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RESEARCH ARTICLE

Prospects of Molecular Characterization of Catla catla in Special Reference to PCR and Sequencing

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ABSTRACT

Conservation genetics or the application of genetics to the preservation of species has received increasing attention in recent years. Molecular DNA tools have been widely applied in different organisms in the past decades. Novel genetic technologies involving the use of DNA based tools are under development for a range of aquaculture species. These gene marker technologies can be used for identifying and monitoring of lines, families, and individuals, monitoring and controlling of inbreeding. Present investigation was aimed for molecular characterization of Catla species using mitochondrial cytochrome C oxidase subunit-I.

KEYWORDS

Catla catla, Gene Marker, Genomic DNA, Fish

INTRODUCTION

India is considered as one of the richest mega biodiversity centers in the world having two important biodiversity hotspots, the Western Ghat and the Eastern Himalayas. Nelson, 1984 estimated 21, 723 extant species of fish under 4,044 genera, 445 families and 50 Orders in the world[1]. Jayaram, 1981 listed 742 freshwater species of fishes under 233 genera, 64 families and 16 orders from the Indian region[2]. Talwar, 1991 estimated 2546 species of fish belonging to 969 genera, 254 familie and 40 orders.[3] The Indian fish population represents 11.72% of species, 23.96% of genera, 57% of families and 80% of the global fishes. It is well known that the Indian sub-region of the Oriental Region has perhaps the largest number of fish genera representing nearly 63% of the 89 primary fresh water genera known.[4,5]

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Biological diversity is 'the variety of life' which refers to variation at all level of biological organization.[6] Diversity includes diversity within species and among species. Traditionally the fish identification was established using a broad range of morphometric and meristic characteristic as, total length, fin characteristics including dorsal, ventral, pectrol, caudal fin height and length, scale's number etc.[7] Morphology remains the cornerstone of taxonomic diagnosis and has enabled the description of an estimated 1.7 million species, there are, however, limitations to relying on morphology in diagnosing life's diversity. The foundation for biodiversity and organic evolution is the genetic variation within species. The genetic variation in and between populations is the outcome of several factors,

such as mutation rate, breeding size of the population, breeding strategy, migration and, above all, selection.[8,9,10]

Jhingran, 1968 described the distribution of Catla catla, which starts from the Ganga river network in the north to the Krishna river down south of India, Pakistan, Bangladesh and Burma.[11] The fingerlings of catla, introduced into the Cuddapah-Kurnool canal from river Godavari in 1909 found their way into river Penna and the connected waters in the Nellore district of the state of Andhra Pradesh. Catla fingerlings were also introduced in Cavery river during 1920s and subsequently into the Bhavani. Later the species was introduced into Periyar lake, Powai lake.[12,13]

Jhingran, 1968 has also mentioned that Catla fingerlings have been also exported to Israel in 1954 and to Japan, and Mauritius in the 1960s. According to FAO Database on Introduction Aquatic Species (DIAS) Catla has been distributed to other countries also such as Zimbabwe, Israel, Bhutan, Philippines, Former USSR, Japan, Sri Lanka, Laos, Pakistan, Malaysia, Thailand, Vietnam and Mauriti.[11]

The limitations in the use of traditional methods necessitated the need for applying modern techniques for genetic identification and characterization of aquatic species.[14] The modern approaches in genetic characterization of aquatic species, as, sequence base studies of mitochondrial gene are COI sequences.[15,16,17,18] The advantages with mt DNA analysis lie with its maternal inheritance, which helps in tracing maternal lineages[19] and its rapid rate of evolution, which is about ten times more as compared to chromosomal DNA.[20] This increases the probability to detect genetic differences in different populations exhibiting mt DNA sequence divergence.[21]

MATERIAL AND METHOD

Sample Collection, Morphometric and Meristic Measurement

Samples of *Catla catla* were collected from small tributaries at Rewa [M.P]. Identification was done at species level that followed by (Talwar, 1991).[3] Fishes were counted and morphometric characteristics such as total length (cm), body weight (gm), body depth (cm) and head length (cm) were measured for taxonomic identification. All fishes caught in the net and measurements were taken. Fish samples were preserved in 95% alcohol and kept in the lab for identification.

