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Genetic divergence in wild population of *Labeo rohita* (Hamilton, 1822) from nine Indian rivers, analyzed through MtDNA cytochrome b region

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Abstract The present study examined partial cytochrome *b* gene sequence of mitochondrial DNA for polymorphism and its suitability to determine the genetic differentiation in wild *Labeo rohita*. The 146 samples of *L. rohita* were collected from nine distant rivers; Satluj, Brahmaputra, Son, Chambal Mahanadi, Rapti, Chauka, Bhagirathi and Tons were analyzed. Sequencing of 307 bp of Cyto *b* gene revealed 35 haplotypes with haplotype diversity 0.751 and nucleotide diversity (π) 0.005. The within population variation accounted for 84.21% of total variation and 15.79% was found to among population. The total Fst value, 0.158 ($P < 0.05$) was found to be significant. The results concluded that the partial cyto *b* is polymorphic and can be a potential marker to determining genetic stock structure of *L. rohita* wild population.

Keywords *Labeo rohita* · Cytochrome *b* · mtDNA · Polymorphism · Genetic divergence

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Introduction

Labeo rohita, an esteemed table fish, has high commercial and aquaculture importance across its native distribution ranging from Indus, Ganges and Brahmaputra river basins [1] and is also one of the choice fish for sustaining culture based capture fishery out of its native distribution. *L. rohita* is popular for polyculture with other Indian major carps in the countries; Myanmar, Bangladesh, Nepal and India up to Pakistan [2]. Aquaculture production of IMC was estimated to 4% of the world production [3], where as in India the production of *L. rohita* was 9,45,233 mt. Whole of the seed demand for culture of this fish is met through hatchery bred stocks and there is considerable progress to genetically improve the domesticated stocks through selective breeding. Like many cultured cyprinids (*Catla catla*, *Cirrhinus mrigala* etc.); wild population of *L. rohita* are at risk of loss of genetic diversity and variability due to extinction of genetically distinct wild stocks and mixing with the farmed accidental escapes or reservoir stock programmes [4, 5]. Natural genetic resources form the basis for selection of the founder stocks for the selection programmes. Knowledge of genetic diversity data could have a vital role in scientific planning of the breeding programmes for genetic improvement and effective management of the wild genetic resources. Identification of polymorphic molecular markers is a critical requirement in the investigation to determine genetic variation and divergence [6].

Mitochondrial DNA analysis is widely used in studying population structure of animal species. MtDNA has fast evolution rate and its maternal mode of inheritance make it a very potential genetic marker system, alone or in combination with other nuclear markers such as microsatellites, for analyzing population structure and phylogenetic studies [7–9]. Variation in mitochondrial cyto *b* region has been

used for population studies in fishes across taxonomic orders such as, *Acipenceriformes* [10]; *Squaliformes* [11]; *Salmoniformes* [12, 13]. The polymorphic cyto *b* region has been used in analyzing and determining genetic variability and diversity in cyprinid fish species [14, 15].

The identification of polymorphic molecular markers is a critical requirement in the investigation to determine genetic variation and divergence. The present study analyzes samples of *L. rohita* from nine riverine populations to determine genetic variation in the partial mitochondrial cyto *b* sequences. The objective is to evaluate the utility of partial cytochrome b sequence in determining genetic differentiation in wild *L. rohita* populations.

Materials and methods

Sample collection

Tissue samples of *L. rohita* were collected from nine different riverine catches including the Indus, Ganges, Brahmaputra and East Coast river system. The samples were collected from various localities, for river Satluj ($n = 25$) which belongs to the Indus river system from Harike patan (31°13′N, 75°12′E) Punjab and Bhakhra dam. For river Brahmaputra ($n = 15$) which originates from south-western Tibet and flows southwest through the valley of Assam and joins Ganga river system, samples were collected at from Kalangpar (26°11′N, 91°47′E) Assam. The samples were collected for river Chambal ($n = 14$) at Gwalior (26°56′N, 78°62′E) Madhya Pradesh, for river Son ($n = 15$) originates near Amarkantak ranges central India from Bansagar dam (24°45′N, 81°85′E) Madhya Pradesh, for river Rapti ($n = 20$) samples were collected from near Gorakhpur Uttar Pradesh (26°13′N, 83°10′E), for river Chauka ($n = 10$) were collected from the place near Tambore, Uttar Pradesh (27°21′N, 81°23′E), for river Bhagirathi ($n = 12$) which is the lower stretch of Ganga, samples were collected from Nabadeep Nadia, West Bengal (23°24′N, 88°23′E), and for river Tons ($n = 12$) at Chakghat, Madhya Pradesh (25°06′N, 81°45′E). The rivers Chambal, Son, Tons, Chauka, Rapti, Bhagirathi are distant tributaries of Ganga river. For river Mahanadi ($n = 23$) which is an independent river originating from central plateau in India and draining into the Bay of Bengal, sampling was done at Cuttack (21°58′N, 86°07′E). For all the samples, the blood was extracted through caudal puncture and fixed in 95% ethanol in 1:5 (blood:ethanol) ratio.

