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UTILITY OF MITOCHONDRIAL ATPASE 8 GENE SEQUENCE IN IDENTIFICATION OF INDIAN CARPS

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Identification of fish species is essential for better broodstock management as well as for the detection of unintentional or fraudulent species substitutions in the market place. Twenty seven individuals from 4 Indian carp species were analyzed to assess the utility of mitochondrial ATPase 8 gene sequences in identifying four cultivable carp species i.e, *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* and *Labeo fimbriatus* from India. Results of sequencing and alignment of the full length ATPase 8 gene of the four species revealed that there were enough species specific signatures which could be used for differentiating these four species. The phylogenetic analysis of Indian carp species by the Kimura 2-parameter method showed that each species forms a separate clade, and *L. rohita* is more closely related to *L. fimbriatus* than *C. catla* and *C. mrigala*. This study using only a very short sequence of ATPase 8 gene may help in identification of carp species as well as detection of fraudulent substitution of fish species.

INTRODUCTION

Biosurveillance and conservation management depends crucially on the correct identification of the specimens (Hebert *et al*., 2003). It is essential for authentication of fish species at the spawn and fry stages for better broodstock management. The possibility of genetic contamination of native and cultured stocks can be a serious problem involving the process when the production and commercialization of hybrids are carried out in the places of parental native species occurrence (Toledo-Filho *et al*., 1994). As escapes of individuals produced in fish farms into the natural environment are very frequent (Orsi and Agostinho, 1999), the occurrence of ecological disequilibrium (Einum and Fleming, 1997), genetic introgression and even the extinction of native species may occur (Allendorf *et al*., 2001; Epifanio and Philipp 2001). But beyond the harmful environmental effects of hybridization, the loss of the native genetic pool of distinct parental species may preclude the development of future breeding programmes and the commercial production of these species.

Indian carps, *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*, *Labeo fimbriatus* and some other species are economically important freshwater fishes in Indian subcontinent. These

species are of commercial significance due to their aquaculture potential and high consumer preference. Identification of fish species is traditionally based on external morphological features, including body shape, patterns of colour, scale size and count, number and type of fins and rays and, various relative measurements of body parts. In some cases morphological features are of limited value for identification and differentiation purposes, even with whole specimens, because they can show either considerable intraspecific variations or small differences between species (Teletchea, 2009; Chen *et al.*, 2012). Molecular identification procedures generally include the analysis of proteins by electrophoretic techniques, such as sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), capillary electrophoresis (CE) or immunoassay techniques (Huang *et al.*, 2008). Several molecular biotechnology methods based on polymerase chain reaction (PCR) have been developed, including single nucleotide polymorphism (SNP) (Itoi *et al.*, 2005), nested PCR (Pardo and Perez-Villareal, 2004; Zhang *et al.*, 2007), multiplex PCR (Lin and Hwang, 2008), real-time PCR (Itoi *et al.*, 2005; Trotta *et al.*, 2005), PCR - restriction fragment length polymorphism (PCR-RFLP) (Zhang *et al.*, 2007) and denaturing gradient gel electrophoresis (DGGE) (Comi *et al.*, 2005). However, compared with proteins, the DNA is more stable for species identification because it is less affected by thermal treatment than the proteins (Lin and Hwang, 2008; Chen *et al.*, 2012). Mitochondrial DNA analysis is the most preferred for taxonomy and evolutionary studies due to its fast evolutionary rate, high number per cell and maternal mode of inheritance (Wirgin *et al.*, 2002; Dowling *et al.*, 2008). It is widely used to determine the variation at interspecific and intraspecific levels (Avise *et al.*, 1986; Avise, 1998). DNA barcoding is a well known approach to identify specimens independent of morphology (Hebert *et al.*, 2003). Many mitochondrial genes like Cyt *b*, 12S rRNA, 16S rRNA have been used as a marker for identification of fish species (Chen *et al.*, 2012; Cawthorn *et al.*, 2012).

Our objective was to evaluate the usefulness of mitochondrial ATPase 8 gene sequences in differentiating four Indian carps at DNA level. Information available on identification of carps is scarce. Our findings will have potential implications for identification, management and conservation of the commercially important carp species.

MATERIALS AND METHODS

Sample collection

A total of 27 samples from four species of carps, *L. rohita*, *C. catla*, *C. mrigala* and *L. fimbriatus* belonging to family Cyprinidae were collected during 2009–10 from the river Mahanadi (Latitude 20.27°N and Longitude 85.52°E). Morphological identification of species was done based on Talwar and Jhingran, 1991.

