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Research Article

Genetic identification of grouper fishes (Perciformes: Serranidae: *Epinephelus*) through DNA barcoding from Nizampatnam coastal waters, India

Darwin Chatla[1,*], Padmavathi Pamulapati[1], Suneetha Kola[1], Muddula Krishna Naranji[2]

[1]Aquatic Biology Laboratory, Department of Zoology and Aquaculture, College of Sciences, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

[2]Laboratory of Ichthyology, Department of Zoology, Institute of Biosciences, University of Federal Do Rio Grande Do Sul, Avenda BentoGnoclaves, Brazil.

[*]Corresponding author e-mail address: darwin.chatla@gmail.com

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Abstract

Groupers (*Epinephelus*) are a wide-ranging group of ecologically and economically significant fishes with a controversial classification due to external morphological overlap. The diversity of marine species was mostly highlighted by barcoding and phylogenetic research from diverse parts of the world to establish taxonomic ambiguities. Here, we concentrated on *Epinephelus* (grouper) species from Nizampatnam coastal waters; a biogeographic region has no *Epinephelus* species that have been genetically identified. The partial gene of mt*COI* was used to identify five *Epinephelus* species. Genetic distance was on average between 0.104 and 0.170. The *COI* gene's average percentage (%) of nucleotide base composition across five species is T – 29.36; C – 27.88; A – 24.83; and G – 17.93. Test of substitution saturation values, ISS: 0.141; ISS.C: 0.734; T value 32.64 and DF value 604. With respective reference sequences, the phylogenetic analysis from the neighbor-joining tree displayed distinct clades for five species of *Epinephelus*. Barcoding Gap Investigation, analysis confirmed that all five sequences represented five taxonomic units (OTUs) and were determined with initial and recursive partitions based on Prior intra-specific divergence value P – 0.0359-0.0599. This study established the first-ever documentation of DNA barcodes for groupers (*Epinephelus*) in this area, as well as expansion for the Indian and global records of barcode.

Keywords: Cytochrome Oxidase I (COI), Phylogenetic, Epinephelus, Nizampatnam

Introduction

Groupers are one of the most commercially valuable marine fishes representing 163 species under 16 genera with a cosmopolitan distribution (Froese & Pauly, 2023). In total, 47 species of *Epinephelus* have been reported from Indian coastal waters (Darwin & Padmavathi, 2020). These highly diverse groupers play a key role in regulating the structural communities in the coral-reef ecosystem and act as an indispensable link to the aquatic food chain (Rao, 2009). The diversity of grouper fishes is displayed astonishingly with a variety of colours and

other unique environmental adaptations. Grouper landings in India have been constantly increasing during the last decade from 2009 to 2018 with an annual average of 37970 tons (CMFRI, 2019). People place this intense fishing pressure is placed on the groupers for food, medicine, and ornamental uses (Vincent, 2006; Sujathaet al., 2015; Darwin et al., 2020). Since groupers are crucial to the organization of coral-reef communities, their depletion could have a profound impact on ecosystems (Sujatha et al., 2015). Out of 163 species recorded worldwide, the IUCN classified twenty species (12%) as being at riskof extinction and twenty-two species (13%) as being close to endangered (Sadovy et al., 2013). Hence, to conserve these coral reef fishes, accurate species identification is important for biodiversity assessment and sustainable management of fishery resources. Unfortunately, species complexity and uncertainties over generic placements among groupers lead to the misidentification of species, which makes fisheries management and conservation challenging (Craig & Hastings, 2007; Schoelinck et al., 2014). Because of erroneous identification, FAO (2016) reported that about 61% of landed groupers were under not the enough information category.

