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Research Article

DNA barcoding and genetic diversity of threadfin breams (Nemipteridae): Unravel the cryptic species from the northern Arabian Sea

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Abstract

The Nemipteridae fish family is a colored marine fish species having elongated bodies and filamentous dorsal fin extensions. They are widely distributed in the Indo-Pacific Oceans and prefer to live in shallow coastal waters, often over muddy and sandy bottoms. This fish family has economic value in Southeast Asia and is widely consumed as food. In this study, we conducted the DNA barcoding of the Nemipteridae fish family. Five different species of Nemipteridae were taken from the marine waters of Pakistan. A total of 33 specimens belonging to five species and three genera were collected from 2019 to 2023. This study delineated two cryptic species (*Nemipterus japonicus* and *Nemipterus randallii*) on the molecular basis. The phylogenetic tree was made and showed genus-wise clustering of the species. A clear sister clade was able to be seen within the *N. japonicus* and *N. randalli*, Moreover, the barcode gap showed a significant genetic gap between these species, validating them as separate species. The present study of DNA barcodes has demonstrated high efficiency in the identification of species. This is the first study that explores the genetic basis of the family Nemipteridae from the coastal area of Pakistan.

Introduction

More than 55,000 species of half of the extant vertebrates are fish. Throughout the history of complex life, fish have been ecologically dominant in the aquatic environment. Each of the species present today has a distinct range of adaptations and an evolutionary history spanning millions of years, encompassing many more ancestral species (Helfman *et al.,* 2009). Moreover, fish are the most varied group of vertebrates, with over 33,000 species documented, and they live in nearly every major aquatic habitat type, performing a wide range of ecological activities in ecosystems (Martinez *et al.,* 2018). Geographically, the tropics have the most diversity. The West Indo-Pacific area, which encompasses the western Pacific, India, along with the Red Oceans, as well as the Red Sea, has the most marine diversity (Helfman *et al.,* 2009).

Threadfin breams (Nemipteridae) are found across the Indo-Pacific. These fish are adapted to live in shallow coastal waters, most commonly over sandy or muddy bottoms. They are carnivorous, feeding on invertebrates like crustaceans and smaller fish, and are known for their schooling behaviour, which aids in defence and feeding (Russell, 1990; Wu, 2008; Amine, 2012). They have good economic and food value and play a major role in daily fish landings. The prime factors, like adaptability to fluctuating surroundings and diverse reproductive modes (protogynous hermaphroditism), have made them resilient. The Japanese threadfin bream, *Nemipterus japonicus* (family Nemipteridae), is found all over the Indian and Pacific Oceans. Two species, *Nemipterus japonicus* and *Nemipterus randalli*, account for 90% of the threadfin bream fishing in Pakistan. Although these two species are very closely related and look similar to each other due to their morphology, it is sometimes difficult to distinguish between them. The main difference between these two species is the colour of the tail end. The *Nemipterus randallii* species has a red colour tail end, and the *Nemipterus japonicus* has a yellow color tail end (Kalhoro *et al.,* 2014). Sometimes, these extra tail ends are broken, and most of the Nemipteridae taxonomy research is based on preserved specimens and does not accurately characterize colour patterns. Moreover, it is difficult to identify the species at larval stages. DNA barcoding can be thought of as the heart of a taxonomic system. The mitochondrial gene cytochrome c oxidase I (COI) has been proven to be the foundation of a universal bioidentification system for animals. Researchers have been experimenting with the concept of identifying species quickly and readily using merely a short DNA sequence, known as a DNA barcode, that indicates a uniform place in the genome (Vohra and Khera, 2013; Khan *et al.,* 2024; Sial *et al.,* 2024; Raza *et al.,* 2025). The potential of DNA barcoding to identify species from a wide range of taxa and to find cryptic species has been amply demonstrated in recent years. The study of taxonomically challenging taxa has benefited from DNA barcoding. The use of DNA barcoding in fast biodiversity assessments has proven to be successful. A crucial component of DNA barcoding is the capability to link programs to museum and herbarium specimens, allowing this new science to gain from centuries of validated taxonomic data investment (Fišer and Buzan, 2014).

All sixty-two currently identified Nemipteridae species have been discussed in various places, with sixty of them being depicted in colour and with line drawings. Out of them, 14 species have been identified: *Nemipterus bipunctatus, Nemipterus japonicus, Nemipterus randalli, Nemipterus peronii, Parascolopsis aspinosa, Nemipterus zysron, Parascolopsis boeseman, Parascolopsis townsendi, Parascolopsis eriomma, Scolops bimaculata, Scolopsis taeniata, Scolopsis ghanam, Scolopsis vosmeri,* and *Scolopsis torquatus* found in Pakistan (Psomadakis, 2015). Commonly, they are known as “Lal-pari”, “Chakori”, “Korora” (Sin); “Kolonto” (Bal) (Psomadakis, 2015).

