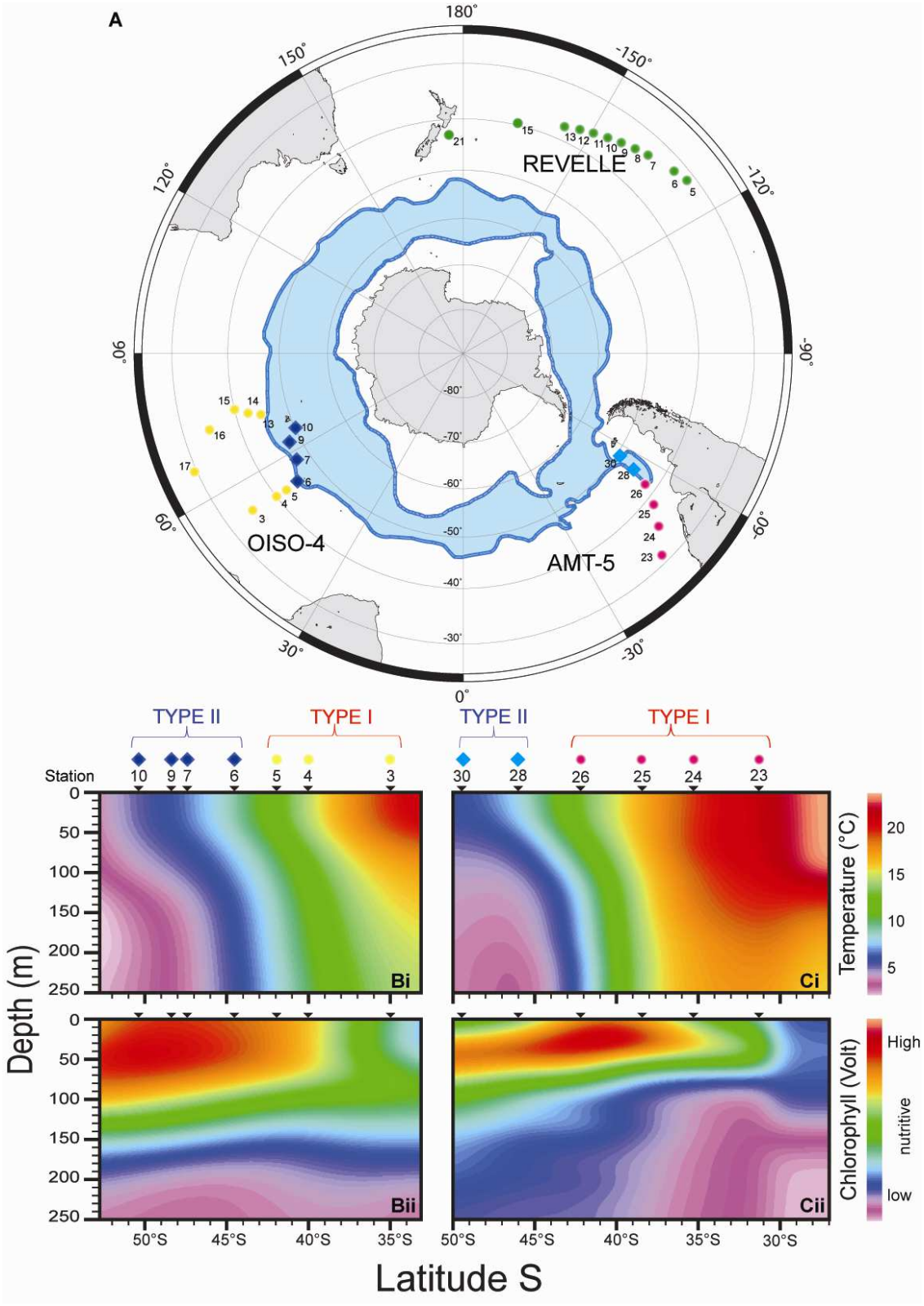

Figure 3: Latitudinal distribution of Types I and II of *Globoconella inflata* in the southern hemisphere (the northern hemisphere contains only Type I representatives). (A) Polar projection of the cruises AMT-5 (September-October 1997), OISO-4 (January-February 2000) and REVELLE (January-february 2004); the position of the Antarctic Circumpolar Current is symbolized in blue (after Carter et al., 2008). Temperature and fluorescence profiles (0-250m) are given for the cruise OISO-4 (Bi and Bii) and AMT-5 (Ci and Cii); occurrences of genotypes (circles for Type I; diamonds for Type II) are given for each station, positioned relative to latitude. Colors as in Fig 2.

2.3. Inter-Hemisphere comparative distribution of G. inflata in surface sediments

Due to the lack of plankton tow samples from northern high latitudes, our dataset fails to illustrate whether the subpolar waters of the northern hemisphere host a specific genotype of *Globoconella inflata*. In order to test this assumption, we compared, within each hemisphere, the overall distribution of our morphospecies in surface sediments samples (Figure 4). We used the Brown University Foraminiferal Database (BFD; Prell et al., 1999) that records the absolute abundances of 37 extant morphospecies over 1265 core-top localities of the world oceans. Thirteen mean annual temperatures measured between 0 and 500 m depths, which correspond to the potential depth-habitats of *G. inflata* at these localities, were extracted from the World Ocean Atlas (WOA; Locarnini et al., 2005). A principal component analysis was performed on these 13 temperatures values × 1265 localities to allow the direct comparison between the synthetic descriptors of the thermal structure of the water column (the resulting principal components) and the abundance of *G. inflata*. The PC1 is a mean thermal state of the first 500 meters of the water column. The PC2 is an indicator of stratification and contrasts the first 125 (negatively) meters to the temperatures recorded between 150 and 500 meters (positively). With the exception of a few (i.e., 11) core-top samples located in the vicinity of the Gulf Stream (Dietrich, 1980), where *G. inflata* is sparsely recorded (1-15 specimens representing less than 3.5% of the total assemblage excepting in one locality that exhibits a highly atypical assemblage including 27% of *G. inflata*), the morphospecies is absent in northern subpolar waters, at thermal conditions where Type II occurs in southern high latitudes. The latitudes at which *G. inflata* disappears in the Northern hemisphere have mean SST (sensu Kucera et al., 2005) values identical to those measured, in the Southern hemisphere, within the biogeographic boundary between Type I and II genotypes. Clearly, these observations argue for an absence of a subpolar genotype in the Northern hemisphere.

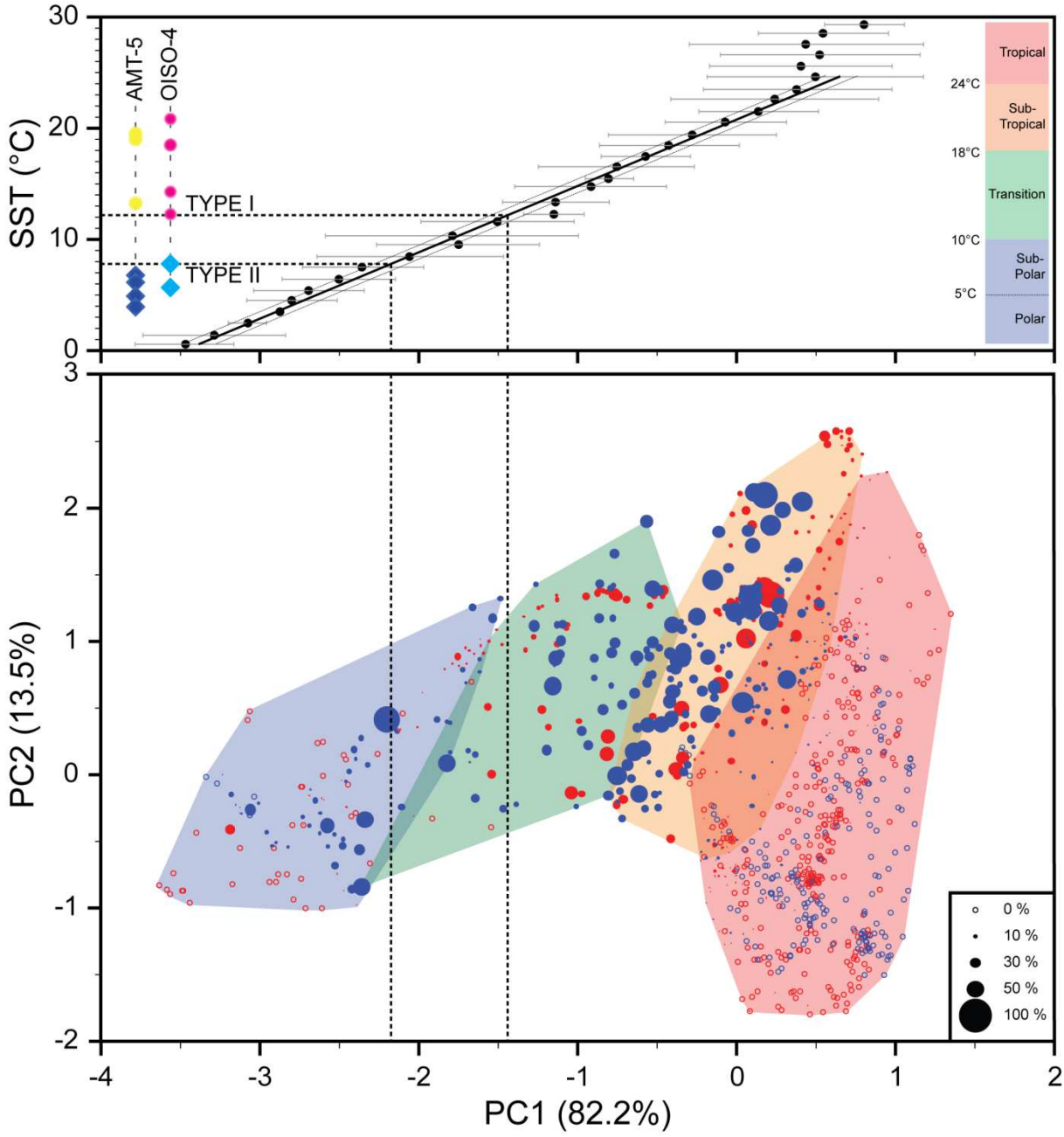

Figure 4: The bubble biplot allow the direct comparison of the relative abundance of *G. inflata* (proportional to circle size) in surface sediments of 1265 localities from the northern (in red) and southern (in blue) hemisphere with the thermal structure of the 500 upper meters of the water column. A least square regression between the mean value of PC1 for each 1 SST-degree interval and SST (Sea Surface Temperature = 10 meters temperature accordingly to Kucera et al. (2005); standard deviation indicated by a gray line) shows the good relation between these two descriptors in the 0-25 °C-SST interval and illustrates the clear thermal boundary (between 8 and 12°C based on the studied samples) between the two genotypes for the cruises AMT-5 and OISO-4 (colors and symbols as in Figure 3).

2.4. Biometry

A preliminary biometric analysis was performed on plankton sample specimens collected along the south Atlantic AMT-5 cruise, a transect that crosses the Antarctic subpolar front. Measurements of overall size and apertural relative size descriptors reveal differences among populations sampled on each side of the front (Figure 5). Based on the procedure by Aurahs et al. (2009), we have estimated the probability to have mixing of Type I and Type II specimens in the *Globoconella inflata* shells sampled north and south of the subpolar front. We find that such a probability remains below 0.035% (95% confident interval). Consequently, the specimens we collected north and south of the front, and then measured, are representatives of Type I and Type II, respectively. A discriminant analysis involving both populations indicates a highly significant differentiation among genotypes (Wilk's $\lambda = 0.858$; $F=25.06$; df = 2, 303; $p = 8.48\times10^{-11}$). At identical overall shell sizes, specimens collected north of the front display a higher apertural/final chamber size ratio than those collected south of the front. The resulting discriminant function allows the correct classification of 66% of the analyzed specimens to one of the two genotypes, making possible a population-level use of this discriminant function in order to indentify both genotypes in fossil assemblages.

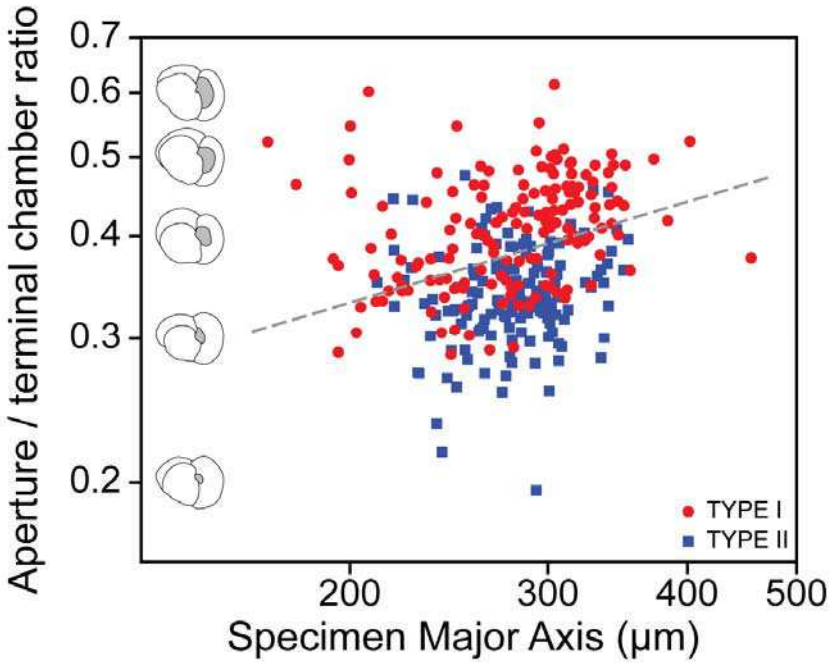

Figure 5: Log-Log Biplot of specimen major axis vs aperture/terminal chamber ratio for 306 specimens collected during the cruise AMT-5 in the south Atlantic. All *Globoconella inflata* specimens collected North of the subpolar front are considered to be representatives of Type I; all others are considered as Type II. The discriminant boundary that maximizes the separation between the two genotypes is represented by a gray dashed line.

3. Discussion

3.1. ITS for Identifying Genetic Variability in Planktonic Foraminifera

"*A phylogenetic tree of species contains smaller trees descending within its branches: the trees of genes*" (Maddison 1997). In the case of our study, such assumption asks whether our phylogenetic reconstruction "truly" reveals the evolutionary history of *Globoconella inflata*. ITS rDNA is a widely used marker for phylogenetic systematics in protist taxa, especially at the species level (Van Open et al., 2005; Behnke et al., 2004; Vanormelingen et al., 2008; Castelyn et al., 2008; Amato et al., 2007; Beszteri et al., 2005). However, ITS rDNA-based phylogenetic reconstructions have to be interpreted very cautiously because intragenomic variations can lead to overestimates of the diversity or to the incorrect finding of cryptic species, as shown for example for the dinoflagellate cluster *Symbiodinium* spp (Thornill et al., 2007). In the case of *G. inflata*, the branch separating the two phylogroups we evidence is highly supported in all datasets analyzed, even in the case of the comITS dataset (see 3.1). In addition, no clone assigned to Type II clustered within the Type I and inversely. This strongly suggests that these two phylogroups evolved as separated evolutionary units that split of during the past.

The use of a single genomic region may be hazardous to delineate biological entities within a single morphospecies (Amato et al., 2007). Indeed, despite recent advances in the exploration of the ribosomal DNA of foraminifera, their biology remains poorly understood, especially because none was able yet to obtain complete life cycles of these organisms in laboratory cultures. It is therefore not possible to conduct mating experiments to test those genotypes for the Mayrian biological species concept (groups of interbreeding natural populations that are reproductively isolated from other such groups). Consequently, the only way to prove the reliability of the ITS-defined phylogroups as different biological species is to combine evidences from different life history traits that directly or indirectly relate to the interbreeding criterion. The highly supported genetic characterization, the complete geographical and ecological disruption, and the morphological discrepancy evidenced among genotypes (Figs. 3, 4, 5) argue together for a biological species-level differentiation among Types I and II. This separation occurred during the dispersion history of *G. inflata*.

