PFR^2: a curated database of planktonic Foraminifera18S ribosomal DNA as a resource for studies of plankton ecology, biogeography, and evolution

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Abstract

Planktonic Foraminifera (Rhizaria) are ubiquitous marine pelagic protists producing calcareous shells with conspicuous morphology. They play an important role in the marine carbon cycle and their exceptional fossil record serves as the basis for past climate reconstructions. A major worldwide sampling effort over the last two decades has resulted in the establishment of multiple large collections of cryopreserved individual planktonic foraminifera samples. Thousands of 18S rDNA partial sequences have been generated, representing all major known morphological taxa across their worldwide oceanic range. This comprehensive data

coverage provides an opportunity to assess patterns of molecular ecology and evolution in a holistic way for an entire group of planktonic protists. We combined all available published and unpublished genetic data to build PFR², the *Planktonic Foraminifera Ribosomal Reference* database. The first version of the database includes 3,322 reference 18S rDNA sequences belonging to 32 of the 47 known morphospecies of planktonic Foraminifera, collected from 460 oceanic stations. All sequences have been rigorously taxonomically curated using a six-rank annotation system fully resolved to the level of morphological species and linked to a series of metadata. The PFR² website, available at http://pfr2.sb-roscoff.fr, allows downloading the entire database or specific sections, as well as the identification of new planktonic Foraminiferal sequences. Its novel, fully documented curation process integrates advances in morphological and molecular taxonomy. It allows for an increase in its taxonomic resolution and assures that integrity is maintained by including a complete contingency tracking of annotations and assuring that the annotations remain internally consistent.

Introduction

Despite their ubiquity and the critical role they play in global biogeochemical cycles, unicellular eukaryotes (protists) remain the most poorly known domain of life (e.g. Pawlowski et al., 2012). Because of their extreme morphological and behavioral diversity, the study of even relatively narrow lineages requires a high degree of taxonomic expertise (e.g. Guillou et al., 2012, Pawlowski and Holzmann, 2014). As a result, the knowledge of protistan ecology and evolution is limited by the small number of taxonomists resulting in scarcity of taxonomically well-resolved ecological data. As an alternative approach, numerous studies have demonstrated the potential of identification of protists by means of short DNA sequences or barcodes (e.g., Saunders, 2005; Sherwood et al., 2007; Hollingsworth et al., 2009; Nossonova et al., 2010;

64Pawlowski and Lecroq, 2010; Hamsher et al., 2011; Stern et al., 2010; Schoch et al., 2012), both
65at the single-cell and metacommunity levels (e. g., Sogin et al., 2006; Logares et al., 2014, de
66Vargas et al., 2015). Such barcoding/metabarcoding approaches critically rely on the fidelity of
67the marker gene with respect to specificity (avoiding ambiguity in identification),
68comprehensiveness (assuring all taxa in the studied group are represented in the reference
69barcode database) and accuracy (assuring that barcode assignments are consistent with a
70coherent, phenotypic taxonomic framework; e. g. Zimmermann et al., 2014). These three pre-
71requisites are rarely found in protists, where classical morphological taxonomy is often
72challenging, DNA extraction and sequencing from a single cell is prone to contamination, and a
73large portion of the diversity in many groups remains unknown (e.g. Mora et al., 2011). In this
74respect, planktonic Foraminifera represent a rare exception.

75 Planktonic Foraminifera are ubiquitous pelagic marine protists with reticulated
76pseudopods, clustering within the Rhizaria (Nikolaev et al., 2004). The group is marked by a
77rather low number of morphospecies (47; Hemleben et al., 1989), which can be distinguished
78using structural characteristics of their calcite shells. Their global geographic distribution,
79seasonal dynamics, vertical habitats and trophic behavior have been thoroughly documented by
80analyses of plankton hauls (e.g., Bé and Hudson, 1977), sediment trap series (e.g., Zaric et al.,
812005) and thousands of surface sediment samples across the world oceans (e.g., Kucera et al.,
822005). Their outstanding preservation in marine sediments resulted in arguably the most
83complete fossil record, allowing comprehensive reconstruction of the evolutionary history of the
84group (Aze et al., 2011). The morpho-taxonomy and phylogeny of the group have been largely
85confirmed by molecular genetic analyses (e.g., Aurahs et al., 2009a) based on the highly
86informative, ~1,000 bp fragment at the 5'end of the 18S rDNA gene. These analyses confirmed

87that the morphological characters used to differentiate planktonic Foraminifera taxa are
88phylogenetically valid both at the level of morphological species and at the level of higher taxa.
89The studied gene fragment contains six hypervariable expansion segments, some unique to
90Foraminifera, providing excellent taxonomic resolution (Pawlowski and Lecroq, 2010). Analyses
91of this fragment revealed the existence of genetically distinct lineages within most of the
92morphospecies, which likely represent reproductively isolated units (Darling et al., 1996, 1997,
931999, 2000, 2003, 2004, 2006, 2007, 2008, 2009; Wade et al., 1996; de Vargas et al., 1997, 1999,
942001, 2002, de Vargas and Pawlowski, 1998; Stewart et al., 2001; Aurahs et al., 2009b, 2011;
95Ujiié et al., 2008, 2009, 2012; Morard et al., 2009, 2011, 2013; Seears et al., 2012; Weiner, 2012,
962014; André et al., 2014). In order to assess the ecology and biogeography of such cryptic
97species, large numbers of rDNA sequences from single-cell extractions collected across the
98world oceans have been generated for most morphospecies (Figure 1). Due to this extensive
99single-cell rDNA sequencing throughout the last decades, the genetic and morphological
100diversity of planktonic foraminifera have been linked together to a degree that now allows for
101transfer of taxonomic expertise. The knowledge of the genetic and morphological taxonomy of
102the group allows the establishment of an exceptionally comprehensive reference genetic database
103that can be further used to interpret complex data from plankton metagenomic studies with a
104high level of taxonomic resolution. Because planktonic Foraminifera are subject to the same
105ecological forcing as other microplankton, including the dominance of passive transport in a
106relatively unstructured environment, huge population sizes, and basin-scale distribution of
107species, they can potentially serve as a model for the study of global ecological patterns in other
108groups of pelagic protists, whose diversity remains largely undiscovered (Mora et al., 2011).

By early 2014, 1,787 partial 18S rDNA sequences from single-cell extractions of planktonic Foraminifera were available in public databases. However, their NCBI taxonomy is often inconsistent, lacking standardization. It includes (and retains) obvious identification errors, as discussed by Aurahs et al. (2009) and André et al. (2014), and their annotation lacks critical metadata. In addition, an equivalent number of rDNA sequences not deposited in public databases have been generated by the co-authors of the present study. Collectively, the existing rDNA sequences from single cells collected throughout the world oceans cover the entire geographic and taxonomic range of planktonic Foraminifera. This collection unites the current morphological, genetic, ecological, and biogeographic knowledge of the group and may serve as a *Rosetta Stone*/*Philae Obelisk* for interpreting metabarcoding data (Pawlowski et al., 2014). To pave the way for future exploitation of this resource, we combined all published and unpublished planktonic Foraminifera rDNA sequence data and curated the resulting database with a semi-automated bioinformatics pipeline. The resulting "Planktonic Foraminifera Ribosomal Reference database" (PFR^2) is a highly resolved, fully annotated and internally entirely consistent collection of 18S rDNA sequences of planktonic Foraminifera, aligned and evaluated in a way that facilitates direct assessment of barcoding markers.

Material and Methods

Primary database assembly

A total of 1,787 18S rDNA sequences of planktonic Foraminifera were downloaded from the GenBank query portal (http://www.ncbi.nlm.nih.gov/; release 201) on the 14th of May 2014. The taxonomic path and metadata for these sequences were extracted from NCBI and supplemented by information in original papers when available. The metadata associated to each sequence

consisted of: (i) their organismal origin (specimen voucher, taxonomic path, infra specific genetic type assignment), (ii) their methodological origin (direct sequencing or cloning), and (iii) their spatio-temporal origin (geographic coordinates, depth, and time of collection). Metadata were described using standard vocabularies and data formats. For 47 sequences, the coordinates of the collection site could not be recovered, in which case the locality was described in words (Supplementary Material 1).

