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Original Article

Molecular insights into the genetic and haplotype diversity among four populations of *Catla catla* from Madhya Pradesh revealed through mtDNA *cyto b* gene sequences

R.K. Garg *, Vaishali Mishra

Centre of Excellence in Biotechnology, M.P. Council of Science and Technology (MPCST) ,Vigyan Bhawan, Nehru Nagar, Bhopal 462003, M.P., India

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ABSTRACT

In the present investigation, the genetic structure of four populations of *Catla catla*, sequences of mitochondrial gene, *cytochrome b* (*cyto b*) from four populations were sequenced and analyzed. The sequences of mitochondrial regions revealed high haplotype diversity and low nucleotide diversity. The lowest 249 polymorphic sites and 0.00 parsimony informative sites were detected in populations of Fish Federation Pond (CCFFB) whereas highest 330 polymorphic sites and 56 parsimony informative sites were detected in populations of Narmada River (CCNRH) in the *cyto b* gene sequences in *Catla catla* populations. The twelve different haplotypes were detected among the four populations studied, lowest population specific haplotype as 2.00 was observed in Fish Federation Pond (CCFFB) and highest was in Population of Narmada River and Tighra reservoir. Sequencing of *cyto b* gene revealed 12 number of haplotypes (*h*) with haplotype (gene) diversity (*Hd*) 0.8736 and nucleotide diversity (π) 0.6474. These data clearly indicated that, feral/wild population showing highest values of polymorphisms, parsimony, haplotype diversity showing good, healthy habitat is lotic water (Narmada River) and lentic water body (Tighra reservoir). The results also concluded that the partial *cyto b* is polymorphic and can be a potential marker to determine ecological habitat based genetic differentiation among the populations.

1. Introduction

The genetic variations and gene flows within population is extremely useful for gathering information on individual's identity, breeding patterns, degree of relatedness and disturbance of genetic pool among them [1]. The genetic variation of a species is distributed both within populations, expressed as differences between individuals, and between populations, expressed as differences in the presence and frequency of alleles.

The development of highly variable molecular DNA marker and statistical methodologies for interpreting genetic data has provided the opportunity to gain an intricate understanding of population characteristics such as dispersal and genetic structure, which is important for the successful management of threatened species both in the wild and in captivity [2–5]. Globally, declines of freshwater animals have been much greater than losses of animals in terrestrial systems, and freshwater fishes are among the world's most endangered vertebrates [6]. Most of the fish used for human consumption is obtained through exploitation of wild populations. India is endowed with rich fish genetic biodiversity i.e., 2,200 fish species and ranks 9th in term of freshwater mega biodiversity [7].

Catla catla is an important species in fisheries and aquaculture [8] which is naturally found in the Indo-Gangetic river system [9]. *Catla catla* is declining mainly due to overfishing and habitat alterations [10]. The *cyto b* region of mt-DNA is one of the preferred mitochondrial genes for studying intraspecific genetic diversity in fishes [11–13]. The detailed comparative studies on natural population, cultured population and population reared by federation is not available, so, the present investigation is aimed to assess the potential of mt-DNA *cyto b* gene based comparative studies for wild and cultured populations collected from four different ecotypes.

2. Materials and methods

Lotic habitat, lentic habitat, man-made Pond (Fish Federation Pond) and Culture Pond were chosen to give a good representation

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* Corresponding author at: Centre of Excellence in Biotechnology, M.P. Council of Science and Technology (MPCOST), (Deptt. of Science & Technology, Govt. of M.P.), Vigyan Bhawan, Nehru Nagar, Bhopal 462003, M.P., India.
E-mail address: rkgarg@mpcost.nic.in (R.K. Garg).

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of ecological and habitat variations (Table 1, Fig. 1) with respect to finding out of genetic traits among *Catla catla* populations. In this study, tried to analyze whether this species showing ecological based genetic variability which performed by mitochondrial DNA sequence (MTCYTOB gene) variations performed by a universal primers as forward: L14841 and reverse: H15149 (Table 2).

