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ECOPHENOTYPES: GENETIC AND ENVIRONMENTAL INFLUENCES ON SHELL MORPHOLOGY IN VENERID CLAM, *PAPHIA MALABARICA*

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Abstract- Phenotypic plasticity in *Paphia malabarica* was analysed using shell morphometry and molecular analysis. Intra-specific variability reflects changing ecophysiological requirements and constraints with body size, alongside genetic and environmental influences on shell morphology. External shell trait measurements in the morphotypes displayed phenotypic plasticity. Molecular analysis using Internal Transcribed Spacers sequence region of nuclear ribosomal DNA revealed that the genetic distance between the *Paphia* populations of the estuary was not significant to consider them as genetic variants. Phylogenetic analyses confirmed that the morphotypes are sister clades with cent percent bootstrap value. Hence, it can be authenticated that the morphotypes are clams exhibiting phenotypic plasticity and they are ecophenotypes resulted from the environmental factors. The morphometrics can quantify a trait of evolutionary significance, and deduce something of their ontogeny or evolutionary relationships by detecting changes in the shape of the organism.

Keywords- Ecophenotypes, morphotypes, morphometric traits, Internal Transcribed Spacers, *Paphia malabarica*, phenotypic plasticity, phylogeny

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Introduction

The family Veneridae comprises macro-benthic, filter-feeding, marine or estuarine bivalves. They enjoy ubiquitous distribution ranging from temperate to tropical waters [1]. They usually burrow in muddy or sandy habitats, but vary considerably in lifestyles, since they live in coral reefs and lagoons, mangrove zones, intertidal flats, bays, estuaries, estuarine lagoons, surf zones and the deep sea [2]. Despite their colonisation on all types of habitat, from coastal to deep-sea areas, Venerids generally exhibit few morphological diversity connected with soft-tissue anatomy. This makes it difficult to identify the cases of morphological parallelism among evolutionarily distant species and of shell diversification among closely related ones.

Morphological variations in bivalve shells are increasingly the focus of diverse studies that bridge palaeontology and ecology. Morphometric descriptions of bivalve shells contain information on phylogenetic relatedness at low taxonomic levels. The morphometrics can quantify a trait of evolutionary significance, and infer something of their ontogeny or evolutionary relationships by detecting changes in the shape of organisms. Morphometrics form tools that allow comparisons to describe complex shapes in an austere fashion, and permits numerical comparison between different forms. Most bivalves are ideal subjects for studying the relationship between body form and ecology. Their shape and growth are directly controlled by habitat specific factors due to the sedentary life of these organisms.

Several studies on the bivalve shells have proved its use in defining both intra and inter-specific variations among different population inhabiting wide geographical range [3-8]. Such variability is likely to reflect changing eco-physiological requirements and constraints with body size, alongside genetic and environmental influences on shell morphology [9-10].

A non-morphological criterion for mollusc species identification was carried out through ribosomal DNA (rDNA). It has both rapidly and slowly evolving regions, and it is particularly useful for phylogenetic analysis [11]. Ribosomal DNA internal transcribed spacer (ITS) sequence variation has generally proven to be a powerful tool for studying phylogenetics and for species identification [12-13]. It has been used in a wide range of invertebrates [14-15] including molluscs [16-17]. Several researchers showed that the ITSs sequences show more divergence than their flanking regions and are easily amplified. Hence they can be used to distinguish related species and to infer phylogenetic relationships from population to families and even higher taxonomic levels [18-19].The difficulty in using these sequences stems from the occurrence of multiple copies per genome [20], which opens the possibility of intra-individual and intra-specific variation.

Morphometric analysis of shell shape and size seems a priori to be a realistic alternative for inter-group discrimination. Morphometric studies yield valuable information for managing fishery resources and understanding environmental changes. The analysis of shape profiles in bivalves can underpin the geographically based studies of morphological variation that occur in individuals of different population. The main objectives of our study were to investigate (1) the extend of intra-species external trait variations in the morphotypes and whether these differences were reflected in the molecular analysis, and (2) phylogenetic structure and evolutionary divergence in the morphotypes.