We next compiled all unpublished 18S rDNA sequences generated by the authors of this paper and linked them with the same suite of metadata. These sequences originate from single-cell extractions of planktonic Foraminifera collected by stratified or non-stratified plankton net hauls, in-situ water pumping, as well as SCUBA diving. After collection, the specimens were individually picked under a stereomicroscope, cleaned, taxonomically identified and transferred into DNA extraction buffer or air-dried on cardboard slides and stored at -20°C or -80°C. DNA extractions were performed following the DOC (Holzmann & Pawlowski, 1996), the GITC* (Morard et al., 2009), or the Urea (Weiner et al., 2014) protocols. Sequences located at the 5' end of the 18S rDNA were obtained following the methodology described in de Vargas et al. (1997), Darling et al. (1996, 1997), Aurahs et al. (2009b), Morard et al. (2011) and Weiner et al., (2014). In total, 820 new planktonic Foraminiferal sequences were analyzed and annotated for this study. In addition, 925 unpublished sequences analyzed in Darling et al. (2000, 2003, 2004, 2006, 2007), Darling and Wade (2008), Seears et al. (2012) and Weiner et al. (2014) were also included. All unpublished sequences, except 177 sequences shorter than 200bp, were deposited in GenBank under the accession numbers KM19301 to KM194582. Overall, PFR^2 contains data from 460 sites sampled during 54 oceanographic cruises and 15 near shore collection campaigns

between 1993 and 2013. It covers all oceanic basins, all seasons, and water depths ranging between the surface and 700 meters (Figure 1; Supplementary Material 1).

Taxonomy

Morphological taxonomy

As the first step in the curation process, the primary taxonomic annotations of all 3,532 18S rDNA sequences gathered from NCBI and our internal databases were harmonized. The identification of planktonic Foraminifera is challenging especially for juvenile individuals, which often lack diagnostic characters (Brummer et al., 1986). Thus, many of the published and unpublished 18S rDNA sequences were mislabelled or left in open nomenclature. In some cases the same taxon has been recorded under different names, reflecting inconsistent usage of generic names, synonyms and misspelling. To harmonize the taxonomy, we first carried out a manual curation of the original annotations to remove the most obvious taxonomic conflicts in the primary database. To this end, the sequence annotations were aligned with a catalog of 47 species names based on the taxonomy used in Hemleben et al. (1989), but adding *Globigerinoides elongatus* following Aurahs et al. (2011) and treating *Neogloboquadrina incompta* following Darling et al. (2006). Thus, the 109 sequences labelled as *Globigerinoides ruber* (pink) and the 63 labelled as *Globigerinoides ruber* (white) were renamed as *Globigerinoides ruber.* The 113 sequences of *Globigerinoides ruber* and *Globigerinoides ruber* (white) attributed to the genotypes II were renamed *Globigerinoides elongatus*. The 12 sequences labelled *Globigerinella aequilateralis* were renamed *Globigerinella siphonifera* following Hemleben et al. (1989). The 7 sequences corresponding to the right-coiled morphotype of *Neogloboquadrina pachyderma* were renamed *Neogloboquadrina incompta*. All taxonomic reassignments were checked by sequence

similarity analyses to the members of the new group. Next, we attempted to resolve the attribution of sequences with unresolved taxonomy and searched manually for obviously misattributed sequences. This refers to sequences which are highly divergent from other members of their group but identical to sequences of other well resolved taxa. Overall, these first steps of manual curation led to taxonomic reassignment of 124 sequences. All corrections and their justification are documented in the Supplementary Material 1.

Molecular taxonomy

In order to preserve the information on the attribution of 18S rDNA sequences to genetic types (potential cryptic species), we harmonized the existing attributions at this level for species where extensive surveys have been carried out and published. A total of 1,356 sequences downloaded from NCBI were associated with a genetic type label, which was always retained. In addition, 19 sequences labelled as *Globigerinoides ruber*, 15 as *Globigerinoides sacculifer*, 36 as *Globigerinita glutinata*, 6 as *Globigerinita uvula*, 9 as *Globorotalia inflata*, 10 as *Neogloboquadrina incompta*, 6 as *Neogloboquadrina pachyderma,* 5 as *Orbulina universa,* 5 as *Pulleniatina obliquiloculata*, 30 as *Hastigerina pelagica* and 32 as *Globigerinella siphonifera* have been analyzed after their first release in the public domain by Aurahs et al. (2009), Ujiié et al. (2012), Weiner et al. (2012, 2014) and André et al. (2013, 2014), and were attributed to a genetic type by these authors. These attributions differ from those in the NCBI label, but were retained in the PFR² database. In case of multiple attributions of the same sequence to different genetic types by several authors, we retained the molecular taxonomy that was based on the study presenting the most resolved and comprehensive attribution. In addition, 877 unpublished sequences belonging to *Orbulina universa, Globigerina bulloides*, *Neogloboquadrina incompta*, *Neogoboquadrina dutertrei*, *Neogloboquadrina pachyderma,* and *Turborotalita quinqueloba*

received a genotypic attribution following de Vargas et al. (1999) and Darling et al. (2004, 2006, 2007, 2008). Most of these sequences have been produced and identified within earlier studies, but were not originally deposited on NCBI. Their PFR² genotypic assignment is therefore entirely consistent with the attribution of the representative sequences of the same genetic type that were deposited on NCBI.

PFR² final taxonomic framework

As a result of the first manual curation and annotation to the level of genetic type, the original 3,532 18S rDNA sequences were re-assigned to 33 species names and 2,276 sequences were annotated to the level of genetic types (Supplementary Material 1). For all sequences, we established a ranked taxonomy with six levels: 1- Morphogroup, 2-Genus, 3-Species, 4-Genetic type level 1, 5-Genetic type level 2, 6-Genetic type 3. For the "Morphogroup" rank we used the taxonomical framework of Hemleben et al. (1989), dividing the extant planktonic Foraminifera species into five clades based on the ultrastructure of the calcareous shell: Spinose, Nonspinose, Microperforate, Monolamellar and Non-spiral. The "Genus" and "Species" ranks follow the primary annotation as described above. For the "Genetic type level 1", "Genetic type level 2" and "Genetic type level 3" ranks, we used the hierarchical levels presented in the labels of the genetic types of *Globigerinoides ruber*, *Globigerinoides elongatus*, *Globigerinella siphonifera*, *Globigerinella calida*, *Hastigerina pelagica*, *Globigerina bulloides*, *Neogloboquadrina dutertrei*, *Pulleniatina obliquiloculata* and *Turborotalita quinqueloba*. Genetic type attributions lacking hierarchical structure were reported in the rank "Genetic type level 1". After this step, the Primary Reference Database (Figure 2) of 3,532 sequences contained 113 different taxonomic paths (Supplementary Material 1).

Sequences partitioning into conserved and variable regions

Because PFR² is a resource not only for taxonomic assignment but also for ecological and biogeographical studies, all planktonic Foraminiferal 18S rDNA sequences were included irrespective of length, as long as they contained taxonomically relevant information. As a result, the length of the sequences included in the annotated primary database ranges between 33 and 3,412 bp. To evaluate their coverage and information content, all sequences were manually aligned using Seaview 4 (Gouy et al., 2010) to the borders of each variable region of the 18S rDNA fragment. The positions of the borders were determined according to the SSU rDNA secondary structure of the monothalamous Foraminifera *Micrometula hyalostera* presented by Pawlowski and Lecroq (2010), except for the region 37/f where a strict homology was difficult to establish for all sequences. Instead, we defined the end of this region by the occurrence of a pattern homologous to the series of nucleotides "CUUUCACAUGA" located at the 3' end of Helix 37. We also noticed that the short conserved fragment located between the variable regions 45/e and 47/f was difficult to identify across all sequences. We thus merged the regions 45/e, 46 and 47/f into a single region that we named 45E-47F (Table1). As a result, the position and length of six conserved (32-37, 37-41, 39-43, 44-45, 47-49, 50) and five variable (37F, 41F, 43E, 45E-47F, 49E) regions were identified for all sequences (Figure 2). The remaining part of the 18S rDNA sequence, only present in sequences EU199447, EU199448 and EU199449 and located before the motive "AAGGGCACCACAAGA" has not been analyzed in this way. All regions fully covered in a sequence and containing sequence motives observed at least twice in the whole dataset were labelled as "complete". Regions fully covered but containing a sequence motive that was observed only once in the whole dataset were labelled as "poor". This is because we consider sequencing/PCR errors as the most likely cause for the occurrence of such unique

sequence motives. We realize that using this procedure, even genuine unique sequences may be discarded from the analysis, but this would be the case only if such sequences deviated in all regions. In all other cases, the regions were labelled as “partial” when only a part of the region was present or “not available” if they did not contain any fragment of the sequence. As a result we obtain the Partitioned Primary Reference Database (Figure 2). The coverage of each individual region in the Partitioned Primary Reference Database is given in Supplementary Material 1, and all sequence partitions are given in Supplementary Material 2.