Abundant sediment data make this morphospecies one of the best known planktonic foraminifera in the fossil record, in terms of origination and paleogeographic distribution (e.g., see Figure 5 in Scott et al., 2007). It initially appeared during the early Pliocene (4.14 Ma; Scott et al., 2007) in the transitional southwest Pacific before definitely invading the transitional and subtropical waters of both hemispheres 2.09 myrs ago (Berggren et al., 1995). Restricted to these environments during the first million of years of its evolution, *G. inflata* expanded its geographic range to the southern subpolar waters at ~700 ka (Kennett, 1970; Keany and Kennett, 1972). Previous workers interpreted this event as an intra-specific migration event associated with an adaptation to cold waters of the high latitudes. Such interpretation implied that the morphospecies exhibits a wider temperature tolerance in the southern hemisphere than in the northern hemisphere, where its distribution does not range as far north as the transitional waters. Our data strongly suggest that this invading datum corresponds to a peripatric (allopatric sensu Norris, 2000) speciation event that led to the separation of an ancestral genotype into two evolutionary significant units.

3.2. Cryptic Diversity as a Tool for Monitoring Past Migrations of the Antarctic Subpolar Front

The geographic co-occurrence of distinct cryptic species of the same morphospecies is one of the main source of bias in paleoclimatic reconstructions (de Vargas et al., 2001; 2002; Darling et al., 2003; 2004; 2006; 2007; Stewart et al., 2001; Aurahs et al., 2009; Morard et al., 2009). Consequently to its evolutionary history, *Globoconella inflata* falls in a unique case because no co-occurrence of genotypes has been observed to date in similar water masses. Thus, the cryptic diversity in *G. inflata* does not affect paleoceanographic reconstructions as long as these latter are conducted within the biogeographic range of a single genotype. Most studies focusing on the ecology of *G. inflata* are based on the analysis of specimens that have been collected within the geographic range of Type I (Erez & Honjo, 1981; Ravelo & Fairbanks, 1992; Ganssen & Kroon, 2000; Kuroyanagi & Kawahata, 2004; King & Howard 2003, 2005; Wilke et al., 2006; Cléroux et al., 2007; Farmer et al., 2007; Keigwin et al., 2007). These studies are consequently not affected by the presence of distinct genotypes with peculiar ecologies.

However, it may have been the case for some studies conducted on each side of the subantarctic front. King and Howard (2005) and Chiessi et al. (2007) reported unexpected

isotopic variations in specimens collected from the subantarctic frontal zone, in the SW Atlantic and SW Pacific, respectively. Based on our results, it seems reasonable that these unexpected differences may be linked, at least partly, to behavioral differences between cryptic species (i.e., trophic strategies and mineralization depth).

The cryptic diversity in *G. inflata* may be a powerful tool to monitor past migrations of the Antarctic subpolar front during the glacial and interglacial stages of the middle and late Pleistocene. Several arguments pled for a direct recognition of the cryptic species in sediments. First, only two extant cryptic species have been described to date, making feasible to transfer the genetic information to the interpretation of the fossil record. Second, since *G. inflata* have permanently inhabited the subantarctic waters since ~700 ka, Types I and II have probably maintained their structural sensitivity to temperature conditions and fluctuations since that time of southern high-latitude colonization. Third, our rough preliminary biometrical analysis clearly suggests that the genotype of shell samples can be established based on the apertural/final chamber size ratio; based on the two first points, application of this approach should work equally in the fossil record. Consequently, identification of cryptic diversity in *G. inflata* should be considered as a valuable tool to precisely track the glacial/interglacial oscillations of the southern subpolar front over the last ~700 ka, a critical parameter for global paleoceanographic and paleoclimatic reconstructions (Martinez-Garcia et al., 2009; 2010).

4. Material and Methods

4.1. Sample Collection

Living *Globoconella inflata* were collected with plankton tows (64 µm or 100µm mesh sizes) from the world ocean. In the Atlantic Ocean, specimens were collected during the cruises AMT-5 (September-October 1997), C-MarZ (April 2006; http://www.cmarz.org/CMarZ_RHBrown_April06/index.htm), FORCLIM 7 (April 2009) and offshore Bermuda. In the Pacific Ocean, specimens were collected during the cruises REVELLE (January-February 2004; http://shipsked.ucsd.edu/schedules/2004/rr_2004/constable/), Kt-06 (June 2006) and offshore San Diego. In the Indian Ocean, they were collected during the cruise OISO-4 (January-February 2000; Metzl, 2000). Additional material has been collected offshore Villefranche-sur-mer (France) in the Mediterranean Sea. All living specimens were individually cleaned with a fine brush, isolated on the day of collection into a DNA extraction buffer (see below) and stored at -20°C. In this study, we genetically analyzed 497 specimens from 49 stations of collection.

Temperature, salinity and chlorophyll-a fluorescence vertical profiles of the 250 m upper water column were analyzed by CTD casts at most of the stations (Table 1). These hydrographic data are used here to characterize surface water mass boundaries throughout each of the sampling transects.

4.2. DNA Extraction, Amplification and Sequencing

DNA extractions of 25 specimens were performed using the classical guanidium isothiocyanate (GITC) DNA extraction buffer (for details see de Vargas et al., 1997). For the remaining 472 specimens, the original composition of the GITC extraction buffer was modified by removing EDTA, which has the property to dissolve $CaCO_3$ (Wade & Garcia-Pichel, 2003; de Vargas et al., 2004). Even in the absence of EDTA the buffer is able to efficiently penetrate in the shell of planktonic foramnifera and preserve the nucleic acids.

In this study, PCR amplifications of whole internal transcribed spacer (ITS, ~1000 base) region containing ITS-1, ITS-2, and 5.8S ribosomal genes were carried out for each individual of *G.inflata*. Methods of PCR amplification, PCR product purification and cloning, as well as the foraminifera-specific primers are described by de Vargas (2001).

The clones were sequenced using the primers *S30f* and *L5f* with an ABI prism sequencer (Applied Biosystel) at the Station Biologique de Roscoff. 135 new individuals sequences were obtained in this study and are deposited in Genbank with accession number XXXXXX to XXXXXX.

4.3 DataSets, Alignement and DNA Sequence Analysis

Because of the relative high rate of evolution of ITS region, we developed a four-step analysis. First, we analysed a dataset of 54 sequences from different specimens and without any ambiguous nucleotides in order to reveal the genetic diversity among this morpho-species (CleITS dataset). Second, we added 51 sequences corresponding to 18, 16, 15 and 2 clones for 4 different specimens (CloITS dataset). This was done to test the role of non-concerted evolution in our results. Third, we added 27 sequences containing several undetermined nucleotides (LarITS) in order to increase our geographical coverage. Finally, we added 3 sequences of the same individual displaying peculiar molecular characteristics (ComITS).

Each datasets were both manually and automatically aligned using MUSCLE with SEAVIEW version 4.0 (Gouy et al., 2010). For both alignment of each dataset, highly variable, poorly aligned regions were removed from the final alignments using GBLOCKS (v. 0.91b; Castresana, 2000) with the less stringent parameters. A consensus of both alignment (manual and automatic) was built to conserve only non ambiguous positions.

Selection of an evolutionary model was done using jModeltest v 0.1.1 (Posada, 2008). The (HKY+I+G) model was selected under the Akaike information criteria for the CleITS and LarITS datasets, the (TrN+G) was selected for the CloITS dataset and the (HKY+G) was favored for the ComITS dataset. Assuming those models of substitution, four discrete categories for the gamma distributions and NNI+SPR tree improvements, most likely topologies were inferred using PhyML v3.0 (Guindon and Gascuel, 2003). All model parameters were optimized during searches. Branch support for the maximum likelihood tree was assessed using non parametric bootstrap with 500 pseudo-replicates.

4.4. RFLP Analysis.

In order to recognize efficiently and rapidly the genotype of the 417 remaining specimens PCR-amplified we developed a RFLP protocol. PCR products digestions were performed with *BstNI* (New England Biolabs), which cuts at the sequence CC/WGG, according to the following protocol: 5 µl of the 1000 pb ITS rDNA were directly digested for 2h at 60°C in a total volume of 10 µl containing 0.1 µl of the enzyme (1 U), 1 µl of the 10 X buffer (New England Biolabs) and 3.9µl of distilled water. Distinct patterns for each genotype were UV-detected after migration of the digested PCR products on 1,5% agarose gel, and ethidium bromide coloration. The Type I produce a two band pattern (each band between 400 and 600 pb), but could be complicated by a third band if the specimen display length polymorphism between ITS copies. The Type II is not cut.

4.5. Spatial and Environmental Distribution of Globoconella inflata

We used the Brown university Foraminiferal Database (BFD; Prell et al., 1999), a planktonic foraminifers faunal dataset based on Parker's (1962) and Bé's (1977) morphospecies definitions. The BFD records the absolute abundances of ~551,600 intact individuals distributed within 37 extant morphospecies over 1265 sample localities in surface sediments(median sample size: 379 individuals; 95% Confidence Interval: 235-1033) distributed in the World Ocean. *G. inflata* is recorded in 566 (44.7%) localities, where it represents an average of 11.4% of the total assemblage (95% C.I.: 0.2%-54.7%).

For each of the 1265 core-tops, we extracted temperature data (annual mean) from the World Ocean Atlas 2005 (WOA; Locarnini et al., 2005) at the following water depths: 0, 10, 20, 30, 50, 75, 100, 125, 150, 250, 300, 400 and 500 m. In 3.27% (561 over 17,710) of the sample locality × water depth couples analyzed, temperature values were not directly available in the WOA for the 1°² target-cell where the BFD sample station is located. In those cases, we interpolated the missing values from the ≤8 1°² cells immediately surrounding the target-cell (forming a 3°×3° surface area). Interpolated values are weighted averages (weighting factor: inverse angular distance to the target-cell).

Based on this directly available and interpolated mean annual temperature dataset, the synthetic descriptors of the thermal structure of the water column for each of the 1265 sample localities were estimated through a correlation matrix-based Principal Component Analysis of

the 13 temperature variables. A bubble biplot of the two first components, representing 95.7% of the overall annual 3D thermal variability, illustrates the distributions of *G. inflata* morphospecies abundance and *G. inflata* genotypes occurrences throughout the main climatic provinces of the world ocean (Figure 4).

4.6. Biometrics

In order to compare the potential morphological differences between *G. inflata* phylotypes, we conducted biometric measurements on 306 non-genetically analyzed specimens collected during AMT-5 cruise. Specimens collected north (n = 154) and south (n = 152) of the subpolar Antarctic front, were assumed to be representatives of TYPE I and TYPE II, respectively. The robustness of such an assumption was estimated based on the procedure by Aurahs et al. (2009) that allows estimation of the probability that the observed distribution is biased by overlooking rare specimens of the presumably absent genotype. We used the equations

$$p < 1 - \sqrt[N]{\frac{1}{N}\left(N - 1 - 1.96\sqrt{1 - \frac{1}{N}}\right)}$$

$$q = 1 - (1 - p)^N$$

Where p is the relative abundance of such a rare type, q is the probability of not having collected a rare genetic type among the sampled material, and N the total number of collected individuals.

All the non-genetically analyzed specimens were mounted on glass cover slips with double side tape and similarly oriented on their umbilical and edge view. The cover slips were mounted on universal stage that permits identical orientation of test and digitized under the microscope using an optical image analyzer (OPTIMAS v. 6.51). The major axis of specimen in edge view (a simpe and robust estimator of individual size) as well as the major axes of the terminal chamber and aperture were extracted by digitizing outlines.

The ratio between aperture and terminal chamber major axes was plotted against individual size in a Log-Log diagram, and differences between the specimens located

northward and southward the subpolar Antarctic front were quantified through a two-group discriminant analysis using PAST v 2.0 (Hammer et al, 2001).

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Chapitre 5 : Impact de la diversité cryptique sur les reconstitutions paléocéanographiques

Après avoir démontré, dans les chapitres 2 à 4, que la reconnaissance morphologique des espèces cryptique était possible, nous avons quantifié l'impact de leur intégration dans les fonctions de transfert paléoenvironnementales utilisées couramment pour reconstituer les variations climatiques du Néogène récent.

Cette étude s'est appuyée sur une collection de 615 individus génotypés, récoltés sur 17 stations de l'Océan Indien, et couvrant 30° de latitude (campagne OISO-4, Janvier-Février 2000). Onze génotypes ont été identifiés dans cette collection parmi les 4 morphoespèces étudiées : 2 génotypes d'*Orbulina universa*, 3 génotypes de *Truncorotalia truncatulinoides*, les 2 génotypes de *Globoconella inflata*, et 4 des 8 génotypes de la morpho-espèce *Globigerina bulloides* (dont un inédit). Sur cette base solide, nous avons estimé les tolérances thermiques restreintes des ces espèces cryptiques et modélisé leur abondances dans les sédiments de surface de l'hémisphère sud suivant la méthode développée par Kucera et Darling (2002). Ainsi, les fonctions de transfert ont été re-calibrées afin de quantifier le gain de l'intégration de la diversité cryptique de ces quatre morpho-espèces.

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The benefit effect of planktonic foraminiferal cryptic diversity on assemblage-based SST reconstructions

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Abstract

The discovery of a significant level of cryptic genetic diversity in modern planktonic foraminifera has challenged the usefulness of shells of these organisms in paleoceanography. Since cryptic genetic species exhibit narrower biogeographic distributions than their related morphospecies, and since they can be distinguished based on their shell morphology, it is possible to improve the resolving power of the paleoceanographic reconstructions that rely on sediment assemblages. Here, using modern genotypes distribution data from the Indian Ocean as constraints, we model the relationships between sea-surface temperatures and abundances of 11 genotypes of *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides*. For this latter morphospecies, a new genotype is identified in the (sub)polar waters of the southern oceans. We apply the model to a database of morphospecies core-top counts (1334 samples) from the southern oceans. This new dataset is used as a training set to calibrate three different transfer functions classically used in assemblage-based SST reconstructions. Trials with the Imbrie and Kipp Transfer Function (IKTF), the Weight Averaging Partial Least Square (WA-PLS) and the Modern Analogue Technique (MAT) show that the splitting of morphospecies into cryptic species resulted in substantial improvements of SST estimates, respectively 6.58%, 20% and 25.15%. For SSTs ranging between 3 and 14°C, the MAT technique prediction error reaches values well below the currently admitted average prediction error rate of ~1°C. This result clearly indicates that the benefit effect of planktonic foraminiferal cryptic diversity in assemblage-based SST reconstructions allows precisions never reached in paleoceanography.

1. Introduction

The fossil record of planktonic foraminifera (Globigerininae) constitutes one of the most informative archive of paleoceanographic changes. In addition to the development of paleoproxies deduced from the chemical composition of calcite in planktonic foraminiferal shells (e.g., Katz et al., 2010), the use of these organisms in paleoceanography relies on quantitative empirical calibrations between the Sea Surface Temperature (SST) and the relative abundance of morphospecies in surface sediments of the Ocean (e.g., Imbrie and Kipp, 1971; Hutson, 1977; Malmgren et al., 2001; Mix et al., 2001; Kucera et al., 2005). Such "transfer functions" allow the prediction of ancient SSTs. They rely on the ground hypothesis that each individual morphospecies of planktonic foraminifera has its own environmental preferences that are stable through time.

The discovery of cryptic genetic diversity in planktonic foraminifera (for a review, see Darling and Wade, 2008) has significant repercussions for the use of such species-specific paleoproxies. Molecular analyses have revealed that each modern morphospecies is composed of a complex of two to seven distinct genotypes, most of them exhibiting a distinct biogeography and/or ecology (de Vargas et al., 1999; 2001; 2002; Morard et al., 2009). As a consequence, the SSTs reconstructions using the morphospecies concept of planktonic foraminifera are based on aggregates of biogeogaphically and ecologically distinct cryptic species and contain significant noise (Kucera and Darling, 2002). On the other hand, this discovery may be a benefit rather than a malaise for paleoceanographers, since recent studies have demonstrated that genotypes exhibit particular shell morphologies that can be recognized based on population-scale morphometric analyses (de Vargas et al., 2001; Morard et al., 2009; Quillévéré et al., submitted; Morard et al., in prep). As a consequence and for several morphospecies, it is feasible to transfer the biological information acquired through genetic analysis to paleoceanography. Kucera and Darling (2002) were the first to model the benefit effect of cryptic diversity on assemblage-based SST reconstructions. After splitting the morphospecies *Globigerina bulloides* into three cryptic species in the Atlantic, these authors showed that the incorporation of the geographic range of each cryptic species resulted in a substantial decrease in error rate of the transfer functions. They also suggested that the benefit for paleoceanographers might be tremendous if all morphospecies could be split into cryptic species with narrow ecological requirement ranges (see also Kucera et al., 2005).

