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DNA barcoding of *Cynoglossus arel* using mitochondrial COI and 16S rRNA genes

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ABSTRACT

DNA barcoding is not a substitute for taxonomy; however, it does provide a powerful tool to aid species identifications and focus on future taxonomic research efforts. In the present study, an attempt is made to identify and validate *Cynoglossus arel* collected from the Mangalore coast by DNA barcoding using mitochondrial COI and 16S rRNA genes. The primer pairs used in the study could successfully amplify 646 bp segment of COI and 616 bp segment of 16S rRNA gene in *C. arel*. The K2P average genetic distance calculated among species in the Cynoglossidae family was 0.22 and 0.09 among COI and 16S rRNA sequences respectively. This study makes an essential contribution to the NCBI database as this work provides the first 16S rRNA gene sequence for *C. arel* in the database. The COI and 16S rRNA gene sequences of *C. arel* and related species in phylogenetic analysis segregated Family Cynoglossidae into one clade and the outgroup (Family Tetraodontidae) as another clade.

Keywords: COI gene, *Cynoglossus arel,* DNA barcoding, 16S rRNA gene

The biological diversity of each country is a valuable and vulnerable natural resource. Thus, sampling, identifying, and studying biological specimens are among the first steps towards protecting and benefiting from biodiversity. Species recognition is a basic need for large scale biodiversity monitoring and conservation. Classical taxonomy falls short in this race to catalogue biological diversity before it disappears. This slow rate of progress in fish identification is mainly due to the lack of trained taxonomists in these regions, which contribute to the 'taxonomic impediment' (Swartz *et al.* 2008). Thus it was felt that there is a need for an easy approach of species identification, which helps in the documentation of biodiversity.

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. This technique is working under the principle that inter-species variations are more significant than the intraspecies variations (Ward *et al.* 2005), allowing one to distinguish the species using nucleotide sequences. Mitochondrial (mt) DNA analyses using conserved genes like 16S rRNA, Cyt b, or cytochrome oxidase subunit I (COI) have emerged as powerful approaches to answer questions of fish taxonomy, species identification, and population genetics (Hebert *et al.* 2003). Rapid barcoding and comparison with the growing databases of gene sequences will increase the speed of identification of newly collected or unknown specimens.

Flatfish comes under the order Pleuronectiformes, which include families Bothidae (flounders), Psettodidae (Indian halibut), Soleidae (soles), and Cynoglossidae (tongue soles). Eleven genera and 25 species of flatfishes contribute to the fisheries along the Indian coast (Vivekanandan *et al.* 2003). The present work was carried out for developing DNA barcodes of *Cynoglossus arel* species belonging to the family Cynoglossidae. This study provides the baseline data for further research on DNA barcoding of *Cynoglossus arel* as there is a lack of information on this aspect in the National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Sample collection: Cynoglossus arel samples were collected from the Mangalore fish landing center, Karnataka. Following collection, the specimens were digitally photographed and identified by using conventional taxonomic methods with suitable reference materials (Munroe 2001). Fin and muscle tissue from each sample was aseptically removed and preserved in absolute alcohol at –20°C for further molecular study. The voucher specimen of *Cynoglossus arel* were preserved for future references.

Genomic DNA isolation, amplification, cloning, and sequencing: DNA was extracted from fin and muscle tissue by following the standard phenol-chloroform method (Sambrook *et al.* 1989). DNA concentration and purity in the sample was estimated by using a spectrophotometer (NanoDrop 2000c Thermo Scientific, USA). Extracted DNA was subjected to PCR amplification using gene-specific universal primers (Table 1) targeting 658 bp fragment of COI and 583 bp fragment of 16S rRNA. Amplification of genes was carried out in a programmable

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Table 1. Primers used for the amplification of COI and 16S rRNA genes of *C. arel*

Gene	Primer	Primer Sequence	Reference
COI	Fish F2	5' TCGACTAATCATAAAGATATCGGCAC 3'	Ward *et al.* (2005)
	Fish R2	5' ACTTCAGGGTGACCGAAGAATCAGAA3'	
16S rRNA	16S F	5'CGCCTGTTTATCAAAAACAT3'	Palumbi (1996)
	16S R	5'CCGGTCTGAACTCAGATCACGT3'	

thermocycler (Bio-Rad Laboratories, USA) with slight modifications of published data of Mohanty *et al.* (2013). The optimized PCR programme consisted of initial denaturation at 95°C for 4 min, followed by 34 cycles of 1 min denaturation at 94°C, 30 sec of annealing at 54°C and 62°C for COI and 16S rRNA primers respectively, 1 min of extension at 72°C and the final extension at 72°C for 15 min. The PCR amplified products were visualized in 1.5% agarose gel by electrophoresis and analyzed using the Gel documentation system (Herolab, Germany).

