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Evaluation and optimization of DNA extraction methods for benthic foraminifera from the Gulf of Kachchh, Gujarat, India

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India has a vast coastline of 7460 km and prolific presence of foraminifers which are significant paleoclimatic proxies. However, no research has been carried out to extract genomic DNA from foraminifer and sequencing it for DNA barcoding. To initiate molecular study on benthic foraminifers from the Gulf of Kachchh, India, we tested out various kits based protocols and extract the foraminiferal DNA from *Ammonia* sp. Though most of the earlier methods yielded moderate amount of mean genomic DNA yield (0.87-9.07 ng/µL) they failed to amplify the DNA. Possibly, this is the first attempt in India wherein standardization of foraminiferal DNA extraction and PCR amplification is performed. This standardized protocol (modified protocol) yielded the highest quantity of mean genomic DNA yield (28.41 ng/µL) and its PCR amplification was also successful.

Keywords: *Ammonia* sp., DNA barcoding, Foraminifers, PCR amplification, rDNA

Foraminifera have been studied for more than two centuries to understand the paleoclimatic conditions, aquatic pollution and to evaluate the overall environmental quality using conventional taxonomic methods[1]. After the invention of DNA barcoding, DNA extraction, PCR amplification and sequencing have become vital tools in modern taxonomy. Accordingly, the need to sequence the DNA of foraminifers in the laboratory has long been felt by the researchers in India. The existing classification system depends on the composition of the test wall, number and arrangement of chambers and the morphology of the aperture which often results in taphonomic bias[2]. Therefore, molecular studies of the foraminifers based on 18S rRNA have been initiated which provides invaluable insights into marine speciation, evolution, phylogenetics and biogeography[3-9].

Significant success has been achieved in sequencing DNA of both planktonic and benthic foraminifers from different parts of the world[7,8,10-12]. Nevertheless, in India which has a vast coastline of 7460 km and prolific presence of foraminifers, no research has been carried out to extract genomic DNA from foraminifer and sequencing it for DNA barcoding. However a study focused on the extraction of 12S mitochondrial DNA which is responsible for the dimorphism and coiling direction of benthic foraminifers has been reported[13]. Realizing these lacunae, we have carried out the DNA extraction and amplification of the foraminifers. Possibly, it is the first report on DNA extraction and amplification of benthic foraminifers collected from the coast of Gulf of Kachchh, India.

Ribosomal genes were used in molecular systematics of foraminifers[14]. Compared with other eukaryotes, foraminiferal ribosomal genes are highly divergent because of numerous substitutions, helping in phylogenetic analyses of this gene[15]. The molecular systematic analysis utilizing mitochondrial DNA has been done for benthic foraminifer, *Ammonia* sp. The objectives for selecting this particular species are: (i) they are highly abundant and results can be replicated easily; and (ii) most of the published molecular techniques are successfully applied on *Ammonia* sp., and hence they can be evaluated and directly applied on them.

Materials and Methods

Reagents

All the chemical reagents were procured from HiMedia unless and otherwise specified. The

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glasswares were acquired from Borosil and the plastic wares from Tarsons. The PCR primers were synthesized by Eurofins Genomics India Pvt. Ltd. The different ready-made kits utilized have been specified wherever used.

Specimen collection & Isolation

Live specimens were collected from Nana Layza, Kachchh (N 22˚50.203', E 069˚13.185') in December 2015. The sediments collected from the top 1-2 cm of intertidal zone were sieved through 1.0 mm sieve to remove micro arthropods, annelids and coarser sand grain. The sediments were again sieved through 100 µm sieve to remove fine silt, clay and sand. The remaining sediments were filled up to 20% in air tight plastic bags which contained 60% seawater and 20% air. They were brought to the laboratory immediately and were transferred to large plastic trays with fresh seawater. The sediments were kept overnight for the live foraminifers to travel to the surface. Specimens with visible and colourful cytoplasm (greenish, brownish, whitish) were considered as alive and were collected under Magnus Stereozoom Microscope with the help of a pasture pipette and transferred to a Petri dish containing sterile seawater and identified based on Loeblich & Tappan[16].