In this paper, we model the effect of splitting four morphospecies of planktonic foraminifera, i.e., *Orbulina universa, Truncorotalia truncatulinoides, Globoconella inflata* and *Globigerina bulloides*, into cryptic species in the South Hemisphere. We first determine the specific water masses preferences of each genetic type along a 30° latitudinal transect in the south Indian Ocean. We then use these observed environmental preferences to build a model that quantifies the benefit effect of cryptic diversity on SST reconstruction techniques based on foraminiferal counts.

2. Material

2.1. Sample collection

Total plankton samples were collected onboard the *Marion-Dufresne* during the cruise OISO-4 (January-February 2000; Metzl, 2000) in the south Indian Ocean (Figure 1; Table 1). Ring net tows (100 µm mesh size) were used between 250 m depth and the sea surface. Samples were filtered through a 2 mm sieve and the finer fraction including the foraminifera was distributed into plastic Petri-dishes with 0.2 µm filtered sea water. Using a dissecting microscope and a fine brush, foraminifera were isolated and transferred into smaller Petri-dishes with filtered sea water 0.2 µm. Specimens were then individually cleaned by brushing in order to remove the organic matter, marine snow, or other organisms that may stick to the shell. They were finally transferred into a 200 µl plastic tube containing 50 µl of the GITC* nucleic acid extraction buffer (see below) and stored at -20°C. The brush was carefully sterilized in boiling distilled water after each individual extraction. The extraction tubes were stored at -20ºC.

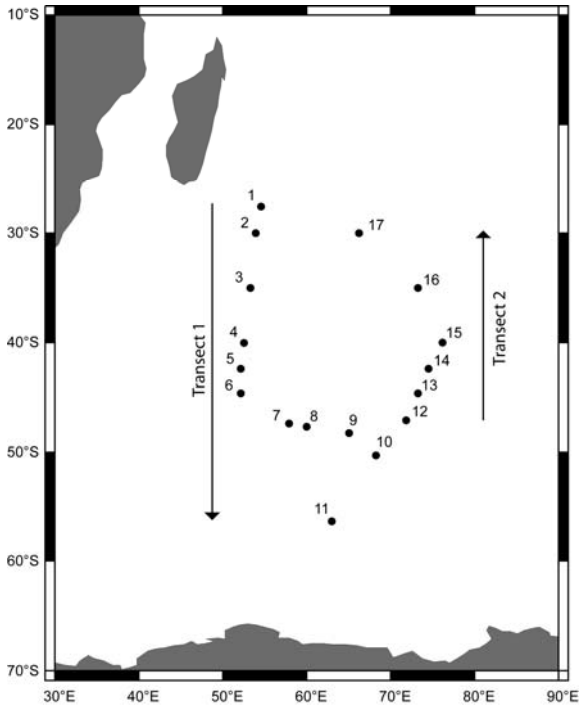

Figure 1: Geographic location and labels of the oceanic stations sampled during the cruise OISO-4 (January-February 2000; Metzl, 2000).

2.2. Core-top and environmental dataset

In order to model the distribution of the cryptic species of *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides* in the south hemisphere, we used the core top dataset of Barrows and Juggins (2005; Figure 2). This dataset records the absolute abundance of 37 extant morphospecies over 1334 localities of the Southern Ocean. For each of the 1334 core-top localities, we extracted mean summer Sea Surface Temperature values (hereafter noted SST) from the World Ocean Atlas (WOA; Locarnini et al., 2005). For 6.07% (81 over 1334) of the sampled localities, SST values were not available in the WOA for the 1°² target cell where the sample station is located. In those cases, we interpolated the missing values from the ≤8 1°² cells immediately surrounding the target-cell (forming a 3°×3° surface area). For 0.59% (6 over 1334) of the samples localities, SST values were not available in the WOA for the ≤8 1°² cells immediately surrounding the target cell. In those cases, we interpolated the missing values from the ≤16 1°² cells surrounding the 8 1°² cells.

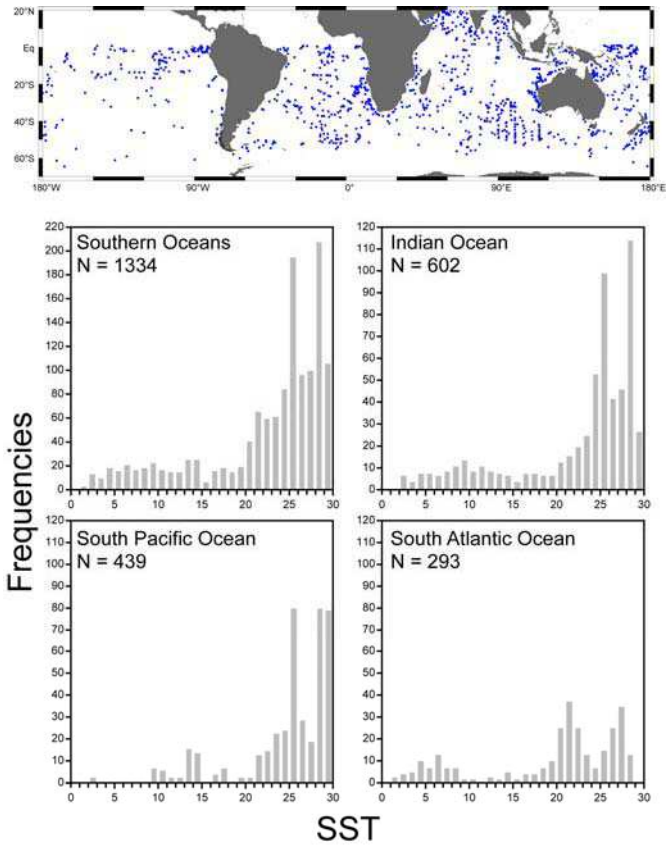

Figure 2: Southern hemisphere map indicating the locations of the 1334 core tops (blue circles) included in Barrows and Juggins, (2005) planktonic foraminiferal dataset. Histograms represent raw frequencies of observed temperature at core top location.

3. Methods

3.1. Molecular analysis

3.1.1. DNA extraction, amplification and genotype identification

We used the GITC* buffer procedure to extract DNA and retain calcareous tests of the specimens (Morard et al., 2009). A fragment of ~620 bp of the SSU of the morphospecies *Orbulina universa* and *Globigerina bulloides* were PCR amplified using the couple of foraminiferal-specific primers S15rf (GTG CAT GGC CGT TCT TAG TTC) – S19f (CCC GTA CRA GGC ATT CCT AG). The whole ITS of *Globoconella inflata* and the ITS-2 of *Truncorotalia truncatulinoides* were PCR amplified using the couples of Globorotaliid-specific primers S30f–L5f and 5.8S12f-L2Taiga (de Vargas et al., 2001), respectively. Restriction Fragment Length Polymorphism (RFLP) approaches were applied to positive PCR products of the morphospecies *Orbulina universa*, *Truncorotalia truncatulinoides* and *Globoconella inflata* following de Vargas et al. (1999), Quillévéré et al. (submitted) and Morard et al. (in prep) in order to efficiently and rapidly identify the collected cryptic species.

No RFLP protocol was available to identify the cryptic species of *Globigerina bulloides*. Prior to the development of such an approach (see below), we sequenced several specimens from all stations of the OISO-4 transect in order to identify the sampled cryptic species. The PCR products were sequenced using the primers *S15rf* and *S19f* with an ABI prism sequencer (Applied Biosystel) at the Station Biologique de Roscoff. 43 new individual sequences were obtained in this study and are available online at http://www.ncbi.nlm.nih.gov/ (accession numbers XXXXXX to XXXXXX).

3.1.2. Phylogenetic analysis of *G. bulloides*

The 43 SSU rDNA sequences of *Globigerina bulloides* were automatically aligned with the sequences available from NCBI (http://www.ncbi.nlm.nih.gov/Genbank) using the online available, up-to-date version of MUSCLE in SEAVIEW v. 4.0 (Gouy et al., 2010). Selection of an evolutionary model was performed using JMODELTETSTY v. 0.1.1 (Posada, 2008). The (GTR+G) model was selected under the Akaike information criteria. Assuming this model of substitution, four discrete categories for the gamma distribution and NNI+SPR tree improvements most likely topologies were inferred using PHYML v. 3.0 (Guindon and

Gascuel, 2003). All model parameters were optimized during searches. Branch support for the maximum likelihood tree was assessed using non parametric bootstrap with 1000 pseudo-replicates.

3.1.3. RFLP analysis of *G. bulloides*

Because we discovered a new genotype (see below) and also because no protocol apart sequencing was available to identify the cryptic species of *G. bulloides*, we performed a RFLP protocol to rapidly discriminate the four genotypes collected through the OISO-4 transect. No restriction enzyme allowed separation of the 4 genotypes at once. Consequently, we developed a 2 steps RFLP protocol for such a characterization. The first step was completed with the enzyme *HhaI* (New England Biolabs) wich cut at sequence 5'…GCG/C…3'. 5 µl of each PCR product was mixed with a solution containing 3.95µl of distilled water, 1µl of NEB buffer 4 solution (New England Biolabs), and 0.05 µl the *HhaI* enzyme, and then incubated 2 hours at 37°C and inactivated at 60°C during 20 minutes. In this first step, the Types IIc and IIf (new) displayed a two bands pattern at 530 and 90 bp, while the Types IIa and IIb were not cut. The second step was achieved with the enzyme *BsiEI* (New England Biolabs) which cut at sequence 5'…CGRY/CG…3'. 5µl of each PCR product was mixed with a solution containing 3.9 µl of distilled water, 1µL of NEB buffer 4 solution and 0.1 µl of *BsiEI* enzyme, and then incubated 2 hours at 60°C during 2 hours and inactivated at 80°C during 20 minutes. In this second step, the Types IIa and IIc displayed a three bands pattern at 130, 210 and 280 pb while the Types IIb and IIf displayed a two bands pattern at 130 and 490 pb.

3.2. Theoretical distribution of genotypes

Based on the observed distributions of the *Orbulina universa, Truncorotalia truncatulinoides, Globoconella inflata* and *Globigerina bulloides* genotypes along the OISO-4 transect, we developed models of abundances of each cryptic species depending on temperature following Kucera and Darling (2002). We used a best-fit Gaussian response model using PAST 2.0 (Hammer et al., 2001) to estimate the optimum and tolerance of each cryptic species based on its relative abundance versus sea surface temperature (Table 2; *sensu* Kucera et al., 2002). In order to estimate the model parameters we assumed that dominant

cryptic species at station 1 (23.7 °C) are also dominant at higher temperatures. Based on these theoretical distributions we calculated the relative abundances of genotypes, for each of the four morphospecies, in surface sediments of the southern oceans. We then used these relative abundances to deconvolve the different genotypes lumped in surface sediments of the southern oceans. In addition, we added a 25% symmetric random noise to the modeled abundances in order to simulate the uncertainties introduced by the model of morphological recognition of cryptic species in surface sediments.

3.3. Training sets

In order to estimate the potential improvement linked to integration of cryptic diversity in assemblage-based SST reconstructions, we compared the relative efficiency of three classical techniques of SST reconstruction in incorporating the theoretical distribution of genotypes in the training sets. We replaced the abundance of morphospecies from the initial dataset of Barrows and Juggins (2005) by their related genotypes to constitute new training sets. We constituted one training set for each morphospecies split in their related genotypes and a fifth training set (termed "UTIB" for "*universa-truncatulinoides-inflata-bulloides"*) combining the genotypes of all four morphospecies

3.4. Transfer functions

We ran each of these training sets with three classical techniques used extensively in assemblage-based SST reconstructions: the Imbrie and Kipp transfer function (IKTF), the Weight Averaging Partial Least Square regression (WA-PLS) and the Modern Analog Technique (MAT), using the C2 software (Juggins, 2007).

3.4.1. IKTF (Imbrie and Kipp, 1971)

This approach is the first mathematical technique achieved to lay faunal composition with environmental variables. It diminishes a matrix of species relative abundances in core-tops into a set of varimax-rotated Q-mode principal components loadings. Several regressions were performed between these loadings and SSTs to achieve the transfer function.

Accordingly to Malmgren et al. (2001), we retained the factors which provided the lowest RMSEP values for the different test sets.

3.4.2. WA-PLS (Ter Braak et al., 1993)

As in the IKTF, the WA-PLS technique establishes a relationship between species composition of an assemblage and environmental variables. This technique maximizes the covariance between components of vector of weight average optimum of species and an environmental variable. Five different components were established and we selected the ones that provided the lowest RMSEP to estimate the SST.

3.4.3. MAT (Prell, 1985)

This method does not build regressions between training sets and environmental variables, but searches the best analogues between fossil and modern assemblages using the square chord distance measure of Prell (1985). In this study, the SSTs are estimated from the observed temperature of the 10 best analogs available in the training set.

3.5. Temperature estimation robustness

In order to test the robustness of the improvement due to the integration of cryptic diversity in training sets, we applied a leave-one out cross-validation on the "UTIB" training set for each technique used. Then, we compared global RMSEP with results previously obtained.

Furthemore, we generated 10 datasets incorporating a uniformly-distributed ±25% symetric random noise in the modeled abundance of cryptic species in order to model errors linked to failure by the morphological recognition model to identify cryptic species. We used each of these datasets as training sets to calibrate transfer function and used this calibration to perform a reconstruction on the 9 remaining noised datasets. We repeated this approach for each possible combination of one vs. nine noised datasets.

4. Results

4.1. Phylogenetic analysis of Globigerina bulloides

Based on the phylogenetic inference, the 48 *Globigerina bulloides* sequences clustered into five distinct types with almost no detectable variation within each type (Figure 3). The comparison of the OISO-4 DNA sequences to those available from NCBI indicates that one of the identified genetic types is identical to the Type IIa of *G. bulloides*. Two genetic types show minor differences with the Types IIb and IIc. The fourth corresponds to a still undiscovered and distantly related genotype named Type IIf. The last cluster is the Type IId, only represented by sequences from NCBI.

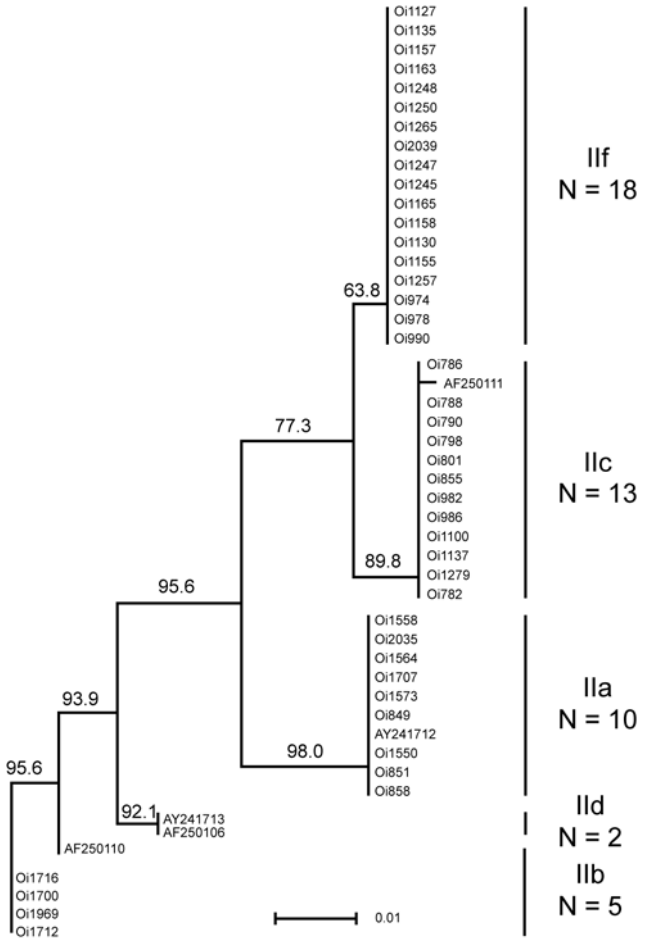

Figure 3: SSU rDNA-based unrooted tree between 51 individual sequences of the *Globigerina bulloides* morphospecies. The Maximum Likelihood inference shows the relationships between the 5 genotypes IIa, IIb, IIc, IId and IIf. The bootstrap scores (1000 replicates) supporting each phylogroup are given next to branches. The scale and branch length are given in % of nucleotide substitution per sites.