Grouper fishes are mostly determined by their coloration and external features (Heemstra & Randall, 1993). However, there is confusion in the identification of distinct species under the *Epinephelus* genus due to their slight dissimilarities in morphological characters (Chatla et al., 2019). Identification of fish species through traditional morphological methods has some limitations. Variation in characters, phenotypic plasticity and problems with cryptic species lead to misidentification of species (Srinu et al., 2019; Darwin et al., 2020). Recently, utilization of molecular methods such as DNA labeling or barcoding and analysis of sequence data through bioinformatics tools has helped in resolving the ambiguity of species identification and their relationships (Desalle & Goldstein, 2019; Sachithanandam & Mohan 2020; Elías - Gutiérrez et al., 2021; Oppen & Coleman, 2022; Mwita et al., 2023). Determining the diversity of marine species was mostly highlighted by barcoding and phylogenetic research from diverse parts of theworld to establish the taxonomic ambiguities (Chakraborty et al., 2017; Srinu et al., 2019; Chatla et al., 2019; Darwin et al., 2020; Sachithanandam et al., 2022; Tang et al., 2023). With species complexity and uncertainties over generic placements, an effort has been made to identify the grouper fishes (Perciformes: Serranidae: *Epinephelus*) from Nizampatnam coastal waters and establish their phylogenetic relationships using DNA barcodingand sequence analysis.

Materials and Methods

Sampling, gDNA extraction and PCR amplification

Fish samples, used in the present study, were collected from the Nizampatnam coastal waters ($15^0 52' 58''$ N and $80^0 38' 18''$), Southeast coast of India, during December 2019 to September 2020, excluding the fishing leisure course (in Andhra Pradesh, April 15th to 14th June). The collected samples were identified using standard taxonomic keys (Heemstra & Randall, 1993).

gDNA (genomic deoxyribonucleic acid) was isolated from the muscle tissues of individual grouper species by using the Macherey-Nagel NucleoSpin® Tissue Kit, followed by the manufacturer's procedures: Muscle tissues were loaded in 1.5 ml microcentrifuge tubes. 25 µl of proteinase K and180 µl of T1 buffer were added, and then the mixture was placed in a water bath at 56^{o}C for incubation until the tissue was completely lysed. Subsequently, 5 µl of RNase A (100 mg/ml) was immersed in the mixture and placed at room temperature for five minutes. Next, 200µl of B3 buffer was loaded and incubated for ten minutes at 70^{o}C. After incubation, 210 µl of ethanol (100%) was added and properly blended using a vortex. Then the mixture was pipetted into a 2 ml collecting tube and subjected to centrifugation for one minute at 11000 rpm, and this solution was shifted to a 2 ml fresh tube and cleaned with a wash buffer of 500 µl by using 600 µl of B5 buffer. The washing step was repeated. Finally, gDNA was eluted out by using 50 µl of elution buffer.

PCR amplification of the mt*COI* gene was performed based on the Fish-F1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and Fish-R1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') primers described by Ward et al. (2005). PCR was set up in a 20µl reaction mixture

made up of about 1µlof template DNA (0.5-lµg); 1ul of each forward and reverse primer (10 nm/µl); 5µl of 2x – Smart Master Mix concentrated PCR buffer (Thermo Fisher™) and finally 12 µl of dh2O (molecular grade). The PCR thermal conditions were as follows: start with an initial step of 3 min at 94°C followed by 35 cycles set at 45s at 94°C (denaturation), 50°C for 45s (annealing), 1 min at 72°C for 30 cycles (elongation), and a final step of 7 min at 72°C holding at 4°C (final-elongation). The amplified products were separated in 1.2 % agarose gel with a molecular standard 2 – log ladder (NEB). The final, successfully clearly visualized amplicon products were sent to the best-known service centre, Ragional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, India for purification and single-direction sequencing using an automated DNA sequencer (ABI systems) following the method of the Big Dyeterminator.

Sequences analysis

The partial gene sequences of mt*COI* obtained for five species were subjected to BLASTn (a nucleotide search tool) to determine the sequence identity. It was further verified in the ExPASy ProtParam tool to identify the stop codons, no indels were observed. The confirmed sequences were submitted in FASTA format to Banklt submission tool of GenBank - NCBI (National Centrefor Biotechnology Information) domain to obtain a valid accession number. To strengthen the position of sequences, the obtained sequence data of five species was complemented with sequences acquired from the GenBank - NCBI. Three factors – identity, query coverage, and E- value were taken into consideration for comparison with NCBI existing sequence data to determine the highest homology. The GenBank - NCBI retrieved and submitted sequences were subjected to evolutionary analysis using MEGAX (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2018). ClustalW analysis tool is used for multiple sequence alignment. Nucleotide composition and percentage of G-C content were calculated by using the computed nucleotide composition selection. Genetic divergence at various hierarchical levels was analyzed by using the K2P (Kimura 2 Parameter) approach (Kimura, 1980).