The most abundant species i.e. *Ammonia* sp. were then isolated and photographed (Fig. 1) using the CMOS camera and stored into 1.5 mL micro centrifuge tubes containing the Tris EDTA buffer (10 µL) for further processing. The samples were then stored at 4℃ until DNA extraction was carried out.

DNA extraction

Extraction was performed using three different types of DNA extraction kits i.e., DNAeasy Blood and Tissue Kit (Qiagen), Prepman Ultra Kit (Applied Biosystems) and Powersoil DNA Extraction Kit (Mo Bio). Reported protocols were also tried with three different lysis buffers namely DOC lysis buffer[11], 4 M GITC buffer without EDTA (without any reducing agent)[12] and 4 M GITC buffer with EDTA[17]. GITC buffer without EDTA[12] was used without crushing the tests of the foraminifers while with EDTA[17] was used after crushing the tests with a homogenizer. The comparison of the protocols is given in Table 1.

A modified protocol was also developed. In this protocol, the ATL lysis buffer from Qiagen was used. The tests were crushed and 100 µL lysis buffer was added to all the MCTs and kept for lysis at 75℃ for 3 h. Then after adding chloroform:isoamyl alcohol (24:1), they were centrifuged for 10 min at 13000 rpm. After retaining the aqueous layer, ice cold ethanol and sodium acetate were added and then precipitation was

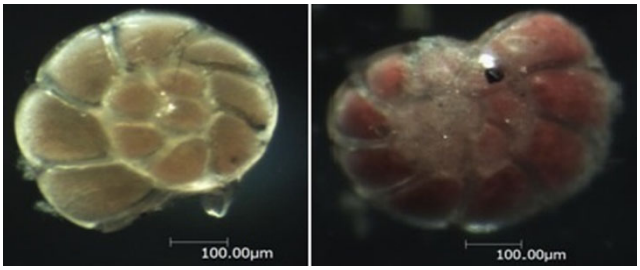

Fig. 1 — *Ammonia sp.* photographed at 40X under Magus stereozoom microscope (MSZ-TR) with CMOS Camera.

Table 1–Comparison of the three manual protocols

Step No.	Protocol name: 4 M GITC buffer without EDTA[12]	4 M GITC buffer with EDTA[17]	Modified Protocol
1	Samples placed in lysis buffer	Samples placed in lysis buffer	Samples placed in ATL lysis buffer
2	Heated to 75℃ for 24 h	Specimens are grinded & heated to 60℃ for 10 min	Specimens are grinded & heated to 75℃ for 3 h
3	Chloroform:Isoamyl Alcohol added & centrifugation performed at 13000 rpm for 10 min	Centrifugation performed at 13000 rpm for 10 min and supernatant extracted out	Chloroform:Isoamyl Alcohol added & centrifugation performed at 13000 rpm for 10 min
4	Aq. layer retained to which ice cold ethanol and 3M NaOAc is added	Isopropanol added to supernatant & centrifugation performed at 13000 rpm for 5 mins	Aq. layer retained to which ice cold ethanol and 3M NaOAc is added
5	Precipitation done overnight at –80℃ and centrifugation performed at 15000 rpm for 10 min at℃ 5℃	Precipitation done overnight at –20℃ and centrifugation performed at 15000 rpm for 10 mins at 5℃	Precipitation done for 3 h at –80℃ and centrifugation performed at 13000 rpm for 10 mins at 5℃
6	Pellet washed with 70 % ethanol & centrifugation performed at 15000 rpm for 5 min	Pellet washed with 70 % ethanol & centrifugation performed at 15000 rpm for 5 min	Pellet washed with 70 % ethanol & centrifugation performed at 15000 rpm for 5 min
7	Remaining ethanol removed & air dried	Remaining ethanol removed & air dried	Remaining ethanol removed & air dried
8	DNA resuspended in Tris buffer and stored at –80℃	DNA resuspended in sterile H_2O and stored at –20℃	DNA resuspended in Tris buffer and stored at –20℃

performed at –80°C for 3 h. After centrifugation for 10 min at 15000 rpm, the pellet was retained and washed in 300 µL ethanol (70%) before being centrifuged again for 5 min at 15000 rpm. The remaining ethanol was removed and the sample was air dried. The DNA was then re-suspended in 30 µL TE buffer and stored at –20°C.

