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Molecular taxonomy of *Littoraria* spp. (Gastropoda: Littorinidae) using mitochondrial DNA cytochrome c oxidase I sequences

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Original Article

Abstract

DNA taxonomy offers advanced possibilities for demarcating species and assessing phylogenetic relationships among them. In this study, among the 39 species recorded worldwide, 5 species of the genus *Littoraria* (Gastropoda: Littorinidae), found along the southern Kerala coast, were barcoded using the cytochrome c oxidase I (COI) gene, encoding COX I. *Littoraria carinifera* was recorded and its presence confirmed for the first time along the Kerala coast. A neighbour-joining tree was created based on 52 mitochondrial COI barcode sequences from 10 species of the genus *Littoraria*, with two outgroups. It revealed monophyly with 1000-replicate bootstrapping using the Kimura two-parameter model. The average nucleotide proportions and GC content at different codon positions were calculated for each species in our study. The AT composition was significantly greater than the GC composition. Transition/transversion rate ratios for purines and pyrimidines were measured, resulting in an overall bias with R=3.876. This study will enable future research on the biogeography and population dynamics of members of this genus and provide valuable insights for conservation purposes if needed.

Keywords: *Cytochrome c oxidase I, DNA barcoding, Littoraria, rocky intertidal shore, Kerala*

Introduction

The genus *Littoraria* (Gray, 1833), a member of the family *Littorinidae* (Children, 1834), is widely distributed in tropical and subtropical regions (Rosewater, 1970). These marine gastropods, commonly known as periwinkles, are abundant and widespread across worldwide seashores (Reid *et al*., 2012). They are either seen as solitary or in clusters. They inhabit various intertidal habitats, including mangroves, estuaries, and rocky shores. It is typically abundant in the elevated areas of rocky shores, where their upper boundaries have been utilised to demarcate the ecological zone known as the supralittoral or littoral fringe (Stephenson and Stephenson, 1949). A total of 39 species belonging to the genus *Littoraria* were identified worldwide, of which 11 species were detected along the Indian coast, and 7 species, specifically from the Kerala coast, were recognised. The intertidal rocky shore is a favourable habitat for littorinids, classified into different zones. These zones include the supralittoral, midlittoral, and sublittoral zones. Despite the harsh environmental conditions in the supralittoral zone, gastropods belonging to the genus *Littoraria* have adapted to retreat into crevices and attach to the substrate to avoid dislodgement due to strong wave action (Katie *et al.,* 2014) and thrive in this challenging habitat (Newell, 1979). These snails play a crucial role in the functioning of mangrove ecosystems, influencing nutrient cycling, primary production, and energy flow (Hasidu *et al.,* 2020). Additionally, they serve as bioindicators, providing valuable insights into the health and stability of coastal environments, making them essential for biomonitoring programs (Syahrial *et al.,* 2021), wherein they contribute to the dynamics of food webs in rocky substrates. The IUCN Red List shows Least Concern (LC) status for *Littoraria undulata*, but does not provide a conservation status for the rest of the species (IUCN, 2025).

Littoraria spp. have properties of grazers, consuming living and decaying plants (Torres *et al.,* 2008), that affect the density of algae and barnacles (Buschbaum, 2000). Another common environment for littorinid species are estuarine and mangrove ecosystems. Their presence on mangrove mud and rocks facilitates energy transfer by providing food for larger organisms, microalgae, and small invertebrates, thereby contributing to the dynamics of mangrove food webs (Syahrial *et al.,* 2021). Mangrove ecosystems themselves provide numerous ecological and economic benefits, including supporting coastal fisheries, storing carbon, improving water quality, and supplying wood products (Idrus *et al.,* 2021; Syahrial *et al.,* 2021). The ecological significance of *Littoraria* spp. extends to their utilisation as bioindicators for assessing the success of mangrove reforestation efforts (Rial *et al.,* 2020). Their distribution patterns are closely linked to their ability to withstand environmental stressors, such as hydric stress, which influences their zonation along the intertidal gradient (Reis *et al.,* 2021). Biological interactions associated with the genus include competition and predation, where the latter is the most prominent, as it keeps the population of *Littoraria* spp. under control (Reid, 1984). *Littoraria* spp. is the only mobile arboreal gastropod in the intertidal region (Reid, 1985), and they exhibit vertical zonation, particularly in mangrove trees.

DNA barcoding is an excellent tool for identifying species and their classification. The molecular identification of organisms is commonly employed using a short standard DNA fragment of the COI gene (Hebert *et al.,* 2003). The COI gene is frequently used as a universal marker in animal DNA barcoding as it possesses a haploid genome, high copy number, low recombination and lacks introns (Hebert *et al.,* 2003; Hajibabaei *et al.*, 2007). The gene length strikes a desirable balance, enabling quick and cost-effective sequencing while providing sufficient sequence length to detect intraspecific variation. This characteristic ultimately leads to accurate species identification (Hlaing *et al.,* 2009). Molecular analysis of an organism is critical in assessing species' morphological variability and diversity (Schiaparelli *et al.,* 2017).