Catla catla populations were caught through commercial catches from above mentioned four sites i.e. Narmada River (n = 04), Tighra Reservoir (n = 04), Fish Federation Pond (n = 07 and Khatik Fish Farm (n = 05). Incisions (not more than 5–6 mm deep) were made and skin flap was removed. Small white muscle pieces were cut using surgical blade or small fine scissors. The muscles were kept on the aluminium foil labelled with fish number held over ice and the aluminium foil was folded as shown below and it was kept on ice temporarily and finally stored at –80 °C in the laboratory till further use for analysis. In the present investigation, molecular research methodologies were adopted to delineate the gene flow and hereditary traits among the *catla* populations.

2.1. Extraction of genomic DNA from tissues samples

Total genomic DNA was extracted as protocol provided by [14,15] with using some modifications. One hundred mg tissue sample was taken in pre-chilled eppendorf tube (1.5 ml capacity) and grinded tissue with the help of micro pestle within the tube. During grinding, added 0.5 ml of digestion buffer (100 mM Tris–HCl with pH 8.0, 10 mM EDTA with 8.0, 1.4 M NaCl, 1% SDS and 0.2% β-Mercaptoethanol) in tubes and added remaining 0.5 ml after grinding. Incubated samples at 50 °C for 30–60 min on dry bath with occasional shaking and then centrifuged at 5000 rpm for 10 min at room temperature. Collected supernatant in a fresh eppendorf tube and added equal volume of phenol:chloroform:iso amyl-alcohol (25:24:1) to the samples. Centrifuged again at 10,000 rpm for 10 min at 4 °C and transferred top aqueous layer to new tube. Added half volume of 7.5 M ammonium acetate and 2 volumes of 100% chilled ethanol. Tues kept in deep freezer for 1–2 h and centrifuged at 10,000 rpm for 10 min at 4 °C. Added 1 ml of 70% ethanol in the tubes for washing and centrifuged for 10 min at 10,000 rpm at 4 °C. Discarded upper aqueous layer and dried the pellet for 1–2 h at room temperature. Added 50 μl Tris–EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) and stayed for 2 h to dissolved the pellets.

Table 2
Primers used for PCR amplification of mitochondrial *cyto b* gene in the present studies on *C. catla* populations.

Primer Name	Sequence (5′ → 3′)
L14841	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA
H15149	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTCA

2.2. Quantification of extracted genomic DNA and integrity checking

The yield of extracted DNA from fish tissues in ng/μl was measured using a UV Spectrophotometer (ND-1000) at 260 nm and 280 nm wavelength. The purity of DNA was determined by calculating the ratio of absorbance at 260–280 nm. The ratio of absorption at 260 nm v/s 280 nm should 1.8 is commonly used to assess the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acid) tends to absorb at 280 nm. The DNA sample is considered as pure when the 260 to 280 ratio comes near 1.8. But the DNA sample having ratio 1.5–2.0

Table 1
Sample collection sites along with coordinates for estimation intraspecific genetic variance among *C. catla* species.

S. No.	Sample Code	Sampling Site	Geographical Coordinates	Samples Size (n)
1.	CCNRH	Narmada River, Hoshangabad	22.75°N 77.72°	04
2.	CCTRG	Tighra Reservoir, Gwalior	26°13′17.11″N 78°00′6.52″E	04
3.	CCFFB	Fish Federation Pond, Bhopal	23.2084°N, 77.3790°E	07
4.	CCKFB	Khatik Fish Farm, Bhopal	23.2437°N, 77.4731°E	05

