Journal of Environmental Biology
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

JEB®

Original Research Paper
Volume 47 Issue 2 March 2026 pp. 287-296
DOI: http://doi.org/10.22438/jeb/47/2/MRN-5821

J. Environ. Biol., 2026

Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Morphometry and molecular insights into three species of Balistidae family from the South-eastern Arabian Sea, India

K.S. Pratiksha[1], R. Mridula[1]*, K.M. Rajesh[2] and N.K. Suyani[3]

[1]*Department of Fisheries Resources and Management, College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Mangaluru-575 002, India*
[2]*Finfish Fisheries Division, ICAR-Central Marine Fisheries Research Institute, Mangalore Regional Centre, Mangaluru-575 001, India*
[3]*Peninsular and Marine Fish Genetic Resources Division, National Bureau of Fish Genetic Resources, Kochi-682 018, India*

Received: 30 June 2025 **Revised:** 26 September 2025 **Accepted:** 25 October 2025

***Corresponding Author Email:** mridularajesh789@yahoo.co.in ***ORCiD:** https://orcid.org/0000-0002-3925-2956

Abstract

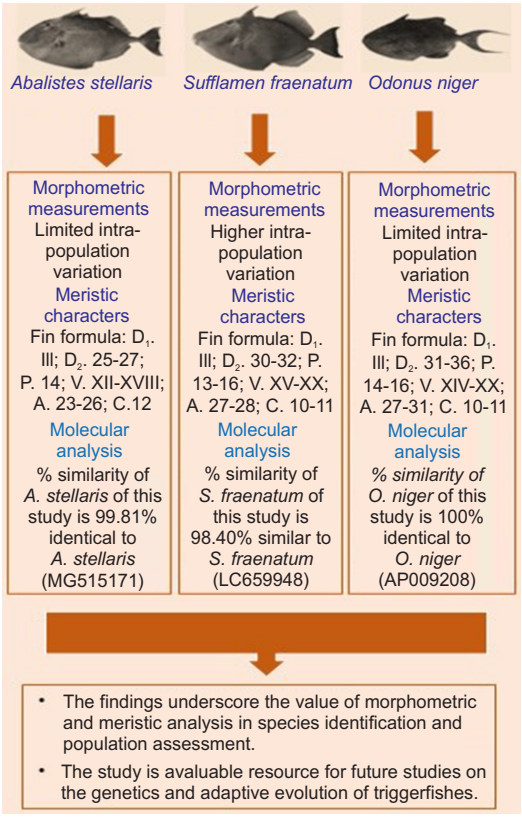
Aim: The present investigation aimed to evaluate the mitochondrial COI and 16s rRNA gene sequences of three balistid species—*Abalistes stellaris*, *Sufflamen fraenatum* and *Odonus niger*—to assess the genetic congruence and phylogenetic relationships from the waters along the south-west coast of India.

Methodology: Three species of balistids—*Abalistes stellaris, Sufflamen fraenatum* and *Odonus niger* procured from three major marine landing centres of Karnataka were examined for morphometry and genetic identification using partial sequences of the COI and 16S rRNA to distinguish between species and understand their relationships.

Results: The morphometric measurements of *Odonus niger* and *Abalistes stellaris* showed a narrow range of coefficient of variation, suggesting minimal or very low intra-population variation, whereas *Sufflamen fraenatum* displayed a wide range, indicating high intra-population variation. The intra- and inter-species sequence divergence values, based on the COI gene, ranged between 0.0034 to 0.0051 and 0.1549 to 0.1824, respectively.

Interpretation: The present analysis clearly distinguished the three balistid species from the south-eastern Arabian Sea, aligning with established Balistidae taxonomy. The findings underscore the value of morphometric analysis in species identification and population assessment, offering a foundation for future genetic and evolutionary studies crucial for effective fisheries management and conservation.

Key words: Arabian Sea, Balistidae, COI, Triggerfish, 16S rRNA



How to cite: Pratiksha, K.S. R. Mridula, K.M. Rajesh and N.K. Suyani: Morphometry and molecular insights into three species of Balistidae family from the South-eastern Arabian Sea, India. *J. Environ. Biol.*, **47,** 287-296 (2026).

Introduction

Members of the Balistidae family, commonly known as triggerfish, are widely distributed across the tropical and subtropical waters of the Pacific, Atlantic, and Indian Oceans. They play an important ecological role in the coral lagoons, coral reef ecosystems, and extrinsic reef slopes (Sribenja *et al.,* 2022). These fishes often occur in aggregations and feed on algae, zooplankton, crustaceans, and sponges. Triggerfishes can grow up to 300 mm in length, with some reaching over 500 mm, and are commonly caught as bycatch in trawl nets and purse seines along the south-west coast of India. Globally, the Balistidae family encompasses twelve genera with 41 valid species, all found exclusively in marine waters (Fricke *et al.,* 2022). Of these, twenty species across 12 genera have been reported in Indian waters (Mohapatra *et al.,* 2020). Triggerfishes are highly regarded by marine aquarists worldwide, especially for their distinctive colour patterns. They are characterized by a moderately compressed, deep body covered in thick, tough skin with large scales, relatively short gill opening, and two dorsal fins, first dorsal fins. The dorsal fin has three distinct spines, with the first spine capable of locking in an erect position by the second spine, forming a 'trigger' mechanism (Bray, 2023).

