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Note

DNA barcoding of *Thais* species (Family: Muricidae) from west coast of India

RAVI KUMAR, A. K. JAISWAR, A. PAVAN-KUMAR. A, S. K. CHAKRABORTY
S. JAHAGEERDAR AND W. S. LAKRA

Central Institute of Fisheries Education (*Deemed University*), *Versova, Andheri West, Mumbai - 400 061 Maharashtra, India*
e-mail: pavanannam@gmail.com

ABSTRACT

Accurate species identification is essential for defining biological entities for sustainable management and conservation. Species identification under the genus *Thais* (Mollusca: Neogastropoda: Muricidae) based on shell morphology often mystify due to the phenotype plasticity. In this study, efficiency of DNA barcodes (mitochondrial cytochrome *c* oxidase subunit I gene - COI gene) was tested to discriminate six species of *Thais* from west coast of India. The COI gene analysis revealed that the average transitional pairs (S_i=56) were less than transversional pairs (S_v=59) with an average ratio of 0.93. The mean intraspecific and interspecific distances were 0.09±0.011 and 0.19±0.012, respectively. The average between species distance was 2 times more than within species distance. However, the DNA barcodes were not able to discriminate all the species with high barcode gap. Further, intraspecific distance values were estimated by including reported sequences from other geographical locations to test the DNA barcode efficiency in delineating conspecific individuals across different geographical locations.

Keywords: Cytochrome *c* oxidase subunit I gene, DNA barcoding, Gastropods,Muricidae, *Thais*

Gastropods and bivalves constitute about 98% of the total population of molluscs. Muricidae is the second largest family in the Neogastropoda and a widespread, speciose group of marine predators found in most parts of the world (Vermeij, 1996). Traditionally species under Muricidae family are identified based on shell morphology, sculpture, micro-structure, radula, anatomical characters, operculum structure and meristic counts (Rao and Rao, 1993; Rajagopal *et al*., 1998; Rao, 2003; Kumbhar and Rivonker, 2012). However, these morphological characteristics are prone to be influenced by environmental factors which in turn lead to misidentification of species.

Accurate and unambiguous species identification is essential for biodiversity documentation, assessment and sustainable management of molluscan resources. DNA based taxonomy using sequence diversity among species can be used to identify and resolve taxonomic ambiguities including the discovery of new or cryptic species (Hebert *et al.,* 2003a). Mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (COI) sequence is the most widely used genetic marker for species identification. The diversity of *Thais* species along the Indian coast has not so far been documented using molecular markers. Hence, the present study was undertaken with the objective to test the efficacy of mitochondrial COI gene in discriminating and documenting selected species of *Thais* from the west coast of India.

Six species of the genus *Thais* representing two subfamilies of Muricidae were collected from across the west coast of India (Table 1). The specimens were provisionally identified upto genus level in the field based on reported literature (Apte, 1998; Tan and Sigurdsson, 1996a, b; Tan, 2000; Tan and Liu, 2001; Fernando and Fernando, 2002). Later, the species identification was confirmed with the help of taxonomic experts from Zoological Survey of India (ZSI), Kolkata. Foot tissue from live specimens were collected under aseptic conditions and preserved in absolute alcohol and stored at -80^{0}C, for further processing.

Total genomic DNA was isolated from preserved foot tissue following SDS-phenol/chloroform method as per Sambrook *et al.* (2001) with modifications. The COI gene was amplified with primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATAT TGG-3′) (Folmer *et al.,* 1994) in 25 µl reaction volume containing 100 ng template DNA, 10 pmol of each specific primer, 200 µM of each dNTPs, 1.0 unit of Taq DNA polymerase and 1x Taq buffer containing 1.5 mM $MgCl_2$. The PCR cycling conditions were set as initial denaturation at 95^{0}C for 3 min followed by 35 cycles of 94^{0}C for 1min, 46^{0}C for 1 min and 72^{0}C for 1 min and final extension at 72^{0}C.

