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Molecular marker (RAPD and RFLP) analysis of Mullet species

I Vidhya[a], N Felix[a], A Kathirvelpandian[b] & E Suresh*[,a]

[a]Institute of Fisheries Postgraduate Studies, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Chennai, Tamil Nadu – 603103, India
[b]ICAR – National Bureau of Fish Genetic Resources, Kochi Centre, Kochi, Kerala – 682 018, India
*[E-mail: sura12@gmail.com]

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Mullet species belongs to the family, Mugilidae. Traditional taxonomic characters failed to differentiate mullet species due to the possibility of cryptic species and morphological similarity among mullet species. This study aimed to differentiate the selected mullet species using molecular markers. A total of 95 mullet samples (*M. cephalus*, *P. macrolepis*, and *P. tade*) were collected from Tamil Nadu and Kerala. For this study, 5 RAPD primers (OPA1, OPA10, OPA11, OPA15, and OPA16) were chosen from a set of 30 universal primers. The Nei's unbiased genetic distance was calculated with the help of the software "POPGEN version 1.32". The genetic distance between *P. tade* and *P. macrolepis* was lower, while the distance between *M. cephalus* and *P. tade* was greater, measuring 43.37 %. For the PCR-RFLP marker, the restriction enzyme was chosen based on *in-silico* analysis. The Alu I enzyme displayed species-specific patterns in *M. cephalus* at 190 bp, 200 bp, and 210 bp for the 16S rRNA gene.

[**Keywords**: 16s rRNA, Markers, Mullet, RAPD, RFLP, Taxonomy]

Introduction

Mullets, found extensively in marine nearshore waters and aquaculture across warm and cool regions, exhibit remarkable adaptability to diverse environments[1-3]. Thriving in freshwater, marine, estuarine, and lagoonal ecosystems, they sustain themselves predominantly on diatoms, microalgae, and desmids[2]. Notably, mullets demonstrate an impressive tolerance to a wide range of salinity (5 to 25 ppt) and temperature (10 to 27 °C), highlighting their resilience in fluctuating environmental conditions[3,4]. Belonging to the family Mugilidae, mullets boast a rich diversity with 26 genera and 78 species globally, of which India hosts 24 species distributed across 10 genera[5,6]. Specifically, Tamil Nadu is home to 10 reported species within the Mugilidae family[7]. However, identifying mullet species within the Mugilidae family presents challenges due to morphological similarities, intraspecific variation, and the presence of cryptic species[8-10]. This difficulty is especially pronounced when dealing with the morphological characteristics of mullet larvae and juveniles[11,12].

Despite these taxonomic challenges, seminal studies by Jayaram[13], Suresh Babu *et al.*[14], and Chandra *et al.*[15] have provided foundational insights into mullet diversity and distribution in the Indian waters. Furthermore, recent molecular investigations by Saad *et al.*[16] and Patel & Patel[17] have deepened our understanding of mullet phylogenetics and genetic diversity in India[14]. Beyond their ecological significance, mullets play a vital economic role in India, with aquaculture production steadily rising and wild capture fisheries supporting coastal communities' livelihoods[15,16]. In 2023 alone, India reported over 35,000 metric tons of mullet aquaculture production, reflecting increasing domestic and international demand. However, to sustainably manage mullet resources, comprehensive taxonomic assessments integrating modern molecular techniques are imperative. Current study aims to bridge this gap by employing an integrative taxonomic approach, contributing to fundamental knowledge and effective conservation management strategies.

DNA markers, termed molecular markers, and DNA sequences serve as invaluable tools in tracking specific locations on chromosomes across generations[17]. Their association with genetic variations facilitates the study of trait inheritance, with Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) emerging as common markers for species identification[18,19]. RAPD, a nuclear DNA marker, offers economic feasibility and flexibility, while RFLP, a mt-DNA marker, provides insights into

Fig. 1 — Sampling location of fishes

polymorphisms based on distinctive fragment sizes[20]. The utilization of these markers underscores their significance in various applications, including species identification, processing industry, and conservation studies[21].

Materials and Methods

Fish specimens

A total of 95 samples were randomly collected from Kovalam, Tamil Nadu (8°22'0.01'' N, 76°59'48.01'' E), and the Vypin fish landing center, Kerala (9°58'59.99" N 76°14'30.00" E) (Fig. 1). The collected samples were stored in 90 % alcohol and transported to the research facility for further analysis and identification. Using the FAO identification sheets[22], the samples were classified into three species: *viz. Mugil cephalus* Linnaeus, 1758, *Planiliza macrolepis* (Smith, 1846), and *Planiliza tade* (Fabricius, 1775) (Fig. 2).

Total genomic DNA isolation and quantification

Genomic DNA was isolated using the cost-effective "Salting-Out Method"[23], which avoids harmful chemicals. The DNA quality and quantity was evaluated using 1 % agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). DNA samples (1 – 2 µl) were measured for concentration and purity, with purity indicated by the 260/280 ratio. The DNA was then diluted to 20 – 50 ng/µl, suspended in TE buffer, and kept at -80 °C, ready for subsequent use.