Species identification is one of the most essential aspects of biological research, particularly in biodiversity analysis and conservation efforts (Dayrat, 2005). Morphological systematics has traditionally been the main method of taxonomy in aquatic biology. However, from a developmental perspective, morphological studies may not always distinguish species, as physical differences are not necessarily distinct, and reproductively isolated groups may appear physically similar (Bhaskar *et al.,* 2021). Consequently, accurate identification of morphologically similar, resemblance, exploited fish species is crucial for fisheries management and stock structure analysis. In case of taxonomic uncertainty, DNA-based molecular genetic markers have proven highly effective (Hebert *et al.,* 2003). Mitochondrial DNA (mtDNA) is a predominant genetic marker that has been extensively studied in fish phylogenetics, particularly the mitochondrial 16S rRNA and the cytochrome C Oxidase subunit I (COI) genes, which are highly conserved (Lakra *et al.,* 2009). These markers are powerful tools for examining intra- and inter-species variations, as well as deeper phylogenetic splits within species (Kvie *et al.,* 2013). Recently, DNA-based fish identification has become a prominent technique, due to applicability across all life history stages of fish (Bineesh *et al.,* 2021).

Molecular identification reports on triggerfish includes *Sufflamen chrysopterum* from the Gulf of Thailand (Sribenja *et al.,* 2022), *Pseudobalistes fuscus* from Chinese waters (Zhang *et al.,* 2016) and *Balistes capriscus* from the southeastern United States (Antoni *et al.,* 2011). Additionally, limited studies have focused on the taxonomic and biological aspects of the species belongs to the Balistidae family from Indian waters (Suyani *et al.,* 2021a; Suyani *et al.,* 2021b; Sahayak *et al.,* 2013;Sahayak *et al.,* 2014; Nair *et al.,* 2013). Although twenty species belonging to twelve genera have been reported from Indian waters, DNA barcoding studies on triggerfish species from this region are still lacking. The present investigation is the first attempt to evaluate the mitochondrial COI and 16s rRNA gene sequences of three balistid species—*Abalistes stellaris, Sufflamen fraenatum* and *Odonus niger*—to assess genetic congruence and phylogenetic relationships from the waters along the *south-west* coast of India.

Materials and Methods

Sample collection: A total of 90 specimens each of *A. stellaris* (17.0-31.2 cm), *S. fraenatum* (15.8-43.0 cm) and *O. niger* (18.8-30.6 cm) were procured from the bycatch landings of commercial high-speed multi-day trawlers operating off Mangalore (12.853°N, 74.833°E), Malpe (13.347°N, 74.701°E) and Karwar (14.801°N, 74.124°E) fishing harbours (Fig. 1) in Karnataka from May to November 2021. Fresh specimens were transported to the Department of Aquatic Animal Health Management laboratory at the College of Fisheries, Mangaluru, in iced conditions for detailed morphological and molecular analyses. All the fishes were identified and differentiated in consonance with Sahayak *et al.* (2014). After identification, tissue samples (fin clips) from the dorsal, anal, and ventral fins were collected, preserved in 95% ethanol, and stored at –20 ºC for subsequent molecular analysis.

Morphometric and meristic characterization: Various morphometric and meristic characters were measured in accordance with Hubs and Lagler (2004). Morphometric measurements—including total length (TL), standard length (SL), pre-dorsal length-1 (PDL1), pre-dorsal length-2 (PDL2), pre-anal length (PAL), body depth (BD), head length (HL), snout length (SnL), and eye diameter (ED)—were taken from the left side of each individual's body with an accuracy of 0.1 cm using an electronic Vernier caliper. Meristic counts, such as the number of spines in the dorsal and ventral fins, the number of rays in the dorsal, pectoral, anal, and caudal fins, and the number of gill rakers, were recorded. Spines and fin rays were counted from both the sides for paired fins such as pectoral and pelvic. All morphometric data, calculated as percentages of TL, were statistically analyzed subjected to determine the mean, standard deviation, and coefficient of variation. Total length was considered the independent variable, while other characters were treated as dependent variables. Additionally, linear regressions were used to estimate parameters and the correlation coefficient (r) for the fitted linear relationships between TL and other morphometric characters. The range, mean, standard deviation, and coefficient of variation for meristic counts were also included in the statistical analysis (Snedecor and Cochran, 1989).

DNA isolation, PCR amplification, and sequencing: DNA extraction was performed on tissue samples (fin clips) preserved in 95% ethanol. Total genomic DNA was isolated for all species using the standard phenol-chloroform-isoamyl alcohol method (Sambrook *et al.,* 1989) with minor modifications. The quality of the extracted genomic DNA was verified through agarose gel electrophoresis (0.8%) with ethidium bromide in a 1 × TAE buffer,

