

## Molecular approach to the identification and phylogenetic biogeography of snail *Telescopium telescopium* using mt-COI gene sequences

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**Abstract:** We aimed to apply DNA barcoding tool for the molecular identification of horn snails *T. telescopium* using mitochondrial cytochrome oxidase I gene (mt-COI) and to investigate their evolutionary relationship along with location-specific bio-geographical variations. The molecular data sets of this study indicate that strong probability of *T. telescopium* species taxonomic confirmation using mt-COI sequences. Results of the phylogenetic analysis suggest that *Telescopium* sp. was monophyletic with disseminated nodes and the evolution of group II originated from group I. The substantial genetic distance among the mt-COI sequences (0.005 to 0.184) were noticed. Large divergence between the south-west coast of India and Australia region population indicates limited gene flow between the two continents. Our study suggests that the genera *Telescopium* is globally ubiquitous but genetically showing inter-region differentiation. We conclude that mt-COI gene can be used to identify gastropod *T. telescopium* species.

**Keywords:** Barcoding, biodiversity, genetics, mollusc, phylogeny, snails, taxonomy

## 1. Introduction

Indian coastal waters are receiving more attention from the scientific community due to their enormous fishery and shellfish potential resources [1]. In the Indian Ocean, Bay of Bengal waters hosts an extent range of molluscan (bivalves and gastropods) faunal distribution, species diversity and they are ecologically important to many communities [2]. However, this group diversity is highly underestimated [3]. Molluscs are the most diverse marine phylum and represent higher species diversity has resulted in a considerable taxonomic problem [4]. Some species in Indian coast remain uncertain; there is a need to incorporate molecular methods. Molecular markers especially mitochondrial DNA cytochrome oxidase I (mt-COI) are considered as a better choice for species discrimination due to non-recombinant and environment independent nature [4]. The application of DNA barcoding brings substantial benefits to other fields, such as border biosecurity [5], biodiversity conservation [6], ecological monitoring [7].

DNA barcoding has successfully utilized for the taxonomic confirmation of marine organisms, the molecular phylogeny of gastropod molluscs is extensively studied using morphometric characteristics and DNA sequences [8]. The standard pairwise distance method such as Neighbour-Joining tree (NJ) based on Kimura 2-parameter distance (K2P) and GC content divergence assessment is currently the principal approach used to analyze patterns of diversity with COI barcode region. It has been informative at the species-level discrimination across the range of several groups from terrestrial, freshwater and marine environment [9]. The accuracy of such results depends particularly on the delineation between intra-specific variation and inter-specific DNA sequence divergence [10]. The strong molecular identification of marine gastropods requires the creation of comprehensive reference library of DNA barcode matching of each specimen to a diagnostic barcode sequence [11].

Gastropod mollusc *Telescopium telescopium* lives in mud-flats of mangrove forest in throughout the coastlines of Indian Ocean waters. Mangrove horn snail or mud whelks *T. telescopium* is the only living organism of this genus from the family Potamididae (Gastropoda), found abundantly and frequently dominated in mud-flats of mangrove forest in West and Central Indo-Pacific regions due in part of its central locations at the meeting of oceanic waters has the greatest diversity of mangrove forest. The distribution of this species is likely impacted locally by the loss of habitat [e.g. mangrove deforestation, marine pollution] [12]. So far, no reliable information available to deal the estimated population of this species in Indian waters. Houbbrick et al. [13] was reported the revised systematics and phylogeny of this taxa, following that Bandel et al. [14] was recognized two sub families, Potamidinae and Telescopinae, the

later on included all the living potamidids except the genus *Potamides* in the family Potamididae. With the recent development of molecular techniques, the evolutionary relationship of Potamididae and their ecological association with mangrove ecosystem has also been studied using mt-COI sequence data [15]. The further research of this genus will reveal to complete understanding of the evolutionary relationship of this species in family Potamididae, ecological association with mangrove forest and their potential application in pollution monitoring [16] and marine bio-discovery program [17, 18]. The DNA sequence of mt-COI gene data facilitate not only the inference the phylogenetic relationships of fauna but also providing an efficient method for species-level identification under the term of molecular taxonomy or DNA Barcoding [19].

