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# Molecular Insights into the Identification, Diversity and Phylogenetics of the Por's Goatfish *Upeneus pori* (Actinopterygii: Syngnathiformes: Mullidae) from Odisha Coast, Bay of Bengal, India

**Bijayalaxmi Sahu[1, 2], Rakesh Kumar Dalai[2], Tapan Kumar Barik[2], Amiya Kumar Patel[1,*]**

[1]Department of Biotechnology and Bioinformatics, Sambalpur University, Jyoti Vihar-768019, Burla, Odisha, India
[2]P.G. Department of Zoology, Berhampur University, Bhanja Bihar-760 007, Berhampur, Odisha, India

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**Corresponding Author**
E-mail: amiya.gene@gmail.com

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## Abstract

Numerous different habitats found in marine environments are often conducive to migration and ecological adaption of various marine organisms. The inherent drawbacks of morphology-based identification systems have made it imperative to develop trustworthy genetic techniques that permit the unambiguous identification of fish species. In the present study, seven specimens of the goatfish *Upeneus pori* Ben-Tuvia and Golani, 1989, were recorded (February 2021) for the first time from the Odisha coast, Bay of Bengal, India. Their DNA was isolated, and mtCOI barcode regions of four specimens were amplified and sequenced successfully. The generated barcode sequences were analyzed using different molecular approaches. *U. pori* exhibited low genetic variation within populations (average K2P distance = 0.0046). The median joining network of *U. pori* was simple, with only five haplotypes, including one haplotype present in this region. The analysis of the variation of the amino acids in the mtCOI barcode sequences suggested that there were no significant variations found among *U. pori* of different geographical locations. The species *U. pori* was diverged from its common ancestor during the late Miocene sub-epoch, which was around 6.38 Mya. These findings provide insights into the population genetics and evolutionary history of *U. pori* in this region.

## Introduction

In addition to a rapid elevation in global trade, habitat modification, climate change, and the development of new trade routes, the second half of the 20th century saw a massive increase in the biological invasion of non-native marine species into various environments. Hence, non-native species' introduction, dispersal, and colonization of new habitats have gained international attention (Havel et al., 2015; Hulme, 2009; Vitousek et al., 1996). Such biological invasions have a major impact on human society, the local ecosystem, and the economy. The Indian Ocean borders the Indian Peninsula from the south, the Arabian Sea from the west, and the Bay of Bengal from the east. Being primarily tropical, this region is home to a wide diversity of fauna and is among the world's megadiverse nations (Raghunathan et al., 2012). With a distinct marine natural system, the Bay of Bengal is the biggest triangular basin in the world, located in the Indian Ocean. Many marine fishes have reportedly been introduced into the Bay of Bengal, causing a community shift away from their natural habitats, according to recent reports (Barik et al., 2021, 2020; 2018a, 2018b, 2018c, 2017). Accurate fish species identification is crucial for estimating the degree of species composition change in a given area. Earlier, species identification involved the use of commercial methods such as immunoassay and cytotaxonomy in conjunction with traditional morpho-taxonomy (Phillips & Ráb, 2001). However, it is known that the primary shortcomings of the previously employed methods for species

identification were frequent alteration of phenotypic features, substantially more expensive procedures, and comparatively lower levels of competence. As a result, developments in DNA-based methodologies during the past ten years have effectively incorporated DNA analysis into the discipline of taxonomy. Among the currently used DNA-based techniques, DNA barcoding has shown to be the most reliable and accurate way to distinguish between various species. The highly conserved mitochondrial cytochrome c oxidase I (COI) gene is widely employed as a barcode identifier for the identification of most animal species, as numerous studies have shown (Hebert et al., 2003a,b). This method has been acknowledged as the most flexible approach for delimitation and identification since it consistently examines high rates of sequence divergence at the species level (Ivanova et al., 2012; Vences et al., 2012). Therefore, DNA barcoding method would also be useful in identifying invasive species of the Mullidae family in the Odisha coast, Bay of Bengal.