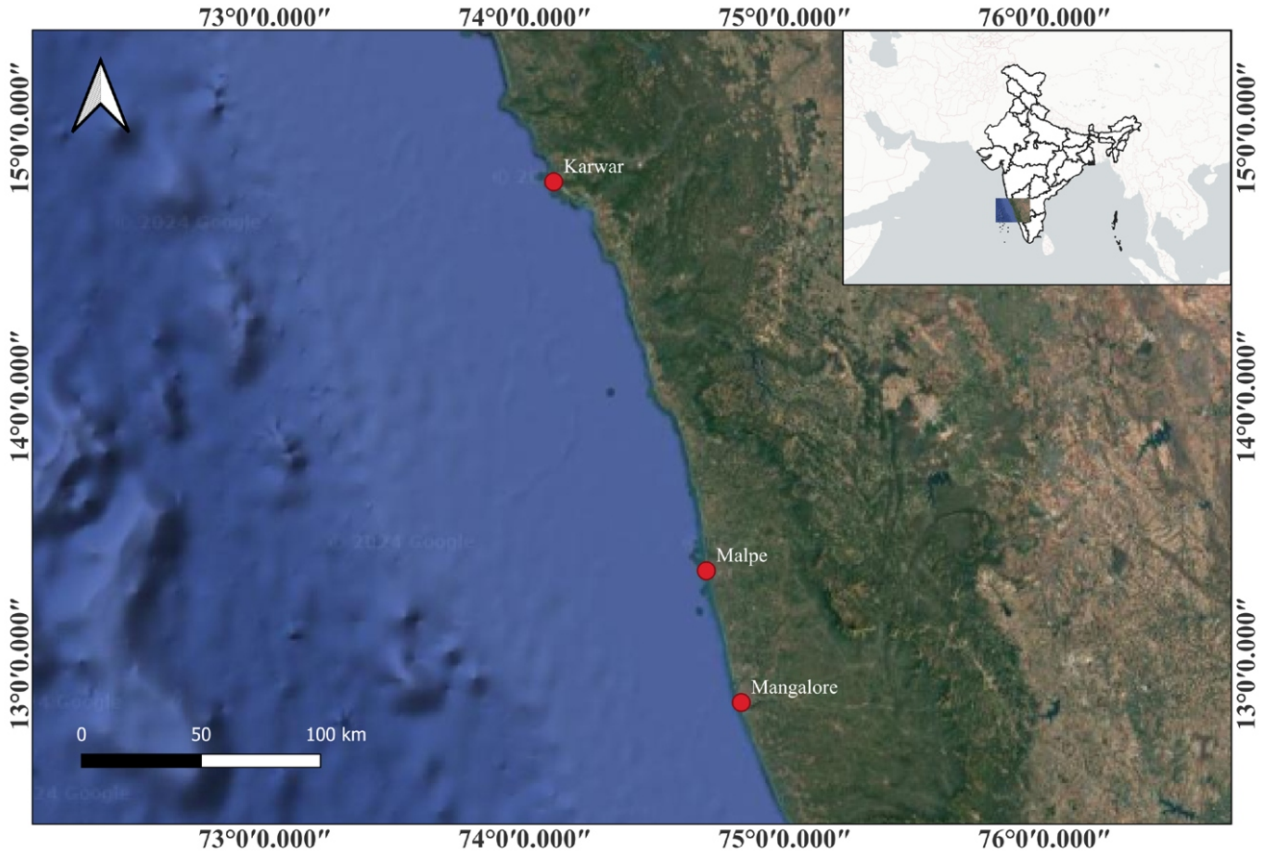


Fig. 1: Sampling locations.

and the final DNA concentration was determined by measuring the optical density at 260 nm using a Nano Drop 2000c Spectrophotometer (Thermo Fisher Scientific, USA).

A partial sequence of the 658 bp region of the COI gene was amplified using the primer pair Fish F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and Fish R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward *et al.,* 2005), while 583 bp fragment of the mitochondrial 16S rRNA gene was amplified using primers 16S F (5'-CGCCTGTTTATCAAAAACAT-3') and 16S R (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.,* 1991). PCR amplifications were performed in a 30 µl reaction mixture consisting 1 × assay buffer (10 mMTris-HCl, 50 mMKCl, 0.01% gelatin, pH 8.3) with 1.5 mM MgCl2 (Genei, Bangalore, India), 10 pmol of each forward and reverse primer for the COI gene and 16s rRNA, 200 µM of each dNTP (Genei, Bangalore, India), 0.9 units of Taq DNA polymerase (Genei, Bangalore, India), and 2 µl of template genomic DNA.

The amplification conditions for the COI gene included an initial preheat at 95ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 35 sec, with a final extension at 72 °C for 5 min. In contrast, the amplification conditions for 16S rRNA involved an initial preheat at 95ºC for 7 min, followed by 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 54.5 ºC for 1 min, and extension at 72 ºC for 30 sec, with a final extension at 72 °C for 10 min. For each sample, 10 µl of the PCR product was electrophoresed at 100-120 V through 1.5 % agarose gels, stained with ethidium bromide, and visualized under UV transilluminator (Bio-Rad Laboratories, Inc., USA). The amplified PCR products were purified using a HiPurA PCR purification kit (HiMedia, Mumbai, India) and sequenced by Sanger method (Barcode Bioscience Pvt. Ltd., Bangalore, India) using specific primers.

Data analysis: The raw DNA sequences obtained were examined and edited manually for miscalls and base spacing errors using BioEdit sequences Alignment Editor software ver. 7.0.5.2 (Hall, 1999). Alignment was performed using the MultAlin program. Sequence differences between species were quantified by averaging pairwise comparisons across all individuals. Sequences for both 16S rRNA and COI genes were submitted to NCBI GenBank with assigned accession numbers. Nucleotide composition, as well as the number of transitions and transversions between species, were determined using MEGA 11. The Kimura-2-parameter (K2P) model was applied to

calculate intraspecific and interspecific divergence values using Molecular Evolutionary Genetic Analysis (MEGA) software ver. 11.0 (Kimura, 1980). Neighbour-Joining (NJ) trees were constructed in MEGA 11 using 1000 bootstrap replications (Costa *et al.,* 2006). Statistical analysis for morphometric data was performed using MS Excel 2013.