Every species has a unique DNA barcode that distinguishes it from other species. Accordingly, the molecular identification of species through DNA barcoding can enable accurate species demarcation, biodiversity assessment, demarcation of cryptic species, and phylogenetic relationships of related taxa (Ward *et al.,* 2005). The effectiveness of this approach has been demonstrated in identifying gastropods and various other organisms, addressing the constraints of relying solely on morphological characteristics for specimen identification (Barco *et al.,* 2010; Layton *et al.,* 2014). Evaluation and monitoring of biodiversity depend on the precise taxonomic identification of species. Here, molecular identification using the DNA barcoding technique was applied to identify and confirm partial COI gene sequences from five members of the genus *Littoraria* spp., collected from selected stations on the southern Kerala coast. This makes it easier to test if DNA barcoding effectively distinguishes between different species and discusses evolutionary relationships within the genus.

Material and methods

Collection of specimens and taxonomic assignment

Specimens of the genus *Littoraria* employed in this investigation, were obtained from the southern Kerala coast, India, from April 2019 – January 2020. A total of three sampling stations (Fig. 1) were identified where diverse *Littoraria* spp. were available: an intertidal rocky shore at Thirumullavaram (8.8994 N 76.5505 E), a barmouth at Neendakara (8.9443 N 76.5402 E), and a mangrove area at Ayiramthengu (9.1262 N 76.4798 E). The collection was done during low tide. Samples of vouchers were transported to the laboratory for identification after being stored in 95% alcohol. The shell colours were recorded and specimens photographed immediately after collection. Specimen identification primarily relied on the morphology of the shell. It was executed following the most recent taxonomic and molecular investigations (Reid, 1984). Molecular systematics was used to correctly identify the unknown specimens to create phylogenetic topologies between recognised species and unknown specimens (Ran *et al.*, 2020).

DNA processing

A molluscan tissue, approximately 200-300 mg, was excised, immersed in 96% ethanol, maintained at a temperature of -20 °C, and subjected to genomic analysis. Subsequent genetic material extraction was performed using the NucleoSpin® Tissue Kit. Agarose gel electrophoresis was employed to assess the DNA's quality. A UV transilluminator (Genei, India) was used to observe the gels. While exposed to UV light, the image was recorded with a Gel documentation system (Bio-Rad, India). The quantity of DNA was measured using a NanoDropTM 2000/c spectrophotometer (Thermo Fisher Scientific, United States). The target region of COI was amplified by PCR using universal forward and reverse primers LCO1490 5' -GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198 5' -TAA ACT TCA GGG TGA CCA AAA AAT CA-3', respectively, designed by Folmer *et al.* (1994).

The target COI gene region was amplified via Polymerase Chain Reaction (PCR) in a 25 µl reaction volume containing 14 µl nuclease-free water, 6.5 µl PCR master mix, 1.25 µl each of forward and reverse primer, 1 µl $MgCl_2$ and 1 µl DNA. The PCR conditions included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 44 °C for 1 minute, 72 °C for 1.5 minutes, with