DNA extraction and PCR amplification

Total genomic DNA was extracted from blood using the phenol–chloroform method, protocol modified by Ruzzante [16]. Cyto *b* region was amplified with universal primers L14841 and H15149 [17]. The amplification consisted of 30 cycle with an initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s; annealing at 55°C for 60 s

Table 1 AMOVA analyses of cytochrome b sequences for nine populations of *L. rohita*

Source of variation	df	Sum of squares	Variance components	% of variation	Fixation index	*P* value
Among populations	8	21.574	0.12621 Va	15.79%	Fst: 0.158	0.00000 ± 0.00000
Within populations	137	92.220	0.67314 Vb	84.21%		
Total	145	113.795	0.79935			

Table 2 Population pair wise Fst (below diagonal), population specific Fst (at diagonal) and *P* values (above diagonal) between nine different populations of *L. rohita*

River	Satluj	Brahmaputra	Son	Chambal	Mahanadi	Rapti	Chauka	Bhagirathi	Tons
Satluj	**0.171**	0.144	0.000	0.000	0.027	0.000	0.000	0.009	0.000
Brahmaputra	0.050	**0.185**	0.000	0.009	0.081	0.108	0.018	0.000	0.000
Son	0.197*	0.118*	**0.065**	0.009	0.000	0.000	0.000	0.000	0.027
Chambal	0.209*	0.171*	0.137*	**0.180**	0.000	0.009	0.009	0.000	0.000
Mahanadi	0.061*	0.029	0.177*	0.173*	**0.177**	0.000	0.009	0.000	0.000
Rapti	0.115*	0.026	0.127*	0.150*	0.087*	**0.157**	0.009	0.000	0.000
Chauka	0.138*	0.163*	0.143*	0.202*	0.156*	0.153*	**0.162**	0.000	0.000
Bhagirathi	0.225*	0.225*	0.140*	0.302*	0.217*	0.202*	0.212*	**0.165**	0.000
Tons	0.240*	0.223*	0.092*	0.272*	0.222*	0.208*	0.229*	0.276*	**0.150**

* $P < 0.05$

Bold values indicate population specific Fst

Table 3 Relative haplotype frequencies between nine populations of *L. rohita*

Haplotype	Satluj (25)	Brahmaputra (15)	Son (15)	Chambal (14)	Mahanadi (23)	Rapti (20)	Chauka (10)	Bhagirathi (12)	Tons (12)
h01	0.64	0.667	0	0.429	0.6	0.667	0	0	0
h02	0.04	0	0	0	0	0	0	0	0
h03	0.2	0	0	0	0	0	0	0	0
h04	0.08	0	0	0	0	0	0.2	0.0833	0
h05	0.04	0	0	0	0	0	0	0	0
h06	0	0.133	0	0.143	0	0	0	0	0
h07	0	0.0667	0	0	0	0	0	0	0
h08	0	0.0667	0.133	0	0	0	0	0	0
h09	0	0.0667	0	0	0	0	0	0	0
h10	0	0	0.2	0	0	0	0	0	0
h11	0	0	0.0667	0	0	0	0	0	0
h12	0	0	0.0667	0	0	0	0	0	0
h13	0	0	0.2	0	0	0	0	0	0
h14	0	0	0.133	0	0	0	0	0	0
h15	0	0	0.0667	0	0	0	0	0	0
h16	0	0	0.133	0	0	0	0	0	0
h17	0	0	0	0.429	0	0	0.1	0	0
h18	0	0	0	0	0.08	0	0	0	0
h19	0	0	0	0	0.0435	0	0	0	0
h20	0	0	0	0	0.087	0	0	0	0
h21	0	0	0	0	0.0435	0	0	0	0
h22	0	0	0	0	0.0435	0	0	0	0
h23	0	0	0	0	0	0.1	0	0	0
h24	0	0	0	0	0	0.05	0	0	0
h25	0	0	0	0	0	0.1	0	0	0
h26	0	0	0	0	0	0.05	0	0	0
h27	0	0	0	0	0	0	0.2	0	0
h28	0	0	0	0	0	0	0.1	0	0
h29	0	0	0	0	0	0	0	0.333	0
h30	0	0	0	0	0	0	0	0.417	0
h31	0	0	0	0	0	0	0	0	0.0833
h32	0	0	0	0	0	0	0	0	0.0833
h33	0	0	0	0	0	0	0	0	0.25
h34	0	0	0	0	0	0	0	0	0.0833
h35	0	0	0	0	0	0	0	0	0.167