Materials and Methods

Morphometric analysis

Random samples of bivalve clam, *Paphia malabarica* were collected from the clam beds of Ashtamudi estuary, a deep estuary. The clams collected were transported to the laboratory and kept in aerated habitat water for twenty four hours for defecation. The clams were grouped in to two sets, set I constituted the clams collected from the upper reaches of the estuary and that of the barmouth was designated as set II. About hundred clams from each set were sacrificed for biometric measurements. The shell length, shell width, shell thickness and inflation of the shells were noted using digital vernier callipers. T-test was carried

out to analyse the mean difference between the shell traits observed in *P. malabarica* morphotypes. Statistical analysis was carried out using SPSS (version 20.0).

Amplification, sequencing and analysis of the Internal Transcribed Spacers (ITSs) in the nuclear ribosomal DNA (nrDNA) of Venerid clam *Paphia malabarica* and its morphotype (hereafter designated as *Paphia malabarica* strain *neendakarayansis*) were carried out to estimate the evolutionary divergence between the morphotypes.

Sample collection and DNA extraction

Live adult clam samples of *Paphia malabarica* and its morphological variants were used for the study. Total genomic DNA was extracted from approximately 200 mg of adductor muscle following modified CTAB protocol [21]. The tissue was incubated for 15 minutes at 55°C in 600 µl CTAB buffer containing 25 µl, 10 mg/ml proteinase K, homogenised with a pestle, and incubated for an additional 60 minutes. Initially the DNA was extracted with saturated phenol and then with chloroform: isoamyl alcohol (24:1). Genomic DNA was ethanol precipitated. DNA pellets were dissolved in 50 µl TE (10 mM HCl, pH8.0, 1 mM EDTA) and stored at -20°C for further use.

PCR amplification and sequencing

The internal transcribed spacer region of nuclear ribosomal DNA was amplified using the following primers. ITS1 F (5'-GGTGAACCTGCGGATGGA -3') and ITS1 R (5'-GCTGGCTGCGCTCTTCAT -3') are primers that annealed to the 3' end of 18S rDNA gene and the 5.8S rDNA gene, respectively. ITS2 region was amplified using ITS2 F (5'-ATGAAGAGCGCAGCCAGC-3') and ITS2 R (5'-GGCTCTTCCCGCTTCACTC-3') as primers that annealed to the 5.8S rDNA gene and the 5' end of 28S rDNA gene. PCR was performed in a total reaction mixture of 50µl of the isolated genomic DNA from clam samples to amplify the ITS region. Two pairs of primers were designed based on sequence information obtained from GenBank [GenBank: AY498751, AF202106, AF131019, AY198756, and AF120559] using Primer Select Software (DNA Star Package Version 5.01). The PCR was performed with the above-mentioned primer pair. The PCR mixture contained 1µl of isolated genomic DNA (50 ng) from each sample, 1 µl of each primer (10 pmol/µl), 2 µl 10 mM deoxyribonucleoside triphoshate, 5 µl 10X PCR buffer containing $MgCl_2$, and 1µ l of 5 U/µl *Taq* DNA Polymerase. The PCR reaction was conducted with the initial denaturation at 94 °C for 2 minutes followed by denaturation at 94 °C for 45 seconds, annealing at 62 °C for 60 seconds and elongation at 72 °C for 2 min. These cycles were then followed by 34 cycles of denaturation, annealing and elongation followed by an extended final elongation step at 72 °C for 10 min. The PCR products were electrophoresed in a 1 % (w/v) agarose gel stained with ethidium bromide and observed on an UV transilluminator. The amplicon was excised from the gel and the DNA was eluted from the gel slice by using Fermentas Gene Jet Gel extraction kit according to the manufacturer's specifications. Sequencing was done using the big dye terminator kit in ABI 3730 XL DNA analyser. The obtained forward and reverse sequences were aligned to get the contig sequence using Sequencher software and were analysed.