RAPD-PCR amplification

Initially, 30 universal primers were screened for RAPD-PCR (Table 1), and five (OPA1, OPA10, OPA11, OPA15, and OPA16) were chosen based on their amplification efficiency and specificity. The genomic DNA was amplified in sterile 0.2 ml PCR tubes using a Thermocycler (Eppendorf Mastercycler nexus Gx2, Germany). The reaction mixture for the OPA1 primer comprised 12.5 µl of master mix (Ampliqon Biotechnology, Denmark), 1.3 µl of primer (Shrimpex Biotech Service Pvt Ltd), 1 µl of $MgCl_2$ (PROMEGA Biotechnology, USA), and 50 ng of genomic DNA. The PCR conditions included initial denaturation at 94 °C for 3 min, followed by 35 cycles comprising 30 sec at 94 °C, 45 sec at 40 °C, and 1 min at 72 °C, followed by a final elongation

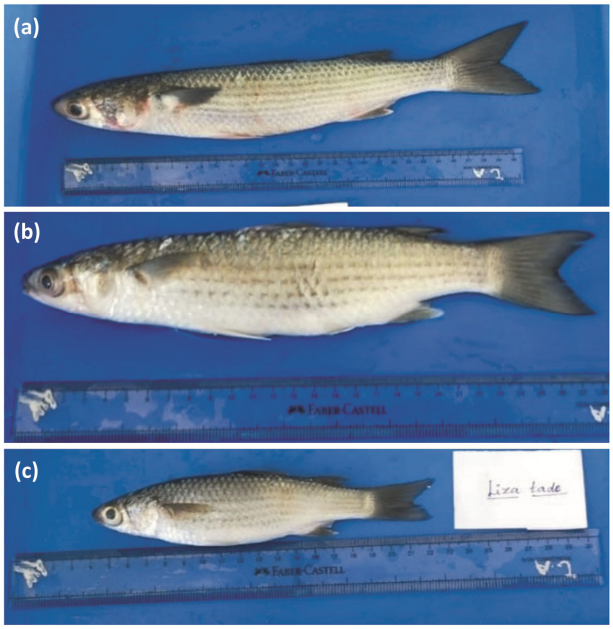

Fig. 2 — Fish samples used in this study: a) *Mugil cephalus*, b) *Planiliza macrolepis*, and c) *Planiliza tade*

step at 72 °C for 10 min. For the remaining four primers, the reaction mixture included master mix (12.5 µl), primer (1 µl), $MgCl_2$ (1 µl), and genomic DNA (50 ng). The amplification conditions were initially denatured at 94 °C for 5 min, followed by 35 cycles comprising of 1 min at 94 °C, 1 min at 40 °C, and 2 min at 72 °C, followed by a final elongation step at 72 °C for 10 min. The PCR products were observed on agarose gels with 2 % concentration, stained with ethidium bromide, and electrophoresed in 1X TAE buffer. The sizes of DNA fragments were determined using a 100bp DNA ladder (Gene DireX, Taiwan). RAPD data was analyzed using the genetic software POPGENE ver 1.31[ref. 24].

Amplification of mitochondrial genes and sequencing

Amplification of DNA was carried out using primers specific to the 16S rRNA and CO1 mitochondrial genes. The sequences of the primers used for the 16S rRNA gene were: 5'CGCCTGTTATCAAAAACAT3' (forward) and 5'CCGGTCTGAACTCAGATCACGT3' (reverse), while for the CO1 gene, the sequences used were 5'TGTAAAACGACGGCCAGTCGAC-TAATCATAAAGATATCGGCAC3' (forward) and 5'CAGGAAACAGCTATGACACTTCAGGGTGACC GAAGAATCAGAA3' (reverse), as described by Palumbi[25] and Ward *et al.*[26]. The 25 µl PCR mixture contained template DNA (20 – 50 ng), master mix (12.5 µl), and each primer (1 µl). The amplification conditions for CO1 gene included denaturation for 4 min at 95 °C, followed by 30 sec at 94 °C for, 30 sec at 54 °C, and 45 sec at 72 °C for 35 cycles, with a final elongation for 10 min at 72 °C. For 16s rRNA gene, conditions were denaturation for 5 min at 95 °C, followed by 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C for 30 cycles, with a final elongation for 10 min at 72 °C.

Table 1 — List of RAPD primers

Sr. No.	Primers	Sequence (5'-3')
1	OPA1	CAGGCCCTTC
2	OPA2	TGCCGAGCTG
3	OPA3	AGTCAGCCAC
4	OPA4	AATCGGGCTG
5	OPA5	AGGGGTCTTG
6	OPA6	GGTCCCTGAC
7	OPA7	GAAACGGGTG
8	OPA8	GTGACGTAGG
9	OPA9	GGGTAACGCC
10	OPA10	GTGATCGCAG
11	OPA11	CAATCGCCGT
12	OPA12	TCGGCGATAG
13	OPA13	CAGCACCCAC
14	OPA14	TCTGTGCTGGC
15	OPA15	TTCCGAACCC
16	OPA16	AGCCAGCGAA
17	OPA17	GACCGCTTGT
18	OPA18	AGGTGACCGT
19	OPA19	CAAACGTCGG
20	OPA20	GTTGCGATCC
21	OPB1	GTTTCGCTCC
22	OPB2	TGATCCCTGG.
23	OPB3	CATCCCCCTG
24	OPB4	GGACTGGAGT
25	OPB5	TGCGCCCTTC
26	OPB6	TGCTCTGCCC
27	OPB7	GGTGACGCAG
28	OPB8	GTCCACACGG
29	OPB9	TGGGGGACTC
30	OPB10	CTGCTGGGAC

