INTEGRATIVE TAXONOMIC ASSESSMENT OF THREE GASTROPOD SPECIES FROM THE MANGROVE HABITAT OF THE VELLAR ESTUARY, TAMILNADU, INDIA

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The Class Gastropoda is highly diverse and ecologically significant, yet many species remain poorly studied in tropical estuarine habitats. This study integrates morphological characters and molecular tools to assess variation among three dominant gastropod genera such as *Clithon*, *Neritina*, and *Nassarius* from the Vellar estuarine mangroves, Tamil Nadu. Morphometric traits, including shell length (SL), shell width (SW), aperture length (AL), aperture width (AW), operculum length (OL), operculum width (OW), and shell weight (S weight), were measured. Statistical analyses (ANOVA, correlation matrices, PCA) revealed significant interspecific variation. Principal components PC1 and PC2 explained 74.55% and 24.60% of the total variance, respectively. PCA score plots distinctly separated *C. oualaniense*, *N. stolatus*, and *N. violacea*, with no overlap among individuals. DNA barcoding using the mitochondrial COI gene confirmed species-level differences, showing closer genetic affinity between *C. oualaniense* and *N. violacea* (K2P = 0.160), while *N. stolatus* formed a distinct clade in NJ tree analysis (K2P = 0.233). Intraspecific K2P distances were consistently lower than interspecific values, supporting the effectiveness of COI for gastropod species delimitation. The findings highlight the importance of broader genetic surveys using multiple markers across estuarine and mangrove ecosystems to strengthen taxonomic resolution and guide conservation efforts.

Keywords: DNA Barcoding, Gastropoda, Mollusca, phylogeny, taxonomy, Vellar Estuary.

INTRODUCTION

Estuaries are dynamic ecosystems formed where rivers meet the sea, creating brackish water that sustains a wide range of plant and animal species adapted to fluctuating salinity (Pritchard, 1967). These habitats including mangroves, coral reefs, marshes, and rocky shores serve as shelter, feeding, and breeding grounds for migratory birds, fishes, prawns, shellfish, and water snails (McLusky, 1981). Mollusks, soft-bodied invertebrates of the phylum *Mollusca*, are abundant in estuaries and are typically protected by calcium carbonate shells secreted by the mantle (Khan *et al*., 2005; Rosenberg, 2014). Among mollusks, gastropods constitute the most diverse

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class, with an estimated 65,000–80,000 species worldwide (Bouchet & Rocroi, 2005), many of which inhabit estuarine and marine ecosystems such as the Vellar Estuary. India harbors about 3,271 mollusk species, including ~1,900 gastropods, with the Gulf of Mannar alone supporting 260 gastropod species (Sakthivel & Fernando, 2004).

Globally, the freshwater gastropod fauna comprises ~4,000 valid species across at least 33–38 independent lineages within Neritimorpha, Caenogastropoda, and Heterobranchia. These species inhabit rivers, lakes, swamps, springs, and even temporary aquatic habitats across all continents except Antarctica (Strong *et al.*, 2008). In India, gastropods are ecologically important components of mangrove ecosystems, with mollusks contributing significantly to nutrient cycling and habitat stability within the country's 4,827 km² of mangrove forests, distributed along the east coast, west coast, and the Andaman and Nicobar Islands (Shanmugam & Rajagopal, 2006; Pawar, 2012). As a key link in estuarine and marine food webs, gastropods serve as prey for many fishes and birds. Their diverse feeding strategies including grazing, browsing, suspension feeding, scavenging, herbivory, and carnivory enable them to occupy a wide range of ecological niches.

Despite their ecological significance, many estuarine gastropod species remain poorly studied in India, particularly with respect to their taxonomy and population variation. Morphological plasticity in shell characters often complicates species identification, especially in dynamic habitats such as estuaries, where salinity, sediment type, and hydrodynamics influence shell form. Recent advances in molecular techniques, such as DNA barcoding using the mitochondrial COI gene, have proven effective for resolving taxonomic ambiguities and complementing traditional morphometric approaches. However, integrative morpho-molecular studies on estuarine gastropods from Indian mangrove ecosystems are scarce.