### 1.1 Evolutionary significant of horn snail

Mangrove horn snail or mud whelks *T. telescopium* is the only living organism of this genus from the family Potamididae (Gastropoda), found abundantly and frequently dominated in mud-flats of mangrove forest in West and Central Indo-Pacific regions due in part of its central locations at the meeting of oceanic waters has the greatest diversity of mangrove forest. The distribution of this species is likely impacted locally by the loss of habitat [e.g. mangrove deforestation, marine pollution] [12]. So far, no reliable information available to deal the estimated population of this species in Indian waters. Houbrick et al. [13] was reported the revised systematics and phylogeny of this taxa, following that Bandel et al. [14] was recognized two sub families, Potamidinae and Telescopinae, the later on included all the living potamidids except the genus *Potamides* in the family Potamididae. With the recent development of molecular techniques, the evolutionary relationship of Potamididae and their ecological association with mangrove ecosystem has also been studied using mt-COI sequence data [15]. The further research of this genus will reveal to complete understanding of the evolutionary relationship of this species in family Potamididae, ecological association with mangrove forest and their potential application in pollution monitoring [16] and marine bio-discovery program [17, 18]. The DNA sequence of mt-COI gene data facilitate not only the inference the phylogenetic relationships of fauna but also providing an efficient method for species-level identification under the term of molecular taxonomy or DNA Barcoding [19].

The aim of this present study, to improve the systematics of the genus *Telescopium* by the integration of morphometric characteristics and DNA barcoding of *T. telescopium* shells found in the Bay of Bengal waters. ii) to determine their evolutionary relationship by biogeographical variations based on mt-COI sequences.

## 2. Material and methods

### 2.1 Collection of samples

Horn snails used in this study were collected in Parangipettai coast, Bay of Bengal - India during March to August 2011 and their taxonomy was identified using [13]. All the specimens (n=10) were preserved in 95% ethanol for DNA molecular analysis.

### 2.2 DNA Isolation and Sequencing

Total DNA was extracted from the muscle tissue/eggs of each specimen using Qiagen DNA Easy blood and tissue kit (Hilden, Germany). Universal Primers LCO 1490 and HCO2198 as described in Folmer et al. [20] were used for the amplification of a fragment of mt-COI gene. PCRs were performed in a 12.5 µl volume with 6.25 µl of 10% trehalose, 1.25 µl PCR buffer [200 mM Tris– HCl (pH 8.4), 500 mM KCl], 2 .85 µl of nuclease free water, 0.625 µl MgCl<sub>2</sub> (50 mM), 0.5 µl of each primer (10 µM), and 1 µl of DNA template. A single soak at 95°C for 2 min was followed by 35 cycles (denaturation at 94°C of 30s, annealing at specific temperature 52°C for 30s) and extension at 72°C for 1 min) and a final extension at 72°C for 10 min on a PCR Thermal Cycler. Further, the PCR products were purified using PCR Purification Kit (Qiagen, Hilden, Germany). The same primers were used for the sequencing reaction, and the PCR products were sequenced using an ICM version 3.1 automated sequencer (MegaBace) Bioserve DNA sequencer, BioServe Biotechnologies, Hyderabad, India.

### 2.3 Data analysis

DNA sequences were edited using Bioedit Sequence Alignment Editor V 1.0, 2005 [21], and an alignment of snail's sequences was performed using CLUSTAL W [22]. For the phylogenetic analysis, we were included 26 sequences of *T. telescopium* were retrieved from variable sites in the data set along with out-group of organisms. Phylogenetic analysis was conducted using Maximum Likelihood (ML) and Bayesian inference (BI). For ML analysis, the best fit jModelTest v 3.06 (Provo UT, USA) used for constructing the phylogenetic tree with genetic distance data were generated in MEGA6 [23]. For BI, general time-reversible model (GTR) of sequence evolution with a gamma distribution and variable set was selected and made with the use of MrBayes v 3.2.1 [24]. The Markov Chain Monte Carlo (MCMC) analysis was run for 50,000, which was sampled every 1000 generations and the first 25% of the tree were discarded as burn-in, the remaining trees were used to construct the final tree and estimate posterior probabilities (Pp).

