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Development Of Molecular Markers For The Study Of Fish Fauna Of Manimala River Of Kerala, India

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Abstract

Molecular level Studies on the freshwater fishes of Kerala is posed a difficulty due to the non availability of molecular markers. In the present paper we describe the development of Cyt b gene sequences of some of the freshwater fishes of Manimala River of Kerala. A total of 16 cyt b sequences were generated in which 37 % of the sequences are deposited for the first time in GenBank. Neighbor joining tree along with the K2P genetic distance were also provided. This may be useful as a baseline data for further studies on the fish fauna of Kerala Rivers.

Key words: Manimala River, fish taxonomy, molecular markers, mitochondrial DNA, cyt b gene sequence

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1. Introduction

Mitochondrial DNA (mt DNA) is a tiny fraction of organismal genome size, considered as the most popular marker in molecular ecology for studying animals in molecular level. Following Avise *et al*., (1987) and Moritz *et al*., (1987), among others, popular geneticists and molecular systematics have adopted this tool with confidence. Virtually every molecular study of animal species in the field involves mt DNA haplotyping at some stage. Not surprisingly, a mitochondrial fragment, CO1, was recently elected as the standardized tool for molecular taxonomy and identification (Ratnasingham and Hebert, 2007). There are many reasons for adoption of mt DNA as marker of choice for molecular ecology studies. Experimentally, mt DNA is relatively easy to amplify because it appears in multilple copies in the cell. Mitochondrial gene content is strongly conserved across animals, with very little duplication, no intron and very short intergenic regions (Gissi *et al*., 2008). Because of its elevated mutation rate, mitochondrial DNA is highly variable in natural populations, which can generate some idea about population history over short time intervals. Mitochondrial DNA having many variable region typically flanked by highly conserved ones helped as to design PCR primers. Clearly mt DNA is the most convenient and cheapest solution when a new species has to be genetically explored in the wild. These practical issues qualify to a large extent the popularity of mt DNA in molecular ecology.

Mitochondrial DNA has a number of specific biological properties such as maternal inheritance; make it an appropriate marker of molecular biodiversity. This considerably simplifies the representation and analysis of within- species variation data. Secondly mt DNA has been supposed to evolve in a nearly neutral fashion. Being involved in basic metabolic functions (respiration), mitochondrial- encoded genes have been considered as less likely than other genes to be involved in adaptive processes. Finally and not independently, the evolutionary rate of mt DNA has been frequently assumed to be clock- like- in the absence of any mutations spreading through positive selection, only neutral (and slightly deleterious) mutations accumulate in time, so that mt DNA divergence levels should roughly reflect divergence times. Clonal, neutral and clock- like: mt DNA apparently stands as the ideal witness of population and species history. Because of its popularity, mt DNA polymorphism and divergence data sets have grown at an impressive rate during the last 3 decades. One of the major hurdles studied in local fish species in India was the non- availability of molecular markers. Here we report the development of mt DNA (Cyt b) marker sequences of some fish species of Manimala River, Kerala, India.

2. Materials and Methods

2.1 Fish collection

Fishes were collected from various locations of Manimala River (Fig.1) using gill nets and caste nets. Cast nets were used in areas where water is not very deep; it is to catch surface dwellers and shallow water species. Gill nets are used in areas where water body is deep. Fishes collected at intervals from different localities during the morning times.

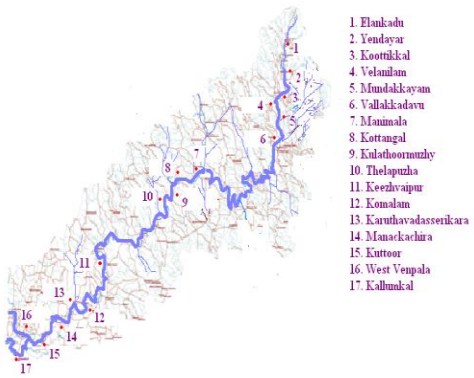

Fig.1: Map of Manimala River showing sites of fish collection

2.2 DNA extraction

Muscle tissue samples (~50 mg) from fishes were collected from the tail region and preserved in absolute alcohol. Total DNA was extracted using Qiagen Dneasy blood and tissue kit according to the manufacturer's instruction. Briefly tissues