The Mullidae family is comprised of six genera, such as *Mulloidichthys*, *Mullus*, *Parupeneus*, *Pseudupeneus*, *Upeneichthys*, and *Upeneus*. which includes 100 species (Fricke et al., 2023), that are distributed in Indian, Atlantic, and Pacific oceans and infrequently found in brackish environments (Chen and Zhang, 2015; Nelson et al., 2016; Uiblein and Gouws, 2014). The family Mullidae was formerly a member of the Perciformes order. As of 2022, it has been classified under the Syngnathiformes order (Nash et al., 2022), which is acknowledged by Fricke et al. (2023). According to Fricke et al. (2023), there are currently 47 legitimate species in the genus *Upeneus*, divided into seven taxonomic groups: japonicus, tragula, moluccensis, stenopsis, margarethae, suahelicus, and pori (Uiblein and Maclaine, 2021). Three genera composing 18 species of goatfish of the marine fish family Mullidae were recorded from the coastal water of India (Barman and Mishra, 2007). Nonetheless, in 2023, records from India included about 14 species belonging to the three genera *Mulloidichthys, Parupeneus*, and *Upeneus* (Vishnupriya and Nair, 2023). According to Vishnupriya and Nair (2023), the genus *Upeneus* has the most variety, with six species, followed by the genus *Parupeneus* with five species and the genus *Mulloidichthys* with three species. Pati et al. (2018) reported ten species of the family Mullidae in the Odisha coast of the Bay of Bengal i.e., *Mulloidichthys flavolineatus* (Lacepède, 1801), *Parupeneus ciliatus* (Lacepède, 1802), *Parupeneus indicus* (Shaw, 1803), *Parupeneus macronemus* (Lacepède, 1801), *Upeneus guttatus* (Day, 1868), *Upeneus moluccensis* (Bleeker, 1855), *Upeneus sulphureus* (Cuvier, 1829), *Upeneus taeniopterus* (Cuvier, 1829), *Upeneus tragula* (Richardson, 1846) and *Upeneus vittatus* (Forsskål, 1775).

*Upeneus pori*, also known as the Por's goatfsh, is a subtropical species that is found in the western Indian Ocean, that expands from southern Oman to the southernmost portion of the Red Sea (Ben-Tuvia and Golani, 1989). This species was first identified as Upenoides (= *Upeneus tragula*) by Kosswig (1950) in the Gulf of Iskenderun (Turkey), where it reached the Mediterranean through the Suez Canal (Golani, 2010). Large amounts of this commercially significant demersal species are captured by trawling in shallow waters (10–40 m) along the shores of the eastern Levantine Sea. It primarily inhabits sandy and muddy substrates, reaching a depth of 50 m (Yemisken et al., 2014; Bilecenoğlu, 2016). Due to the genus *Upeneus* and the Mullidae family in general, and this species in particular, being so diverse, there are difficulties in identification. Therefore, in addition to morphotaxonomy, molecular taxonomical identification is crucial for reconfirmation. The first appearance of the species *U. pori*, on the Odisha coast of the Bay of Bengal in 2021, has been discussed on the substructure of morphological diagnostic features and putative molecular characteristics (operational taxonomic units, evolutionary species, and phylospecies) by utilizing various tools on the substratum of mitochondrial COI gene.

## Materials and Methods

### Collection, Classification and Storage of Samples

On the Gopalpur-on-Sea (Lat 19.25° N, Lon 84.90° E), Odisha coast, Bay of Bengal, India (Figure.1), during the year 2021, seven specimens of the marine fish species resembling the genus *Upeneus* (family: Mullidae) were collected (February 2021) from the local fishermen during their daily fishing activities. The specimens were brought to the laboratory under freezing conditions inside the icebox as soon as possible. Additionally, isolated muscle tissue samples and fin clips were kept in 100% ethanol at -20°C in deep freezer. The specimens were identified by an examination of the taxonomic keys and the meristic characteristics stated in many leading taxonomic guides (Talwar and Kacker, 1984; Rao, 2003; Randall, 2001). The specimens were classified according to Eschemeyer's Catalogue of Fishes (Fricke et al., 2023) and designated to their species level.

### Isolation, Amplification and Sequencing of Mitochondrial COI Gene

Genomic DNA was extracted from the muscle tissue of the four collected specimens using the salting out approach (Sambrook and Russell, 2001). Using specific primers (Ivanova et al., 2007), a fragment of the cytochrome c oxidase subunit I (COI) gene was amplified. In a 25 µl reaction volume with 100 ng of template DNA, 0.2 M of each primer, 0.2 mM of dNTPs mix, 1 U of Taq DNA polymerase, and 1X PCR assay buffer, the mtCOI gene was amplified in the PCR conditions consisting of an initial step at 95°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30


**Figure 1.** Map of study site (rectangle shape), Gopalpur-on-Sea, Odisha coast, Bay of Bengal, India

seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR products were purified with PCR purification kit (Qiagen, USA) following the manufacturer's protocol and outsourced for Sanger sequencing.

