Journal of Earth Science, *Vol. XX*, *No. XX*, *p. 1−11*, *Online 2022* *ISSN 1674-487X*
Printed in China
https://doi.org/10.1007/s12583-022-1609-1

Proteome-Based Clustering Approaches Reveal Phylogenetic Insights into *Amphistegina*

Marleen Stuhr*[1], Bernhard Blank-Landeshammer[2, 3], Achim Meyer[1], Vera Baumeister[4], Jörg Rahnenführer[5], Albert Sickmann[2, 6, 7], Hildegard Westphal[1, 8, 9]

1. *Leibniz Centre for Tropical Marine Research* (*ZMT*), *Bremen*, *Germany*
2. *Leibniz-Institut für Analytische Wissenschaften -ISAS- e.V.*, *Dortmund*, *Germany*
3. *Austrian Competence Center for Feed and Food Quality*, *Safety and Innovation*, *Wels*, *Austria*
4. *Chrestos Concept GmbH & Co KG*, *Essen*, *Germany*
5. *Department of Statistics*, *TU Dortmund University*, *Dortmund*, *Germany*
6. *Medizinisches Proteom-Center* (*MPC*), *Ruhr-Universität Bochum*, *Bochum*, *Germany*
7. *Department of Chemistry*, *University of Aberdeen*, *Old Aberdeen*, *Scotland*, *UK*
8. *Department of Geosciences*, *Bremen University*, *Bremen*, *Germany*
9. *King Abdullah University of Science and Technology* (*KAUST*), *Thuwal*, *Saudi Arabia*

Marleen Stuhr: https://orcid.org/0000-0000-0001-9155-9464

ABSTRACT: Foraminifera are highly diverse and have a long evolutionary history. As key bioindicators, their phylogenetic schemes are of great importance for paleogeographic applications, but may be hard to recognize correctly. The phylogenetic relationships within the prominent genus *Amphistegina* are still uncertain. Molecular studies on *Amphistegina* have so far only focused on genetic diversity within single species and suggested a cryptic diversity that demands for further investigations. Besides molecular sequencing-based approaches, different mass spectrometry-based proteomics approaches are increasingly used to give insights into the relationship between samples and organisms, especially as these do not require reference databases. To better understand the relationship of amphisteginids and test different proteomics-based approaches we applied *de novo* peptide sequencing and similarity clustering to several populations of *Amphistegina lobifera*, *A. lessonii* and *A. gibbosa*. We also analyzed the dominant photosymbiont community to study their influence on holobiont proteomes. Our analyses indicate that especially *de novo* peptide sequencing allows to reconstruct the relationship among foraminiferal holobionts, although the detected separation of *A. gibbosa* from *A. lessonii* and *A. lobifera* may be partly influenced by their different photosymbiont types. The resulting dendrograms reflect the separation in two lineages previously suggested and provide a basis for future studies.

KEY WORDS: large benthic foraminifera, *de novo* peptide sequencing, tandem mass spectra clustering, LC-MS/MS runs, proteomics, symbiont diversity, phylogeography, Fragilariales.

0 INTRODUCTION

Foraminifera are a highly diverse group of unicellular protists that have a long evolutionary history. They represent key bioindicators in paleontological and environmental studies, but may be hard to differentiate and identify correctly due to their small size and high phenotypic plasticity (Narayan et al., 2022; Macher et al., 2021; Prazeres et al., 2020a; Bhatt and Trivedi, 2018; Hallock et al., 2003). Reconstructions of their phylogenetic and evolutionary schemes are of great importance for paleogeographic applications. Yet, as for decades these were solely based on diagnostic characters of their morphology, modern and fossil foraminiferal taxa have an extensive history of taxonomic revisions (Pawlowski and Holzmann, 2008; Haynes, 1992; Loeblich and Tappan, 1988; Loeblich et al., 1957). The application of molecular tools to identify recent species and resolve their phylogenetic relationship has therefore in recent years become increasingly prominent and often revealed a high genetic diversity at the cryptic level (Bhatt and Trivedi, 2018; Pawlowski et al., 2014; Pawlowski and Lecroq, 2010; Pochon et al., 2006; Flakowski et al., 2005; Pawlowski and Holzmann, 2002). However, foraminifera have fast evolving ribosomal genes (Pawlowski and Holzmann, 2002) and a wide occurrence of intragenomic variability (Weber and Pawlowski, 2014). Genetic markers do not always yield satisfying results, and recent studies tend to combine molecular with morphological approaches (Macher et al., 2021; Morard et al., 2019; Pawlowski et al., 2013). Yet, the phylogenetic relationships within

*Corresponding author: marleen.stuhr@leibniz-zmt.de

Manuscript received November 9, 2021.
Manuscript accepted December 30, 2021.

Stuhr Marleen, Blank-Landeshammer Bernhard, Meyer Achim, Baumeister Vera, Rahnenführer Jörg, Sickmann Albert,Westphal Hildegard , 2022. Proteome-Based Clustering Approaches Reveal Phylogenetic Insights into *Amphistegina*. *Journal of Earth Science*, XX(XX): 1–11. https://doi.org/10.1007/s12583-022-1609-1. http://en.earth-science.net

one of the most prominent and abundant foraminiferal genera, *Amphistegina*, have not been sufficiently revisited using modern methodologies, so uncertainties still exist (Renema, 2018; Langer and Hottinger, 2000).

Amphistegina is the only extant genus of the family Amphisteginidae and belongs to the group of large benthic foraminifera (LBF), which live in symbiosis with photosynthesizing microalgae (Prazeres et al., 2021; Boudagher-Fadel, 2018; Renema, 2018; Lee, 2006; Leutenegger, 1977). It has a circum-global distribution and can be found in all (sub-)tropical oceans, and it also has one of the widest latitudinal extensions among the LBF, often dominating assemblages of shallow marine ecosystems from one to more than 100 m depth (Renema, 2019; Weinmann et al., 2013; Hallock, 1985; Hansen and Buchard, 1977). Moreover, *Amphistegina* spp. are currently invading into new higher latitudinal habitats such as the Mediterranean Sea (Mouanga and Langer, 2014; Langer et al., 2013; Triantaphyllou et al., 2009) and are predicted to further extend their distribution range with climate change (Guastella et al., 2019; Weinmann et al., 2013). Amphisteginids can locally occur in very high densities and thereby contribute significantly to coastal carbonate sediment production around several atolls and reef islands, especially in the western Pacific (Doo et al., 2012; Renema, 2003; Langer et al., 1997). *Amphistegina* species are also relatively easy to culture and have been found to bleach in response to environmental changes associated to climate change (Schmidt et al., 2011; Hallock, 1999; Hallock et al., 1993, 1986), which has on the one hand led to their increased application as bioindicators for coral reef condition and on the other hand to intense laboratory experiments using them for e.g. physiological or calcification studies (Stuhr et al., 2017; Prazeres et al., 2015; Bentov et al., 2009; Talge and Hallock, 1995; Hallock et al., 1986; ter Kuile and Erez, 1984; Hallock and Hansen, 1979; Zmiri et al., 1974). Many modern approaches are first tested and applied on these foraminifera, especially *A. lobifera* (Titelboim et al., 2021; Ross and Hallock, 2018; Stuhr et al., 2018a; Glas et al., 2012; Prazeres et al., 2011). Thus, they can be regarded as a model organism for modern diatom-bearing perforate hyaline LBF.

The genus *Amphistegina* was first described by d'Orbigny (d'Orbigny, 1826) and then Ehrenberg (Ehrenberg, 1838). Current studies suggest that at least 11 extinct relatives (Larsen, 1978) have been widely distributed since the Eocene and throughout the Miocene (Todd, 1976), and it contains a minimum of six to seven commonly described recent morphospecies (Förderer and Langer, 2019; Renema, 2018; Debenay, 2013; Murray, 2006; Langer and Hottinger, 2000; Hottinger et al., 1993; Larsen, 1978). These are generally grouped in two morpho-groups, with lineage I (Larsen, 1978), or the *A. radiata*-group (Renema, 2018) containing the more symmetrical forms *A. radiata* and *A. papillosa*, as well as, according to Larsen (1978), also *A. gibbosa* (shown in Fig. 3) and *A. bicirculata*. Lineage II or the *A. lessonii*-group has widely spaced curved septa and is therefore more asymmertrical in axial view (Renema, 2018). It contains *A. lessonii*, *A. lobifera* (both shown in Fig. 3) and, according to e.g. Renema (2018), also *A. bicirculata* and *A. quoi*, of which the latter is rarely recognized by others. *A. gibbosa* is not described by Renema (2018), however, briefly mentioned as "western Atlantic sister species of *A. lobifera* and *A. lessonii*". Hohenegger (2014) distinguishes between the *A. lessonii* kinship and the *A. radiata* kinship like Renema (2018), though he does not mention the existence of *A. gibbosa*. Besides these, also *A. madagascariensis* is occasionally described (Förderer and Langer, 2019; Murray, 2006; Zmiri et al., 1974), but assumed to be a variety of *A. lessonii* (Förderer and Langer, 2019).