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were homogenized and treated with buffer ATL and proteinase K and incubated in 56^0c for digestion. After the complete digestion of the tissues RNAs A was added to remove RNA from the sample and processed with buffer AL and absolute alcohol. The mixture was subjected to a series of centrifugation steps with washing buffers and finally eluted with buffer AE. The eluted solution contains high molecular weight genomic DNA which was subjected to agarose jel electrophoresis and spectrophotometry to check the quality and quantity and visualized under UV-trans illuminator.

Table -1: List of fishes used for the study of development of molecular markers

Sl No	Name of fish	Order	Family	Place of collection	GenBank Acc.No
1	*Megalops cyprinoides*	Elopiformes	Megalopidae	Manackachira	KJ442603
2	*Salmostoma boopis*	Cypriniformes	Cyprinidae	Kottangal	KJ442587
3	*Barilius bakeri*	Cypriniformes	Cyprinidae	Koottikkal	KJ442580
4	*Devario malabaricus*	Cypriniformes	Cyprinidae	Velanilam	KJ442590
5	*Cyprinus carpio communis*	Cypriniformes	Cyprinidae	West Venpala	KJ442595
6	*Puntius mahecola*	Cypriniformes	Cyprinidae	Komalom	KJ442600
7	*Dawkinsia filamentosa*	Cypriniformes	Cyprinidae	Kulathurmoozhy	KJ442582
8	*Gonoproktopterus kurali*	Cypriniformes	Cyprinidae	Kottangal	KJ442586
9	*Cirrhinus mrigala*	Cypriniformes	Cyprinidae	Kuttoor	KJ442596
10	*Garra mullya*	Cypriniformes	Cyprinidae	Elankadu	KJ442584
11	*Mesonoemacheilus triangularis*	Cypriniformes	Balitoridae	Yendayar	KJ442611
12	*Horabagrus brachysoma*	Siluriformes	Bagridae	Thelapuzha	KJ442591
13	*Mystus gulio*	Siluriformes	Bagridae	Keezhvaipur	KJ442606
14	*Ompok bimaculatus*	Siluriformes	Siluridae	Karuthavadasserikkara	KJ442604
15	*Wallago attu*	Siluriformes	Siluridae	Vallakkadavu	KJ442594
16	*Clarias dussumeiri*	Siluriformes	Claridae	Manimala	KJ442598
17	*Hyrhamphus limbatus*	Beloniformes	Hemirhamphidae	Kallumkal	KJ442593
18	*Xenentodon cancilla*	Beloniformes	Belonidae	Velanilam	KJ442583
19	*Macrognathus guentheri*	Synbranchiformes	Mastacembelidae	Komalom	KJ442597
20	*Parambassis dayi*	Perciformes	Chandidae	Velanilam	KJ442585
21	*Pristolepis malabarica*	Perciformes	Nandidae	Mundakkayam	KJ442581
22	*Etroplus maculatus*	Perciformes	Cichlidae	Koottikkal	KJ442579
23	*Etroplus suratensis*	Perciformes	Cichlidae	Keezhvaipur	KJ442588