All the protocols were tried in triplicates and in each individual trial three specimens of the *Ammonia* sp. were taken for DNA extraction. Genomic DNA yield was quantified using QIAxpert System (Qiagen) at 260 nm and a mean value for the triplicate of each method was calculated.

Amplification of DNA

Amplification was performed using a set of different primers as shown in Table 2. In total, 6 different forward primers and 6 different reverse primers were used. Different combinations were tried along with different PCR conditions in Applied Biosystems™ Veriti™ Thermal Cycler. The annealing temperature was shifted based on the melting temperature (T_m) of the primers. But only a particular set of primer and nested primer yielded positive result. In this method, for the first round of PCR 5 µL of DNA, 10 µL Sigma Master Mix, 1.2 µL $MgCl_2$, 1 µL Forward primer and 1 µL Reverse primer was added in steps. Thermal cycles used were 95°C for 3 min (1 cycle); 95°C for 45 s, 50-55°C for 45 s and 72°C for 2 min (35 and 25 cycles for first round (1˚) and nested (2˚) PCR); 72°C for 7 min. For 2˚ PCR, 3 µL of 1˚ PCR product was added to 10 µL Sigma Master Mix, 1.2 µL $MgCl_2$ and 1.0 µL Forward and Reverse primers. Later the amplified product was visualized on 2% agarose gel using Gel Doc XR+ system.

Table 2 — The primers used for the amplification

Name & Reference	Sequence (5'-3')	Length (bp)	Type
s14F3[19]	ACGCAXGTGTGAAACTTG	18	Forward
sB[19]	TGATCCTTCTGCAGGTTCACCTAC	24	Reverse
s14F1[19]	AAGGGCACCACAAGAACGC	19	Forward
C5[12]	GTAGTATGCACGCAAGTGTGA	21	Forward
138[12]	TGATCCTGCAGGTTCACCTAC	21	Reverse
2082F[12]	TGAAACTTGAAGGAATTGACGGAAG	25	Forward
2514R[12]	GGCATCACAGACCTGTTATTGCC	23	Reverse
S15R[17]	GTGGTGCATGGCCGT	15	Forward
S21F1[17]	CCTTGTTACGACTTCTC	17	Reverse
S18[17]	TAACAGGTCTGTGATGCC	18	Forward
S20R[17]	GACGGGCGGTGTGTACAA	18	Reverse
newB[20]	TGCCTTGTTCGACTTCTC	18	Reverse

Results

Three kits were used for DNA extraction from *Ammonia* sp. namely DNeasy Blood & Tissue Kit (Qiagen), Prepman Ultra Kit (Applied Biosystems) and Powersoil DNA Extraction Kit (Mo Bio); however no significant yield was obtained as shown in Table 3. The nested PCR amplification of these extracted DNA did not show any band in the 2% agarose gel.

The use of different reported protocols based on the differences in the buffer composition showed variable yields of genomic DNA extract. The DOC lysis buffer[11] yielded the least amount of genomic DNA (2.87 ng/µL) even after breaking the tests. Moderate amount of genomic DNA (8.4 ng/µL) was obtained when the 4 M GITC buffer without EDTA[12] was applied without crushing the tests with 24 h incubation time at 75°C. Higher yield of genomic DNA (9.07 ng/µL) were obtained by 4 M GITC buffer with EDTA[17] when the foraminiferal tests were crushed using homogenizer.