**Sequence Data Analysis**

With the aid of the program CodonCode Aligner v.11.0.2 (www.codoncode.com), the generated chromatograms' quality was examined, and high-quality continuous sequence reads of bases (quality value > 20) were taken into consideration for additional investigation. Using the software Bioedit v7.2.5, the forward and reverse chromatograms were both evaluated. The accurate barcode sequences were created, aligned, and further consensus sequences were constructed as per the method described by Hall (1999). Using expasy analysis tool, the protein-coding sequences were converted to amino acids, and no stop codons were discovered. In order to determine whether the sequence belonged to the locus targeted, the NCBI web server's BLASTN program was used (Altschul et al., 1990). The segment that showed 100% alignment with no gap or indel (insertion or deletions) was chosen.

Forty-one COI gene sequences of the species *U. pori* from all available reference sequences from NCBI representing different locations in the NCBI database were retrieved. The Kimura two-parameter (K2P) distance, nucleotide composition, codon usage bias and transition/transversion bias of the sequences of the species were as determined by MEGA X (Kimura, 1980; Tamura et al., 2004; Kumar et al., 2018). Both a comparison of overall diversity and a pairwise comparison between different places were done in order to obtain the haplotype diversity. To ascertain the ancestry of haplotypes, a median-joining network (Bandelt et al., 1999) was built in PopART v1.7 (Leigh and Bryant, 2015). DnaSp v6.12.03 was used to determine the gene sequences' intra-population polymorphism (Rozas et al., 2017). Additionally, analyses were done on the number of variable sites, nucleotide diversity (Jukes and Cantor, 1969), haplotype diversity (Nei and Tajima, 1983), and parsimony informative sites.

The amino acid sequences of respective nucleotide sequences along with the sequences of the same species from different locations, were downloaded from the NCBI database. Thereafter, the amino acid sequences were aligned with ClustalW using MEGA X software (Kumar et al., 2018). Entropy (uncertainty; H(x)) values were determined for each place in BioEdit. Entropy has a value of 0 when there is zero variation, and it rises as variability rises. On the basis of the resultant values, the variable amino acids were then further separated into

four (arbitrary) groups according to increasing entropy: H(x) 0.5-0.7, 0.71-0.9, 0.91-1.1, and >1.1. Entropy values below 0.5 were deemed non-variable amino acid positions, and residues with zero variation were defined as conserved. We grouped the amino acids into five standard groups in order to identify their chemical characteristics at each site: nonpolar aliphatic (G, A, V, L, M, I); polar uncharged (S, T, C, P, N, Q); aromatic (F, Y, W); positively charged (K, R, H); and negatively charged (D, E) by referring to the earlier study by Pentinsaari et al. (2016). We regarded the site as non-variable and treated it as equal to those sites with entropy<0.5 when the amino acids at a given position exhibited variation exclusively among amino acids within such groups.

Phylogenetic analysis was carried out by constructing the maximum likelihood (ML) tree using MEGA X (Kumar et al., 2018). COI gene sequences of 50 individuals from 21 species belonging to the family Mullidae, including the sequences of the species *U. pori* from Gopalpur-on-Sea were analysed, whereas one sequence from the family Callionymidae serving as an outgroup for the phylogenetic analysis. The support for the nodes in the ML tree was assessed using 1000 bootstrapping iterations. The interspecies and intergeneric Kimura-two parameter (K2P) distance, a standard for barcoding investigations, was also computed using MEGA X (Kimura, 1980; Kumar et al., 2018). The best-fit model for the maximum likelihood tree's development was identified.

The RelTime with ML technique in MEGA X (Kumar et al., 2018) was used to estimate the divergence time of the species. The divergence time was calculated using the sequences of the mtCOI genes from the species as well as some other sequences of the Mullidae family obtained from GenBank. Since the RelTime method only needs the minimum and/or maximum calibration boundaries, we selected the following fossil evidence time boundaries from the original studies: (1) fossil evidence of the genera *Parupeneus* and *Upeneichthys* (2.09 Mya-13 Mya) for the entirety of the family Mullidae (Rabosky et al., 2013, 2018). The outgroup clade was automatically eliminated from the analysis under the presumption that the equivalent rates of evolution between the in-group and out-group sequences could not be tested (Kumar et al., 2018).

## Results

### Genetic Diversity

In the present study, the COI sequences of the species *U. pori* from various locations available in the NCBI database, including the generated sequences from this study, were considered for genetic diversity analysis. Out of the seven specimens of *U. pori* collected in this study, COI sequences of four specimens were successfully amplified and sequenced. Sequences bearing accession numbers ON182865 (700bp), OR782112 (704bp), OR593385 (700bp), and OR593299 (700bp) were generated from this study.