The current study aimed to determine the genetic relationship among different species, including (*Nemipterus japonicus, Nemipterus randalli*, *Scolopsis vosmeri, Scolopsis bimaculata*, *Parascolopsis bimaculata*) of the Nemipteridae fish family. Here, we validated the existence of these species on a morphological and molecular basis. Together with the delineation of two cryptic species from the coastal area of Pakistan. Moreover, we unravelled the genetic diversity, barcode gap, and phylogenetic relationship within the family Nemipteridae based on the COI gene.

Materials and methods

Fish specimens and DNA extraction

Five different fish species of the family Nemipteridae were collected from 2019 to 2023 in marine water sites in Pakistan. In total, 33 specimens were collected from the daily fish landing facilities in Pakistan. The exact collection sites are shown in Figure 1. The samples were then brought to the Centre of Excellence in Marine Biology (CEMB) for taxonomic identification. The identification of species was carried out by the FAO Fish Identification Sheets (Psomadakis, 2015). After taking the photographs, the samples were transported to the Department of Biotechnology, University of Sargodha, for further molecular analysis. Extraction of tissue was done from the muscle or the caudal fin in sterile conditions and kept at -20°C in 95% ethanol until used. DNA was extracted by the manual method and by following the protocol of the organic method of DNA extraction. The DNA was eluted in 30 µL of TE buffer and was stored in the freezer for further processing.

PCR amplification

A total amount of 25µL of the master mix was used for PCR to amplify the COI gene. In total, 1.5µL of genomic DNA was used with 2.5 µL of *Taq* buffer (KCl+ NaOH), 0.25µL of *Taq* DNA polymerase, 2µL of MgCl2, 2.5µL of DNTPs, 0.25 µL of each forward and reverse primer, respectively, and 15.75µL of ddH2O was added. The universal primers (Fish F1, R1) were used for the amplification of the COI gene: Fish F1 5′ TCAACCAACCACAAAGACATTGGCAC 3′, Fish R1 5′ TAGACTTCTGGGTGGCCAAAGAATCA 3′ (Ward *et al*., 2005). The PCR conditions consisted of an initial step of 4 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. The PCR products were visualized on 1.5% agarose gels, and the most intense products were selected for sequencing. Products were sequenced commercially using the Sanger sequencing method. After that, the samples were sent to CCDB (Canadian Centre for DNA barcoding) for Sanger sequencing.