The tissue samples were collected from the fishes with a forceps and scalpels and fixed in 95% alcohol in 1:5 tissues: alcohol ratio. The tissue samples were transported to laboratory at room temperature and stored at 4°C till analysis.

Fin Formula

D iii-iv 14-16; A iii 5; P i 20; V i 8

Characteristics

The distinguishing characteristics shared by ***Catla catla*** are: Body deep, its length 2.5 to 3 times in standard length. Head enormously large, Mouth wide and unturned, with a prominent protruding lower jaw. Pectoral fins long, extend to pelvic fins, Scales conspicuously large; lateral line with 40 to 43 scales.

Colour

Greyish on back and flanks, silvery-white below, fins dusky. Generally those inhabiting weedy or turbid ponds have a darker colour.

Isolation of Genomic Dna From the Ethanol Fixed Tissue

- Approximately 50 mg of muscle/fin tissue was taken in 1.5 ml eppendorf tube having 0.5 ml high T.E. The tissue sample was centrifuged at 10,000 rpm for 10 min at **4°C.** The high T.E. was decanted and the same step is repeated again.
- Tissue sample was suspended in 0.5 ml incubation buffer and sample and stored at **-40^{0}C** for overnight incubation.
- The tissue sample was homogenised on next day with the help of a micro pestle and simultaneously preparation of Incubation buffer was done (Incubation buffer contains 10 mg SDS per sample, and was kept at **37°C** until SDS dissolved completely) and

10µl (per sample) of Proteinase K was added at last to the Incubation buffer. 0.5 ml of above Incubation buffer was added to each of the homogenised tissue sample.

- The homogenised tissue were then transferred to the 50ml Oakridge tubes and kept for overnight incubation at **37^0 C** in water bath.
- After taking out the sample from the water bath 1 ml of incubation buffer was added in each tube for proper lysis of the samples.
- 2 ml of Tris-saturated phenol (pH 8.0) was added in each tube and was shaken (by repeatedly inverting tubes) slowly on rocker for 10 min. Then 2 ml Chloroform: Isoamyl alcohol (24:1 v/v) was added to each tube and again shaken slowly for 10 min on rocker, for the proper mixing of the solutions.
- Sample tubes were centrifuged at 10, 000 rpm for 10 min at **20°C**, supernatant was pipetted out, avoiding interface white layer. 2 ml of chloroform: Isoamyl alcohol (24:1) was added to supernatant in each tube, and was shaken slowly for 10 min. The sample tubes were then centrifuged at 10,000 rpm for 10 min at 20°C.
- Clear supernatant was pipetted out and 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 times ice-cold absolute ethanol (kept in freezer) were added to the supernatant. Slowly mixing of the tube was done and then tubes were kept on the ice for one hour. The samples were centrifuged at 10,000 rpm for 10 min at **4°C** and supernatant was poured out and pellet was marked out.
- 2ml of 70% ethanol was added to each tube and centrifuged at 10,000 rpm for 10 min at 4°C. Ethanol was discarded and the pellet was air dried for 10-15 minutes.
- After drying, TE (pH 8.0) was added and pellets were dislodged from the tube walls. Isolated DNA was kept at **4°C** for overnight.
- 1µl of RNAase was added in each sample tube and samples were kept at **37°C** in water bath for 2 hours. And then stored at **4°C.**

Determination of Quality and Quantity of Isolated DNA

Estimation of the DNA concentration was done on 0.7% agarose gels in submarine gel casting units (Wealtec, USA). The qualitative and quantitative estimation was done by observing the bands in ultraviolet light on UV transilluminator (GE Healthcare) with UV shield and UV protective goggles. The DNA was diluted to get a final concentration of 50 ng/µl. Stepwise procedure is given below.