4.2. RFLP identification of genotypes

In total, 615 specimens have been successfully amplified and genetically identified by sequencing or RFLP. We identified 11 different genotypes within the 4 morphospecies genetically analyzed. We identified the Caribbean (n = 35 specimens) and Mediterranean (n = 114) species of *Orbulina universa*, the Types I (n = 70), III (n = 79) and IV (n = 26) of *Truncorotalia truncatulinoides*, the Types I (n = 75) and II (n = 21) of *Globoconella inflata* and the Types IIa (n = 57), IIb (n = 34), IIc (n = 59) and IIf (n = 45) of *Globigerina bulloides* (Table 1).

4.3. Distribution of genotypes

The OISO-4 transect covers a large geographic and environmental range. It crossed four different oceanic currents from warm and oligotrophic subtropical waters to cold and nutrient-rich supolar waters (Figure 4). Our dataset is sufficiently large and detailed to highlight the distribution of the cryptic species of the four morphospecies studied.

The Caribbean species of *Orbulina universa* has been sampled in oligotrophic stratified subtropical water masses whereas the Mediterranean species occupies the more nutrient-rich water masses where higher levels of chlorophyll-a concentration in the mixed layer are recorded (Morard et al., 2009). The Type I of *T. truncattulinoides* occurs in warm subtropical waters whereas the Types III and IV occurred in transitional and subpolar water respectively. Remarkably, genotypes co-occur only at boundary stations located in water masses in-between the subtropical and subtropical frontal zones (Figure 4, Quillévéré et al., submitted). The Type I of *Globoconella inflata* is present from subtropical to transitional water masses. The Type II is restricted to subpolar water masses. No co-occurrence has been observed between these two genotypes (Morard et al., in prep). The cryptic species distribution of *G. bulloides* is more complex because the 4 genotypes identified in OISO-4 transect display a high degree of overlapping (Figure 4). The types IIb has been sampled in subtropical and transitional water masses. The types IIa and IIc display the same distribution related to transitional to subpolar water masses. However, the Type IIc distribution is slightly shifted in cooler waters. The Type IIf seems to be mostly restriced to cold polar water masses.

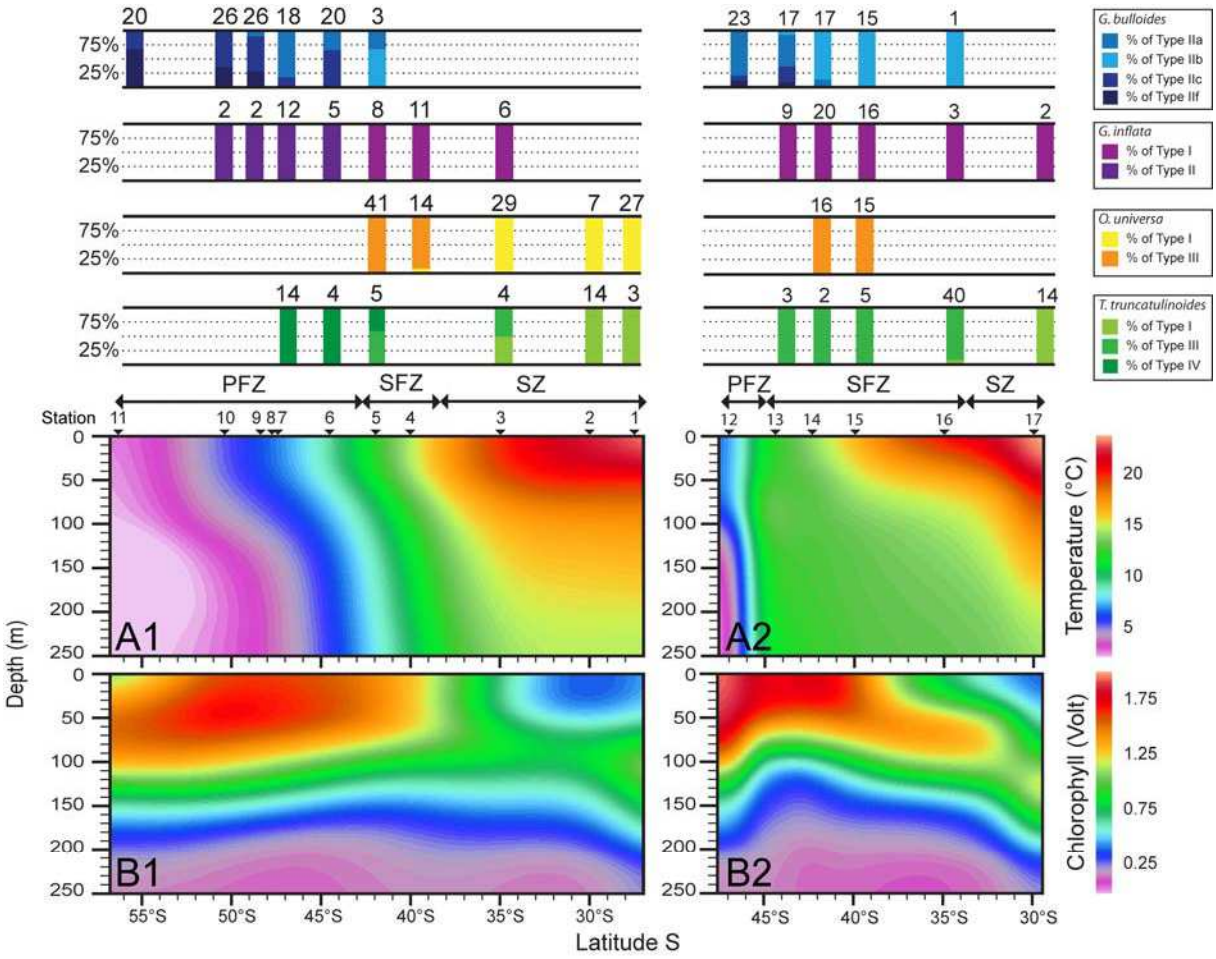

Figure 4: Latitudinal distribution of cryptic species of *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides* morphospecies along the temperature (A) and fluorescence (B) profiles (0 to 250m) of transects 1 and 2 (figure 1) of the cruise OISO-4 in the south Indian Ocean (January – February 2000). The total number of genotyped specimen and % of each cryptic species is given for each station, positioned along the latitudes with a black triangle. Position of the subtropical front from Belkin and Gordon (1996); SZ = Subtropical Zone; SFZ = Subtropical frontal Zone; PFZ = Polar Frontal Zone

4.4. Modeled abundances

By transferring the theoretical (Gaussian) distributions of cryptic species into surface sediment morphospecies assemblages (Table 2; Figures 5-8), we assume that, in spite of some noise in abundance distributions due to various factors (e.g., salinity, productivity, pH), such distributions are primarily controlled by SST (Kucera and Darling, 2002).

4.4.1. *Orbulina universa*

Whereas three cryptic species have already been described in *O. universa*, only two of them, the Mediterranean and Caribbean ones were encountered in the studied samples, making impossible at this time to model the distribution of the third one (Sargasso species).

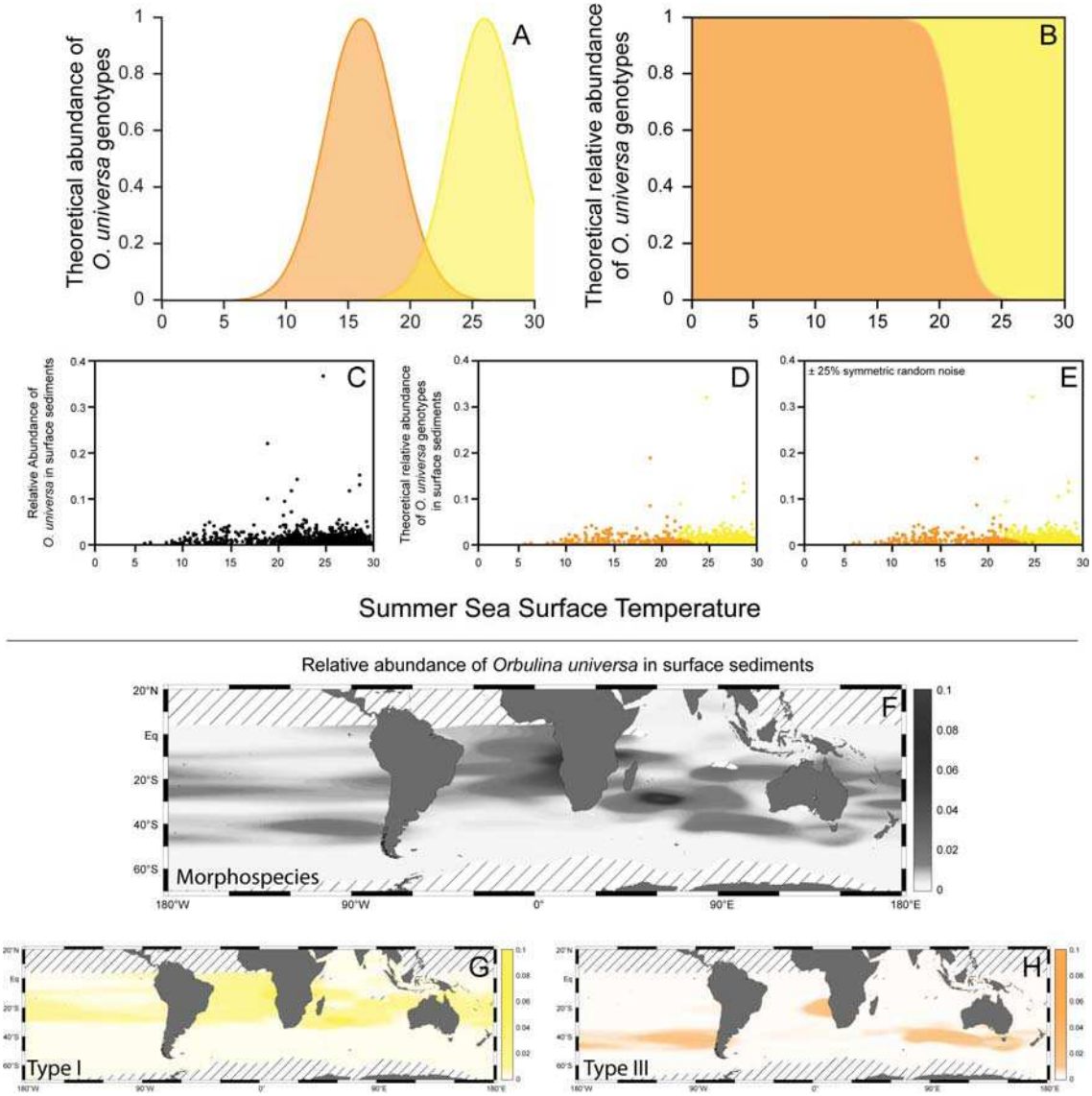

Figure 5: Distribution of the genotypes of the morphospecies *Orbulina universa* inferred from their sampled abundancies in OISO-4 (Figure 1 and 4; Table 1) and transferred into Gaussian theoretical abundance Curves (A, B; See table 2 for model parameters). This model is used to deconvolute the different cryptic species in core-top of southern Oceans against sea surface temperature (C, D). A component of random noise has been added to theoretical abundance of cryptic species (E). The abundance of morphospecies as well as theoretical abundance of cryptic species has been plotted against geography (F-H).

The geographic distribution of cryptic species of *Orbulina universa* is indirectly correlated with SST as a consequence of a primary control by the productivity level of the surface waters (de Vargas et al., 1999; Morard et al., 2009). This makes the use of a SST prediction model based on *O. universa a priori* legitimate, at least in an open-ocean oceanographic context where SST and productivity strongly co-vary. However, using this model is more difficult in more complex oceanographic contexts such as upwelling areas. Accordingly to the geographic distribution observed by de Vargas et al. (1999) and Morard et al. (2009), the Mediterranean species is dominant in highly productive systems, whereas the Caribbean species dominates oligotrophic areas. Nevertheless, the SST prediction model, while unable to predict the observed co-occurrences of both genotypes in the western equatorial Pacific (de Vagas et al., in prep: figure 4), suggests such co-occurrence in the Benguela system, where no empirical evidences are available so far to validate or refute this hypothesis (Figure 5G and 5H). Even if such co-occurrence seems unlikely (Morard et al., 2009: figure 9), These results clearly indicate that in complex oceanographic contexts mixing contrasted water masses, the SST prediction model derived from *O. universa* distribution in OISO-4 fails to safely predict genotypes' distributions and abundances.

4.4.2. *Truncorotalia trucatulinoides*

The distribution of *Truncorotalia truncatulinoides* genotypes is related to the thermal stratification of the water column (Quillévéré et al., submitted) and can be accurately predicted by SST. The modeled distribution of at least three of the five cryptic species of *T. truncatulinoides* follows the expected geographic distribution. Two distinct genotypes have not been encountered in the studied samples: the Type II restricted to the Atlantic Ocean (de Vargas et al., 2001), and the Type V, found only in the NW Pacific (Ujiié and Lipps, 2009; Quillévéré et al., submitted). As the Types I, II and V show the same ecological characteristics, we lumped Types I and II in the modeled distributions of the Atlantic Ocean and Types I and V in the modeled distribution of the Pacific Ocean.

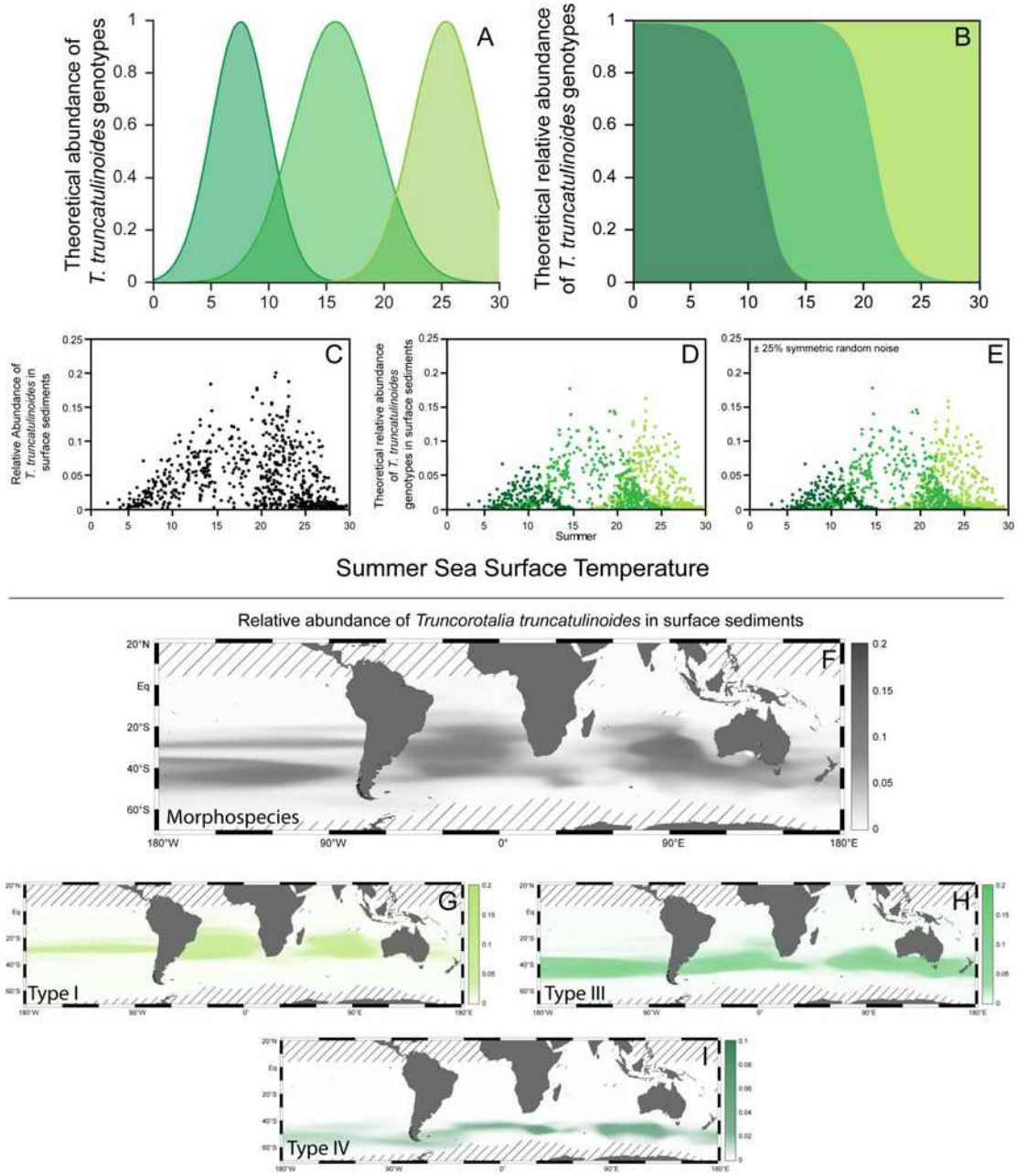

Figure 6 : Distribution of the genotypes of the morphospecies *Truncorotalia truncatulinoides* inferred from their sampled abundancies in OISO-4 (Figure 1 and 4; Table 1) and transferred into Gaussian theoretical abundance Curves (A, B; See table 2 for model parameters). This model is used to deconvolute the different cryptic species in core-top of southern Oceans against sea surface temperature (C, D). A component of random noise has been added to theoretical abundance of cryptic species (E). The abundance of morphospecies as well as theoretical abundance of cryptic species has been plotted against geography (F-I).