The present study addresses this gap by examining morphological variation and genetic divergence among three dominant gastropod genera like *Clithon*, *Neritina*, and *Nassarius* from the Vellar Estuary, Tamil Nadu. Through morphometric analyses and COI-based DNA barcoding, this study aims to assess species-level differences, provide insights into their taxonomic resolution, and emphasize the need for broader genetic surveys across Indian estuarine and mangrove habitats.

MATERIALS AND METHODS

Sampling

Specimens of 20 individuals from three Gastropod species, namely *Clithon oualaniense*, *Nassarius stolatus*, and *Neritina violacea*, were collected from the mangroves of Vellar Estuary (11º29´26´´N 79º45´57´´E), Tamil Nadu, India, by hand picking and transferred live to the laboratory. The samples were washed thoroughly with tap and distilled water before being preserved in 95% ethanol for further molecular studies.

Morphometric measurement and analysis

Morphometric characters of *C. oualaniense*, *N. stolatus*, and *N. violacea* were measured, and the values are presented as minimum (MIN), maximum (MAX), average (AVG), and standard deviation (STDEV). The parameters included shell length (SL), shell width (SW), aperture length (AL), aperture width (AW), operculum length (OL), operculum width (OW), shell weight (S WEIGHT), and relative width percentages [SW (%SL), AW (%AL), OW (%OL)]. Measurements were recorded in millimeters (mm) for linear traits and milligrams (mg) for shell weight. These morphological data was subjected to one way analysis of variance (ANOVA) for finding the variation between species at the p-value of 0.05. Principal Components Analysis (PCA) of covariance matrices was used on the morphometric variables to determine variability among and within the samples suggested by Wiley (1981). This technique, which quantifies shape differences independent of size, as previously been used to distinguish many species (Bowers & Stauffer, 1993). Morphometric measurements were then log transferred to preserve allometrics, standardize variance and produce a scale invariant covariance matrix before analysis. To ensure comprehensive analyses of the data for more powerful discrimination between species, loadings of PC1 were scattered against the PC2 in PAST (V3.0).

DNA isolation

Genomic DNA was isolated from tissue samples using the standard method as described by Miller *et al.* (1988) and the quality of the DNA was assessed by 260 and 280 nm in a UV spectrophotometer. The DNA was made-up with TAE buffer for a final concentration of 100 ng⁄µL.

PCR and sequencing

The COI gene was amplified in a 50 µL PCR mix with 5 µL of 10X Taq polymerase buffer, 2 µL of MgCl2 (50 mM), 0.25 µL of each dNTP (0.05 mM), 0.5 µL of each primer (0.01 mM), 0.6 U of *Taq* polymerase and 5 µl of genomic DNA. The universal primers *LCO1490* 5´- G GTCAACAAATCATAAAGATATTGG-3´ and *HCO2198* 5´- TAAACTTC AGGGTGACCA AAAAATCA-3´ (Folmer *et al*., 1994) for the amplification of the COI gene. The thermal cycling profile consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 54°C for 40 s, and extension at 72°C for 1 min 10 s, with a final extension at 72°C for 10 min. PCR products were visualized on 1.5% agarose gels, and the most intense amplicons were selected for sequencing. Purified PCR products were sequenced through a commercial facility (RGCA, Sirkazhi, India).

