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DNA barcoding and preliminary phylogenetic analysis of few gastropods (Family: Potamididae and Nassariidae) in Vellar estuary mangroves, India by *COI* and *18S rRNA* genes

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Class Gastropoda is one of the widely studied invertebrates throughout the globe. But, even now most of the species have been identified only by conventional morphology. DNA barcodes have been greatly useful to delimit the cryptic species. In this study, we have analyzed cytochrome c oxidase I (*COI*) and *18S rRNA* gene sequences from 12 specimens belonging to four species to barcode and examine the molecular variation in gastropods of the Vellar estuary mangroves of Tamil Nadu, India. From the results, it is revealed that, among the four genera, *Terebralia*, *Telescopium*, and *Cerithidea* are closely related than genus *Nassarius* based on the *COI* and *18S rRNA* gene sequences. The neighbour joining trees also confirmed four distinct clades harbouring four genera. As expected, the within species K2P genetic variation is lesser than between species K2P genetic variation in both the genes. An intensive survey is needed to assess the genetic variation of all gastropods in the manmade Vellar estuary mangroves and nearby natural Pichavaram mangroves with additional molecular markers.

[**Keywords**: Gastropods, Genetic distance, Mangrove, mtDNA genes, Phylogeny]

Introduction

Mollusks contribute the 2nd largest invertebrate group on the earth next to Arthropods. India has a wealth of 3,271 species of mollusks belonging to 220 families, 591 genera and about 1,900 species that come under gastropods. About 484 species of mollusks were reported in Gulf of Mannar region. Out of which, 260 species are gastropods[1]. The Vellar estuary (11°29″ N; 79°46″ E) in India forms a widespread setup of backwater with branches of the Cauvery river. The estuary bank consists of manmade mangroves and thus becomes suitable habitat of various crustaceans, mollusks and fish species. In this mangrove system, 10 species of gastropods were reported in an earlier study[2]. Throughout the globe, the species description, and evolutionary interactions among and within several gastropods are weakly resolved[3,4]. Identification of the type of specimens and delineation into some gastropods are through conventional morphological characters[5]. Taxonomical studies based only on morphological characters sometimes lead to overestimation of some species because they exhibit high phenotypic variability, *e.g.* *Littorina saxatilis*[6]. The chance of a genetic change may reflect its phenotypic characters according to their habitat conditions[7]. On the other hand, some phenotypic traits hide the presence of their evolutionary lineage or cryptic species, and finally lead to underestimation of the species count[8]. The misidentification of any species may give false representation in biodiversity of a particular environment, which always needs precise taxonomy[9].

DNA barcoding technique has been effectively used in more number of gastropod families to conquer the sole morphological identification problems[5,10]. The successful molecular level identification of species need a complete reference library of DNA sequences for matching each sample to a diagnostic DNA sequence or sequences array[11]. To accomplish that idea, a worldwide accessible DNA barcode database (BOLD system)[12] that includes reference libraries has been developed to check and validate the query sequence. Mitochondrial DNA (mtDNA) has been widely used to assess the molecular relationships among individuals, populations and various species[13]. Within the mitochondrial genome, the *COI* gene is

broadly used due to its slowest mutation and lower substitution rates than other mtDNA genes[14]. In the case of Vellar estuary harboured gastropods, no report was available about molecular identification and their genetic distance. To fill the paucity, this work was initiated by choosing four species namely, *Telescopium telescopium, Cerithidea cingulata, Nassarius festivus* and *Terebralia palustris* for molecular identification and phylogenetic analysis using COI and 18S rRNA genes.