Semi-automated iterative curation pipeline for optimal taxonomic assignment

The consistency of taxonomic assignments within the annotated database of partitioned sequences was assessed using a semi-automated process (Figure 2 and 3). All “complete” regions of sequences with the same taxonomic assignment at the morphospecies level were automatically aligned using global pairwise alignment (Needleman & Wunsch 1970), as implemented in the software *needle* from the Emboss suite of bioinformatics tools (Rice et al., 2000). To detect annotation inconsistencies, mean pairwise similarities were computed for each “complete” region of each sequence against all other sequences with the same taxonomic assignment from the finest annotation level “Genetic type level 3” to the rank “Species level”. Results are provided in Supplementary Material 1 and were visualized using R (R Development Core Team, 2014) and the ggplot2 library (Wickham, 2009). The resulting plots are given in Supplementary Material 3. If all annotations are consistent and there is no variation within taxa, each sequence within the analyzed taxon should only find an exact match and the mean pairwise similarity for that taxon should be 1. However, there are several reasons why the mean pairwise similarity within a taxon may be lower. First, if a sequence has been assigned the wrong name, its similarity to all other sequences labelled with that name will be low and the resulting mean

pairwise similarity decreases. Second, if a sequence has been assigned to the correct taxon, but the taxon comprises multiple sequence motives, that sequence will find a perfect match within the taxon but the mean pairwise similarity may also be lower than 1.

In order to deconvolve the different sources of sequence variability within taxa, we followed a three-step iterative approach, which was repeated for each of the 11 ‘complete’ regions of the analyzed SSU rDNA fragment. First, we considered the distribution of mean pairwise similarities for all sequences within each region assigned to one taxon at the finest rank of “Genetic type level 3”. Assuming that misidentifications are rare and result in large pairwise distances, we manually searched for sequences whose mean pairwise similarity deviates substantially from the rest of the sequences within the taxon. Such sequences were initially “invalidated”, whereas all other sequences analyzed at this level were “validated”. We then repeated the same procedure for the higher ranks of “Genetic type level 2”, “Genetic type level 1” and at the “Species level”, always starting with the full database (Figure 2 and 3A). Thus, at each level, we expected a misidentified sequence to have a lower pairwise similarity from the mean than any pairwise similarity between correctly assigned sequences (Figure 3B). This procedure had to be repeated for every rank, because not all sequences in the database are assigned to all ranks. Once “validated”, sequences cannot be “invalidated” during analyses of higher rank taxa, because they represent known variability within that taxon. In taxa where all sequences within a region show low mean pairwise similarities all attributions are initially invalidated (this would be typically the case for a “wastebasket taxa”, Figure 3C).

In the second step, all sequences invalidated during step 1 were reconsidered based on their pairwise similarities with ‘validated’ sequences from the same region. The main goal of the curated taxonomy being to achieve correct taxonomic assignment at the species level, the

pairwise comparison was carried out at this rank. If the best match is a ‘validated’ sequence with the same initial species attribution as the invalidated sequence, this sequence is “validated” at the species level and its assignment at the level of genetic type is then deleted. Such a situation can only occur when the sequence was initially assigned to the wrong genetic type within the correct species. If the pairwise comparisons of all regions analyzed match sequences with different but consistent species attributions than the invalidated sequence, the sequence is reattributed to that species. If the pairwise comparisons indicate that the analyzed sequence has no close relative in the validated part of the database, the initial attribution is retained, provided that the initial attribution is not yet in the validated dataset. This case occurs when all sequences of one species have been initially invalidated because the same species name was associated with highly divergent sequences. When the sequence has no close relative but its initial attribution is represented in the validated part of the dataset, the initial attribution is discarded and the sequence receives an artificial attribution derived from the nearest higher rank that matches the pairwise comparisons. In all cases, the erroneous attributions are replaced by the corrected ones in the database (Figure 2, Supplementary Material 1) and in the third step, sequences that received new attributions were reanalyzed as described in step 1. If inconsistencies in the distribution of mean pairwise similarities remain, steps 2 and 3 are repeated until no inconsistency is observed.

As a final diagnosis, to evaluate the robustness and potential limitations of the curated taxonomy, we performed a leave-one-out BLAST analysis and a monophyly validation by NJ on long sequences. First, each individual sequence included in the first version of PFR² was blasted against the remaining part of the database including n-1 sequences using SWIPE (Rognes, 2011). The sequences among the “n-1 PFR² database” returning the highest score were retrieved and

312their taxonomic attribution compared to the one of the blasted sequence (Supplementary Material
3131). Second, we retrieved all sequences covering the 5 variable and 6 conserved regions and
314divided them according to their assignment to higher taxa (here simplified by the morphogroups
315Monolamellar, Non-Spinose, Spinose and Microperforates + Benthic). Each subset was
316automatically aligned using MAFFT v.7 (Katoh et al., 2013) and the subsequent alignments were
317trimmed off on the edge to conserve only homologous fragments. For each alignment, a
318phylogenetic tree was inferred using a Neighbor-Joining approach with Juke and Cantor distance
319while taking into account gap sites as implemented in SEAVIEW 4 (Supplementary Material 4)
320with 100 pseudo-replicates. The scripts used to perform the different curation steps are available
321as Supplementary Material 5.

322**Results**

323Of the 3,532 planktonic Foraminiferal 18S rDNA partial sequences analyzed, 3,347 contained at
324least one gene region that was considered “complete” and could be subjected to the curation
325process. The remaining 185 sequences included 33 singletons (rare motives or poor quality
326sequences) and 152 sequences that were too short to cover at least one region (Supplementary
327Material 1). Amongst the 3,347 curated sequences, the taxonomic assignment of 84 was initially
328invalidated. Of these, 3 represent cases where the morphospecies attribution was correct, but the
329attribution to a genetic type was erroneous. In 46 cases, the invalidated sequences found a perfect
330match with a different taxon and thus their taxonomic assignment was changed. In all of these
331cases, the novel taxonomic assignment corresponded to a morphologically similar
332morphospecies, explaining the original misidentification of the sequenced specimen. In 14 cases,
333the original assignment was retained because the sequences did not find any match and their
334original attribution did not appear in the validated part of the dataset. All of these sequences were

labelled as *Hastigerinella digitata*. This species name had been entirely invalidated in the first step because of inconsistent use of the homonymous species name *Beella digitata*. Finally, 17 sequences received an unresolved artificial assignment. These represent six different sequence motives diverging substantially from all sequences in the validated part of the database and also between each other. Because the original attribution upon collection was obviously wrong, we could not reassign these sequences to the species level. In two cases, we could identify the most likely generic attribution, but four sequences are left with an entirely unresolved path. Finally, our procedure captured one sequence with a spelling error in its path and three sequences that appear to have been attributed correctly but represent small variants within species. After resolution of the 84 conflicts described above, the re-annotated dataset was subjected to a second round of the curation process for verification. All sequences were validated.

Having established an internally consistent taxonomic annotation for all 3,347 18S rDNA sequences from individual planktonic Foraminifera, we generated the *Planktonic Foraminiferal Ribosomal Reference* or PFR^2 database. Of the 3,347 sequences, 25 were shorter than 200 bp, and could not be deposited in NCBI (see Supplementary Material 1). The PFR^{2}1.0 database thus includes 3,322 reference sequences assigned to 32 species and 6 taxa with unresolved taxonomy (Figure 2), and contains 119 unique taxonomic paths when including all three levels of genetic types.