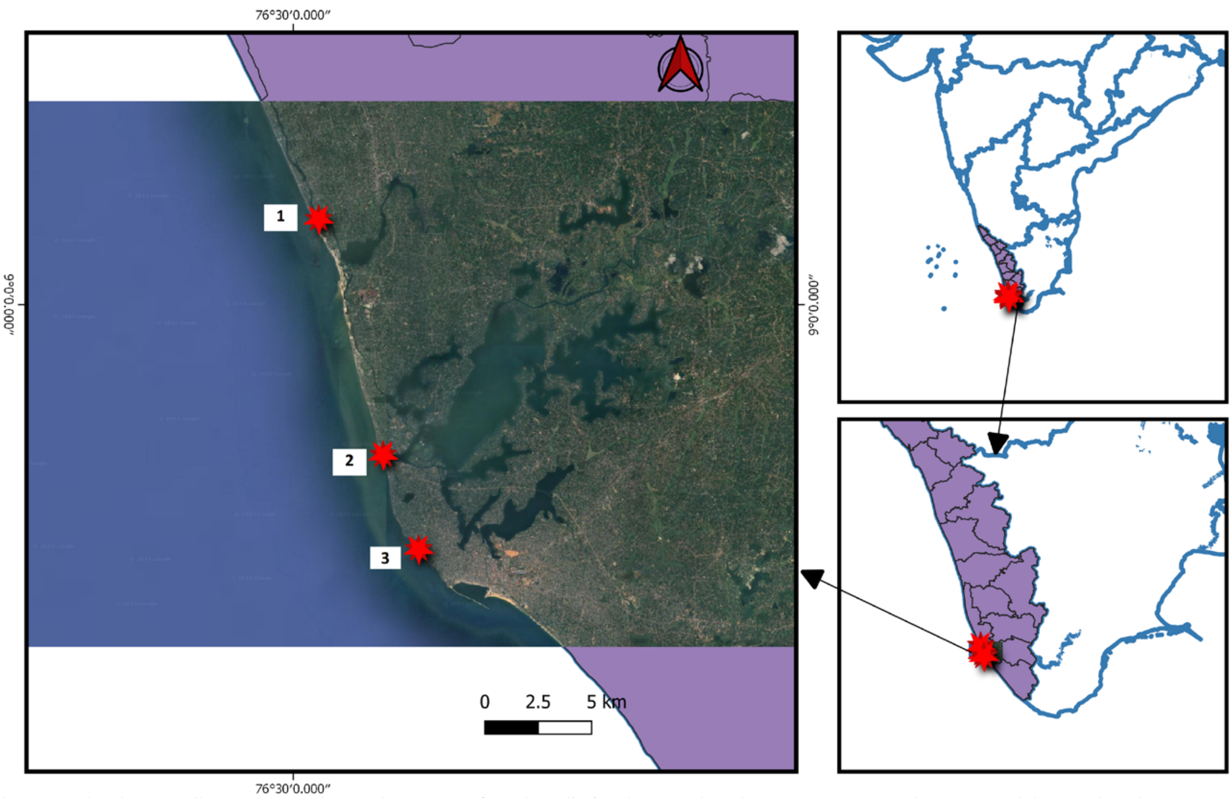

Fig. 1. Map showing sampling stations at the Southern coast of Kerala, India (Station 1 – Ayiramthengu Mangrove, Station 2 – Neendakara and Station 3– Thirumullavaram)

a final extension at 72 °C for 7 minutes. The PCR products were visualised on a 0.8% agarose gel prepared in 50× Tris-Acetate-EDTA (TAE) buffer and stained with ethidium bromide, run at 90 V and 3.51 mA for approximately 20 minutes. A Gel Documentation System with UV light was used to visualise gel images. PCR products were stored at 4 °C for subsequent use in sequencing. Applied Biosystems Sequence Scanner Software v1.0 (United States) evaluated the quality of sequences. Sequence alignment and necessary modifications of the acquired sequences were performed with the assistance of Geneious Pro v5.1 (Drummond *et al.,* 2010).

Data analysis

The 15 generated unidirectional sequences were first visually verified using the sequence editor BioEdit v. 7.0.9.0 (Hall, 1999), which was also used to eliminate primer sequences, and later align the sequences using Clustal W. Stop codons, insertions and deletions were removed from all examined sequences by manually editing each sequence to maintain accurate protein-coding sequences, using the online translation tool (https://insilico.ehu.es/translate/). Furthermore, the sequences were subjected to homology analyses, using the BLAST tool, obtaining a species identity of > 97%, and were finally deposited in GenBank, wherein accession numbers were obtained post-submission.

The software MEGA 11 (Tamura *et al.,* 2021) was utilised to analyse the 15 sequences generated in this study, specifically assessing the nucleotide polymorphism features, such as the number of conserved sites, singleton sites, variable sites, and parsimony-informative sites. The remaining two sequences were designated as outgroups and excluded from this analysis. Basic nucleotide sequence statistics like nucleotide composition, frequencies of A, T, G and C base pairs, AT content, GC content of the first, second and third codon positions, overall transition/transversion ratio, pairwise genetic distances (intraspecific and interspecific) and overall mean distance using the Kimura-2 parameter model (Kimura, 1980) were calculated using the partial deletion option in MEGA 11 (Tamura *et al.,* 2021).

The nucleotide composition and nucleotide substitution pattern were assessed, utilising a statistical framework based on the Maximum Composite Likelihood approach. The overall transition/transversion bias R, which is defined as:

$$R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]$$

Here, k1 represents the transition rate between purines, while k2 corresponds to the transition rate between pyrimidines. The analysis employed the Tamura-Nei method to calculate the transition/transversion rate ratios of purines (k1) and pyrimidines (k2).

The phylogenetic analysis was performed using the Neighbour-Joining (NJ) method (Saitou and Nei, 1987) with the Kimura-2-parameter model (Kimura, 1980) in MEGA 11 (Tamura *et al.,* 2021). The dataset comprised 52 nucleotide sequences, including 15 newly generated COI sequences and 37 homologous sequences from related species retrieved from NCBI (Table 1). Sequences were aligned, and positions with less than 95% site coverage were removed using the partial deletion option, allowing no more than 5% gaps, missing data, or ambiguous bases. This resulted in a final dataset of 598 positions. *Planaxis sulcatus* and *Nerita plicata* were selected as outgroups to root the

Table 1. Analysed samples and the GenBank accession numbers of the COI mtDNA sequences