The nucleotide composition of the sequence of *U. pori* from the study site were found to be 28.9% (T), 29.9% (C), 22.1% (A), and 19.1% (G), respectively, whereas the average nucleotide composition of all 41 sequences was found to be 29.3% (T), 29.6% (C), 21.8% (A) and 19.3% (G) respectively. The nucleotide composition of the COI gene sequences of *U. pori* showed that the AT content (51%) was found to be higher compared to the GC content (49%). The transition/transversion rate ratios of the 41 sequences were estimated to be $k_1$=69.487 (purines) and $k_2$=37.525 (pyrimidines). The overall transition/transversion bias (R) was estimated to be 25.526. The ratio of transition and transversion showed that transitions were more recurrent compared to transversions. The average intraspecies K2P distance of the *U. pori* was found to be 0.0046. In the codon usage bias analysis of *U. pori,* T content was less compared to A, G, and C in the first codon position. In second codon position, T content was high compared to A, G, and C respectively. In third codon position, G content was very less compared to A, T, and C, respectively (Figure 2).

The median joining network of *U. pori* was simple, with only five haplotypes among 41 sequences available from different locations (Figure 3). There were five polymorphic sites with 5 mutations, out of which four were parsimony informative sites and one was singleton variable site. Out of the five haplotypes (Table 1), four were shared haplotypes. The COI sequences of the collected specimens from Gopalpur-on-Sea represented one shared haplotype (Hap_4) with the sequences from India (Tamil Nadu), Israel and Madagascar. Hap_1 was the dominant haplotype distributed in Israel, Egypt, Lebanon, Turkey, and China. The nucleotide diversity ($\pi$) and haplotype diversity (*Hd*) of *U. pori* populations were estimated to be 0.002 and 0.637, respectively. The higher *Hd* and lower $\pi$ indicated the modest level of genetic variability in the populations under study.

### Amino acid Variation

The distribution of amino acids in the COI sequence of *U. pori* is presented in Figure 4. For the study of the amino acid variations in the COI sequences of *U. pori,* 235 amino acids were considered. The study indicated that there were three variations found in the *U pori* amino acid sequences, which were considered non-variable regions due to the minimal value of entropy less than 0.5. Thus, the analysis suggested that there were no significant variations in the amino acid sequences found among *U. pori.*

### Phylogenetic Analysis

The average interspecies K2P distance within the genus *Upeneus* of the family Mullidae was found to be 0.14, whereas the average intergenus K2P distance was determined to be 0.18. Besides, the overall mean