Sequencing the PCR product

The amplified regions of CO1 and 16s rRNA genes were sequenced via the Sanger Sequencing method. Sequence quality was checked with "Seq Scanner" software, and alignment and editing of DNA sequences was conducted using "BioEdit Sequence Alignment Editor" software. Species identification was confirmed with GenBank data (https://blast.ncbi.nlm.nih.gov/). To ensure accuracy, sequences were searched using specific keywords and accession numbers corresponding to *M. cephalus*, *P. macrolepis*, and *P. tade*. Cross-referencing with peer-reviewed studies, such as

Thomson[27], Harrison & Senou[28], Durand *et al*.[29] and Durand & Borsa[30] ensured validity. Any sequences showing inconsistencies or low similarity scores were excluded. The submission of CO1 and 16S rRNA sequences to GenBank obtained the following accession numbers: *M. cephalus* (MW684708, MW677958), *P. macrolepis* (MW674647), and *P. tade* (MW684707, MW677957). Genetic distances and dendrograms were computed using Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0.

PCR-RFLP

Restriction site analysis of the nucleotide sequences was performed using the NEBcutter V2.0 program (http://nc2.neb.com/NEBcutter2/). Based on the results obtained from NEBcutter, the enzyme Alu I was selected for its ability to digest the DNA fragment at multiple sites. According to the software, Alu I digested the 16s rRNA gene at 150 bp, 300 bp, and 310 bp in *M. cephalus*; at 150 bp, 185 bp, and 310 bp in *P. macrolepis*; and at 150 bp, 180 bp, and 310 bp in *P. tade*. The selected 16s rRNA gene was treated with the Alu I restriction enzyme following the NEB instructions with slight modifications. Specifically, 5 µl of the PCR product was treated with enzyme (0.5 µl) for 30 min at 50 °C. The digested products were subsequently observed on 2 % agarose gels stained with ethidium bromide and run on 1X TAE buffer. The size of the DNA fragments was calculated using a 100 bp DNA ladder (Gene DireX, Taiwan).

Data analysis

Following amplification, PCR products were separated on a gel. Scoring was performed manually for the three mullet species. Each lane bands was compared with bands in other lanes, and reliable bands were recorded for present and absent as 1 and 0 respectively. RAPD markers, considered dominant markers, were analyzed under two assumptions: alleles derived from distinct loci do not migrate together to identical positions on a gel, with each fragment representing a Mendelian locus. The visible 'dominant' marker allele is anticipated to be in Hardy-Weinberg equilibrium, co-occurring with a null recessive allele or the absence of a fragment[24]. DNA fragment sizes were determined using a 100 bp DNA ladder (Gene DireX, Taiwan). The RAPD data were analyzed using the POPGENE version 1.31. Dendrograms were constructed using Nei's[31] and Reynolds *et al*.[32] genetic distances, using the unweighted pair-group method with arithmetic averages (UPGMA) of Sneath & Sokal[33].

Results

Genomic DNA

Total genomic DNA was extracted from 95 individuals of mullet species. All the samples had DNA in the range between 1.8 and 2.0 (A260/A280). The samples showing OD ratio between 1.7 – 2.0 were considered as good quality DNA. The quantity of the DNA concentration ranged from 40 – 250 ng/µl.

RAPD profile for mullet

All five primers analyzed in this study exhibited polymorphism. A total of 69 alleles were identified (Table 2). For *M. cephalus*, OPA1 primer revealed 11 alleles, ranging in size from 480 bp to 1600 bp. The OPA10 primer yielded 9 alleles, ranging from 400 bp to 1600 bp. Four alleles were detected using the OPA11 primer, with sizes ranging from 1000 bp to 1400 bp. OPA15 and OPA16 identified 8 alleles each, with sizes ranging from 550 bp to 1900 bp. In total, 10 unique bands were observed for *M. cephalus*. With the OPA1 primer, three unique bands were detected at 1500 bp, 1200 bp, and 850 bp. OPA10 revealed three unique bands at 1500 bp, 1300 bp, and 1000 bp. The OPA11 primer produced two unique bands at 1300 bp and 1200 bp, while OPA16 resulted in unique bands at 1200 bp and 1000 bp.

For *P. macrolepis*, the OPA1 primer identified 8 alleles ranging from 500 bp to 1600 bp. OPA10 yielded 7 alleles ranging from 400 bp to 1600 bp. OPA11 revealed 11 alleles ranging from 350 bp to 1800 bp. OPA15 and OPA16 produced 5 and

Table 2 — Number of amplified and monomorphic loci

Sr. No.	Primer code	Total number of alleles	No. of alleles			No. of polymorphic bands	Polymorphism (%)
			M. cephalus	*L. macrolepis*	*L. tade*		
1	OPA1	14	11	8	7	5	35.71
2	OPA10	14	9	7	6	8	57.14
3	OPA11	15	4	11	11	5	33.33
4	OPA15	14	8	5	12	9	64.29
5	OPA16	12	8	8	8	4	33.33
Total		69	40	39	44	36	43.37