Sl. No.	Species and Type locality	No. of specimens (n)	Collection site	GenBank accession number	
				References	In this study
1.	*L. carinifera* (Menke, 1830): Blinjoe, Indonesia	2	India: Neendakara Kollam, Kerala		OQ511508 OQ519643
		3	Malaysia: Gaya Island,	FN557085	
			Indonesia:Lembar,	FN557084	
			Malaysia:Tanjung Rhu,	FN557083	
2.	*L. undulata* (Gray, 1839): Oceania, Australia	6	India: Neendakara, Kollam, Kerala		OQ518909 OQ519642 OQ519890 OQ519888
			India: Thirumullavaram, Kollam, Kerala		OM780260 OM780261
		4	Papua New Guinea: Milne Bay	MZ559686	
			India: Kappad Beach, Kozhikode	FN557150	
			Philippines: Samar Island	AJ488635	
			China: Hainan Island	MN389027	
3.	*L. angulifera* (Lamarck, 1822): Leiden, Liberia	5	Brazil:Caraneia,	FN557072	
			Senegal:Sine-Saloum Delta	FN557073	
			USA	MH809401	
			Jamaica: Fort Charlotte	FN557071	
			USA	MH809400	
4.	*L. bengalensis* (Reid, 2001): Hare Island, Tamil Nadu	2	India: Ayiramthengu Mangrove, Kayamkulam, Kerala		OQ519854 OQ519889
		2	Malaysia: Kuah, Langkawi	FN557081	
			India: Hare I., Gulf of Mannar	FN557082	
5.	*L. melanostoma* (Gray, 1839): Djakarta Bay, Indonesia	5	China: Hainan Island	MN389028	
			Vietnam: Tuan Chau	FN557119	
			Hong Kong: Sheung Pak Nai	HE590830	
			Malaysia: Matang Forest Reserve, Perak	HE590829	
			Singapore: Sarimbun	FN557118	
		1	India: Neendakara, Kollam, Kerala	x	OQ519887
6.	*L. intermedia* (Philippi, 1846): Lombok: Indonesia	5	India: Hare I., Gulf of Mannar	FN557109	
			Egypt: Nabq, Gulf of Aqaba	FN557107	
			Philippines: Magellan Bay	FN557105	
			USA: Hawaii, Oahu, Kaneohe Bay	FN557103	
			French Polynesia: Moorea Island	KT149308	
7.	*L. vespacea* (Reid, 1986): Sumatra, Indonesia	1	Singapore: Sungi	FN557158	
8.	*L. pallescens* (Philippi, 1846): Jawa, Indonesia	4	India: Ayiramthengu Mangrove, Kayamkulam, Kerala		OR037303 OR042805 OR042803 OR042804
		4	Madagascar	MG826531	
			Madagascar	MG826530	
			Madagascar	MG826529	
			Madagascar	MG826528	
9.	*L. glabrata* (Philippi, 1846): Dautzenberg, South Africa	4	Sri Lanka: Weligama	FN557101	
			Fiji: Namaqaqua	FN557092	
			Papua New Guinea: Milne Bay	MZ559695	
			Tanzania: Mangapwani	FN557100	
10.	*L. subvittata* (Reid, 1986): South Africa	2	Mozambique	MG826672	
			Tanzania	MG826597	
11.	*Planaxis sulcatus* (Born, 1778): Moluccas, Indonesia	1	Madagascar: Nosy Ankazoberavina	MZ470583	
12.	*Nerita plicata* (Linnaeus, 1758): Sunda, Indonesia	1	Malaysia: Pantai Pandak.	OP884633	

phylogenetic tree. Tree reliability was evaluated using 1,000 bootstrap replicates (Felsenstein, 1985), with support values reported at each node. Evolutionary distances were calculated using the Kimura-2-parameter model and the tree was drawn to scale, with branch lengths representing the number of base substitutions per site.

Results

Five species of *Littoraria* were identified along the Kerala coast: *Littoraria undulata* (Gray, 1839), *L. intermedia* (Philippi, 1846), *L. bengalensis* (Reid, 2001), *L. pallescens* (Phillippi, 1846), and *L. carinifera* (Menke, 1830) (Fig. 2).

Morphological description of *L. carinifera*

Littoraria carinifera features a sturdy, cone-shaped shell, ranging from 12 to 24 mm in height, with six to seven whorls. The spire is gently convex, with whorls that are almost flat and sutures lacking clear definition. A distinctive keel-shaped ridge marks the periphery, accompanied by a wide columella with a slight, shallow depression. The shell's surface bears seven to nine main grooves and 15 to 30 ribs on the final whorl, some with a keel-like form, while finer sculptural details are faint or absent. The colouration is grey, with 13 to 18 thin, orange or brown vertical stripes on the last whorl, and the aperture displays dark brown bands aligning with the external keels. Closer inspection reveals delicate spiral lines on the ribs and vertical lines within the grooves. Beyond its type locality, Reid (1984) documented this species in India at sites including Bandra in Bombay, Vingurla near Goa, and Netravati in Mangalore.