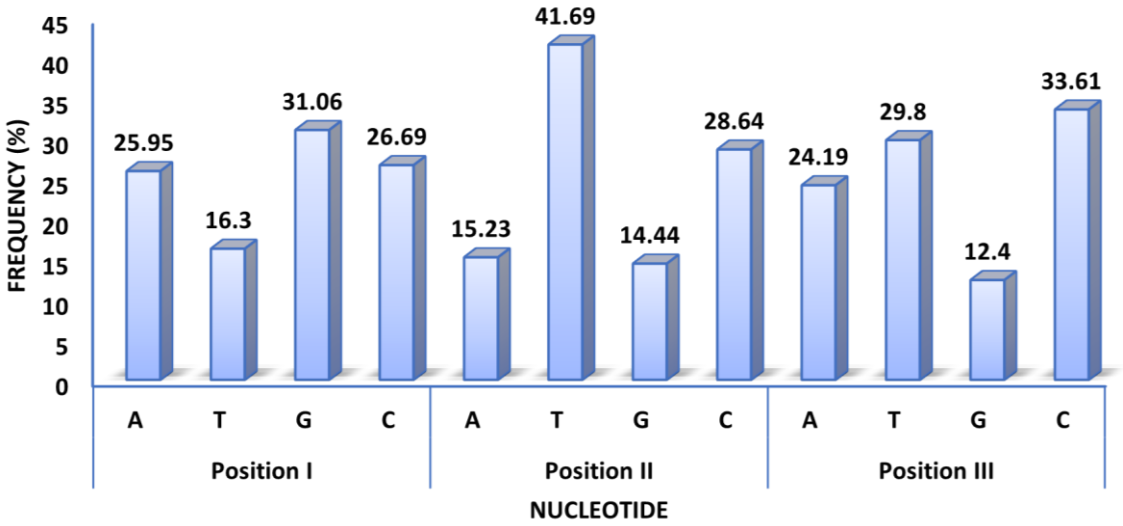


**Figure 2.** Distribution of nucleotides within codons in the barcodes of the fish species *Upeneus pori*

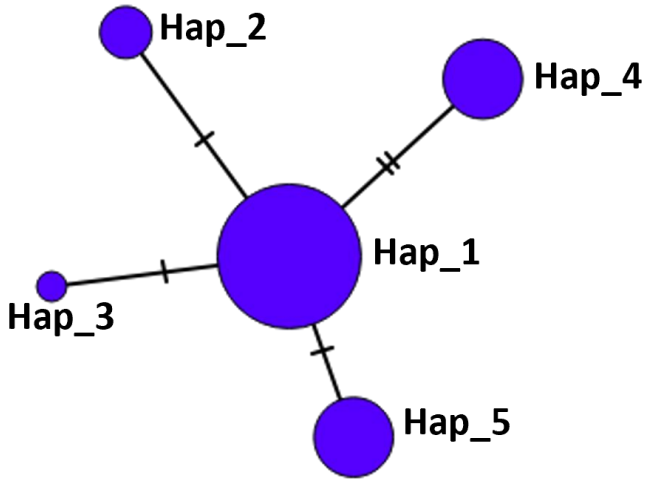


**Figure 3.** Median-joining haplotype networks of COI gene of the species *Upeneus pori.* Size of the circles designates the number of shared sequences in the haplotype. Mutations are represented by lines and the rate of mutation is shown by dashes along the lines.

**Table 1.** Details of the haplotypes of *Upeneus pori* present in different geographical locations

| Haplotype | Accession Numbers | Country |
|---|---|---|
| | LC543943-45, LC572156, KT823810-11 | Egypt |
| | KM538624, KM538626, KM538628, KM538630-36, KF564319 | Israel |
| **Hap_1** | KY176690 | Turkey |
| | KR861568 | Lebanon |
| | MW922385-88 | China |
| **Hap_2** | KM538625 | Israel |
| | OL409597, OL409258 | Madagascar |
| **Hap_3** | KM538627 | Israel |
| | KM538629 | Israel |
| **Hap_4** | OL409211 | Madagascar |
| | MT656560 | India |
| | ON182865, OR782112, OR593385, OR593299(Generated in this study) | India |
| **Hap_5** | OP860955-57, OP860960, OP860969, OP860971, OP860976 | Iran |

distance was estimated, which varied from 0 to 0.21 between 49 sequences of the species from six different genera in the Mullidae family. For maximum likelihood phylogenetic tree construction, the best fit model test for the family Mullidae was identified as HKY+G+I with BIC value of 13785.796. In the ML phylogenetic trees (Figure 5), the new sequences from *U. pori* species generated in this study were clustered with their respective species. The analysis clearly suggested that *U. pori* species, as well as the whole *Upeneus* genus, were monophyletic. Moreover, it was inferred from the phylogenetic analysis that *U. pori* revealed a sister lineage with the species *Upeneus lombok*¸ which was followed by *Upeneus australiae*.

**Molecular Dating**

It was predicted that the family Mullidae originated around 48.95 Mya during the early Eocene sub-epoch. Although, the divergence time of the genus *Upeneus* was estimated to be during the late Eocene sub-epoch, which was around 34.65 Mya, the species of the genus *Upeneus* were diverged from each other around 26.48 Mya. The species *U. pori* diverged from its common ancestor during the late Miocene sub-epoch, which was around 6.38 Mya. The species *Upeneus margarethae, Upeneus tragula, Upeneus spottocaudalis, Upeneus sundaicus, Upeneus supravittatus* and *Upeneus suahelicus* of the genus *Upeneus* were originated in less than 1 Mya. However, the species *Parupeneus insularis, Parupeneus crassilabris, Parupeneus bifasciatus* and *Parupeneus trifasciatus* originated in the present epoch Holocene which was less than 0.01 Mya (Figure 6).

## Discussion

The stability of the biosphere is due to the continuous interaction between the different life forms residing in it. The interaction may be direct or indirect, but every life form in the biosphere is connected to one another in certain ways. So, the conservation of different life forms is necessary in order to maintain the stability of the biosphere. The goatfish species, *U. pori* Ben-Tuvia and Golani, 1989, were recorded for the first time in February, 2021 from the waters of the Odisha coast, Bay of Bengal, India.