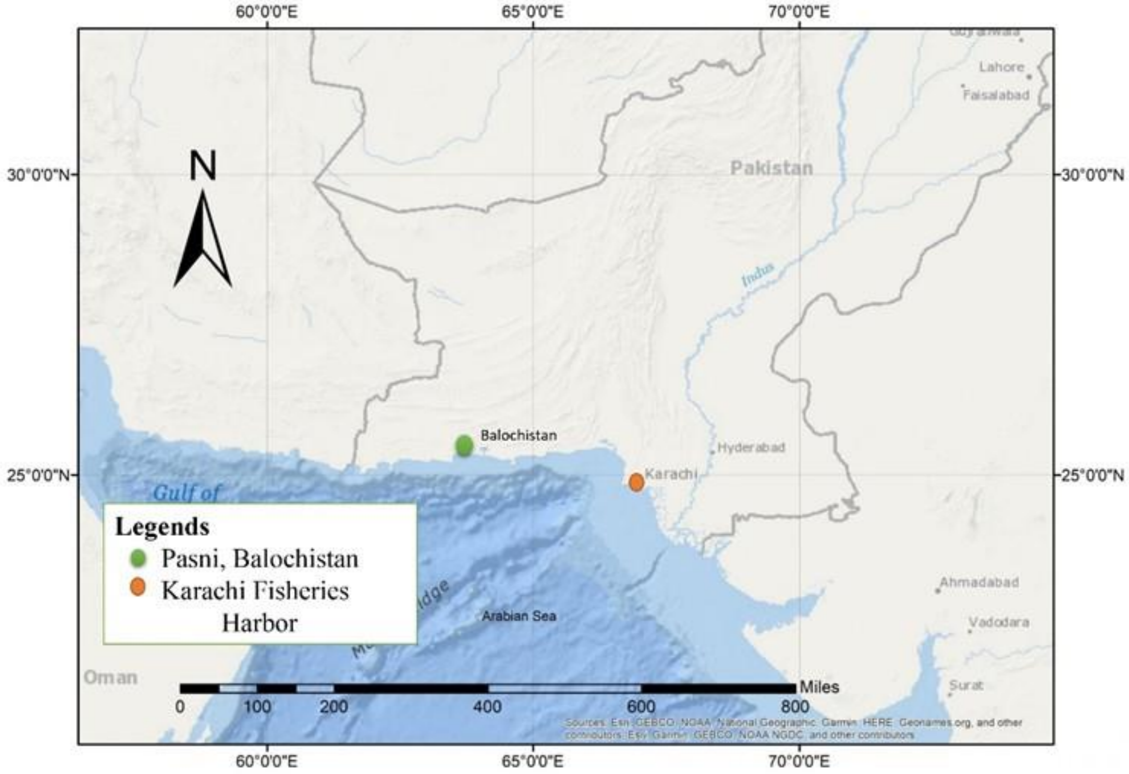

Figure 1: Showing the exact sampling sites. The green spot represents the Balochistan site, and the Orange colour represents the Karachi Fisheries harbour site.

Phylogenetic analysis

Sequences were aligned using MEGA, and the sequence difference between species was calculated by averaging pairwise comparisons of sequence difference across all individuals. The COI sequences of the five individuals of each species were aligned to yield a final sequence of 655 bp. The pairwise evolutionary distance among haplotypes was determined by the Kimura 2-Parameter method (Kimura, 1980) using the software program MEGA X (Molecular Evolutionary Genetics Analysis) (Kumar *et al.,* 2004). The best DNA/protein model (HKY) was found by using MEGA, and the maximum-likelihood tree was made considering the bootstrap analysis with 1000 replicates.

Genetic distances, barcode gap, cumulative curve, and nucleotide diversity

The pairwise-genetic distances were calculated by MEGA, and a heatmap was formed by using an R script. All of the sequences were uploaded to the BOLD systems database. This sequence information was further used to infer the cumulative curve, Barcode gap analysis, and nucleotide diversity analysis of the species (Ratnasingham and Hebert, 2007).

Results

Genetic distances

In total, 33 specimens collected from the daily fish landing facilities in Pakistan were considered for genetic distance. The detailed information is given in Table 1. In total, 33 specimens collected from the daily fish landing facilities in Pakistan were considered for genetic distance. The detailed information is given in Table 1. The obtained sequences and pictures of the fish are provided in supplementary file 1.

Table 1: GenBank accession number of Nemipteridae family species

Species	BOLDsystems Voucher no.	Accession no
Nemipterus japonicus	MAK-03	PQ358471
Nemipterus japonicus	MAK-03B	PQ358472
Nemipterus japonicus	MAK-03C	PQ358473
Nemipterus japonicus	MAK-81	PQ358474
Nemipterus japonicus	MAK-131A	PQ358475
Nemipterus japonicus	MAK-131B	PQ358476
Nemipterus japonicus	MAK-81B	PQ358477
Nemipterus japonicus	MAK-131G	PQ358478
Nemipterus japonicus	MAK-131H	PQ358479
Nemipterus japonicus	MAK-131I	PQ358480
Nemipterus randalli	MAK-130A	PQ359428
Nemipterus randalli	MAK-130B	PQ359429
Nemipterus randalli	MAK-130G	PQ359430
Nemipterus randalli	MAK-130H	PQ359431
Nemipterus randalli	MAK-130I	PQ359432
Parascolopsis aspinosa	MAK-167A	PQ358485
Parascolopsis aspinosa	MAK-167B	PQ358486
Parascolopsis aspinosa	MAK-167C	PQ358487
Parascolopsis aspinosa	MAK-167D	PQ358488
Scolopsis bimaculata	MAK-122A	PQ358919
Scolopsis bimaculata	MAK-122B	PQ358920
Scolopsis bimaculata	MAK-161A	PQ358921
Scolopsis bimaculata	MAK-161B	PQ358922
Scolopsis bimaculata	MAK-161D	PQ358923
Scolopsis bimaculata	MAK-122J	PQ358924
Scolopsis bimaculata	MAK-122K	PQ358925
Scolopsis bimaculata	MAK-122L	PQ358926
Scolopsis vosmeri	MAK-89B	PQ358498
Scolopsis vosmeri	MAK-89C	PQ358499
Scolopsis vosmeri	MAK-96B	PQ358500
Scolopsis vosmeri	MAK-96C	PQ358501
Scolopsis vosmeri	MAK-89	PQ358502
Scolopsis vosmeri	MAK-96	PQ358503