The leave-one-out BLAST evaluation applied on the first version of PFR² to assess its robustness returned an identical taxonomic path for 2,509 sequences. For 614 sequences, the BLAST-determined taxonomic paths were identical between the “morphogroup” and “species” rank but displayed a different resolution between the ranks “genetic type level 1” and “genetic type level 3”. This reflects a situation where some sequences belonging to one species are annotated to the

358level of a genetic type, whereas others are not. Finally, 19 sequences were assigned to the correct
359species but to a different genetic type. This illustrates the case of genetic types represented by
360only one sequence in the database, which were assigned to the closest genetic type within the
361same species by the leave-one-out procedure. Thus, 94.5 % of the sequences in the PFR^2 database
362find a nearest neighbor with a correct taxonomic assignment at the target level of species. For the
363remaining 180 sequences, the returned taxonomic path was inconsistent at the level of species. In
364two cases, the sequences were assigned to a sister species, which is morphologically and
365phylogenetically close (*Globorotalia ungulata* and *Globorotalia tumida*), reflecting insufficient
366coverage in the database for these species. Two cases involved singleton sequences with
367unresolved taxonomy, which find no obvious nearest neighbor. Finally, 176 cases of inconsistent
368identification refer to sequences of *Globigerinella calida* and *Globigerinella siphonifera*, whose
369species names have been used mutually interchangeably (Weiner et al., 2014) and the clade has
370been shown to be in need of a taxonomic revision (Weiner et al., 2015). The leave-one-out
371evaluation thus reveals excellent coverage of PFR^2 and confirms that the curated taxonomy is
372internally entirely consistent. To further confirm the validity of morphospeceis level taxonomy,
373we constructed NJ phylogenies for the four major clades including only the long sequences
374(Supplementary Material 4). This analysis confirmed the monophyly of all morphospecies,
375except the *Globigerinella calida/Globigerinella siphonifera* plexus. All clades were strongly
376supported except for the sister species *Globorotalia tumida* and *Globorotalia ungulata* and the
377monolamellar species *Hastigerina pelagica* and *Hastigerinella digitata*. In the first case, the poor
378support reflects the lack of differentiation between the two species in the conserved region of the
379gene which decreases the bootstrap score and the in the second case the extreme divergence of

380the two genetic lineage of *Hastigerina pelagica* renders the phylogenetic reconstruction difficult
381(Weiner et al., 2012).

382An analysis of the taxonomic annotations retained in PFR^2 reveals that the database covers at
383least 70-80% of the traditionally recognized planktonic Foraminiferal species in each clade. The
384species represented in PFR^2 constitute the dominant part of planktonic Foraminifera assemblages
385in the world oceans. Compared with a global database of census counts from surface sediments
386(MARGO database, Kucera et al., 2005), the species covered by PFR^2 account globally for >90%
387of shells larger than 150 μm found in surface sediments (Figure 4). In cold and temperate
388provinces, PFR^2 species account for almost the entire assemblages, while in warmer subtropical
389and tropical waters, only up to 4% of the sedimentary assemblages are not represented in PFR^2.
390Evidently, PFR^2 reference sequences cover most of the ecologically relevant portion of the
391morphological diversity and the taxa that are not yet represented in PFR^2 are small, rare or
392taxonomically obscure. It is possible that some of these taxa may correspond to the six sequences
393with unresolved taxonomy. If so, PFR^2 may be considered to cover up to 38 of the 47 recognized
394species.

395Finally, for each species present in PFR^2, we evaluated the ecological coverage of the global
396sampling effort (Figure 4). Morphospecies of planktonic Foraminifera are known to be
397distributed zonally across the world oceans, reflecting the latitudinal distribution of sea surface
398temperature (e.g., Bé and Tolderlund, 1971). A comparison between the temperature range of
399each species as indicated by their relative abundance in surface sediment samples (Kucera et al.,
4002005) and the temperatures measured at sampling localities shows that a large portion of the
401ecological range of the species is covered by the reference sequences in PFR^2 (Figure 4).

The PFR² web interface

To facilitate data download and comparative sequence analyses, PFR² has been implemented into a dedicated web interface, available at http://pfr2.sb-roscoff.fr. The website provides:

(1) a search/browse module, which allows the user to download parts of the database either by taxonomic rank (morphogroup name, genus name, species name), geographic region (e.g., North Atlantic, Mediterranean Sea, Indian Ocean) or collection (cruise name) ;
(2) a classical BLAST/Similarity module that facilitates identification of unknown sequences;
(3) a map module displaying the localities for all sequences present in PFR² and facilitating download of all data from each single locality;
(4) a download section with direct access to all data included in PFR². All sequences and sequence partitions are available in FASTA format and the metadata are available in a tabulated file.

Discussion

Comprehensive databases of ribosomal RNA sequences with curated taxonomy are available for Protists (Protist ribosomal reference database, *PR²*; Guillou et al., 2013) and for the major domains of life (SILVA, Yilmaz et al., 2013), and these databases also include sequences of planktonic Foraminifera. However, those databases are used mainly as benchmarks to annotate complex environmental datasets (e.g. de Vargas et al., 2015) at the level of morphological species. In contrast, PFR² has been designed and implemented in a way that facilitates other applications.

First, we note that because of the structural limitations, PFR² contains “only” 402 sequences of planktonic Foraminifera (Based on Released 203 of GenBank, October 2014), compared to PFR², which contains for now 3322 SSU rDNA sequences. Second, 2276 of the sequences

426present in PFR² have an assignation to the level of the genetic type and as far as possible, the
427sequences are associated with metadata related to the origin of each specimen and the conditions
428where it was collected, thus forming a basis for ecological modelling. Third, very importantly,
429using planktonic Foraminifera as a case study, we propose and implement an annotation scheme
430with unmatched accuracy and full tracking of changes. This is only possible because of the
431relatively "small" size of PFR² combined with high-level expert knowledge of their taxonomy.
432The fidelity of the annotations will facilitate a qualitatively entirely different level of analysis of
433eDNA libraries.

434For example, the design of PFR² allows to incorporate advances in classical and molecular
435taxonomy, particularly at the level of genetic types (e. g. André et al., 2014), which can be re-
436evaluated depending of the criteria used to delineate molecular OTUs. Further, by retaining
437information on clone attribution to specimens (vouchers), PFR² allows to evaluate intra-genomic
438polymorphism, which offers excellent opportunity to identify the phylogenetically relevant level
439of variability (Weber and Pawlowski, 2014). Finally, the modular structure of PFR^2 (i.e., its
440partitioning into variable and conserved regions) is particularly suitable for the evaluation of
441existing barcodes or the design of new barcoding systems needed to capture total or partial
442planktonic foraminiferal diversity within complex plankton assemblages. An examination of the
443length polymorphism in the 11 regions of the 18S rDNA fragment that have been aligned for all
444PFR^2 sequences reveals that next to the variable 37F region identified as a barcode for benthic
445Foraminifera (Pawlowski and Lecroq, 2010), several other regions would be suitable as targets
446for barcoding of planktonic Foraminifera (Figure 5).

447The main difference between PFR² and classical databases is in the association of sequence data
448with environmental and collection data. Such level of annotation is not feasible in large

databases, which have to rely on the completeness and level of detail of metadata provided in GenBank. The association of metadata to PFR² sequences facilitates an assessment of biogeography and ecology of genetic types (potential cryptic species). This is important for studies of evolutionary processes in the open ocean such as speciation and gene flow at basin scale, but also for paleoceanography, which exploits ecological preferences of planktonic Foraminfera species to reconstruct climate history of earth (e. g. Kucera et al., 2005). Modeling studies showed that the integration of cryptic diversity into paleoceanographic studies may improve their accuracy (Kucera and Darling, 2002; Morard et al., 2013). Together with the MARGO database (Kucera et al., 2005) which records the occurrence of morphospecies of planktonic Foraminifera in surface sediments and the CHRONOS/NEPTUNE database (Spencer-Cervato et al., 1994; http://www.chronos.org/) which records their occurrence through geological time, PFR² represents the cornerstone to connect genetic diversity to the fossil record in an entire group of pelagic protists.

Conclusion and perspectives

The PFR^2 database represents the first geographically and taxonomically comprehensive reference barcoding system for an entire group of pelagic protists. Therefore it constitutes a pivotal tool to investigate the diversity, ecology, biogeography, and evolution in planktonic Foraminifera as a model system for pelagic protists. In addition, the database constitutes an important resource allowing reinterpretation and refinement of the use of Foraminifera as markers for stratigraphy and paleoceanography. In particular, PFR^2 can be used to: (i) annotate and classify newly generated 18S rDNA sequences from single individuals; (ii) study the

biogeography of cryptic genetic types; (iii) design rank-specific primers and probes to target any group of planktonic Foraminifera in natural communities; (iv) assign accurate taxonomy to environmental sequences from metabarcoding or metagenomic datasets. This last point is particularly important. Future global metabarcoding of planktonic Foraminifera covering comprehensive spatio-temporal scales will likely reveal the full extent and complexity of species diversity and ecology in the group, serving as a model system for studies of the dynamics of the plankton and its interaction with the Earth system.