Most of these extant species are widely distributed throughout the Indo-Pacific and show a depth-related niche partitioning that is related to shape trends (Hohenegger, 2004; Hallock and Hansen, 1979), but partly overlaps, resulting in locally mixed assemblages (more details on species descriptions and distributions can be found in Förderer and Langer, 2019; Renema, 2018; Debenay, 2013; Murray, 2006; Loeblich and Tappan, 1994, 1988; Hottinger et al., 1993; Larsen, 1978; Hansen and Buchard, 1977). *A. gibbosa*, in contrast, is generally assumed to be the only *Amphistegina* species present in the Atlantic, specifically the Caribbean region. This is however controversial (Hallock, 1999) and some authors identified *Amphistegina* found along the coast of Brazil as *A. lessonii* instead (Alves and Júnior, 2020; Prazeres et al., 2012, 2011). In light of their ubiquitous appearance in shallow warm-water carbonate systems it is even more surprising that species boundaries and numbers remain widely uncertain (Renema, 2018) and that therefore a reassessment of their phylogenetic and evolutionary relationships is long overdue.

Molecular studies on *Amphistegina* have so far only focused on genetic diversity within single species. Schmidt et al. (2016) showed a distinction between *A. lobifera* from Israel (Mediterranean and Red Sea) and Australia (Great Barrier Reef). This was confirmed by a detailed study of Prazeres et al. (2020b), who differentiated between six genotypes of *A. lobifera* that could indeed represent at least four separate species and have distinct distribution ranges structured along a longitudinal gradient. Subsequently, Ramadhani et al. (2022, under review) determined three distinct genetic types of *A. lessonii* across its global range, with several of these co-occurring in the centre of its distribution. This further suggests a high cryptic diversity within the genus and demands for broader investigations of these prolific foraminifera using modern approaches.

Besides RNA and DNA sequencing-based molecular approaches, different mass spectrometry (MS)-based proteomics approaches are increasingly used to give insights into the relationship between samples and organisms (Aebersold and Mann, 2003). The most common method is to first generate short peptides and determine their mass-to-charge (m/z) ratios by MS. These measured mass spectra are then compared to a given database to find the matching peptide sequences and in turn identify the corresponding proteins. However, this method fails to recognize peptides that do not have existing sequence data in the given database, or for organisms that do not have a suitable reference database at all (MacCoss, 2005). This has limited the application of proteomics methods largely to the analysis of model organisms. However, in recent years instrumental and methodological advances enabled the adaption of these methods to a wider range of samples (Heck and Neely, 2020). In cases where reference databases are unavailable, *de*

novo peptide sequencing, i. e., direct inference of peptide sequences from tandem mass spectra, is often the only option. Because this may have some limitations, a novel synergistic approach optimizes *de novo* peptide sequencing by combining the outcomes of several commonly available algorithms (Blank-Landeshammer et al., 2017). Another MS-based approach is to cluster similar peptide mass spectra by similarity, using different algorithms (Rieder et al., 2017b). These commonly used clustering approaches can be further improved by the DISMS2 filter, which allows to also consider precursor mass, precursor charge or retention time. By comparing thousands of peptide spectra, without *de novo* annotation or reference database, the similarities among sample proteomes are calculated and clustered, which may indicate their phylogenetic proximity (Rieder et al., 2017a).

To better understand the relationship of recent *Amphistegina* and at the same time test the different proteomics-based approaches on species with no appropriate reference database, we applied and compared the results of *de novo* peptide sequencing as well as clustering with DISMS2 to three recent *Amphistegina* species. These were collected from different sites, partly repeatedly over several years, to test for the influence of spatial and temporal variations. LBF are holobionts, i.e., a combination of the foraminiferal host organism, their photosymbiotic algae and microbiome. As these associated organisms were included in the proteomics workflow, they are also contained in the resulting MS data. We therefore applied a DNA sequencing approach to analyse the dominant photosymbiont community, and their potential influence on the holobiont similarity clusters.

1 MATERIALS AND METHODS

1.1 Sample Collection

Samples were collected by snorkelling or SCUBA diving during different field campaigns (Stuhr et al., 2021, 2018b) following standard protocols in all locations (Red Sea (RS), Eilat, Israel; Western Indian Ocean (WIO), Zanzibar, Tanzania; Western Atlantic (WA), Florida Keys, USA), and either directly frozen at -80 °C or cultured at ZMT for several months (Table 1), in which case LBF were kept in sterilized seawater for three days prior to freezing in order to limit contamination by the microbiome within the culture dishes. They were then either extracted for DNA analysis in Bremen or transported on dry ice to ISAS, Dortmund, for proteomic analyses.

1.2 Proteomic Analyses

1.2.1 Sample preparation

Eight specimens per replicate were pooled and processed as described previously (Stuhr et al., 2018a; Rieder et al., 2017a). After protein digestion and quality-control, amino acid analysis was performed on all samples. Thus, sample amounts were corrected to compensate for systematic errors and to guarantee equal starting material for the subsequent LC-MS analysis.

1.2.2 LC-MS/MS analysis

Using an Ultimate 3000 nano-RSLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (both Thermo Scientific), 330 ng per sample were analysed. A 100 µm × 2 cm C18 trapping column was used for preconcentration of peptides for 10 min using 0.1% TFA (v/v) with a flow rate of 20 µL/min followed by separation on a 75 µm × 50 cm C18 main column (both PepMap RSLC, Thermo Scientific) with a 90 min LC gradient ranging from 3%–35% of buffer B: 84% acetonitrile, 0.1% formic acid at a flow rate of 250 nL/min. The Orbitrap Fusion Lumos MS was operated in data-dependent acquisition mode and MS survey scans were acquired from m/z 300 to 1 500 at a resolution of 120 000 using the polysiloxane ion at m/z 445.12002 as lock mass. Isolation of precursors was performed by the quadrupole with a window of 0.4 m/z. Acquisition was performed in top speed mode, selecting the most intense signals within a cycle time of 3 s between survey scans and subjecting them to higher energy collisional dissociation with a normalized collision energy of 32% at a resolution of 15 000, taking into account a dynamic exclusion of 15 s. Automated gain control target values were set to 2×10^5 for MS and 5×10^4 MS/MS. Maximum injection times were 120 and 250 ms, respectively. Precursor ions with charge states of +1, > +7 or unassigned were excluded from MS/MS analysis and monoisoptic peak determination was set to 'peptide'.

1.2.3 Proteome-wide distance measure DISMS2

DISMS2 is a flexible algorithm that computes proteome-wide distances between MS/MS runs consisting of thousands of unidentified spectra, without additional information (Rieder et al., 2017a). Here, the ProteoWizard tool MSConvertGUI

Table 1 Site, depth, and year of collection of foraminifera populations analysed in this study

Species	Site	Year	Depth (m)	Collection	Abbreviation
Marginopora vertebralis	Zanzibar, WIO	2014	3	Culture	*M. vertebralis* WIO
Amphistegina lessonii	Eilat, RS	2016	3	Culture	*A. lessonii* RS16
Amphistegina lessonii	Eilat, RS	2016	3	Field	*A. lessonii* RS*16
Amphistegina lessonii	Zanzibar, WIO	2015	3	Culture	*A. lessonii* WIO15
Amphistegina lessonii	Zanzibar, WIO	2014	10	Culture	*A. lessonii* WIO14
Amphistegina lobifera	Eilat, RS	2016	3	Culture	*A. lobifera* RS16
Amphistegina lobifera	Eilat, RS	2016	3	Field	*A. lobifera* RS*16
Amphistegina lobifera	Zanzibar, WIO	2015	3	Culture	*A. lobifera* WIO15
Amphistegina gibbosa	Florida, WA	2015	18	Culture	*A. gibbosa* WA15
Amphistegina gibbosa	Florida, WA	2014	18	Culture	*A. gibbosa* WA15

Foraminifera frozen immediately after collection (field) are marked with an asterisk *.

(Chambers et al., 2012) was used to convert Thermo RAW files into mzXML files. The open data format mzXML can be read with the R package readMzXmlData (Gibb, 2015). The washing step of the liquid chromatography was excluded. Thus, only spectra of the first hundred minutes are part of the analysis. First, distances for all these spectra were computed. Second, in each run 2 000 spectra with highest total ion signal were extracted. The preselection of spectra has already been used before (Palmblad and Deelder, 2012).