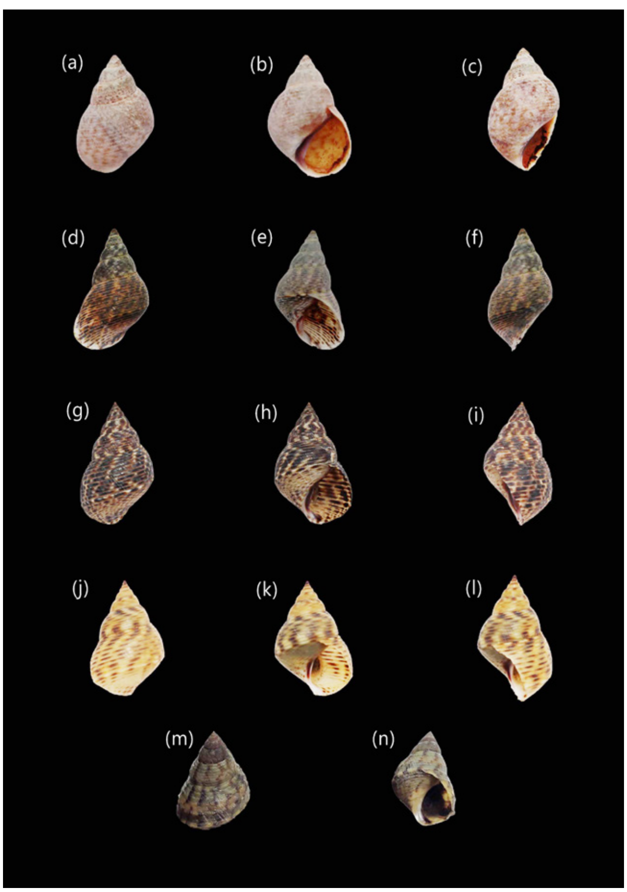

Fig. 2. Shell morphology of *Littoraria* spp., collected from the Southern Kerala coast depicted in dorsal, ventral and side views: (a-c) *L. undulata*, (d-f) *L. bengalensis*, (g-i) *L. intermedia* (j-l) *L. pallescens* and (m-n) *L. carinifera*

COI gene analysis

Following sequence alignment and quality trimming (including removal of stop codons, indels, and ambiguous regions), all 50 sequences were standardised to a conserved 606 bp length for phylogenetic analysis. The numbers of conserved sites, singleton sites, variable sites, and parsimony-informative sites were recorded as 414/606, 9/606, 192/606, and 183/606, respectively. A, T/U, C, and G had average nucleotide proportions of 25.59%, 33.9%, 22.58% and 17.94%, respectively. The base composition assessment found that the average Thymine proportion exceeded all other bases, while the average Guanine percentage was the lowest. Compared to the GC, the AT composition (average 59.6%) was more significant (Table 2). *L. undulata* (41.67%) and *L. intermedia* (39.32%) had the greatest and lowest GC compositions, respectively. Variations in GC content have an impact on distinct positions within codons. Analysis of GC composition within COI sequences of Littoraria spp. revealed a non-uniform distribution. The highest GC content (56.2%) was observed at each codon's first (5'-most) nucleotide position. This value progressively declined to 42.1% at the

Table 2. The base composition and AT-GC content percentages and GC content of each of the three codon positions of COI sequences of the genus *Littoraria*

Littoraria spp.	T(U)	C	A	G	GC	AT	1st	2nd	3rd
L. carinifera	35.28	21.59	25.00	18.12	39.71	60.29	57.34	42.21	19.62
L. melanostoma	35.02	21.19	25.21	18.58	39.77	60.23	55.45	42.08	21.78
L. vespacea	33.83	22.44	26.07	17.66	40.10	59.90	56.44	42.08	21.78
L. glabrata	34.53	21.09	25.77	18.61	39.70	60.30	54.27	42.08	22.66
L. undulata	32.34	23.78	25.98	17.90	41.67	58.33	56.71	42.15	26.17
L. intermedia	34.70	21.66	25.98	17.67	39.32	60.68	55.33	42.08	20.56
L. bengalensis	33.30	22.84	25.75	18.11	40.95	59.05	55.75	42.18	24.94
L. angulifera	34.10	22.09	25.09	18.72	40.81	59.19	57.38	42.08	22.97
L. pallescens	34.02	22.59	25.55	17.83	40.42	59.58	56.92	42.00	22.44
L. subvittata	32.76	23.51	25.66	18.07	41.58	58.42	56.44	42.08	26.24

second and 22.92% at the third (3'-most) nucleotide position within each codon. The transitional substitution rates ranged from 8.15 to 35.44, while those of transversional substitutions ranged from 1.9 to 3.59 (Table 3). In the dataset, purines had a transition/transversion rate ratio (k1) of 4.293, and pyrimidines had a rate ratio (k2) of 9.875. The overall bias for transitions/transversions was calculated as R = 3.876.

Based on mitochondrial COI gene sequences, Table 4 presents the pairwise genetic distances (K2P%) within and between species of the genus *Littoraria*. With a mean value of 0.67%, the intraspecific genetic distance varied between 0% and 1.3%. In contrast, the interspecific genetic distance varied from 5.6% to 19.4%, averaging 14.86%. The highest interspecies distance occurred between *L. subvittata* and *L. melanostoma*, while the lowest was between *L. intermedia* and *L. bengalensis*. The average distance within the genus across all comparisons was calculated to be 0.14.