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Author contribution

KFD, CdV, YU, RM, TdG, AKFW, HS, MK, AA, MS participated in sample collection, CdV, MK, KFD, CMW, CJD, FQ, GE, TdG provided laboratory infrastructure, KFD, YU, RM, AKFW, AA, HS participated in laboratory work. FM and RM conceived and designed the bioinformatics pipeline, FM performed the computational work, SA built the website. RM wrote the manuscript with help from MK and CdV. All authors read, edited and approved the final manuscript.

Data Accessibility

Sequences, NCBI accession numbers and metadata are available in Supplementary Material 1 and 2 and on PFR² website at http://pfr2.sb-roscoff.fr. The custom scripts used to perform the curation procedure are available in Supplementary Material 5, the results of the curation process are given in Supplementary Material 1 and 2.

Figures

Figure 1

Sampling Map. Location of the 460 oceanic stations sampled over 20 years for single-cell genetic studies of planktonic Foraminifera. Each symbol corresponds to a scientific cruise or near shore collection site. Cruise names and dates of the collection expeditions are indicated in the legend. Grey shading shows ocean bathymetry.

Figure 2

Workflow to constitute PFR². In step "I" the sequences, metadata and taxonomic information are retrieved from public databases and literature or from the internal databases of the authors to constitute the Primary Reference Database. In step "II", the coverage of each sequence is evaluated by alignment with structural regions of the 18S RNA secondary structure derived for the species *Micrometula hyalostera* (Pawlowski and Lecroq, 2010). In step "III", the consistency of the annotation is checked from the most exclusive level of annotation "genetic type 3" until the species level (Phase 1) to detect annotation inconsistency (See Figure 3). Sequences with wrong annotation are invalidated, compared to the validated part of the dataset (Phase 2) and re-annotated depending on the best hit out of the valid dataset. The consistency of all annotations is then checked again following the same procedure as in Phase 1 (Phase 3), to ensure that no taxonomic inconsistency remains. In step IV, all sequences which have been subjected to the curation process are integrated in the Planktonic Foraminifera Ribosomal Reference database (PFR²). The results of all steps are given in Supplementary Material 1.

Figure 3

Annotation inconsistency detection. The procedure followed to identify annotation inconsistency is exemplified by three cases. Each graph represents variability in pairwise similarities observed across each region of all sequences sharing the same annotation level. The names of the taxon and annotation level are given above the plot with the number of sequences in parenthesis. Each vertical line represents one region with the variability represented as dot plot, the number of "complete" regions is given at the bottom of the line. The case "A" describes the annotation validation process starting from the most exclusive rank of "genetic type level 3" to the "species" rank. After the validation at one rank level, the sequences with valid annotation are merged in a taxonomic unit of a higher rank. This now includes multiple sequence motifs decreasing the level of identity in each region, leading to a high variability in higher ranks. Case "B" represents the occurrence of obvious outliers at the species level, which are invalidated. Case "C" represents the co-occurrence of divergent sequences under the same taxonomic attribution, which are consequently all invalidated. The dot plots for all ranks can be found in Supplementary Material 4 and the pairwise similarities calculated for each taxonomic level are given in Supplementary Material 1.

Figure 4

Taxonomic and ecological coverage of PFR². For each morphogroup (Spinose, Non-Spinose, Microperforates, Monolamellar and Non-Spiral) the number of species included in PFR² is given in the filled bar while the number of species not present is indicated in the adjacent open bar. The relative abundance in the sediments of each species included in PFR² is given in log value against mean Sea Surface Temperature (SST) at the sampling station. Relative abundances in sediments are derived from the MARGO database (Kucera et al., 2005) and the mean annual SST from the World Ocean Atlas (Locarnini, 2005). The grey dots highlight the mean annual SST at the location where the living planktonic foraminifera yielding sequences were sampled. The number of sequences available for each species as well as the number of taxonomic paths above the species level is shown next to the graphs. Also shown is the cumulative mean relative abundance in the sediments of all species included in PFR² plotted against the mean annual SST in discrete 1°C intervals. Vertical bars represent 95% confidence intervals for each 1°C bin.

Figure 5

Length polymorphism. Each rectangle represents the length polymorphism within each region of the analyzed 18S rDNA fragment across all resolved taxonomic units in PFR². The regions are based on the rRNA secondary structure and are named following Pawlowski and Lecroq (2010).

Supplementary Material.

Supplementary Material 1.

Information on all consecutive steps followed to constitute the PFR². All fields are explained in the file.

Supplementary Material 2

FASTA files of sequences used to build the PFR². FASTA files are provided for the full sequences and individual partitions.

Supplementary Material 3

Dot plots showing pairwise similarities for each taxonomic level. See Figure 3 for explanations of the content of the plots.

Supplementary Material 4

Neighbor-joining trees showing the monophyly of each morphospecies.

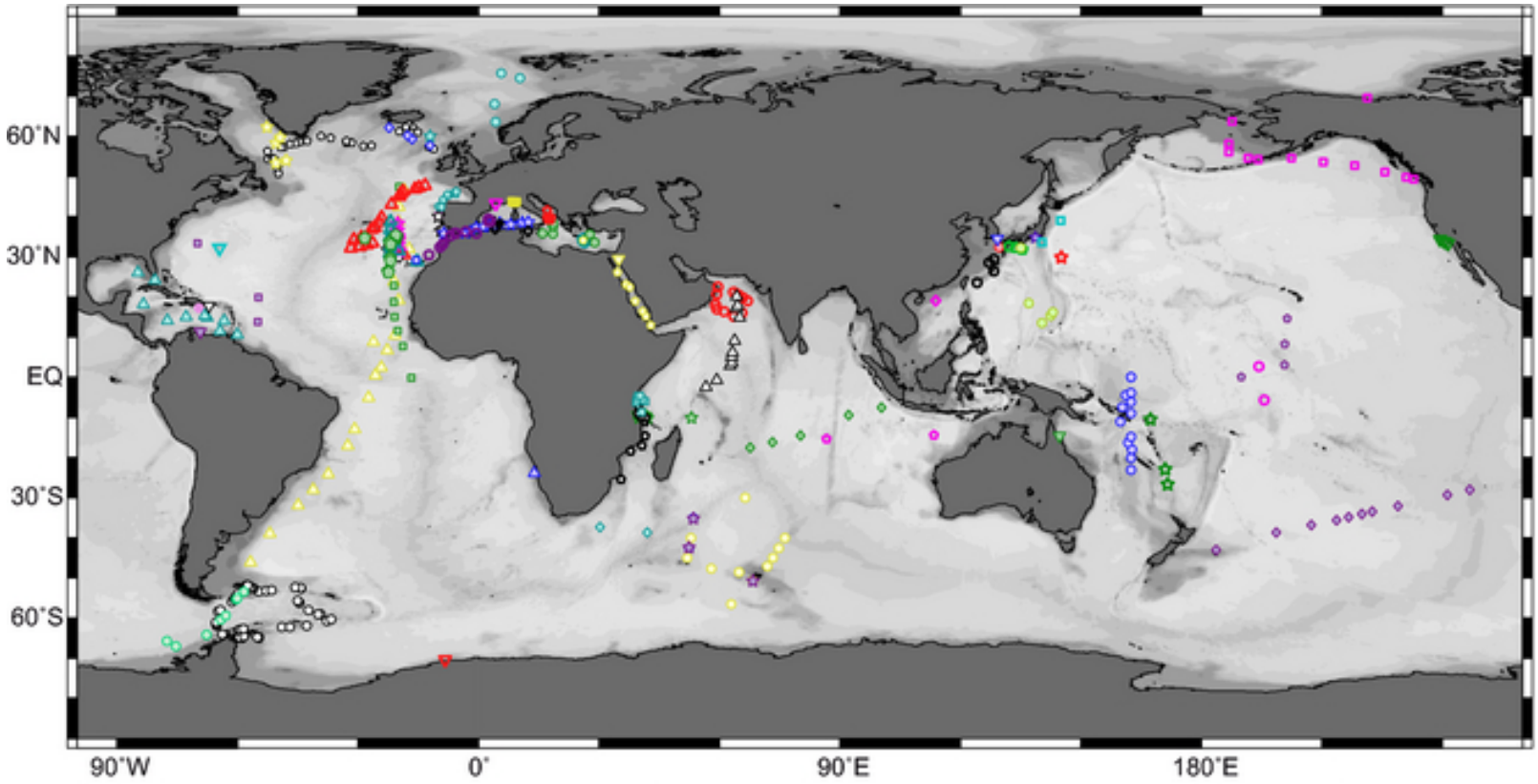
Supplementary Material 5

Custom scripts used to perform the different curation steps.