The rate of substation saturation for individual nucleotide genes was assessed in the DAMBE7 software package (Xia, 2018) by plotting transitions (S) and transversions (V) against pairwise genetic distance (F84 distance model). Homoplasy due to multiple substitutions was assessed by the index of substitution (ISS). The Neighbor-Joining tree was constructed by the K2P model with a suitable out-group (Saitou & Nei, 1987). Bootstrap analysis with one thousand pseudoreplicates was used to validate the robustness of the internal nodes of the NJ tree (Felsenstein,1985).

To identify the species delamination between the mt*COI* sequences, the Automatic Barcode Gap Investigation (ABGD) method was used. (Puillandre et al., 2012). ABGD provides a two-phase approach that splits the sequences into operational taxonomic units (OUTs) as initial and recursivepartitioning (Kekkonen & Herbert, 2014; Kekkonen et al., 2015). The analysis was run based on a K2Pmatrix model with the following parameters: minimum relative gap width of 1.5 (X value); Pmin-0.001 to Pmax 0.1 (intra-specific divergence); 20 steps and 20 Nb bins. The maximum intra-specific divergence was plotted against the minimum inter-specific divergence.

Results and Discussion

Systematics

All the observed samples' DNA quality was determined to be satisfactory (Figure 1) and was used for sequencing analysis. After alignment, the total 609 base pair (bp) mt*COI* gene sequence for all five *Epinephelus* species was retrieved. The registered sequences at GenBank – NCBI were confirmed with valid accession numbers MT154688 (*E. coioides*), MT154689 (*E. radiatus*), MT154690 (*E. latifasciatus*), MT154691 (*E. bleekeri*) and MT154692 (*E. areolatus*).

Results revealed that the range of mean genetic distance was found between 0.104 and 0.170 (Table 1), *E. latifasciatus* and *E. coioides* had the closest (0.104), whereas *E. areolatus* and *E.*

coioides had the farthest genetic distance. Within the five *Epinephelus* species, the total percentage of mean genetic distance was observed at 0.15 ± 0.01. This pattern agrees with previous grouper DNA barcoding investigations reported in India (Basheer et al., 2017) and Indonesia (Fadli et al., 2021).