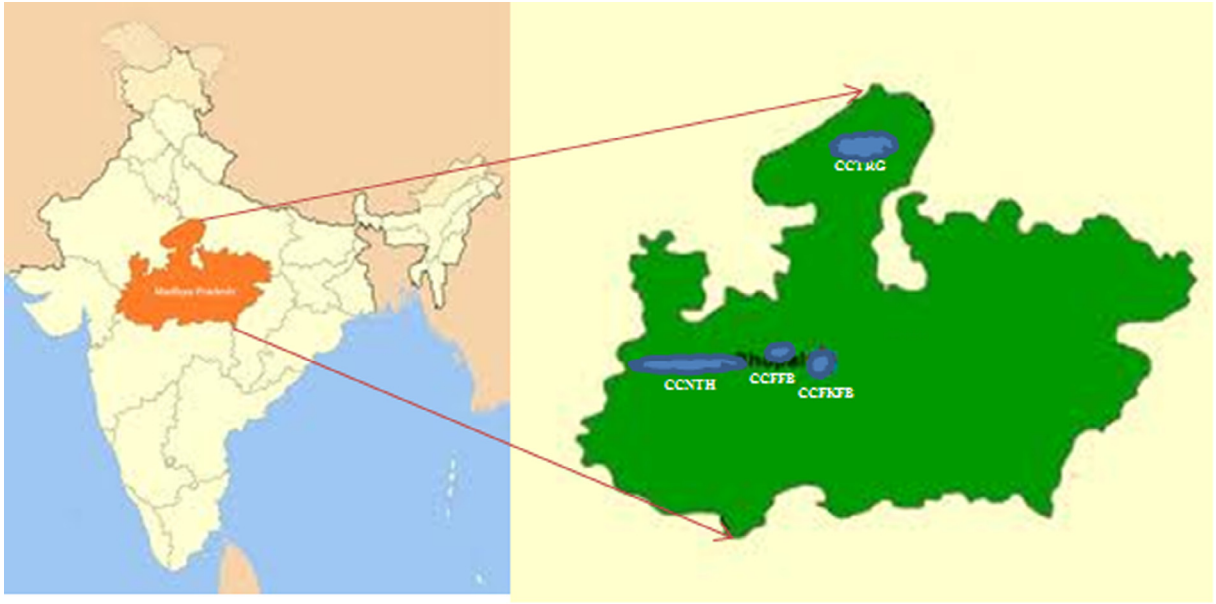

Fig. 1. Map showing samples collection sites i.e. Narmada River (CCNRH), Tighra Reservoir (CCTRG), Fish Federation Pond (CCFFB), Khatik Fish Farm (CCKFB).

can be easily used for PCR. After checking quality and quantity of DNA, the dilutions were made as desired for PCR amplification as 50 ng/μl or samples were treated with proteinse-K or RANase to get the pure DNA in the samples [16].

2.3. PCR programming for mtDNA cyto b gene amplification

Total genomic DNA was extracted from fish muscles tissues using the phenol-chloroform method [17]. Polymerase Chain Reactions (PCR) for mtDNA gene amplification was carried out in 25 μl reaction mixture which comprising 9 μl distilled water, 15 μl 2 × PCR master mixes, 3 μl templates DNA, 3 μl forward primers and 3 μl reverse primer. The amplification consisted of 30 cycles with an initial denaturation at 940 C for 5 min, denaturation at 940 C for 30 s, annealing at 550 C for 60 s and extension at 720 C for 90 s per cycle and final extension at 720 C for 10 min was done [18]. Mitochondrial DNA exhibits several properties that make it a useful tool in the study of phylogenetics, molecular evolution and even conservation genetics, due to its relatively simple genetic structure, maternal mode of inheritance (in most situations), and high rate of evolution/polymorphisms. Mitochondrial DNA (mtDNA) is characterized by its maternal inheritance, plain constitution, infrequent recombination, small molecular weight and elevated mutation rate in contrast to various nuclear markers. Therefore, mtDNA sequences have been very significant for studying of genetic analysis, investigation of quantitative trait loci, molecular evolution, disease identification [19,20].

2.4. DNA Sequencing and their analyses

The DNA fragment was excised from gel with sharp scalpel and cleaned. Weight the gel slice (desired fragment) and transferred to a 1.5 ml microfuge tube. 400 μl of binding buffer was added in 100 mg of gel slice in a tube (@ 0.40 μl/1 mg gel slice) and incubated at 50–60 °C for 10 min with occasionally shaking until agarose is completely dissolved. However, more concentration of the binding buffer (@ 0.70 μl/1 mg gel slice) may be use for high concentration gel (1.5–2.0%). Loaded above mixture in column (MX-10) and left stand for 2 min. Then, centrifuged at 10,000 rpm for 2 min and discarded the flow through in the tube. Added 500 μl of wash solution, and centrifuged at 10,000 rpm for 1 min and discarded flow through from the tube and repeated same procedure again to remove any residual wash buffer. Placed column in a cleaned microfuge tube with 1.5 ml capacity and added 30–50 μl of elution buffer at the centre part of the column then incubated at room temperature for 2 min. Samples centrifuged at 10,000 rpm for 2 min for elution of DNA.

