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A Preliminary Investigation To Explore The Diversity Of Recent Benthic Foraminifera Using Taxonomical And Molecular Approach: A Case Study From Northwestern Part Of The Arabian Gulf, Kuwait.

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Abstract: A taxonomic classification system based on the composition of the test wall, the number and arrangement of chambers, and the morphology of the aperture was used to identify 45 species from 24 genera. Principally Ammonia, Elphiedium, Rosalina, and Quinquloculina represented groupings. The described species' taxonomic position is still up for debate. In the current study, we sequenced the 18S ribosomal RNA (rDNA) gene, a widely used genetic marker that can assist with species-level analysis and biodiversity studies. Majority of Rotallidae and Nummulitidae foraminifera groupings were further identified using a molecular technique. Molecular findings matched similar diversity dominated by the Phylum Retaria in the Order Rotaliida and the Family Nummulitidae. Possibly, the DNA region applied does not reflect enough discriminatory power to differentiate amongst closely related foraminifera species. Therefore, different markers, such as 28S rDNA and ITS rDNA, showing high discriminatory powers should be tested in future.

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1. Introduction

Foraminifera are a diverse group of single-celled amoeboid protozoa belonging to the phylum Granuloreticulosa, with more than 10,000 species found worldwide mainly living as marine organisms (Adl et al., 2007). The first Foraminifera classification was proposed by d'Orbigny in 1826 based on morphological features. Foraminifera are considered as one of the most difficult taxonomic groups in describing new species, due to high similarities in morphological features (Murray, 2007). Although foraminifera lack proper taxonomic description, they are used as environmental quality indicators (Alves et al., 2015) and markers of past environmental changes (Zillen et al, 2008). In the Arabian Peninsula, foraminifera are studied and used as indicator for environmental changes through studying the biogeochemical analysis and the distribution of metal concentration in its shell. Studies on foraminifera environment, morphology, biodiversity, molecular and metabarcoding investigation attracted many scientists locally and globally in the past half a century (Anber, 1974; Al-Abdul-Razzaq et al., 1983; Al-Abdul-Razzaq and Bhalla, 1987a & 1987b; Al-Zamel et al., 1996; Al-Shuiabi, 1997; Cherif et al., 1997; Khader, 1997; Al-Enezi, 2002; Al-Eneziand and Frontalini, 2015; Al-Enezi et al., 2020 & 2022). The main taxonomic problem being faced by protistology's is the morphological description of the foraminiferal external shell that has led to the misidentification of closely related species.

Brinkmann et al. (2023), demonstrates the usefulness of eDNA metabarcoding in surveying community differentiation between ecosystems of certain contrasting environmental conditions. Nevertheless, several aspects of eDNA metabarcoding require further analyses before standardized protocols can be implemented for routine applications. Such analyses include dedicated comparisons of technical as well as biological replicates regarding diversity, and the relation to in-situ foraminiferal morpho-communities. This may allow us to elucidate the function of extraction-kit type and targeted foraminiferal groups.

The two most prevalent benthic foraminiferal genera globally are Ammonia and Elphidium (Murray, 1991). From tropical to polar locations, as well as from the intertidal zone to the continental slope, Elphidium genera are largely distributed (Murray, 2007). Furthermore, one of the most well investigated taxa, Ammonia may be found from the subtidal to the outer continental shelf. The genera Ammonia and Elphidium both play a significant role in faunas of the benthic foraminifera. These genera make up a moderate fraction of the foraminiferal assemblages in the shallow marine of Kuwait and the Arabian Gulf.

There are about 5,000 species of modern (living) foraminifera and more than 50,000 fossil species (Debenay et al. 1996). Almost all these species have been described based on morphological characters of their test (shell). Compared to many other protists, biological features such as cell structures or life cycles are usually not considered in foraminiferal systematics (Pawlowski and Lee 1992).

