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PCR-RFLP and sequence analysis of 12S and 16S rRNA mitochondrial genes of Grey mullets from East coast of India

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Present study consists PCR-RFLP and sequence analysis of mitochondrial genes (12S and 16S rRNA) on four species of Mugilidae, collected from east coast of India. Three and four restriction enzymes were found informative for 12S rRNA mitochondrial gene for *Liza macrolepis* and *Liza tade* respectively. Whereas, eight, seven and eight restriction enzymes were found informative for *Liza macrolepis*, *Liza parsia* and *Liza tade* respectively for 16S rRNA mitochondrial gene. Sequence analysis of 12S rRNA and 16S rRNA revealed that *Liza macroplepis* was distant from the other two *Liza* species, *L. parsia* and *L. tade*. *Mugil cephalus* was found to be the most distinct amongst the mullet species analyzed based on sequence analysis of 16S rRNA mitochondrial gene.

[**Keywords** :Gene, marine, mullet, phylogenetic, estuarine.]

Introduction

Grey mullets widely distributed worldwide both in tropical and temperate regions inhabit marine, estuarine, and freshwater environments. The family of Mugilidae (Pisces, Mugiliformes) has 14 genera and 64 valid species which mainly represent *Mugil* and *Liza* having 12 and 23 species respectively[1]. However, in spite of these revisions, the systematic classification and identification of some species and genera within the family is still not clear. For any fisheries management and breeding program, precise species identification is extremely important. In case of mullets, the conservative external morphology, lack of useful taxanomic characters and homogeneous karyological structure leads to difficulty in establishment of their phylogenetic relationships and identification[1,2]. The Mugilid species constitutes an economically important species in fisheries sector of many countries including India. However, one of the major concerns is elucidating the phylogenetic relationships among the *Liza* species and the monophyletic origin of the genus *Liza*. *Liza* as genus was not recognized and was included within *Chelon*[3], later *Chelon* was recognized as a valid genus, derived from *Liza*[1].

Mitochondrial genome analysis is widely used for studying phylogenetic and systematic relationships. The main advantages of using mitochondrial genes are its maternal inheritance and higher nucleotide substitution rate when compared to the nuclear genome[4]. The molecular techniques such as PCR-RFLP analysis have been widely used as species specific markers. The restriction patterns generated in this technique serve as a diagnostic marker for taxonomic and phylogenetic analysis. This is particularly useful and important for species such as mullets where identification at the larval and fingerling stages is difficult as the morphological and physiological characters do not show significant differences[5]. Mitochondrial DNA divergence, phylogenetic relationships and genetic identification in species belonging to Mugilidae has been reported using mitochondrial DNA (mtDNA) fragments such as, cytochrome b and 12S rRNA genes[5], 2S/16S rRNA and ND3/ND4L/ND4 genes[6], 5S rRNA gene[7], 16S rRNA and cytochrome b genes[8], 12S, rRNA, 16S rRNA and COI genes[9, 10].

M. cephalus and other Mugilid species from the east and west coast of India have never been studied from the perspective of taxanomic identification using molecular tools. In the present study, four Mugilid species were collected from Indian east coasts. Three congeneric [*Liza parsia* (Hamilton & Buchanan, 1822), *Liza tade* (Forsskal, 1775) and *Liza macrolepis* (Smith, 1849)] and one noncongeneric (*Mugil*

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cephalus) species were analysed, at the molecular level by PCR-RFLP of mitochondrial DNA (mtDNA) in order to characterize them.

Material and Methods

Mitochondrial DNA extraction

Extraction of mitochondrial DNA was carried out in four and three samples of *Liza macrolepis* collected from Puri (Orissa) and Chennai (Tamil Nadu) respectively. Seven samples each of *Liza parsia* and *Liza tade* were collected from Kakdwip (West Bengal). Five samples of *Mugil cephalus* was collected from Chennai (Tamil Nadu). All these sampling sites are located in east coast of India. The white muscle tissue from dorsal musculature were collected and stored in absolute alcohol till processed. Mitochondrial DNA was extracted from 100 mg of muscle tissues from these fishes. The muscle tissues after washing in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) were homogenized with 500 µl of ice cold homogenization buffer (30 mM Tris-HCl, 30 mM EDTA, 15mM NaCl, pH 7.8) containing 100 µg/ml proteinase K. Equal volume of freshly prepared solution of 1% SDS and 0.2 N NaOH were added followed by gentle mixing and incubation in ice for 2 min. Equal volume of ice cold potassium acetate (Potassium 3M, acetate 5M) was added and mixed gently . The sample was stored in ice for 5 min. and centrifuged at 12000 × g for 10 min. The supernatant containing mtDNA was extracted with Tris saturated phenol chloroform isoamyl solution (25:24:1). mtDNA was precipitated by adding 2 volumes of ethanol and storing the sample at -20°C for 2 hours. The pelleted mtDNA obtained by centrifugation at 12000 × g for 10 min at 4°C was washed in 70% ethanol, air dried and resuspended in 50 µl of distilled water.