Sequence analysis

Partial COI gene sequences of the four gastropod species were edited using BioEdit Sequence Alignment Editor (Hall, 1999), aligned with CLUSTAL-W, and manually checked for accuracy. Identical sequences were grouped under the same haplotype identity, and a single representative sequence of each haplotype was used for phylogenetic analysis, assuming shared evolutionary origin among identical haplotypes. Haplotype definitions were deposited in NCBI GenBank (Accession Nos.: OQ421462, OQ421463, OQ421467, OQ421468, OQ421822, OQ422170). Nucleotide diversity, genetic variation, nucleotide composition, and pairwise evolutionary distances among haplotypes were estimated using the Kimura 2-Parameter model (Kimura, 1980) in MEGA v6.1 (Tamura *et al*., 2013). A neighbor-joining (NJ) tree was constructed in MEGA v6.1, and the robustness of tree topology was assessed through bootstrap analysis with 1,000 pseudoreplications.

RESULTS AND DISCUSSION

Taxonomy and classification

Clithon oualaniense (R. P. Lesson, 1831)

Phylum: Mollusca
Class: Gastropoda
Family: Neritidae
Genus: *Clithon*
Species: *C. oualaniense*
Common name: Dubious nerite snail or Guamanian Nerite

Habitat: Clithon oualaniense snails are often found in either brackish or freshwater habitats. They are usually found in huge numbers "near monsoon drains, among seagrasses and seaweeds, mangrove streams, muddy sand banks". They can also be found in and estuaries.

Size: Shell size varies between 5 mm and 15 mm

Description: Whorls are not compressed, the columella is slightly calloused, the aperture is semilunar, the color is bright and highly variable, ranging from yellow to green, and it is frequently embellished with dark bands or axial streaks. The shell is small, ovate, moderately thin, and the spire is compressed. The surface is smooth and glossy.

Pattern: The shell of the *Clithon oualaniense* has a variety of designs. The patterns might take the shape of spiral, zigzag, axial (transverse), or triangle-shaped lines and patterns. Hence, the visibility of the background color and these line patterns affects the overall shell pattern. The distance between the axial lines can vary, ranging from closely packed designs to more dispersed ones. The tongues vary in size and density; they may be completely missing from the shell or perhaps cover a large portion of it. Even certain tongues and axial lines in certain shell designs are organized in spirals.

Color pattern: Background colours are usually yellow, olive, or greenish.

Nassarius stolatus (Gmelin, 1791)

Phylum: Mollusca
Class: Gastropoda
Family: Nassariidae
Genus: *Nassarius*
Species: *N. stolatus*
Common name: The Nassa mud snails or dog whelks

Habitat: Highly occur in Intertidal, sandy substates.

Size: The length of the shell varies from 10mm to 20 mm.

Description: Shell small, thick, and ovately conical, with a pyramidal spire composed of six to seven convex whorls. Body whorl inflated, spirally sculptured, and printed with faint axial ribs that form nodules below the sutures. Spiral grooves become more prominent toward the base of the body whorl. Whorls overlap distinctly at the sutures. The aperture is oval, with a small apical notch present near the aperture margin. Outer lip thickened and supported by a prominent dorsal varix, which extends above the penultimate whorl. Columella provided with callus deposits and distinct denticles. The shell surface exhibits bumpy ridges on the spire, with occasional broad brown bands on the anterior portion of the body whorl. Longitudinal folds are prominent on the upper whorls but less evident on the body whorl, and crossed locally by deep transverse striae, particularly on the basal and apical whorls; these striae become finer and more regular on successive whorls. The right side of the body whorl is typically smooth and devoid of longitudinal folds. Ecologically, these whelks are active scavengers, often observed foraging in intertidal pools during tidal changes. They are attracted to carrion, such as dead crabs or fishes, and congregate rapidly at such food sources.

Color pattern: Smooth shell, brown to olive green, with only a few spiral ridges towards the front. An area of deep red surrounds the suture, and a wider, browner ring also encircles the centre of the body whorl.