- The casting unit was setup according to instructions of manufacture (WEALTEC, Electrophoresis unit)
- 210 mg agarose was dissolved in 3 ml of 5X TAE and 27ml double distilled water. Agarose solution was boiled for few seconds until the agarose get dissolved.
- 1.2µl of ethidium bromide is added before it gets cooled. The above agarose solution was cooled down to approximately 50ºC, and then it was poured in the casting plate with already adjusted gel comb. It was then left for solidification at room temperature for nearly 30 minutes.
- Cold 0.5 X TAE as gel running buffer was used. 2µl of DNA solution with bromophenol blue was loaded in the wells along with known quality of DNA in adjacent wells. It was run at 100mA/70 V for 15 to 20 min and the DNA band was observed with ultraviolet transilluminator.
- Visualization: DNA can be visualized in the gel with ethidium bromide. It is entrapped into the grooves of double stranded DNA and fluoresces in UV light. The high concentration of DNA in a band on a gel will fluoresces brightly a back ground of diffuse ethidium bromide in the gel matrix.

Polymerase Chain Reaction (PCR)

It is a technique in molecular biology by which a small fragment of deoxyribonucleic acid (DNA) can be rapidly cloned, or duplicated, to produce multiple DNA copies. PCR can be used to identify individuals from minute amounts of tissue or blood, to diagnose genetic diseases, and to research evolution. The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation [94•C]

During the denaturation, the double strand melts open to single stranded DNA.

Annealing [54•C]

The primers are jiggling around, caused by the Brownian motion. Primer length and sequence are of critical importance melting temperature increases with length and increasing (G+C) content. A common approximation of Tm for short sequences is

$$Tm = 4\ (G+C) + 2\ (A+T)$$

The annealing temperature for PCR depends directly on length and composition of the primers.

Extension [72•C]

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. The universal set of primers FishF1& FishR1 (Ward *et al*., 2005) were used to amplify the mitochondrial genes cytochrome c oxidase I (COI).

A 50 µl PCR amplification of mitochondrial genes COI was performed with 2 µl of each template DNA. The reagents procured from the Bangalore Genei (Bangalore) used in PCR reactions were 5 µl of 10X *Taq* polymerase buffer, 2 µl of $MgCl_2$ (50mM), 0.25 µl of each dNTP's (0.05mM), 0.5 µl of each primer (0.01mM) and 0.6 U of *Taq* polymerase. Thermal cycler MJ 21 Research PTC-200 (Watertown, MA) was used for PCR amplification. PCR products were visualized on 1.2% agarose gels documented using Gel Documentation system (Biorad). Products with concentration between 50 to 100ng per micro litre were selected for sequencing.

Sequencing Procedure

DNA sequencing is a very important technique by which the precise order of nucleotides in a piece of DNA can be determined. There are two different techniques: the chain termination method by F. Sanger and A.R. Coulson in U.K, and the chemical degradation method A. Maxam and W. Gilbert in the U.S.A., both techniques allow DNA sequences of several kb in length to be determined in the minimum of time. The DNA sequence is now the first and most basic type of information to be obtained about a cloned gene.

Once target sequences were selected and successfully amplified, sequence reactions were performed. Sequencing was performed following the dideoxynucleotide chain termination method, using automated techniques in ABI 3730 sequencer. Once target sequences were selected and successfully amplified, Sequencing PCR Products were labelled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc).The composition of sequencing reaction is as follows-:

Table 1: List of primers used for amplification for cytochrome oxidase subunit- I mitochondrial regions

Sr. No	Mitochondrial region	Primer name	Primer sequence (5' - 3')	Length (bp)	Reference
1	Cytochrome c oxidase -I	FishF1	TCAACCAACCACAAAGACATTGGCAC	26	Ward *et al*., 2005
		FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	26	

Ethanol Precipitation

1. 1µl of 7.5 M Ammonium acetate was added to each reaction tube.
2. 27.5µl of 100% Ethanol was added to each reaction tube & mixed by inverting the tube several times. The final concentration of ethanol should be 70%.(This is a critical step & final ethanol concentrations < 65% produce weak signals while concentrations 75% result in sequences with "blob" artifacts due to precipitation of unincorporated dye terminators.)
3. The tubes were centrifuged at either room temperature or 4°C in a micro centrifuge for 15 minutes at ~12 000 rpm.
4. The supernatant was removed from each micro centrifuge tube by aspiration to prevent dye blobs.
5. DNA pellets were washed with 70% ethanol and the tubes were briefly centrifuged.