Phylogenetic analysis

The forward and reverse sequences were assembled using SeqManII software in DNA Star Package version 5.01 to obtain ITS1 and ITS2 sequences. Obtained ITS1, 5.8s, ITS2 and 28s sequences were deposited in NCBI GenBank. Each newly determined sequence was checked against existing haplotypes using DNA star and then the sequence was registered as a new haplotype. Since ITS1-R and ITS2-F primers anneal to the same region in 5.8S rDNA, the complete sequence of 5.8S ribosomal RNA gene was produced by assembling ITS1 and ITS2 into an ITS. The boundaries of coding and spacer regions were determined by comparison with the sequence information of *Arctica islandica* [GenBank: AF202106]. The sequences were edited and analysed using programme EdiSeq. ITS sequence containing the 5.8S rDNA gene across the species was aligned using the ClustalW (EMBL-EBI).

The phylogenetic trees were constructed using MEGA version 5 [22] in the two different phylogenetic modes, Maximum-Parsimony (MP) [23] and Minimum-Evolution (ME) [24]. Maximum- Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm [25]. The phylogenetic tree by Minimum-Evolution method was constructed by applying Maximum Composite Likelihood (MCL) approach [26]. Complete deletion options were used for handling gaps in all analysis. The bootstrap values [27], that indicate the robustness of nodes in Maximum- Parsimony trees were inferred from 500 replications. The molecular clock test was performed by comparing the Maximum Likelihood (ML) value for the given topology with and without the molecular clock constraints [28].

Sequence availability

The nucleotide sequence data of the study have been submitted to GenBank Nucleotide Sequence Library under the following accession numbers *Paphia malabarica,* JX997826 and *Paphia malabarica* strain *neendakarayansis*, KC416612.

Results

Intra-species variation in the external shell morphometry: External shell morphometry studies in *P.malabarica* revealed variations in external shell traits [Fig-1]. The clams collected from the population near the barmouth of the estuary (Type II) was found to be smaller than the clams collected from the upper reaches of the estuary (Type I). They exhibited improper shell formation, uneven thickness and with slightly bean shaped ventral outline [Fig-1b].The statistical analysis of shell traits confirmed the phenotypic plasticity among the *Paphia* population.

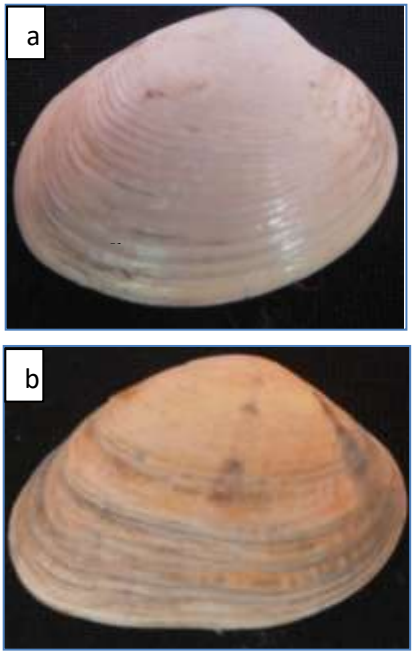


Fig-1 Morphotypes of *Paphia malabarica* in the estuary morphotype I (b) morphotype II.

Sequence alignment

The sequences obtained were identified using NCBI-BLAST [29] [Fig-2] and rechecked using CLUSTAL X [30]. Besides these sequences, 19 sequences were retrieved from GenBank for multiple sequence alignment (MSA).

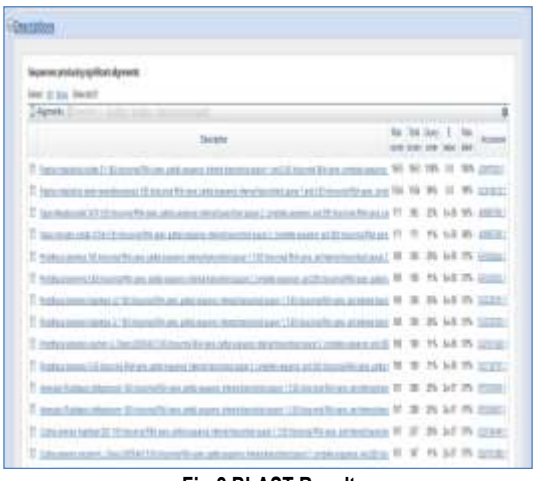
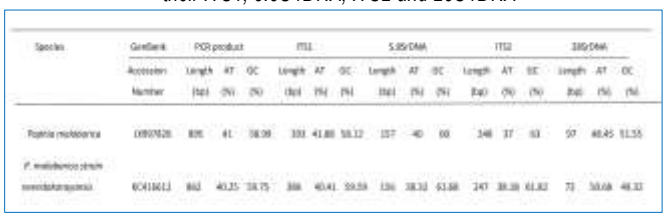


