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DNA barcoding of selected cephalopods from Indian coast

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ABSTRACT

Biodiversity characterization is essential for sustainable utilization of resources and to define biological entities for conservation. India is blessed with rich biodiversity but few species have been formally documented till now. DNA barcoding, i.e. the use of 655bp region of mitochondrial cytochrome c oxidase I (COI) gene was adopted as a global bio-identification system for animals in recent years. In this study, 14 individuals of Cephalopods comprising 5 species, 3 orders and 3 families were barcoded. The average genetic distance within species, genus, family and order was 0.002, 0.106, 0.171, and 0.208, respectively. In addition to barcode-based species identification system, allopatric divergence values among different geographically distant populations were estimated. The Neighbour-Joining tree revealed distinct clusters shared by the species of same genera.

Key words: DNA Barcoding, Cephalopods, Cytochrome c oxidase I (COI)

Characterization of the diversity up to species level is essential for sustainable utilization of biodiversity. However, it is generally agreed that till now, only a small fraction of all species i.e., 1.5–1.8 million out of an estimated 10 million in the world (Wilson 2003) has been formally described. In the face of dwindling numbers of trained taxonomists and phenotypic plasticity in the species discrimination characters, an accurate and fast identification method is needed to assist in species inventories.

Hebert *et al*. (2004) demonstrated that the COI region is appropriate for discriminating closely related species across diverse animal phyla and this has been used for marine as well as freshwater fishes (Hajibabaei *et al.* 2005, Steinke *et al.* 2005, Ward *et al.* 2005, Hubert *et al.* 2008 and Lakra *et al.* 2011). These results have prompted international efforts to standardize screening of species diversity and to accelerate the process of cryptic species identification.

India has vast marine biodiversity due to its large coastline of 8,120 km^2, with 2.02 million km^2 of EEZ and 0.5 km^2 of continental shelf. Cephalopods comprise the important components of marine biodiversity and represent one of the most diverse metazoan groups from morphological and ecological point of view. Morphological identification of these groups is difficult, time-consuming and very often requires highly trained taxonomists.

The present work was carried out with an objective of developing DNA barcodes for selected Cephalopods from Indian coast. This study provides the baseline data for further research on DNA barcoding of Cephalopods as there is a lack of information on this aspect.

MATERIALS AND METHODS

Taxon sampling: The cephalopod samples comprising 5 species, 3 orders and 3 families (Table 1) were collected from fish landing centres of Ratnagiri, Mandapam, Rameswaram, Pamban, Chennai and Mumbai from September 2011 to March 2012. The collected samples were transported to the laboratory in ice boxes. The specimens were identified based on morphological characters using FAO species identification sheets (2006) and preserved in absolute alcohol for further molecular study.

Genomic DNA isolation, amplification and sequencing: Total genomic DNA was isolated from the muscle tissue according to the SDS-phenol/chloroform method of Sambrook *et al.* (2001) with some modifications. The concentration of isolated DNA was estimated using an UV spectrophotometer. The cytochrome c oxidase subunit I (COI) gene was amplified in a 50µl volume with 100 ng template DNA, 10 pmol of each specific primer, 200 µM of each dNTPs, 1.0 units of Taq DNA polymerase and 1× Taq buffer containing 1.5 mM $MgCl_2$. The universal primers LCOI 1490F1-5'GGTCAACAAATCATAAAGATATTGG3'

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Table 1. List of species DNA barcoded along with Genbank accession numbers

S.No	Order	family	Genus	Species	No. of Individuals	Accession Number
1	Octopoda	Octopodidae	Cistopus	indicus	03	KC409357-KC409359
2	Sepiida	Sepiidae	Sepia	pharaonis	03	KC409360-KC409362
3			Sepiella	japonica	01	KC409394
4	Teuthide	Loliginidae	Uroteuthis	edulis	05	KC409364-KC409368
5				duvauceli	02	KC409385-KC409386

and HCOI 2198 - 5'TAAACTTCAGGGTGAC CAAAAAATCA3' (Folmer *et al.* 1994), were used to amplify the COI gene. The PCR conditions were initial denaturation at 94°C for 3 min followed by 35 cycles of 45 sec at 94°C, 30 sec at 51°C, 60 sec at 72°C and final extension at 72°C for 10 min. The PCR products were visualized on 1.5% agarose gels and the amplicons were purified by Gel extraction kit following the manufacture's protocol. The purified products were sequenced commercially.