The results from genetic distance analysis are shown in Figure 2. The genetic distance analysis revealed pairwise distances ranging from 0 (indicating identical sequences, as seen between PQ358472-PQ358473, PQ358475-PQ358476, PQ358478-PQ358479, and PQ359428-PQ359429, among others) to a maximum divergence of 0.279 between PQ358926 and OQ387335, suggesting substantial genetic differentiation between these samples. Several sequences showed extremely close relationships, particularly PQ358498-PQ358501 and PQ358503-PQ358501, with distances as low as 0.0015. The sample OQ387335 emerged as the most divergent, consistently exhibiting the highest distances across multiple comparisons. This variation highlights both shared haplotypes (evidenced by zero distances) and significant genetic divergence within the dataset, with the maximum distance reflecting deep differentiation between the most distantly related sequences.

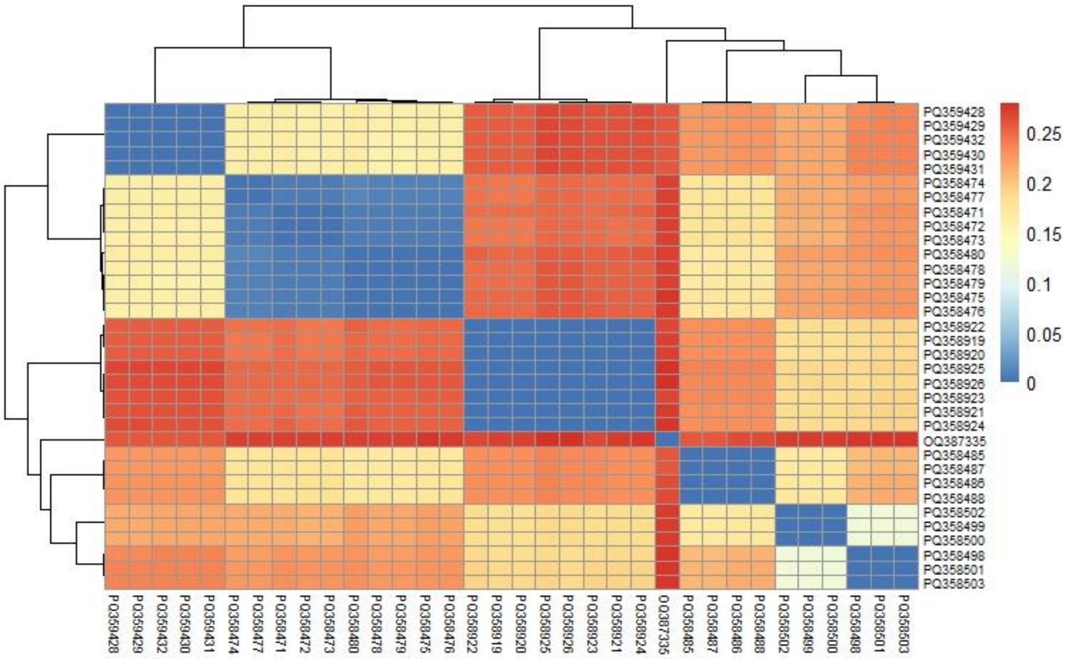

Figure 2: Pairwise distance using Kimura-2-Parameter among Serranidae species. Each number on the sides represents the GenBank/Accession number of the species as illustrated in Table 1. The scale bar on the right side represents values given in the heatmap.

Accumulation curve

The cumulative curve (Fig. 3) shows the efficiency of identification based on the sequenced DNA barcode and the number of species identified. As we move from left to right on the graph, there is an increase in the line that shows the initial rapid species identification, together with the addition of the sequences. At a specific point of 33 on the X-axis, the curve becomes equilibrium, depicting no new identification of the species, even with the addition of the new sequences. Most of the species are already identified at that point, which is known as the saturation point in the cumulative curve.

Barcode gap analysis

The results from the Barcode Gap Analysis are depicted in Figure 4. The Barcode Gap Analysis (Table 2) revealed a clear species delineation within the family: Nemipteridae. Considerably, *Nemipterus japonicus* and *Nemipterus randalli* illustrated a distance of 15.5%. On the other hand, *Parascolopsis aspinosa* displayed zero of the intra-species differences, however, they had a nearest neighbour distance of about 15.72% to *Nemipterus japonicus*. Moreover, *Scolopsis vosmeri* illustrated the highest intraspecific variation (6.93%) and showed the separation from the nearest neighbour, *Parascolopsis aspinosa*, with a 16.39% distance, depicting a considerable genetic distance.