and extension at 72°C for 90 s per cycle and final extension at 72°C for 10 min. Amplification was carried out in 50 µl reaction mixture which comprising 7.5 µl distilled water, 5 µl 10× PCR buffer, 4 µl template DNA, 2 µl primer, 0.5 µl $MgCl_2$, and 1 µl Taq DNA polymerase.

Purification of double-stranded PCR products

Double stranded PCR product was purified using gel elution method from low melting agarose, in this the samples having sharp, clear and bright bands were separate out by cutting it without primer from the gel, after that the samples were again processed with phenol–chloroform method, protocol modified by Ruzzante [16].

DNA sequencing

The purified PCR amplicon was used in setting up sequencing reaction with same set of primers using Mega Bace ET Terminator Dye kit. The sequencing PCR was done as per recommendation of GE and comprised of 30 cycles of: 95°C for 10 s; 50°C for 20 s; 60°C for 2 min. PCR products were precipitated using ethanol and ammonium acetate and were dissolved in MegaBace loading

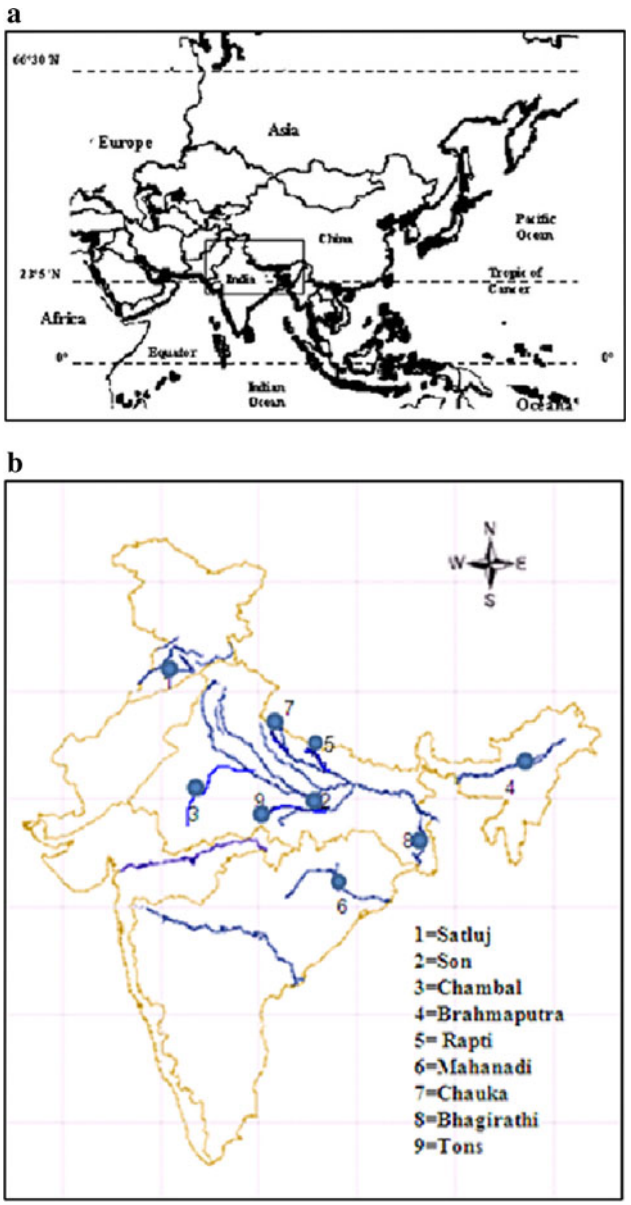

Fig. 1 **a** General map of the region, study area is located within the *box*. **b** Different collection sites for natural population of *L. rohita*

buffer. The DNA sequencing was done in an automated DNA sequencer, MegaBace 500 (GE Healthcare) using manufacturer's recommendations.