Sequences were subjected to BLAST at the National Centre for Biotechnology (NCBI), website (www.ncbi.nlm.nih.gov/blast). All sequences of the mt-DNA (*cyto b* gene) of *C. catla* planning to submit in Gene- bank. Amplified *cyto b* gene was sequenced in both the directions to check the validity of the sequences data. All DNA sequences were aligned using CLUSTAL-W [21] and sequence composition was estimated using MEGA 5.0 ver software [22]. However, further molecular parameters of genetic diversity such as genetic differentiation values, nucleotide diversity, haplotype diversity, etc. were calculated by MEGA ver 5.0, DNASP ver 5.0 software [23].

3. Results and discussion

The *Catla catla* (Hamilton, 1822) fish is widely distributed in major rivers of India especially in gangetic river systems and their tributaries and comes under order Cypriniformes and family Cyprinidae. It is good for food for economically weak communities due to low price in the market. It has enormous potential for high productivity and is known for its nutritive and therapeutic qualities.

In the present investigation, we executed to find out the "Gene flow and hereditary traits depletion in *Catla catla* genotypes identified through mitochondrial DNA (*cyto b*) sequences variations" to assed the threats who effected on the gene and their diversity and conservation aspects. In this investigation the field level and performed *cyto b* gene sequences variations and tried to analyze whether this species showing ecological manipulation based genetic variability or not. Consecutively, we also worked on the nuclear DNA phenotypes variations based studies using random molecular primers for all four populations genotypes and obtained good results as expected.

The purity of extracted nuclear DNA samples was calculated from O.D. 260/O.D. 280 ratios [24]. When DNA shows ratio of absorbance values at wavelengths 260/280 as 1.8 it indicates the purity of DNA, When DNA shows ratio of absorbance values at wavelengths 260/280 as more than 1.8 it indicates the contamination of RNA in the DNA, when DNA shows ratio of absorbance values at wavelengths 260/280 less than 1.8 it indicates the contamination of protein in the DNA. During polymerase chain reactions (PCR) amplification, it is needed to have the DNA concentration close to the 50 ng/μl, therefore, all yielded DNA were diluted and adjusted the concentration 40 to 50 ng/μl using sterile Milli-Q water for further molecular experimental work.

Amplicons of the targeted gene were obtained as desired molecular sizes 700 bp (Fig. 2). Sequences of mt-DNA *cyto b* were examined in *Catla catla* samples from Narmada River, Tighra reservoir, Fish Federation Culture Pond and Khatik Fish Farm and tried to analyze whether the species is showing distance based genetic variability or not. Consecutively, we also worked on the DNA polymorphisms based on mt-DNA sequences variations for all four populations and obtained excellent results as expected.

In the present investigation, 20 genotypes of *Catla catla* and obtained single band of gene interest with molecular weight 375 bp which shows that the *cyto b* gene having good molecular weight when compared with Mandal [25]. Mean haplotype diversity (*Hd*) was found to be minimum 0.28571 in Fish Federation Pond population and variance of HD was 0.04351. The lowest variance of haplotype diversity 0.03125 was found in Narmada river and Tighra reservoir, while nucleotide diversity (π) was lowest 0.22975 was found in Fish Federation Pond (CCFFB) population. Haplotype diversity ranged from 0.28571 (Fish Federation Pond) to 1.00 (Narmada river and Tighra reservoir) and nucleotide diversity (π) from 0.22975 in Fish Federation Pond to 0.65750 in Narmada river (Table 2). DNA sequences of the mtDNA *cyto b* gene of CCFFB (n = 5) with accession number KU183619, KU183620, KU183621, KU183622, KU183623 and CCFFB (n = 7) with accession KJ462126, KJ462127, KJ462128, KJ462129, KJ462130, KJ462131, KJ462132 were submitted to NCBI, USA are available as reference sequences for public domain uses (Table 3).

The *cyto b* region was found to be polymorphic and the pattern of variation of nucleotide diversity congruent with pattern of haplotype diversity. Higher haplotype diversity and low nucleotide diversity indicated in Fish Federation population studied recently diverged from each other [26]. Such gene flow can potentially reduce genetic differentiation and be responsible for high within-population variations as observed in the *C. catla* samples between the Indus and Ganga river system [27]. The average frequencies of four nucleotide for all samples were 29.30 (T), 21.70 (C), 28.70 (A) and 20.30 (G) and were found to be A + T rich (58.00%). The observed A + T rich nature of nucleotide sequences of *cyto* b in *C. catla* was similar to that reported for many other fish species [28].