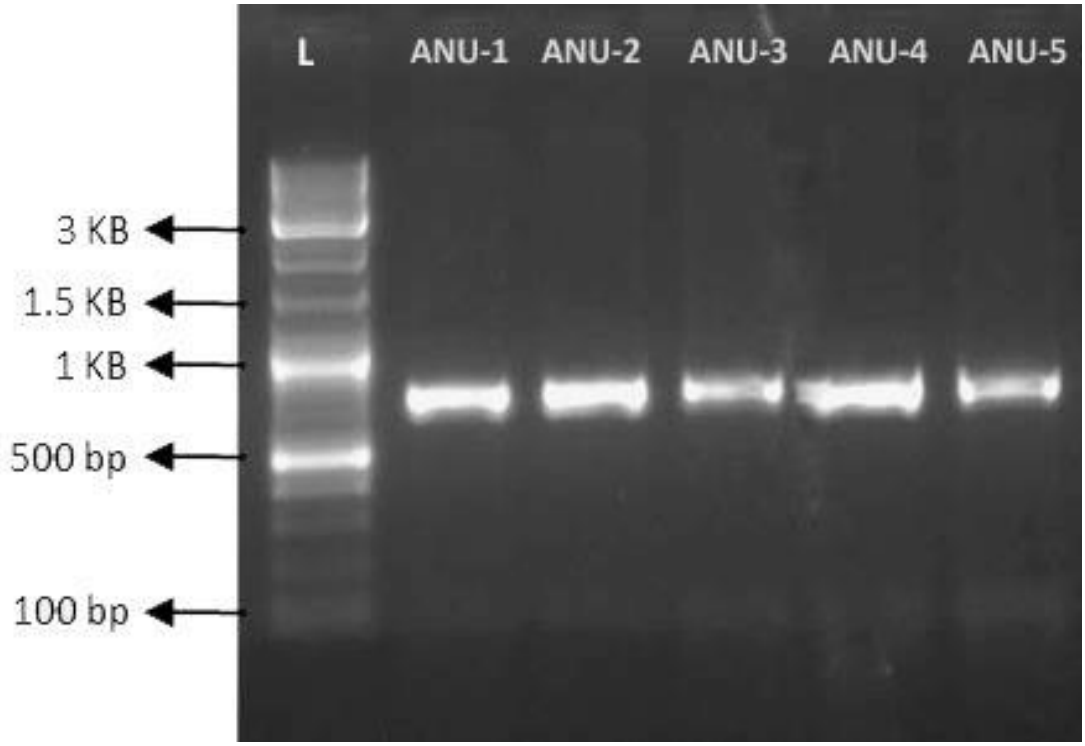

Figure 1. PCR amplified product of mt*COI* gene from five *Epinephelus* spp. *L: 2-Log DNA ladder; ANU-1: *E. coioides;* ANU-2: *E. radiatus;* ANU-3: *E. latifasciatus;* ANU-4: *E. bleekeri;* ANU-5: *E. areolatus.*

Table 1. Genetic distance of five *Epinephelus* species (*Below diagonal: genetic distance within the five species; above diagonal: standard error).

Species	*E. coioides*	*E. radiatus*	*E. latifasciatus*	*E. bleekeri*	*E. areolatus*
E. coioides	-	0.016	0.014	0.017	0.018
E. radiatus	0.130	-	0.017	0.017	0.018
E. latifasciatus	0.104	0.147	-	0.018	0.019
E. bleekeri	0.148	0.148	0.159	-	0.015
E. areolatus	0.170	0.164	0.169	0.117	-

The mean frequencies of nucleotide compositions with a G-C concentration of 45.82% were found by sequence analysis of five different *Epinephelus* species. The highest percentage of 47.6% G-C content was found in *E. areolatus* while the lowest percentage of 45% was observed in *E. bleekeri.* The *COI* gene's average percentage (%) of nucleotide base composition across fives pecies is T - 29.36; C – 27.88; A – 24.83; and G – 17.93. The significance level of value is slightlygreater than that found in grouper individuals from the Philippines (45.16%) and Malaysia (44.59%) (Alcantara & Yambot, 2016; Aziz et al., 2016). Various aspects including the size of the genome, the requirement for oxygen, temperature, and environmental factors, may be related to the difference in G-C composition (Wu et al., 2012).

Estimated transitions and transversions were not linear for the gene sequences plotted against the F84 distance model (Figure 2). This pattern indicates that it may be associated with the number of gene sequences that still retain adequate phylogenetic signals for the mt*COI* gene (Pavan et al., 2015; Viswambharan et al., 2015). The test of substitution saturation values was exhibited as follows: ISS (index of substitution saturation) is 0.141, while ISS.C (critical index of substitution saturation) is 0.734, with a T value of 32.64 and a DF value of 604. These values indicated that ISS.C was shown to be significantly than ISS. This significance pattern

is in agreement with earlier research on molecular phylogeny conducted by Pavan et al. (2013).