Amplification of mitochondrial genes

Polymerase chain reaction (PCR) was carried out using primers for mitochondrial 12S rRNA and 16S rRNA genes as described previously[11]. The sequence of the primers used were, 12S rRNA I: 5' AAACTAGGATTAGATACCCTATTAT 3' and 12S rRNA II: 5' AAGAGCGACGGGCGATGTGT 3'; 16S rRNA I: 5' CGCCTGTTTAACAAAAACAT 3' and 16S rRNA II: 5' CCGGTCTGAACTCAGATCATGT 3'.

The PCR reaction mixture included all 4 dNTPs (200 µM), 30 pmoles of each primer concentration, 1 unit of Taq polymerase, 1 × polymerase buffer containing 1.5 mM $MgCl_2$ (Bangalore Genei, India.) and 50 ng of template DNA. The thermal program was for 93°C for 1 min followed by 30 cycles of 93°C for 30s, 48°C for 30s, 72°C for 30s and 72°C for 10 min as final extension cycle.

Restriction Fragment Length Polymorphism

PCR-RFLP was carried out on the 12S and 16S rRNA mitochondrial genes of these fishes. Unpurified amplified PCR products of approximately 430 bp and 630 bp size of the 12S and 16S rRNA respectively of mtDNA of fish samples were digested with various restriction enzymes in 20 µl reaction volume following the conditions specified by supplier (New England Biolabs). The restriction enzymes used for RFLP analysis are shown in Tables 1 and 2. Agarose gel analysis was carried out after 4 h of incubation with restriction enzymes at specified temperature.

Sequence analysis

The PCR products obtained for 12S and 16S rRNA were sequenced by Bangalore genei Ltd (India), using both forward and reverse primers of the respective mitochondrial genes. Sequence homology search was carried out using BLAST algorithm (www. ncbi. nlm.nih.gov). Sequence alignment was done using Clustal W program at (http://www.ebi.ac.uk) web site. The sequence was aligned and edited using BioEdit Sequence Alignment Editor. The nucleotide sequences were analyzed for restriction sites by submitting the sequences to NEBcutter V2.0 programme (http://tools.neb.com/NEBcutter2/index. php). The nucleotide frequencies and genetic distance values (Jukes-Cantor) were estimated using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Table 1—Restriction enzyme analysis of mitochondrial 12s rRNA PCR product showing estimated restriction fragment size (bp)

Restriction Enzymes	*Liza macrolepis*	*Liza parsia*	*Liza tade*
Fat I		430	280 150
Hind III	230 200		
*Hha*I		430	280 150
*HinP*1I		430	280 150
*Nla*III		430	280 150
*Ssp*I	350		
*Fau*I	230 200		

Results and Discussion

PCR product of approximately 430 bp amplification was observed using 12S rRNA primers in fish species *Liza macrolepis*, *Liza parsia* and *liza tade* as shown in Figure 1. Whereas, PCR amplification of approximately 630 bp size was observed using the primers for 16S rRNA mitochondrial gene in all the four species, *Liza macrolepis*, *Liza parsia* and *Liza tade* and *Mugil cephalus* as shown in Figure 2. The amplification achieved for these two mitochondrial genes in mullets were in the same size range as reported earlier in our previous studies on other brackishwater finfishes[12]. Similar size range of amplified PCR products for 12S and 16S rRNA mitochondrial genes using the universal primers[13] have been reported by other workers. For example, the sizes of PCR-amplified mtDNA segments for the five mugilid species (*L. saliens*, *L. aurata*, *L. ramada*, *M. cephalus* and *C. labrosus*) were found to be about 450 bp for 12s rRNA mitochondrial gene. In case of 16s rRNA the amplified product size was found to differ among the five species, with *M. cephalus* having a segment of about 630 bp and the other species exhibiting a segment of about 600 bp[9]. However, in the present study we observed similar size range of about 630 bp of amplified product for 16S rRNA mitochondrial genes in all the four mullet species analyzed.