Neritina violacea Gmelin, 1791

Phylum: Mollusca
Class: Gastropoda
Family: Neritidae
Genus: *Neritina*
Species: *N. violacea*
Common name: Violet nerite

Habitat: Widely distributed in Estuarine and mangrove mud flats

Size: The shell size varies from 15mm to 25 mm

Description: The shell of a crepidula is laterally elongate-ovate, smooth on the oblique side, and without spiral sutures. Spire second. 7–10 denticles on the columellar plate. Operculum has a smooth, semi-lunar surface. When living, the shell has a skin on top (periostracum), which is often coated with silt and algae. Columellar plate has a brownish orange tint, while the shell is yellowish brown. The flat bottom might be anything from vivid brick red to pale with an orange hue. frequently having a thin, black rim. Oval, massive, thick shell with a spire that has sunk. The shell has a "skin" (periostracum) that is generally coated with silt and algae when it is living. There may be no "teeth" or only a few small ones in the centre of the straight edge at the shell entrance. Operculum is thick, smooth, and has black spots and patches that are similar in colour to the underside. Although those saw had light skin with dark markings and a black foot, the body was described as being orange with black patches.

Color pattern: Body typically orange with irregular black patches, although examined specimens appeared pale with dark spots and a distinctly black foot. Operculum thick, smooth, and similar in coloration to the underside of the shell, marked with dark patches and blotches. The basal surface of the shell varies from vivid brick red to pale orange, often bordered by a thin black rim. Shell coloration, though appearing dark overall, exhibits subtle purple streaks and characteristic tent-like markings across the surface.

Morphometric variation

Morphometric variables of *C. oualaniense*, *N. stolatus*, and *N. violacea* are given in Table 1. The one way- ANOVA result shows all *p*-values were above 0.05 (SL: 2.13, SW(%SL): 1.36, AL: 3.24, AW(%AL): 6.22), indicating statistically significant differences among the species. Correlation analysis between shell length

vs shell width, shell length vs shell weight, aperture length vs aperture width in all the three gastropod species shows a good positive R^2 value and the results are given in Figures 1–3.

Table 1

Morphometric variation in three gastropod species

Characters	***Clithon oualaniense***	***Neritina violacea***	***Nassarius stolatus***
SL (mm)	7.48±0.57[a]	15.80±1.44[b]	13.99±0.84[c]
SW(%SL)	74.26±2.73[a]	69.14±2.69[b]	54.05±2.86[c]
AL (mm)	5.52±0.55[a]	12.91±0.79[b]	7.35±0.45[c]
AW (%AL)	60.07±2.40[a]	84.79±2.58[b]	53.35±3.89[c]

Note: The common superscripts not sharing the row is significantly different at p=0.05 level; SL=Shell length; SW=Shell width; AL= Aperture length; AW= Aperture width.