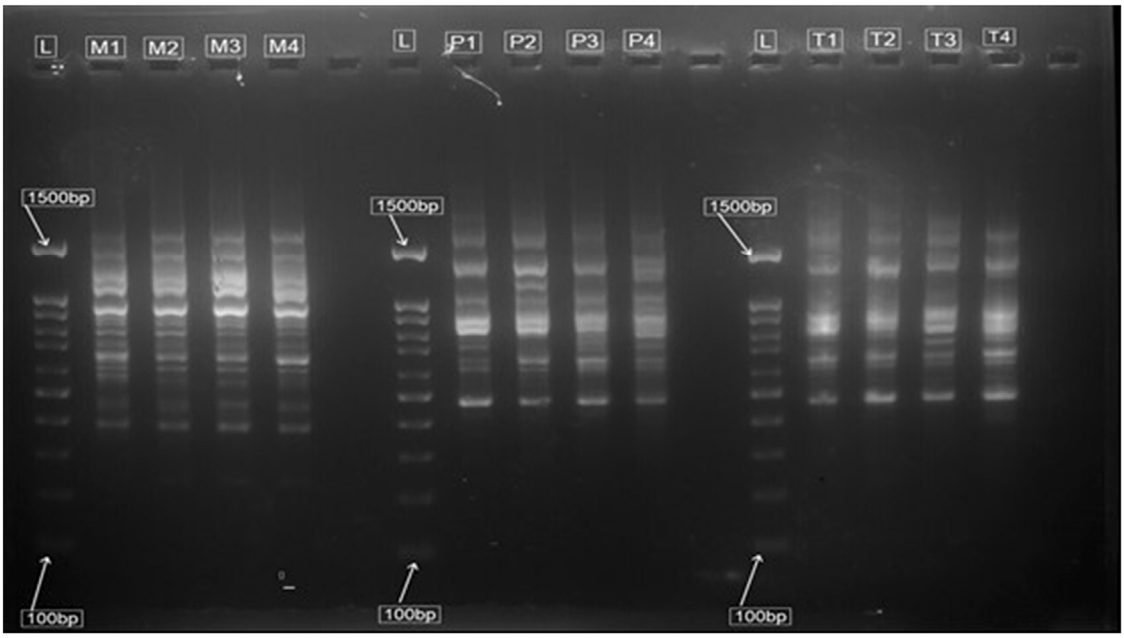

Fig. 3 — OPA1 primer amplification. L - Ladder, M1 to M4 - *M. cephalus*, P1 to P4 - *P. macrolepis*, T1 to T4 - *P. tade*

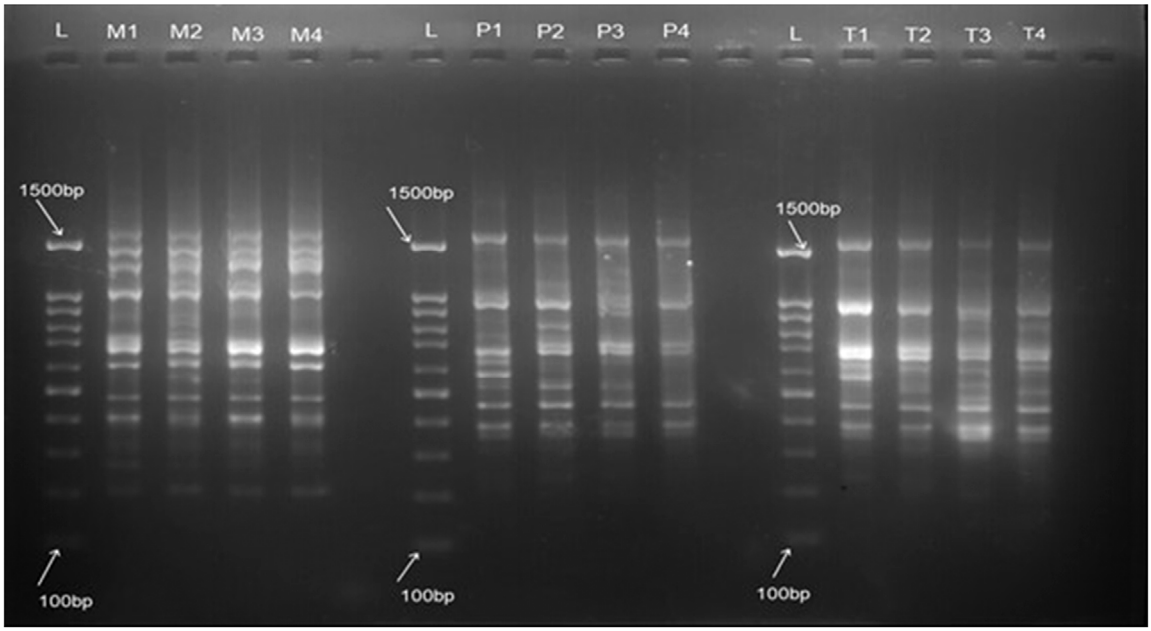

Fig. 4 — OPA10 primer amplification. L - Ladder, M1 to M4 - *M. cephalus*, P1 to P4 - *P. macrolepis*, T1 to T4 - *P. tade*

6 alleles, respectively, with sizes ranging from 650 bp to 2000 bp and 470 bp to 1600 bp, respectively. For *P. tade*, the OPA1 primer detected 7 alleles ranging from 480 bp to 1600 bp. OPA10 revealed 6 alleles ranging from 380 bp to 1600 bp. OPA11 identified 12 alleles ranging from 350 bp to 1800 bp. OPA15 detected 12 alleles, and OPA16 identified 7 alleles, with sizes ranging from 450 bp to 2000 bp and 500 bp to 1600 bp, respectively (Fig. 3 – 7).