Fig-2 BLAST Result

Analysis of PCR amplicons

The entire ITS region was amplified from 10 individuals each of the two morphotypes. In all cases PCR yielded a single band of sequence approximately 900 bp in length for *P. malabarica* and its morphological variant. Using the sequence information of *Arctica islandica* (GenBank: AF202106], the boundaries of coding and spacer regions were determined. The total length of the sequence in *Paphia malabarica* was found to be 895bp with an AT and GC content of 41% and 58.99% respectively whereas its morphological variant, *Paphia malabarica* strain *neendakarayansis* rendered only 862bp with 40.25% AT content and 59.75% GC content [Table-1].

Table-1 *Species name, total length, GC content, AT content of PCR product and their ITS1, 5.8S rDNA, ITS2 and 28S rDNA*



ITS1 sequence of *P .malabarica* produced 393bp with 41.88% of AT content and 58.12% of GC content. Whereas in the morphological variant, the ITS1 region rendered 386bp with 40.41% AT content and 59.59% GC content. ITS1 region lacked microsatellites. The 5.8s rDNA portion of *Paphia malabarica* furnished 157bp with AT and GC content respectively as 40% and 60%. But the 5.8s region in the morphologic variant yielded only 156bp with 38.32% of AT content and 61.68% of GC content. The ITS2 region generated 248bp with 37% AT content and 63% GC content in *Paphia malabarica* and in the morphological variant the same region generated 247bp with 38.18% AT content and 61.81% GC content. The 28s rDNA region in *Paphia malabarica* generated 97bp with 48.45% AT content and 51.55% GC content whereas its morphological variant produced only 73bp with 50.68% AT content and 49.32% GC content [Table-1].

In both, the ITS2 region possessed three dinucleotide microsatellites. First set was formed of three repeat units of CG from 564 to 569bp in *Paphia malabarica* and from 555 to 560bp in its morphological variant. Second set of microsatellite was formed of four repeat units of CT and were located from 606 to 613bp in *Paphia malabarica* and from 597 to 604bp in morphological variant. Third set of microsatellite consisted of three repeats units of GC and were located from 704 to 709bp in *Paphia malabarica* and from 695 to 700bp in its morphological variant [Table-2].

Table-2 *Microsatellites in the ITS2 region of sequences.*

Species	GenBank accession No.	Location (bp)	Microsatellites
P. malabarica	JX997826	564-569 606-613 704-709	CGCGCG CTCTCTCT GCGCGC
P.malabarica strain *neendakarayansis*	KC416612	555-560 597-604 695-700	CGCGCG CTCTCTCT GCGCGC

Sequences of conserved motifs were located in both the ITS1 and ITS2 regions [Table-3].Two conserved motifs were identified in the ITS1 region. The first conserved motif contained 30 bp .The second conserved motif was constituted by 25 bp. The ITS2 region depicted two conserved motifs. The first conserved motif was with 20 bp. The second conserved motif was with a size of 21 bp.

Phylogenetic analysis

For the 21 species analysed, 210 sites were found to be phylogenetically informative Using *Arctica islandica* as out group, the MP analysis generated the MP tree [Fig-3] and the ME tree obtained by MCL method is represented in [Fig-4]. Results of the MP tree were coherent with those of ME tree. The differences in topology between the MP and ME tree were limited to weakly boot straps supported branching points. The phylogenetic trees generated indicated that the members were constituted by two well differentiated clades having three well resolved groups. The estimated evolutionary divergence between sequences is depicted in [Table-7]. Average Kimura 2- parameter genetic difference between populations indicated the pair wise distance between the *P.malabarica* and its morphological variant was a negligible value (0.010).