Genetic diversity is linked to adaptability, and the loss of genetic variability raises the risk of population extinction. Previous studies have suggested that genetic variety may be significant in influencing an individual's health (Acevedo-Whitehouse et al., 2003; Hoffman et al., 2009; Liu et al., 2018). The nucleotide composition analysis of the COI gene sequences of the studied fish species *U. pori* revealed that the sequences were AT rich and the minimal occurrence of nucleotide 'G', as observed in all the fish species, which was reported by several workers (Bej *et al.,* 2012, Swain *et al.,* 2014; Sahoo *et al.,* 2019). In the codon usage bias analysis, least use of T, highest use of T and a clear anti-G bias was observed in the first, second and third codon positions respectively. The similar patterns have also been observed in other studies (Barik et al., 2021; Bingpeng et al., 2018; Wang et al., 2014). Base use bias in codon positions resulted from different degrees of base-mutation selection pressure applied to mitochondrial gene codon sites during species evolution. The combinatorial impact of both neutral and selection pressure on a genome is ascertained by the codon use analysis.

The average intraspecies genetic (K2P) distance of the fish *U. pori* was 0.0046 and five haplotypes were observed in *U. pori* populations available in different geographical locations globally. The species exhibited relatively higher haplotype diversity and lower nucleotide diversity, which indicated the modest level of genetic variability with recent demographic expansion of the populations under study (de Jong et al., 2011; Song et. al., 2014; Garg et. al., 2018). The preservation of genetic diversity is essential for both individual's probability of survival as well as the population's capacity for evolution (Keller and Waller, 2002; Frankham, 2005; Höglund, 2009). Although there is a link between genetic diversity and population health (Spielman *et al.,* 2004; Höglund, 2009), there are instances of communities persisting for a long period of time despite having low genetic variability (Nichols et al., 2001; Johnson et al., 2009). *U. pori* haplotype from Odisha coast represented a shared haplotype with *U. pori* from India (Tamil Nadu), Israel and Madagascar. Therefore, the *U. pori* population from Tamil Nadu coast might be expanded to Odisha coast, Bay of Bengal.

The phylogenetic trees of the Mullidae family supported the monophyly of both the Mullidae family and the genus *Upeneus*, which was substantiated by previous studies (Nash et al., 2022). The clade formation of different species of the genus *Upeneus* in the phylogenetic trees of the present study of the Mullidae family was also supported by earlier studies (Nash *et al.,* 2022). *U. pori* had a sister lineage with the species *U. lombok* with lowest K2P distance of 0.06, compared to other species of the genus *Upeneus*. *Upeneus* genus was separated from its common ancestor approximately around 26.48 Mya, which was supported by an earlier study (Nash *et al.,* 2022) in which the divergence time of the genus *Upeneus* from its common ancestor was between 17.0 and 27.7 Mya. The *Upeneus* genus was split into two major clades. The first clade is made of the paraphyletic genus *Upeneus*, which includes the genus *Mulloidichthys* inside the clade, which is subdivided into two subclades within the *Upeneus* that diverged around 21.91 Mya during Miocene epoch, as supported by previous studies (Nash *et al.,* 2022) where it was 18.3 Mya. The study evidenced that the grouping of some species had very small branch lengths between taxa, making it challenging to resolve the relationships at the tip level among some species.