Species confirmation and relatedness among mullet species

Nei's genetic distance among the three mullet species varied from 0.4286 to 0.9583. The smallest distance was noted between *P. tade* and *P. macrolepis*, while the largest distance occurred between *M. cephalus* and *P. tade*. Nei's genetic identity ranged from 0.3835 to 0.6514, with the lowest identity observed between *M. cephalus* and *P. tade*, and the highest between *P. macrolepis* and *P. tade* (Table 3).

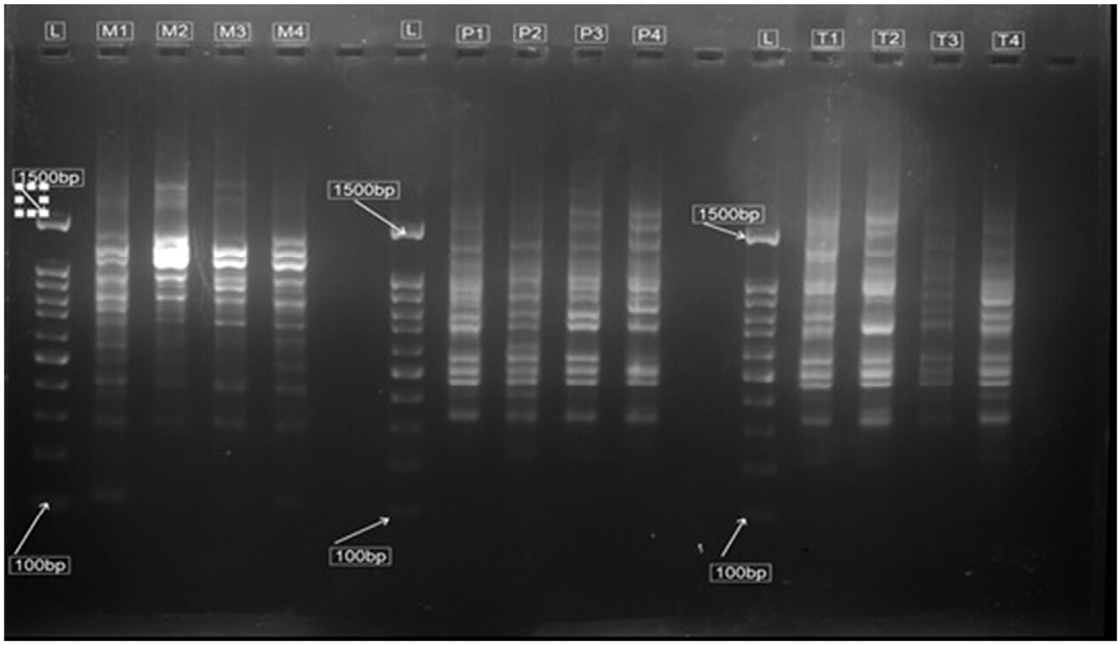

Fig. 5 — OPA11 primer amplification. L - Ladder, M1 to M4 - *M. cephalus*, P1 to P4 - *P. macrolepis*, T1 to T4 - *P. tade*

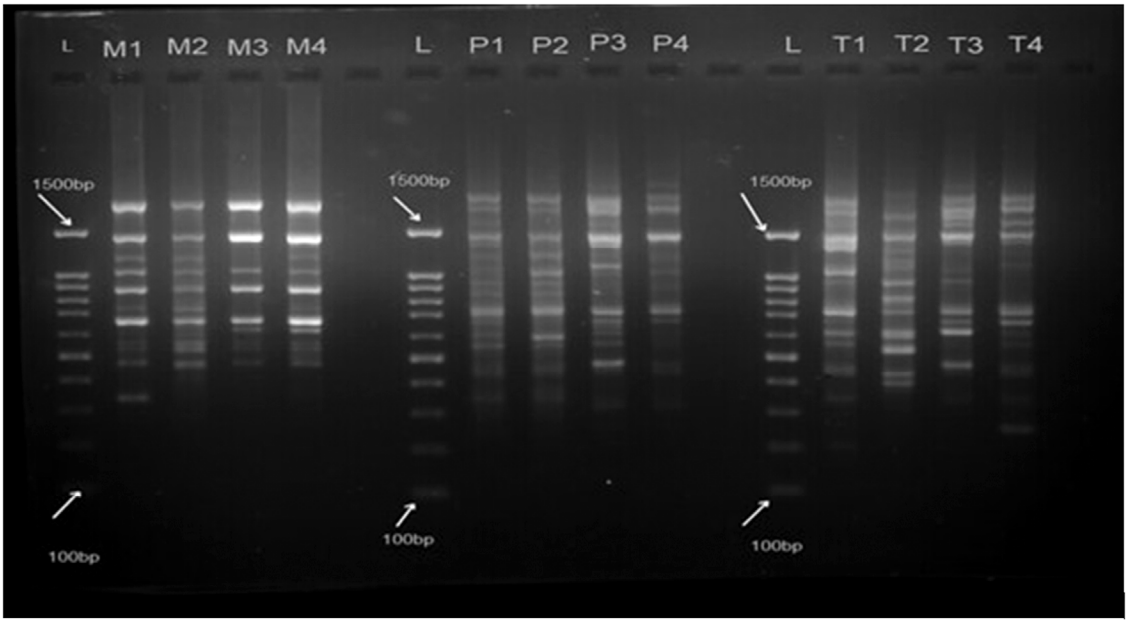

Fig. 6 — OPA15 primer amplification. L - Ladder, M1 to M4 - *M. cephalus*, P1 to P4 - *P. macrolepis*, T1 to T4 - *P. tade*

A dendrogram based on Nei's genetic distance depicts the relationships among the three mullet species. It revealed two distinct clades, with *P. macrolepis* and *P. tade* grouped together, while *M. cephalus* forms a separate cluster (Fig. 8).