Results and Discussion

Morphometric measurements are widely used as robust tools for detecting morphological variations among populations and for classifying closely related species based on shapes (Costa *et al.,* 2006). The statistical parameters for each morphometric measurements, including the mean, standard deviation (SD), range, and coefficient of variation (CV) of *A. stellaris, S. fraenatum* and *O. niger* are represented in Table 1. The data evaluation indicated that the maximum CV was observed in snout length for all the three species: *A. stellaris* (15.85%), *S. fraenatum* (21.64%), and *O. niger* (12.78%) indicating greater variability in this trait within populations. Conversely, the lowest CV values were recorded for eye diameter in *A. stellaris* (10.56%) and *S. fraenatum* (9.29%), and for standard length in *O. niger* (8.42%), suggesting stable traits with minimal variability. The morphometric measurements of *O. niger* and *A. stellaris* indicated an extremely narrow range of coefficient of variation (CV), indicating limited intra-population variation. These findings are consistent with earlier report from the South-east Arabian Sea (Suyani *et al.,* 2021a). In contrast, *S. fraenatum* exhibited a wider range of CVs, from 9.29% (eye diameter) to 21.64% (snout length), indicating greater intra-population variation, likely attributable to ecological influences such as habitat heterogeneity that expose populations to diverse selective pressures and help maintain genetic diversity. Correlation analysis between total length and other morphometric characters revealed strong positive relationships (high correlation coefficients, *r*) for most traits across all three species, except for eye diameter. This indicates a high degree of interdependence among morphometric traits, with eye diameter showing comparatively weaker association with overall body size.

Comparative analysis of morphometric characters with total length was carried out for three balistid species using correlation coefficients (*r*) and regression parameters (a and b). In *A. stellaris,* the standard length (r = 0.9855), pre-anal length (r = 0.9655), and pre-dorsal length-1 (r = 0.9348) showed strong correlations with total length, while the remaining traits also exhibited good correlations (Table 2). In *Sufflamen fraenatum*, all morphometric traits were highly correlated with total length, except for snout length and eye diameter, which showed good and moderate correlations, respectively. For *Odonus niger,* the eye diameter was the only trait with a weak correlation, while all other characters demonstrated moderate to high correlations with total length (Table 2). Regression analysis revealed that most morphometric traits in *A. stellaris* and *O. niger* exhibited negative allometric growth ($p < 0.01$), with the exception of standard length, pre-anal length, and snout length in *A. stellaris,* and snout length in *O. niger,* which showed isometric or near-isometric growth. In contrast, *S. fraenatum* displayed predominantly isometric growth for most traits, except for pre-dorsal length, head length, and eye diameter, which followed negative allometric patterns ($p < 0.01$). The regression coefficient (*b*) values support these observations, highlighting species-specific growth patterns. The pronounced differences in allometric growth among the three species underscore their taxonomic relevance and suggest that allometric

Table 1: Statistical analysis of different morphometric characters of three species of family Balistidae landed along the Karnataka coast

Statistical parameters	*Abalistes stellaris*		*Sufflamen fraenatum*		*Odonus niger*	
	Mean ± SD (cm)	CV (%)	Mean ± SD (cm)	CV (%)	Mean ± SD (cm)	CV (%)
Total length (TL)	31.81 ± 4.64 (15.8-43.0)	14.59	23.40 ± 3.74 (17.0-31.2)	15.98	24.35 ± 2.42 (18.8-30.6)	9.92
Standard length (SL)	24.48 ± 3.70 (12.2-33.6)	15.11	18.46 ± 2.86 (13.0-25.3)	15.48	16.46 ± 1.39 (14.3-19.8)	8.42
Pre-dorsal length-1 (PDL1)	8.63 ± 1.14 (4.5-11.6)	13.17	6.81 ± 1.09 (5.2-9.0)	16.05	5.17 ± 0.50 (4.4-6.5)	9.62
Pre-dorsal length-2 (PDL2)	15.44 ± 2.09 (8.3-21.0)	13.57	11.94 ± 1.79 (8.5-16.5)	14.96	8.82 ± 0.76 (7.3-10.8)	8.66
Pre-anal length (PAL)	15.18 ± 2.21 (7.6-20.3)	14.57	11.79 ± 1.93 (9.0-15.6)	16.36	9.81 ± 0.86 (8.2-12.5)	8.77
Body depth (BD)	11.23 ± 1.54 (5.9-14.4)	13.69	9.79 ± 1.77 (7.5-13.5)	12.99	7.85 ± 0.94 (5.8-10.5)	11.97
Head length (HL)	9.11 ± 1.37 (5.2-13.3)	15.02	7.16 ± 0.93 (5.5-9.0)	18.10	6.18 ± 0.75 (4.6-8.8)	12.08
Snout length (SnL)	5.55 ± 0.88 (2.6-7.5)	15.85	4.45 ± 0.96 (3.1-6.6)	21.64	3.70 ± 0.47 (2.8-4.7)	12.78
Eye diameter (ED)	1.73 ± 0.18 (1.1-2.0)	10.56	1.18 ± 0.11 (1.0-1.5)	9.29	0.91 ± 0.10 (0.8-1.0)	10.69

*Values in parenthesis indicates range (cm); CV, coefficient of variation

Table 2: Linear regression analysis of different morphometric characters on total length

Statistical parameters	*Abalistes stellaris*			*Sufflamen fraenatum*			*Odonus niger*		
	a	***b***	***r***	***a***	***b***	***r***	***a***	***b***	***r***
Standard length (SL)	0.7554	1.0052	0.9855	0.8951	0.9600	0.9914	1.6548	0.7195	0.8599*
Pre-dorsal length-1 (PDL1)	0.4468	0.8559	0.9348*	0.3484	0.9427	0.9426	0.4670	0.7528	0.7904*
Pre-dorsal length-2 (PDL2)	0.9610	0.8024	0.8701*	0.6847	0.9069	0.9710*	0.9834	0.6871	0.7963*
Pre-anal length (PAL)	0.5420	0.9631	0.9655	0.5171	0.9916	0.9661	0.9864	0.7194	0.8364*
Body depth (BD)	0.5874	0.8527	0.8934*	0.3360	1.0688	0.9331	0.5576	0.8275	0.6924*
Head length (HL)	0.4933	0.8422	0.8542*	0.5981	0.7877	0.9529*	0.7119	0.6759	0.5695*
Snout length (SnL)	0.1782	0.9931	0.8963	0.1047	1.1866	0.8678	0.1368	1.0321	0.7976
Eye diameter (ED)	0.2069	0.6139	0.8597*	0.3432	0.3931	0.6791*	0.2077	0.4605	0.4220*

a, intercept; b, slope; r, correlation coefficient; *p < 0.01

Table 3: Statistical analysis of different meristic characters of three species of Balistidae family landed along the Karnataka coast