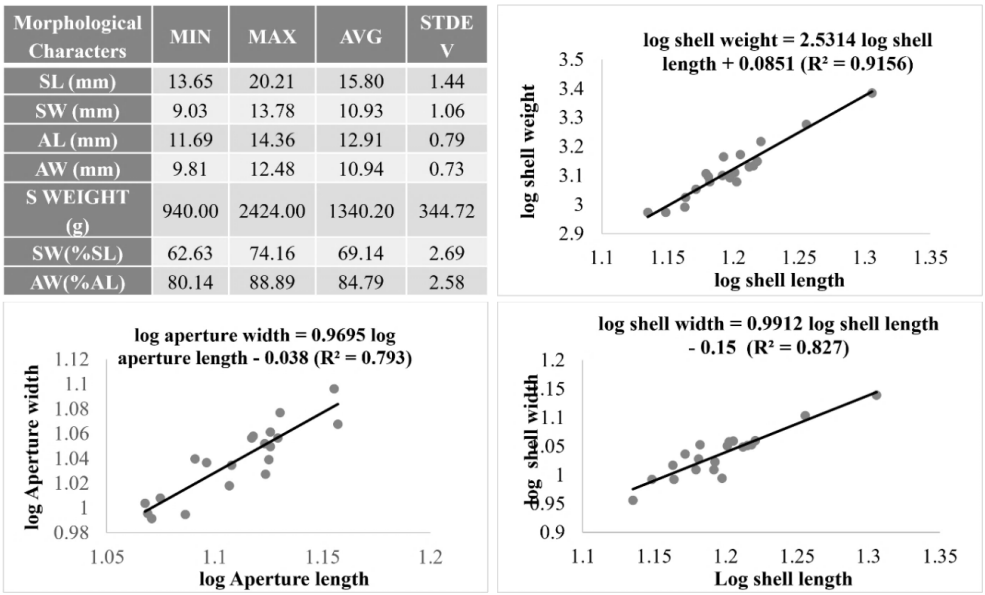

Fig. 1. Morphological characters and their correlation pattern in *Neritina violacea*.

Morphological Characters	MIN	MAX	AVG	STDEV
SL	6.64	9.11	7.48	0.57
SW	4.96	6.76	5.55	0.45
AL	4.77	6.64	5.52	0.55
AW	2.72	4.11	3.32	0.42
S WEIGHT (g)	130.00	290.00	185.50	48.28
SW(%SL)	70.71	78.84	74.26	2.73
AW(%AL)	56.78	65.59	60.07	2.40

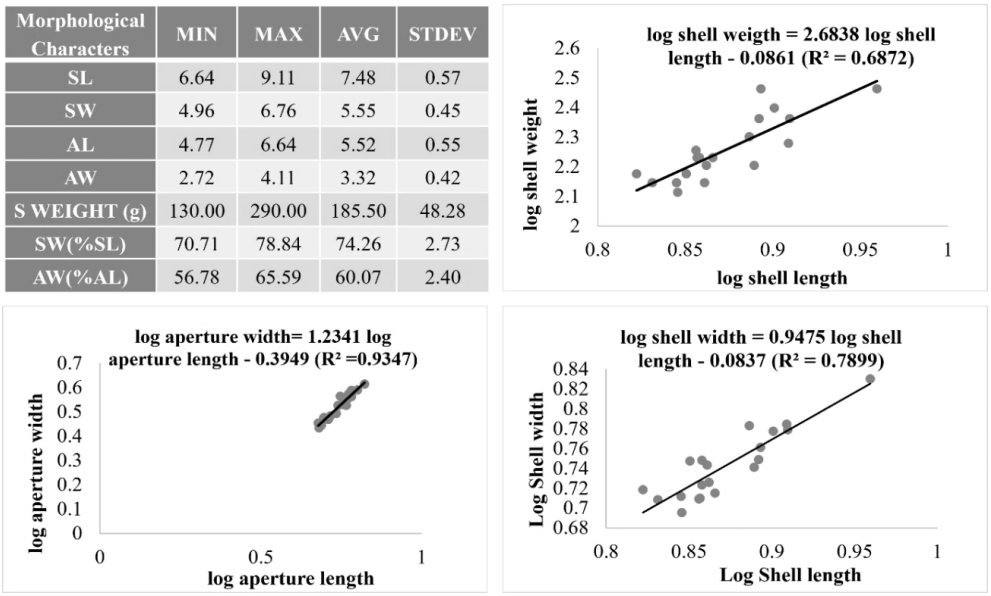

Fig. 2. Morphological characters and their correlation pattern in *Clithon oualaniense.*

Morphological Characters	MIN	MAX	AVG	STDEV
SL (mm)	12.40	15.53	13.99	0.84
SW (mm)	6.89	8.23	7.55	0.35
AL (mm)	6.52	8.32	7.35	0.45
AW (mm)	3.12	4.56	3.92	0.37
S WEIGHT (mg)	320.00	600.00	471.50	81.71
SW(%SL)	50.42	60.30	54.05	2.86
AW(%AL)	47.49	58.61	53.35	3.89