Materials and methods

The gastropod species were collected from the Vellar estuary mangrove forest by hand picking. They were morphologically identified as *Cerithidea cingulata, Nassarius festivus, Telescopium telescopium* and *Terebralia palustris* based on their morphological characters described in previous report by Poutiers *et al*[15]. Muscle tissue was dissected out from the entire specimen and stored in 95 % ethanol for DNA isolation. Total genomic DNA was extracted from the stored muscle by adopting a standardized protocol[16]. The *COI* gene was amplified by PCR with the primers *LCO 1490* 5´- GGTCAACAAATCATAAAGATATTGG-3´ and *HCO 2198* 5´- TAAACTTCAGGGTGACCAAAAA ATCA-3´(ref. 17). PCR amplification was conducted in a total volume of 20 µL containing 0.16 µL of *Taq* DNA polymerase (5 units/µL), 2.0 µL of 10X Taq buffer, 1.6 µL of dNTP mixture (2.5 mM each); 1.0 µL of each primer (10 mM) and 1.0 µL of template. PCR conditions comprised 35 cycles of denaturation (94 °C, 30 s), annealing (45 °C, 30 s) and extension (72 °C, 60 s). The 18S rRNA gene was amplified by the primers *18S A*5´-AACCTGGTTGATCCTGCCAGT-3´ and *18S B*5´-TGATCCTTCCGCAGGTTCACCT-3´[18]. PCR amplification was conducted in a total volume of 20 µL containing 0.16 µL of *Taq*DNA polymerase (5 units/µL), 2.0 µL of 10X Taq buffer, 1.6 µL of dNTP mixture (2.5 mM each); 1.0 µL of each primer (10 mM) and 1.0 µL of template. PCR conditions comprised 35 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 30 s) and extension (72 °C, 60 s) in a thermal cycler (Techgene, UK). Amplified products were checked in a 1.5 % agarose gel with 0.5 mg/mL ethidium bromide. Subsequent DNA sequencing was performed at Eurofins (Bangalore, India). Sequencing reactions followed the manufacturer's suggested protocol. All of the newly obtained sequences were deposited in the NCBI GenBank (accession numbers KT270932–KT270936, KT336812-KT336820, KU512837-KU512846). Subsequently, both COI and 18S rRNA genes of the four species were analysed in NCBI BLAT for sequence similarity. Genetic variation and pairwise evolutionary distance among sequences were estimated by Kimura 2-Parameter (K2P) method[19] using the software program MEGA 6[20]. The neighbour-joining (NJ) trees for *COI* and *18S rRNA* were constructed and to verify the robustness of the internal nodes of these trees, bootstrap analysis was carried out using 1000 pseudoreplications.

Results and discussion

A total of 24 sequences of four gastropod species were analyzed in the present study. According to the *COI* gene sequence variation, the genetic distance was measured by K2P parameter and results are shown in Table 1. The maximum K2P distance (0.260) was found between *Terebralia palustris* and *Nassarius festivus* whereas the minimum distance (0.213) was observed between *Cerithidea cingulata* and *Telescopium telescopium.* However, based on *18S rRNA* gene sequences, the maximum K2P distance (0.069) was observed between *Nassarius festivus* and *Cerithidea cingulata* (Table 2) whereas the minimum distance of (0.027) was observed between *Telescopium telescopium* and *Terebralia palustris.* Figures 1 and 2 show the phylogenetic trees constructed by COI and 18S rRNA genes of gastropod species using neighbor-joining algorithm with distance scale.

As expected standardized extraction method for DNA isolation and PCR amplification of *COI* and *18S rRNA* were perfectly worked on these species as well with previous reports[16-18]. The BLAST results showed

Table 1 — K2P genetic distance of between (below diagonal) and within species (bold) based on COI gene sequence

Species	*Telescopium telescopium*	*Cerithidea cingulata*	*Nassarius festivus*	*Terebralia palustris*
Telescopium telescopium	**0.073**			
Cerithidea cingulata	0.213	**0.000**		
Nassarius festivus	0.249	0.250	**0.000**	
Terebralia palustris	0.239	0.251	0.260	**0.000**

Table 2 — K2P genetic distance of between (below diagonal) and within species (bold) based on 18S rRNA gene sequence