2.3 Amplification of mt Cyt b gene by PCR

The PCR was performed using universal primers flanking the mt Cyt b gene. In a 25 µL reaction volume, based on the initial trials, the PCR reaction mixture was optimized as follows:
2.5 µ L of 10 X assay buffer (160 mM (NH4)2SO4, 670 mM Tris- HCL, pH 8.8, 0.1% tween- 20, 25n M MgCl2fom Bioron, Gmbh, Germany), 0.5 µ L (200 µM each) of dNTP mix (solution salts of dATP, dCTP, Dgtp and DTTP 10 Mm each in water, i.e., 40mM total Ph 7.5 from Promega, Madison, WI USA), o.5 µL or 20 Picomoles of forward ((AAAAAGCTTCCATCCAACATCTCAGCAT GATGAAA;
ACTGCAGCCCCTCAGAATGATATTTGTCCT CA) 1 U Taq DNA polymerase (DFS- Taq DNA polymerase, Bioron GmbH, Germany), 50 ng of purified DNA and autoclaved NFW to make up the volume. The PCR tube containing the reaction mixture was flash spun and amplification was performed in a thermal cycler (Applied Biosystems.PCR amplification were performed with 35 cycles of the following steps: 94˚ C for 40 s, 50˚ for 45 s and 72˚ C for 1 min, proceeded by 5 min at 95˚C and followed by 10 min at 72 ˚C.

2.4 Sequencing of the PCR products

The PCR products together with a 2- log DNA ladder (NEB) were separated by electrophoresis in 1.2 % agarose gel stained with ethidium bromide, inTBE and visualized in GelDoc (syngene). PCR products were cleaned up using exosap- IT (USB), sequenced bidirectionally using the BigDye Terminator v3 kit and run on an ABI 3500 DNA analyser (ABI). The forward and

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reverse sequences were subjected to CAP3 Sequence Assembly Programme (Huang and Madan, 1999) to obtain contig sequences.

2.5 Data analysis

The sequences were aligned using ClustaIW and potentially misaligned sequences were excluded. Pair wise evolutionary distance was determined by the Kimura-2-parameter method using the soft ware programme MEGA 5. The number of polymorphic sites and nucleotide diversity (pi), haplotype diversity and nucleotide composition were determined. Gaps were considered as missing data on the phylogenetic reconstructions. Neighbor Joining (NJ) tree was constructed using the sequences generated in the present study. The new sequences are deposited in Gene Bank and the details are provided in Table 1.

3. Result and Discussion

A total of 16Cyt B sequences were generated representing 16 species belonging to 6 orders and 12 families. All the sequences were trimmed at 417 bp. Number of polymorphic sites (S) were determined as 201. Invariable sites and singleton variable sites are 216 and 29 respectively. Parsimony informative sites were found as 172 with 16 haplotypes. Haplotype diversity was found to be 1.00 and Nucleotide diversity was 0.211. Nucleotide composition analysis revealed the mean frequencies for the complete data set to be 26.22 %for T, 32.93 % for C, 28.49 % for A and12.96%.for G. The percentage GC ratio was found to be 45.3%.

Table -2: Genetic distance (%) calculated using Kimura 2-parameter model based on Cyt b sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Gonoproktopterus_Kurali															
2	Salmophasia_Boopis	0.243														
3	Etroplus_Suratensis	0.312	0.296													
4	Devario_Malabaricus	0.271	0.293	0.358												
5	Horabagrus_Brachysoma	0.189	0.256	0.291	0.288											
6	Hyporhamphus_Limbatus	0.281	0.305	0.269	0.338	0.267										
7	Wallago_Attu	0.232	0.301	0.291	0.322	0.209	0.302									
8	Cyprinus_Carpio	0.130	0.223	0.290	0.276	0.207	0.273	0.253								
9	Cirrhinus_Mrigala	0.147	0.256	0.302	0.278	0.247	0.280	0.256	0.118							
10	Macrognathus_Guentheri	0.262	0.301	0.222	0.314	0.291	0.256	0.270	0.252	0.266						
11	Clarias_Dussumieri	0.191	0.248	0.308	0.264	0.167	0.305	0.213	0.188	0.203	0.258					
12	Puntius_Mahecola	0.155	0.228	0.338	0.279	0.243	0.331	0.299	0.141	0.155	0.255	0.203				
13	Megalops_Cyprinoides	0.206	0.250	0.265	0.275	0.223	0.259	0.253	0.209	0.232	0.241	0.192	0.216			
14	Ompok_Bimaculatus	0.239	0.282	0.312	0.298	0.163	0.272	0.164	0.232	0.222	0.267	0.194	0.253	0.210		
15	Mystus_Gulio	0.234	0.272	0.352	0.300	0.173	0.328	0.236	0.240	0.270	0.324	0.185	0.232	0.252	0.234	
16	Mesonoemacheilus_Triangularis	0.176	0.275	0.298	0.266	0.246	0.306	0.305	0.205	0.193	0.284	0.254	0.230	0.225	0.284	0.261