We used the same full factorial design as in the original paper (Rieder et al., 2017a) to find optimal parameter settings for all spectra. Out of in total 81 factor combinations the combination with the highest partial R squared (0.618 3) was chosen. Partial R squared is a goodness of fit measure that was calculated based on adonis, an ANOVA like approach for distance matrices. Optimized parameters correspond to the values from the original paper. In the pre-processing step no selection of highest peaks (*topn* = ∞) is made and binning is performed with binsize *bin* = 0.2. In the step that includes checking of constraints the size of retention time window is *ret* = 3 000 and the accepted precursor mass shift limit is *prec* = 10 ppm. The cosine distance (*dist* = d_{cos}) is chosen as distance measure between spectra and the cutoff for distance of spectra is *cdis* = 0.3.

1.2.4 *De novo* peptide sequencing data analysis

Conversion of generated. raw files and subsequent data analysis by *de novo* peptide sequencing was performed as described by Blank-Landeshammer et al. (2017). Only full *de novo* sequences of agreeing spectrum annotations by PEAKS, pNovo+ and Novor—fulfilling an expected false discovery rate (FDR) of 5%—were used for further analysis.

For distance calculation, first the similarity of peptide identifications was determined by calculating the Sørensen-Dice coefficient (Sørensen, 1948; Dice, 1945) for all sample pairs, where $|X|$ and $|Y|$ represent the number of unique peptides in the samples.

$$\mathrm{QS} = \frac{2|X \cap Y|}{|X| + |Y|}$$

For comparison of populations, all identified peptides within the replicates were pooled and compared as described above.

All statistical analyses were performed and figures were produced with the statistical programming software R (R Core Team, 2016), Version 4.1.0. Package readMzXmlData (Palmblad and Deelder, 2012) was used for reading MS data, and packages ape (Paradis et al., 2004) and phytools (Revell, 2012) were used for plotting dendrograms. In each case, dendrograms were generated by using complete linkage hierarchical clustering and triplicates of *M. vertebralis* WIO14 as an outgroup. The dendrograms of two different approaches were visualized facing each other for direct comparison.

1.3 Genetic Analyses

1.3.1 Molecular analysis of photosymbionts

Symbiont DNA was extracted and prepared for 18S sequencing as previously described in Stuhr et al. (2018b). Briefly, 36 foraminifera (*n* = 4 per population) were crushed with a micropestle and digested in a CTAP/protein kinase K lysis buffer, and subsequently extracted in phenol-chloroform (Green and Sambrook, 2012). After ethanol washing, a 443-bp fragment of the 3′end of the photosymbionts' 18S rDNA was amplified using primers SymFS1 and 1528R (Schmidt et al., 2015) and Taq polymerase using previously described cycling conditions (Stuhr et al., 2018b). PCR products were SAP digested and Sanger sequenced (Starseq, Mainz) without cloning. The sequences were trimmed for primers and with a quality threshold of 0.01 (1% error probability) in Geneious Prime 2021.1.1. The resulting contigs were deposited at ENA (project accession number PRJEB49701) and aligned after adding sequences with close BLAST hits or from diatoms previously described or sequenced from LBFs extracts (Prazeres et al., 2021; Stuhr et al., 2021, 2018b; Barnes, 2016; Lee, 2006). Alter (Glez-Peña et al., 2010) was used to condense haplotypes with identical sequences. MEGA X (Kumar et al., 2018) was used to determine the best fitting substitution model and finally the taxonomic assignment was done by calculating maximum-likelihood phylogenetic trees in RAxML (Kozlov et al., 2019).

2 RESULTS

2.1 Proteome-Based Clustering

The comparison of dendrograms resulting from proteome-wide distance measures DISMS2 on either all or only the top 2 000 spectra (Fig. 1), illustrating distances between MS/MS runs of different LBF populations and their technical replicates, showed relatively similar overall results, grouping all samples according to species. However, if all spectra were included in the analysis (left side), the uncultured *A. lessonii* RS16 samples and one *A. lessonii* WIO15 sample grouped together with all *A. lobifera*, instead of with the remaining *A. lessonii*. In contrast, the *A. lessonii* WIO14 samples formed a separate cluster when using only the top 2 000 spectra for DISMS2 (right side), yet these did not merge with samples of other species. Samples also cluster more according to their collection site and year using this approach, e. g. with all *A. lobifera* triplicates grouping by their population and then by location. *A. gibbosa* formed a separate cluster in both approaches and hence clustered dominantly by population and then collection year. The average distance between technical replicates of the populations was between 40% and 60% when all spectra were considered and between 35% and 50% for the top 2 000 spectra. Nodes connecting all samples of each species were at ~80% and around 50%–70% distances, respectively.

Since DISMS2 with the top 2 000 spectra showed a very coherent result regarding grouping of triplicates and populations, this was considered as the more reliable approach and further compared to the output of a *de novo* annotation approach. Complete linkage hierarchical clustering of *de novo* peptide sequences resulted in widely similar grouping (Fig. 2). One sample of *A. lessonii* WIO15 grouped more closely with *A. lobifera* than the other *A. lessonii* samples, which however clustered closer together without building a separate group for the population sampled in 2014. Moreover, within *A. lobifera* the samples of the cultured RS16 and WIO15 populations grouped first together, and then the uncultured RS16 samples. Apart from these minor differences, populations and triplicates

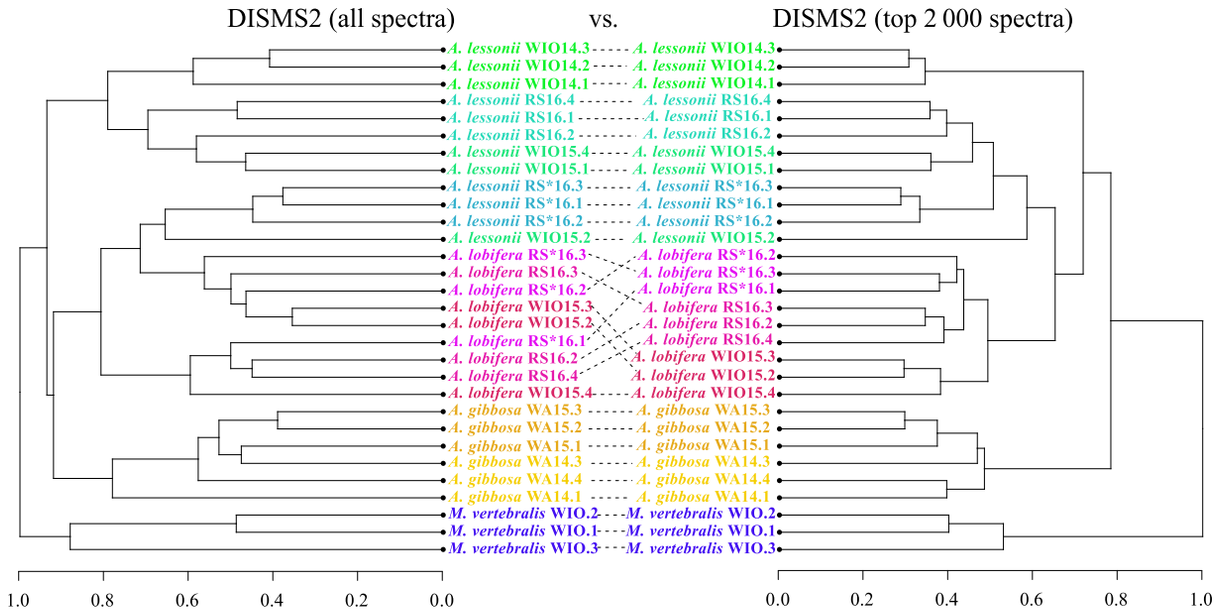

Figure 1. Proteome-wide distance measure clusters computed via DISMS2 with optimized parameters based on either pairwise distances of all spectra (left side) or only the top 2 000 spectra (right side) produced by 30 MS/MS runs of triplicates of different population of large benthic foraminifera, using complete linkage hierarchical clustering.