By integrating molecular methods, through applying Next Generation Sequencing (NGS) technique, using the gene 18S ribosomal RNA (rDNA) widely used to characterize taxonomic diversity. The combination of NGS with the traditional taxonomy findings (i.e., based on species description and morphological identification) aids in identifying and differentiating closely related species (Hebert et al., 2003). Furthermore, molecular analysis of short, sequenced regions using phylogenetic tree approach can assign species to a taxonomic group, also, aid in resolving misidentification taxonomic problems in Foraminifera (Morard et al., 2016). The molecular systematics efforts of the Kingdom Protista are being coordinated by an international organization known as the Consortium for the Barcode of Life Protist Working Group (CBOL-ProWG) and their main objective is to establish a universal criterion for barcode-based species identification using gene markers: 18S rDNA, 28S rDNA and ITS rDNA (Pawlowski et al., 2012).

2. Materials and Methods

Study area

Kuwait is located northwest of the Arabian Gulf (Lat. 28°30′ – 30°05′ N and Long. 46°33′ – 48°30′ E), with a coastline running from north to south of Kuwait City, featuring an important Bay which is narrow oval-shaped embayment (Figure 1). The Shatt-Al-Arab River (northern of Kuwait Bay), dust storms, surface runoff wastewater from industrial operations, and other human-triggered pollution all contribute to nutrient enrichment in the Kuwaiti Bay, making it an unfavorable place for marine ecosystems.

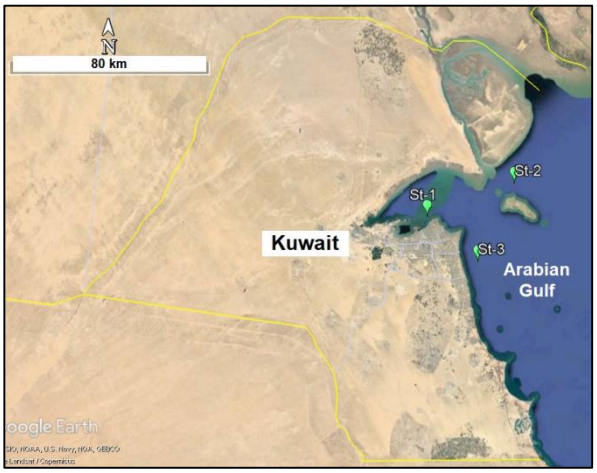


Figure 1. Map of Kuwait showing the study stations, (Google Maps, 2023)

Sediment samples

Throughout several expeditions from January to October 2019, bottom sediment samples have been collected from Kuwait's Bay. Three stations were included in the current preliminary study. One station inside the Bay (St-1), the second at the entrance of the Bay (St-2) and the third (St-3) along Kuwait's coastline opposite 'Al Badaa' area (Fig. 1). Using a grab sampler, sediment samples of 50 cm^3 were collected (0-1 cm depth) and placed in tightly sealed plastic containers, stored at -20° C in the Lab for further investigation. Under a light stereomicroscope, the protoplasm's natural coloring and the presence of pseudopodial activity were observed to distinguish between alive and dead foraminifera. Specimens showed signs of movement and alive were cleaned, separated by paintbrush, placed in Eppendorf tubes, and brought to room temperature for eDNA investigations.

Classification and Taxonomical identification

Shoenfeld et al. (2012) procedures were used for foraminiferal separation and sampling. Foraminifera were identified morphologically and taxonomically using the monographs of Cimerman and Langer (1991), Hottinger et al. (1993), Loeblich and Tappan (1994), Cherif et al. (1997), Hayward et al. (2004), Parker (2009), and Amao et al. (2016, 2018a, 2018b, 2019).

Molecular approach

Applying molecular techniques for eDNA sequencing, we used the gene region 18S ribosomal RNA (rDNA). To keep the accuracy and reliability of sequencing data, quality control (QC) is performed at each step of the procedure, from DNA extraction to final sequence analysis. The eDNA workflow is shown in Figure 2.

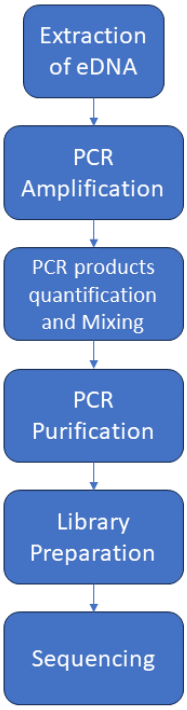


Figure 2. Molecular workflow from eDNA sample to sequencing.