4.4.3. *Globoconella inflata*

The two cryptic species of *Globoconella inflata* have been encountered in the studied samples in two different water masses. The Type I occupies the transitional and subtropical water masses (11.7-23.7°C) whereas the Type II is restricted to subpolar water masses (4.94-6.78°C) (Table 1). These contrasted geographic occurrences can be accurately predicted by SST. However, no co-occurrence of the two genotypes has been sampled in the available dataset. For these reasons, the geographic and thermal structuring of genotypes occurrences within the 8-12 °C transitional interval still remains to be further substantiated.

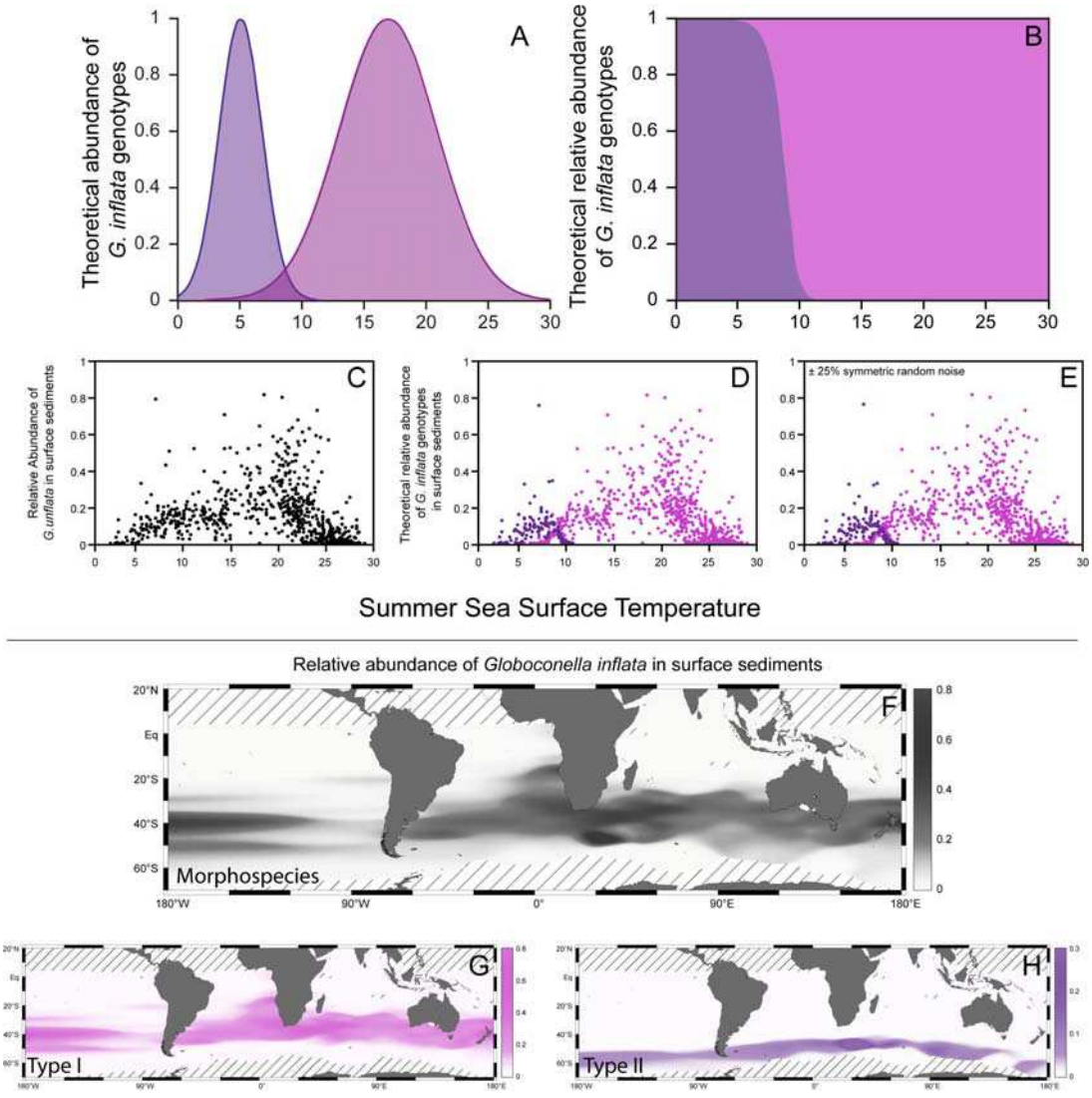

Figure 7: Distribution of the genotypes of the morphospecies *Globoconella inflata* inferred from their sampled abundancies in OISO-4 (Figure 1 and 4; Table 1) and transferred into Gaussian theoretical abundance Curves (A, B; See table 2 for model parameters). This model is used to deconvolute the different cryptic species in core-top of southern Oceans against sea surface temperature (C, D). A component of random noise has been added to theoretical abundance of cryptic species (E). The abundance of morphospecies as well as theoretical abundance of cryptic species has been plotted against geography (F-H).

4.4.4. *Globigerina bulloides*

The cryptic species of *Globigerina bulloides* display the most complex biogeographic distribution because of a high degree of overlap between genotypes (Figure 4).

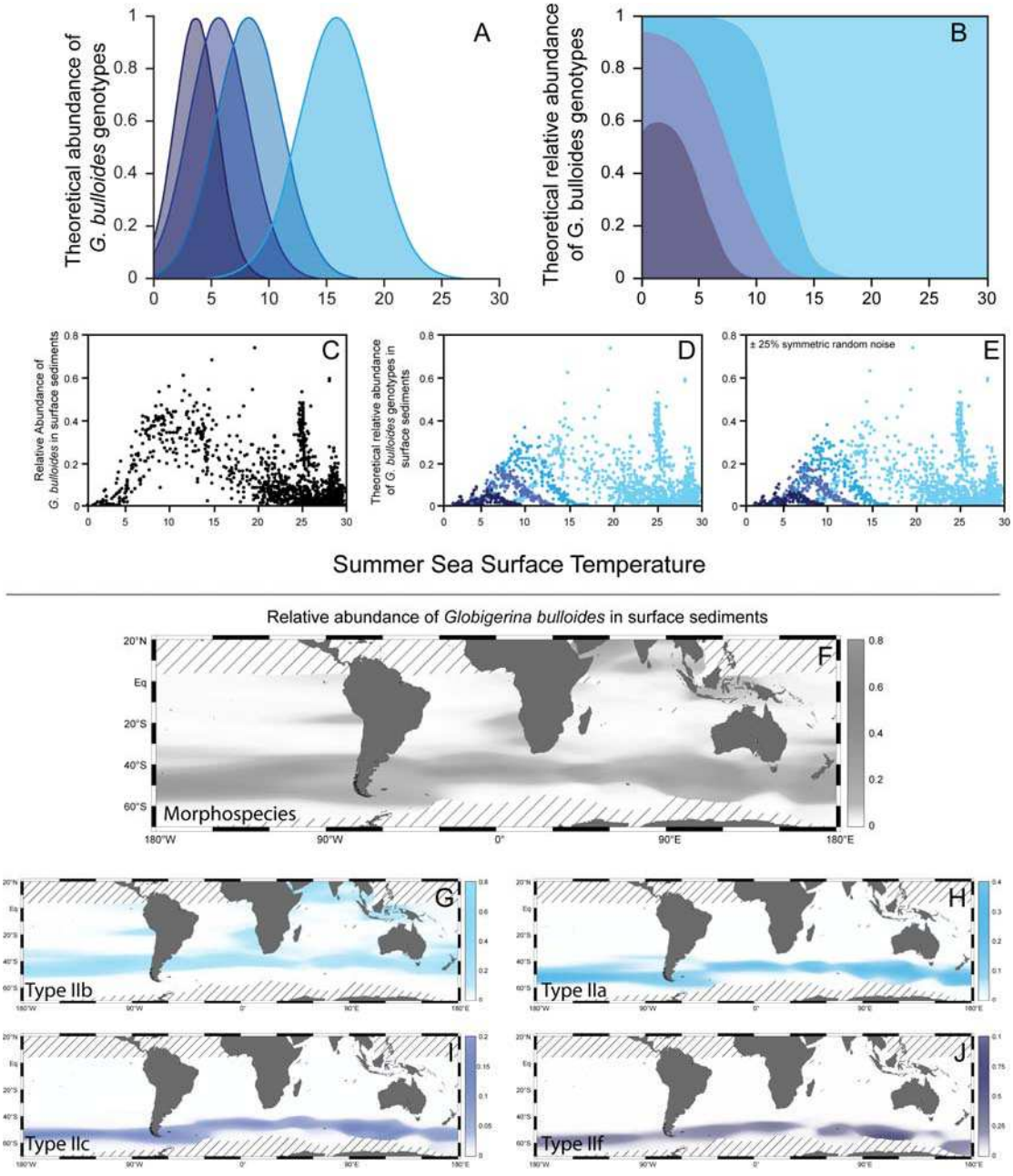

Figure 8: Distribution of the genotypes of the morphospecies *Globigerina bulloides* inferred from their sampled abundancies in OISO-4 (Figure 1 and 4; Table 1) and transferred into Gaussian theoretical abundance Curves (A, B; See table 2 for model parameters). This model is used to deconvolute the different cryptic species in core-top of southern Oceans against sea surface temperature (C, D). A component of random noise has been added to theoretical abundance of cryptic species (E). The abundance of morphospecies as well as theoretical abundance of cryptic species has been plotted against geography (F-J).

Only 4 out of the 8 identified genotypes of *G. bulloides* have been encountered in the studied samples. Among the 4 missing genotypes, 3 have been only observed in the Northern hemisphere (Types Ib, IIe and IId), and thus are not expected in the studied samples. However the Type Ia has been observed in tropical and subtropical areas such as the Indian and the Pacific Oceans (Darling and Wade, 2008). Consequently, the modeled distribution of Type IIb is a likely aggregate of Types Ia and IIb (Figure 8G).

4.5. Improvement of assemblage-based SST reconstructions

To evaluate the improvement of the error rate due to the integration of cryptic species into the training set, we compared reconstructions performed using the initial training set made of 37 morphospecies with the training set including cryptic diversity of *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and/or *Globigerina bulloides*. For all three SST prediction techniques (IKTF, WA-PLS and MAT), the integration of cryptic diversity into the transfer function does improve the overall quality of the predicted temperature (Figure 9; Table 3). However, some discrepancies are observed.

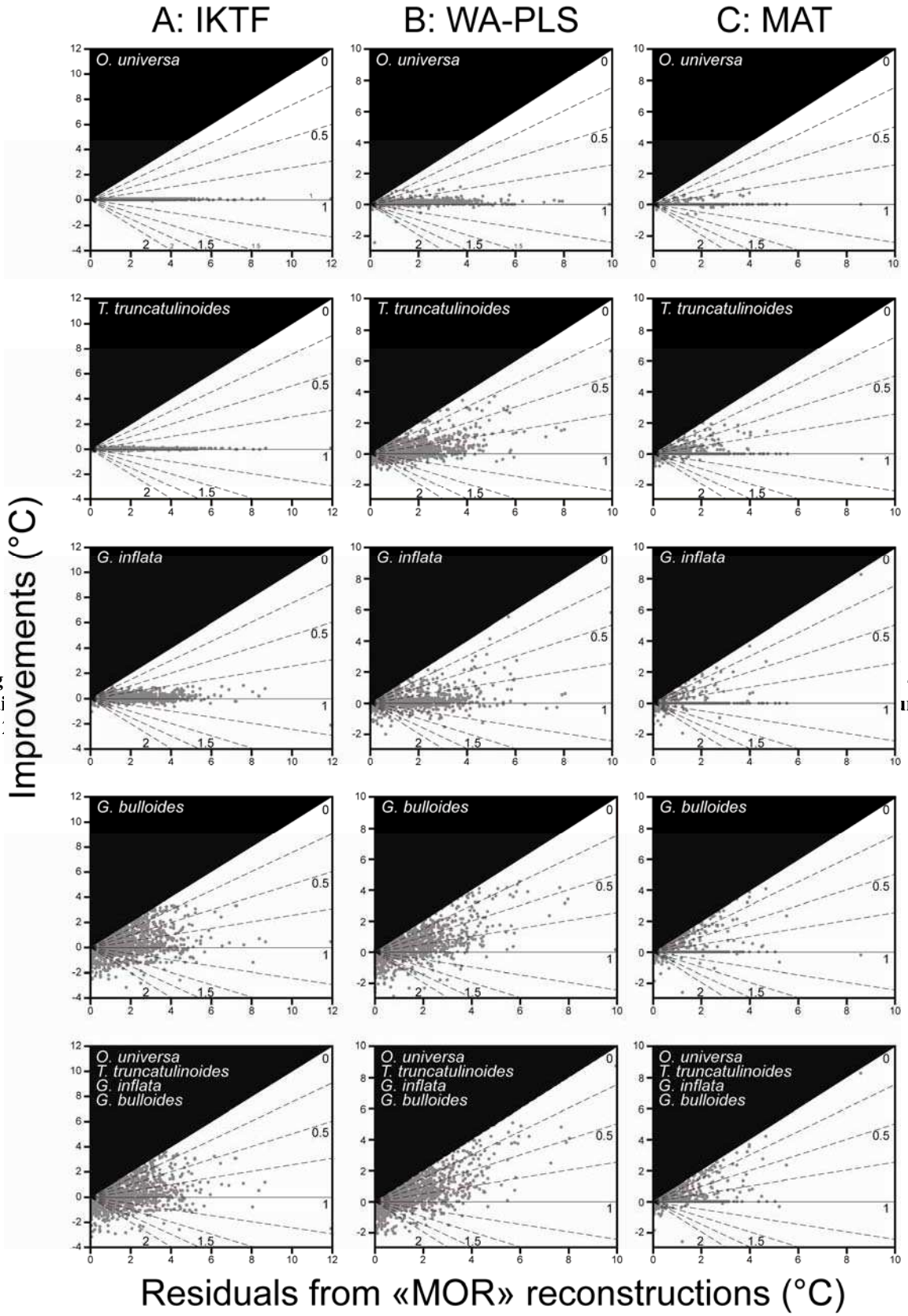

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Figure 10: Biplots between residuals of reconstructions performed with the "MOR" training set (δ_{MOR}) vs. improvements defined as the differences between residual of temperature estimated with "MOR" and those with the "UTIB" training set (δ_{UTIB}) for the three transfer functions. IKTF (A), WA-PLS (B) and MAT (C). Black dashed lines indicate error coefficients values defined as $\delta_{UTIB}/\delta_{MOR}$.

The contribution of *Orbulina universa*, *Truncorotalia truncatulinoides* and *Globoconella inflata* are negligible in IKTF (Figure 10). The improvement of IKTF, mostly due to *Globigerina bulloides* (Figure 10A), is concentrated in the North and the South East of the Indian Ocean and offshore Indonesia (Figure 11E and 11J), where the core tops are the more densely sampled (Figure 2).