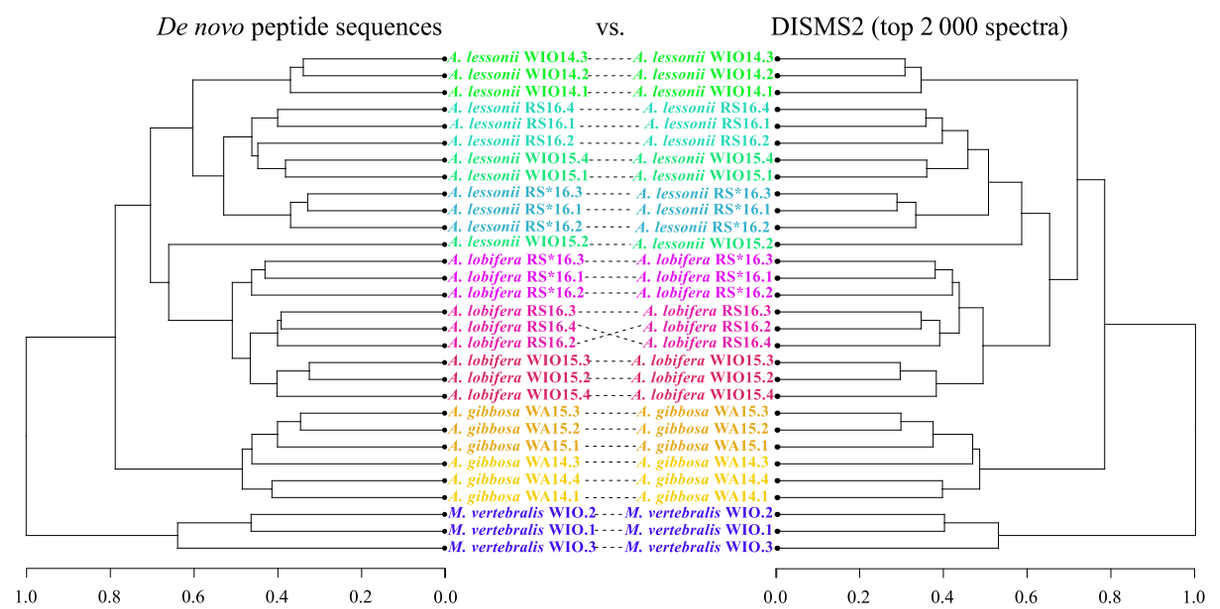

Figure 2. Proteome-wide distance measure clusters computed via de novo peptide sequences (left side) or DISMS2 on top 2 000 spectra with optimized parameters (right side) produced by 30 MS/MS runs of triplicates of different population of large benthic foraminifera, using complete linkage hierarchical clustering.

within all LBF, including *A. gibbosa* and *M. vertebralis*, showed widely analogous clusters. In both approaches, *A. lobifera* and *A. lessonii* are clearly more similar to each other than they are to *A. gibbosa*. The average distance between technical replicates was between 35% and 50% for *de novo* peptide sequences. Nodes connecting all samples of each species were around 50%–60% distances.

Because only one sample did not cluster with the remainder of its population and species, we regarded the *de novo* approach as providing the most representative results. When triplicates of each population were pooled, the resulting hierarchical cluster (Fig. 3) grouped robustly by LBF species, with *A. lessonii* and *A. lobifera* being most similar to each other, followed by *A. gibbosa*. Within species, no dominant grouping by collection site of year could be identified.

2.2 Photosymbiont Barcoding

To understand whether the similarity patterns of the tested LBF reconstructed by proteomics-based approaches may reflect their different photosymbiont communities, we applied a widely used DNA barcoding approach and calculated phylogenetic a tree of the resulting ~450 bp 18S rDNA sequences. This molecular fingerprinting of all samples (n = 24, Fig. 4) revealed that all *A. gibbosa* (n = 5) contained the same diatom

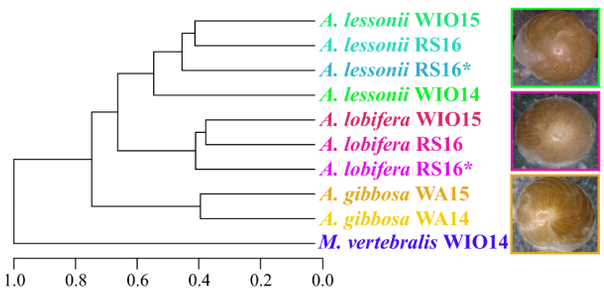

Figure 3. Proteome-wide distance measure clusters computed via *de novo* peptide sequences produced by 30 MS/MS runs of pooled triplicates of different population of large benthic foraminifera, using complete linkage hierarchical clustering. Images on the right side show from top to bottom representative specimens of *Amphistegina lessonii*, *A. lobifera* and *A. gibbosa*. *Marginopora vertebralis* is not displayed.

symbiont sequence, while *A. lessonii* (n = 11) yielded seven haplotypes and *A. lobifera* (n = 8) yielded five haplotypes. All these sequences fall within the order Fragilariales that cluster closely together with sequences obtained from formerly sequenced *A. lobifera* from Israel, Indonesia, Australia, the Maldives and Papua New Guinea (Prazeres et al., 2021; Stuhr et al., 2021), *A. lessonii* from Zanzibar (WIO), Palau, Papua New Guinea, Australia and Eilat (RS)(Ramadhani et al., 2022, under review; Prazeres et al., 2021; Stuhr et al., 2018b) and *A. gibbosa* from Florida (WA) (Stuhr et al., 2018b). In contrast, *A. radiata* from Papua New Guinea (Prazeres et al., 2021) host endosymbionts from Triceratiales instead, another order of diatoms. Although the obtained sequences (443 bp) are too short for a taxonomic assignment to the species level, our results indicate some variability in the diatom assemblages, especially within *A. lessonii*. The resulting dendrogram also showed a separation of *A. lobifera* endosymbiont sequences from Greece and *A. gibbosa* photosymbionts from the other samples, while *A. lessonii* and *A. lobifera* photosymbionts grouped weakly by collection sites. The minor division of greek *A. lobifera* from the remaining samples could be due to a change in their main associated pennate diatoms during the spreading of the species on its northwestward expansion in the Mediterranean (Guastella et al., 2019; Langer et al., 2013; Triantaphyllou et al., 2009) as an adjustment to varying environmental conditions or native diatom communities. While Schmidt et al. (2016) found no systematic difference in the photosymbiont community of Red Sea and Mediterranean populations, the diatom population in other areas, potentially more marginal in terms of *A. lobifera*'s temperature range, are likely to bear a different local diatom community with which the foraminifera can associate. Moreover, it is not clear whether all *A. lobifera* in the Mediterranean are really Lessepsian invaders or might have actually been present in lower densities already before the opening of the Suez canal (Langer, 2008), in which case they could have adjusted their photosymbiont community to the different environmental conditions over much longer time scales.

3 DISCUSSION

Large benthic foraminifera are among the most biodiverse and ecologically intriguing calcium-carbonate producers worldwide (Narayan et al., 2022; Förderer and Langer, 2019; Förderer et al., 2018; Doo et al., 2012; Langer et al., 1997). Acting as bioindicators, they let us characterize recent and reconstruct paleoenvironments (Prazeres et al., 2020a; Emrich et al., 2017; Pisapia et al., 2017; Narayan et al., 2015; Carnahan et al., 2009; Hallock et al., 2003; Hallock and Glenn, 1986), based on our knowledge of their physiology, geochemistry, biogeography, ecology and evolutionary biology. Furthermore, their evolutionary history and proliferation through geological time inspires research and ideas on the various modes of adaptation, extinction, and radiation during periods of environmental change (Prazeres and Renema, 2019; Hohenegger, 2014; Pomar and Hallock, 2008). Although with the development of modern methods, we are now able to reveal cryptic species and their underlying phylogenetic relationships, the actual diversity and natural history of some of the most prominent LBF remain undiscovered (Stulpinaite et al., 2020; Bhatt and Trivedi, 2018; Renema, 2018; Pawlowski and Holzmann, 2002). By combining new MS-based techniques with advanced *de novo* peptide sequencing or similarity clustering algorithms we here present a pilot study, delineating three *Amphistegina* species commonly found in association with coral reefs.

The results of both our proteomics-based approaches showed a separation of the three species. However, as DISMS2 applied to all spectra inadequately grouped the uncultured *A. lessonii* RS16 samples and one *A. lessonii* WIO15 sample with *A. lobifera*, this approach gives the least reliable results. This is further indicated by the highest distances between population replicates as well as nodes connecting all samples of the same species (excluding described faulty grouped samples).

Although these distances were lower when using the top 2 000 spectra only, the *A. lessonii* WIO14 formed a group separated from the other *A. lessonii* and also the *A. lobifera* samples. Hence, the *de novo* peptide sequences-based dendrogram represented the most trustworthy outcome, with all but one sample (*A. lessonii* WIO15.2) clustering correctly by species, and principally also the technical triplicates by population, with comparably low distances. This might be because the *de novo* peptide sequencing algorithms act as efficient filters to separate true (i. e., biologically relevant) spectra from background spectra and those stemming from laboratory contaminations compared to unsupervised clustering. We therefore demonstrate that the approach of Blank-Landeshammer et al. (2017) is capable of comprehensively delineating LBF taxa without a reference database. Yet, as multiple algorithms can be easily applied to the same MS/MS dataset, a combination with DISMS2 (Rieder et al., 2017a) applied to top spectra may serve to further confirm the outcome and boost reliability.