RFLP analysis for mullet species

The NEB DNA cutter software facilitated the selection of a suitable restriction enzyme. Sequences from three species were uploaded into the software, generating a list of enzymes capable of digesting different parts of the sequences. From these options, an enzyme that could digest the genes of the three species at different sites was chosen. The enzyme "Alu I" was selected for the 16s rRNA gene based on this criterion. Alu I proved capable of digesting the 16s rRNA region of mullet species, cleaving at three different sites in *M. cephalus* (190 bp, 200 bp, and 210 bp). In

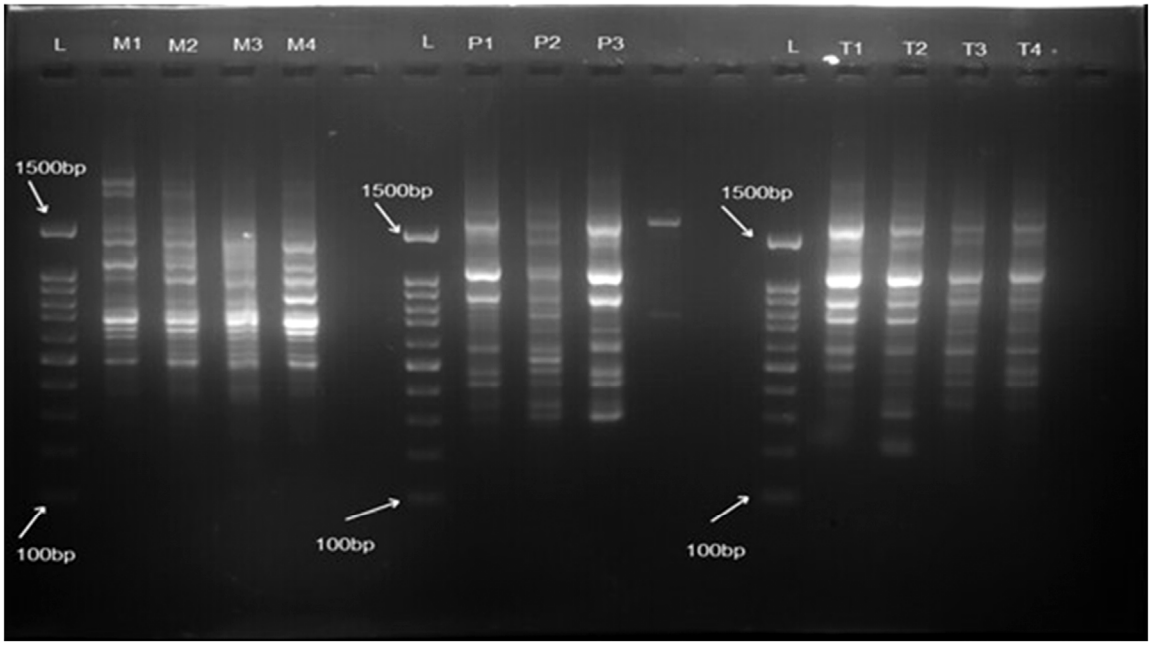

Fig. 7 — OPA16 primer amplification. L - Ladder, M1 to M4 - *M. cephalus*, P1 to P4 - *P. macrolepis*, T1 to T4 - *P. tade*

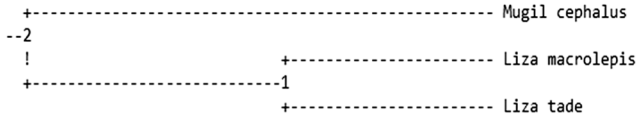

Fig. 8 — Dendrogram using Nei's unbiased genetic distance

Table 3 — Nei's genetic distance for three mullet species=

Species	*M. cephalus*	*L. macrolepis*	*L. tade*
M. cephalus	****		
L. macrolepis	0.9349	****	
L. tade	0.9583	0.4286	****

P. macrolepis and *P. tade*, Alu I trimmed the sequences at 130 bp, 210 bp, and 250 bp. Notably, Alu I differentiated *M. cephalus* from *P. macrolepis* and *P. tade* but did not distinguish between *P. macrolepis* and *P. tade* (Fig. 9).