Neighbour-joining (NJ) tree

The COI sequences of all individuals were used to create the condensed form of the evolutionary NJ tree (Fig. 3) where all of the specimens under study were seen in delineated groups. *Planaxis sulcatus* and *Nerita plicata* were chosen as outgroups. The phylogenetic tree was represented by five well-defined clades as follows:

Table 3. Estimation of the nucleotide substitution pattern based on the maximum composite likelihood model

	A	T	C	G
A	-	*3.59*	*2.39*	8.15
T	*2.71*	-	23.6	*1.9*
C	*2.71*	35.44	-	*1.9*
G	11.63	*3.59*	*2.39*	-

Note: R-value 3.876, Transversional substitution rates are indicated in italics, while transitional substitution rates are displayed in bold

Clade I: *L. intermedia*, *L. bengalensis*, *L. angulifera* and *L. pallescens*
Clade II: *L. subvitatta*
Clade III: *L. undulata*
Clade IV: *L. vespacea, L. carinifera and L. melanostoma*
Clade V: *L. glabrata*

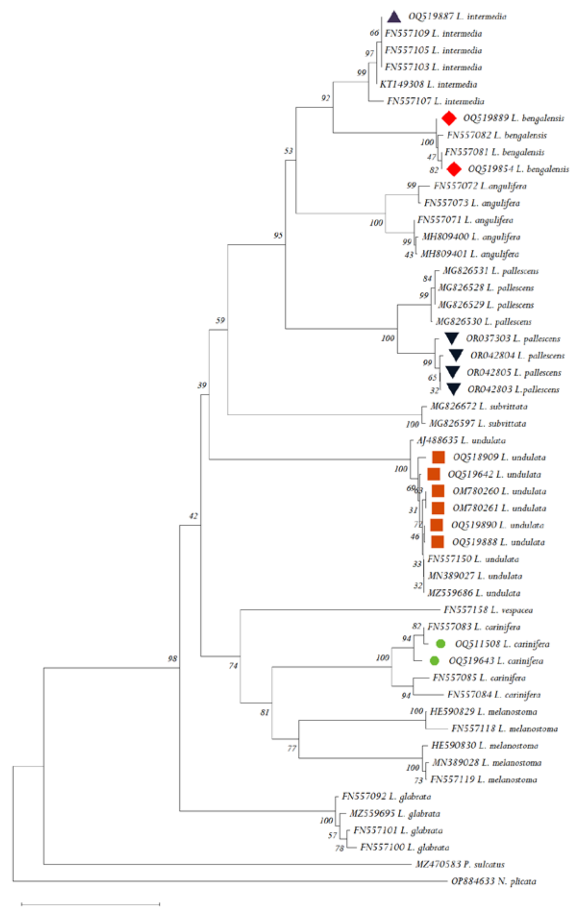

Fig. 3. NJ tree of *Littoraria* spp. obtained through the partial sequences of Cytochrome c Oxidase subunit I (COI) using MEGA 11

Table 4. Mean genetic distances (K2P%) within and between *Littoraria* spp. based on COI gene sequences

Littoraria spp.		1	2	3	4	5	6	7	8	9	10
1.	*L. carinifera*	2.0									
2.	*L. melanostoma*	11.3	0.9								
3.	*L. vespacea*	14.8	13.3	0.0							
4.	*L. glabrata*	16.7	17.3	15.7	0.6						
5.	*L. undulata*	16.6	19.6	15.7	14.3	0.2					
6.	*L. intermedia*	15.2	15.7	17.5	14.3	13.9	0.3				
7.	*L. bengalensis*	17.5	17.6	16.7	14.9	14.9	5.6	0.1			
8.	*L. angulifera*	16.7	15.5	17.1	15.8	16.8	7.5	10.0	1.0		
9.	*L. pallescens*	15.6	15.9	15.7	14.6	16.3	9.0	11.4	9.9	1.3	
10.	*L. subvittata*	18.4	19.4	18.2	16.0	14.6	12.5	13.6	14.0	15.2	0.3

The NJ tree shows the percentage of associated taxa clustered together next to its branches. Since barcodes of the same species were consistently grouped in the same clade, it indicates that barcodes of the same species exhibit minimal variation. Two closely related specimens formed unified groups, clearly distinguished from each other in the NJ tree. Each species within the genus formed distinct monophyletic clusters based on the COI gene. Bootstrap values greater than 70% supported major branching nodes, with branch lengths scaled to 0.05 nucleotide substitutions per site.

Discussion

The present study documented the presence of five species, namely *L. undulata, L. intermedia, L. carinifera, L. bengalensis*, and *L. pallescens*, through both conventional and molecular taxonomy. *L. carinifera* was identified for the first time on the Kerala coast. However, *L. glabrata* and *L. scabra* were not recorded among the species collected in the present study. Based on the reports of Bijukumar (2012), Franklin and Laladhas (2014), and Ravinesh *et al.* (2021), five *Littoraria* spp. were recorded from the Kerala coast (*L. undulata*, *L. scabra*, *L. intermedia*, *L. bengalensis*, and *L. glabrata*). Bharti and Shanker (2021) authenticated *L. undulata*, *L. bengalensis,* and *L. pallescens* from the Kerala coast. Until now, only these species have been primarily attempted to be identified using conventional taxonomic methods, standard keys, and references.