The resultant dendrograms provide first hints into the phylogeny of the three studied *Amphistegina* species. Clearly, the Indopacific species *A. lobifera* and *A. lessonii* are more closely related to each other than they are to their counterpart *A. gibbosa* in the Atlantic (Figs. 1 to 3). This confirms that the morphologically relatively similar and therefore sometimes confused species *A. lessonii* and *A. gibbosa* are indeed separate species, and agrees with their phylogenetic separation suggested by Larsen (1978) into relatively widely separated lineages. This pattern is reflected in the photosymbiotic diatoms harbored by

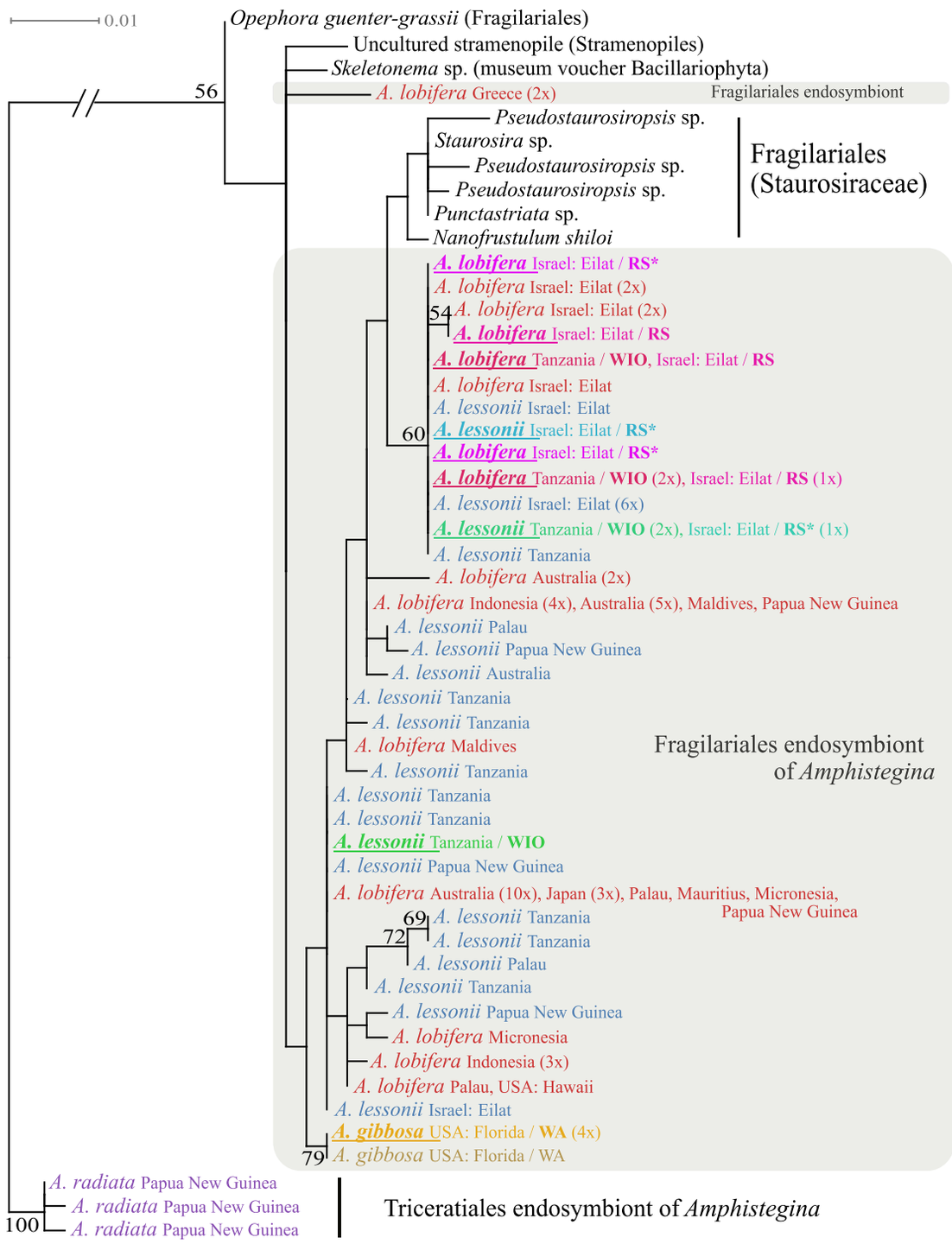

Figure 4. Phylogenetic assignment of diatom-symbiont sequences from *Amphistegina lobifera* and *A. lessonii* collected in Zanzibar, Tanzania, and Eilat, Israel, or *A. gibbosa* from the Florida Keys, USA. These new sequences are highlighted in bold and underlined. Bootstrap support above 50% is given at the respective nodes.

these populations (Fig. 4). Hence, the differences detected for the LBF holobionts may be influenced and partly based on the differences of the photosymbiont communities. Overall, as previously recognized, *A. lessonii* and *A. lobifera* contain a more diverse community of Fragilariales (Ramadhani et al., 2022, under review; Prazeres et al., 2021; Stuhr et al., 2021, 2018b; Barnes, 2016; Schmidt et al., 2016), while *A. gibbosa* contains only one strain of a Fragilariales species (Stuhr et al., 2018b; Barnes, 2016), fairly distinct from those harboured by the aforementioned species. Interestingly, also *A. radiata* appears to have a higher specificity in its photosymbiont assemblages, which is a member of Triceratiales instead of Fragilariales (Prazeres et al., 2021; Barnes, 2016). Whiles this may be an adaptation to their usually more light-reduced habitat (Prazeres et al., 2021), the variation in symbiont flexibility could likewise reflect their phylogenetic distinction into the two lineages sug-

gested by Larsen (1978), with more restricted associations in lineage I (*A. radiata-A. gibbosa*) and highly flexible associations in lineage II (*A. lessonii-A. lobifera*). In this regard, it would be highly interesting to examine the photosymbiont communities of *A. papillosa*, which is generally assumed to belong to lineage I or the *A. radiata* group/kinship, to see whether it also shows a relatively high symbiont specificity.

Despite a potential effect of the global symbiont community on the proteome-based similarity clusters, the well-defined separation of morpho-species signifies the LBF host as the major driver. If the symbionts had an overriding impact, *A. lobifera* and *A. lessonii* as well as samples with morpho-species should cluster more mixed, as specimens from different collection sites or years showed similar symbiont sequences. In contrast, recent environmental conditions appear to have a detectable influence. For example, *A. lobifera* and *A. lessonii* from the Red Sea that were cultured prior to sampling (RS16) are more similar to their likewise cultured counterparts from the western Indian Ocean (WIO15) than they are to subsamples of the same Red Sea population sampled immediately without culturing (RS16*). Since the proteome is largely influenced by environmental factors and may thereby influence evolutionary processes itself (Baer and Millar, 2016) it appears reasonable that populations cultivated under alike conditions will have more similar proteomes. Conversely, *A. lessonii* WIO14 was in all dendrograms slightly disjointed from the remaining *A. lessonii* populations. Notably, it is the only *A. lessonii* population that was sampled in at least 10 m depth, while all others originated from 2–3 m depth. Whether this distinction in *A. lessonii* from Zanzibar may thus represent a genotypic differentiation related to their original habitat, or if this population was somehow differently affected by culturing, will have to be clarified in future studies. Conditions such as the microbiome or phenological phases can potentially vary between cultures, even under similar light, temperature, and water geochemistry, especially if these have been developing over long time.

4 CONCLUSIONS

Our proteome-based clustering approaches along with phylogenetic analyses of their photosymbiont communities indicate that especially *de novo* peptide sequencing provides a reliable tool to reconstruct the evolutionary relationship among foraminiferal holobionts. Although the wide separation of *A. gibbosa* from *A. lessonii* and *A. lobifera* along with their distinctive type of Fragilariales symbionts may indicate a partial influence of the photosymbiont communities, the well-defined species separation in between *A. lobifera* and *A. lessonii* contradicts their highly widely overlapping symbiont communities. This difference between proteome-based clusters and the phylogenetic dendrogram of the photosymbionts therefore signifies that the symbiont community cannot be the major clustering driver. This further confirms that proteomics approaches allow us to draw phylogenetic information, which here reproduces the separation in two lineages as suggested by Larsen (1978). This study provides a basis for future studies that should include the remaining *Amphistegina* morpho-species and include populations from their wide spatial distribution, across depth and their entire geographic range, but should moreover also be extended to other genera of foraminifera. Shedding light onto the true (cryptic) diversity and unravelling the evolutionary history of the prominent genus *Amphistegina* will help ecologists and palaeontologists alike towards understanding paradigms in the face of changing environmental conditions, past and future adaptions, or potential radiation events.