Analysis of DNA sequences

Amplified cyto b regions were sequenced in both the directions to check the validity of the sequence data. All DNA sequences were aligned using ClustalW [18] and were further analysed for determining parameters of population genetic variation. MEGA 4.1 [19] was used to estimate parameters of genetic variation. Sequence composition, molecular diversity indices, genetic differentiation and Fst values were calculated using Arlequin 3.11 [20] and haplotype diversity and nucleotide diversity was estimated using DnaSP 4.5 [21].

Results

Partial cyto *b* fragment (307 bp) was sequenced in 146 individual samples collected from nine different rivers to determine the genetic variability. The Genbank accession numbers of the mtDNA cyto *b* sequences are HQ315788–315821, JF717362–717380, JN020246–020272, JN034914, JN034915, JN034916, GU131016–131042 and GU292051–292080. A total of 29 positions were found to be variable with 35 haplotypes and 20 parsimony informative sites (Table 1, supplementary data). The average frequencies of four nucleotides for all the samples of *L. rohita* are A: 28.00%; T: 29.20%; C: 28.30%, G: 14.50%; Nucleotide sequences of Cytochrome b were A + T rich (57.30%) with transition to transversion ratio (Ts:Tv) was 4.825. Transitional substitutions were detected more commonly than transversional ones.

The nucleotide diversity (pi) for the all the nine populations was found to be 0.005 and haplotype diversity (Hd) was 0.751 with variance 0.001 ± 0.039. Hd for river Satluj was 0.563 with variance 0.01 ± 0.1, for river Brahmaputra it was 0.562 with variance 0.0206 ± 0.143, for river Son it was 0.914 with variance 0.001 ± 0.043, for river Chambal, Hd was 0.659 with variance 0.005 ± 0.072, for river Mahanadi, Hd was 0.518 with variance 0.014 ± 0.122, for river Rapti, Hd was 0.511 with variance 0.016 ± 0.128, for river Chauka was 0.822 with variance 0.009 ± 0.09, for river Bhagirathi it was 0.742 with variance 0.007 ± 0.084 and for river Tons Hd was 0.848 with variance 0.005 ± 0.074. The nucleotide diversity (π) for the populations Satluj, Brahmaputra, Son, Chambal, Mahanadi, Rapti, Chauka, Bhagirathi and Tons was 0.003, 0.002, 0.013, 0.003, 0.003, 0.004, 0.004, 0.004 and 0.005, respectively.

Out of total variation, only 15.79% was attributed to among population differences and 84.21% was due to within populations (Table 1). The Fst value was found to be significant 0.158 ($P < 0.05$). Population pair wise Fst values ranged from 0.000 to 0.265 (Table 2). The mean diversity for the entire population was 0.005 and the coefficient of differentiation for all nine populations was 0.106. The population mean distance within groups was ranged from 0.002 (Brahmaputra) to 0.013 (Son) and is in conformity with pattern revealed from haplotype diversity.

The most common haplotype h01, was present in all the populations except Son. Haplotype h04 was shared between Satluj and Bhagirathi samples; haplotype h06 was shared between rivers Chambal and Brahmaputra samples,