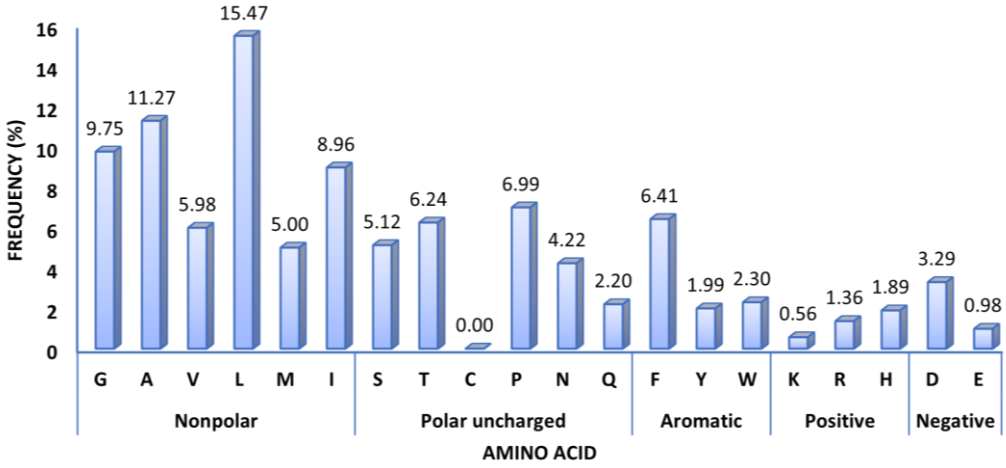


**Figure 4**. Distribution of the amino acids within the barcode of *Upeneus pori.*

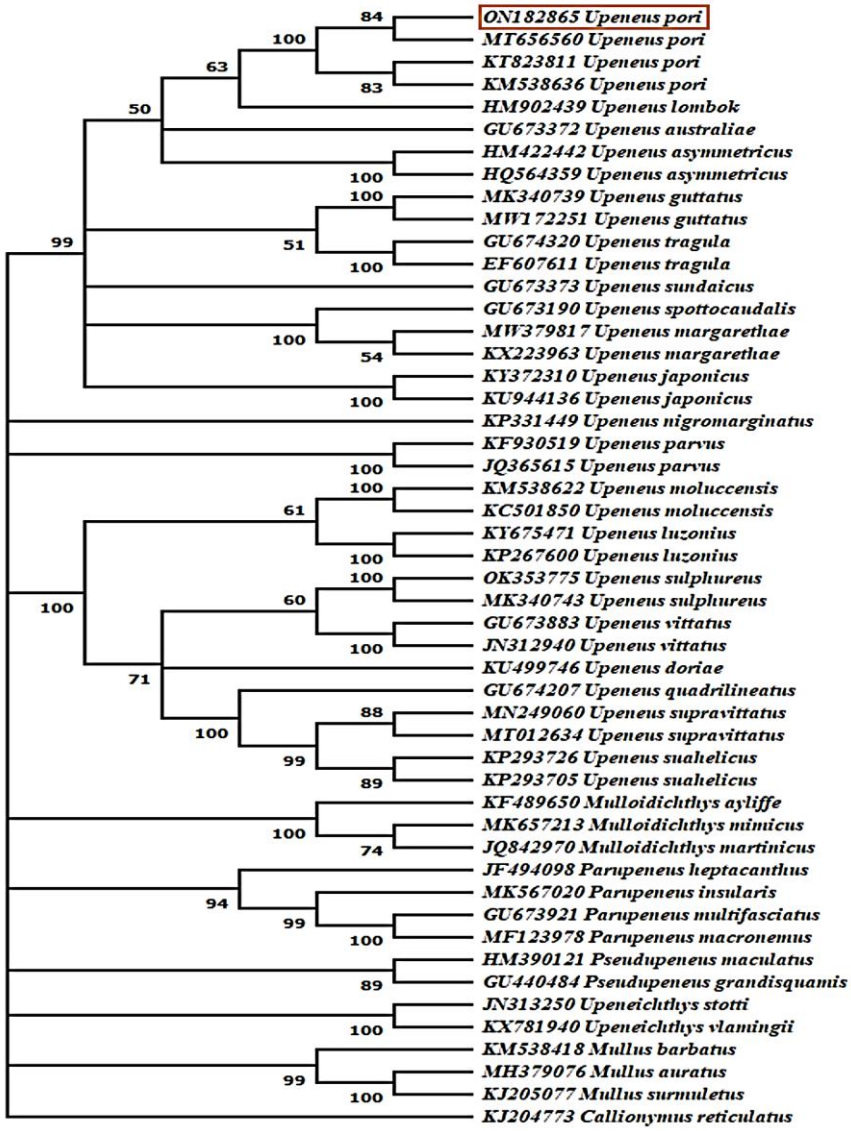


**Figure 5**. Maximum likelihood phylogenetic tree of the family *Mullidae* using Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). The discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4299)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 31.05% sites). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Accession No. ON182865 was generated from the present study