Meristic characters	*Abalistes stellaris*		*Sufflamen fraenatum*		*Odonus niger*	
	Mean ± SD	**CV (%)**	**Mean ± SD**	**CV (%)**	**Mean ± SD**	**CV (%)**
Dorsal fin I	3 ± 0.00 (3)	0.00	3 ± 0.00 (3)	0.00	3 ± 0.00 (3)	0.00
Dorsal fin II	25.87 ± 0.51 (25-27)	2.28	30.46 ± 0.45 (30-32)	1.75	33.28 ± 1.19 (31-36)	3.97
Pectoral fin	14 ± 0.00 (14)	0.00	14.54 ± 0.58 (13-16)	4.47	14.28 ± 0.45 (14-16)	2.56
Ventral spines	14.56 ± 1.05 (12-18)	8.15	16.84 ± 1.37 (15-20)	5.02	16.92 ± 1.00 (14-20)	6.35
Anal fin	24.71 ± 0.73 (23-26)	3.42	27.69 ± 0.44 (27-28)	1.16	29.29 ± 0.99 (27-31)	3.64
Caudal fin	12 ± 0.00 (12)	0.00	10.33 ± 0.50 (10-11)	4.84	10.33 ± 0.50 (10-11)	4.84
Gill rakers	33.42 ± 1.79 (30-37)	6.06	27.77 ± 1.65 (25-33)	6.27	31.64 ± 2.04 (19-35)	6.16

*Values in parenthesis indicates range (numbers); CV, coefficient of variation

analysis is a useful tool for investigating intra- and inter-specific morphological variation. Fish are known to be highly responsive to environmental conditions, often adapting by altering their body proportions. These variations in growth patterns may therefore reflect ecological influences and adaptive responses, in addition to genetic factors. Meristic analysis revealed that the number of spines on the first dorsal fin (D1:3) remained constant across all specimens of three balistid species. In *A. stellaris,* the highest CV was observed in ventral fin spines (8.15%) followed by the number of gill rakers (6.06%) and anal fin rays (3.42%). For *S. fraenatum,* the gill rakers showed the highest CV (6.27%), while the anal fin rays had the lowest (1.16%). In *O. niger,* the ventral fin spines (6.35%) and pectoral fin rays (2.56%) showed the highest and lowest CVs, respectively (Table 3).

The fin formula recorded were: *A. stellaris*: D1. III; D2. 25-27; P. 14; V. XII-XVIII; A. 23-26; C. 12; *S. fraenatum*: D1. III; D2. 30-32; P. 13-16; V. XV-XX; A. 27-28; C. 10-11 and *O. niger*: D1. III; D2. 31-36; P. 14-16; V. XIV-XX; A. 27-31; C. 10-11. These findings align with earlier reports (Suyani *et al.,* 2021a; Sahayak *et al.,* 2013), with minor variations in ventral spines and caudal rays. Such meristic plasticity may reflect genetic divergence, environmental conditions and anthropogenic influences (Pinheiro *et al.,* 2005; Ullah *et al.,* 2022).

Molecular analysis: Partial sequencing of the mitochondrial COI and 16S rRNA genes from seven individuals representing *Abalistes stellaris, Sufflamen fraenatum,* and *Odonus niger* yielded 10 sequences (Table 4). These sequences, along with reference haplotypes from NCBI, were used to assess the intraspecific, interspecific, and intergeneric genetic distances within the Balistidae family (Order: Tetraodontiformes). Combined sequence data were analyzed to evaluate genetic variation and percentage similarity, and phylogenetic trees were constructed to determine species relationships.

DNA barcoding relies on the principle that interspecific genetic divergence exceeds intraspecific variation, enabling

Table 4: Details of locations and GenBank accession numbers of three species of Balistidae family landed along the Karnataka coast

Species	Collection locality	GenBank Accession Number	
		COI sequence	16S rRNA sequence
Abalistes stellaris	Mangalore	Om924063	-
Abalistes stellaris	Malpe	-	Om924064
Sufflamen fraenatum	Mangalore	Om924065	-
Sufflamen fraenatum	Malpe	-	Om924066
Odonus niger	Mangalore	OM943168	Om943169
Odonus niger	Malpe	OM943170	Om943171
Odonus niger	Karwar	OM943172	On040616

- Indicates COI x and 16rRNA sequences not done

Table 5: Sequence divergence values (above diagonal) and nucleotide differences (below diagonal) among three species of Balistidae family based on partial COI sequence

Species (with location)	1	2	3	4	5
1. *O. niger* (Malpe)	–	0.0051	0.0051	0.1824	0.1566
2. *O. niger* (Karwar)	3	–	0.0034	0.1172	0.1652
3. *O. niger* (Mangalore)	3	2	–	0.1790	0.1549
4. *A. stellaris* (Mangalore)	106	103	104	–	0.1772
5. *S. fraenatum* (Mangalore)	91	96	90	103	–

Table 6: Percentage similarities of three species of Balistidae based on COI sequences with NCBI haplotype