The amplified PCR products were purified by using the GenElute PCR Clean-Up Kit (Sigma–Aldrich). The amplified PCR products of COI and 16S rRNA genes were ligated into the Genei T vector for cloning by following the manufacture's protocol (GeNei INSTANT cloning kit). The frozen competent *E. coli* strains (DH5α cells) were used for the transformation of COI and 16S rRNA, followed by the screening of recombinant transformants. The purified recombinant plasmids having COI and 16S rRNA gene inserts were sequenced to determine the nucleotide sequences by outsourcing them to Chromous biotech, Bengaluru using gene-specific primers.

Sequence analysis: The sequences obtained were subjected to BLAST analysis and aligned using ClustalW (Thompson *et al.* 1994). The sequences determined were submitted to the GenBank, NCBI. The phylogenetic analysis was performed using MEGA version 4.0 (Tamura *et al.* 2007). The nucleotide composition and the number of transition and transversion between species were determined by DnaSp ver3. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.* 2004). The Neighbour Joining tree was constructed using the Kimura -2 parameter model with 1000 bootstrap replicates to test the efficacy of barcodes in discriminating the species for analysis using a highly reliable outgroup species from the family Tetradontidae.

RESULTS AND DISCUSSION

Morphometric identification of Cynoglossus arel: Morphometric characters are significant key aspects of conventional taxonomical identification. So it is imperative to identify the fish through the classical taxonomical approach and further use DNA barcoding as a confirmative molecular tool. Morphometric evaluation of *C. arel* was done by using taxonomic keys in the FAO species identification sheet for fishery purposes. The diagnostic characters of *C. arel* (Fig. 1) species collected include an elongated body with dorsal and anal fins joined to the caudal fin.

Eyes on the left side of the body, two lateral lines on eyed side, and lips which are not fringed confirmed it as *Cynoglossus* spp. Two lateral lines on the eyed side but none on the blind side and eyes with a small scaly interorbital space confirmed the species as *C. arel.* The total length of species collected ranged between 17.5 cm to 23 cm. In the present study, taxonomic keys were used systematically for confirmation of all the species since it is essential to identify the fish through the Linnaean taxonomy and then proceed with molecular tools. The meristic and morphologic classification should be revised using molecular techniques (Infante *et al.* 2004). The trend which appears to be that DNA barcoding needs to be used alongside traditional taxonomic tools and alternative forms of molecular systematics so that taxonomic ambiguities can be resolved (Emery *et al.* 2009).

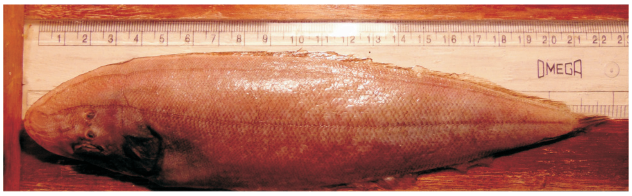

Fig. 1. Photograph of *Cynoglossus arel.*

Amplification of COI and 16S rRNA gene by using PCR and cloning: Sequencing of the mitochondrial COI gene is an established methodology with an international campaign directed at barcoding of all fishes (Puckridge *et al.* 2013), as it helps not only faster identification of species, but also in identifying eggs, larvae or fragments of a species which could not be identified by conventional taxonomic approach. DNA concentration measured showed a purity range from 1.74 to 1.91 in the samples, which were in conformity with the purity range of ~1.8 in 260/280 ratio. The size of the COI amplified PCR product was about 650 bp for *C. arel*, and the amplification of 16S rRNA for *Cynoglossus arel* yielded a product size of about 580 bp.

The purified PCR products of the gene coding for COI and 16S rRNA *C. arel* were ligated into the Genei T vector for cloning. The positive clones were confirmed by PCR using gene-specific primers, and all the positive clones were archived at –80°C in the glycerol stock solution.