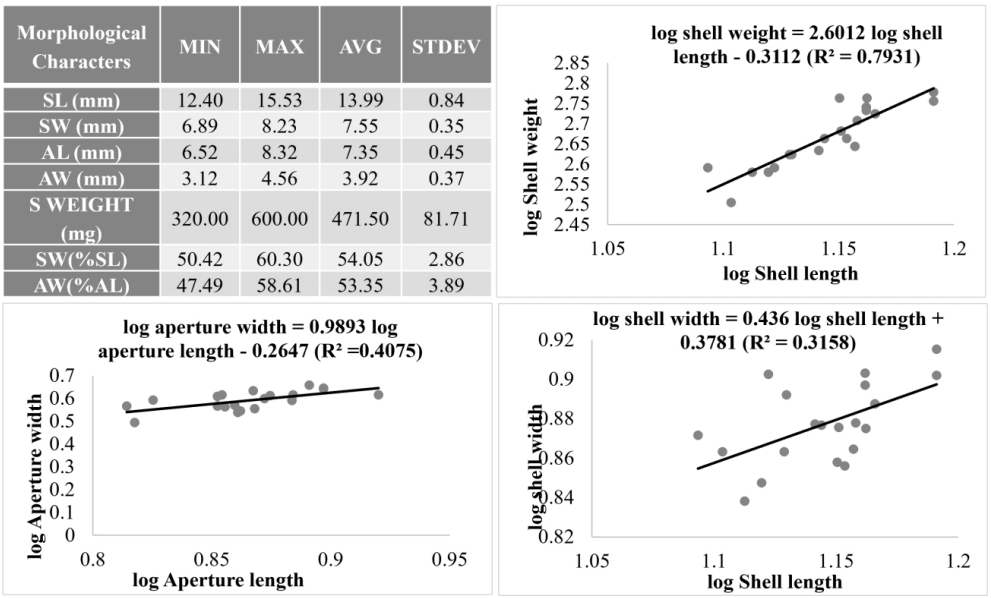

Fig. 3. Morphological characters and their correlation pattern in *Nassarius stolatus.*

Principal Component Analysis (PCA) identified key traits contributing to species discrimination. PC1 and PC2, with loadings shown in Table 2, revealed high positive weightage for SW (%SL) and AW (%AL) in PC1, while SL showed a

strong negative weightage in PC2. Higher eigenvalues indicate stronger trait influence. The clustering pattern of PCA score plot clearly differentiated the three species i.e. *C. oualaniense*, *N. stolatus*, and *N. violacea*, with no overlap among individuals (Fig. 4). PC1 and PC2 explained 74.55% and 24.60% of the total variance, respectively. Shell width relative to shell length (SW%SL) and aperture width relative to aperture length (AW%AL) contributed most to PC1, while shell length (SL) showed strong negative loading in PC2, highlighting key traits responsible for interspecies variation.

Table 2

Morphometric variable loadings for the first and second principal components of three species of gastropods

Loadings	**PC1 (74.55%)**	**PC2 (24.60%)**
SL	0.0902	- 0.3829
SW (%SL)	0.3399	0.8668
AL	0.1742	-0.1938
AW (%AL)	0.9197	-0.2467
Eigen value	224.611	74.1307