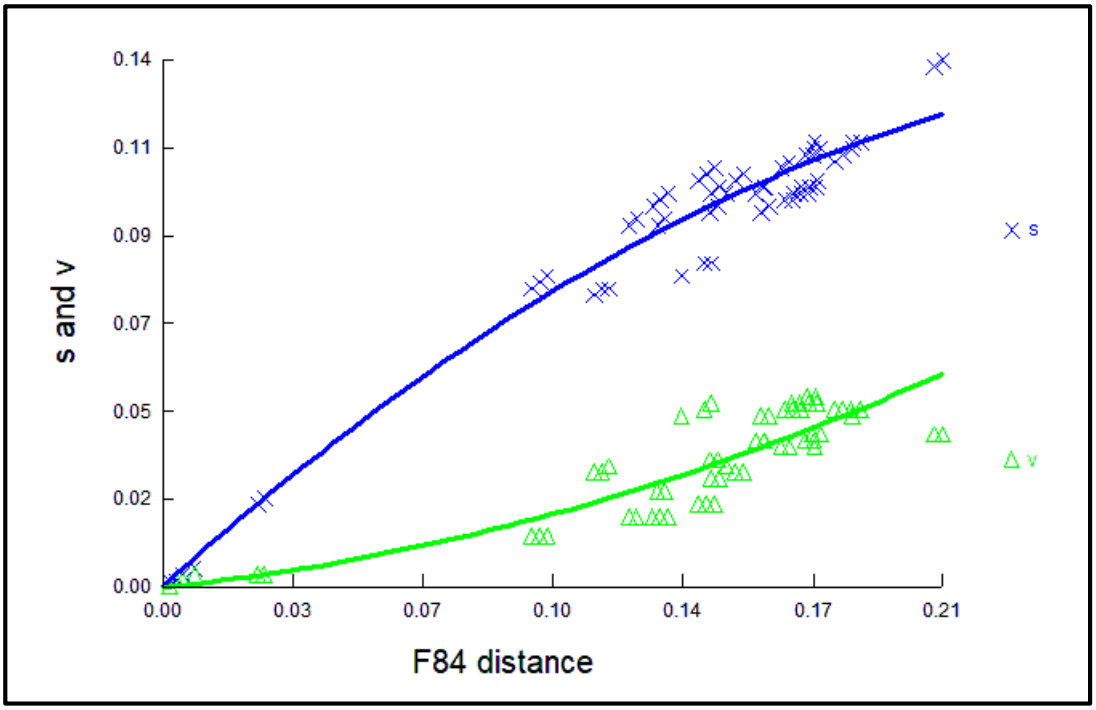

Figure 2. Sequence saturated plots of Transitions (S) and transversions (V)

The phylogenetic analysis for five *Epinephelus* species with their corresponding reference mt*COI* sequences revealed distant clades from the NJ tree (Figure 3). The *E. coioides* clades were formed from the sequences of species from the India with those from China and Vietnam coastal seas. *E. radiatus* clades were displayed within the samples from Indian oceans. *E. latifasciatus* clades were exhibited with the respective sequences of samples from Indian and Indonesian coastal waters. *E. bleekeri* clades were formed with the sequences of samples from the coastal waters of the United Arab Emirates, Bangladesh, and Saudi Arabia. *E. areolatus* forms a clade with those sequences of samples from the United Arab Emirates and Saudi Arabia. The outgroup *Cephalopholis sonnerati* showed a separate clade from the genus *Epinephelus.*

In concordance with species delimitation and Barcoding Gap investigation, ABGD analysis confirmed that all 5 sequences (apart from 21 sequences (20 - *Epinephelus*; 1 Outgroup -*Cephalopholis*) of GenBank) represented five taxonomic units (OTUs) and was validated by the recursive partition, with the initial partition focusing on prior intra-specific divergence value P = 0.0359 - 0.5999 (Figure 4). The outcome of ABGD at partition 8 is represented in Table 2. The results displayed that one sequence each from group 1 to 5 representing *E. coioides, E. radiatus, E. latifasciatus, E. bleekeri* and *E. areolatus* respectively are from the present study, and the remaining four sequences of five groups each and one sequence of the 6th group are from the NCBI database.

Conclusion

Identification of groupers species based on morphological traits may be inconclusive, moreover, not even a single systematic external morphological feature has been demonstrated to be significant in differentiating these commercially important groupers. Therefore, in this study, an effort has been made to use DNA barcoding as a potentially effective molecular method to distinguish between species and successfully sequenced the mt*COI* gene from five different *Epinephelus* species, in order to resolve the taxonomical ambiguity brought on by the overlapping characters. The barcodes established in this work are the foremost sequences

from Nizampatnam coastal waters to be submitted to the GenBank – NCBI and significantly expands the Indian and global fish barcode entries. From the present study, it can be concluded that the applicability of these primers to five *Epinephelus* species is significant and thus DNA barcoding could be used as a global standard for identifying grouper species.