The phylogenetic parameters were observed using Figtree v.1.4.0 (<http://beast.bio.ed.ac.uk/FigTree>). The molecular phylogeny was built to understand the inter-relationship of genetic variation between the species, and genetic variance by bio-geographical locations. The biogeographic distribution of this horn snail was determined for the species as widely distributed in the Indian Ocean and Indo-pacific regions, following that the classification of biogeographic regions and taking into account the previous report of this species distribution.

### 3. Results and Discussion

#### 3.1 Taxonomic assignment

*Description:* Shell is very thick and solid, black coloured at first glance, originally reddish-brown colour. Length of the shell size was varied from 80–145 mm, large and conical shaped with numerous flat-sided whorls sculptured with spiral grooves. Shell aperture narrowly ovate and outer lip curved toward centrally placed, short siphonal canal. Very long snout with small buccal mass and taenioglossate radula. Mantle with siphonal light sensory organ. The zygonerous nervous system was observed, the egg capsules deposited in gelatinous strings.

*Distribution:* Widely distributed in the Central Indo-pacific, West- Indo-pacific regions. Native to India, Australia, Indonesia, Philippines, Malaysia, Thailand, Singapore, Japan and Madagascar.

*Material examined:* Vellar estuary tidal flats, 2008; Pondicherry mangroves, September 2010, Central Marine Fisheries Research Institute, Kochi – India, museum Repository (D.B. 8.3.1).

The present study was designed to identify the mangrove mollusc *T. telescopium* using mt-COI gene sequences. Morphological data were compared to molecular results to illustrate major trends encountered. Morphologically identified taxa and corresponding molecular identifications using mt-COI gene of *T. telescopium* are listed in Table 1. Morphological assessments identified *T. telescopium* of ten samples are shown in Figure 1. The COI data of genera *Telescopium* was significantly correlated with morphological assessment. Our results confirmed the mt-COI based molecular identification of *T. telescopium* to support previous suggestions of [15].

#### 3.2 Molecular Phylogenetic analysis

The phylogenetic tree was constructed along with twenty-six sequences retrieved from variable sites in the data set (Table 2). Indian mangrove snails exactly formed a phylogenetic group with same species referred from GenBank in the phylogenetic tree of mt-COI (Figure 2),

and these results supported the validity of morphological identification of *T. telescopium* species. The topological tree derived from the best-fit models of nucleotide substitution for individual data based on AIC selected by jModel Test with criterion (GTR) was calculated. The variation in nucleotide composition was studied using GC content analysis. GC content is also varies taxonomically within mitochondrial genome, although all mitochondrial genomes sequenced to date are poor, and widely applied in systematics [25, 26]. The mean GC content variation of Indian snails *T. telescopium* 41.79%, but showed minor variation (41.49 to 42.27 %) are given in Table 1. A chi-square test of homogeneity demonstrated significant variation in nucleotide frequencies among individual species ( $p < 0.001$ ). In the previous study, Layton et al., (2014) reported the mean GC content of Canadian molluscs 36.9% (range 24.5-46.5 %), and 37.62 % in north western pacific molluscs varied substantially among molluscan orders [27]. Generally, it is higher in mammals, birds and teleost fish (range 32-46%), following that molluscs (29-40%) and found lowest in nematodes and insects ranging from 15-35% [25].