Species (with GenBank Acc. No.)	*O. niger* (Mangalore)	*O. niger* (Malpe)	*O. niger* (Karwar)	Species (with GenBank Acc. No.)	*A. stellaris* (Mangalore)	*Species* (with GenBank Acc. No.)	*S. fraenatum* (Mangalore)
O. niger (AP009208)	98.46%	99.83%	99.31%	*A. stellaris* (MT076494)	98.12%	*S. fraenatum* (Lc659948)	97.77%
O. niger (FJ459558)	99.24%	99.62%	99.43%	*A. stellaris* (KU170631)	98.85%	*S. fraenatum* (Ap004456)	97.42%
O. niger (FJ459554)	96.68%	99.06%	98.87%	*A. stellaris* (MH235602)	97.94%	*S. fraenatum* (Kt601033)	97.36%

species discrimination using a threshold value (Hebert *et al.,* 2003). Its effectiveness in marine species identification has been well established (Lakra *et al.,* 2010). Molecular identification offers a reliable tool for accurate species-level classification, aids in stock structure assessment, and supports stock-specific fisheries management and conservation (Basheer *et al.,* 2015). This study is the first to report mitochondrial DNA sequences for these three balistid species from Indian waters. Phylogenetic analyses based on both COI and 16S rRNA genes showed identical topologies, clearly separating *A. stellaris, S. fraenatum,* and *O. niger* into distinct clades with no shared haplotypes or overlap, confirming their taxonomic distinction and supporting the utility of DNA barcoding in balistid species identification.

Data analysis of mitochondrial COI gene sequence: A total of 697 nucleotide base pairs of aligned COI gene sequences were generated for all species. There were no insertions, deletions, or stop codons in any of the sequences. The analysis showed average nucleotide frequencies of 25.4% A, 29.9% T, 16.8% G, and 27.6% C. Nucleotide frequencies for all three species from different locations are presented in Fig 2. The transition/ transversion bias (*R*) was 2.09, and sequence divergence among the studied species was also estimated (Table 5).

In this study, the highest intraspecific sequence divergence (0.51%) was observed between *O. niger* (Malpe) and *O. niger* (Mangalore), and the lowest (0.34%) between *O. niger* (Mangalore) and *O. niger* (Karwar). The maximum and minimum interspecies sequence divergence values were recorded between *O. niger* (Malpe) and *A. stellaris* (Mangalore) (18.24%), and between *O. niger* (Mangalore) and *S. fraenatum* (Mangalore) (15.49%), respectively. The pairwise genetic distance of COI sequences based on the K2P model represents the highest genetic distance (0.2358) between *A. stellaris* (Mangalore) and *Aluterus monoceros* (MN549717), and the lowest genetic distance (0.01399) between the species *O. niger* (Karwar) and *A.*

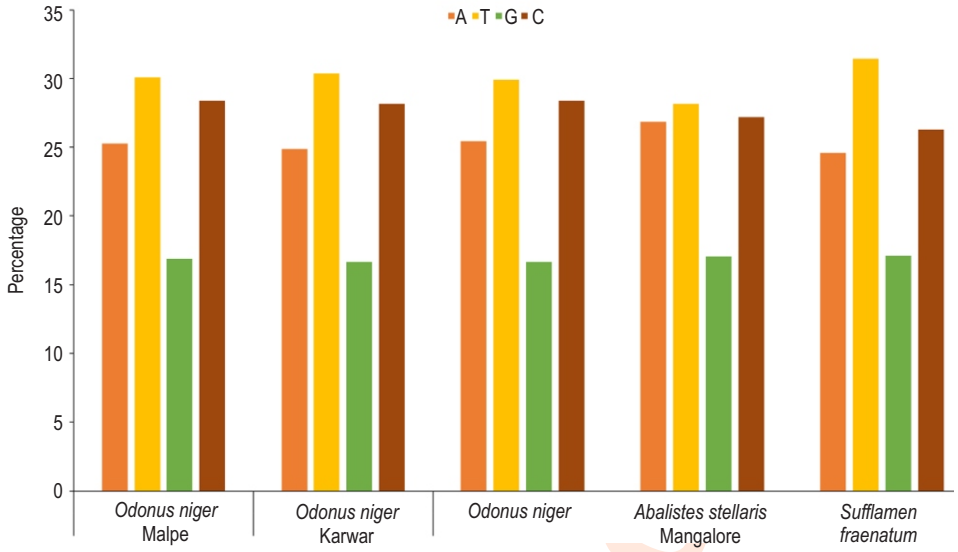


Fig. 2: Nucleotide frequencies of three species of balistidae from different locations based on COI sequences.

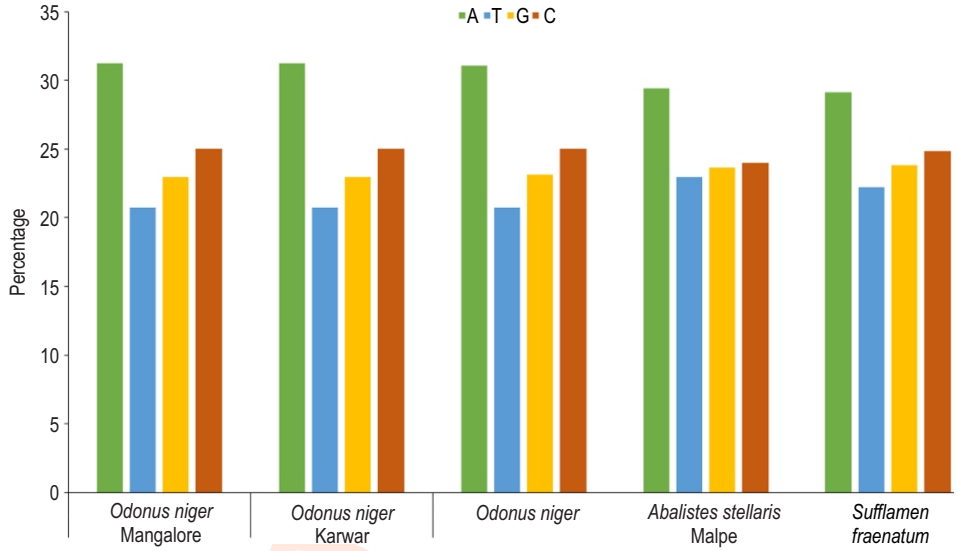


Fig. 3: Nucleotide frequencies of three species of balistidae from different locations based on 16S rRNA sequences.