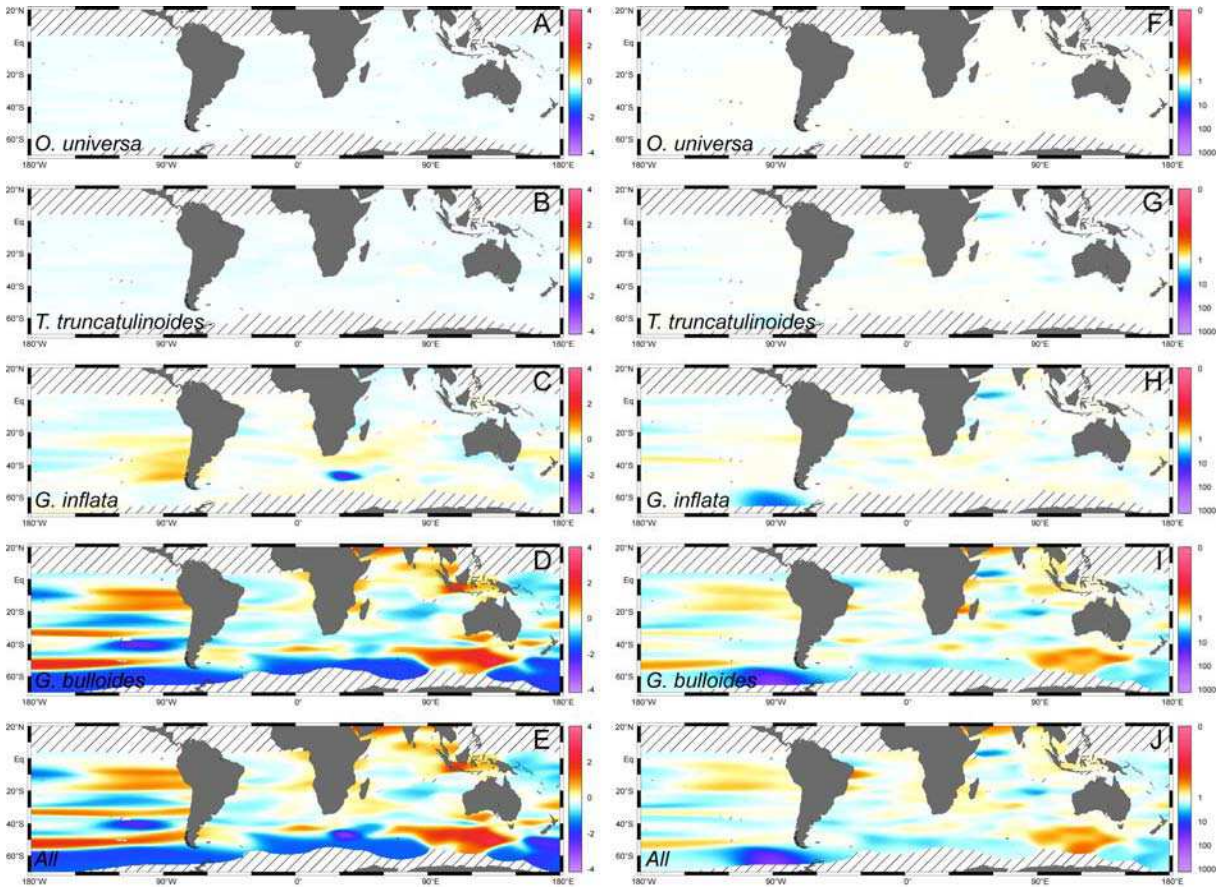

Figure 11: Geographic distribution of the reconstruction improvements with the UTIB training set when using the IKTF transfer function. Intensity of improvement is given in Celsius degrees (A-E) and error coefficient values (F-J) for each morphospecies split into its related genotypes integrated in the training set (A-D, F-I) and the UTIB training set (E, J).

In WA-PLS, the contribution of *O. universa* is also negligible However, replacement of the relative abundances of *T. truncatulinoides* and *G. inflata* by their related genotypes leads to a global improvement of WA-PLS (Figure 10B). This improvement is mostly concentrated in frontal zones and more sparsely in tropical regions. Even if the WA-PLS is globally improved, noise is generated by the integration of cryptic diversity due to: (1) the artificial mix of distinct genotypes (e. g. Types Ia and IIb in subtropical and tropical waters), and (2) the very nature of this regression-based prediction technique (Figure 10B and 12).

Thus, even if the raw reduction of RMSEP is the highest of the three techniques considered here (25%; Table 3), the prediction error is actually only partially improved (Figure 12F-J).

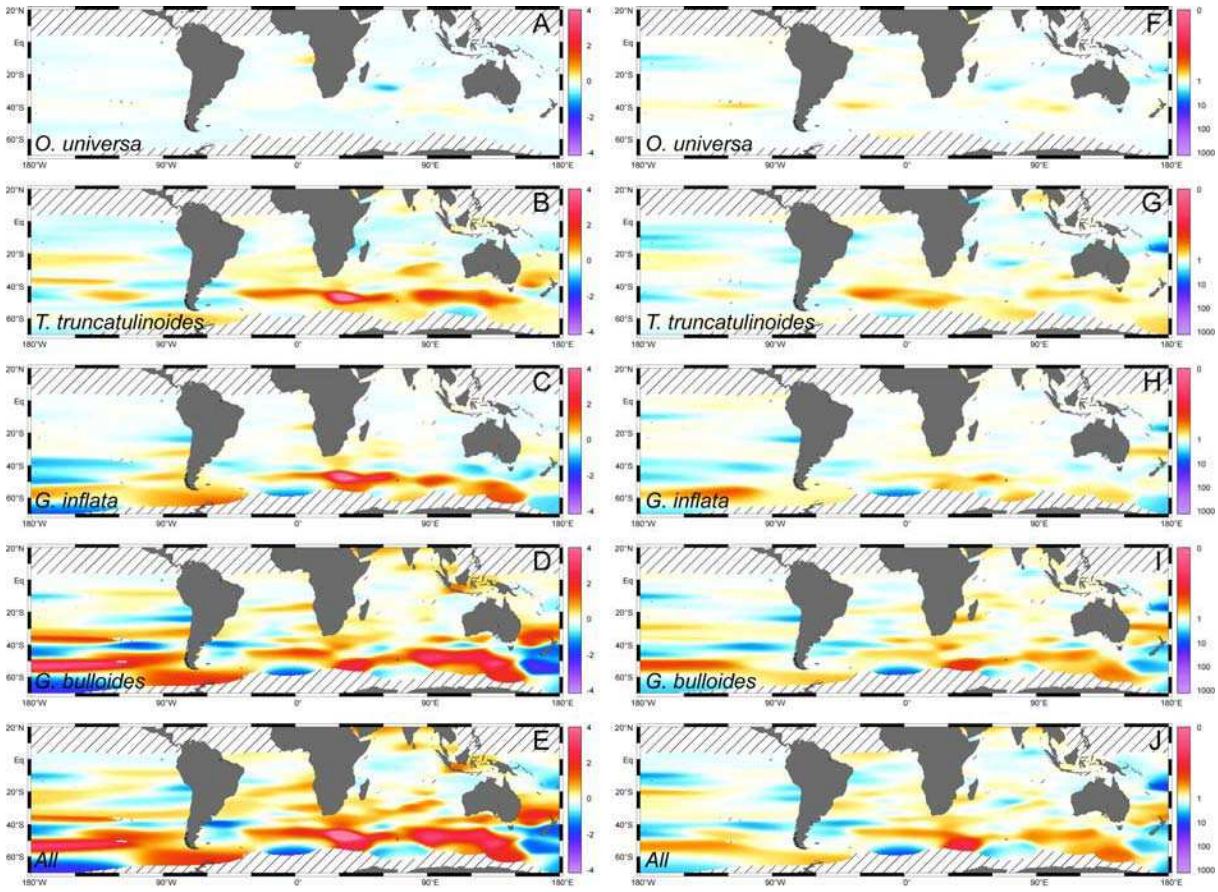

Figure 12: Geographic distribution of the reconstruction improvements with the UTIB training set when using the WA-PLS transfer function. Intensity of improvement is given in Celsius degrees (A-E) and error coefficient values (F-J) for each morphospecies split into its related genotypes integrated in the training set (A-D, F-I) and the UTIB training set (E, J).

The MAT appears to be the most sensible to integration of cryptic diversity. As for IKTF and WA-PLS, the gain introduced by *O. universa* genotypes is negligible (Figure 10C). The reduction is actually concentrated in frontal zones where the residuals are inferior to 1°C (Figures 6C-2), with very few exceptions. The integration of cryptic diversity did not generate supplementary noise, even in the case of the lump of different genotypes in modeled distribution (Type IIb and I of *G. bulloides*). The few cases where error increased are caused by the lack of modern analog in the training set.

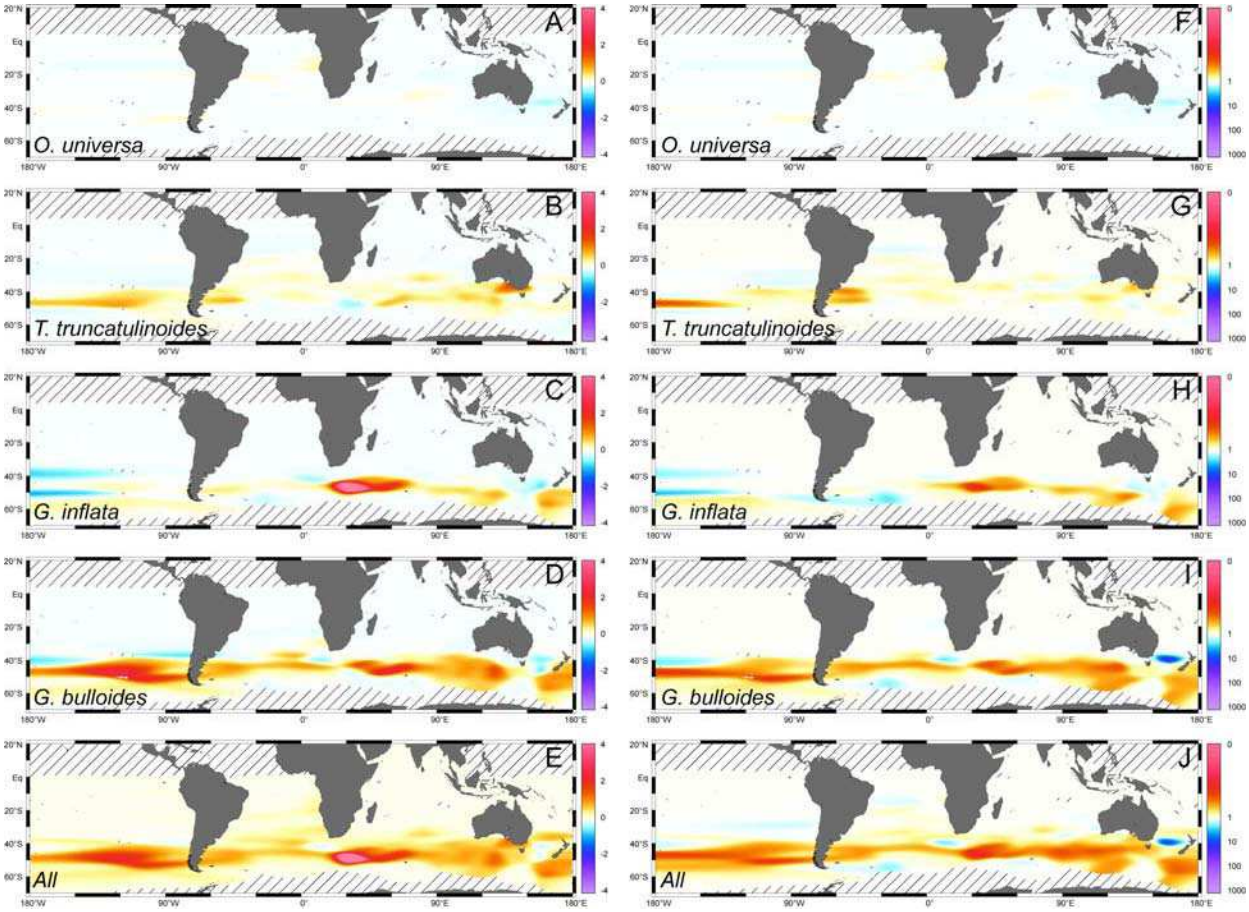

Figure 13: Geographic distribution of the reconstruction improvements with the UTIB training set when using the MAT transfer function. Intensity of improvement is given in Celsius degrees (A-E) and error coefficient values (F-J) for each morphospecies split into its related genotypes integrated in the training set (A-D, F-I) and the UTIB training set (E, J).

4.6. Robustness

First, the leave-one-out cross-validation procedure shows the temperature estimates of all three techniques to be highly robust, with an average RMSEP-value increased by 0.66% for IKTF, 3.25% for WA-PLS and less than 0.01% for MAT, representing a maximal deviation-value at the 99% confidence level of 0.0560°C, 0.2799°C and 0.0031°C, respectively.

Second, the reconstructions performed on noisy datasets also illustrate the high level of robustness of the three techniques. Indeed, the 11 × 11 RMSEP-values obtained for each couple of training dataset × reconstructed dataset returned centered Gaussian distributions with means and standard deviations of $3.5\times10^{-5} \pm 1.56\times10^{-3}$ for IKTF, $9.7\times10^{-5} \pm 3.51\times10^{-4}$

for WA-PLS, and $4.8\times10^{-4} \pm 2.59\times10^{-3}$ for MAT, representing a maximal deviation-value at the 99% confidence level of 0.297°C, 0.435°C, and 0.440°C in MAT). In other words, uniformly distributed random fluctuations in a ±25%-interval around the UTIB genotype abundances never generate a supplementary noise in the predicted values higher than ~0.5°C

5. Discussion

5.1. Improvement of the transfer function

In this study, we showed that the integration of cryptic diversity into the transfer functions leads to a global improvement of the SST-estimates for all three prediction techniques (IKTF, WA-PLS, MAT; Figures 9-14).

However, the improvement of SST-estimates obtained by using IKTF is mainly linked to the heterogeneous spatial distribution and abundances of the 4 *G. bulloides* genotypes, leading to strong prediction improvements in densely sampled areas with highly abundant *G. bulloides* genotypes.Consequently, the total RMSEP-value remains high (Figure 9A-4) even if the error level is partially decreased in a few regions (North and South East of the Indian Ocean; Figure 11J).

Despite relatively high overall error level (Figure 9B), the highest improvement (~25 %; Figure 14; Table 3) is obtained with the WA-PLS. This method searches for Gaussian distributions of species in the training set to predict any environmental variable (Ter Braak et al., 1993). Thus, by modeling the distribution of cryptic species with Gaussian models (Figures 4-8), we incidentally produce the optimum dataset conditions for WA-PLS accurate prediction. Therefore, the improvement generated by the integration of cryptic diversity in the WA-PLS transfer function is maximized

Improvements with MAT induced by integrating cryptic diversity is maximal between transitional and polar climatic zones (Figure 13C) where the RMSEP are < 1°C (Figure 14, Table 3), below the currently admitted average prediction error rate of ~1°C (Malmgren et al.,2001). Integrating cryptic species in MAT lowers the RMSEP under this presumably final limit, which now clearly appears as the spurious artifact of lumping genotypes with distinct temperature requirements (Kucera & Darling, 2002: p. 696).