Polymorphic sites were obtained as 16 for CCNRH, 5 for CCTRG, 62 for CCFFB and 16 for CCKFB which clearly indicates that invariable sites is highest in CCFFB and lowest in CCTRG. Similarly, vari-

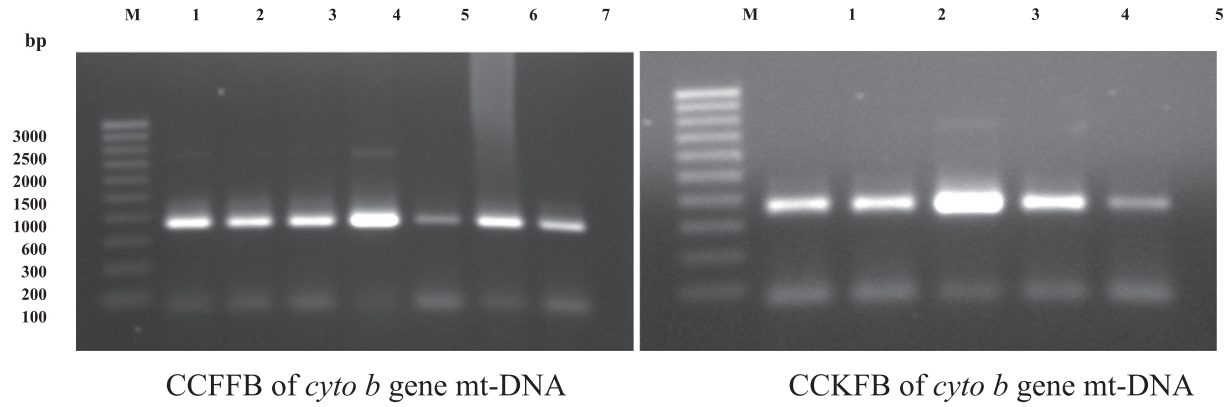

Fig. 2. Representative gel images of PCR amplified *cyto b* gene products in *Catla catla* populations using L14841 & H15149 primers.

Table 3
Sequences used for phylogenetic analysis, their accession numbers obtained from NCBI, and source of samples.

S. No.	Specimen code	Sample type	Source	Gene accession number	GI number
1.	CCKFB	Isolate-A	Khatik Fish Farm, Bhopal	KU183619	1023511449
2.	CCKFB	Isolate-B	Khatik Fish Farm, Bhopal	KU183620	1023511451
3.	CCKFB	Isolate-C	Khatik Fish Farm, Bhopal	KU183621	1023511453
4.	CCKFB	Isolate-D	Khatik Fish Farm, Bhopal	KU183622	1023511455
5.	CCKFB	Isolate-E	Khatik Fish Farm, Bhopal	KU183623	1023511456
6.	CCFFB	Isolate-A	Fish Federation Pond, Bhopal	KJ462126	602182695
7.	CCFFB	Isolate-B	Fish Federation Pond, Bhopal	KJ462127	602182697
8.	CCFFB	Isolate-C	Fish Federation Pond, Bhopal	KJ462128	602182699
9.	CCFFB	Isolate-D	Fish Federation Pond, Bhopal	KJ462129	602182701
10.	CCFFB	Isolate-E	Fish Federation Pond, Bhopal	KJ462130	602182703
11.	CCFFB	Isolate-F	Fish Federation Pond, Bhopal	KJ462131	602182705
12.	CCFFB	Isolate-G	Fish Federation Pond, Bhopal	KJ462132	602182707

able sites are highest in CCTRG and lowest in CCFFB. However, Singleton variable sites are higher in CCNRH and lower in CCFFB and CCKFB which clearly shows that Singleton sites is lower in culture ponds. In the present investigation *C. catla*, the *cyto b* region was found to be polymorphic and the pattern of variation of nucleotide diversity congruent with the pattern of haplotype diversity. Higher haplotype diversity and low nucleotide diversity indicated that the studied populations recently divergent from each other [29].

In Parsimony informative sites it shows that it is lowest in culture ponds than in Narmada River ad Tighra Reservoir. In similar way, total number of mutations is greater in Narmada River and Tighra Reservoir than in Fish Federation Culture Pond and Khatik Fish Farm which indicates correct study in *C. Catla* populations. Sequence conservation as 0.212 and monomorphic sites as 62 in CCFFB clearly indicated that Fish Federation Pond showing highly conserved population since it was cultured population (see Table 4).