stellaris (KU170631). Furthermore, the COI sequence developed in this study for *O. niger* (Malpe) showed 99.83% similarity to the COI sequence of *O. niger* (AP009208). Similarly, *A. stellaris* (Mangalore) indicated 98.85% identity to *A. stellaris* (KU170631), and *S. fraenatum* (Mangalore) exhibited 97.77% identity to *S. fraenatum* (LC659948) (Table 6).

Data analysis of mitochondrial 16S rRNA gene sequence: Sequencing of the 16S rRNA gene generated 625 nucleotide base pairs. No stop codons were found in any of the sequences. The average nucleotide frequencies were 30.4% A, 21.5% T, 23.3% G, and 24.8% C. The nucleotide frequencies of 16S rRNA gene sequences are presented in Fig. 3. The overall transition/transversion bias (R) was 1.40. Pairwise nucleotide sequence divergence values based on the 16S rRNA are provided in Table 7. The intraspecific nucleotide sequence divergence between *O. niger* (Malpe) and *O. niger* (Mangalore and Karwar) was 1 (0.17%). The highest interspecific nucleotide sequence divergence value (37, 6.57%) was recorded between *O. niger* (Mangalore and Karwar) and *A. stellaris* (Malpe). The pairwise genetic distance of 16S rRNA sequences based on the K2P model was highest between *A. stellaris* (MG515171) and *A.*

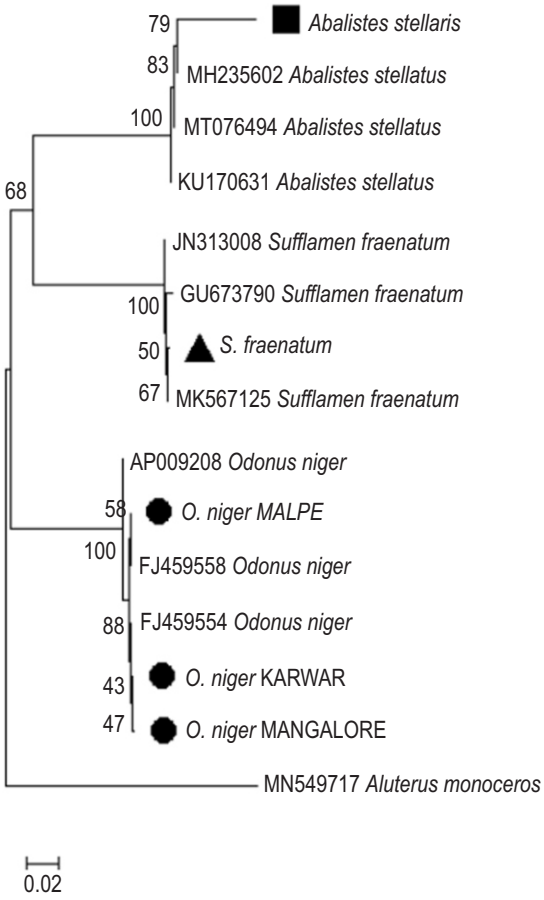


Fig. 4: Neighbour joining (NJ) tree representing phylogenetic relationship of three species of Balistidae inferred from partial COI sequences. Numbers at nodes indicate the bootstrap values. Scale bar represents 0.02 substitution per nucleotide position. The bold square, triangle and circle highlighted the studied triggerfish in this article.

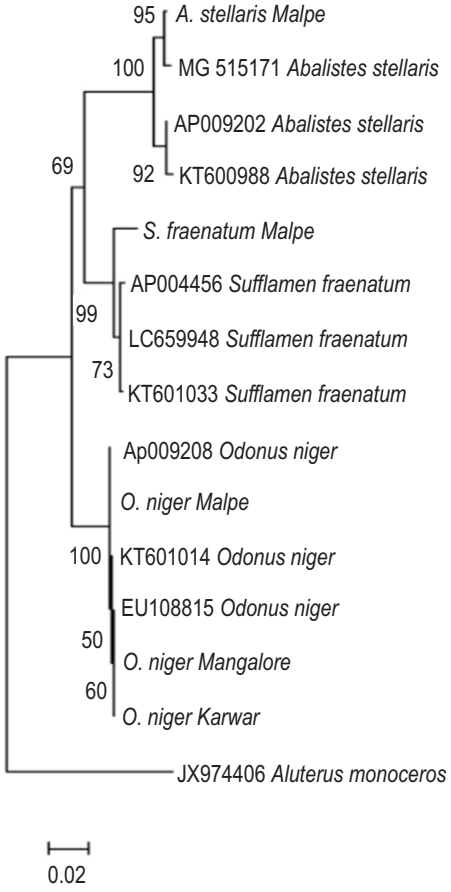


Fig. 5: Neighbour joining (NJ) tree representing phylogenetic relationship of three species of Balistidae inferred from partial 16S rRNA sequences. Numbers at nodes indicate the bootstrap values. Scale bar represents 0.02 substitutions per nucleotide position. The studied triggerfish are indicated based on location.

monoceros (MN549717) (0.1499) and lowest between *O. niger* (Malpe) and *S. fraenatum* (LC659948) (0.0392). The percentage similarity of *O. niger* (Mangalore) in the present study was 100% identical to *O. niger* (AP009208). Additionally, *A. stellaris* and *S. fraenatum* from Malpe showed 99.81% and 98.40% similar to *A. stellaris* (MG515171) and *S. fraenatum* (LC659948), respectively (Table 8).