DNA isolation

Fin clipping was done from each individual fish, preserved in 95% ethanol and stored at -20 ^{0}C until DNA extraction. Total DNA was isolated from fin tissue by proteinase K digestion followed by standard phenol and chloroform extraction (Sambrook *et al.*, 1989). The DNA samples were then resuspended in 1X TE buffer. The concentration and purity of isolated DNA was estimated at wavelength 260/280 nm using a UV spectrophotometer.

Amplification and sequencing

The complete ATPase 8 gene was PCR amplified in a 25 µl reaction volume with 1X PCR buffer (Genei, India), 0.25 mM of dNTP mix, 10 pmol of each primer, 0.25U of Taq polymerase and 50 ng genomic DNA using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA). The primer pairs used for PCR were L8331 (5′AAAGCRTTRGCCTTTTAAGC 3′) and H9236 (5′ GTTAGTGGTCAKGGGCTTGGRTC 3′) (Thai *et al.*, 2004). The PCR temperature profile used was 1 cycle of initial denaturation at 94 °C for 4 min followed by 34 cycles (denaturation: 94 °C for 30 s, annealing: 55 °C for 1 min and extension: 72 °C for 2 min) and a 7 min final extension at 72 °C. PCR products were checked on 1% agarose gels and the most specific and intense products were selected for sequencing. Amplified PCR products were purified using the PCR purification kit (Qiagen, Netherlands) and 200 ng of purified PCR product was used for cycle sequencing reaction. The same forward and reverse PCR primers were used for sequencing to get complete sequence of the fragment. Sequencing was done with an automated DNA sequencer (ABI Prism 310 genetic analyzer, USA). Raw sequence data were edited manually and aligned using the software Bioedit version 7.0.9.0 (Hall, 1999) to get a consensus sequence of individual gene fragment.

Sequence analysis

The consensus nucleotide sequence of each gene fragment gathered from 27 individuals in four species was aligned using CLUSTALW (Thompson *et al.*, 1994). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 165 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION

Fish are the largest group of vertebrates which exhibit a remarkable diversity of morphological attributes and biological adaptations (Eschmeyer *et al.*, 1998; Nelson, 2006). Species are typically circumscribed based on the existence of fixed diagnostic morphological characters which distinguish them from other species (Wiens and Servedio, 2000). But for fishes, there are a large number of intraspecific variants or interspecific overlapping, so fish identification is challenging for taxonomists when facing rich biota. The limitations inherent in morphology-based identification systems and the dwindling pool of taxonomists call for the molecular approach to species recognition (Steinke *et al.*, 2009; Zhang and Hanner, 2011).

In this study, a total of 7 individuals of *L. rohita*, 7 of *C. catla*, 6 of *C. mrigala* and 7 of *L. fimbriatus* were used for sequence analysis of ATPase 8 gene. All the sequences generated in this study have been submitted to GenBank (Table 1). The 165bp nucleotide sequence of ATPase 8 was analyzed. The nucleotide frequencies observed were; A=35.6%, T=26.7%, C=26.6% and G=11.0%. In ATPase 8 gene, the most frequently occurring amino acids were Proline (14.8%) and Isoleucine (11.11%) whereas least frequently occurring amino acids were Aspartate, Glycine, Methionine and Glutamine (1.85%) each, followed by Cysteine, Arginine and Tyrosine (0.00%).

Table 1. List of species and GenBank accession numbers

Species	Accession number
Labeo rohita	JN859643 – JN859649
Catla catla	JN859671 – JN859677
Cirrhinus mrigala	JN859755 – JN859760
Labeo fimbriatus	KC990473 – KC990479

Among *L. rohita* population, no variation was found throughout the gene. However, 5, 12 and 2 variations were observed in *C. catla*, *C. mrigala* and *L. fimbriatus*, respectively (Fig. 1). As ATPase 8 gene is a conserved sequence, the variations found are unique to that particular species. By designing species-specific diagnostic primers, it would be a powerful and convenient technique for identification of fish species because of its simplicity, specificity, sensitivity and related inexpensiveness.