Following the isolation of foraminifera samples from the sediments, the selected samples are stored directly in DNA extraction buffer and frozen at -20ºC until ready for DNA extraction. Genomic DNA was extracted for each sample separately following single-cell genetic analysis approach (Weiner et al., 2016). The Wizard® Genomic DNA Purification Kit was used for the isolation of DNA from the foraminifera cells following the manufacturers protocol. For the Library Construction, Quality Control and Sequencing, the PCR amplification of targeted region (18S ribosomal RNA) was performed by using specific primers connecting with barcodes. The PCR products with proper size were selected by 2% agarose gel electrophoresis. The same amount of PCR products from each sample was pooled, end-repaired, A-tailed, and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads. The experimental procedures of DNA library preparation are shown in Figure 3.

Finally, Bioinformatics and data analysis

Sequences analysis were performed using Uparse software (Uparse v7.0.1090, see details http://drive5.com/uparse/) (Magoc et al., 2011) using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same OTUs.

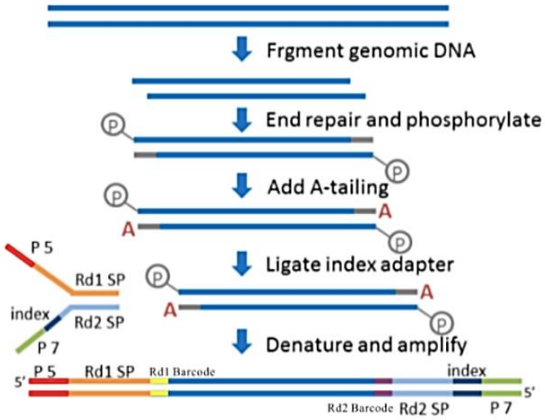


Figure 3. Molecular workflow of library construction

Representative sequence for each OTU was screened for further annotation. Sequences analyses were performed using Qiime (Version 1.7.0, details: http://qiime.org/scripts/assign_taxonomy.html) (Bokulich et al., 2013) in RDP method and Silva database (see details http://www.arb-silva.de/) (Caporaso et al., 2010) for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species) (Threshold:0.6~1). To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE software (Edgar, 2013) (Version 3.8.31, details: http://www.drive5.com/muscle/) can compare multiple sequences rapidly.

3. Results

Forty-five species were recorded in the study stations classified systematically depending on morphological characteristics using stereomicroscope and Scanning Electron Microscope (SEM). The most important and significant recorded benthic species belong to the order Rotaliida, represented by 17 species belonging to 9 genus (Figure 4 and plate 1 & 2). One of the main controversial issues in conventional morphology-based taxonomy of foraminifera is the identification of species Pawlowski and Holzmann (2008). The studied individuals were identified by using the references of the type specimen description enlisted in Loeblish and Tappan (1988 & 1994) and Ellis and Messina Catalogues (Back Matter, 2000), Cimerman and Langer (1991) and Pawlowski et al. (2003, 2013). Rotaliida is the most diversified order of modern benthic foraminifera, comprising 73 extant families (Holzmann, M. and Pawlowski, 2017).

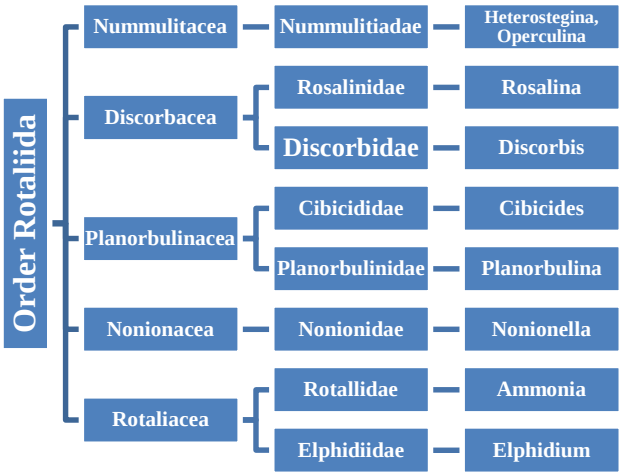


Figure 4. Diagram showing the taxonomic positions of the Order Rotaliida, superfamilies, families and the most abundant genera in studied stations.