Species	*Telescopium telescopium*	*Cerithidea cingulata*	*Nassarius festivus*	*Terebralia palustris*
Telescopium telescopium	**0.000**			
Cerithidea cingulata	0.032	**0.000**		
Nassarius festivus	0.054	0.069	**0.005**	
Terebralia palustris	0.027	0.032	0.064	**0.000**

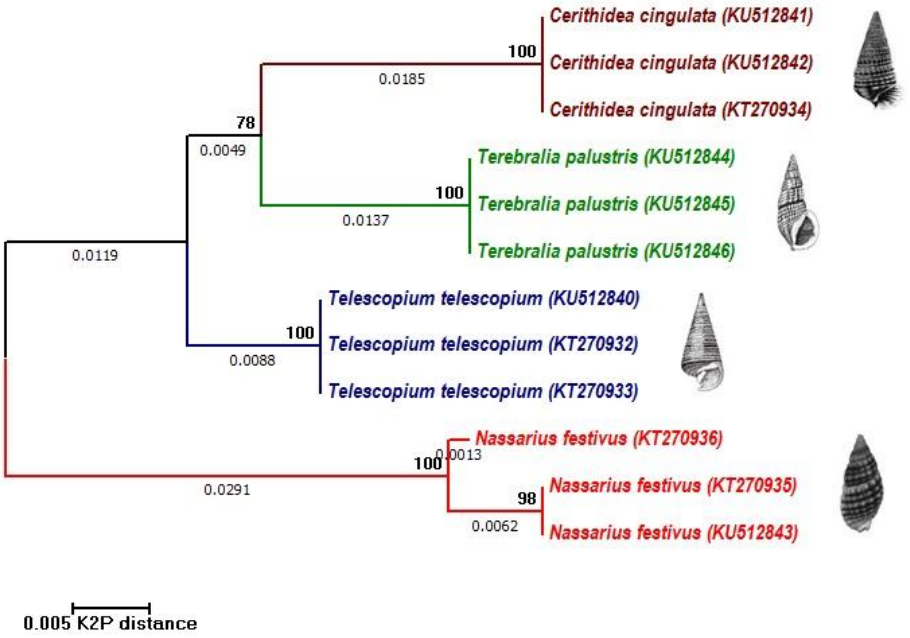

Fig. 1 — Neighbour joining tree of studied gastropod species based on COI gene sequences

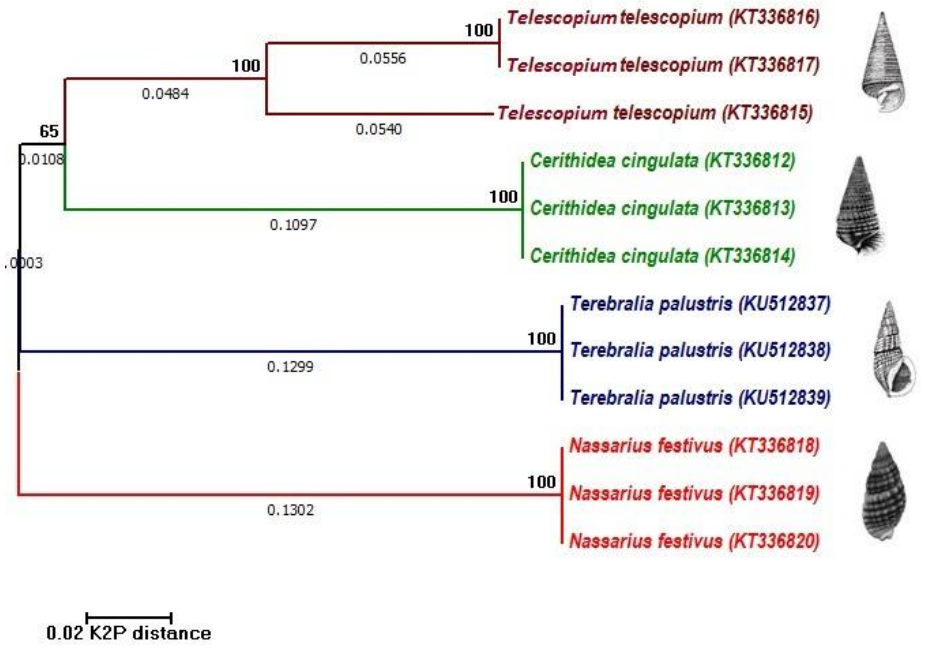

Fig. 2 — Neighbour joining tree of studied gastropod species based on 18S rRNA gene sequences

significantly more query coverage, similarity and very less "*E*" value (0.000) and confirmed them as *Cerithidea cingulata, Telescopium telescopium, Nassarius festivus* and *Terebralia palustris.*