Analysis of Conserved DNA region shows 1–353 for all four populations and total number of sites for all four populations as 353 and number of polymorphic sites is higher in CCNRH and CCTRG than in CCFFB and CCKFB which clearly indicates that number of variable/polymorphic sites is lower in culture ponds. Sequence conservation (C) is higher in culture ponds than in Narmada River and in Tighra Reservoir. Total number of variable sites clearly shows higher in CCNRH and CCTRG than in CCFFB and CCKFB. Haplotype diversity is higher in CCNRH and CCTRG than in CCFFB and CCKFB and nucleotide diversity is also greater in CCNRH and CCTRG than in culture ponds. Sampling variance is higher in culture ponds than in other two populations. Nucleotide diversity (π) was found to be greater in CCNRH and CCTRG than in other two populations of *C. catla.* Theta (per sequence) from Eta gives the maximum values for CCNRH and CCTRG than in population of culture ponds.

Haplotype distribution is also calculated as number of haplotypes (h) is more in CCNRH and CCTRG (4 and 4 respectively) and lesser in CCFFB and CCKFB (3 and 3 respectively)., haplotype diversity (Hd) shows good result in CCNRH and CCTRG as expected. The Gene Flow and Genetic Differentiation were analyzed, number of haplotypes (h) was 12, haplotype diversity (Hd) was 0.87368 whereas the average number of nucleotide differences (Kt) is 188.40000 and nucleotide diversity (π) was found 0.64742. The level of lower level of genetic divergence of *C. catla* observed in this study was slightly lower in Fish Federation pond population might be due to highly culture type of habitat (Tables 5and 6).

Table 4
Population wise genetic polymorphism and F-statistics detected in four different populations of *C. catla.*

S. No.	Statistical Data Name	CCNRH	CCTRG	CCFFB	CCKFB
1.	Monomorphic sites	16	5	62	16
2.	Polymorphic sites	330	342	249	275
3.	Parsimony informative sites	56	48	0	53
4.	Sequence conservation (C)	0.049	0.020	0.212	0.053
5.	Nucleotide differences (k)	227.167	258.00	71.381	156.00
6.	Number of segrating sites (S)	277	287	234	275

Table 5
Haplotype/nucleotide diversity detected in four different populations of *C. catla.*

S. No.	Statistical Data Name	CCNRH	CCTRG	CCFFB	CCKFB
1.	Number of haplotypes (*h*)	4	4	3	3
2.	Haplotype diversity (*Hd*)	1.000	1.000	0.524	0.700
3.	Variance of Haplotype diversity	0.03125	0.031	0.043	0.047
4.	Standard Deviation of Haplotype diversity	0.177	0.177	0.209	0.218
5.	Nucleotide diversity (π)	0.657	0.740	0.229	0.536
6.	Nucleotide diversity with JC (PiJC)	2.301	2.796	0.00	1.146

Table 6
Overall Haplotype/nucleotide diversity detected in four different populations of *C. catla.*

S. No.	Statistical Data Name	Total Data Estimates
1.	Number of sequences	20
2.	Number of segregating sites (S)	291
3.	Number of haplotypes (h)	12
4.	Haplotype diversity (Hd)	0.87368
5.	Average number of nucleotide differences (Kt)	188.40000
6.	Nucleotide diversity (π)	0.64742

The baseline information on genetic variation and the evidence of population sub-structuring generated from this study would be useful for planning effective strategies for conservation and rehabilitation of this highly endangered species. The overall genetic differentiation among 20 genotypes reflected that Hs as 0.62738 with Hst as 0.28191 obtained with degree of freedom df = 33.00 and p value of Chi 0.0117. Both data shows that, *catla* population studied having restricted gene flow and genetic variance as compared with populations studied by various scientists such as [28].

Molecular phylogenetic tree suggested that cultured and natural populations of *catla* species are genetically distinct to each other (Fig. 3). Four populations clearly distributed in two major groups with their respective individuals as Fish Federation Pond (CCFFB) and Khatik Fish Farm (CCKFB) grouped in a sub-tree and Tighra reservoir (CCTRG) and Narmada River (CCNRH) distributed in another sub-tree (Fig. 3). Cytochrome *b* gene of mtDNA showed that this type of molecular insights among the population of *catla* which is supported by other studies carried by mitochondrial gene [13,29].