Molecular findings

Amplicon was sequenced on Illumina paired-end platform to generate 250 bp paired-end raw reads (Raw PE), and then merged and pre-treated to obtain Clean Tags. The chimeric sequences in Clean Tags were detected and removed to obtain the Effective Tags which can be used for subsequent analysis. The summarizations obtained in each step of data processing are shown in Table 1.

Table 1. A summary of sequence processing

Sample Name/ (Station #)	Raw PE(#)	Raw Tags(#)	Clean Tags(#)	Base(nt)	Avg Len	Q20	GC%	Effective%
M7 (St-1)	104,539	77,876	72,979	21,548,761	295	98.05	63.35	69.77
SP2 (St-2)	160,198	135,488	134,636	27,692,251	206	99.23	59.84	84.04
SP4 (St-3)	176,537	145,894	48,220	15,407,154	320	98.68	60.11	27.31

OTU analysis and taxonomic annotation

To study the foraminifera community composition in each sample, Operational Taxonomic Units (OTUs) were obtained by clustering with 97% identity on the Effective Tags of all samples, and then identified. In the process of constructing OTUs, basic information of different samples were collected, such as Effective Tags data, low-frequency Tags data and Tags annotation data (Table 1). A total of 733 OTUs and 160,094 reads were obtained by generating OUT tables after noise reduction and chimeric removal. The summary of OTUs and Tags number are shown in Table 1, and Figure 5.

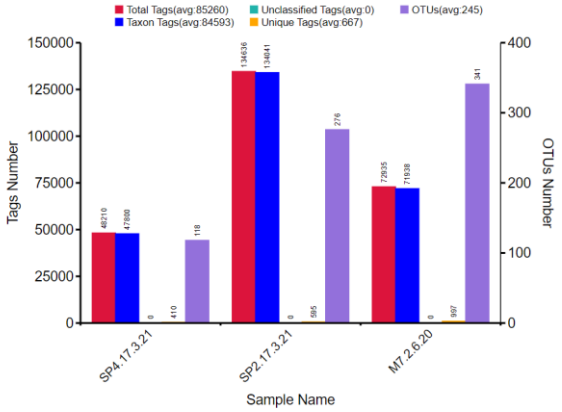


Figure 5. A summary of the tags and OTUs number of each sample

Molecular approach showed that OTUs processed from the three stations showing in Figure 1 contained similar diversity dominated by the Phylum Retaria in the Order Rotaliida and the Family Nummulitidae. Also, monophyletic group Euglenoza was present in all samples, except for SP2 samples which included species from the Phylum of Arthropoda Table 2 and Figure 6.

Table 2. Sequences with ≥ 97% similarity to the investigated samples

	Sequence Samples (Station No.)		
Sequence percentage match with ≥ 97%	**SP4 (St-1)**	**SP2 (St-2)**	**M7 (St-3)**
< 1 %	Euglenoza (Monophyletic group)	Euglenoza (Monophyletic group)	Euglenoza (Monophyletic group)
< 1 %	Retaria (Phylum)	Retaria (Phylum)	Retaria (Phylum)
< 1 %	Rotaliida (Order)	Rotaliida (Order)	Rotaliida (Order)
< 1 %	Nummulitidae (Family)	Nummulitidae (Family)	Nummulitidae (Family)
< 1 %		Arthropoda (phylum)	