The within species K2P distance value was lower than between species value. As per the earlier study based on *COI* gene by Borges *et al*.[21] using 34 gastropod species the range of within species K2P genetic distance was 0.0–6.38 and the mean intraspecific distances was 0.8. In our study, the range of within species K2P genetic distance was 0.0–0.073. In their study, the intraspecific distance of 1.9 was observed in *Nassarius incrassatus*. But in this study, *Nassarius festivus* showed the intraspecific distance of only 0.073.

Here, the maximum K2P distance (0.260) was found between the genus *Terebralia* and *Nassarius* whereas, the minimum distance (0.213) was observed between *Cerithidea* and *Telescopium.* The present results also supported the previous report[21], where the within genus genetic distance was lesser than between genus genetic distance. The maximum K2P distance (0.069) was found between the genus *Nassarius* and *Cerithidea* whereas the minimum distance (0.027) was observed between *Terebralia* and *Telescopium.* However, the maximum K2P distance within genus was observed in *Nassarius* (0.005) and minimum distance of 0.000 was observed in the other entire genus based on 18S rRNA gene sequences. This proves the efficacy of COI and 18S rRNA genes in species identification.

Phylogenetic relationships among the genus of *Cerithidea, Telescopium,* and *Terebralia* (family: Potamididae) and *Nassarius* (family: Nassariidae) were inferred based on *COI* gene and *18S rRNA* sequences by Neighbor-joining algorithm showed a significant difference in clade pattern (Figs. 1 and 2). It may be due to the difference in mutation rate among the two genes and may also be the reason of small sampling size. The phylogram sugeested two major clades *i.e*., Potamididae and Nassariidae. In the present observation, the monophyletic *Cerithidea* and *Terebralia* are closely clustered. *Cerithidea, Telescopium,* and *Terebralia*are distinctly placed as sub-clades. It clearly demonstrates that the sequence variation is more in Potamididae family.

But in *18S rRNA* tree *Telescopium telescopium* was closely related with *Terebralia palustris* followed by *Cerithidea cingulata. Nassarius festivus* (Nassariidae) formed a separate clade in *18S rRNA* based tree also. It is clearly confirmed that this species belongs to another family Nassariidae. In the present study, family Potamididae and Nassariidae form a close relationship with a bootstrap of 65 – 100 % (Figs. 1 and 2). The results were similar to the previous study in gastropods[22]. They reported moderate support (57-100 % bootstrap) is enough for a sister relationship between marine gastropods based on *COI* gene. These two markers in this study showed different type of cluster in the phylogram, and this may due to the independent mutation rate of each gene.

The preliminary assessment does not give a clear and complete account of the genetic diversity in gastropods such as family Potamididae and Nassariidae. Owing to limited number of specimens, it is impossible to demonstrate all aspects of genetic variation between the species of two families. However, this study with two markers (*COI* and *18S rRNA*) may provide some information about the phylogeny of this group, and is likely to provide a platform for future examination. The sequences of *COI* and *18S rRNA* is enough to discriminate each species. Finally, it is suggested that the molecular identification results confirm the gastropods of Vellar estuary as *Cerithidea cingulata, Telescopium telescopium, Terebralia palustris* and *Nassarius festivus* without ambiguity.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

Sample collection was done by MT, DA, TR and RK. DNA isolation and PCR by MT. Data analysis and paper writing by MT, DA, TR, RK and VR.

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