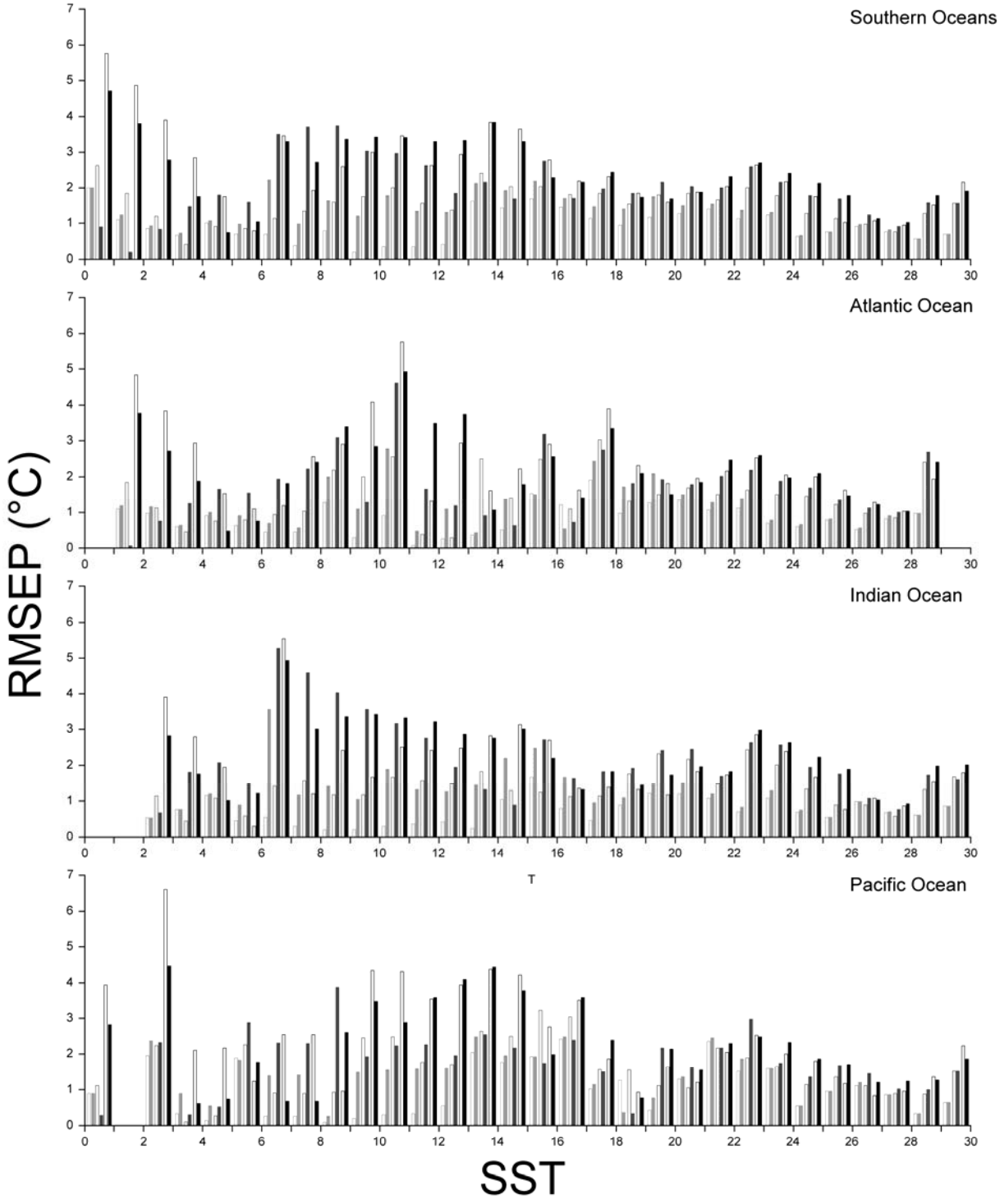

Figure 14: Thermal distribution of root mean square errors of predictions (RMSEP) for all transfer functions used in southern oceans and South Pacific, Atlantic, and Indian Oceans. Filled bars: reconstructions based on the "MOR" training set; open bars: reconstruction based on the "UTIB" training set. Light Gray: MAT; Dark gray: WA-PLS; Black bars: IKTF.

It is worth noting that the strong improvement obtained with MAT in Southern Ocean middle to high latitudes is not associated here to an error increase at lower latitudes. The reason is that MAT does not rely on linear functions to perform assemblage-based SST reconstructions. On the other hand, the accuracy of MAT is highly improved by the density of geographically co-occurring genotypes within each morphospecies: the higher, the better MAT will be, provided enough analogue assemblages are available in the training dataset. The taxonomic richness integrating cryptic species does not increase in tropical and subtropical climatic zones, because the morphospecies *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides* are only replaced in the training set by one of their related genotypes (Figure 5 to 8). Lumping genotypes (e.g., Types Ia and IIb of *Globigerina bulloides*) does not affect the SST prediction, an interesting quality of MAT when morphometric approach fails to fully discriminate cryptic diversity in sediments.

Finally, the quality of the MAT SST-estimates decreases in the southwest Pacific and southeast Atlantic Oceans (Figure 13E and 13J), a direct consequence of the lack of modern analogs in the training set. Since increasing the assemblage-level taxonomic richness leads to a decreasing number of close analogs in the training dataset, this problem, independent of the procedure of genotype integration , can only be solved by enlarging the training set.

5.2. Contribution of the cryptic species

All cryptic species do not equally contribute to the improvement of SST-estimates. For instance, integrating the genotypes of *O. universa* does not improve the transfer functions for at least two reasons. First, their genotype distribution appears to be controlled by the productivity of surface waters rather than by the SST (de Vargas et al., 1999; Morard et al., 2009). Second, the total abundance of *O. universa* in sediments is usually low (<4.5% in 99% of the assemblages, median abundance: 0.4%; Figure 5C), leading to high relative abundance uncertainties. Consequently, the cryptic species of *O. universa* should be used very cautiously in order to predict SST-values.

On the other hand, integrating cryptic diversity from *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides* is clearly more beneficial for paleo-reconstruction in the southern oceans. As discussed above, the estimations of temperature improve with the increase of the taxonomic richness of the assemblage: the higher the degree

of overlap between genotypes, the more the transfer function will be improved. This explains why the integration of the cryptic species of *G. bulloides* and, to a lesser extent, *T. truncatulinoides*, contribute to the highest improvement (Figure 9), especially in MAT. The potential contribution of *G. inflata* cryptic diversity in SST reconstruction is more difficult to estimate at this time. Indeed, no co-occurrence between the two known genotypes has been observed so far (Morard et al., in prep), making the overlap between genotypes inferred in this work theoretical, as still unobserved (see figure 6A). On the other hand, the abrupt transition between the two *G. inflata* genotypes at ~10°C (Figure 4) could be a very useful tool to track past oscillations of the subpolar water front (Morard et al., in prep.).

Incidentally, the cryptic diversity of *G. inflata* could be useful information to accurately predict SST in the case of samples affected by dissolution. A good example for this is the assemblage of the core top V16-058, located south of Africa (46.50°S; 31.27°E). This assemblage, which is heavily affected by dissolution, is strongly dominated by *G. inflata* (79%). The estimation of the temperature based on the initial dataset of Barrows and Juggins (2005) gives an error of 8,6°C (Figure 8C). This sample is located in the thermal range of theoretical overlap of *G. inflata* cryptic species (Figure 7A and 7B). Consequently, the SST-estimate based on the training set integrating the cryptic diversity of *G. inflata* is partially corrected when using the WA-PLS (Figure 10B) and completely corrected with the MAT (Figure 10C). Indeed, the integration of cryptic diversity enables to rectify errors induced by dissolution, a factor of artificial decrease in the taxonomic richness of assemblage.

6. Conclusion

The integration of cryptic diversity into transfer functions will lead to a global improvement of planktonic foraminiferal assemblage-based SST reconstructions. In the case of the MAT, the RMSEP-values fall below the hitherto-thought final lower limit of 1°C for SST ranging between 14°C and 3°C (Malmgren et al., 2001; Kucera and Darling, 2002). However, all cryptic species do not equally participate to the SST-estimate improvement, as illustrated by the negligible contribution of the genotypes of *O. universa* in this process (Figure 10). Finally, it is worth noting that the errors in SST-estimates ranging between 14°C and 3°C have been improved using the genotypes of only 3 morphospecies over the 37 constituting the initial set of Barrows and Juggins (2005). Therefore, the complete morphological deciphering of all genotypes for all morphospecies of planktonic foraminifera appears useless in order to significantly improve the transfer functions accuracy. Rather, focus should be put on genotypes whose distributions are best explained by SST. Indeed, improving SST-reconstructions will not only depend on our ability to recognize cryptic species in sediments, but on our aptitude to accurately estimate the mix of several genotypes related to single morphospecies, and thus to increase assemblage's taxonomic richness.

The recognition of cryptic diversity based on morphological characters and biometrical data is now feasible (Morard et al., 2009; Quillévéré et al., submitted; Morard et al., in prep). Even if the process of recognition is still time consuming, techniques for automatic measurements now exist (Bollman et al., 2005). Soon, conducting biometric analyses on large collections of foraminifera to quickly identify cryptic diversity will be possible. From such data, the results discussed in this paper clearly show that a significant improvement in the accuracy of SST-reconstructions can be expected.

Acknoledgments

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Conclusions

Les foraminifères planctoniques constituent l'un des objets les plus étudiés et utilisés d'abord par les biostratigraphes puis par les paléoceanographes. Cette exploitation s'est accrue à partir des années 1950 avec l'utilisation des isotopes stables (Emiliani, 1954) ainsi qu'avec l'établissement des premières fonctions de transfert (Imbrie & Kipp, 1971). Dès lors, la finesse des techniques employées que cela soit en chimie isotopique ou dans l'établissement de fonctions de transfert n'a cessé de s'accroître. En parallèle, les connaissances sur la biogéographie et l'écologie des foraminifères n'ont cessé de s'accumuler (voir pour une revue Arnold & Parker, 1999). Cependant, des données tardives en provenance de la biologie moléculaire ont remis en cause l'utilisation routinière de ces organismes (Huber et al., 1997). L'utilisation des foraminifères planctoniques comme marqueurs paléocéanographiques repose sur l'hypothèse que chaque espèce morphologique correspond à une espèce biologique caractéristique d'un habitat spécifique. Or, l'observation systématique de diversité cryptique sur l'ensemble des morpho-espèces analysées (à l'exception de *Globigerinoides sacculifer* ; André, 2010) remet en cause ce postulat (voir pour une revue Darling et Wade., 2008). Certains auteurs choisirent une stratégie de contournement en restreignant la couverture géographique des calibrations des fonctions de transfert, les espèces cryptiques ayant une distribution géographique et environnementale plus restreinte que leur morpho-espèce (Kucera et al., 2005). Cependant, cette approche ne résout que partiellement le problème car les génotypes ont des degrés de recouvrement plus ou moins importants.

L'unique solution repose donc sur la reconnaissance morphologique des espèces cryptiques. Dans ce travail, nous avons quantifié la variabilité génétique, écologique et morphologique de 4 morpho-espèces clefs de la paléoceanographie : *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* et *Globigerina bulloides* (uniquement des données morphologiques préliminaires pour cette dernière, Figure 1). Cette approche repose sur la caractérisation tridimensionnelle de spécimens uniques, rendue possible par l'utilisation du GITC*, protocole d'extraction d'ADN non destructif pour la coquille. Des analyses moléculaire ont permis de confirmer les patterns biogéographiques des espèces cryptiques à l'échelle mondiale ainsi que de découvrir des génotypes inédits. Ainsi les formes

de spécimens préalablement génotypés ont été quantifiées par le biais d'analyses morphométriques. Couplé à des analyses statistiques rigoureuses, ces mesures ont servi de base à l'établissement de modèles de reconnaissance morphologique applicables à l'échelle populationnelle.

De cette manière, un modèle de reconnaissance morphologique à pu être établi pour reconnaître les trois espèces cryptiques de la morpho-espèce *Orbulina universa* (définies sur la base de variations de la SSU) basé sur la porosité et la distribution des surface de pores de la loge terminale. Ce modèle, établi à partir d'une collection de 306 individus génotypés permet d'assigner correctement de 71% à 91% le génotype des individus avec la distribution des surfaces de pores. Ainsi, l'espèce Caribéenne ou Type I (n = 100), est caractérisée par une densité de pores moindre et une surface de pores plus importante que les espèces Sargasse ou Type II (n = 36) et Méditerranéenne ou Type III (n = 170 ; 89% et 91% de reconnaissance respectivement). Ces deux autres espèces ont une variabilité morphologique plus recouvrante, avec une densité de pores plus importante et une taille moindre, ne permettant de reconnaître que 71% des individus en se basant sur leur morphologie. Cependant l'espèce Sargasse est caractérisée par un test moins épais (6,51 +/- 4,47 µm) que l'espèce Méditerranéenne (11,75 +/- 6,07 µm) et Caribéenne (14,25 +/- 7,16 µm). Ainsi, avec deux caractères, il est possible de faire la distinction entre ces trois génotypes. Les espèces Caribéenne et Sargasse ont des préférences écologiques similaires, et préfèrent les masses d'eau oligotrophiques des gyres (sub)tropicales, alors que l'espèce Méditerranéenne préfère les eaux plus eutrophes des masses d'eau tempérées à subtropicales. Ainsi la diversité cryptique de ce taxon constitue un outil pour reconstituer les variations de productivité des eaux de surface.

Le cas de la diversité cryptique chez *Truncorotalia truncatulinoides* est différent. Les espèces cryptiques ont été définies sur la base de variations de l'ITS et 5 différents génotypes ont été reconnus (de Vargas et al., 2001 ; Ujiié et al., 2009 ; ce travail). Les Types I (n = 184), II (n = 338) et V (n = 17), ont été identifiés dans les eaux subtropicales de l'hémisphère Sud, de l'Atlantique et du Pacifique Nord, respectivement, et les types III (n = 248) et IV (n = 26) ont été identifiés dans les eaux de transition et subpolaires de l'hémisphère Sud, respectivement. Cependant, l'échantillonnage utilisé dans ce travail est clairement déséquilibré, les Types IV et V étant sous-échantillonnés. Malgré cela, il a été possible d'établir un modèle de reconnaissance morphologique, non pas sur des espèces cryptiques individuelles comme cela a été le cas pour *Orbulina universa* mais sur deux clusters de génotypes constitués de génotypes phylogénétiquement proches. Le cluster 1 est constitué des Types I, II et V, tous présents au Nord du front nord subtropical des océans de l'hémisphère

sud alors que le cluster 2 est constitué des Types III et IV présents uniquement au sud du même front. La morphologie plus complexe de ce taxon (enroulement trochospiralé, loges carénées) a permis d'établir un modèle de reconnaissance morphologique tridimensionnelle, basé sur les contours de tests en vues latérale ombilicale et ombilicale, pour maximiser sa robustesse. Le modèle résultant permet une assignation génétique de 80 % des spécimens, c'est-à-dire lorsque un spécimen est classifié correctement avec la vue latérale et ombilicale, 17% d'assignation ambigüe c'est-à-dire lorsque un individu est classé dans un cluster différent par les deux vues, et enfin 3% d'assignation incorrecte. Le cluster 1 est caractérisé par une throchospire haute en vue latérale ombilicale, et par une forme allongée en vue ombilicale. Le cluster 2 est quand à lui caractérisé par une throchospire basse et biconvexe en vue latérale ombilicale ainsi qu'une forme circulaire en vue ombilicale. Ce modèle à été testé avec succès sur des individus collectés lors des campagnes mais non génotypés. Ce modèle, séparant les deux clusters permet de reconstituer les migrations du front nord subtropical de l'hémisphère Sud.

La morpho-espèce *Globoconella inflata* n'ayant pas été étudiée, une étape d'acquisition de séquences a due être réalisée pour identifier la variabilité génétique de ce taxon. Le séquençage de la SSU n'ayant révélé aucune variation, l'ITS fut le seul marqueur susceptible de révéler la présence d'une diversité cryptique. Ainsi, ces analyses ont dévoilé la présence de deux génotypes au sein de cette morpho-espèce. Le premier, dominant, est nommé Type I et est présent dans les environnements subtropicaux et tempérés des deux hémisphères alors que le second génotype (Type II) présente une distribution restreinte aux environnements subpolaires de l'hémisphère Sud. Les deux génotypes ont une distribution allopatrique car aucune co-occurrence n'a été observée dans le jeu de données. A l'instar d'*Orbulina universa* et *Truncorotalia truncatulinoides*, des analyses morphométriques on été réalisées pour établir un modèle de reconnaissance morphologique. Cependant, sur les 477 spécimens génotypés, seulement 26 étaient de Type II et pour certains, la coquille n'a pu être préservée (collecte antérieure à l'utilisation du GITC*). En l'absence d'identification génétique directe, des mesures biométriques simples (ratio de la taille de l'ouverture et de la chambre terminale) ont été conduites sur 306 spécimens non génotypés collectés dans l'Atlantique sud. Grâce à la distribution disjointe des deux espèces cryptiques, il a été possible d'assigner un génotype à ces individus : Type I pour ceux collectés au Nord du front subpolaire et Type II pour ceux collectés au sud du front subpolaire. Sur la base de ces mesures simples mais robustes, il a été possible d'établir un modèle de reconnaissance morphologique assignant correctement 66% des génotypes. Ce modèle discriminant préfigure

l'établissement d'un modèle développé sur la base de comparaisons morpho-génétiques directs, permettant de reconstituer les oscillations du front subpolaire de l'hémisphère sud. De futures collections seront réalisées dans le cadre des missions OISO (Océan Indien Service d'Observation) courant 2011 en hautes latitudes, ces collectes permettront de prélever suffisamment de spécimens de *G. inflata* pour développer ce modèle.