Results of this study showed three major strong clades and a significant distinction of this species diversified from all over the Indian ocean in the newly reconstructed phylogenetic tree. In Bayesian tree method, our molecular phylogenetic hypothesis showed genera *T. telescopium* to be a monophyletic group with high affinity of Posterior probabilities, (1.0Pp) and Maximum likelihood bootstrap value (Mlb) of 99Mlb along with disseminated nodes. Our results exhibited a consistent strong monophyletic clade (1.0Pp, 100Mlb) of the individual snail (AM932795; JX390723.1) collected from Goa and Kochi waters (West coast of India) formed a separate clade (Clade I) with maximum genetic distance (13.6–18.7%) in Figure 2. Due to the lack of additional sample and mt-COI sequence data of this species we could not reveal the entire clade's structure.

In clade II the other individuals were separated into two groups (Group I & II) by biogeographic site variation except for samples (KT336816, 17). Group I clade was scattered with many deep nodes (0.57 to 0.98Pb, 52-98Mlb), and observed the good indication of subdivision in all the three major groups (Table 3). Additionally, in clade II (group I) have formed a separate cluster with the firm affinity of samples collected in Indian and Australian waters (1.0Pb, 92Mlb). Particularly, the snails were reported in Australian waters forming a separate subclade with the significant affinity (0.95Pp, 94Mlb) to the Indian individuals. Furthermore, the group II formed a deep node comparing to group I clade with strong support (0.97Pp, 99Mlb). While remaining horn snail formed several clade nodes with huge divergence with Indian species. The results of this present study confirm that genera *T. telescopium* have an intra-species diversification between the species reported in various

sites in Indian Ocean waters. In the previous study, Lydeard et al. [28] were reported moderate support (bootstrap 87%) for a sister relationship between *Telescopium* and *Terebralia* sp, later Reid et al. [15] have reported limited support of posterior probability (93%) using mt-COI, 16S and 28S sequence data sets. As well, Reid et al. [15] have reported the higher value of genetic distance up to 16% (used Kimura two-parameter model) of *T. telescopium* collected in various sites and mt-COI sequence of this species formed a complex phylogenetic structure. Similar to this previous study, we have observed the maximum value of genetic distance in between the species up to 18.7% after additionally adding our Indian snails mt-COI sequence data in data set. The results of this present study confirm that *Telescopium* snails reported in Australian and Indian waters are very close relatives or from same population stock. The individual snails were collected in Bay of Bengal waters formed many subclades with the higher value of genetic divergence (1.0–10.5%), these results demonstrated that more intra-subpopulation of horn snail was appeared and diversified in the new mangrove environment shortly and evolved. Similar results of this study were reported by [15], the results of this present study confirmed that the potamidae horn snail's origin and radiation were within the Indo-West Pacific region.

As presently understood, horn snail *T. telescopium* is the only living organism from the Potamidae occupying a distinct range of microhabitats of mangrove ecosystem [13,29]. The detailed biogeographic history of Potamidae is not yet studied completely, the evolution of the Potamidae molluscs has been very well connected with that of mangrove ecosystem ever since, while few genera (*Cerethedia* spp, *Terebralia* sp) lost their dependence with mangrove fauna [15]. Based on the previous research report, *T. telescopium* have been widely distributed in Indian waters (Bay of Bengal, Arabian Sea), Indian Ocean ecoregions (Philippines, Indonesia, Malaysia and Thailand), Indo-pacific (Australia) and completely absent in mangroves of Lakshadweep Islands, West coast of India [30]. The analysis of this study is extending our knowledge of the biogeographic distribution of this horn snails and confirmed that *T. telescopium* found only in Indian Ocean waters. Remarkably, these potamid snails widely distributed in Indian waters and West Indo-Pacific regions (Figure 3). In phylogeny analysis, the snails were collected in the Arabian Sea (Goa and Kochi Waters) formed a separate clade (Clade I), and the remaining samples gathered in Bay of Bengal waters and other study sites formed a separate clade (Clade II) from this group (Figure 3). Further, we concentrated the study on how does the inclusion of bio-geographically separated populations influences DNA barcoding. As given the results in Table 3, the expansion of geographical coverage significantly increased intra-specific divergences. Intra-specific sequence divergences ranged from 0 to 18%, the evolutionary distance between the sequences of bio-geographic