Resuspension of Samples

1. Pellet was dissolved in 10µl of Mega BACE loading solution & vortex vigorously for 10-20 seconds to ensure complete resuspension.
2. Tubes were briefly centrifuged to collect the samples at the bottom of the well & to remove bubbles.

Detection on an Automated Sequencer

The fluorescently labelled fragments that migrate through the gel are passing a laser beam at the bottom of the gel. The laser exits the fluorescent molecule, which sends out light of a distinct color. That light is collected and focused by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a CCD camera (**c**harge **c**oupled **d**evice). Each base has its own color, so the sequencer can detect the order of the bases in the sequenced gene.

PCR amplified products of mitochondrial COI gene, were run on the 1.2% Agarose gel, along with, marker.

RESULTS AND DISCUSSION

Isolation of Genomic DNA from the Ethanol Fixed Tissue

Total genomic DNA was extracted from the ethanol fixed tissue of different species of Genus ***Catla catla*** (Cypriniformes), with Proteinase-K and phenol-chloroform method[22], which removes proteins and other cellular components from the nucleic acids and pure genomic DNA was obtained is shown in figure 1 in lane 1 and 7.

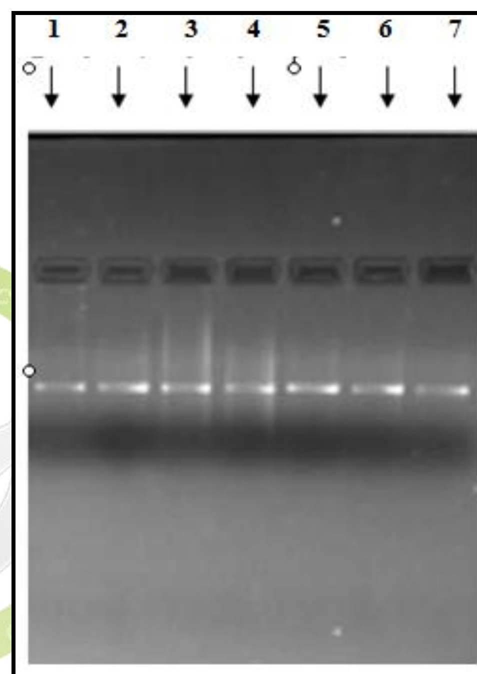

Figure 1: COI mitochondrial gene PCR amplified products (Lane 1& 7: *Catla catla)*

Cytochrome C Oxydase I

The samples of *Catla catla* were sequenced in the present study for cytochrome c oxidase I (COI) gene while the sequences of four species i.e., *Neolissochilus hexagonolepis, Barbonymus altus, Carassius auratus and Cyprinus carpio* were taken from NCBI-Genbank (accession no: EU714099.1, EF609294.1, EF609306.1 and EU524554.1) for comparative studies. There were a total of 655 positions in the final dataset.[23]

Nucleotide Sequence Variation Analysis

Out of 655 positions analyzed in cytochrome c oxidase subunit I (COI) gene, 38 sites were

observed as conserved while 617 sites were observed as variable and 151 as parsimoniously informative. Only 466 site was singleton i.e. observed in one sequence only. The sequences showed 25 four-fold degenerate sites representing all the synonymous changes. The overall average base composition was Thymine (T) 0.28%, Cytosine (C) 0.277%, Adenine, (A) 0.268 % and Guanine (G) 0.175%.[24,25]