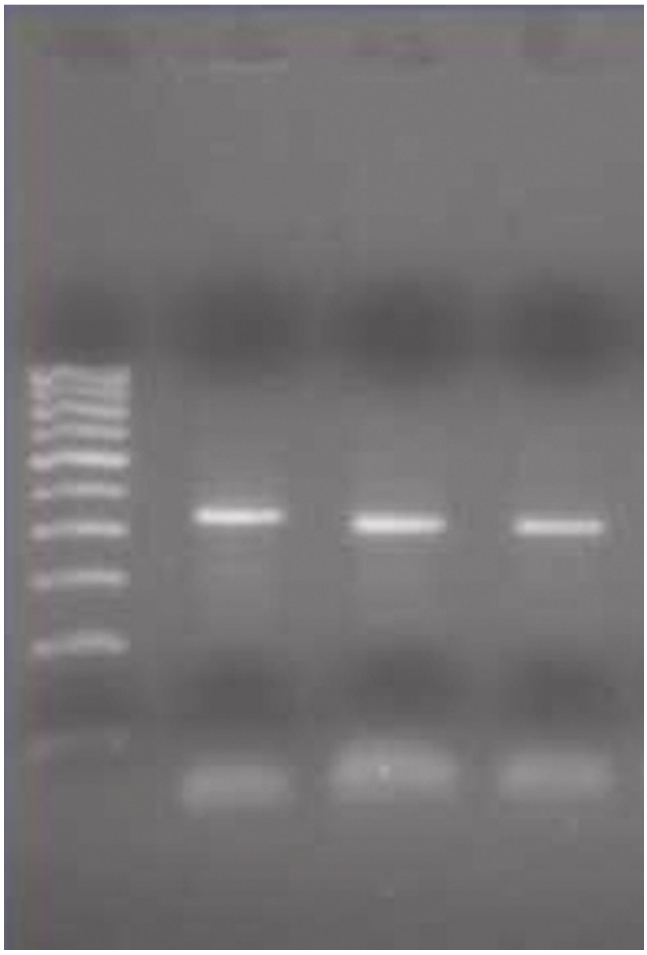

Figure 1—Amplified PCR product of 12S rRNA mitochondrial genes. Lane 1. 100 bp marker. Lane 2. *Liza macrolepis* Lane 3. *Liza parsia* Lane 4. *Liza tade*

Amplified DNA segments of 12S rRNA and 16S rRNA mitochondrial genes of these species were digested with at least 7 and 14 restriction enzymes respectively to check for the presence of restriction sites. Fragment patterns produced by each restriction enzyme for the two mtDNA segments are presented in Tables 1 and 2. *Liza macrolepis* and *Liza tade* revealed recognition sites for three and four restriction enzymes for 12S rRNA mitochondrial gene (Table 1). Whereas, restriction enzyme analysis of mitochondrial 16s rRNA PCR product showed recognition sites for four, seven, six and five restriction enzymes for *Liza macrolepis*, *Liza parsia, Liza tade* and *Mugil cephalus* respectively (Table 2).

Table 2—Restriction enzyme analysis of mitochondrial 16s rRNA PCR product showing estimated restriction fragment size (bp)

Restriction Enzymes	*Mugil cephalus*	*Liza macrolepis*	*Liza parsia*	*Liza tade*
*Alu*I	250	250		
	180	220		
		130		
*Hha*I	400	400		
	160	150		
*Mbo*I	200	250		
	150	210		
*Mbo*II	400	300		
	200	200		
*Dde*I	320			
	280			
*Apo*I			300	300
*Eco*RI			300	300
*Nco*I			280	330
			240	280
*Pho*I			550	520
*Nsp*I			380	630
			250	
*Fau*I		400		
		200		
*Tsp*509I		400		
		200		
*Btg*I		550	270	550
			260	
*Sty*I		450	450	350
		150	150	150