Table 1. Selected *Thais* species from Indian west coast and other geographical locations

Subfamily	Species (number)	Location/country (lat; long.)	Accession no.
	Thais tissoti (Petit de la Saussaye, 1852) (04)	16.98^0 N; 73.3^0 E 18.97^0 N; 72.59^0 E South Africa	KF246688 – 89[a] HE584381-82[b]
Rapaninae	*Purpura rudolphi* (Lamarck,1822) (03)	16.98^0 N; 73.3^0 E 18.97^0 N; 72.59^0 E China	KF246694[a] GU188256[b] HQ834096[b]
	Thais bufo (Lamarck, 1822) (09)	16.98^0 N; 73.3^0 E; 18.97^0 N; 72.59^{0}E; 15.49°N; 73.8278° E China South Africa	KF246690 – 93[a] KC466598-601[b] HE584386[b]
	Indothais blanfordi (Melvil,1893) (02)	18.97^0 N; 72.59^0 E	KF906133[a] KF246687[a]
	Indothais lacera (Born,1778) (03)	18.97^0 N; 72.59^0 E China South Africa	KF906134[a] KC466631-38 [b] HE584335[b]
Ergalataxinae	*Orania subnodulosa* (Gravely, 1942) (01)	18.97^0 N; 72.59^0 E; 15.49°N; 73.8278° E	KF246695[a]

[a] Sequenced in the present study
[b] Reported sequences downloaded from NCBI, GenBank

The PCR amplification products were purified with gel extraction kit (Fermentas) following the manufacturer's protocol and the purified PCR products were sequenced directly using PCR primers. Sequencing was performed in both directions for better accuracy using ABI Big DYE terminator method (Eurofins Lab, Bangalore, India). These sequences were edited and aligned using Clustal W program (Thompson *et al.,* 1997) with default settings implemented in MEGA v 5.0 software (Tamura *et al.,* 2011). To estimate the degree of increase in intraspecific divergence values, sequences of species used in the present study and reported from different geographical locations downloaded from NCBI, GenBank were used (Table 1).

Around 29 (present study: 11; reported: 18) COI sequences representing six species of *Thais* were used for analysis. The pairwise evolutionary distance values were estimated by Kimura 2 parameter method using MEGA v 5.0 software. The Neighbour-joining (NJ) tree based on cytochrome *c* oxidase subunit I gene K2P distance values was constructed with 1000 bootstrap replications. Automatic Barcode Gap Discovery (ABGD) online tool was used to delineate the species based on COI gene divergence values. The relationship between geographical distance and intraspecific genetic divergence value was analysed using the Mantel test (Mantel, 1967) implemented in XLSTAT (version 2013.3.02; Addinsoft, Inc., NY, USA).

A total of 11 individuals belonging to six species of *Thais* were barcoded using the fragment of COI gene with an average length of ~570-600 base pairs. Additionally, 18 reported sequences of *Thais* spp. from different locations were also included and all the sequences were checked for the presence of insertions, deletions and stop codons. The lack of stop codons and *indels* was consistent with all the COI sequences suggesting that NUMTs (Nuclear DNA sequences originating from mitochondrial DNA sequences) were not sequenced/reported. Overall GC content was 35.8% and the variation in GC content for codon third base position was higher than for position 1. The GC content variation at position 2 was very limited which showed the absence of synonymous mutations at position 2. The average transitional pairs (S_i =56) were less than transversional pairs (S_v =59) with an average ratio of 0.93.

For Indian haplotypes, the average intraspecific and interspecific distance values were 0.09±0.011 and 0.19±0.012, respectively. Specifically, the distance values between *Thais tissoti* and *Orania subnodulosa* and between *Indothais blanfordi* and *O. subnodulosa* were less than the average intraspecific distance values (Table 2) and it was also substantiated by Neighbor-Joining tree analysis (Fig. 1). The Intraspecific genetic distance values increased more than 2 folds when conspecific sequences from other geographical locations were included in the analysis (Table 3). Significant correlation between geographic distance and genetic divergence for *Thais bufo* and *Indothais lacera* with coefficients of 0.486 and 0.471 ($p<0.001$) respectively was further revealed by Mantel test (Fig. 2).

As the genetic distance/divergence values among certain species were less than the intraspecific values, it would be difficult to delimit the species using traditional threshold method. To circumvent this problem, online

Table 2. Genetic distance (K2P model) values of COI gene across *Thais* spp.