Sequence analysis: Sequences were aligned using ClustalW (Thompson *et al.* 1997) and submitted to NCBI Genbank. The COI sequences of five individuals of each species were aligned to yield final sequences of 600 bp. The genetic divergence among the species was estimated (Kimura 1980) using the software MEGA V.5.10 (Kumar *et al.* 2004). The neighbour – joining tree was constructed with 1000 bootstrap replications to test the efficacy of barcodes in discriminating the species.

RESULTS AND DISCUSSION

DNA barcoding, a tool that obtains species specific DNA signature, is based on the idea that sequence diversity within small stretches of the organism's genome can provide a 'biological barcode' to enable identification of any organism at the species level. Several mitochondrial genes like cytochrome b oxidase, cytochrome c oxidase subunit I, NADH subunits, tRNAs etc have been sequenced and analysed for species identification purposes (Simmons and Weller 2001 and Banks *et al.* 2003). The cytochrome c oxidase subunit I (COI) gene has got special attention due to the availability of universal primers and its ability to represent a greater range of phylogenetic signal than any other mitochondrial gene (Hebert *et al.* 2003).

In the present study, 14 cephalopod samples comprising 5 species, 3 orders and 3 families were barcoded using partial cytochrome c oxidase subunit I gene. All sequences were checked for the presence of insertions, deletions, stop codons and the absence of these elements proved that NUMTS were not amplified. The sequence analysis revealed average nucleotide frequencies as A = 29.3%, T = 37.3%, G = 15.3% and C = 18.1%. The average transversional pairs (S_v=33) were slightly more than transitional pairs (S_i=31) with an average ratio of 0.96. It was reported earlier that cytochrome c oxidase partial gene of the invertebrates has relatively more

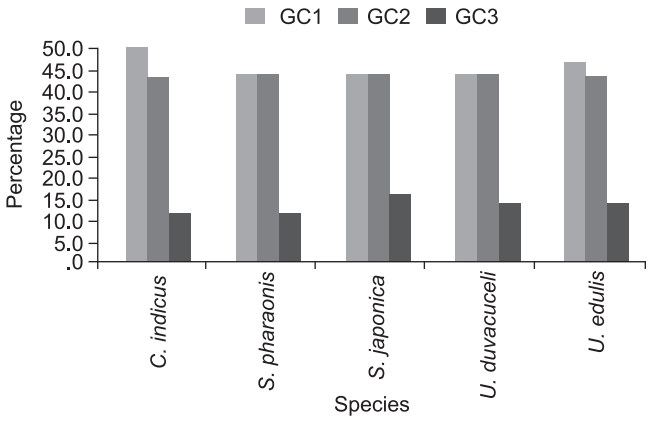

Fig. 1. Variation in GC content for COI gene among cephalopods

transversional pairs than the transitional pairs (Smith *et al.* 2005). Among different species, variation in GC content for codon third base position was higher than for position 1 while the variation at position 2 was very limited (Fig. 1).

The mean sequence length was 600 bp, although for some individuals less than 550 bp sequence was obtained. Each of these sequences clustered tightly with conspecific sequences. Hajibabaei *et al.* (2006) showed that even the DNA barcodes of 200 and 100 bp can be effective in identifying specimens. The average GC content was (33.5%) lower than the teleost's (47.10%, Ward *et al.* 2005). Substantial nucleotide changes were observed at the third base position of codon followed by first base position. This variation is due to the fact that most synonymous mutations occur at the third base position, a few at the first base position and none in second position.

The barcode sequence clearly discriminated taxonomic status of all 5 species examined. In this study, the average intra-specific and intra-generic K2P distance was estimated as 0.002 and 0.106, respectively (Table 2). There was more than 20–fold sequence difference accrued among congeneric

Table 2. Summary of COI genetic divergence (K2P) within various taxonomic levels

Comparisons	Minimum	Maximum	Average	Standard error
Within Species	0.00	0.083	0.002	0.021
Within genera	0.138	0.163	0.106	0.085
Within families	0.163	0.187	0.171	0.032
Within orders	0.181	0.219	0.208	0.015