ACKNOWLEDGMENTS

This project was funded by the Leibniz Association (No. SAW-2014-ISAS-2) awarded to Hildegard Westphal, Albert Sickmann and Jörg Rahnenführer. Sampling in the Florida Keys was conducted under the Research Permit (No. FKNMS-2015-026). Samples from Zanzibar were collected under the Revolutionary Government of Zanzibar 434 (S.M.Z.) Research Permit (No. 0671). We sincerely thank Pamela Hallock and Gita R. Narayan for their sampling support, and the staff of the MAREE at ZMT for help with foraminifera cultures. The final publication is available at Springer via https://doi.org/10.1007/s12583-022-1609-1.

REFERENCES CITED

Aebersold, R., Mann, M., 2003. Mass Spectrometry-Based Proteomics. *Nature*, 422(6928): 198–207. https://doi.org/10.1038/nature01511

Alves, B. M., Júnior, M. N., 2020. Foraminiferal Assemblage Structure from Brazilian Tropical Urbanized Beaches (~7° S). *Anais da Academia Brasileira de Ciências*, 92(2): 1 – 15. https://doi. org/10.1590/0001-3765202020190486

Baer, B., Millar, A. H., 2016. Proteomics in Evolutionary Ecology. *Journal of Proteomics*, 135: 4–11. https://doi.org/10.1016/j.jprot.2015.09.031

Barnes, K. H., 2016. Diversity and Distribution of Diatom Endosymbionts in *Amphistegina* spp. (Foraminifera) Based on Molecular and Morphological Techniques: [Dissertation]. University of South Florida, St. Petersburg, Florida. 160

Bentov, S., Brownlee, C., Erez, J., 2009. The Role of Seawater Endocytosis in the Biomineralization Process in Calcareous Foraminifera. *Proceedings of the National Academy of Sciences of the United States of America*, 106(51): 21500–21504. https://doi.org/10.1073/pnas.0906636106

Bhatt, K. A., Trivedi, M. H., 2018. Molecular Studies on Foraminifers: Past, Present, and Future. *Journal of Foraminiferal Research*, 48(3): 193 – 209. https://doi.org/10.2113/gsjfr.48.3.193

Blank-Landeshammer, B., Kollipara, L., Biß, K., et al., 2017. Combining de Novo Peptide Sequencing Algorithms, a Synergistic Approach to Boost both Identifications and Confidence in Bottom-up Proteomics. *Journal of Proteome Research*, 16(9): 3209 – 3218. https://doi. org/10.1021/acs.jproteome.7b00198

Boudagher-Fadel, M. K., 2018. Evolution and Geological Significance of Larger Benthic Foraminifera. Second Edition. UCL Press, London. 693. https://doi.org/10.2307/j.ctvqhsq3

Carnahan, E. A., Hoare, A. M., Hallock, P., et al., 2009. Foraminiferal Assemblages in Biscayne Bay, Florida, USA: Responses to Urban and Agricultural Influence in a Subtropical Estuary. *Marine Pollution Bulletin*, 59(8–12): 221–233. https://doi. org/10.1016/j. marpolbul. 2009. 08.008

Chambers, M. C., Maclean, B., Burke, R., et al., 2012. A Cross-Platform Toolkit for Mass Spectrometry and Proteomics. *Nature Biotechnology*, 30(10): 918–920. https://doi.org/10.1038/nbt.2377

d'Orbigny, A. D., 1826. Tableau Méthodique de la Classe des

Céphalopodes. *Annales des Sciences Naturelles*, 7: 96–314

Debenay, J.-P., 2013. A Guide to 1 000 Foraminifera from Southwestern Pacific, New Caledonia. Muséum National d'Histoire Naturelle, Paris. 384

Dice, L., 1945. Measures of the Amount of Ecologic Association between Species. *Ecology*, 26(3): 297–302. https://doi.org/10.2307/1932409

Doo, S. S., Hamylton, S., Byrne, M., 2012. Reef-Scale Assessment of Intertidal Large Benthic Foraminifera Populations on one Tree Island, Great Barrier Reef and Their Future Carbonate Production Potential in a Warming Ocean. *Zoological Studies*, 51(8): 1298–1307. https://doi.org/10.1007/s00435-012-0164-1

Ehrenberg, C. G., 1838. Über die Bildung der Kreidefelsen und des Kreidemergels Durch Unsichtbare Organismen. Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin, Berlin. 59–147

Emrich, K., Martinez-Colon, M., Alegria, H., 2017. Is Untreated Sewage Impacting Coral Reefs of Caye Caulker, Belize? *The Journal of Foraminiferal Research*, 47(1): 20–33. https://doi. org/10.2113/gsjfr.47.1.20

Flakowski, J., Bolivar, I., Fahrni, J., et al., 2005. Actin Phylogeny of Foraminifera. *Journal of Foraminiferal Research*, 35(2): 93 – 102. https://doi.org/10.2113/35.2.93

Förderer, M., Langer, M. R., 2019. Exceptionally Species-Rich Assemblages of Modern Larger Benthic Foraminifera from Nearshore Reefs in Northern Palawan (Philippines). *Revue de Micropaléontologie*, 65: 100387. https://doi.org/10.1016/j.revmic.2019.100387

Förderer, M., Rödder, D., Langer, M. R., 2018. Patterns of Species Richness and the Center of Diversity in Modern Indo-Pacific Larger Foraminifera. *Scientific Reports*, 8(1): 8189. https://doi. org/10.1038/s41598-018-26598-9

Gibb, S., 2015. readMzXmlData: Reads Mass Spectrometry Data in mzXML Format. [2021-11-10]. https://github.com/sgibb/readMzXmlData/

Glas, M. S., Fabricius, K. E., de Beer, D., et al., 2012. The O_2, pH and Ca^{2+} Microenvironment of Benthic Foraminifera in a High CO_2 World. *PLoS One*, 7(11): e50010. https://doi.org/10.1371/journal.pone.0050010

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., et al., 2010. ALTER: Program-Oriented Conversion of DNA and Protein Alignments. *Nucleic Acids Research*, 38: W14–W18. https://doi. org/10.1093/nar/gkq321

Green, M. R., Sambrook, J., 2012. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2028

Guastella, R., Marchini, A., Caruso, A., et al., 2019. "Hidden Invaders" Conquer the Sicily Channel and Knock on the Door of the Western Mediterranean Sea. *Estuarine*, *Coastal and Shelf Science*, 225: 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Hallock, P., 1985. Why are Larger Foraminifera Large? *Paleobiology*, 11(2): 195–208. https://doi.org/10.1017/s0094837300011507

Hallock, P., 1999. Symbiont-Bearing Foraminifera. In: Sen Gupta, B. K., ed., Modern Foraminifera. Kluwer Academic Publishers. 123–368

Hallock, P., Forward, L. B., Hansen, H. J., 1986. Influence of Environment on the Test Shape of Amphistegina. *The Journal of Foraminiferal Research*, 16(3): 224–231. https://doi.org/10.2113/gsjfr.16.3.224

Hallock, P., Glenn, E. C., 1986. Larger Foraminifera: A Tool for Paleoenvironmental Analysis of Cenozoic Carbonate Depositional Facies. *PALAIOS*, 1(1): 55–64. https://doi.org/10.2307/3514459

Hallock, P., Hansen, J., 1979. Depth Adaptation in Amphistegina: Change in Lamellar Thickness. *Bulletin of the Geological Society of Denmark*, 27: 99–104. https://doi.org/10.37570/bgsd-1978-27-10

Hallock, P., Lidz, B. H., Cockey-Burkhard, E. M., et al., 2003. Foraminifera as Bioindicators in Coral Reef Assessment and Monitoring: The Foram Index. *Environmental Monitoring and Assessment*, 81(1/2/3): 221–238. https://doi.org/10.1023/a:1021337310386

Hallock, P., Talge, H. K., Smith, K., et al., 1993. Bleaching in a Reef-Dwelling Foraminifer, Amphistengina Gibbosa. *Proceedings of the 7th International Coral Reef Symposium*, 1: 44–49

Hansen, H. J., Buchard, B., 1977. Depth Distribution of *Amphistegina* in the Gulf of Elat, Israel. Utrecht *Micropaleontological Bulletins*, 1977: 205–224

Haynes, J. R., 1992. Supposed Pronounced Ecophenotypy in Foraminifera. *Journal of Micropalaeontology*, 11(1): 59–63. https://doi.org/10.1144/jm.11.1.59

Heck, M., Neely, B. A., 2020. Proteomics in Non-Model Organisms: A New Analytical Frontier. *Journal of Proteome Research*, 19(9): 3595–3606. https://doi.org/10.1021/acs.jproteome.0c00448

Hohenegger, J., 2014. Species as the Basic Units in Evolution and Biodiversity: Recognition of Species in the Recent and Geological Past as Exemplified by Larger Foraminifera. *Gondwana Research*, 25(2): 707–728. https://doi.org/10.1016/j.gr.2013.09.009