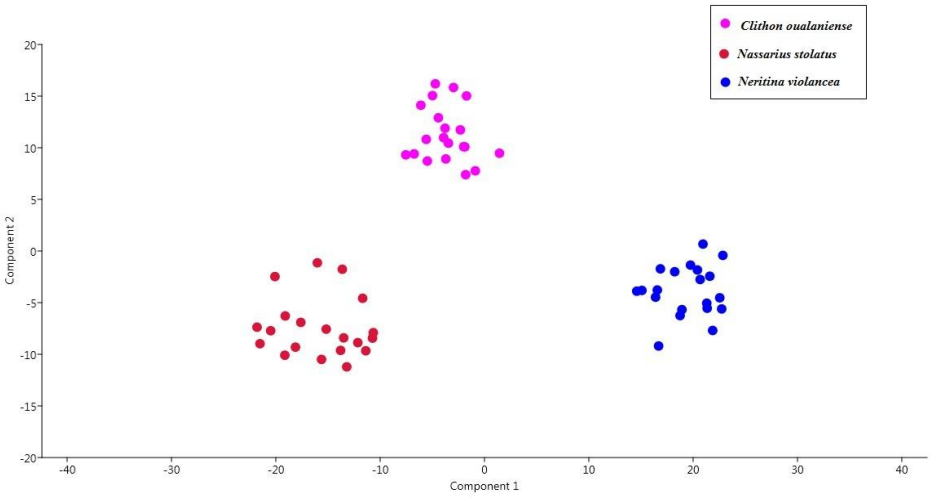

Fig. 4. PCA score plot for three gastropod species.

Genetic variation

Genetic divergence among three gastropod species was analyzed using MEGA 6.0 with 650 aligned positions and the results are given in Table 3. The Kimura 2-parameter model revealed the highest genetic distance (0.233) between *N. violacea* and *N. stolatus*, and the lowest (0.160) between *N. violacea* and *C. oualaniense*. Intraspecific variation was lowest in *N. stolatus* (0.002) and highest in *N. violacea* (0.089), with an overall mean genetic distance of 0.173. A Neighbor-Joining tree (Fig. 5) based on COI sequences confirmed phylogenetic relationships. *C. oualaniense* and *N. violacea* clustered together, indicating their classification under the family Neritidae, while *N. stolatus* formed a separate clade under Nassariidae with strong bootstrap support (100). The overall tree distance was 0.02.

Table 3

K2P Genetic distance between species (below diagonal) and within species (bold) based on COI gene sequence

Species	***Clithon oualaniense***	***Neritina stolatus***	***Nassarius violacea***
C. oualaniense	**0.030**		
N. stolatus	0.227	**0.002**	
N. violacea	0.160	0.233	**0.089**
Overall mean	0.173		

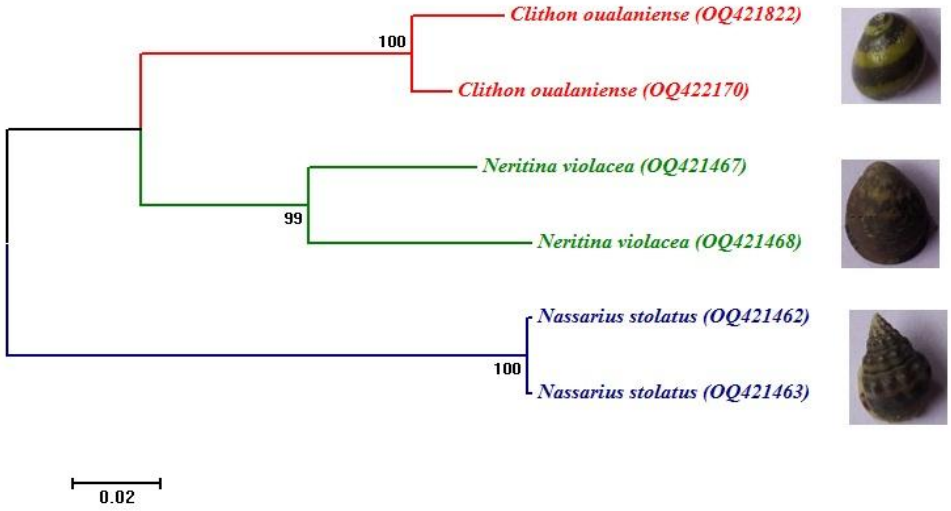

Fig. 5. Neighbour joining tree of three gastropod species based on COI gene sequence.

This study highlights morphological and genetic differentiation among three gastropod species i.e., *C. oualaniense, N. stolatus* and *N. violacea* inhabiting the Vellar estuarine mangroves. Morphometric analysis revealed distinct size and shape characteristics among the species, supported by low standard deviation values and species-specific variations in shell and aperture dimensions. Correlation studies showed strong positive relationships among morphometric traits, particularly between aperture length and width in *C. oualaniense* and operculum traits in *N. violacea*. ANOVA and PCA confirmed significant morphometric divergence, with SW (%SL) and AW (%AL) contributing most to species separation.