When representative Cyt B sequences for the 16 species were compared with existing data, 63% of species shared 97- 100 % identity with existing gene bank database entry. The remaining 37 % species were not compared with existing entries as they were the first submissions for the species. They are *Salmostoma boopis*, *Hyporhamphus limbatus*, *Wallago attu*, *Macrognathus guentheri*, *Mystus gulio* and *Mesoneomocheilus triangularis.* The NJ tree including 16 species was given in Fig. 2. All the species were clustered separately with considerable genetic distance (Table 2). No taxonomic deviation was detected at the species level.

Cyt b gene has been considered as one of the most useful genes in studying the phylogenetics and molecular systematics. In Kerala, irrespective of the high diversity of freshwater fish species, availability of molecular markers are very less which may hinder the molecular level studies on fish fauna of Kerala.

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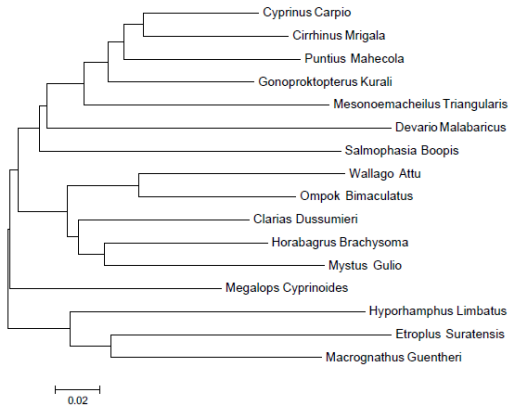

Fig.2: NJ tree of Cyt b sequences of 16 species used in the present study

3.1 Conclusion

In this study, we successfully amplified a 417 bp long Cytochrome b sequences from 16 common fishes found in the Manimala River of Kerala, India. The universal primer pair we used in this study was efficient to amplify the target region. Gnetic relationship between species was shown in NJ tree. Each species was associated with a specific DNA sequence cluster.

Mitochondrial DNA sequences are preferred for forensic, zoological and molecular analysis of vertebrates (Berlin *et al*., 2007) because of the desirable features such as high copy number (2-10 copies per cell and as many as 1,000 mitochondria per somatic cell) compared to just a single (or a few) copy of nuclear gene(s). The recent developments in the molecular biology has revolutionized the molecular taxonomic studies by comparing the DNA sequences obtained from a bit of tissue or a drop of blood with a reference database without sacrificing the entire animal (Galtier *et al*., 2009). Further, PCR amplification and sequencing of mitochondrial DNA using universal primers has been a reliable tool for the forensic analysis of samples in many laboratories (Kocher *et al*., 1989; Sandeep, 2012). Unfortunately, mitochondrial DNA sequences of most bird species of India are not available in Gene bank (http://www. Ncbi.nlm. nih. gov). The limitation of this approach could be the non-availability of the sequence of a rare fish species in the database. The developments of mt DNA markers for the Indian fishes are limited (Persis *et al*., 2009; Lakra *et al*., 2009; Krishna *et al*. 2012). Hence prior authentic availability of mt DNA gene sequence in the data base is a mandatory criterion.

The population of many species has declined and hence their status has reached critical limits. In order to strengthen legislation and assists in conservation of Indian fish fauna we need to have reliable taxonomic analysis tools. In this direction the approach given in this paper will provide a comprehensive insight for the identification and differentiation of unknown fish species. To conclude, PCR amplification of the mt DNA marker, sequencing and analysis would help to solve the problem of identification of fish species unambiguously from a bit of tissue.

4. Acknowledgement

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