Hohenegger, J., 2004. Depth Coenoclines and Environmental Considerations of Western Pacific Larger Foraminifera. *The Journal of Foraminiferal Research*, 34(1): 9–33. https://doi.org/10.2113/0340009

Hottinger, L., Halicz, E., Reiss, Z., 1993. Recent Foraminiferida from the Gulf of Aqaba, Red Sea. Slovenian Academy of Sciences and Arts and Swiss Academy of Natural Sciences, Ljubljana. 181

Kozlov, A. M., Darriba, D., Flouri, T., et al., 2019. RAxML-NG: A Fast, Scalable and User-Friendly Tool for Maximum Likelihood Phylogenetic Inference. *Bioinformatics*, 35(21): 4453–4455. https://doi.org/10.1093/bioinformatics/btz305

Kumar, S., Stecher, G., Li, M., et al., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35(6): 1547–1549. https://doi. org/10.1093/molbev/msy096

Langer, M., 2008. Foraminifera from the Mediterranean and the Red Sea. In: Aqaba-Eilat, the Improbable Gulf: Environment, Biodiversity and Preservation. Magnes Press, Jerusalem. 397–415

Langer, M. R., Hottinger, L., 2000. Biogeography of Selected "Larger" Foraminifera. *Micropaleontology*, 46: 105–126

Langer, M. R., Silk, M. T., Lipps, J. H., 1997. Global Ocean Carbonate and Carbon Dioxide Production: The Role of Reef Foraminifera. *The Journal of Foraminiferal Research*, 27(4): 271–277. https://doi. org/10.2113/gsjfr.27.4.271

Langer, M. R., Weinmann, A. E., Lötters, S., et al., 2013. Climate-Driven Range Extension of Amphistegina (Protista, Foraminiferida): Models of Current and Predicted Future Ranges. *PLoS One*, 8(2): e54443. https://doi.org/10.1371/journal.pone.0054443

Larsen, A. R., 1978. Phylogenetic and Paleobiogeographical Trends in the Foraminiferal genus *Amphistegina*. *Revista Española de Micropaleontología*, X: 217–243

Lee, J., 2006. Algal Symbiosis in Larger Foraminifera. *Symbiosis*, 42(2): 63–75

Leutenegger, S., 1977. Ultrastructure de Foraminifères Perforés et Imperforés Ainsi Que Leurs Symbiotes. *Cahiers de Micropaléontologie*, 3: 5–7

Loeblich, A. R. Jr., Tappan, H., 1988. Foraminiferal Genera and Their Classification, Vol. 1. Van Nostrand Reinhold. 2115

Loeblich, A. R. Jr., Tappan, H., 1994. Foraminifera of the Sahul Shelf and Timor Sea. Cushman Foundation Special Publications No. 31. 666

Loeblich, A. R. Jr., Tappan, H., Beckmann, J. P., et al., 1957. Studies in Foraminifera. *United States National Museum Bulletin*, 215: 412

MacCoss, M. J., 2005. Computational Analysis of Shotgun Proteomics Data. *Current Opinion in Chemical Biology*, 9(1): 88 – 94. https://doi.org/10.1016/j.cbpa.2004.12.010

Macher, J. N., Prazeres, M., Taudien, S., et al., 2021. Integrating Morphology and Metagenomics to Understand Taxonomic Variability of Amphisorus (Foraminifera, Miliolida) from Western Australia and Indonesia. *PLoS One*, 16(1): e0244616. https://doi.org/10.1371/journal.pone.0244616

Morard, R., Füllberg, A., Brummer, G. J. A., et al., 2019. Genetic and Morphological Divergence in the Warm-Water Planktonic Foraminifera Genus Globigerinoides. *PLoS One*, 14(12): e0225246. https://doi.org/10.1371/journal.pone.0225246

Mouanga, G. H., Langer, M. R., 2014. At the Front of Expanding Ranges: Shifting Community Structures at Amphisteginid Species Range Margins in the Mediterranean Sea. *Neues Jahrbuch Für Geologie Und Paläontologie-Abhandlungen*, 271(2): 141–150. https://doi.org/10.1127/0077-7749/2014/0381

Murray, J., 2006. Ecology and Applications of Benthic Foraminifera. *Palaeogeography*, *Palaeoclimatology*, *Palaeoecology*, 95: 1 – 426. https://doi.org/10.1017/cbo9780511535529

Narayan, G. R., Reymond, C. E., Stuhr, M., et al., 2022. Response of Large Benthic Foraminifera to Climate and Local Changes: Implications for Future Carbonate Production. *Sedimentology*, 69(1): 121 – 161. https://doi.org/10.1111/sed.12858

Narayan, Y. R., Lybolt, M., Zhao, J. X., et al., 2015. Holocene Benthic Foraminiferal Assemblages Indicate Long-Term Marginality of Reef Habitats from Moreton Bay, Australia. *Palaeogeography*, *Palaeoclimatology*, *Palaeoecology*, 420: 49–64. https://doi.org/10.1016/j.palaeo.2014.12.010

Palmblad, M., Deelder, A. M., 2012. Molecular Phylogenetics by Direct Comparison of Tandem Mass Spectra. *Rapid Communications in Mass Spectrometry*, 26(7): 728–732. https://doi.org/10.1002/rcm.6162

Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of Phylogenetics and Evolution in R Language. *Bioinformatics*, 20(2): 289–290. https://doi.org/10.1093/bioinformatics/btg412

Pawlowski, J., Holzmann, M., 2008. Diversity and Geographic Distribution of Benthic Foraminifera: A Molecular Perspective. *Biodiversity and Conservation*, 17(2): 317 – 328. https://doi. org/10.1007/s10531-007-9253-8

Pawlowski, J., Holzmann, M., 2002. Molecular Phylogeny of Foraminifera a Review. *European Journal of Protistology*, 38(1): 1 – 10. https://doi.org/10.1078/0932-4739-00857

Pawlowski, J., Holzmann, M., Tyszka, J., 2013. New Supraordinal Classification of Foraminifera: Molecules Meet Morphology. *Marine Micropaleontology*, 100: 1–10. https://doi.org/10.1016/j.marmicro.2013.04.002

Pawlowski, J., Lecroq, B., 2010. Short rDNA Barcodes for Species Identification in Foraminifera. *Journal of Eukaryotic Microbiology*, 57(2): 197–205. https://doi.org/10.1111/j.1550-7408.2009.00468.x

Pawlowski, J., Lejzerowicz, F., Esling, P., 2014. Next-Generation Environmental Diversity Surveys of Foraminifera: Preparing the Future. *The Biological Bulletin*, 227(2): 93 – 106. https://doi. org/10.1086/bblv227n2p93

Pisapia, C., Kateb, A. E., Hallock, P., et al., 2017. Assessing Coral Reef Health in the North Ari Atoll (Maldives) Using the FoRAM Index. *Marine Micropaleontology*, 133: 50 – 57. https://doi. org/10.1016/j.marmicro.2017.06.001

Pochon, X., Montoya-Burgos, J. I., Stadelmann, B., et al., 2006. Molecular Phylogeny, Evolutionary Rates, and Divergence Timing of the Symbiotic Dinoflagellate Genus Symbiodinium. *Molecular Phylogenetics and Evolution*, 38(1): 20-30. https://doi.org/10.1016/j.ympev.2005.04.028

Pomar, L., Hallock, P., 2008. Carbonate Factories: A Conundrum in Sedimentary Geology. *Earth-Science Reviews*, 87(3/4): 134 – 169. https://doi.org/10.1016/j.earscirev.2007.12.002

Prazeres, M., Martínez-Colón, M., Hallock, P., 2020a. Foraminifera as Bioindicators of Water Quality: The FoRAM Index Revisited. *Environmental Pollution*, 257: 113612. https://doi. org/10.1016/j.envpol.2019.113612

Prazeres, M., Morard, R., Roberts, T. E., et al., 2020b. High Dispersal Capacity and Biogeographic Breaks Shape the Genetic Diversity of a Globally Distributed Reef-Dwelling Calcifier. *Ecology and Evolution*, 10(12): 5976–5989. https://doi.org/10.1002/ece3.6335

Prazeres, M., Renema, W., 2019. Evolutionary Significance of the Microbial Assemblages of Large Benthic Foraminifera. *Biological Reviews*, 94(3): 828–848. https://doi.org/10.1111/brv.12482

Prazeres, M., Roberts, T. E., Ramadhani, S. F., et al., 2021. Diversity and Flexibility of Algal Symbiont Community in Globally Distributed Larger Benthic Foraminifera of the Genus Amphistegina. *BMC Microbiology*, 21(1): 243. https://doi.org/10.1186/s12866-021-02299-8