Ces collections nous permettront également d'enrichir nos connaissances concernant la diversité cryptique de *Globigerina bulloides*. Les analyses moléculaires conduites sur les spécimens collectés lors de la campagne OISO-4 ont révélé la présence d'un génotype inédit. Des photographies prises au microscope électronique à balayage ont révélées que ce génotype possède une microstructure singulière (Figure 1) également visible à la loupe binoculaire. Cette microstructure contraste largement avec celle des autres types génétiques échantillonnés dans l'océan Indien. Ainsi, la reconnaissance de ce génotype pourrait être réalisée uniquement sur ce critère sans l'utilisation d'un modèle de reconnaissance morphologique.

Les travaux conduits sur ces quatre morpho-espèces, ont permis de démontrer que les informations environnementales contenues dans la diversité cryptique sont transférables vers le registre fossile. Afin de quantifier l'impact de l'intégration de la diversité cryptique dans les reconstitutions paléocéanographiques, les fonctions de transfert couramment utilisées ont été re-calibrées en intégrant les distributions restreintes des génotypes d'*Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* et *Globigerina bulloides* dans les océans de l'hémisphère Sud. Dans ce cas, les analyses montrent que les erreurs associées à l'estimation des températures passent en-dessous de la barre du degré °C, précision jamais atteinte avec ces méthodes. Deux faits sont importants dans ces analyses. Premièrement, la contribution d'*O. universa* dans l'amélioration des reconstitutions est quasi-nulle. Cela est certainement dû à la sous-représentation de ce taxon dans les sédiments de surface et au contrôle de la distribution des génotypes d'*O. universa* par la productivité, plutôt que par la température. Deuxièmement, la contribution significative des espèces cryptiques de 3 morpho-espèces de foraminifères suffit à résoudre les erreurs dans les reconstitutions. Ainsi, cibler quelques morpho-espèces clefs de la paléoceanographie et abondantes dans les sédiments (à l'instar de *G. inflata* et *G. bulloides*), suffira à améliorer la résolution des erreurs.

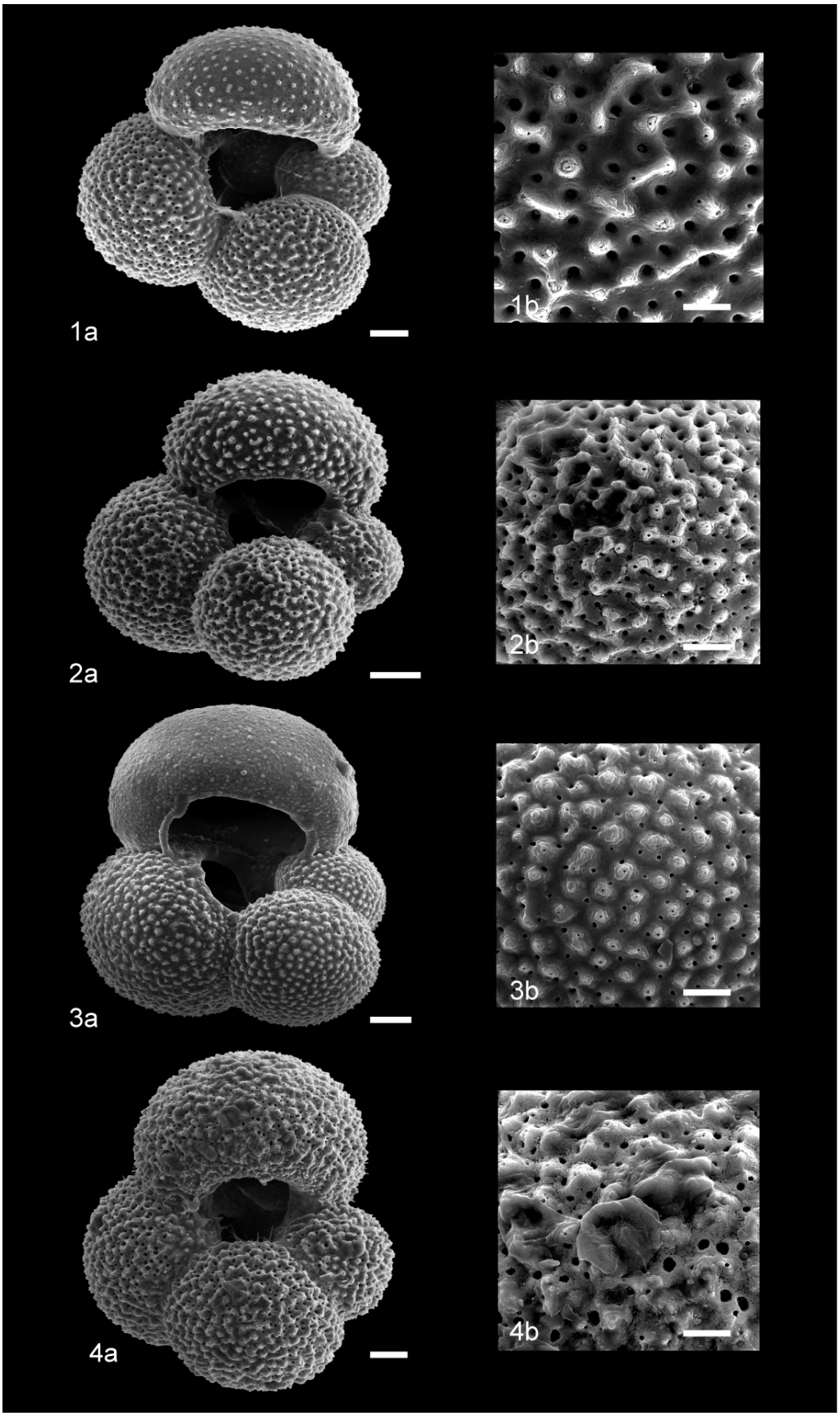

Figure 1 : Photos au microscope éléctronique à balayage (MEB) de spécimens (a) et de la microstructure de 4 spécimens de *Globigerina bulloides* collectés lors de la campagne OISO-4. Chacun des spécimens ayant subi une extraction d'ADN, leur identité génétique est connue : 1: Type IIa, 2 : Type IIb, 3: Type IIc, 4 = Type IIf.

Ce travail a démontré que l'utilisation de la diversité cryptique dans les reconstitutions était non seulement possible, mais qu'elle permet d'aboutir à un degré de précision et de finesse jamais atteints jusqu'à ce jour. Néanmoins, il reste une longue route à parcourir des résultats présentés dans ce manuscrit jusqu'à une utilisation routinière de la diversité cryptique dans les reconstitutions paléoceanographiques.

Premièrement, malgré la robustesse des modèles de reconnaissance établis pour les espèces cryptiques d'*Orbulina universa* et *Truncorotalia truncatulinoides,* leur application repose sur des analyses morphométriques fines, sensibles aux différences de calibration en plus d'être chronophages. De fait, la reproductibilité des mesures, condition *sine qua non* de l'application de ces modèles n'est pas pleinement assurée.

Deuxièmement, dans le cadre de l'intégration de la diversité cryptique, l'amélioration des reconstitutions repose sur des assemblages dont la spécificité est accrue, c'est-à-dire avec plusieurs génotypes appartenant à la même morpho-espèce. Malgré la robustesse des estimations au bruit, l'intensité de l'amélioration dépendra de notre capacité à identifier les mélanges de génotypes dans les sédiments, ainsi que leurs proportions relatives. Les modèles établis dans ce travail sont performants pour la discrimination des génotypes. Il reste à déterminer leur efficacité pour évaluer les mélanges de génotypes. Pour palier ces problèmes, il sera nécessaire d'établir des modèles basés sur des mesures biométriques simples (par exemple, des rapports de longueur pour mesurer la conicité de *Truncorotalia truncatulinoides* ou encore le rapport ouverture/loge terminale chez *Globoconella inflata*). L'utilisation de méthodes d'acquisition automatique permettra de réaliser ces mesures sur des collections d'individus conséquentes (plusieurs milliers) dans un délai raisonnable. Il sera possible d'établir des modèles robustes, résistant aux problèmes de calibration. Puis à partir de la collection d'individus, des jeux de données artificiels intégrant des mélanges de génotypes seront utilisés pour calibrer le modèle et reconnaître les mélanges de génotypes dans les sédiments.

Troisièmement, la stabilité temporelle des caractères morphologiques caractéristiques des espèces cryptiques reste à être établie. En effet, les seules estimations de la longévité des espèces cryptiques sont dérivées de l'utilisation de l'horloge moléculaire. Les reconstructions phylogénétiques établissent des relations entre lignées à partir de leurs représentants actuels. De fait, ces analysent renvoient une image plus juste de la diversité actuelle des foraminifères planctoniques mais ne fournit qu'une estimation minimale de la diversité cryptique passée (de Vargas et al., 2004). Sur ces seules données, il nous est impossible d'affirmer ou d'infirmer si les espèces cryptiques actuelles sont stables écologiquement et spatialement au cours du

temps et si la diversité cryptique fossile est supérieure à celle observée aujourd'hui. Pour lever l'incertitude sur ces questions il faudra opter pour une approche stratophénétique. Cela consiste à quantifier les variations morphologiques d'une lignée dans des séries stratigraphiques continues avec un pas d'échantillonnage serré pour repérer les changements évolutifs (Wei, 1987). Dans notre cas, il faudra appliquer cette approche dans des carottes à l'aplomb d'environnements stables au cours des derniers millions d'années pour vérifier si les populations fossiles correspondent morphologiquement aux types génétiques attendus. Renaud et Schmidt et (2003) ont apporté des résultats convaincant quant à la stabilité temporelle des espèces cryptique de *Truncorotalia truncatulinoides* et leur utilisation comme traceur des migrations des fronts océaniques sur les 140 000 dernières années. Cette approche nous permettra de (1) valider l'utilisation de nos modèles dans le fossile et leur fenêtre temporelle d'application et (2) évaluer si la diversité cryptique fossile est supérieure à celle observée dans l'actuel. Cette étape est un préalable incontournable à l'utilisation de la diversité cryptique pour faire des reconstructions paléocéanographiques.

Depuis les travaux précurseurs de Berger (1969) et Imbrie et Kipp (1971) les paléocéanographes n'ont pas cessé d'accumuler des connaissances sur l'écologie et la biogéographie des foraminifères et d'améliorer les techniques de reconstructions paléoceanographiques pour se heurter à une limite apparemment infranchissable dans la qualité des reconstructions. Toutes les techniques employées n'ont jamais pu fournir des estimations de température déviant de moins de 1°C des températures attendues. Récemment, Kucera et al. (2005), tout en reconnaissant le caractère prévalant de la diversité cryptique chez les foraminifères planctoniques et le gain potentiel que représenterait son intégration dans les reconstitutions, ont estimé qu'il ne serait pas possible de l'utiliser via l'identification des génotypes et optèrent pour une stratégie de contournement en réduisant la couverture géographique des jeux de données de calibration (les espèces cryptiques ont un degré d'endémicité supérieur aux morpho-espèces). Bien que limitant les biais liés à la diversité cryptique, cette approche ne suffit pas à passer outre de manière in équivoque cette barrière des 1°C. Ce travail a non seulement démontré que les espèces cryptiques sont reconnaissables sur la base de critères morphologique mais aussi qu'il n'est pas nécessaire d'identifier *tous* les génotypes de *toutes* les morpho-espèces pour amener le taux d'erreur des reconstitutions en dessous de cette barrière. La diversité cryptique est donc un atout indéniable et pratique qui aménera la paléoceanographie à un degré de résolution jamais atteint.

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Résumé

L'utilisation des coquilles carbonatées de foraminifères planctoniques comme marqueurs paléocéanographiques repose sur l'hypothèse fondamentale que chaque espèce morphologique correspond à une espèce biologique caractéristique d'un habitat spécifique. Cette relation empirique a été récemment remise en cause par des analyses moléculaires qui ont révélé la présence systématique de plusieurs espèces génétiques (espèces cryptiques) au sein des morpho-espèces actuelles. Il a été suggéré que ces espèces cryptiques ou génotypes, présentaient (1) des préférences biogéographiques et écologiques restreintes par rapport à leur morpho-espèce respective et (2) des différences morphologiques. Dans ce travail, nous avons caractérisé la diversité génétique, morphologique et écologique de 4 morpho-espèces clefs de la paléocéanographie, i.e. *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* et *Globigerina bulloides.* Cette étude repose sur le développement d'un protocole d'extraction ADN non destructif pour la coquille calcaire, permettant l'analyse conjointe de la variabilité génétique et morphologique d'un même individu. Les variations de forme ou de porosité de chacun des génotypes des morpho-espèces ont été quantifiées. Il apparaît que le fort degré de plasticité morphologique largement documenté chez les foraminifères planctonique et jusqu'alors interprété comme écophénotypique, est au moins en partie la conséquence du regroupement de plusieurs génotypes présentant des morphologies et préférences écologique particulières. Sur la base des ces observations, des modèles de reconnaissance morphologique permettant d'identifier les génotypes à partir de la morphologie de la coquille, utilisables à l'échelle populationnelle, ont été développés. Afin de quantifier l'impact de l'intégration de la diversité cryptique dans les reconstitutions paléocéanographiques basées sur les assemblages de foraminifères planctoniques, les fonctions de transfert couramment utilisées ont été re-calibrées en intégrant les distributions restreintes des génotypes d'*O. universa*, *T. truncatulinoides*, *G. inflata* et *G. bulloides*. Ces re-calibrations conduisent à un degré de précision jusqu'alors jamais atteint dans les reconstructions paléocéanographiques basées sur les assemblages de foraminifères planctoniques.

Mots clefs : Foraminifères planctoniques, diversité cryptique, SSU rDNA, ITS rDNA, biogéographie, morphométrie, fonctions de transfert.

Abstract

The usefulness of calcareous shells of planktonic foraminifera as a paleoceanographic proxy relies on the key hypothesis that each morphospecies corresponds to a biological species with a specific habitat. This empirical relationship has been challenged since molecular analyses have revealed a significant level of cryptic genetic diversity among modern morphospecies of planktonic foraminifera. Previous workers have suggested that the cryptic species or genotypes (1) display narrower biogeographic and ecological ranges than their related morphospecies, and (2) exhibit shell morphological differences. In this work, we have characterized the genetic, morphological and ecological diversity among four planktonic foraminiferal morphospecies of significance in paleoceanography, i.e. *Orbulina universa*, *Truncorotalia truncatulinoides*, *Globoconella inflata* and *Globigerina bulloides*. Our study relies on the development of a new single-cell DNA extraction protocol that retains the shell, allowing direct morpho-genetic comparisons. Shape or porosity variations within each genotype have been quantified. It appears that the high degree of morphological plasticity widely documented in planktonic foraminifera and classically seen as ecophenotypy, is at least partly the spurious consequence of lumping several genotypes that display morphological and environmental preferences. Based on these observations, we developed several population-scale models, which allow recognition of the cryptic species based on their shell morphology. Finally, in order to quantify the impact of integrating cryptic diversity in assemblage-based paleoceanographic reconstructions, we have re-calibrated transfer functions based on the ecological ranges of the genotypes of *O. universa*, *T. truncatulinoides*, *G. inflata* and *G. bulloides* in the southern oceans. Such re-calibrations led to a great, previously never reached improvement in the accuracy of the assemblage-based paleoceanographic reconstructions.

Keywords: Planktonic foraminifera, cryptic diversity, SSU rDNA, ITS rDNA, biogéography, morphometry, Transfer function.