In this study, species such as *L. intermedia*, *L. bengalensis* and *L. carinifera* are recognisable by their shell colour and sculpture (Reid, 1986a, 1999a; 2001). But *L. undulata* and *L. pallescens* exhibited inconsistent shell patterns. In such cases, relying on morphological species keys alone makes it difficult to differentiate species. Thus, DNA barcoding is a reliable method for species identification within the genus *Littoraria*. This identification method improved the accuracy of species identification through the integration of morphological and DNA barcoding approaches.

The phylogenetic reconstruction exhibited branch support values exceeding 75% at critical nodes, indicating that these species represent the most robust clades within the tree. All littorinid species analysed in this study were closely related, forming well-supported monophyletic groups. Based on several ancestral anatomical features and character coding, *L. undulata* was included in the broader clade comprising both *L. glabrata* and *L. intermedia* (Reid, 1999b). The NJ tree aligns with Reid *et al.* (2010), grouping *L. intermedia*, *L. bengalensis* and *L. angulifera* as sister species, while *L. carinifera*, *L. vespacea* and *L. melanostoma* are clustered together in a broader clade. Similarly, *L. pallescens* is closely related to *L. angulifera*, supporting the broader taxonomic relationships within the genus *Littoraria*. The separation of *L. subvittata* (Clade II) as a distinct lineage suggests potential cryptic diversity or unique evolutionary pressures, warranting further investigation. The low COI divergence (≤0.05 substitutions/site) within species supports the efficacy of this marker for barcoding applications, and robust bootstrap values reinforce the NJ method's reliability for genus-level phylogenies.

COI was chosen as the standard barcode gene as it is present in many species, which demonstrates little overlap between intraspecific and interspecific genetic distance, with the K2P model indicating that intraspecific genetic distances of animals are often less than 1% and never higher than 2% (Hebert *et al.,* 2003). A 3% molecular threshold was the species delimitation's most used cut-off value (Carpenter and Niem, 1999). The use of a fixed empirical threshold of 3% for species delimitation was criticised by Zhang *et al.* (2017), suggesting that it could potentially overestimate species diversity in insects (Zhang and Bu, 2022), which shows substantial intraspecific genetic variation (>3%). According to Hebert *et al.* (2004), the threshold for animal species identification was the mean interspecific difference, ten times greater than the mean intraspecific difference. The results of this study supported this finding. Geographical positions significantly influence evolutionary divergence (Aguillon *et al.,* 2017), where an increase in geographic distance between populations often corresponds to pronounced evolutionary differences.

The genetic variety of these clades has been revealed by phylogenetic and genetic differentiation research, suggesting the potential existence of cryptic species. The intraspecific genetic distances of COI sequences conform to the intraspecific threshold values detected in other molluscs (Feng *et al.,* 2011).

Variability in GC composition

The analysis of nucleotide distribution within the mitochondrial COI sequences of the studied *Littoraria* spp. revealed that the AT content was higher than the GC content, which proved consistent with the study by Sun *et al.* (2012). The GC content variation exerts an effect on distinct positions within codons. In *Littoraria* spp., the average concentration of GC was 56.2% in the first codon positions of COI sequences. At the same time, 42.1% and 22.92% were for the second and third codon positions, respectively. The average GC content in Indian snails of the species *Telescopium* sp. was 41.79%, with values typically falling between 41.49% and 42.27% (Palanisamy *et al.,* 2020). Lower values averaging 36.9% were observed in Canadian molluscs (Layton *et al.,* 2014) and 39.24% for gastropods from Bangladesh (Mahjabin *et al.,* 2023). The degree of selective constraint reflected the differences between these codon positions; hence, GC content offers crucial information about the types of selected forces influencing nucleotide usage

(Clare *et al.,* 2008). The codon positions of mitochondrial genes were affected by base-mutation pressure throughout species evolution; base use bias resulted from base-mutation pressure in codon positions. Furthermore, several biological processes, such as gene expression, have been demonstrated to correlate with GC concentration (Quax *et al.,* 2015).

Conservation status of Littoraria spp.

Littoraria spp. are classified as continental and oceanic (Reid, 1985), corresponding to mangrove dwelling (eg, *L. pallescens*, *L. angulifera* and *L. articulata*) and rock–dwelling (eg, *L. undulata* and *L. intermedia*), respectively. Rocky shore species such as *L. undulata* (Gray, 1839) were found to be the most abundant species (3133 nos) by Karnaver *et al.* (2023) along the intertidal rocky shores of the Thiruvananthapuram coast, while *L. articulata* (Philippi, 1846) was found abundant in the Azheekal coast (Dhanyaraj *et al.,* 2024). *L. undulata* and *L. angulifera* are listed as Least Concern on the IUCN Red List, indicating a low risk of extinction globally (IUCN, 2025). In contrast, mangrove-associated species such as *L. carinifera*, *L. intermedia*, *L. bengalensis*, and *L. pallescens* remain 'Not Evaluated' by the IUCN, highlighting a significant data deficiency in their conservation status, which might not reflect local or regional population trends.