Species	*Indothais blanfordi*	*Thais bufo*	*Purpura rudolphi*	*Indothais lacera*	*Thais tissoti*	*Orania subnodulosa*
Indothais blanfordi		0.024	0.022	0.018	0.014	0.010
Thais bufo	0.236		0.020	0.023	0.019	0.022
Purpura rudolphi	0.270	0.242		0.020	0.018	0.020
Indothais lacera	0.188	0.246	0.246		0.015	0.015
Thais tissoti	0.159	0.212	0.220	0.176		0.010
Orania subnodulosa	0.072	0.204	0.220	0.128	0.081	

* Lower diagonal values indicate average divergence and upper diagonal values indicate standard error

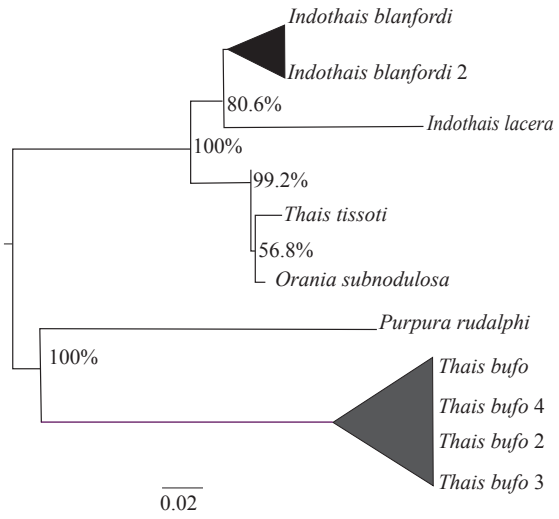

Fig. 1. Neighbor-Joining tree of *Thais* species collected from west coast of India

bioinformatics tool Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.,* 2012) was applied for species delimitation analysis for Indian haplotypes. The tool has partitioned all the six species into three groups based on the pair-wise genetic distance values (Fig. 3)**.** However, addition of reported sequences from different geographic locations resulted in partition of six species into eleven groups (Fig. 4).

Mollusca is one of the largest phylum in marine habitat, however, the group diversity is highly underestimated (Bouchet, 2006). With more than 2500 species, Muricidae family is one of the taxonomically complex groups (Merle, 2005). Furthermore, the complex larval stages, phenotypic plasticity in shell morphology imposed difficulties in species identification at field level (Drent *et al.,* 2004; Johnson *et al.,* 2008; Marko and Moran, 2009; Zou *et al.,* 2011). Molecular markers especially mitochondrial markers are considered as better

Table 3. Maximum intraspecific distances of *Thais* species with barcode records from India and other nations

Species	Maximum intraspecific distance values		Countries with matches	Mantel correlation statistics for geographical *vs*. genetic distances (α =0.05)
	India	Combined		
Thais bufo	0.049 (06)	0.112 (10)	China, England	r= 0.486 p<0.001
Thais tissoti	0.015 (02)	0.170 (04)	China, England	r = 0.471 p<0.001
*Purpura rudolphi**	--	0.321 (03)	China	--
*Indothais lacera**	--	0.241 (10)	China, England	--
*Thais blanfordi***	0.042 (02)	--	--	--

* Species have only one specimen from India
** Species from India only, reported sequences not available
Within brackets, number of specimens

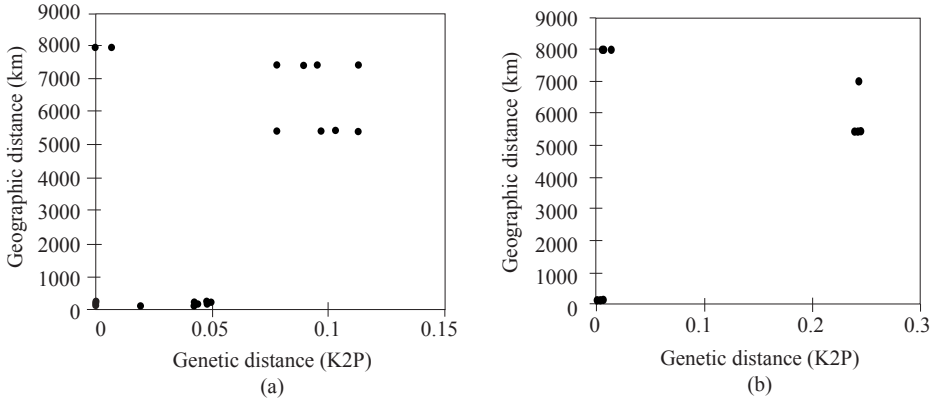

Fig. 2. Correlation between genetic distance (K2P) and geographical distance with Mantel tests for (a) *Thais bufo* (b) *Indothais lacera*