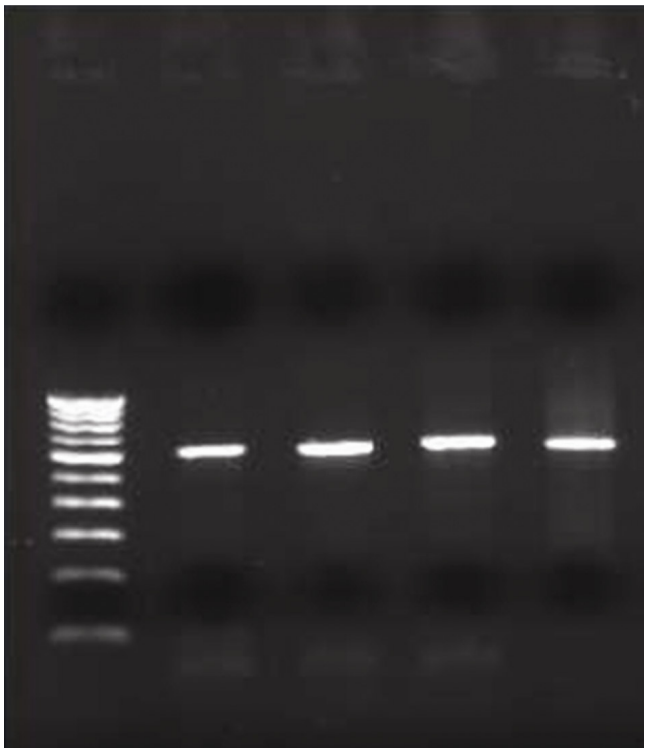

Figure 2—Amplified PCR product of 16S rRNA mitochondrial genes. Lane 1. 100 bp marker. Lane 2. *Liza macrolepis* Lane 3. *Liza parsia* Lane 4. *Liza tade* Lane 5. *Mugil cephalus*

Similar observation of ten and eight restriction enzymes having at least one recognition site at 12s rRNA and 16s rRNA mitochondrial genes, respectively has been reported[9]. Several restriction enzymes produced distinct restriction patterns. For example, after digestion of the 12s rRNA gene segment with *Fat*I, *Liza parsia* showed no restriction site whereas, *Liza tade* showed restriction pattern generating two restriction bands of 280 bp and 150 bp size. These species-specific patterns can be taken into consideration either individually or in various combinations, to distinguish the four mullet species under study.

Sequences of 12S and 16S rRNA mitochondrial genes were submitted to GenBank (www.ncbi.nlm.nih.gov). The accession numbers for 12S rRNA mitochondrial genes are GQ168551, GQ168553 and GQ168555 for *Liza parsia*, *Liza macrolepis* and *Liza tade* respectively. The accession numbers for 16S rRNA mitochondrial genes are GQ168552, GQ168554, GQ168556 and DQ185446 for *Liza parsia*, *Liza macrolepis, Liza tade* and *Mugil cephalus* respectively.

Sequence analysis by alignment of 12S rRNA gene sequence of three *Liza* species produced a consensus sequence length of 275 base pairs. Of 275 sites, 263 were monomorphic and 12 were polymorphic (all were singleton variable sites). At all except one singleton variable sites, *Liza macroplepis* was found differing from *Liza parsia* and *Liza tade*. The analysis revealed nucleotide frequencies as A = 29.5%, T = 21.7%, G = 21.8% and C = 270%. On an average, there were 267 identical, 6 transitional and 2 transversional pairs of nucleotides. The 'R' value (transition/transversion ratio) of 3 indicated that the transitions were more frequent than transversions (the value of R should be 0.5 when there is no bias towards either transitional or transversional substitution). The transition/transversion rate ratios were estimated by maximum composite likelihood method as k_1 = 62.086 (purines) and k_2 = 7.457 (pyrimidines). The average genetic distance among three species was estimated as 0.03. Pair-wise distances (Jukes - Cantor) between species showed that *Liza macroplepis* was distant from the other two species, *Liza parsia* (0.041) and *Liza tade* (0.045). The average number of nucleotide differences per site between two sequences was estimated as 0.029.

Sequence alignment of 16S rRNA mitochondrial gene of three *Liza* species produced a consensus sequence length of 550 base pairs. Of 550 sites, 531 were monomorphic and 17 sites were polymorphic (singleton variable sites). Two of the sites were polymorphic as alignment gaps. The analysis revealed nucleotide frequencies as A = 28.4%, T = 23.6%, G = 22.5% and C = 25.5%. On an average, there were 537 identical, 10 transitional and 1 transversional pairs of nucleotides. The transitions were more frequent than transversions as indicated by R value (7.5). The transition/transversion rate ratios are estimated by maximum composite likelihood method as k_1 = 1.393 (purines) and k_2 = 61.953 (pyrimidines). The average genetic distance among three species was estimated as 0.021. Jukes - Cantor distances between species showed that *Liza macroplepis* was distant from the other two species, *Liza parsia* (0.03) and *Liza tade* (0.028). The distance between *Liza parsia* and *Liza tade* was estimated as 0.005. The average number of nucleotide differences per site between two sequences was estimated as 0.02.