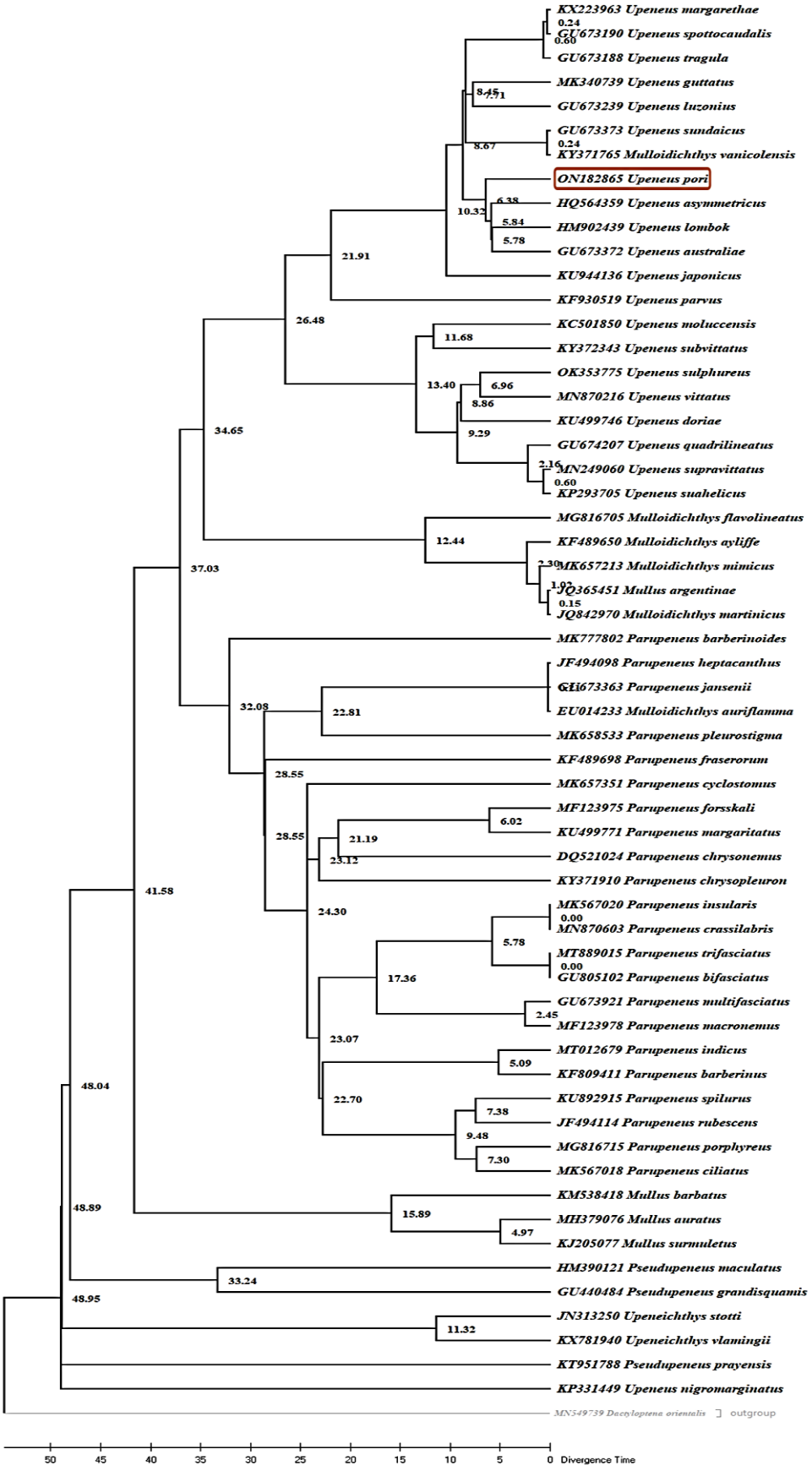


**Figure 6**. Time-calibrated phylogenetic tree of family *Mullidae*. Time tree inferred by applying RelTime method (Tamura et al., 2012; 2018). to user-supplied phylogenetic tree whose branch lengths were calculated using the Maximum likelihood (ML) method and Hasegawa-Kishino-Yano substitution model (Hasegawa et al., 1985). Estimated log likelihood value of the tree is -8613.61. Discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.9283)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 29.99% sites). Numbers on branches represent the time (in Mya) of different nodes. Accession No. ON182865 was generated from the present study.

## Conclusion

Based on morphological traits and DNA barcodes, the currently published study validates a new record of *U. pori* from the Odisha coast, Bay of Bengal. This record broadens the distributional range of *U. pori* in the world's oceans and enhances the species composition and biodiversity of the Odisha coast, Bay of Bengal. This study also emphasizes how important it is to conduct more taxonomic research in order to monitor marine fisheries in the Bay of Bengal and better safeguard species diversity along the Odisha coast, which has a high species variety. This molecular analysis enables us to collect more knowledge about the phylogeny of *Upeneus*, which also contributes to coastal ecosystem management measures and provides the basis for the protection of local species diversity. It was demonstrated that the fish species *U. pori* had lower genetic variation among populations of the species from different geographical locations. The species *U. pori* diverged from its common ancestor during the late Miocene sub-epoch. The population genetic characterization of the natural populations is necessary to establish suitable management practices and genetic improvement programs taking into account different biological phenomena such as gene flow, the impact of mutation, migration, selection, and genetic drift.

## Ethical Statement

Since this study did not disturb environmental populations, ethical approval was not required.

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## Author Contribution

Conceptualization: BS, TKB & AKP, Methodology: BS & TKB, Validation: BS, TKB & AKP, Formal analysis: BS & RKD, Investigation: BS, RKD & TKB, Resources: TKB, Original Draft Preparation: BS Review & Editing: BS, TKB & AKP, Visualization: BS, TKB & AKP, Supervision: TKB & AKP.

## Conflict of Interest

The authors declare that they have no conflicts of interest.

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