Table-3 *First conserved motif in ITS1 region of the sequences.*

Species	Location in ITS1	Sequences
P. malabarica	46-75	CGGCGGCGAC CGGCCGTCCA CAGAGGCGTT
P.malabarica strain *neendakarayansis*	37-66	CGGCGGCGAC CGGCCGTCCA CAGAGGCGTT

Table-4 *Second conserved motif in ITS1 region of the sequences.*

Species	Location in ITS1	Sequences
P. malabarica	344-368	CCGCCTGTGT TGCGCGGGCG GCAGA
P.malabarica strain *neendakarayansis*	336-360	CCGCCTGTGT TGCGCGGGCG GCAGA

Table-5 *First conserved motif in ITS2 region of the sequences.*

Species	Location in ITS2	Sequences
Paphia malabarica	552-571	GCGTTGGCGA GTCGCGCGGG
P.malabarica strain *neendakarayansis*	543-562	GCGTTGGCGA GTCGCGCGGG

Table-6 *Second conserved motif in ITS2 region of the sequences.*

Species	Location in ITS2	Sequences
P.malabarica	578-598	CCCGCTCGTC CGCCGAAGAA T
P.malabarica strain *eendakarayansis*	569-589	CCCGCTCGTC CGCCGAAGAA T

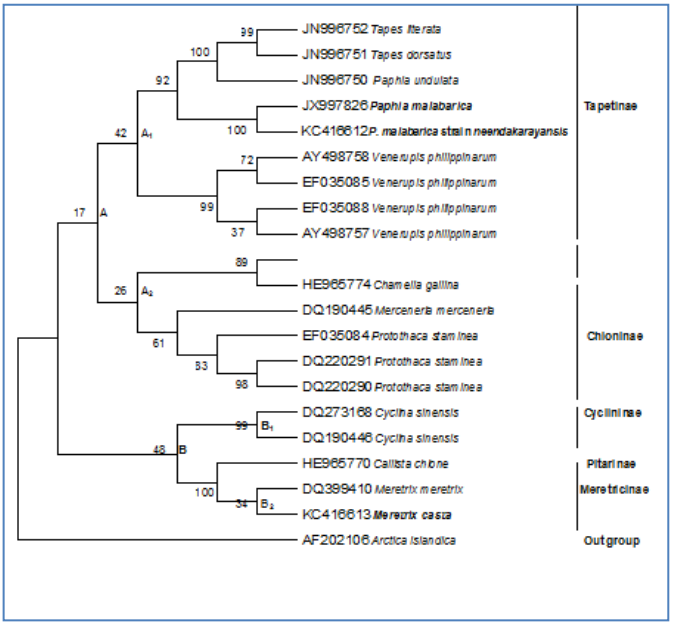


Fig-3 Evolutionary relationships of 21 taxa inferred using the Maximum-Parsimony method

Table-7 *Estimates of Evolutionary Divergence between sequences of sampled clams.*

GenBank Accession No.	JX997826	KC416612
JX997826		
KC416612	0.010	

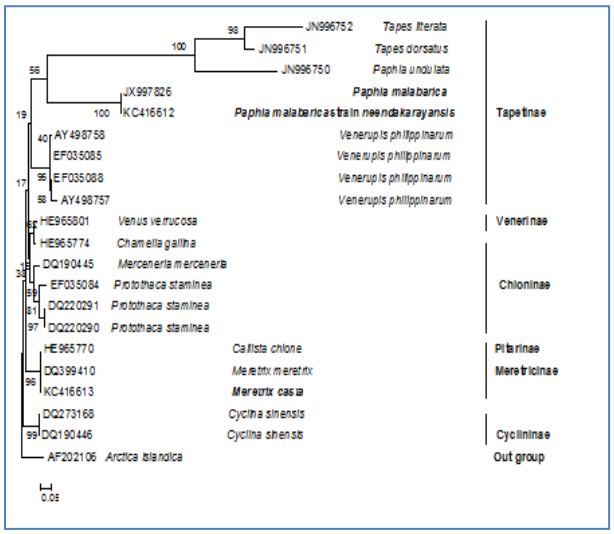


Fig-4 Evolutionary relationships of 21 taxa inferred using the Minimum-Evolution method.