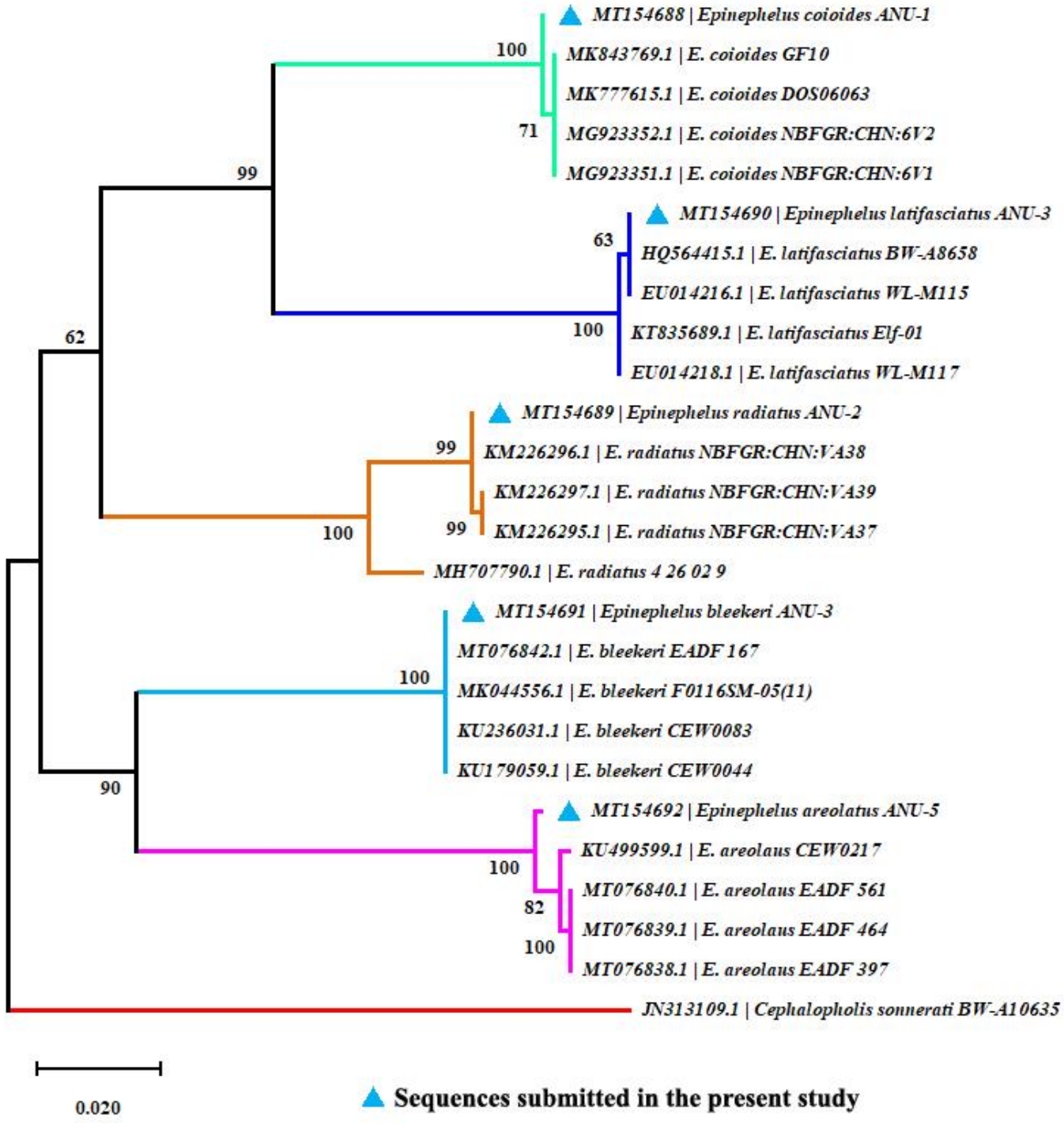

Figure 3. N-J tree of mt*COI* gene sequences for five species of *Epinephelus*

Table 2. Genetic distance of five Epinephelus species (*MT154688, MT154689, MT154690, MT154691 and MT154692 registered accession numbers at GenBank – NCBI).