The phylogenetic results of the present study are comparable with those reported by Wang *et al*. (2019), who analyzed 20 mitogenomes of *Neritina violacea* with *Owenia fusiformis* as an outgroup using the maximum likelihood method. Their phylogenetic tree, supported by 1,000 bootstrap replications, revealed two distinct clades within Neritidae, with *N. violacea* clustering more closely with *N. usnea* and *Theodoxus fluviatilis*, both capable of inhabiting freshwater and brackish environments. Furthermore, *N. violacea* showed a genetic relationship with *Potamopyrgus antipodarum*, a small freshwater snail, suggesting that *N. violacea* may have evolved from a freshwater lineage. The availability of the complete mitogenome of *N. violacea* provides valuable information for understanding neritid phylogeny and the co-evolution of this species in mangrove ecosystems.

In a previous study, Thangaraj *et al*. (2020) used COI and 18S rRNA sequences to infer phylogenetic relationships among the genera *Cerithidea*, *Telescopium*, and *Terebralia*. They reported that the maximum K2P distance occurred between *Terebralia* and *Nassarius*, and their phylogram revealed two major clades, with Potamididae forming the dominant cluster. Within this family, *Cerithidea* and *Terebralia* were closely grouped, whereas *Telescopium* was distantly placed. A second clade contained *N. festivus*, highlighting differences in clade patterns likely due to varying mutation rates between markers and limited sampling.

In the present study, COI sequences were analyzed to assess genetic diversity among three gastropod species. The maximum K2P distance was found between *N. violacea* and *N. stolatus*, indicating distant relatedness, whereas *N. violacea* and *Clithon oualaniense* were more closely related. Interestingly, the greatest within-genus divergence was observed in *N. violacea*, suggesting considerable genetic diversity within this species, while *N. stolatus* exhibited minimal intraspecific divergence, reflecting genetic similarity within the genus. The overall mean genetic distance among the three species was 0.173, indicating substantial interspecific diversity, likely influenced by geographic isolation, ecological adaptation, and evolutionary processes.

Neighbor-joining analysis revealed two well-defined clades. The first, corresponding to the family Neritidae, formed a monophyletic group containing *Clithon* and *Neritina*, which clustered closely together. The second clade contained *N. stolatus*, representing the Nassariidae lineage. Bootstrap support confirmed the robustness of these clusters. These results demonstrate clear genetic separation among the studied taxa, consistent with their morphological distinctness.

Taken together, the present findings, along with earlier phylogenetic studies (Wang *et al*., 2019; Thangaraj *et al*., 2020), confirm that the three gastropod species examined are both morphologically and genetically distinct. This integrative approach provides a robust taxonomic framework for future ecological, evolutionary, and conservation studies in estuarine and mangrove habitats.

CONCLUSIONS

The present study provides valuable insights into the evolutionary relationships among the genera *Clithon*, *Neritina*, and *Nassarius* in the Vellar estuary. By integrating morphometric characters with COI-based molecular analyses, the study highlights the effectiveness of combining traditional taxonomy with modern genetic tools for accurate species identification and classification. The COI gene proved reliable in resolving interspecific and intraspecific variation, offering a clearer understanding of evolutionary history and genetic diversity in estuarine gastropods. These findings contribute to marine biodiversity research and establish a baseline for future studies on gastropod evolution, taxonomy, and conservation. The genetic information generated here is particularly relevant for conserving species diversity and understanding ecological roles and adaptations within dynamic estuarine and mangrove ecosystems. In the context of growing threats such as habitat loss, climate change, and overexploitation, this integrative approach provides a foundation for developing effective management and conservation strategies.

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