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Assessing impact of dams on genetic diversity of native fish *Mastacembelus armatus* in river Yamuna using mitochondrial DNA cytochrome-b sequences as a molecular marker

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Abstract

Several dams have been constructed in Uttarakhand. In the present study an investigation on impact of dams on genetic diversity of native fish species *Mastacembelus armatus* at the Asan barrage on river Yamuna near Vikasnagar, Uttarakhand in India was done. Partial sequence of mitochondrial Cytochrome-b (Cyto-b) gene was used to determine the genetic variation in the population of *Mastacembelus armatus*. DNA was extracted from *Mastacembelus armatus* (n=33) samples, collected from river Yamuna and its tributary Asan from fin and fishes were released back in their habitat. Cytochrome c oxidase I (COI) was used to ascertain the species of fish along with morphometric characters. Analysis of 324 bp mtDNA fragment of Cyto-b revealed the presence of 06 haplotypes with nucleotide diversity, value ranged from 0.0172 to 0.0021 low pair wise Fst value was observed negative (-0.00125) when compared between Asan barrage and Kalsi site. No genetic subdivisions between the population were found after or before the dam sites. Tajima's D value for river Asan, site A1 Mirzapur (before dam) was -0.1167 (a negative value). Negative Tajima D value can be indicative of recent selective sweep or population expansion after a recent bottleneck and linkage to a swept gene. Our data shows that fragmentation of habitat by dams does not have any impact on the genetic diversity of non-migrating *Mastacembelus armatus* fish species.

Keywords: ***Anthropogenic activity, COI, Cyto-b, Garhwal Himalaya, Mastacembelus armatus***

Introduction

Fragmentation of river systems by dams is increasing every day because of human needs. Dam related anthropogenic activities are severe but underappreciated threat to aquatic biodiversity (Araújo *et al*. 2018). Because of time lag and interactions with other factors (e.g. climate change), impacts of aquatic habitat fragmentation on aquatic ecosystems are not visible immediately, and therefore these impacts are underestimated. Managing freshwater biodiversity thus requires deliberate management of aquatic habitat fragmentation (Mantel *et al*. 2017). Studies of aquatic habitat fragmentation in river networks assume importance and these studies advance our general understanding of ecological fragmentation. Effects of river fragmentation due to various reasons are well known on migratory fishes (Brown *et al*., 2013; Leeuwen *et al*. 2018). Sometimes, naturally occurring waterfalls also impact the geographic distributions of aquatic species. Anthropogenic activity like damming, road building, water withdrawal, Hydro Electric Power (HEP) project divided aquatic habitats of rivers into smaller patches. Human actions influence almost every aquatic ecosystem across the globe and only a few rivers remain untouched (Benke, 1990). In some cases it has been so severe that even the dams had to be removed or alternative measures like fish ladders were made mandatory. At some places anthropogenic fragmentation is developing rapidly and this rapid pace sometimes does not provide enough time to aquatic species to adjust and evolve alongside thus affecting the rich biodiversity that is harbored within freshwater ecosystems (Fischer & Lindenmayer, 2007). Changes in aquatic habitat also result in alteration in aquatic communities like macro invertebrates and aquatic flora that occur in the channel of natural rivers. Aquatic flora and

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fauna function in various ways and act as major force determining the species that can colonize particular aquatic ecosystem and following disturbances can alter drastically altering the biomass or, in extreme cases, eliminate species (James & Barko, 1990).