A major disadvantage of mtDNA sequencing is that the mitogenome is inherited maternally in most animals, and so hybrids possess only the genetic signature of the maternal parent. In cases where natural hybridization occurs mitochondrial data must be supplemented with molecular tools based on biparentaly inherited nuclear genes. Independent verification of such mtDNA identifications requires analysis of nuclear genetic loci, but this is technically more difficult than standard mtDNA sequencing (Rastogi *et al.*, 2007). Alternatively, mtDNA based markers are more suitable than nuclear markers. This superiority can be attributed to the fact that mtDNA evolves

Fig. 1. Alignment of mitochondrial ATPase 8 gene sequences in 4 Indian carp species

relatively rapidly at the sequence level because of inherent mitochondrial inefficiency to repair replication errors and DNA damage than nuclear DNA resulting in the accumulation of differences between closely related species (Brown *et al*., 1979). Mitochondrial DNA is used by evolutionary biologists to study the variations between human and other species in order to specify the possible relationships between them. The section most commonly used for these studies is cytochrome b gene which is considered to be useful for species identification and phylogenetic studies because it demonstrates high interspecies conservation and at the same time is variable enough to allow intraspecies differentiation. The present study, using four Indian carp species, demonstrated that ATPAse 8 gene could also be useful for identification of closely related carp species.

Phylogenetic relationship

NJ and UPGMA tree of the ATPase 8 gene from the 27 individuals of 4 Indian carp species were reconstructed (Fig. 2). In the complete ATPase 8 gene sequences (165 bp), 16 were variable sites, 16 informative sites for parsimony, 149 conserved sites, no singleton sites, 106 zero-fold degenerate sites, 35 two-fold degenerate sites and 21 four-fold degenerate sites. The phylogenetic tree indicated that each species forms their separate clade and *L. rohita* was more closely related with *L. fimbriatus* followed by *C. catla* and *C. mrigala*. The same result was shown in both NJ and UPGMA trees.

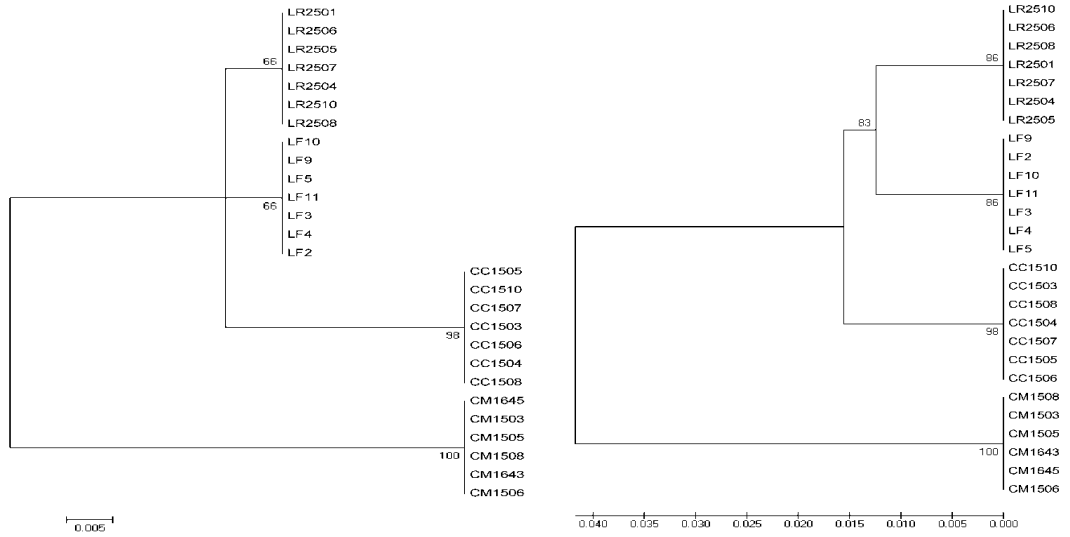

Fig. 2. Phylogenetic trees- NJ (left) and UPGMA (right) of mitochondrial ATPase 8 gene in rohu (LR), catla (CC), mrigal (CM) and fimbriatus (LF). The numbers at nodes indicate percent bootstrap values considering 50% majority rule consensus with 1000 replications.

In this paper, we report an alternative and efficient approach for species identification, which requires only a small amount of PCR product and sequencing. The ATPase 8 sequence can be used as a potential marker for identification of carps. This may be effective as a diagnostic marker, as it demonstrated consistent differences among closely related species and exhibited very limited intraspecific variation. The efficiency of such a DNA based identification system is promising with increasing taxon coverage of gene databases and availability of cheaper sequencing techniques. This study may fulfill the objective of identification of the Indian carp species.

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