The proportion of conserved sites observed among the three *Liza* species DNA segments in this study was 95.6% for 12S rRNA and 96.5% for 16S rRNA. The high sequence homology results are in close agreement to the observations of 84.6% for 12s rRNA

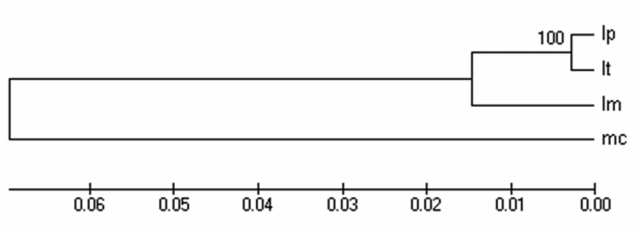

Figure 3—Dendrogram analysis of muglids. lp = *Liza parsia*; lm = *Liza macrolepis*; lt = *Liza tade* and mc = *Mugil cephalus*.

and 86.5% for 16s rRNA in among five species of the Mugilidae family[10]. In our earlier study, we have observed similar highly conserved nature of 16S rRNA mitochondrial gene in penaeid shrimps[14]. This may be attributed to the slower evolutionary rate of 12S and 16S rRNA genes as compared to other mitochondrial genes such as CO I[10].

Alignment of 16S rRNA sequences of *Liza* species with that of *Mugil cephalus* showed consensus sequence of 575 base pairs with 25 gaps at nucleotide positions 238, 337-356 and 408-411. Both *Liza parsia* and *Liza tade* had 20 base pair (at positions 337-356) insertion/deletion whereas, *Liza macroplepis* had 19 base pair insertion/deletion (at positions 338-356). However, the 4 base pair insertion/deletion were present in all the three *Liza* species. The 20 base pair insertion/deletion indicates usefulness of this region in designing molecular markers to distinguish *Mugil cephalus* from *Liza* species analyzed in this study. Three methods of phylogenetic tree construction (Neighbor-Joining, Maximum parsimony and UPGMA) placed *Mugil cephalus* in a separate branch from three *Liza* species. The distance between *Mugil cephalus* and *Liza parsia, Liza macroplepis* and *Liza tade* was estimated as 0.129, 0.127 and 0.127 respectively. The phylogenetic trees produced in our study Figure 3 are in general agreement with those presented by other workers who have reported *Mugil cephalus* as a distant species. For example, the phylogenetic trees analysis by UPGMA and Neighbor-Joining methods, revealed *Mugil cephalus* to be the most genetically distinct species, while the other species are clustered in two separate groups, one comprising *L. aurata* and *L. ramada*, the other comprising *C. labrosus* and *L. saliens*[5,9]. Using different mitochondrial genes (12S, 16S, rRNA, COI, cytochrome b and ND3/ND4L/ND4 genes), similar type of studies, have also indicated *Mugil cephalus* as the most genetically distinct species. The level of divergence observed between *Mugil cephalus* and *Liza aurata* has been reported to be in the range of 22%[6], 14.2%[9] and 17.2%[5] nucleotide substitutions. The phylogenetic analysis based on the 16S rRNA and cytochrome b mitochondrial genes suggest that *Mugil liza* and *Mugil platanus* should be treated as a single species or possibly populations of *Mugil cephalus*[8]. *Mugil cephalus* as the most distinct species among other species of the Mugilidae family, *Chelon labrosus, Liza aurata, Liza ramada,* and *Liza saliens* has also been revealed by sequencing of three mtDNA segments (12S rRNA, 16S rRNA, and CO I)[10]. Similar observation has been reported showing *Mugil cephalus* as the most divergent species of Mediterranean mugilids based on phylogenetic analysis of the partial 16S rRNA mitochondrial gene[15].

Conclusion

The molecular characterization of the grey mullets by sequence analysis of mitochondrial genes (12S and 16S rRNA) revealed that *Liza macroplepis* was distant from the other two species, *Liza parsia* and *Liza tade*. Phylogenetic analysis showed *Liza* species formed a separate cluster and *Mugil cephalus* was found to be the most distinct amongst the mullet species analyzed from east coast of India.

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