Groups (No.)	ABGD groupings at partition 8	
1 (n = 5)	**MT154688**	***Epinephelus coioides* ANU-1**
	MK843769.1	*E. coioides* GF10
	MK777615.1	*E. coioides* DOS06063
	MG923352.1	*E. coioides* NBFGR:CHN:6V2
	MG923351.1	*E. coioides* NBFGR:CHN:6V1
2 (n = 5)	**MT154689**	***Epinephelus radiatus* ANU-2**
	KM226296.1	*E. radiatus* NBFGR:CHN:VA38
	KM226297.1	*E. radiatus* NBFGR:CHN:VA39
	KM226295.1	*E. radiatus* NBFGR:CHN:VA37
	MH707790.1	*E. radiatus* 4_26_02_9
3 (n = 5)	**MT154690**	***Epinephelus latifasciatus* ANU-3**
	HQ564415.1	*E. latifasciatus* BW-A8658
	EU014216.1	*E. latifasciatus* WL-M115
	KT835689.1	*E. latifasciatus* Elf-01
	EU014218.1	*E. latifasciatus* WL-M117
4 (n = 5)	**MT154691**	***Epinephelus bleekeri* ANU-4**
	MT076842.1	*E. bleekeri* EADF_167
	MK044556.1	*E. bleekeri* F0116SM-05(11)
	KU236031.1	*E. bleekeri* CEW0083
	KU179059.1	*E. bleekeri* CEW0044
5 (n = 5)	**MT154692**	***Epinephelus areolatus* ANU-5**
	KU499599.1	*E.areolaus* CEW0217
	MT076840.1	*E. areolaus* EADF_561
	MT076839.1	*E. areolaus* EADF_464
	MT076838.1	*E. areolaus* EADF_397
6 (n = 1)	JN313109.1	*Cephalopholis sonnerati* BW-A10635

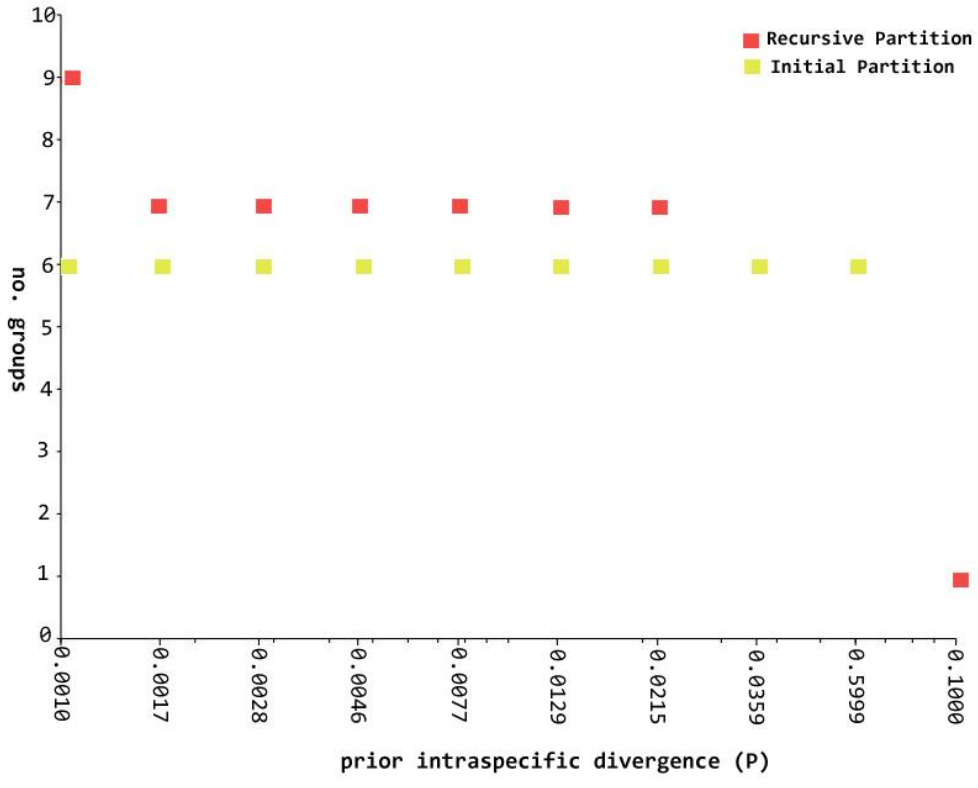

Figure 4. Genetical distant group values generated by ABGD based on K2P matrix model

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