samples from (Thailand, Philippine, Malaysia and Indonesia) are equal 0.08 (8%). The regression between sample size and mean divergence is insignificant ( $p=0.32$ ;  $R^2 = 0.005$ ) as well as the regression between sample size and maximum divergence ( $p=0.67$ ;  $R^2 = 0.0009$ ). The outcome of this study revealed that could be possible of two different population stocks of *T. telescopium* in the Indian Ocean. Considerable divergence between the south west coast of India and Australia populations indicates limited gene flow between two the continents. In future research, further adding of mt-COI sequence data in dataset samples collected from Kenya, Madagascar and West coast of Karachi waters may re-confirm these results. These datasets are potentially useful for the assignment of unknown individuals and accurate documentation of molluscs using DNA barcoding method, also provide the foundation for the biogeographic analysis and species diversity on a global scale.

The deforestation of mangrove ecosystem, discharge of industrial and sewage waste through small tributaries and channels into the Bay of Bengal waters is threatening natural biota, especially the biodiversity of invertebrates (e.g., crabs, molluscs) in Indian waters [2, 7, 12]. Remarkably, Genus *Telescopium* sp and *Cerithedia* spp. losing their dependence with mangrove fauna, particularly the population of horn snails is greatly declined in estuaries and mangroves (Lakshadweep and Puducherry) due to the higher amount  $H_2S$  from deeper sediments [12,30]. The immediate need is to maintain the existing sewage treatment plants so that effluent discharge has a minimum of suspended solids. Molluscan fauna in Indian mangrove ecosystem under sewage pollution threat need conservation. Thus, estuaries and mangroves in the south-east coast of India need urgent monitoring.

#### 4. Conclusions

The conclusions of this study are in agreement with the phylogenetic analysis conducted by [15], as well as with the latest morphological update of the genus [29]. The outcome of this study established a significant contribution to DNA barcode reference sequences of horn snails and demonstrated the inter-generic relationship for effectively monitoring their ecological association with mangrove fauna. In addition, we provided a newly constructed phylogenetic structure using mt-COI sequence data, which suggests that *T. telescopium* is monophyletic. However, it is important to note that some of the cases of deep intra-species genetic divergence showed a biogeographic partition between lineages on the Indian Ocean waters, and mainly found in the Indo-Pacific and Indian Ocean waters, but genetically showing inter-region differentiation. Results of this study suggest that an integrative taxonomy of morphological and molecular methods represents a power tool, when used appropriately, and serve as a point of

reference for future genetics research of *T. telescopium*, which will be vital for all consideration regarding the evolution of family potamid mollusca.

**Conflict of Interest:** Authors do not have any conflict of interest.

**Author Contributions;** Authors Satheesh and Prasanna designed this study, Methodology and lab work; Prasanna, Data Analysis; Satheesh, Purushoth and Umamaheswari; Writing-original draft preparation; Satheesh, Purushoth and Umamaheswari; Writing-Review, Editing;