Table 1. Flanking conserved sequences of the five variable regions in planktonic foraminifera. The minimum and maximum length of each region are given as well as their coverage in the database (see details in the text)

Region	Specificity	Beginning	End	Min length	Max length	Not available	Partial	Poor	Complete
32–37	Eukaryotes	—	—	—	—	949	2583	0	0
37F	foraminifera	5′-GGAUUGACA	CUUUCACAUGA-3′	38	132	800	272	249	2211
37–41	Eukaryotes	—	—	68	72	547	403	138	2444
41F	foraminifera	5′-AAUUGCG	GCAACGAA-3′	58	322	349	346	282	2555
39–43	Eukaryotes	—	—	27	29	460	34	57	2981
43E	Eukaryotes	5′-CUUGUU	AACUAGAGGG-3′	33	195	401	263	265	2603
44–45	Eukaryotes	—	—	113	123	487	1288	136	1621
45E–47F	Eukaryotes–Forams	5′-CAGUGAG	GGUGGGG-3′	179	312	1660	187	386	1299
47–49	Eukaryotes	—	—	140	148	1827	425	152	1128
49E	Eukaryotes	5′-GUGAG	CGAACAG-3′	27	127	2251	130	125	1026
50	Eukaryotes	—	—	—	—	2389	1143	0	0

Fig 1



Scientific cruise

- Alis, GYRAFOR-A (Jun 2008)
- Charles Darwin, CD148 (July 2003)
- Charles Darwin, CD159 (July 2004)
- Discovery, D262 (Apr 2002)
- Discovery, D286 (Dec 2005, Jan 2006)
- Garcia del Cid, Iberia-Forams (Sept 2012)
- Hakuho-maru, KH04-2 (Jun-Jul 2009)
- Hakuho-maru, KH10-4 (Aug 2011)
- James Clark Ross, AMT-5 (Sept-Oct 1997)
- James Clark Ross, AMT-8 (Apr Jun 1999)
- James Clark Ross, JR 19 (Mar 1997)
- James Clark Ross, JR 48 (Feb-Mar, 2000)
- Maria S. Merian, MSM09-2 (Aug-Sep 2008)
- Maria S. Merian, MSM15-5 (Jul 2010)
- Marion Dufresne, GYRAFOR-B (Jul-Aug 2007)
- Marion Dufresne, OISO2011 (Jan 2011)
- Marion Dufresne, OISO-4 (Jan-Feb 2000)
- Melville, Melville (June 2003)
- Meteor, M37-2a (Apr 1997)
- Meteor, M69-1 (Aug 2006)
- Meteor, M71-2 (Dec 2006 - Jan 2007)
- Meteor, M71-3 (Jan-Feb 2007)
- Meteor, M74-1a (Sep 2007)
- Meteor, M74-1b (Sep-Oct 2007)
- Meteor, M75-2 (Feb 2008)
- Meteor, M78-1 (Feb-March 2008)
- Mirai, MR02-K01 (Jan 2002)
- Mirai, MR10-06 (Nov 2010)
- Pelagia, 64PE303 - GLOW (Feb-Mar 2009)
- Pelagia, 65PE304 (Mar 2009)
- Polarstern, Arktis XV/1-2 (Jun 1999)
- Poseidon, P247 (Jan 1999)
- Poseidon, P283-2 (Feb-Mar 2002)
- Poseidon, P308 (Mar 2004)
- Poseidon, P321 (May 2005)
- Poseidon, P334 (Mar-Apr 2006)
- Poseidon, P349 (Apr 2007)
- Poseidon, P411 (Apr 2011)
- Poseidon, P413 (May 2011)
- Professor Logachev, Denmark Strait (Sept 1997)
- Roger Revelle, Revelle (Jan 2001)
- Ron Brown, CMarZ (April 2006)
- Sarmiento de Gamboa, FORCLIM-7 (Apr 2009)
- Seriora, Amakusa (Sep 2009)
- Sir Wilfried Laurier, CCGS (July 2007)
- Sonne, SO-221 (May 2012)
- Sonne, SO-226 (Mar 2013)
- Tansei-maru, KT02-15 (Oct 2002)
- Tansei-maru, KT07-14 (Jun 2007)
- Tansei-maru, KT06-11 (Jun 2006)
- Tansei-maru, KT06-30 (Nov 2006)
- Welwitschia, NatMIRC (Nov 2001)

Near-Shore Collection

- Bermuda (Apr 1996)
- Curaçao (Feb 1993)
- Eilat (Feb 2011)
- Ekstrom Ice Shelf-Atka Bay (Jan 2001)
- Lizard Island, GBR (Aug 1993, Sep 1997)
- Puerto Rico (Mar 1995)
- Santa Barbara Chanel (Feb 1998, Jan-Sep 1999)
- Tsugaru strait
- Villefranche sur Mer (Dec 1995)