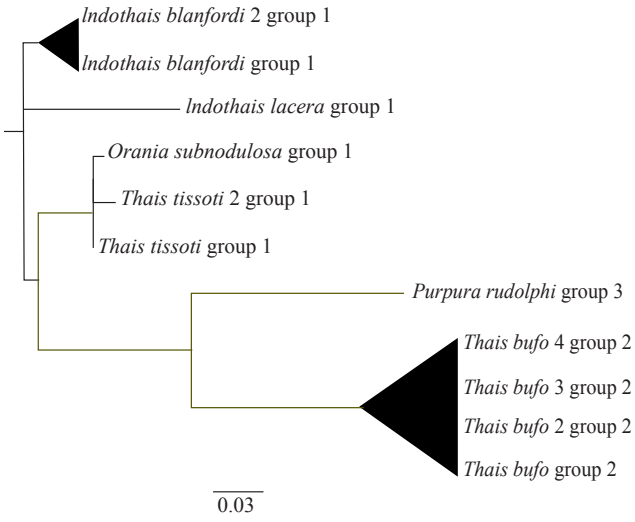

Fig. 3. Automatic Barcode Gap Discovery analysis of *Thais* species collected from west coast of India

biodiversity. In the present study, the GC content of the partial mitochondrial COI region was found to be 35%. Various studies have reported less number of transitional mutations than the transversional changes for COI gene, (Brown *et al.,* 1982; Gojobori *et al.,* 1982; Curtis *et al*., 1984; Wakeley, 1994). However, for some of the invertebrates, transversional pairs were more than the transitional pairs (Badhe *et al.,* 2013), which was observed in the present study also. In DNA barcoding, species is discriminated by estimating divergence values within species which is then compared with interspecific divergence value. In this study, the mean intraspecific value was 0.09±0.011, a value higher than that reported for marine fishes (0.003) and decapods (0.004) (Ward *et al.,* 2005, Costa *et al.,* 2007, Carr *et al.,* 2011). The average interspecific distance value (0.19±0.012) was 2 times more than the intraspecific distance. Hebert *et al.* (2003b) proposed a standard barcode sequence threshold