Genomic DNA yield obtained was subjected to amplification using various primer combinations as shown in Table 2 but none of the above mentioned protocols were able to amplify DNA. Hence, a modified protocol was developed using ATL lysis buffer and the combination of other reported

Table 3—Genomic DNA extracted by different extraction protocols

Protocol & Reference	Genomic DNA yield (ng/µL)	Mean genomic DNA yield (ng/µL)
Qiagen Blood & Tissue Kit	0.6 1.2 0.8	0.87
Applied Biosystems Prepman Ultra Kit	1.4 1.7 1.5	1.53
Mo Bio Powersoil Kit	0.9 0.5 1.0	0.8
DOC lysis buffer[11]	2.4 3.5 2.7	2.87
4 M GITC buffer with EDTA [15]	9.5 10.1 7.6	9.07
4 M GITC buffer without EDTA [10]	6.4 10.3 8.5	8.4
Modified Protocol (This Paper)	29.47 27.1 28.67	28.41

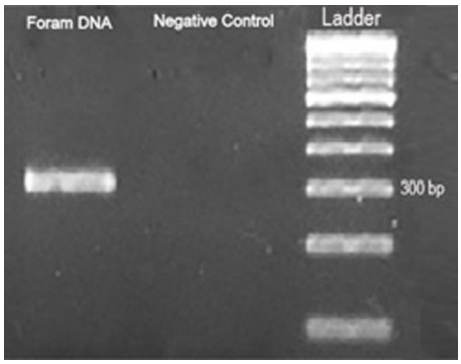

Fig. 2 — Two per cent agarose gel showing PCR products obtained by DNA extracted via the modified method using Gel Doc XR+ system. [The 1˚ PCR was carried out using primer pairs C5 and 138 while 2˚ PCR was carried out using S15R and S20R primer pair]

protocols[12,17]. There was significant increase in the mean genomic DNA yield (28.41 ng/µL). This standardization of the method also leads to successful amplification (~310 bp band) after nested PCR as shown in Fig. 2.

Discussion

The effectiveness of various protocols was tested using genomic DNA yield and the presence of bands on agarose gel. No significant yield was obtained from the ready-made kits which may be due to the fact that they are not designed specifically for DNA extraction from foraminifers. Lecroq[17] also recommended that DNA extraction from single cells should not be performed using commercially available kits.

Different lysis buffers varied greatly in their performance in terms of efficiency in yield of genomic DNA and success in PCR. Seears & Wade[12] also reported that the different lysis buffers tested in their study varied greatly in performance in terms of efficiency in removing cellular material from within foraminiferal shells, yield of crude DNA gained, success in PCR, and shell integrity at the end of the process. Of the reported buffers tested in this study, 4 M GITC with EDTA[15] buffer gave overall favourable results, producing high yields of genomic DNA as compared to DOC lysis buffer[11] and 4 M GITC without EDTA[12]. This might be due to the fact that GITC is a strong protein denaturant[18] as well as DNase inhibitor and tests were crushed in 4 M GITC with EDTA[17] while they were kept as it is in 4 M GITC without EDTA[12]. But the quantity of crude genomic DNA was very low as compared to that obtained by Seears & Wade[12] wherein they used intact foraminiferal shells. This seemed to be the reason for PCR amplification failure of genomic DNA extracted after using all the three reported protocols as Akbari *et al.*[21] also observed that low amount of DNA template in the PCR mix provide no or error prone amplification.

The modified protocol based on ATL lysis buffer developed in this study provided the highest quantity of genomic DNA as well as success in PCR amplification. As shown by Ishimura *et al.*[22], benthic foraminifers show inter-individual isotopic variations because of geographical distances. These variations are useful for exploring which species are most appropriate to use as paleo-indicators in paleo-environmental studies. Similarly, these variations might also be necessitating changes in DNA extraction and PCR amplification protocols which has to be subjected to further studies before final conclusions.

Conclusion

Due to easy and vast availability of *Ammonia* sp., it was used for the present study. Various kits based and reported protocols were tried for DNA extraction, but the modified protocol gave the best genomic DNA yield (28.41 ng/µL) along with successful amplification (~310 bp) of its extracted DNA.

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