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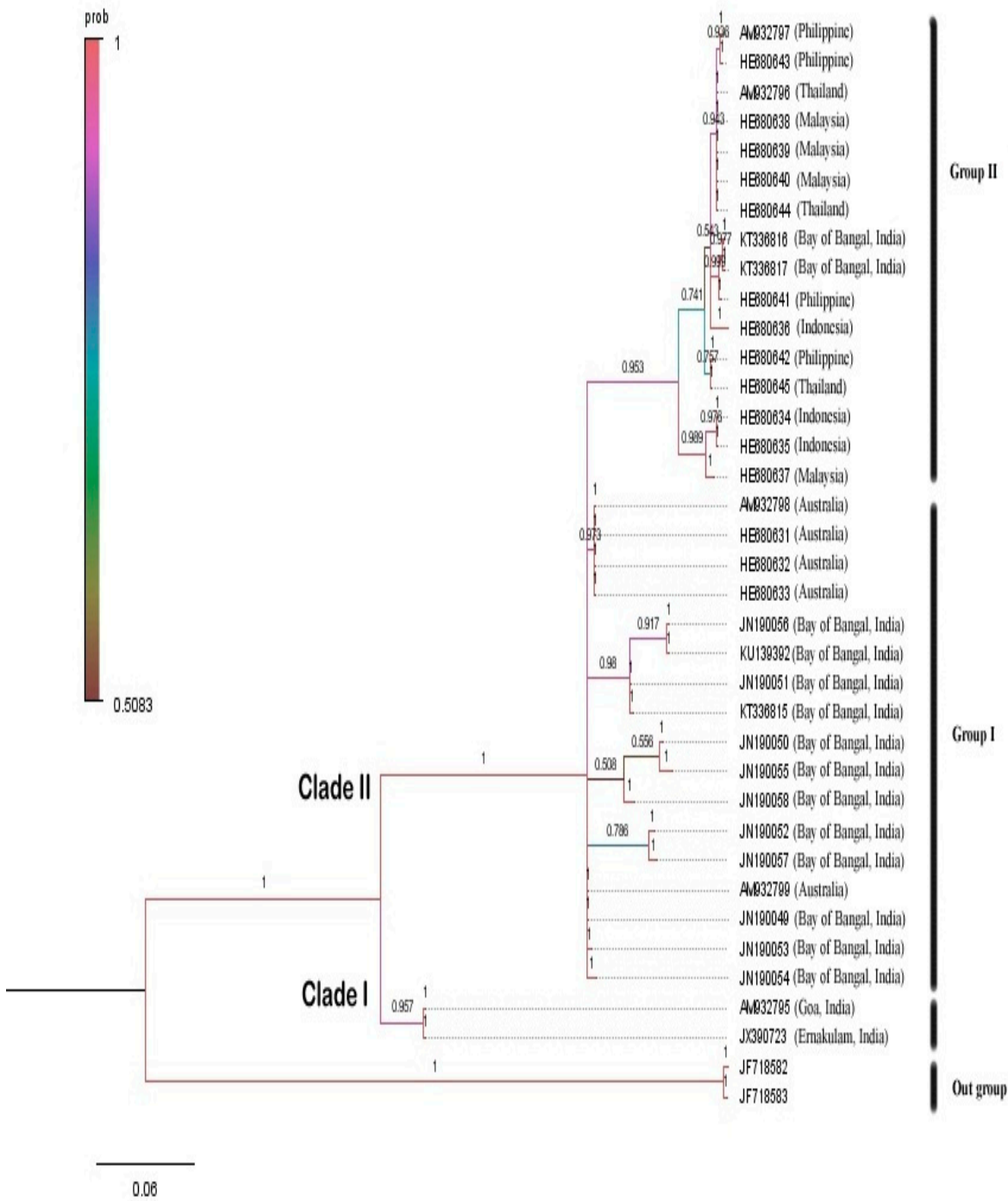
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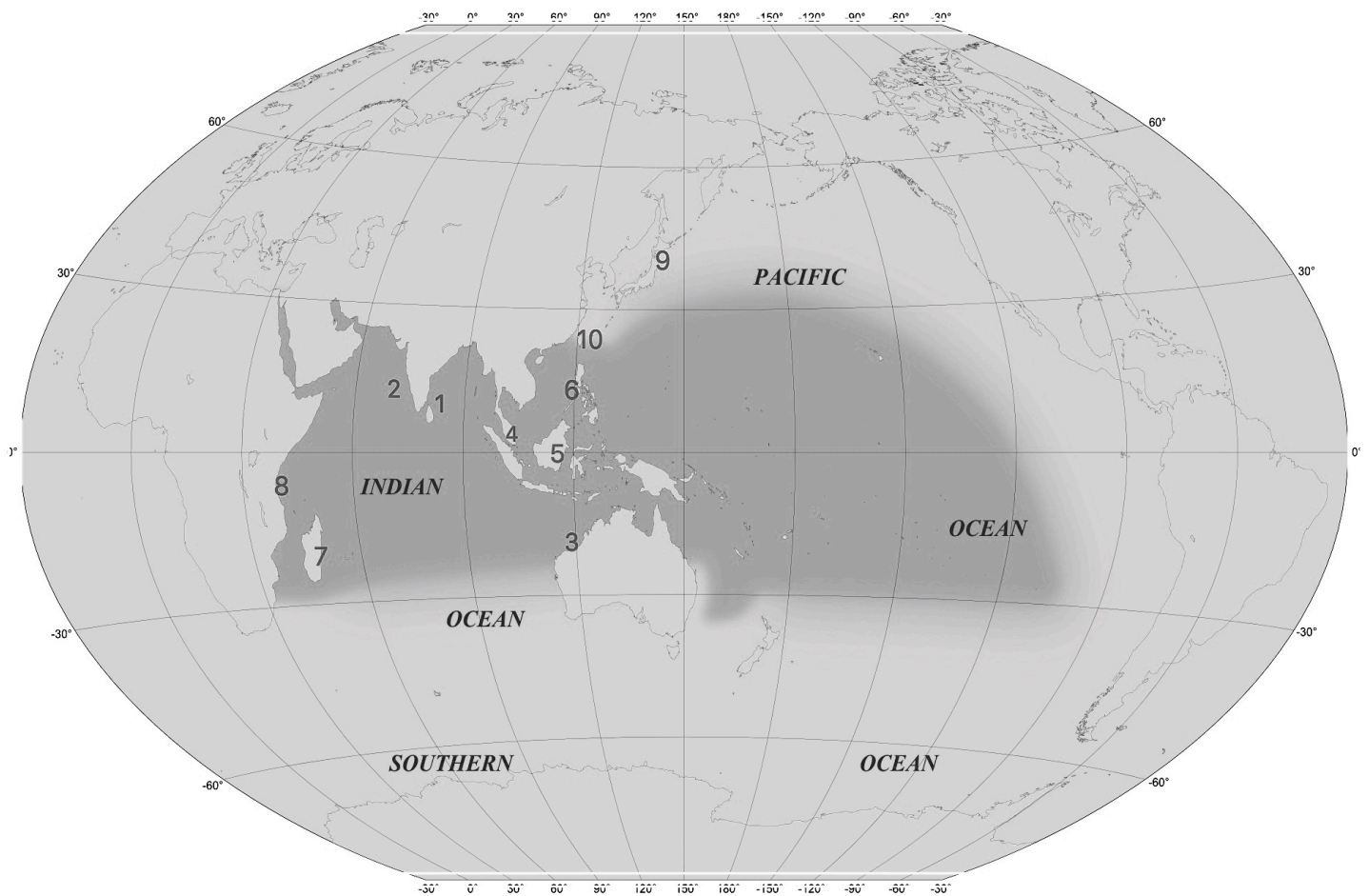
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**Figure 1.** Photographs of Mudwhelk *Telescopium* species. a). Mudwhelk assemblage in Mangrove soil; b). *Telescopium* species from Vellar Mangroves, India; c). harder and bigger size of basal outer lip; d) basal view of *Telescopium*.