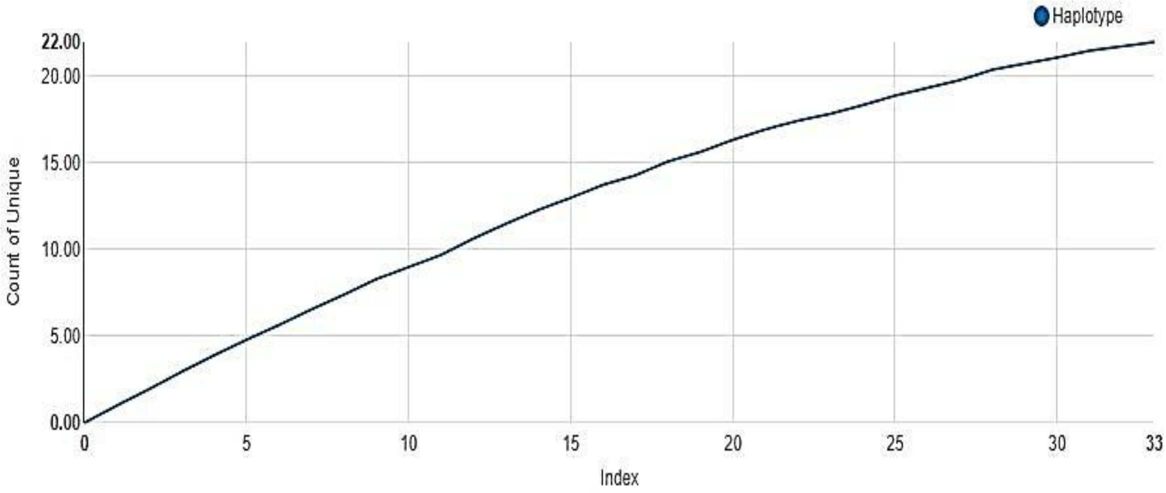

Figure 3: The accumulation curve for DNA barcoding.