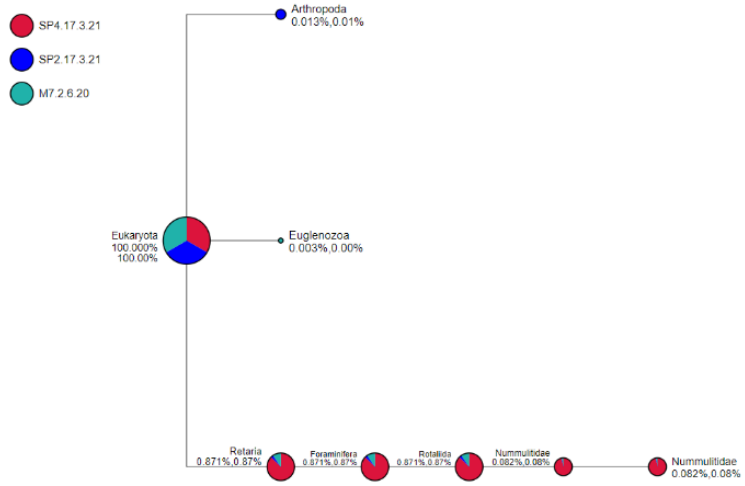


Figure 6. Molecular tree of sequence results

4. Discussion And Conclusion

The Linkage between characteristics of benthic foraminifera and foraminiferal eDNA was explored in this study of molecular investigation and morphological variety of foraminifera in the Arabian Gulf. From the benthic foraminiferal sites, twenty-four genera representing forty-five species were identified. Order Rotaliida and family Nummlitidae showed the most significant molecular data. Elphidium, Ammonia, Challengerella, Astrorotalia, Rosalina and Amphestigena were the most common species recorded, with a relative abundance of approximately 75% of the total foraminiferal numbers. The most frequently observed species less than 125µ in size were broken, the test was filled with sediment and glauconites, and cyanobacteria were also recorded with a slight test modification (diagenesis). A great number of ostracods with open shells were also detected in the studied stations, and a rare arenaceous species (Textularids) were observed. Quinquloclinids, Adelosinids, and Spiroloculinids all have a significant number of species. Small pelecypod shells showed a tiny pore on the shell as evidence of predation.

The species that have been discovered are often found in shallow water on the bottom surface, in silt, or sandy clay sediments in tidal and subtidal zones, whereas Quinqueloculina showed a significant increase in sand sediments at St-3 station. The difficulty in establishing evolutionary relationships between large groupings characterized primarily by morphological criteria and the enormous number of species concerned were the fundamental reasons why higher-level classifications of benthic did not advance (Pawlowski et al., 2012).

The molecular investigation using the universal marker 18S ribosomal RNA (rDNA) managed to identify the dominant groups of foraminifera represented by Rotallidae and Nummulitidae. According to the traditional morphological findings in this study, many of