Sequencing and analyses of COI and 16S rRNA genes of C. arel*:* The generated sequences were submitted in Genbank, NCBI under the accession numbers KT323970,

Table 2. Nucleotide composition of *Cynoglossus arel*

Cynoglossus arel	Gene bank Accession No:	Percentage base composition				GC content (%)	Total length
		A	T	G	C		
COI gene	KT323970	25.2	32.4	17.8	24.6	42.4	646
16S rRNA gene	KT323971	30.7	25	19.6	24.7	44.3	616

KT323971 respectively for COI and 16S rRNA genes of *C. arel*. Sequence alignment of the COI gene in *C. arel* produced 646 nucleotide base pairs, and the 16S rRNA gene in *C. arel* yielded 616 nucleotide base pairs. Since most of the global biodiversity remains unknown, molecular barcoding can only hint at the existence of new taxa, but not delimit or describe them (DeSalle 2006, Rubinoff 2006). Multiple sequence alignment of COI gene sequences of *C. arel* and 16S rRNA gene sequences of *C. arel* with other related species from NCBI were done to find the nucleotide substitutions.

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution

Base	COI				16S rRNA			
pairs	A	T	C	G	A	T	C	G
A	–	6.1	4.89	8.42	–	5.36	4.83	14.26
T	5.10	–	17.90	3.55	6.54	–	10.29	4.21
C	5.10	22.3	–	3.55	6.54	11.43	–	4.21
G	12.11	6.1	4.89	–	–	5.36	4.83	14.26

Nucleotide composition, frequency and substitutions: The COI and 16S rRNA gene sequence analysis revealed the nucleotide composition of *C. arel,* as shown in Table 2. Min and Hickey (2007) reported a strong correlation between the GC composition of the COI gene and the entire mitochondrial genome. The GC content of COI in *C. arel* is 42.4%, which is in conformity with the expected range of 42.2– 47.1% in COI (Ward *et al.* 2005, Lakra *et al.* 2011,Viswambharan *et al.* 2013).

In this study, considering the nucleotide pair frequencies, the average number of transitional and transversional pairs was calculated. The average transitional pairs (si=71) were more than transversional pairs (sv=51) with an average ratio of 1.4 in the COI gene of *C. arel.* The nucleotide pair frequencies of the 16S rRNA gene of *C. arel* revealed an average transitional pair (si=52), which are more than transversional pairs (sv=35) with an average ratio of 1.49. The average number of transitional pairs outnumbered the transversional pairs in accordance with the previous reports on mtDNA in fish (Vinson *et al.* 2004,Chakraborty and Ghosh 2014). Generally, for teleost mtDNA, a more significant excess of transitions related to transversion is typically observed (Ward *et al.* 2005). The maximum composite likelihood model shows the probability of nucleotide substitution from one base (row) to another base (column) instantaneously. The values are given in the tabular format, where only entries within a row should be compared. Rates of different transitional substitutions are shown in bold, and those of transversional substitutions are shown in italics (Table 3). The overall transition/

Table 4. Pair-wise genetic distances (Kimura 2-parameter) of *Cynoglossus arel* and related species based on COI gene sequences

Accession Number	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EU541319	*Cynoglossus Macrolepidotus*	–														
JQ639062	*C. itinus*	0.09														
FJ347912	*C. macrostomus*	0.23	0.21													
JQ349003	*C. puncticeps*	0.21	0.19	0.16												
JX983279	*C. cynoglossus*	0.22	0.22	0.20	0.19											
JQ738572	*C. puppureomaculatus*	0.23	0.21	0.19	0.21	0.25										
HM180553	*C. robustus*	0.23	0.21	0.19	0.21	0.24	0.00									
KT323970	*Cynoglossus arel*	0.23	0.22	0.20	0.22	0.24	0.14	0.13								
GQ380410	*C. abbreviatus*	0.23	0.24	0.23	0.21	0.23	0.19	0.19	0.21							
GQ380409	*C. semilaevis*	0.24	0.23	0.20	0.19	0.23	0.22	0.22	0.22	0.12						
EU513630	*C. canariensis*	0.24	0.22	0.23	0.22	0.23	0.21	0.21	0.21	0.21	0.20					
FJ347908	*C. dubius*	0.31	0.29	0.27	0.25	0.29	0.31	0.31	0.30	0.28	0.25	0.23				
JQ349000	*C. bilineatus*	0.25	0.24	0.23	0.20	0.24	0.25	0.26	0.25	0.23	0.20	0.19	0.15			
EU513627	*C. browni*	0.26	0.25	0.23	0.22	0.25	0.22	0.22	0.25	0.24	0.22	0.21	0.23	0.17		
JQ348998	*C. sinicus*	0.25	0.25	0.25	0.25	0.25	0.24	0.24	0.26	0.22	0.24	0.24	0.25	0.21	0.20	–