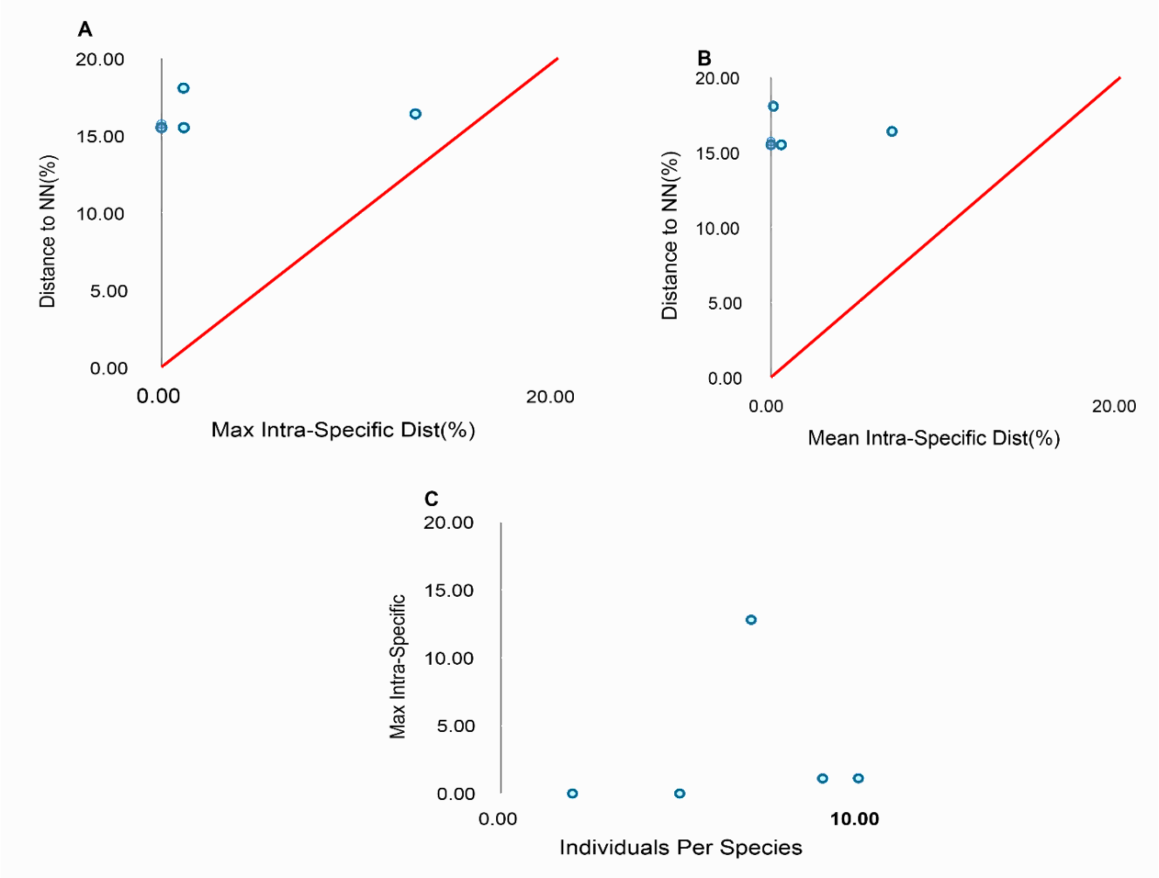

Figure 4: Barcode Gap analysis. (A) Max Intraspecific vs. Nearest Neighbours: the maximum intraspecific distance compared to the nearest neighbour of the species. (B) Mean Intraspecific vs. Nearest Neighbours: the average intraspecific distance in comparison to the distance between nearest neighbours. (C) Individuals per Species: the individual genetic distances between each species, highlighting the variation within and between species.

Table 2: Barcode gap between Nemipteridae species.

Order	Family	Species	Mean Intra-Sp	Max Intra-Sp	Nearest Species	Nearest Neighbour	Distance to NN
Spariformes	Nemipteridae	*Nemipterus japonicus*	0.59	1.13	*Nemipterus randalli*	SUFIS951-23	15.50
Spariformes	Nemipteridae	*Nemipterus randalli*	0.00	0.00	*Nemipterus japonicus*	SUFIS954-23	15.50
Spariformes	Nemipteridae	*Parascolopsis aspinosa*	0.00	0.00	*Nemipterus japonicus*	SUFIS955-23	15.72
Spariformes	Nemipteridae	*Scolopsis bimaculata*	0.14	1.11	*Scolopsis vosmeri*	SUFIS277-21	18.06
Spariformes	Nemipteridae	*Scolopsis vosmeri*	6.93	12.80	*Parascolopsis aspinosa*	SUFIS590-23	16.39

Nucleotide diversity

It shows a higher average of AC than the GC content (Table 3). The GC content ranged from 43% to 48%. The codon position 1 exhibited the highest GC content (up to 57.69%), while other codon positions had various percentages with values ranging from 32.35% to 44.71%.

Table 3: Nucleotide diversity of experimental species.

A %	C %	G %	T %	GC %	GC %Codon Pos 1	GC %Codon Pos 2	GC %Codon Pos 3
23.19749	26.80251	18.33856	31.66144	45.14107	57.54717	43.66197	34.2723
23.00613	26.84049	17.94479	32.20859	44.78528	57.14286	42.85714	34.40367
23.00613	26.84049	17.94479	32.20859	44.78528	57.14286	42.85714	34.40367
23.06502	26.47059	18.42105	32.04334	44.89164	57.2093	43.25581	34.25926
22.53086	27.62346	19.44444	30.40123	47.0679	56.01852	43.05556	42.12963
24.22840	25.0000	18.98148	31.79012	43.98148	56.01852	43.05556	32.87037
22.53086	27.62346	19.44444	30.40123	47.0679	56.01852	43.05556	42.12963
22.37654	27.62346	19.59877	30.40123	47.22222	56.01852	43.05556	42.59259
24.22840	25.00000	18.98148	31.79012	43.98148	56.01852	43.05556	32.87037
24.22840	25.00000	18.98148	31.79012	43.98148	56.01852	43.05556	32.87037
23.83901	26.00619	19.50464	30.65015	45.51084	55.09259	43.25581	38.13953
23.83901	26.00619	19.50464	30.65015	45.51084	55.09259	43.25581	38.13953
23.06502	26.16099	18.57585	32.19814	44.73684	55.09259	43.25581	35.81395
23.06502	26.16099	18.57585	32.19814	44.73684	55.09259	43.25581	35.81395
23.11321	26.41509	18.23899	32.2327	44.65409	56.60377	43.86792	33.49057
23.24493	26.52106	18.09672	32.13729	44.61778	56.54206	43.45794	33.80282
22.88000	28.48000	20.32000	28.32000	48.80000	57.69231	44.01914	44.71154
23.06502	26.47059	18.42105	32.04334	44.89164	57.20930	43.25581	34.25926
23.73418	25.94937	19.93671	30.37975	45.88608	54.50237	44.28571	38.86256
23.61331	25.99049	19.96830	30.42789	45.95880	54.76190	44.28571	38.86256
24.07975	28.52761	18.40491	28.98773	46.93252	58.52535	42.85714	39.44954
24.08875	28.05071	19.01743	28.84311	47.06815	58.57143	44.28571	38.38863