Fig 2

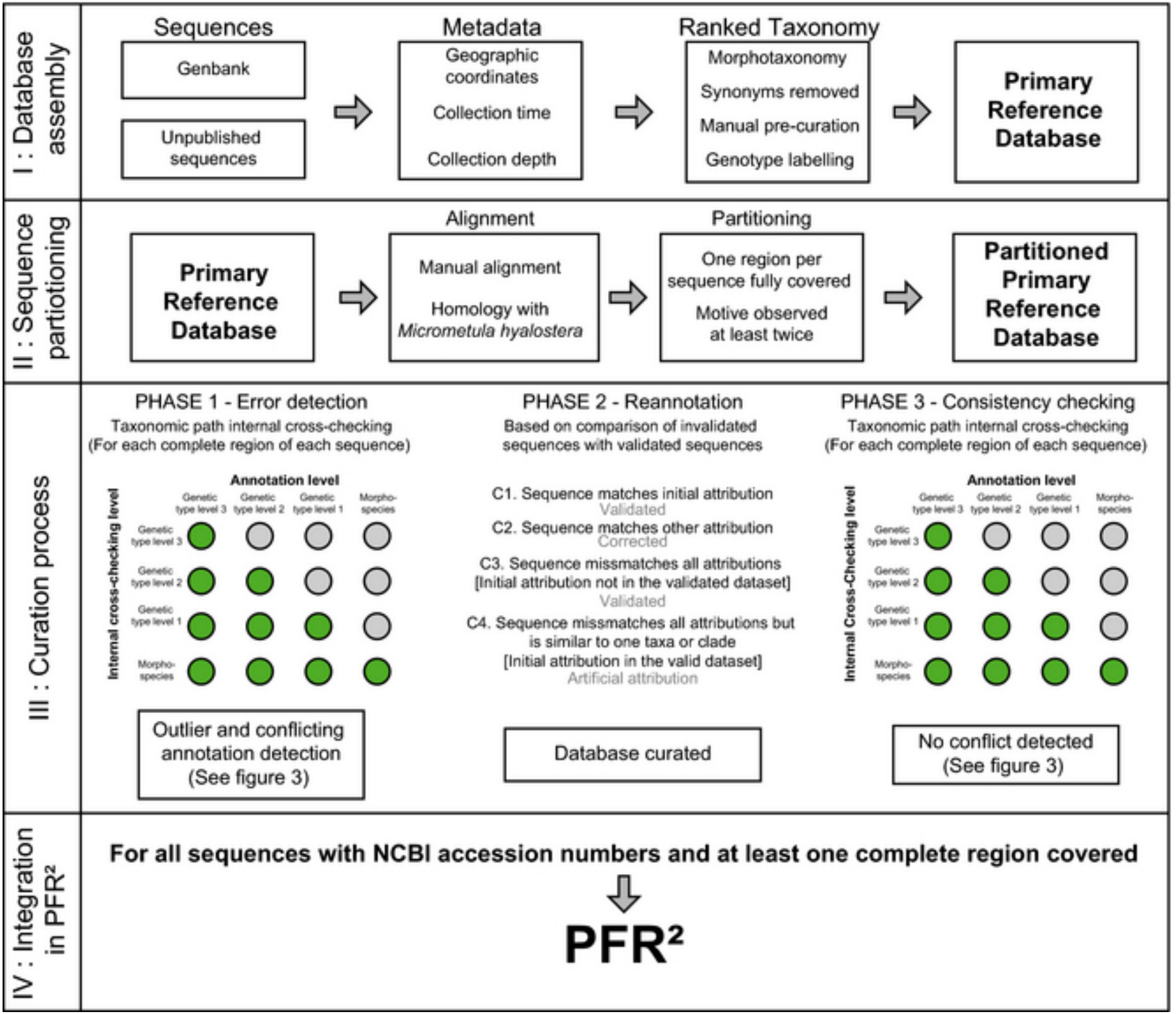

I : Database assembly
Sequences
Genbank
Unpublished sequences
Metadata
Geographic coordinates
Collection time
Collection depth
Ranked Taxonomy
Morphotaxonomy
Synonyms removed
Manual pre-curation
Genotype labelling
Primary Reference Database
II : Sequence partiotioning
Primary Reference Database
Alignment
Manual alignment
Homology with Micrometula hyalostera
Partitioning
One region per sequence fully covered
Motive observed at least twice
Partitioned Primary Reference Database
III : Curation process
PHASE 1 - Error detection
Taxonomic path internal cross-checking
(For each complete region of each sequence)
Annotation level
Genetic type level 3
Genetic type level 2
Genetic type level 1
Morpho-species
Internal cross-checking level
Outlier and conflicting annotation detection (See figure 3)
PHASE 2 - Reannotation
Based on comparison of invalidated sequences with validated sequences
C1. Sequence matches initial attribution
Validated
C2. Sequence matches other attribution
Corrected
C3. Sequence missmatches all attributions
[Initial attribution not in the validated dataset]
Validated
C4. Sequence missmatches all attributions but is similar to one taxa or clade
[Initial attribution in the valid dataset]
Artificial attribution
Database curated
PHASE 3 - Consistency checking
Taxonomic path internal cross-checking
(For each complete region of each sequence)
Annotation level
Internal Cross-Checking level
No conflict detected (See figure 3)
IV : Integration in PFR²
For all sequences with NCBI accession numbers and at least one complete region covered
PFR²

Fig 3

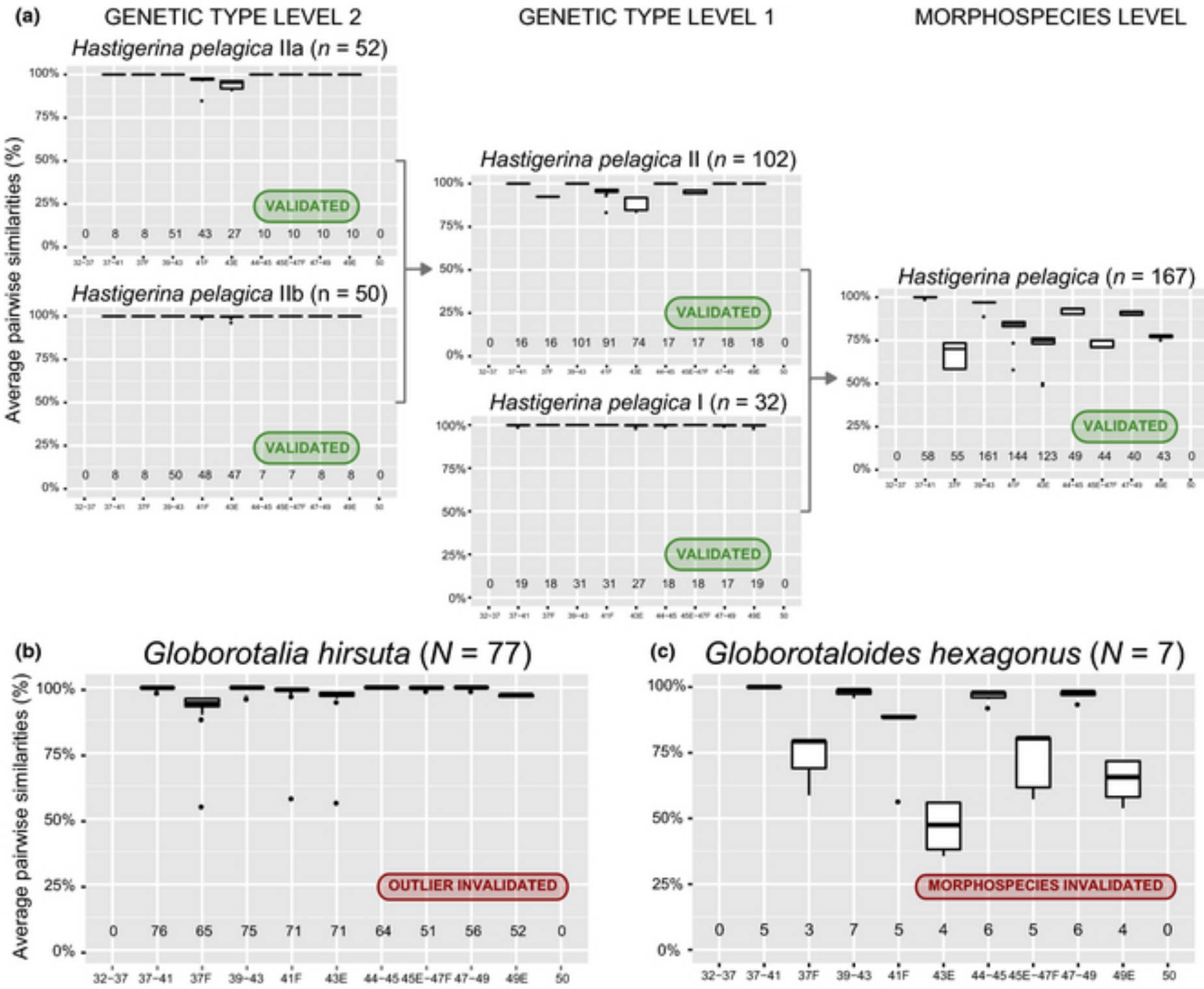

(a)
GENETIC TYPE LEVEL 2
GENETIC TYPE LEVEL 1
MORPHOSPECIES LEVEL
Hastigerina pelagica IIa (n = 52)
Hastigerina pelagica IIb (n = 50)
Hastigerina pelagica II (n = 102)
Hastigerina pelagica I (n = 32)
Hastigerina pelagica (n = 167)
Average pairwise similarities (%)
VALIDATED
0 8 8 51 43 27 10 10 10 10 0
0 8 8 50 48 47 7 7 8 8 0
0 16 16 101 91 74 17 17 18 18 0
0 19 18 31 31 27 18 18 17 19 0
0 58 55 161 144 123 49 44 40 43 0
(b)
Globorotalia hirsuta (N = 77)
OUTLIER INVALIDATED
0 76 65 75 71 71 64 51 56 52 0
(c)
Globorotaloides hexagonus (N = 7)
MORPHOSPECIES INVALIDATED
0 5 3 7 5 4 6 5 6 4 0
100%
75%
50%
25%
0%
32-37 37-41 37F 39-43 41F 43E 44-45 45E-47F 47-49 49E 50