Prazeres, M., Martins, S. E., Bianchini, A., 2011. Biomarkers Response to Zinc Exposure in the Symbiont-Bearing Foraminifer Amphistegina Lessonii (Amphisteginidae, Foraminifera). *Journal of Experimental Marine Biology and Ecology*, 407(1): 116–121. https://doi.org/10.1016/j.jembe.2011.07.015

Prazeres, M., Martins, S. E., Bianchini, A., 2012. Assessment of Water Quality in Coastal Waters of Fernando de Noronha, Brazil: Biomarker Analyses in Amphistegina Lessonii. *The Journal of Foraminiferal Research*, 42(1): 56–65. https://doi.org/10.2113/gsjfr.42.1.56

Prazeres, M., Uthicke, S., Pandolfi, J. M., 2015. Ocean Acidification Induces Biochemical and Morphological Changes in the Calcification Process of Large Benthic Foraminifera. *Proceedings Biological Sciences*, 282: 8–11. https://doi.org/10.1098/rspb.2014.2782

Ramadhani, S. F., Roberts, T. E., Stuhr, M., et al., 2022. Patterns of Host-Symbiont Genetic Diversity and Specificity in the Reef-Dwelling Large Benthic Foraminifera *Amphistegina lessonii*. *Coral Reefs*, Under Review

Renema, W., 2003. Larger Foraminifera on Reefs around Bali (Indonesia). *Zool Verh Leiden*, 345: 337–366

Renema, W., 2018. Terrestrial Influence as a Key Driver of Spatial Variability in Large Benthic Foraminiferal Assemblage Composition in the Central Indo-Pacific. *Earth-Science Reviews*, 177: 514–544. https://doi.org/10.1016/j.earscirev.2017.12.013

Renema, W., 2019. Large Benthic Foraminifera in Low-Light Environments. *Mesophotic Coral Ecosystems*, 553–562. https://doi.org/10.1007/978-3-319-92735-0_31

Revell, L. J., 2012. Phytools: An R Package for Phylogenetic Comparative Biology (and other Things). *Methods in Ecology and Evolution*, 3(2): 217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x

Rieder, V., Blank-Landeshammer, B., Stuhr, M., et al., 2017a. DISMS2: A Flexible Algorithm for Direct Proteome-Wide Distance Calculation of LC-MS/MS Runs. *BMC Bioinformatics*, 18(1): 148. https://doi. org/10.1186/s12859-017-1514-2

Rieder, V., Schork, K. U., Kerschke, L., et al., 2017b. Comparison and Evaluation of Clustering Algorithms for Tandem Mass Spectra.

Journal of Proteome Research, 16(11): 4035 – 4044. https://doi. org/10.1021/acs.jproteome.7b00427

Ross, B. J., Hallock, P., 2014. Chemical Toxicity on Coral Reefs: Bioassay Protocols Utilizing Benthic Foraminifers. *Journal of Experimental Marine Biology and Ecology*, 457: 226–235. https://doi.org/10.1016/j.jembe.2014.04.020

Ross, B. J., Hallock, P., 2018. Challenges in Using Cell Tracker Green on Foraminifers that Host Algal Endosymbionts. *PeerJ*, 6: e5304. https://doi.org/10.7717/peerj.5304

Schmidt, C., Heinz, P., Kucera, M., et al., 2011. Temperature-Induced Stress Leads to Bleaching in Larger Benthic Foraminifera Hosting Endosymbiotic Diatoms. *Limnology and Oceanography*, 56(5): 1587–1602. https://doi.org/10.4319/lo.2011.56.5.1587

Schmidt, C., Morard, R., Almogi-Labin, A., et al., 2015. Recent Invasion of the Symbiont-Bearing Foraminifera Pararotalia into the Eastern Mediterranean Facilitated by the Ongoing Warming Trend. *PLoS One*, 10(8): e0132917. https://doi.org/10.1371/journal.pone.0132917

Schmidt, C., Morard, R., Prazeres, M., et al., 2016. Retention of High Thermal Tolerance in the Invasive Foraminifera Amphistegina Lobifera from the Eastern Mediterranean and the Gulf of Aqaba. *Marine Biology*, 163(11): 1 – 13. https://doi. org/10.1007/s00227-016-2998-4

Sørensen, T., 1948. Method of Establishing Groups of Equal Amplitude in Plant Sociology Based on Similarity of Species Content and Its Application to Analyses of the Vegetation on Danish Commons. *Kongelige Danske Videnskabernes Selskab*, *Biologiske Skrifter*, 5: 1–34

Stainbank, S., Spezzaferri, S., Beccari, V., et al., 2020. Photic Stress on Coral Reefs in the Maldives: The Amphistegina Bleaching Index. *Ecological Indicators*, 113: 106257. https://doi.org/10.1016/j.ecolind.2020.106257

Stuhr, M., Blank-Landeshammer, B., Reymond, C. E., et al., 2018a. Disentangling Thermal Stress Responses in a Reef-Calcifier and Its Photosymbionts by Shotgun Proteomics. *Scientific Reports*, 8: 3524. https://doi.org/10.1038/s41598-018-21875-z

Stuhr, M., Meyer, A., Reymond, C. E., et al., 2018b. Variable Thermal Stress Tolerance of the Reef-Associated Symbiont-Bearing Foraminifera Amphistegina Linked to Differences in Symbiont Type. *Coral Reefs*, 37(3): 811–824. https://doi.org/10.1007/s00338-018-1707-9

Stuhr, M., Cameron, L. P., Blank-Landeshammer, B., et al., 2021. Divergent Proteomic Responses Offer Insights into Resistant Physiological Responses of a Reef-Foraminifera to Climate Change Scenarios. *Oceans*, 2(2): 281–314. https://doi.org/10.3390/oceans2020017

Stuhr, M., Reymond, C. E., Rieder, V., et al., 2017. Reef Calcifiers are Adapted to Episodic Heat Stress but Vulnerable to Sustained Warming. *PLoS One*, 12(7): e0179753. https://doi. org/10.1371/journal. pone. 0179753

Stulpinaite, R., Hyams-Kaphzan, O., Langer, M. R., 2020. Alien and Cryptogenic Foraminifera in the Mediterranean Sea: A Revision of Taxa as Part of the EU 2020 Marine Strategy Framework Directive. *Mediterranean Marine Science*, 21: 719–758. https://doi.org/10.12681/mms.24673

Talge, H. K., Hallock, P., 1995. Cytological Examination of Symbiont Loss in a Benthic Foraminifera, Amphistegina Gibbosa. *Marine Micropaleontology*, 26(1/2/3/4): 107 – 113. https://doi. org/10.1016/0377-8398(95)00015-1

ter Kuile, B., Erez, J., 1984. *In situ* Growth Rate Experiments on the Symbiont-Bearing Foraminifera Amphistegina Lobifera and Amphisorus Hemprichii. *The Journal of Foraminiferal Research*, 14(4): 262–276. https://doi.org/10.2113/gsjfr.14.4.262

ter Kuile, B., Erez, J., Padan, E., 1989. Mechanisms for the Uptake of Inorganic Carbon by Two Species of Symbiont-Bearing Foraminifera. *Marine Biology*, 103(2): 241–251. https://doi.org/10.1007/bf00543354

Titelboim, D., Sadekov, A., Blumenfeld, M., et al., 2021. Monitoring of Heavy Metals in Seawater Using Single Chamber Foraminiferal Sclerochronology. *Ecological Indicators*, 120: 106931. https://doi.org/10.1016/j.ecolind.2020.106931

Todd, R., 1976. Some Observations of *Amphistegina* (Foraminifera). Progress in Micropaleontology, Micropaleontology Press, New York. 382–394

Triantaphyllou, M. V., Koukousioura, O., Dimiza, M. D., 2009. The Presence of the Indo-Pacific Symbiont-Bearing Foraminifer Amphistegina Lobifera in Greek Coastal Ecosystems (Aegean Sea, Eastern Mediterranean). *Mediterranean Marine Science*, 10(2): 73–86. https://doi.org/10.12681/mms.111

Weber, A. A. T., Pawlowski, J., 2014. Wide Occurrence of SSU rDNA Intragenomic Polymorphism in Foraminifera and Its Implications for Molecular Species Identification. *Protist*, 165(5): 645–661. https://doi.org/10.1016/j.protis.2014.07.006

Weinmann, A. E., Rödder, D., Lötters, S., et al., 2013. Heading for New Shores: Projecting Marine Distribution Ranges of Selected Larger Foraminifera. *PLoS One*, 8(4): e62182. https://doi.org/10.1371/journal.pone.0062182

Zmiri, A., Kahan, D., Hochstein, S., et al., 1974. Phototaxis and Thermotaxis in some Species of Amphistegina (Foraminifera). *The Journal of Protozoology*, 21(1): 133 – 138. https://doi. org/10.1111/j.1550-7408.1974.tb03626.x