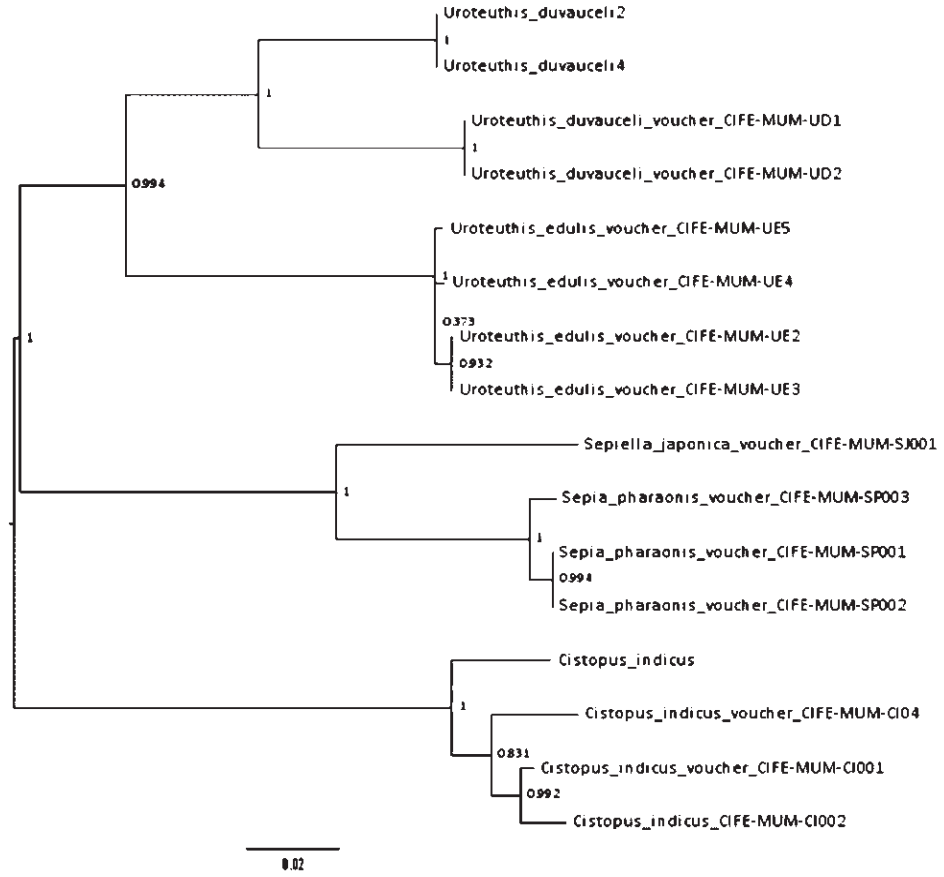

Fig. 2. Neighbour joining tree of cytochrome c oxidase I (COI) gene sequences derived from cephalopods using K2P distances

species than the conspecific individuals. Hebert *et al.* (2004) proposed a standard barcode sequence threshold of 10X the mean intraspecific divergence for flagging genetically divergent specimens as provisional species. This barcoding gap is sufficient to discriminate the cephalopods to their taxonomic position. The COI genetic variation among cephalopods increased from lower taxonomic level to higher taxonomic levels and supported a marked change of genetic divergence at the species boundaries. This observation supports the previous findings (Hubert *et al.* 2008). The Neighbour Joining tree revealed distinct clades where similar species were clustered under same nodes while dissimilar species were clustered under separate nodes. The major nodes were supported by high bootstrap values (70–100%) (Fig. 2).

In this study, intraspecies barcode variability among different populations of *Uroteuthis edulis* and *Cistopus indicus* was assessed to test whether DNA barcoding is indeed an effective tool for species identification on a global scale. In *Uroteuthis edulis*, the COI divergence among the haplotypes reported from Pacific Ocean (East China Sea, Sea of Japan) and Arabian Sea (India) was 0.070 while within haplotypes was 0.007 (Table 3). The degree of variation was equal to the proposed threshold level (10X) for defining species boundaries. This indicates the presence of two lineages or cryptic diversity or allopatric divergence in *U.*

Table 3. Uroteuthis edulis mean K2P distance with multiple samples from different geographical locations

Species	Locality	No.	Distance		Ratio (B/W)
			Within locality	Between locality	
U. edulis	India	05	0.007	India-Shangai: 0.070	10
	India-Japan: 0.071				
	Shangai (EU349452-56)	05	0.001	Shangai-Japan: 0.003	3
	Japan (EU349447-51)	05	0.00	---	