Test of the Molecular Clock

Molecular tree of 21 taxa studied is depicted in [Fig-5]. Maximum Likelihood method comparing trees in which the clock was both relaxed and enforced are presented in [Table-8]. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated.

Table-8 *Result from a test of molecular clocks using the Maximum Likelihood method*

	lnL	Parameters	(+G))	(+I)
With Clock	-3111.046		n/a	n/a
Without Clock	-2744.724		n/a	n/a

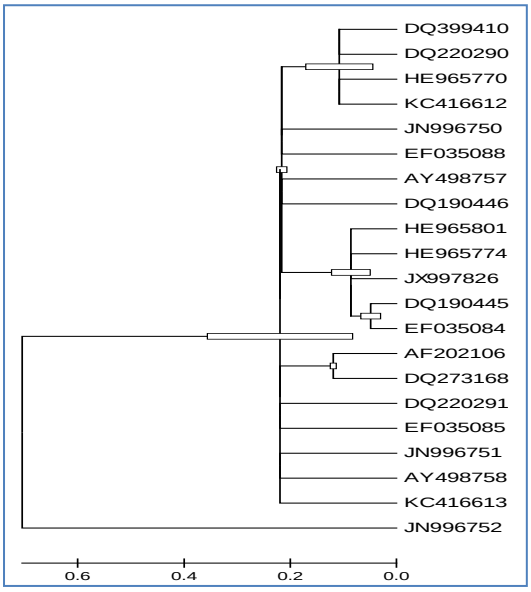


Fig-5 Molecular tree of 21 taxa constructed using Maximum Likelihood method

Phylogenetic inference

Both the trees produced were supported by very high bootstrap values (60 % to 100%). The trees were bifurcated into two major clades. The rooted MP tree delineated two clades, clade A and clade B. The clade A clustered the members of subfamilies Venerinae, Chioninae and Tapetinae. The clade A in turn gets divided into two clades A1 and A2. The clade A1 includes the members of subfamily Tapetinae. The clade A2 includes the members of subfamilies Venerinae and Chioninae. The clade B includes the members of subfamily Cyclininae, Pitarinae and Meretricinae. Thus all the tree analysis point to the presence of three principal groups. First group consists of taxa *Venus verrucosa* (Venerinae), *Chamelia gallina, Merceneria merceneria* and *Prototaca staminea* (Chioninae). The second group formed of taxa *Tapes literata, Tapes dorsatus, Paphia undulata, Paphia malabarica, Paphia malabarica* strain *neendakarayansis* and *Venerupis philippinarum* (Tapetinae). The third group is constituted by the taxa *Cyclina sinensis* (Cyclininae), *Callista chione* (Pitarinae), *Meretrix meretrix* and *Meretrix casta* (Meretricinae). The Venerid clams *Paphia malabarica* and its morphological variant *Paphia malabarica* strain *neendakarayansis* formed sister clades with high bootstrap values and are clustered with *Paphia undulata*, *Tapes dorsatus* and *Tapes literata* with very higher bootstrap values and were included in the sub family Tapetinae. The clam *Meretrix casta* formed sister clades with *Meretrix meretrix* and were included in the subfamily Meretricinae.

Discussion

Genetic information in biological molecules such as proteins and DNA can be used to address numerous aspects of behaviour, life histories and evolutionary relationships of organisms. Ribosomal DNA (rDNA) sequences have been aligned and compared in a number of living organisms and this approach yielded a wealth of information about phylogenetic relationships in bivalve molluscs. The systematic versatility and numerous rates of evolution among different regions of rDNA make it capable to address phylogenetic problems.

The characteristics and variations in the nucleotide sequences of ITS1, 5.8S, ITS2 and 28S regions of ribosomal DNA of *Paphia* morphotypes (*P.malabarica, P.malabarica* strain *neendakarayansis*) were demonstrated through PCR amplification and sequencing.