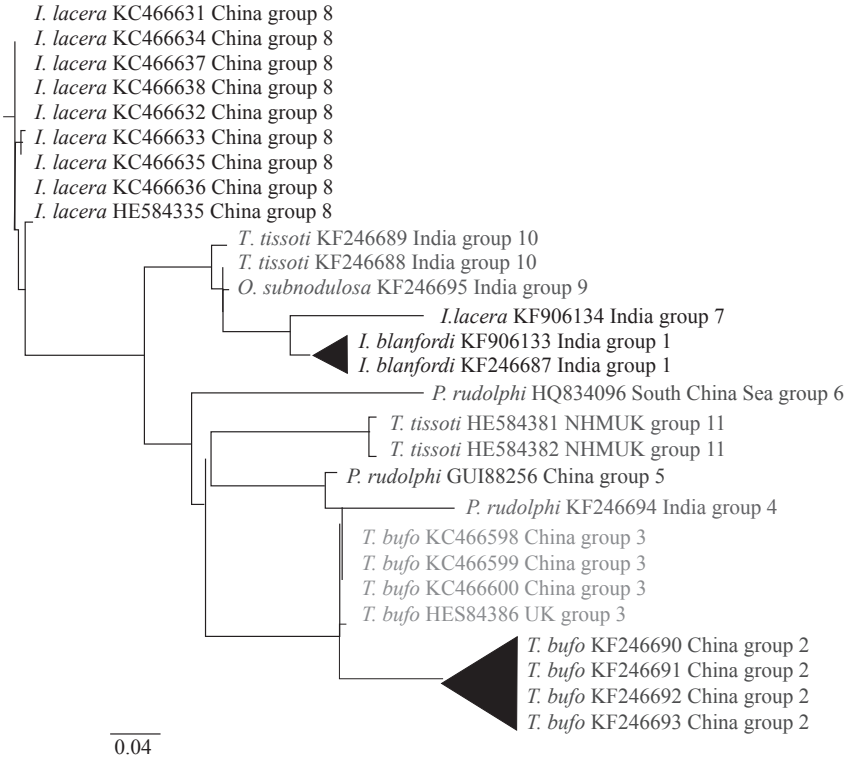

Fig. 4. Automatic Barcode Gap Discovery analysis of *Thais* species from different locations/countries

choice for species discrimination due to non-recombinant and environment independent nature (Tautz *et al.,* 2003; Blaxter, 2004; Rubinoff, 2006).

Several researchers have successfully validated the ability of mitochondrial cytochrome c oxidase subunit I gene to diagnose species in different taxonomic groups (Hebert *et al.,* 2003; 2004). Clare *et al.* (2007) showed the GC content of COI region among different lineages can be analysed and used as measures of nucleotide of 10X than the mean intraspecific divergence value to treat genetically divergent specimens as provisional species. If this threshold level is considered for calculating the interspecific distance value, then it could be more than 0.9 (0.09 X 10). However, the distance values between different *Thais* (specifically *T. tissoti* - *O. subnodulosa, I. blanfordi* - *O. subnodulosa*) species are less than this threshold value. This might be due to recent divergence of taxa or incomplete lineage sorting.

Addition of conspecific sequences from other geographic locations increased the intraspecific distance and the correlation was significant. In the present study also two species *viz*., *T. bufo* and *I. lacera* showed higher intraspecific distance values as more sampling locations were included. Bergsten *et al.* (2012) observed increase in intraspecific genetic variation in beetles of the tribe Agabini, with increase in geographical scale of sampling whereas, Hebert *et al.* (2004, 2010), reported that genetic and geographical distance are uncorrelated based on studies of smaller geographical scales or concerned with more dispersive organisms such as birds. For less mobile/ sessile organisms such as *Thais* species, single gene and the traditional distance value threshold algorithms might not be suitable for species discrimination while multiple gene and other algorithms such as character and phylogeny (monophyly) would be more appropriate and effective for species delimitation. Zou *et al.* (2011) compared the usefulness of two genes (COI and 16S rRNA) and three methods (distance, character and monophyly) for discriminating Neogastropoda species and reported that character and phylogeny based methods can delimit species efficiently rather than distance based methods.

Automatic Barcode Gap Discovery (ABGD) method detects the breaks in the distribution of genetic pairwise distances, referred to as the 'barcode gap', and uses it to partition the data. This method proposes a standard definition of the barcode gap and partition the data set into candidate species. In the current study, ABGD analysis partitioned all specimens from India into three groups *viz*., *T. bufo* (group 2)*, P. rudolphi (*group 3) and *I. lacera, T. tissoti, Thais blanfordi* and *O. subnodulosa* (group 1). The clustering of different species as group 1 might be due to lack of sufficient DNA barcode gap among these species or the specimens identified as *T. tissoti*, *I. lacera*, *T. blanfordi* and *O. subnodulosa* might be belonging to same species. However, after adding conspecific sequences reported from different locations, ABGD tool partitioned the sequences into 11 groups as per their geographic locations. *T. tissoti* and *P. rudolphi* showed polyphyletic nature. This could be due to the allopatric divergence, introgression hybridisation and incomplete lineage sorting. Meyer and Paulay (2005) reported lack of DNA barcoding gap in some of the invertebrates specifically in cowrie species. It is necessary to increase the sampling size rather than concluding that lack of sufficient DNA barcoding gap in molluscs (*Thais* species) might be the reason for clustering of different species in one group as per the present study. More number of markers with additional specimen data would resolve the taxonomic ambiguity among these species.

The present work has generated DNA barcodes for six species of *Thais* distributed from west coast of India. These barcodes will be helpful in identification, assessment and in preparing conservation and management strategies for *Thais* spp. Further studies on the biology, anatomy and taxonomy with more molecular markers are needed in order to provide vital information to assist conservation efforts.

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