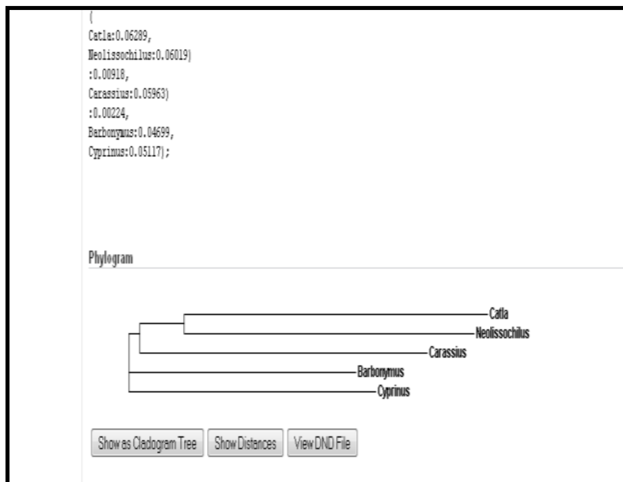

Figure 2: Cladogram and phylogram

All results are based on the pair wise analysis of 5 sequences. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Kimura 2-parameter method in MEGA4 (Kimura M., 1980, Tamura K et al., 2000). Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 655 positions in the final dataset.[26]

The number of base substitutions per site from averaging over all sequence pairs within each species (divergence within species) was calculated based on the pairwise analysis of 12 sequences. Standard error was obtained by a bootstrap procedure (1000 replicates). The estimate of average evolutionary divergence over sequence pairs between species (divergence between species) was calculated. The detail of divergence between the species is shown in given table (Standard errors are in square brackets).

	1	2	3	4	5
1. Catla catla					
2. Neolissochilus hexagonolepis	36.981				
3. Barbonymus altus	30.699	7.893			
4. Carassius auratus	11.656	7.095	0.118		
5. Cyprinus carpio	10.802	6.995	0.108	0.128	

Figure 3: Pairwise distance calculation of COI region

Phylogenetic Analysis

The optimal tree with the sum of branch length = 0.32685086 is shown in given figure. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

The Maximum Parsimony method was also used to construct the phylogenetic tree. The consistency index is 0.805687(0.646552), the retention index is 0.349206(0.349206), and the composite index is 0.281351 (0.225780) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The codon positions included were 1st+2nd+3rd. All positions containing gaps and

missing data were eliminated from the dataset (Complete Deletion option). There were a total of 655 positions in the final dataset, out of which 151 were parsimony informative.

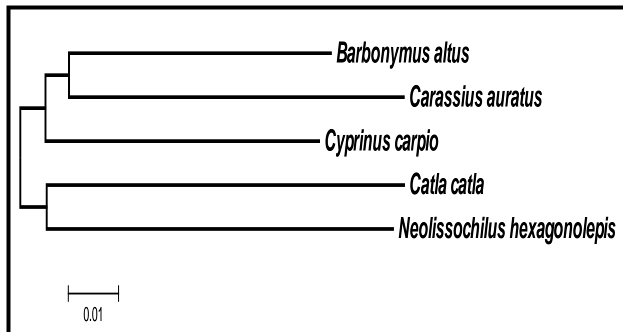

Figure 4: Neighbor-Joining phylogenetic tree

In the aligned sequences of the mitochondrial regions viz. COI a stop codons and indels were absent. Well defined electropherogram peaks were observed and co-amplification of nuclear pseudo genes did not occur. Base frequencies at the three positions of the codon were in accordance with the expectations of the vertebrate COI gene. Thus the sequences are believed to represent true mitochondrial gene sequences rather than numts. In COI, average guanine content is comparatively lower than the other three nucleotides. Within group average divergence of all the five species shows very less variation in the sequences that are taken into account.[27]

The phylogenetic trees drawn using both neighbor-joining and maximum parsimony gave consistent results (consistency indices for all sites in maximum parsimony analysis are 0.874074, 0.745455, and 0.750000 for COI. Tree constructed using neighbor joining method of all COI gave similar topology. These trees reflect their genetic relationship. On the whole this leads to the conclusion that Catla species is closely related to *Neolissochilus hexagonolepis, Barbonymus altus, Carassius auratus and Cyprinus carpio.*[28]

CONCLUSION

At present there is a large-scale aquaculture production of Catla species among the carps throughout the country. Various stressors pose a threat to the natural environment leading to loss in genetic diversity of species. It is very much important to know the genetic variance of a species before embarking on breeding programs and for taking necessary conservation measures. In this context these results demonstrates that mitochondrial DNA can be used as an effective technique for assessing gene variance and differences at species level.

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