The sequences of 20 individuals from four populations of *Catla catla* were matched with the sequences in NCBI and found 99% similarity in maximum number of individuals of *Catla catla.* The results demonstrate that the partial mt-DNA *cyto b* fragment (375 bp) can be used as a potential marker for studying variation both within as well as among populations of *C. catla* and can be useful in determining the distribution and pattern of genetic variation across a wide distributional range of native *C. catla* populations (see Table 7).

It is concluded that *cyto b* sequence is highly variable among wild population that is fishes from Tighra reservoir and Narmada River where genetic distance between them is more as compared to cultured populations fish federation pond (CCFFB) and khatik fish form (CCKFB). However, cultured populations showed highly

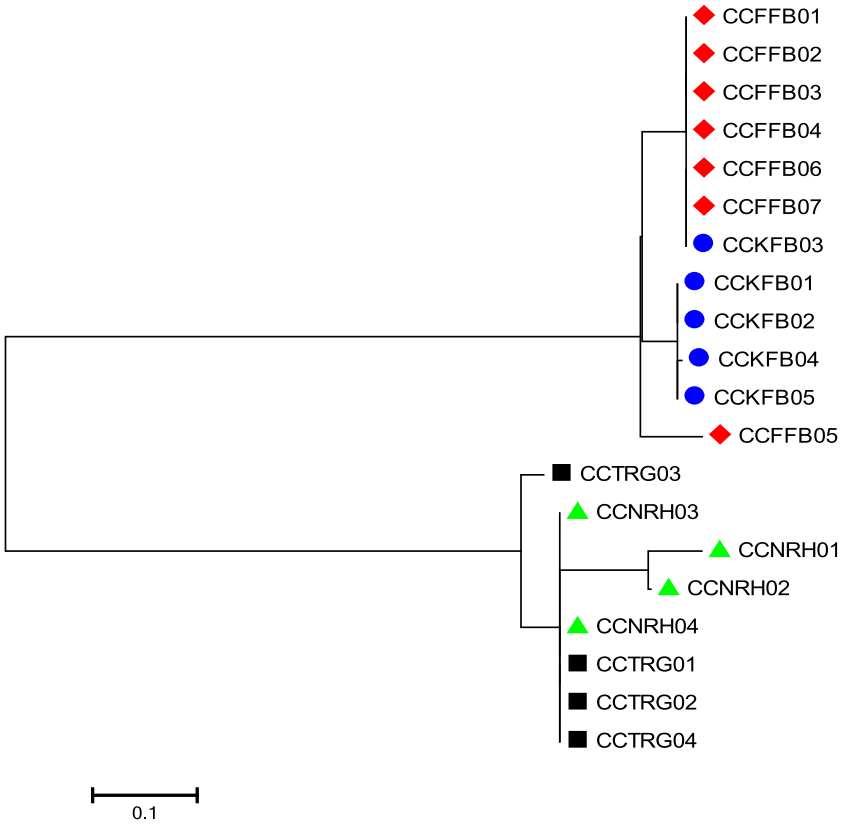

Fig. 3. Phylogenetic tree depicted for genetic relatedness among wild and cultured of populations of *C. catla* using *cyto b* region of mitochondrial DNA.

Table 7
Genetic differentiation among four populations *C. catla* populations.

S. No.	Population 1	Population 2	HS	GST	FST	DA
1.	Narmada River	Tighra Reservoir	1.000	0.000	−0.007	−0.005
2.	Narmada River	Fish Federation Pond	0.489	0.236	0.418	0.318
3.	Narmada River	Khatik Fish Farm	0.820	0.082	0.199	0.148
4.	Tighra Reservoir	Fish Federation Pond	0.489	0.236	0.345	0.255
5.	Tighra Reservoir	Khatik Fish Farm	0.820	0.082	0.164	0.125
6.	Fish Fed Pond	Khatik Fish Farm	0.441	0.263	0.364	0.219

conserved sequences of *cyto b* gene where the genetic distance between them is almost absent or present at low values. On other hand, phylogenetic tree showed the usefulness of *cyto b* sequences in the differentiation between catla different populations who were ecologically dissimilar and these advantages can differentiate mixed populations sources.

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