Discussion

Mullets, are prevalent fishes with numerous morphological characters that are utilized for their identification. Key identification features include mouth anatomy[34], the pharyngobranchial organ[35], lower jaw outline[36], anal fin ray counts[11], body shape and pigmentation[10], and the pyloric caeca[12]. Notably, characters used for identifying fry are often unsuitable for adults due to changes in morphology between life stages[11]. Additionally, some morphological assessments require sacrificing the specimen. To address these taxonomic ambiguities, genetic markers function as a valuable mechanism for systematic ichthyologists and fishery biologists[37]. In this study, RAPD and RFLP markers were employed to resolve taxonomic ambiguities among selected mullet species. Morpho-meristic characters, such as fin ray counts, scale counts, and body proportions, have traditionally been used in mullet identification due to their utility in distinguishing between closely related species. For instance, the count of soft rays in the dorsal and anal fins, the scale morphology, and the relative lengths of body parts have been key diagnostic features in taxonomic keys for mullets[13]. However, relying exclusively on morpho-quantitative traits can result in incorrect identification or oversimplification of species delineation, particularly in cases of morphologically indistinguishable species or those exhibiting phenotypic flexibility. Considering the complexity of species identification in the Mugilidae family, particularly due to the possibility of cryptic species, it is crucial to account for this cryptic nature before assigning a scientific name to a specimen. Regarding *P. macrolepis*, Rajan *et al.*[6] noted the presence of *Planiliza cf. macrolepis* sp. in the Indian region. This designation highlights the uncertainty and potential distinction of this specimen from *P. macrolepis sensu stricto* (Smith 1846), which has its original location in South Africa and potential specimens from the same locality (JQ060425–26) as referenced by Durand *et al.*[29] and Durand & Borsa[30]. The geographic separation and genetic divergence suggest that these

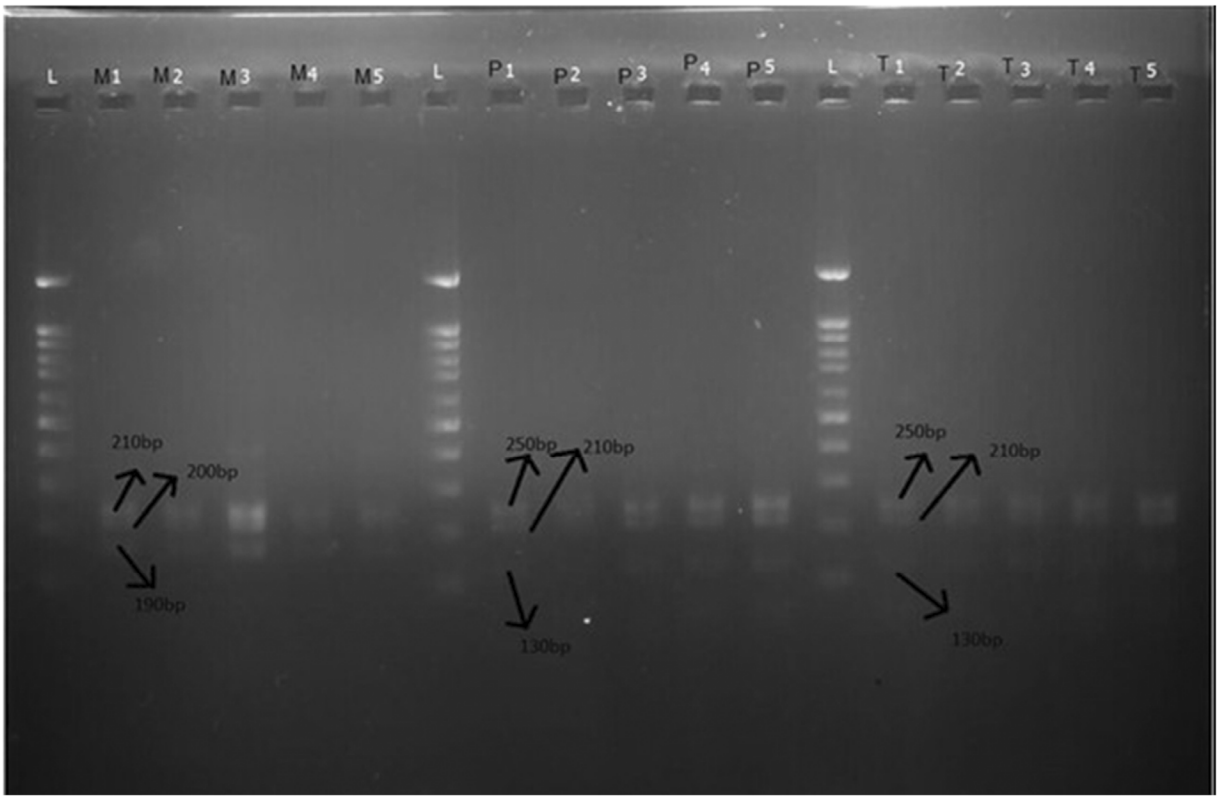

Fig. 9 — Gel image of Alu I enzyme digestion. L - Ladder, M1 to M5 - *M. cephalus*, P1 to P5 - *P. macrolepis*, T1 to T5 - *P. tade*