Phylogenetic inference

COI gene: The phylogram constructed through neighbour-joining (NJ) method was highly reliable, as the out-group (*Aluterus monoceros*) was distinctly separated in the tree, and two distinct clades were formed between the species (Fig. 4). *Odonus niger* (Mangalore, Malpe, and Karwar) formed a discrete clade supported by high bootstrap value (≈100%). Similarly, *Abalistes stellaris* and *Sufflamen fraenatum* formed a distinct branch within another clade with significant bootstrap values.

16S rRNA gene: The NJ tree of 16S rRNA gene revealed an identical phylogenetic relationship among the species, consistent with that inferred from COI gene sequence data. *O. niger* formed a separate branch within one clade, supported by a high bootstrap value (≈100%). In contrast, *A. stellaris* and *S. fraenatum* were grouped together in another clade (Fig. 5).

In the present study, the primer pairs used for the amplifying partial COI sequences were originally developed for invertebrates (Ward *et al.,* 2005), but successfully amplified an approximately 695bp segment in all species. The 16S rRNA gene, known for its low mutation rate compared to other mtDNA genes, is one of the most extensively studied genes within mitochondrial DNA. It has proven useful for analyzing families, species, and populations (Bineesh *et al.,* 2015). The genetic divergence values recorded in the current study are comparable to those reported in other fish species such as deep water basslets (Bineesh *et al.,* 2015), bigeyes (Bineesh *et al.,* 2016), mackerels (Basheer *et al.,*

Table 7: Sequence divergence values (above diagonal) and nucleotide differences (below diagonal) among three species of Balistidae based on partial 16S rRNA sequence

Species (with location)	1	2	3	4	5
1. *O. niger* (Malpe)	_	0.0017	0.0017	0.0639	0.0515
2. *O. niger* (Karwar)	1	_	0	0.0657	0.0532
3. *O. niger* (Mangalore)	1	0	_	0.0657	0.0532
4. *A. stellaris* (Malpe)	36	37	37	–	0.0639
5. *S. fraenatum* (Malpe)	29	30	30	36	–

Table 8: Percentage similarities of three species of family Balistidae based on 16S rRNA sequences with NCBI haplotype

Species (with GenBank Acc. No.)	*O. niger* (Mangalore)	*O. niger* (Malpe)	*O. niger* (Karwar)	Species (with GenBank Acc. No.)	*A. stellaris* (Malpe)	*Species* (with GenBank Acc. No.)	*S. fraenatum* (Malpe)
O. niger (AP009208)	100%	99.83%	99.83%	*A. stellaris* (AP009202)	99.48%	*S. fraenatum* (LC659948)	98.40%
O. niger (EU108815)	99.81%	99.81%	99.81%	*A. stellaris* (MG515171)	98.81%	*S. fraenatum* (AP004456)	98.25%
O. niger (KT601014)	99.25%	99.25%	99.25%	*A. stellaris* (KT600988)	99.25%	*S. fraenatum* (KT601033)	97.50%

2015), and sciaenids (Lakra *et al.,* 2009) using COI and 16S rRNA sequence data. The transition versus transversion ratio calculated for the balistid species aligns with the ratios observed in other fishes, such as snakeheads and murrels (Lakra *et al.,* 2010) and damselfishes (Strandberg and Salter, 2004). This transition versus transversion ratio helps aid in estimating divergence and phylogenetic relationships and will also serve as an indirect measure of saturation (loss of phylogenetic information).

Investigation of the COI gene among the balistid species revealed a high degree of K2P nucleotide divergence in the interspecies range (13.99-17.38%), suggesting the gene's ability to adequately represent inter-relationships among triggerfish species. Consistent with this, genetic divergence values based on COI (0.7-16.6%) within the *Abudefduf vaigiensis* from the South-east coast of India has been reported (Rajesh *et al.,* 2022), while divergence in *Channa* spp. (COI; 4.9-24.60%) was reported from the North-eastern India (Lakra *et al.,* 2010). DNA barcoding based on COI partial gene sequence has been widely utilized for species identification in finfish and shellfish (Spies *et al.,* 2006; Chowdhury *et al.,* 2019). The findings of the present study demonstrate that COI gene sequence barcoding is an effective marker for identifying closely related fish species. Phylogenetic analysis using NJ tree approach with the COI and 16S rRNA data revealed two significant clades with strong bootstrap support.

Also, the findings of this study, clearly differentiated all three balistid species collected from the South-eastern Arabian Sea, aligning with the taxonomic categories of the Balistidae family. The findings of the present study thus provide a valuable resource for future studies on the genetics and adaptive evolution of trigger fishes.

Acknowledgments

The authors express sincere gratitude to Dr. Malathi Shekar, Dr. Girisha S. K. (Assistant Professors), and Dr. Puneeth (Research Scholar) from the Department of Aquatic Animal Health Management, College of Fisheries, Mangaluru, for their invaluable support and provision of laboratory facilities.

Authors' contribution: K.S. Pratiksha: Sample collection, investigation, data analysis; **R. Mridula:** Conceptualization, Methodology, Data analysis, Writing–review and editing; **K.M. Rajesh:** Conceptualization, Methodology, Writing–review and editing; **N.K. Suyani:** Data analysis and writing- original draft.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial,or not-for-profit sectors.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Ethical approval is not required as the research was carried out using deceased specimens.

Conflict of interest: The authors have no competing interests to declare that are relevant to the content of this article.

Data availability: All data generated or analyzed during this study are included in this article.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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