**Figure 2.** Phylogenetic tree of *T. telescopium* obtained by MEGA based on the mt-COI sequences.





**Figure 3.** Biogeographic distribution of *T. telescopium* in Indian Ocean waters. In Figure 3, Number 1 represents Bay of Bengal Sea (Parangipettai and Puducherry), 2: West coast of India (Ernakulam and Goa) and West coast of Karachi; 3: Australian waters; 4: Malaysia; 5: Indonesia; 6: Phillipines; 7: Madagaskar; 8: Kenya; 9: Japan; 10: Taiwan waters.

**Table 1.** GC content variation of Indian mangrove snail *T. telescopium*

S.No	Morphologically identified taxa, their accession number	Gene Length(bp)	G	C	G+C (%)	Family
1	<i>T. telescopium</i> JN190049.1	646	125	145	41.78	Potamididae
2	<i>T. telescopium</i> JN190050.1	644	121	147	41.6	
3	<i>T. telescopium</i> JN190051.1	652	127	147	42.01	
4	<i>T. telescopium</i> JN190052.1	641	125	146	42.27	
5	<i>T. telescopium</i> JN190053.1	646	123	146	41.64	
6	<i>T. telescopium</i> JN190054.1	641	124	142	41.49	
7	<i>T. telescopium</i> JN190055.1	637	121	144	41.59	
8	<i>T. telescopium</i> JN190056.1	647	123	147	41.73	
9	<i>T. telescopium</i> JN190057.1	642	126	143	41.89	
10	<i>T. telescopium</i> JN190058.1	637	119	148	41.91	

**Table 2.** List of samples used in this study and accession number for phylogeny analysis

S. No	Taxa	Collection Information	Accession Number	NCBI Reference
1	<i>T. telescopium</i>	Vellar Estuary, India	JN190049.1	This study
2			JN190050.1	This study
3			JN190051.1	This study
4			JN190052.1	This study
5			JN190053.1	This study
6			JN190054.1	This study
7			JN190055.1	This study
8			JN190056.1	This study
9			JN190057.1	This study
10			JN190058.1	This study
11		Visakhapatnam, India	KU139392.1	Thamalapakula and Kondamudi 2015
12		Vellar Estuary, India	KT336817.1	Thangaraj et al. 2014
13		Vellar Estuary, India	KT336816.1	Thangaraj et al. 2014
14		Vellar Estuary, India	KT336815.1	Thangaraj et al. 2014
15		Kochi, West Coast of India	JX390723.1	Neelima et al. 2012
16		Goa, West coast of India	AM932795.1	Reid et al. 2008
17		Phillipines	HE680643.1	Reid and Claremont 2012
18		Phillipines	HE680642.1	Reid and Claremont 2012
19		Phillipines	HE680641.1	Reid and Claremont 2012
20		Malaysia	HE680640.1	Reid and Claremont 2012
21		Malaysia	HE680639.1	Reid and Claremont 2012
22		Malaysia	HE680638.1	Reid and Claremont 2012
23		Malaysia	HE680637.1	Reid and Claremont 2012
24		Indonesia	HE680636.1	Reid and Claremont 2012
25		Indonesia	HE680635.1	Reid and Claremont 2012
26		Indonesia	HE680634.1	Reid and Claremont 2012
27		Australia	HE680633.1	Reid and Claremont 2012
28		Australia	HE680632.1	Reid and Claremont 2012
29		Australia	HE680631.1	Reid and Claremont 2012
30		Australia	AM932799.1	Reid et al. 2008
31		Australia	AM932798.1	Reid et al. 2008
32		Australia	AM932797.1	Reid et al. 2008
33		Thailand	AM932796.1	Reid et al. 2008
34		Thailand	HE680644.1	Reid and Claremont 2012
35		Thailand	HE680645.1	Reid and Claremont 2012

**Table 3.** Average K2P distances of COI sequences between locations

	<b>South west</b>	<b>South east</b>	<b>Australia</b>	<b>Thailand</b>	<b>Philippine</b>	<b>Malaysia</b>	<b>Indonesia</b>
<b>South west</b>	-						
<b>South east</b>	0.151						
<b>Australia</b>	0.184	0.052					
<b>Thailand</b>	0.142	0.080	0.169				
<b>Philippine</b>	0.144	0.080	0.112	0.005			
<b>Malaysia</b>	0.145	0.086	0.110	0.010	0.010		
<b>Indonesia</b>	0.146	0.082	0.100	0.028	0.040	0.031	-

South west: South West coast of India; South east: South East coast of India