Table 3 (continued):

A %	C %	G %	T %	GC %	GC %Codon Pos 1	GC %Codon Pos 2	GC %Codon Pos 3
24.09241	25.74257	20.13201	30.03300	45.87459	54.95050	44.05941	38.61386
24.04643	25.87065	19.9005	30.18242	45.77114	54.72637	44.27861	38.30846
24.83660	25.65359	17.81046	31.69935	43.46405	55.88235	42.15686	32.35294
23.70031	25.99388	19.26606	31.03976	45.25994	55.04587	42.66055	38.07339
24.20886	25.94937	19.46203	30.37975	45.41139	55.2381	43.12796	37.91469
23.58491	25.78616	19.81132	30.81761	45.59748	53.77358	44.33962	38.67925
22.78287	26.14679	18.34862	32.72171	44.49541	55.04587	42.66055	35.77982
22.78287	26.14679	18.34862	32.72171	44.49541	55.04587	42.66055	35.77982
22.78287	26.14679	18.34862	32.72171	44.49541	55.04587	42.66055	35.77982
23.08869	26.75841	17.73700	32.41590	44.49541	56.88073	42.66055	33.94495
23.08869	26.75841	17.73700	32.41590	44.49541	56.88073	42.66055	33.94495
23.65079	27.14286	17.61905	31.58730	44.76190	57.14286	42.85714	34.28571

Phylogenetic analysis

The Phylogenetic tree exhibited significant relationships within the studied species (Fig. 5). As expected, the species (*Lethrinus nebulosus*) formed a distinct clade, showing its function as an outgroup. The *Nemipterus japonicus* and *Nemipterus randallii*, were clustered under the same clade, *Scolopsis vosmeri* and *Scolopsis bimaculata* were clustered under the same clade, while *Parascolopsis bimaculata* formed a distinct clade.

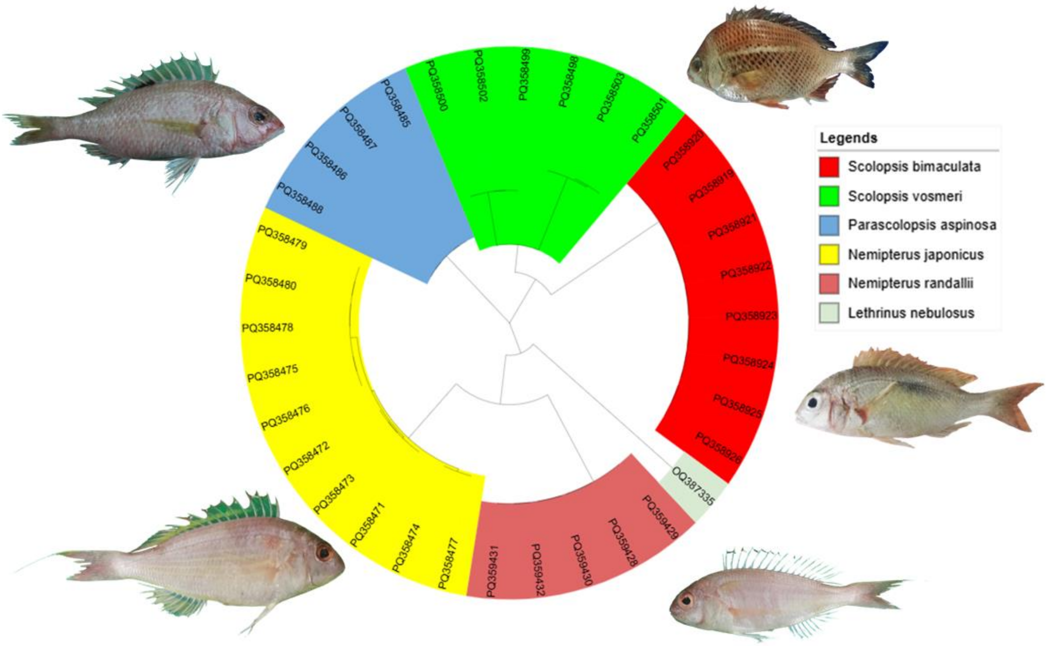

Figure 5: Phylogenetic relationship of Experimental species. Each species is denoted with a different colour. The text at the end of the nodes represents the GenBank accession number of the species.