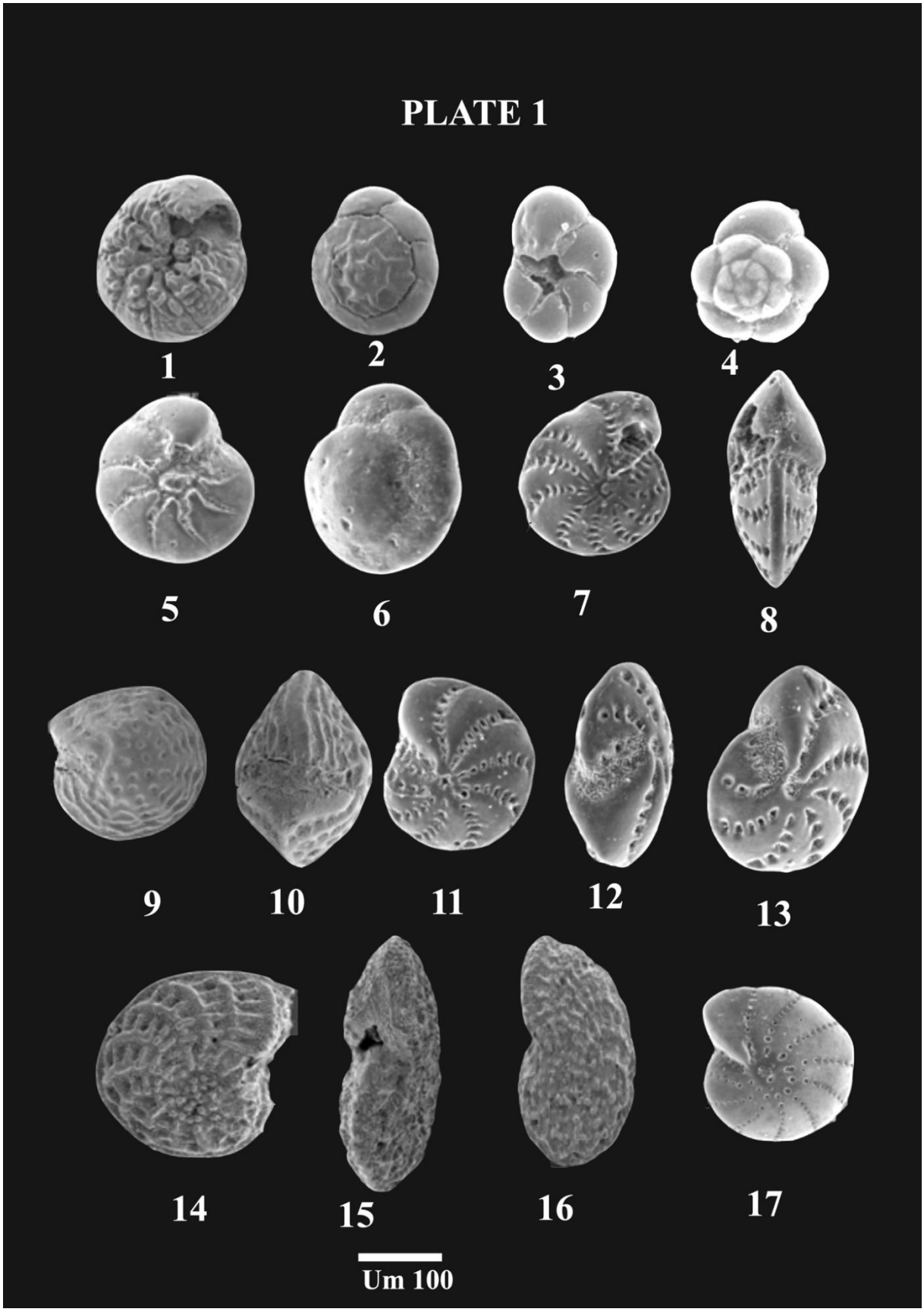
the foraminiferal individuals that were observed and identified by light microscope or SEM were not detected by molecular analysis, possibly the DNA region used are highly conserved and does not have enough discriminatory power to differentiate amongst closely related species. Therefore, different markers showing high discriminatory powers should be tested in future for instance, 28S rDNA and ITS rDNA (Pawlowski et al., 2012).

Plate 1
SEM images of the most abundant foraminiferal species recorded at this study.
Scale bar = 100 µ

1. *Ammonia beccarii* - umbilical view
2. *Ammonia beccarii* - spiral view
3. *Ammonia tepida* - umbilical view
4. *Ammonia tepida* - spiral view
5. *Ammonia parkinsoniana* - umbilical view
6. *Ammonia parkinsoniana* - spiral view
7. *Elphidium cf. E. advenum* - umbilical view
8. *Elphidium cf. E. advenum* - side view
9. *Elphidium craticulatum* - umbilical view
10. *Elphidium craticulatum* - side view
11. *Elphidium williamsoni* - umbilical view
12. *Elphidium williamsoni* - side view
13. *Elphidium williamsoni* - umbilical view
14. *Elphidium jenseni* - umbilical view
15. *Elphidium jenseni* - side view
16. *Elphidium jenseni* - spiral view
17. *Cribroelphidium poeyanum* - umbilical view

Plate 2
SEM images of the most abundant foraminiferal species recorded at this study.
Scale bar = 100 µ