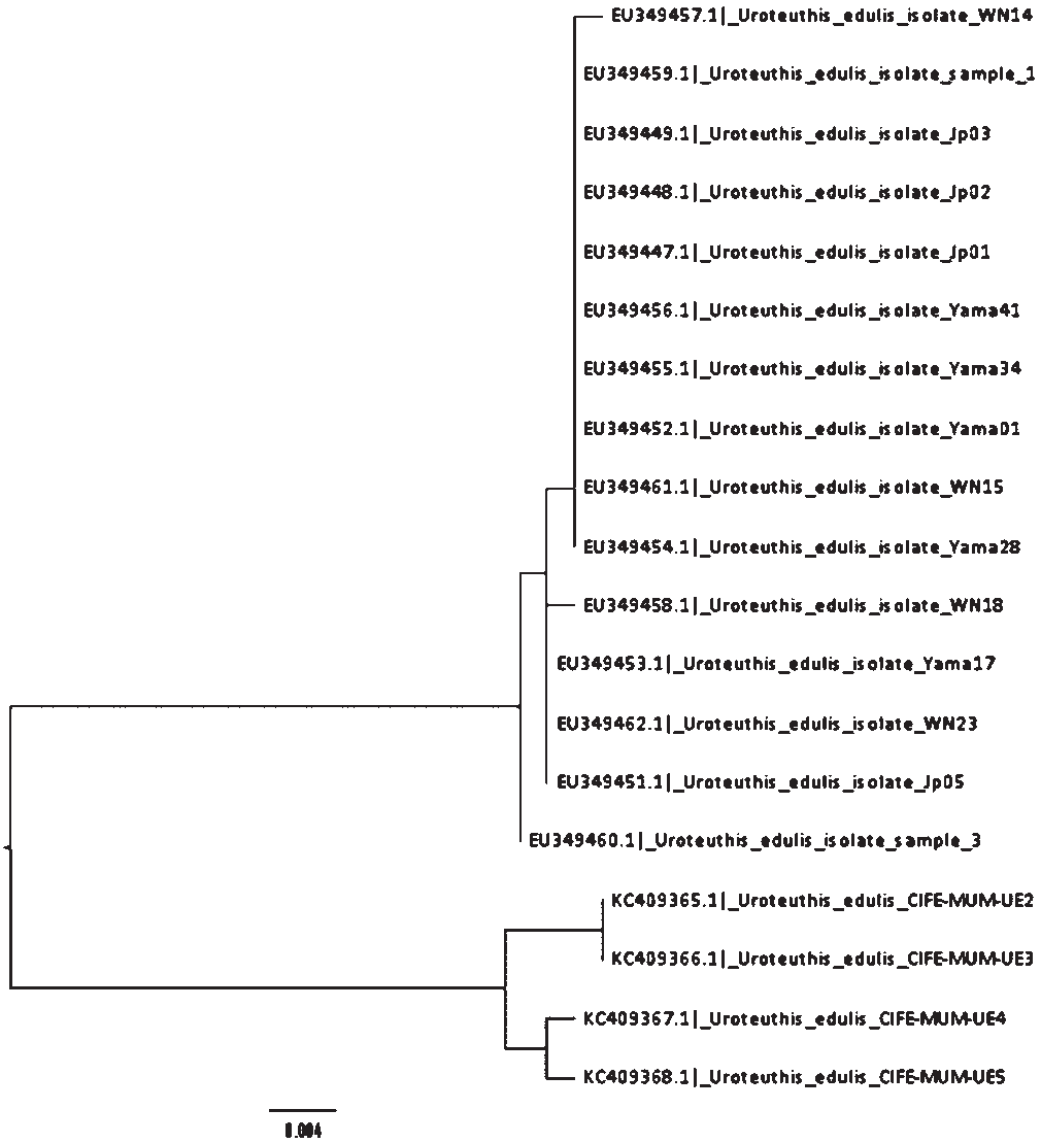

Fig. 3. Neighbour joining tree of cytochrome c oxidase I (COI) gene sequences derived from *Uroteuthis edulis* using K2P distances

edulis populations. However, for defining new species some more molecular markers might be necessary for accurate description. Recently Ward *et al.* (2008) reported a second species of Asian sea bass based on COI sequence divergence. The COI divergence (K2P model) among *Cistopus indicus* individuals from Indian coast varied from 0.012 to 0.027 while the divergence values of *C. indicus* haplotypes from India and Australia showed 0.050. This indicates the presence of two lineages or cryptic diversity or allopatrie divergence in *U. edulis* populations. The NJ tree clearly separated two populations and showed two clusters with significant bootstrap value (Fig. 3).

In conclusion, the results from our data clearly discriminated all the species and were congruent with the taxonomic divisions of the cephalopods. This study also highlighted the usefulness of COI barcodes for studying allopatric divergence among different geographically distant populations. DNA barcoding has the potential to deliver a genomics-era solution to the taxonomic impediment. It can be efficiently used in complementing the conventional taxonomic studies, in securing IPRs for important taxa and identifying biological entities for formulating conservation measures.

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