Fig 4

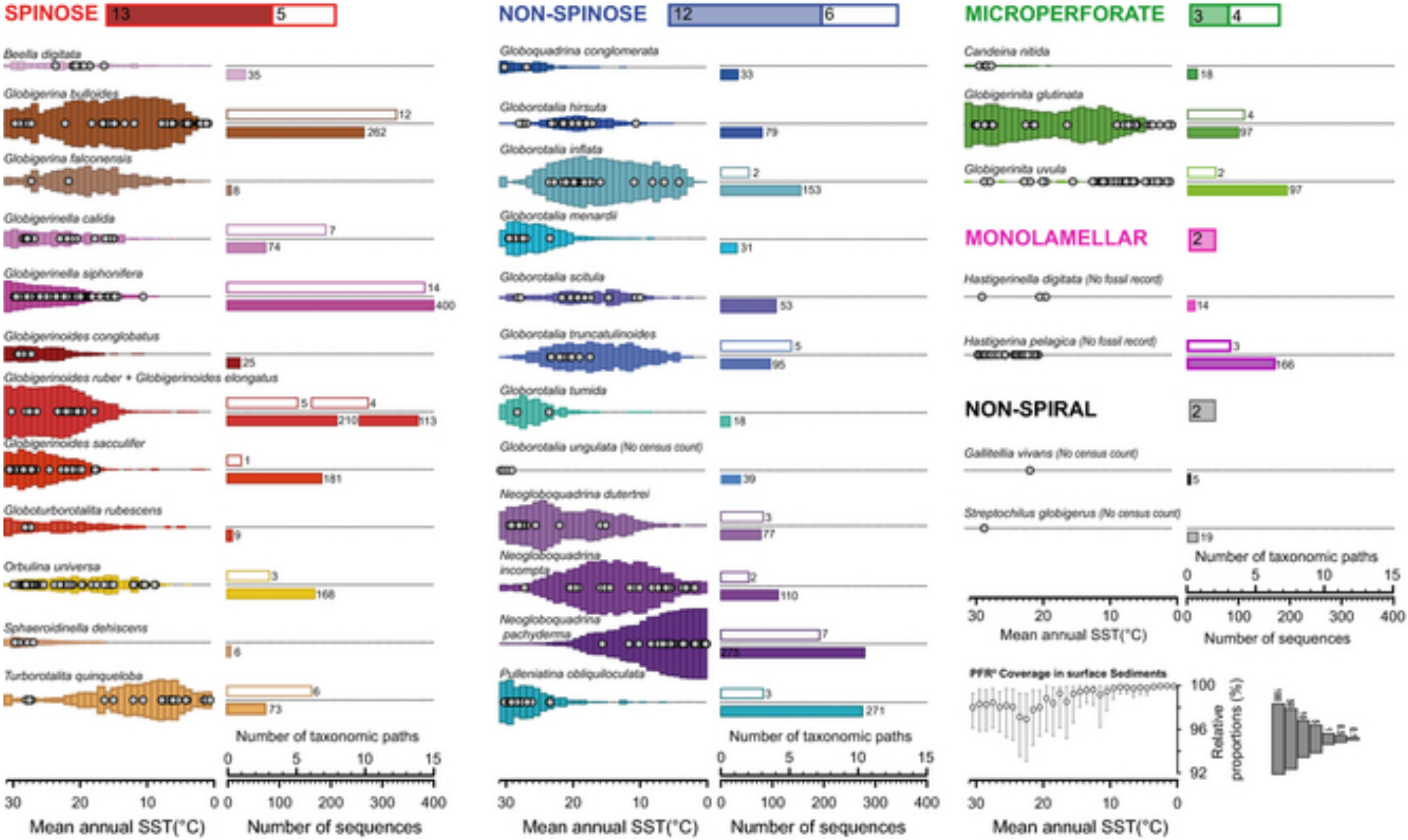
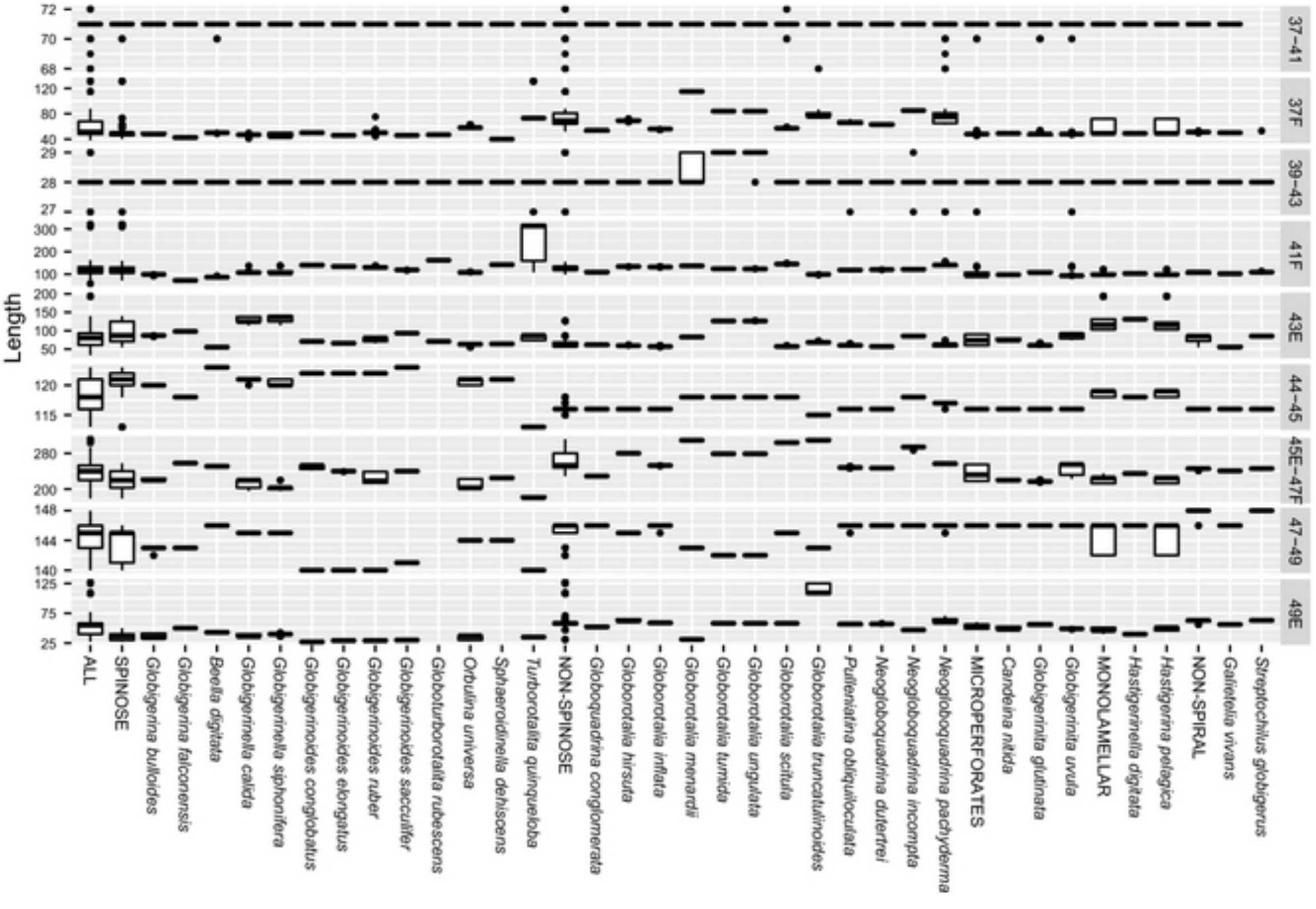

SPINOSE 13 5
NON-SPINOSE 12 6
MICROPERFORATE 3 4
MONOLAMELLAR 2
NON-SPIRAL 2
Beella digitata
Globigerina bulloides
Globigerina falconensis
Globigerinella calida
Globigerinella siphonifera
Globigerinoides conglobatus
Globigerinoides ruber + Globigerinoides elongatus
Globigerinoides sacculifer
Globoturborotalita rubescens
Orbulina universa
Sphaeroidinella dehiscens
Turborotalita quinqueloba
Globoquadrina conglomerata
Globorotalia hirsuta
Globorotalia inflata
Globorotalia menardii
Globorotalia scitula
Globorotalia truncatulinoides
Globorotalia tumida
Globorotalia ungulata (No census count)
Neogloboquadrina dutertrei
Neogloboquadrina incompta
Neogloboquadrina pachyderma
Pulleniatina obliquiloculata
Candeina nitida
Globigerinita glutinata
Globigerinita uvula
Hastigerinella digitata (No fossil record)
Hastigerina pelagica (No fossil record)
Gallitellia vivans (No census count)
Streptochilus globigerus (No census count)
Number of taxonomic paths
Mean annual SST(°C)
Number of sequences
PFR² Coverage in surface Sediments
Relative proportions (%)

Fig 5


37–41
37F
39–43
41F
43E
44–45
45E–47F
47–49
49E
Length
72
70
68
120
80
40
29
28
27
300
200
100
200
150
100
50
120
115
280
200
148
144
140
125
75
25
ALL
SPINOSE
Globigerina bulloides
Globigerina falconensis
Beella digitata
Globigerinella calida
Globigerinella siphonifera
Globigerinoides conglobatus
Globigerinoides elongatus
Globigerinoides ruber
Globigerinoides sacculifer
Globoturborotalita rubescens
Orbulina universa
Sphaeroidinella dehiscens
Turborotalita quinqueloba
NON-SPINOSE
Globoquadrina conglomerata
Globorotalia hirsuta
Globorotalia inflata
Globorotalia menardii
Globorotalia scitula
Globorotalia truncatulinoides
Globorotalia tumida
Globorotalia ungulata
Neogloboquadrina dutertrei
Neogloboquadrina incompta
Neogloboquadrina pachyderma
Pulleniatina obliquiloculata
MICROPERFORATES
Candeina nitida
Globigerinita glutinata
Globigerinita uvula
MONOLAMELLAR
Hastigerinella digitata
Hastigerina pelagica
NON-SPIRAL
Gallitellia vivans
Streptochilus globigerus