The size of ITS is species dependent and the difference could be significant among species [31]. The length of ITS2 was shorter than ITS1. The length of ITS2 region in *P. malabarica* and *P. malabarica* strain *neendakarayansis* was found to be similar and with similar AT and GC content. But the ITS1 region showed length variation. The number of base pairs and the AT content of *P. malabarica* was more than that in its morphologic variant whereas the GC content was found to be more in the variant. In *P. malabarica* length of 5.8S rDNA was consistent with that reported earlier in other Venerids, but it was 1 base pair less in its morphological variant. The conserved motifs in the ITS region of the nucleotide sequences indicate that these motifs might be involved in certain nucleic acid-related function, such as rRNA processing [32]. The dinucleotide microsatellites (CG), (CT), (GC) and (GA) present in the ITS sequence can be used as good markers. The dinucleotide and trinucleotide microsatellites were reported in ITS sequences in clams belonging to Veneridae [33-34].

Intra-species sequence divergence in the ITS region were recorded earlier, but the sequence can be considered different only if sequence divergence is more than 0.9 % [35]. Intra-species sequence divergence was observed in *P. malabarica*. The two sequences exhibited 1% divergence in BLAST. The value of average Kimura 2-parameter genetic difference between the populations gives a quantitative measure of DNA change due to deletion and insertion. A small value of the parameter is an indication of the resemblance of the sequence. The relatively smaller value between the sequences of *Paphia* population indicates that they are closely related. Further Phylogenetic analysis revealed that the genetic distance between the two *Paphia* population of the estuary was not significant to consider them as genetic variants. Even though the morphometric study revealed significant difference, these variations were not reflected in the molecular studies. The study revealed discrepancy between the morphological

differences and genetic similarity. Therefore, it is likely that different morphotypes result from phenotypic plasticity caused by environmental factors. This was corroborated by the findings of earlier researchers [36-37].

ITS (ITS1 and ITS2) of nrDNA are widely and routinely used in the analysis of species relationship using the constructed phylogenetic trees. Phylogenetic analysis using Internal Transcribed Spacers (ITS) of nrDNA of twenty one species belonging to six of the twelve known subfamilies of Veneridae indicated the monophyly of the family.

Eventhough *C.gallina* and *M.merceneria* are members of the subfamily Chioninae; they never formed sister clades. Instead *C.gallina* always grouped as sister clade with *V. verrucosa*. Thus Chioninae and Venerinae, long considered to be closely related, fall within the same clade. This condition is supported by the fact that these two species, which form a homogenous cluster with a high bootstrap value, share a similarly small genetic distance. The same situation was reported earlier [38] and also warrants their attribution to two distinct subfamilies. Pitarinae (*C.chione*) and Meretricinae (*M.meretrix, M.casta*) sustained by high bootstrap values, include Venerids showing ancestral features. The findings of the present study commensurate with many of the earlier findings [39-40].

The divisions of Veneridae into three groups were in line with the earlier findings [41-42]. Several earlier researchers supported the monophyly of Veneridae [43-48].

Conclusion

The molecular analysis of the nucleotide sequences of ITS1, 5.8S, ITS2 and 28S regions of nuclear ribosomal DNA of Venerid clams *P.malabarica* revealed that ITS is species dependent and the difference could be significant among species. Hence, it can be utilized for species identification.

Even though the initial molecular analysis indicated the genetic difference between the *Paphia* populations in the estuary, further phylogenetic analysis ruled out the existence of genetic difference between them. The discrepancy between morphological differences and genetic similarity can be attributed to environmental factors influencing morphology and thus resulting in ecophenotypes reflecting phenotypic plasticity. The phenotypic plasticity can be explained by ecophenotypic variation along a depth gradient with uneven shell thickness and shorter form in deeper part of the estuary near barmouth where wave action and tidal currents were higher and with longer and deeper shells in shallow upper reaches of estuary.

Phylogenetic analysis of the nucleotide sequences confirmed that the sub-families are monophyletic as their family Veneridae. The MP and ME trees were dichotomous resulting in three principal clusters. The first cluster is constituted by members of Venerinae and Chioninae. The second cluster is formed of the members of Tapetinae and the third cluster consisting of members of Cyclininae, Meretricinae and Pitarinae.

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