1. *Challengerella bradyi* - umbilical view
2. *Challengerella bradyi* - spiral view
3. *Asterorotalia milletti* - umbilical view
4. *Asterorotalia milletti* - spiral view
5. *Asterorotalia gaimardii* - umbilical view
6. *Asterorotalia gaimardii* - side view
7. *Asterorotalia gaimardii* - spiral view
8. *Rosalina bradyi* - umbilical view
9. *Rosalina bradyi* - spiral view
10. *Rosalina* spp. - spiral view
11. *Rosalina suezensis* - umbilical view
12. *Amphistegina papillosa* - umbilical view
13. *Amphistegina papillosa* - spiral view
14. *Asterigerinata mamilla* - umbilical view
15. *Asterigerinata mamilla* - spiral view
16. *Neoeponides bradyi* - umbilical view
17. *Neoeponides bradyi* - side view
18. *Neoeponides bradyi* - spiral view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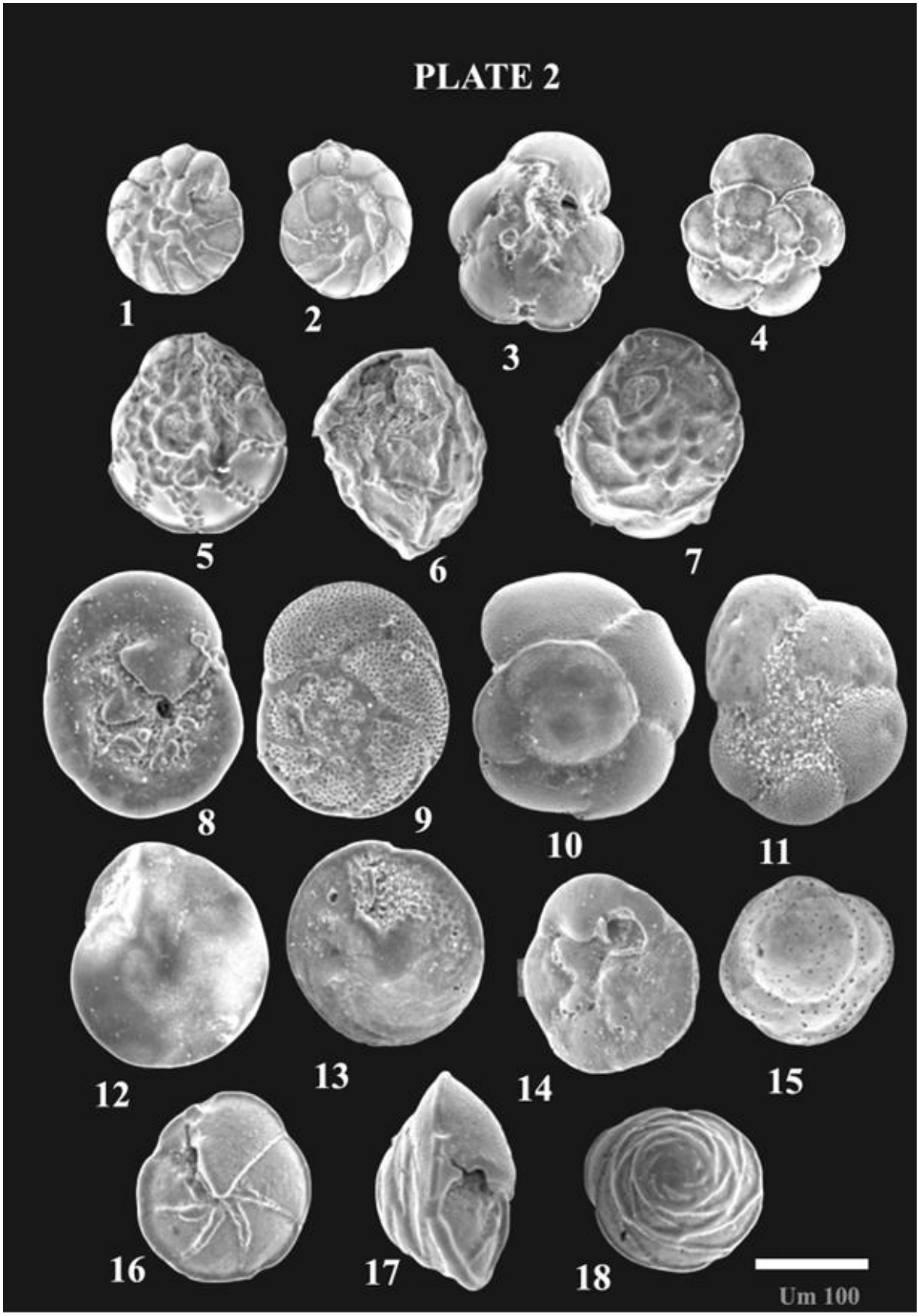
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Conflict Of Interest

The authors declare that they have no conflict of interest.

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