Discussion

The largest class of vertebrates is fish, which display an astonishing range of physical characteristics and biological adaptations. Species are often defined by the presence of unchanging, distinctive morphological traits that set them apart from other species. However, fish have several intraspecific invariants and interspecific overlaps, making it difficult for taxonomists to identify fish in rich biotas. For that purpose, scientists considered the DNA barcode, which is truly a helpful tool (Hebert *et al.,* 2003; Ravitchandirane *et al.,* 2012; Shen *et al.,* 2016; Khan *et al.,* 2024; Sial *et al.,* 2024). In the present study, the marine water fish family of Nemipteridae was observed, and DNA barcodes were generated. All the sequences were amplified using a functional mitochondrial COI sequence. Genetic distances were also observed in the study. More specifically, the two species *Nemipterus japonicus* and *Nemipterus randallii* exhibit common morphological characteristics and can lead to misidentification when their tail end is broken or they have lost their body colour. Here we delineated these two species on a molecular basis.

The nucleotide diversity analysis illustrated the distinct biases in the studied taxa. The higher AC content compared to the GC content hints at the distinct selection pressures. It showed a consistent selection of base pairs and the potential selective pressures that may have influenced codons' usage, displaying the potential selective evolutionary dynamics within the studied taxa. The cumulative curve was formed by using the online platform BOLD systems. It showed the efficacy of DNA barcoding to identify the species within the studied family, Nemipteridae. At the start, the graph showed an inclined line depicting the identification of new species with the addition of sequences. However, upon reaching a maximum value of X=33. There was no more incline, and the curve became a plateau. This showed that upon reaching that level, the addition of new sequences was not able to identify the new species. This saturation point illustrated that the sequencing captured the majority of species diversity within the family (Ugland *et al.,* 2003).

Barcode analysis has the power to delineate species boundaries. By using DNA barcodes, the present study shows the importance of DNA barcoding research as a protocol for fish identification (Čandek and Kuntner, 2015). Our study considered the five threadfin bream species, focusing on describing two cryptic species (*Nemipterus Japonicus* and *Nemipterus randallii*). The barcode gap analysis showed a significant genetic gap (15.05%) between these species, delineating cryptic species on a molecular basis and supporting their classification as separate species. *Parascolopsis aspinosa* illustrated a lack of intraspecific variations and showed a high genetic variability of 15% with other neighbor species (*Nemipterus japonicus*). *Scolopsis vosmerii* showed a high intraspecific and significant distance of (16%) from *Parascolopsis aspinosa,* suggesting complex evolutionary dynamics involving cryptic diversity or speciation. The present results demonstrate DNA barcoding effectiveness in delineating the species to resolve the taxonomic uncertainties and highlight the importance of genetic data in species identification.

The phylogenetic tree based on the COI gene was made, which delineated distinct relationships among the five species. The *Lethrinus nebulosus* was taken as an outgroup that forms a completely separate clade. Notably, *Nemipterus japonicus* and *Nemipterus randallii* were clustered together, depicting their close morphological characters and shared evolutionary relationships within the genus *Nemipterus*. However, we can still differentiate between them based on the phylogenetic tree as they form sister clades. Similarly, *Scolopsis vosmerii* and *Scolopsis bimaculata* formed one clade, illustrating the close evolutionary relationships as they belong to the same genus. *Parascolopsis aspinosa* appeared as a distinct clade, reinforcing its unique phylogenetic position within the family Nemipteridae. The present findings support the monophyly of genera *Nemipterus* and *Scolopsis* (Ning *et al.,* 2015; Hung *et al.,* 2017) and provide valuable insights into the phylogenetic dynamics of these crucial marine fish species.

The use of the COI marker proves that it is a reliable marker for the differentiation among the species. The obtained barcode has been developed by utilizing this marker. Further, different bioinformatics tools were used to clarify that genetic divergence among species is lower as compared to values obtained between different species. The genetic distance keeps on increasing as we move further across the species, family, or order. The current study can be utilized with its wide range of applications in successful management strategies in the fisheries resources of Pakistan.

Conclusions

The present study revealed that cryptic species can be identified with the use of COI barcoding. The obtained results confirmed that DNA barcoding coupled with morphological identification can be used for the precise and accurate identification of fish species, as we developed the detailed DNA barcode of the Nemipteridae fish family. Here, we delineated the cryptic species *Nemipterus japonicus* and *Nemipterus randallii* for effective fisheries management. Moreover, the inter- and intra-specific divergence between species was also inferred. This will play a great role for the fisheries resources and ecologists, for the fish identification through molecular approaches. The data obtained from this study will provide beneficial knowledge for further research in Pakistan.

Acknowledgements

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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