populations may have evolved into distinct species, necessitating comprehensive genetic and morphological studies to confirm their identities and taxonomic statuses. Furthermore, the *M. cephalus* complex illustrates the prevalence of cryptic species within the Mugilidae family. According to Tran *et al.*[38], this complex includes several species groups considered "putative species." Rajan *et al.*[6] identified two such putative species in the Indian waters, which are distinct from *M. cephalus sensu stricto* (type locality: European Sea). The presence of genetically distinct species along the southwest and southeast coasts of India underscores the necessity for distinct nomenclature. *Mugil cephalotus* stands as the oldest available name; however, its revalidation requires a comprehensive morphological analysis using specimens from the type locality and existing syntypes. In recent years, molecular approaches, such as DNA barcoding and phylogenetic analysis, have provided additional insights into mullet taxonomy by examining genetic variation among populations. These molecular techniques have revealed cryptic diversity and evolutionary relationships that were not apparent based on morpho-meristic characters alone. However, conflicts between morpho-meristic and molecular approaches in species identification have also been reported, with some studies suggesting discordance between morphologically defined species and molecularly defined clades[39]. This study provides a snapshot of the taxonomic classification and description of *M. tade* based on the FAO sheets. However, recent advancements in mugilid fish taxonomy, as demonstrated in studies such as Bogorodsky *et al.*[40], suggest revisions to the genus assignment and taxonomic hierarchy. The reclassification of *M. tade* under the genus *Moolgarda* proposed in Bogorodsky *et al.*[40] contrasts with the genus assignment in this study's FAO sheets. Additionally, further descriptions of *M. crenilabis*, *M. seheli*, and *M. tade* utilizing specimens sourced from the region between Africa and India offer updated morphological descriptions compared to the information available in the FAO sheets[36]. Additionally, the genetic diversity within the *Moolgarda* genus provides a more nuanced understanding of mugilid fish diversity and distribution, highlighting the importance of integrating recent research findings into taxonomic databases to ensure accurate and up-to-date species classifications and descriptions.

Random amplified polymorphic DNA (RAPD)

In current study, among 30 RAPD arbitrary primers, only OPA1, OPA10, OPA11, OPA15, and OPA16 showed reproducible results, yielding distinct banding patterns, while the rest were excluded due to inconsistency in amplification. Similar results were documented by Bhat *et al*.[37], who screened eight reproducible primers out of 40 arbitrary primers, producing a total of 69 alleles, with 31 being polymorphic[35]. The polymorphism ranged from 8.7 to 43.37 %, with each primer yielding fragments ranging from 5 to 12, and fragment sizes ranging from 400 to 2000 bp. OPA15 exhibited the highest level of polymorphism, whereas OPA11 and OPA16 showed the lowest. Additionally, in studies on Parrotfish and Synodontis species, RAPD markers revealed varying levels of polymorphism[41,42]. Unique bands observed in this study and in earlier researches were found useful in identifying fish species, with 10 unique bands differentiating *M. cephalus* from *P. macrolepis* and *P. tade*[42]. Genetic distance analysis using RAPD markers revealed ranges between different genera, such as *Mugil* and *Liza*, and within species groups, like *Channa* species[35] and *Schilbe mystus*, *Bagrus bajad*, and *Clarias gariepinus*[42]. The phylogenetic tree constructed using RAPD loci in current study showed distinct clustering among mullet species, with *M. cephalus* forming a separate clade from *P. macrolepis* and *P. tade*. Similar phylogenetic relationships were observed in Synodontis species[43] and Indian Snakehead[44], indicating the utility of RAPD markers in genetic analysis.

Restriction Fragment Length Polymorphism (RFLP)

The nucleotide lengths of COI and 16s rRNA mitochondrial regions in mullet species ranged from 655 bp to 688 bp and 562 bp to 585 bp, respectively, consistent with previous studies[33]. The enzyme Alu I was chosen based on *in-silico* analysis and successfully digested the 16s rRNA gene, producing distinct patterns for *M. cephalus*, *P. macrolepis*, and *P. tade*. This enzyme has been widely used in various genetic analyses, including in mullet species differentiation[45]. Current study aligns with previous research, such as Smith *et al*.[39], who observed genetic distances between mullet species using RAPD analysis, supporting current findings. Similarly, Johnson *et al*.[46] highlighted the limitations of traditional taxonomic characters and emphasized the utility of molecular markers in resolving taxonomic uncertainties in mullet species, echoing current observations[46]. However, discrepancies may exist with certain studies, such as Patel & Patel[47], who reported slightly different fragment sizes for the Alu I enzyme in *M. cephalus*. These differences could stem from regional variations in mullet populations or methodological nuances in marker selection and analysis[42]. While the use of RAPD and RFLP molecular markers has provided valuable understandings of the genetic variability and evolutionary relationships among selected mullet varieties, challenges remain in the accurate taxonomic identification of mugilid species. Achieving a precise and comprehensive understanding of mullet species identification on a global scale is a complex task that cannot be fully addressed within a single scientific study. We acknowledge this limitation in the current research.

Conclusion

In conclusion, study highlights the importance of molecular markers like RAPD and PCR-RFLP in resolving the taxonomic and identification challenges within the Mugilidae family. The study successfully differentiated between *M. cephalus*, *P. macrolepis*, and *P. tade*, emphasizing the importance of molecular approaches, especially for cryptic species. While RAPD markers showed promising results in distinguishing mullet species and yielding unique bands for potential species-specific markers, the RFLP marker faced limitations in distinguishing *P. macrolepis* and *P. tade*. Further exploration of mitochondrial genes could enhance species differentiation. The findings contribute to advancing the understanding of mullet taxonomy, though discrepancies with previous studies highlight the need for continued research and refinement of molecular techniques. This research is crucial for informing conservation efforts and management strategies for mullet species worldwide.

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Conflict of Interest

There is no conflict of interest among the authors.

Author Contributions

IV conducted the experiments. NF helped to collect the samples and conduct the experiments. AK analyzed the data, and ES conceived idea and performed overall coordination to conduct the research.

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