Neendakara, situated at the mouth of the Ashtamudi estuary (Johnson and Muthu, 2022), along with Ayiramthengu mangrove forest, an environmental hotspot (Praseetha and Rajani, 2015), are under severe threat from multiple sources. Pollution from boat-related oil, grease, and industrial effluents introduces heavy metals and contaminants into the ecosystem, and runoff from nearby slaughter houses has heavily polluted Neendakara harbour (Sajeev and Subramanian, 2003; Jayakumar and Chackacherry, 2011). Habitat loss, driven by reclamation for commercial and industrial use, fragments and disrupts natural processes (Wu *et al.,* 2018; Paravat *et al.,* 2009) in mangroves. These pressures are intensified by violations of Coastal Regulatory Zone (CRZ) regulations, undermining efforts to safeguard coastal ecosystems (Ramachandran *et al.,* 2005; Vincent and Owens, 2021).

The Management Action Plan (MAP) for Wetland, funded by the Ministry of Environment and Forests (MoEF), Government of India, focuses on key initiatives, for the conservation of *Littoraria* habitats (Jayakumar and Chackacherry, 2011), which include: catchment treatment (including afforestation and soil/water conservation), flora and fauna preservation, pollution control, sustainable wetland fisheries management and community awareness programs. They also address legal protections in India, such as the inclusion of mangroves in the CRZ-1 category and the Kerala Conservation of Paddy Land and Wetland Act (2008), which bans mangrove destruction or conversion (Hema and Devi, 2014). The Kerala State Coastal Zone Management Authority regulates mangrove-related projects, while the Department of Forests and Wildlife supports conservation initiatives on private lands (Hema and Devi, 2014). Despite these measures, enforcement remains challenging, particularly on private properties (Muraleedharan *et al.,* 2009). The conservation status of unevaluated *Littoraria* spp., requires urgent research to assess population dynamics and distribution, potentially using tools like remote sensing and GIS (Sreelekshmi *et al.,* 2021). Effective conservation also hinges on community participation, as local involvement is critical for success (Barbier, 2008; Stone *et al.,* 2008)

Thus, while *L. undulata* and *L. angulifera* are 'Least Concern' globally; the unevaluated status of other *Littoraria* spp., coupled with threats to the Ashtamudi wetland, signals a need for robust conservation strategies. Legal frameworks offer a starting point, but their success relies on filling data gaps, enforcing regulations, and engaging communities. Further research and participatory approaches are essential for the survival of these ecologically significant species.

Conclusion

This study offers valuable insights into the genetic and phylogenetic characteristics of *Littoraria* spp., along the Kerala coast, by integrating morphological and molecular methods to improve species identification accuracy. Including COI gene sequences has significantly enriched the genetic database for the genus *Littoraria*, providing essential observations on nucleotide composition, genetic variability, and phylogenetic relationships. The findings indicate that the average AT content surpasses the GC content across all species, with GC distribution varying at different codon positions. Phylogenetic analysis confirmed the monophyletic nature of *Littoraria* and identified three distinct clades characterised by minimal intraspecific genetic variation and clearly defined interspecific differentiation. Additionally, the results underscore the effectiveness of COI barcoding for species delimitation, aligning with established thresholds for genetic distances and supporting the identification of cryptic species within the genus.

The study documented the presence of *L. carinifera* along the Kerala coast for the first time, providing new biogeographic records for the region. The observed variability in GC composition and nucleotide usage patterns across codon positions offers valuable insights into the evolutionary pressures and selective constraints shaping the mitochondrial genome of *Littoraria* spp. These findings emphasise the significance of integrating morphological taxonomy with molecular barcoding to enhance species identification and phylogenetic analysis. Universal mollusc primers facilitated the identification of various species through barcode sequences by incorporating additional sequences using alternative primers for all *Littoraria* spp. Species taxonomy could be refined in the current database, and ecological diversity could be more accurately represented. The data generated by this study contribute to a deeper understanding of the genetic diversity, evolutionary relationships, and biogeographic distribution of *Littoraria* spp., thus laying the groundwork for future research on their ecological and evolutionary dynamics.

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Author contributions

Conceptualisation: AKAK, BH, SJ; Methodology: AKAK, BH; Data collection: AKAK; Data analysis: AKAK, BH, SJ; Writing original draft: AKAK; Writing review and editing: BH, SJ; Supervision: BH, SJ

Data availability

The data are available and can be requested from the corresponding author.

Conflict of interest

The authors declare that they have no conflict of financial or non-financial interests that could have influenced the outcome or interpretation of the results.

Ethical statement

No ethical approval is required as the study does not include activities that require ethical approval or involve protected organisms/ human subjects/ collection of sensitive samples/ protected environments.

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