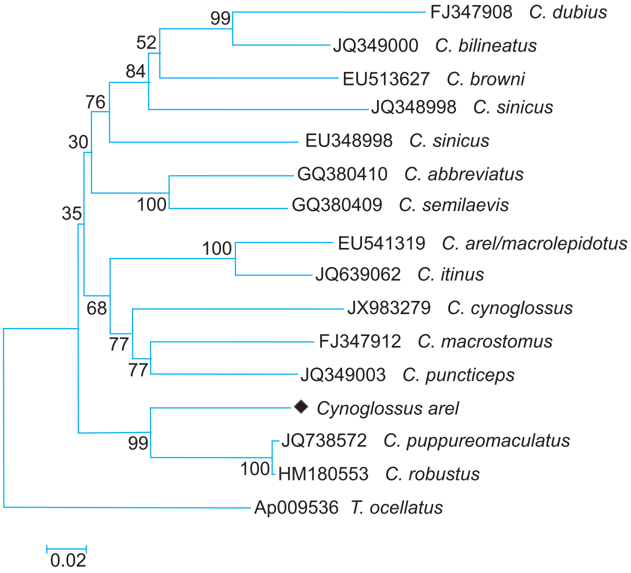

Fig. 2. Neighbour joining tree of COI gene sequences of *Cynoglossus arel* and related species with *Tetradon ocellatus* as outgroup constructed with bootstrap value 1000, scale bar: 0.02 substitution per site.

transversion bias (*R*) *value* obtained with Maximum Composite Likelihood estimate is 1.412 and 1.11 for the COI and 16S rRNA gene of *C. arel* respectively.

Genetic distance: The comparison of pairwise genetic distance for COI and 16S rRNA gene sequences of the Cynoglossidae family are given in Table 4 and Table 5, respectively. The average genetic distance among species in the Cynoglossidae family was 0.22 and 0.09 among COI and 16S rRNA sequences, respectively. In case of the COI gene, *Cynoglossus arel* had the highest intraspecies distance (0.24) with *C. cynoglossus* and lowest intraspecies distance (0.13) between *C. robustus.* With 16S rRNA, *C. arel* is having the highest intraspecies distance (0.11) with *C. sinicus and C. bilineatus* and lowest intraspecies distance (0.08) between *C. puncticeps*. The barcoding studies conducted by the usage of K2P genetic distance for analyzing the data exposed that barcode variations keep on increasing from species to genera, family (Ward *et al.* 2005, Hajibabei *et al.* 2007 and Lakra *et al.* 2011). Usually, interspecific divergence should be about ten times higher than intraspecific divergence (Hebert *et al.* 2004). The mean K2P distance values obtained in the case of 16S rRNA in *C. arel* was 0.09, which is relatively low was likely attributable to the highly conserved nature of the mitochondrial 16 S rRNA gene in vertebrates (Di Finizio *et al.* 2007, Kitano *et al.* 2007).

Evolutionary relationships using phylogram: The phylogenetic analysis provides the most accurate reconstruction of evolutionary relationships and distances between nucleotide sequences. The phylogram generated using MEGA 4 through the Neighbour-Joining method (Saitou and Nei 1987) was used to study the evolutionary relationship between related species. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The Neighbour Joining (NJ) tree illustrated COI based genetic divergence among related species present in NCBI as different branches in *Cynoglossus arel.* Phylogenetic relationships based on morphological and molecular characters are mostly concordant (Ward *et al.* 2005). The ability of the 16S rRNA gene to differentiate two genera was evident through the phylogram as different clades in *C. arel*. The phylogram generated through the neighbour joining method using K2P distance was highly reliable as the outgroup used was clearly segregated in the phylogram. The evolutionary relationship between the COI gene sequence of *C. arel* and other species in the same family (Fig. 2) were represented using *Tetradon ocellatus* (Family Tetraodontidae) as an outgroup. The 16S rRNA gene sequences of *C. arel* and related species (Fig. 3) were segregated into one clade and the outgroup *Tetradon abei* (Family Tetraodontidae) into another clade. Phylogenetic analyses indicated the evolutionary distance between species in the various family such as Cynoglossidae, Soleidae, Bothidae, Paralichythidae, Citharidae, Pleuronectidae, and Psettodidae belonging to the same order Pleuronectiformes. Pleuronectiformes and Tetrodontiformes are derived orders from Perciformes, and

Table 5. Pair-wise genetic distances (Kimura 2-parameter) of *Cynoglossusarel* and related species based on 16S rRNA gene sequences