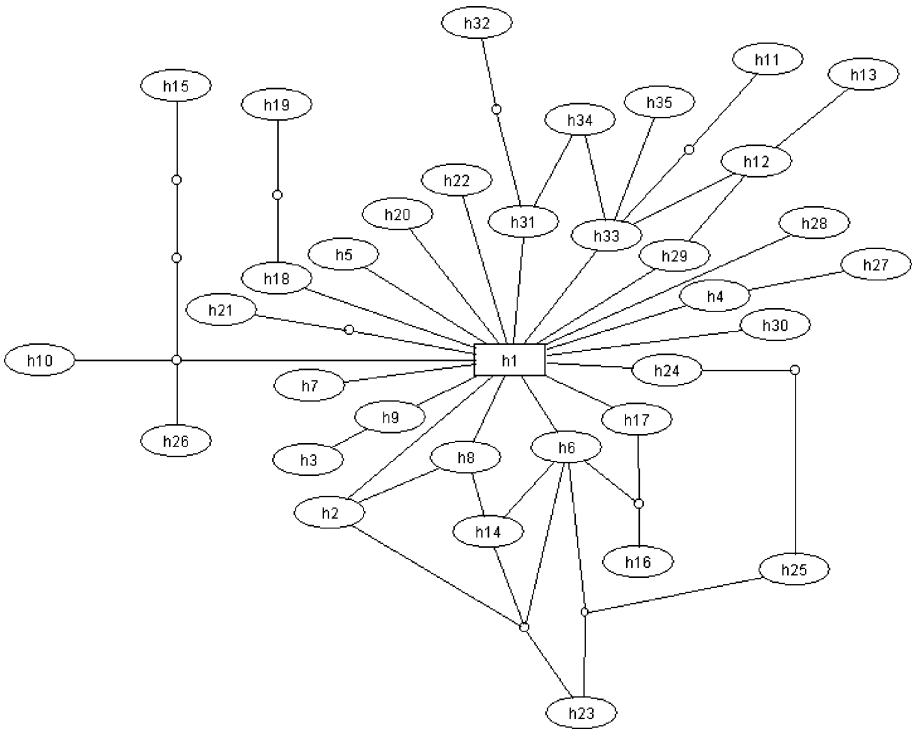

Fig. 2 Haplotype network obtained between nine different populations of *L. rohita*

haplotype h08 was also shared between rivers Son and Brahmaputra samples and haplotype h17 was shared between rivers Chambal and Chauka samples, Son exhibited with maximum numbers of eight haplotypes, followed river Mahanadi and Tons with five haplotypes each, and in river Satluj, Brahmaputra, Rapti and Chauka four haplotypes were observed in each. In river Bhagirathi three haplotypes were observed and two haplotypes were observed in river Chambal (Table 3). Haplotype network (Fig. 1) demonstrated formation of a single clade and all the haplotypes originated from the haplotype h01 directly or through subsequent mutations (Fig. 2).

Discussion

The population structure analysis of any species gives us that important information which is very useful for development the strategies for conservation and effective management for the natural and vulnerable fish population and in the present study the results clearly showed that for analyzing variation both within as well as among populations in *L. rohita* the partial mtDNA cyto *b* fragment of 307 bp is found to be a potential marker. The mtDNA cyto *b* sequences, analysed in the present study revealed moderate level of genetic differentiation in *L. rohita* wild population from nine different rivers and high within population variation. Nucleotide sequences of cyto *b* in *L. rohita* were found to be A + T rich (57.30%), which is similar to many other fishes [22]. The Transition and Transversion ratio (Ts:Tv) was also within the range reported for the cyprinids. The characteristics are concordant to that reported for fish cyto *b* genes [23].

The investigation was based on mtDNA to determine genetic variability and differentiation. Fragmented populations are expected to exhibit high genetic differentiation especially in freshwater species [26, 27]. The differentiation of haplotypes in *L. rohita* is supported by the results of AMOVA, for which it is found to be significant for genetic structuring into the nine populations. The Fst value was found significant 0.158 ($P < 0.05$) and the percentage of variation was expressed in the proportions; 15.79% among populations and 84.21% within populations. The populations analysed for *L. rohita* could have diverged recently from each other as it is evident from the haplotype diversity which was higher than that of nucleotide diversity [24].

The haplotype h01 was the most common and likely to be the ancestral and might be the source of origin from which all the haplotypes have originated, except Son, as the most common haplotype did not observed in Son. According to the distribution pattern and haplotype frequencies of these nine populations, except the most common haplotype h01 and shared haplotype h04, h06, h08 and h17, rest of the haplotypes are likely to be originated

independently through mutations. Sharing of haplotypes was found in h04, h06, h08 and h17 between Satluj and Bhagirathi, Brahmaputra and Chambal, Brahmaputra and Son and Chambal and Chauka respectively, this may be due to homoplasy where the convergence or parallelism takes place and the observed similarity evolves independently from different features in their common ancestor [25]. The observed moderate level of genetic differentiation, despite those populations from different river basins are fragmented, probably indicated the common ancestry in prehistoric period. This was followed by the possible exchange of individuals that could resulted in gene flow between populations in different river basins [28].

The results demonstrated polymorphism and the utility of partial cyto b mtDNA sequence to determine intraspecific genetic diversity and discriminate genetic stocks in wild population of *L. rohita*. The population genetics data thus generated will have wide application in planning breeding programme for aquaculture importance and conservation strategies.

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