Accession Number	Species	1	2	3	4	5	6	7	8	9	10	11	12	13
JQ348998	*Cynoglossussinicus*	-												
JQ349000	*C. bilineatus*	0.04												
JQ349002	*Paraplagusiablochii*	0.11	0.12											
JQ639066	*P. japonica*	0.11	0.11	0.03										
JQ349001	*P. bilineata*	0.11	0.11	0.04	0.04									
JQ349003	*Cynoglossuspuncticeps*	0.10	0.10	0.06	0.06	0.06								
KP057581	*C. trigrammus*	0.11	0.12	0.09	0.09	0.09	0.08							
JQ639062	*C. itinus*	0.11	0.11	0.11	0.11	0.11	0.10	0.11						
KT323971	*Cynoglossusarel*	0.11	0.11	0.09	0.10	0.10	0.08	0.10	0.10					
GQ380410	*C. abbreviatus*	0.09	0.09	0.09	0.10	0.09	0.09	0.09	0.09	0.09				
DQ112680	*C. puppureomaculatus*	0.09	0.09	0.09	0.10	0.09	0.09	0.10	0.09	0.09	0.00			
GQ380409	*C. semilaevis*	0.08	0.09	0.10	0.10	0.10	0.09	0.09	0.09	0.09	0.33	0.33		
DQ112683	*C. lighti*	0.10	0.10	0.09	0.09	0.10	0.08	0.11	0.10	0.09	0.07	0.07	0.06	-

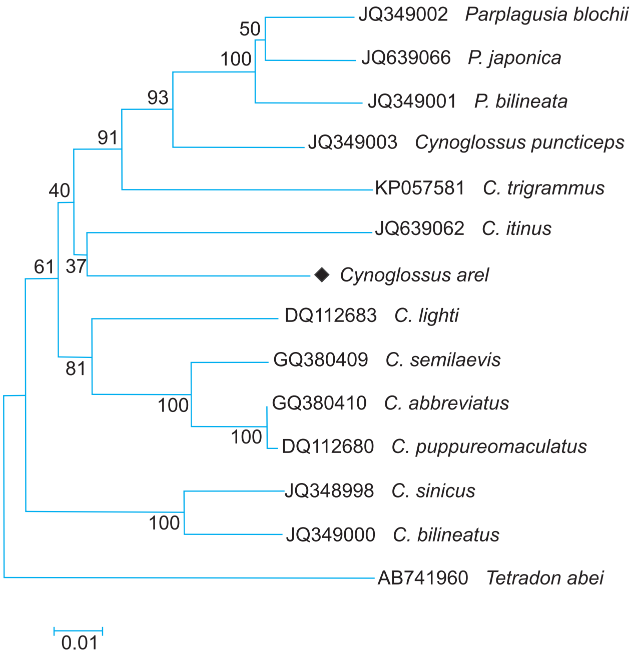

Fig. 3. Neighbour-joining tree of 16S rRNA gene sequences of *Cynoglossus arel* and related species with *Tetradon abei* as outgroup constructed with bootstrap value 1000, scale bar: 0.01 substitution per site.

thus a relationship between species of family Tetradontidae was visible in the phylogram.

This study provides the first 16S rRNA gene sequence for *C. arel*. Chakraborty *et al.* (2006) indicated the usefulness of the 16S rRNA gene sequence for accurate identification of species. Partial sequences of both COI and 16S rRNA genes provided sufficient phylogenetic information to identify *C. arel,* indicating the usefulness of the mtDNA-based approach in species identification. The DNA barcodes developed in this study could be useful for assessing flatfish diversity, and the sequences reported in this study will supplement the previous reports for estimating phylogenetic diversity among flatfishes.

India being a maritime country, its rich fishery resources, hold the key to food security and economic development. Species recognition is a basic need for large scale biodiversity monitoring and conservation. The present study revealed that morphological characteristics and DNA barcoding using COI and 16S rRNA gene clearly distinguish the identified fish species from the Mangalore coast as *C. arel.* This work provides the first 16S rRNA gene sequence for *C. arel*. Partial sequences of both COI and 16S rRNA genes provided sufficient phylogenetic information to identify *C. arel,* indicating the usefulness of the mtDNA-based approach in species identification. This study has supported the claim of the robustness of universal primers for 16S rRNA and COI genes. The phylogenetic position of *C. arel* was analyzed based on COI and 16S rRNA gene sequences to find out the evolutionary relationship and exhibited identical phylogenetic resolution. Pleuronectiformes and Tetrodontiformes are derived orders from Perciformes, and thus a relationship between species of family Tetradontidae was visible in the phylogram.

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