Garhwal Himalaya (Latitude: 30.8728 Longitude: 79.0593), located in Northern part of India is a part of Indian Himalayan Region (IHR, extending from Jammu & Kashmir to West Bengal of Indian Republic, (http://gbpihedenvis.nic.in/indian_him_reg.htm). This region has several fresh water systems and one of the aquatic biodiversity components is represented by fishes. Fish populations located in the remote regions of IHR are among the unique populations as they have dwelled in these segregated geographical niches since the origin of Himalayan range. Various researchers have reported hypothesis related to fish species diversity & richness patterns (Colwell & Coddington 2000; Mora *et al*. 2014; Rodrigues *et al*. 2017). In recent years, ecologists and conservationists have unanimously accepted that humans are also impacting species due to their various activities (Goudie, 2018). Habitat pressure have been said to cause genetic diversity / variations in populations. In this context, anthropogenic activity during construction of dams is a major activity that creates severe pressure on aquatic habitat along with its inhabitants (Waples *et al.* 2008). In Garhwal Himalayan region, a phenomenal number of dams have been constructed and several are under construction and still more are in planning phase (Thapliyal *et al*., 2019). As no long term study, using molecular tools, has ever been carried out to study the population dynamics of fish species in the area therefore in present study we tried to investigate the genetic diversity of native fish species *Mastacembelus armatus (Fig. 1)* around Asan Barrage in river Yamuna.

Fig. 1 Mastacembelus armatus

Material and Methods

Sampling site:

Samples were collected from river Yamuna and river Asan from various sampling sites using nets during April, 2012 - February, 2015 after due permission. At least 06 samples were sampled from each sampling station located at Mirzapur (code A1, $29^0$58'N & $78^0$11'E), before (code A2) and after (code A3) Asan Barrage ($30^0$43'N & $77^0$67'E), Kalsi (code Y1, $30^0$51'N & $77^0$84'E), Poanta Sahib (code Y2, $30^0$44'N & $77^0$61' E) and merger point of river Asan and river Yamuna (code Y3, $30^0$43' N, & $77^0$65' E). A small piece of fin was taken and preserved in 80% alcohol until laboratory analysis. After sampling fishes were released into their natural habitat without harm. Samples size was standard, n=6, but this was in compliance with our efforts to protect biodiversity.

DNA extraction, PCR and DNA sequencing:

Total genomic DNA was isolated from fish fin by using the standard phenol-chloroform isolation protocol as used earlier by Thapliyal *et al*. 2015. In short, DNA was isolated from sample and checked on 1.5% Agarose Gel. 10 Micro L of isolated DNA was used to quantify the amount of DNA in sample by using UV spectrophotometer (Nanodrop). Isolated DNA from samples was used as templates to amplify specific target gene, COI, by Polymerase Chain Reaction (PCR) using Primers: Fish F1 (5'–TCA ACC AAC CAC AAA GAC ATT GGC AC–3') and Fish R1 (5'– TAG ACT TCT GGG TGG CCA AAG AAT CA – 3') (Ward *et. al* 2005). For population genetics, primers used were FP (5-CACTGTAAAGCTAACTTAGC-3), RP (5-AGAATGACAGTACTGCGG-3) which targeted for specific region of mitochondrial Cytochrome-b gene. PCR amplifications were performed using MJ research thermo-cycler (model - PTC-200) in a 50µl reaction consisting of: 5µl of 10X buffer (100mM Tris, pH 9.0, 500mM KCl, 15mM $MgCl_2$, 0.1% Gelatin) (Genei, India), 200µM each nucleotide (dNTP, Genei, India), 5pmole of each primer (Sigma Genosys, USA), 1.5µl high fidelity taq polymerase (Genei, India) and 1-2µl of total DNA. The thermal regime consisted of an initial step of 3 min at 95°C followed by 34 cycles of 50s at 94°C, 1 min at 54°C and 45s at 72°C, followed in turn by 10 min at 72 °C. PCR products were stored at 4ºC. All PCR products were electrophoreses on

1.5% of Agarose gel followed by ethidium bromide staining and visualized under UV illumination (Fig 2a and 2b) in the Gel-Doc system (UVP) and purified using GeNeiTM Quick PCR purification kit (Genei, Bangalore, India). The PCR amplified fragments were cleaned for sequencing by using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). The sample was then finally sequenced bi-directionally using an ABI 3130 Genetic analyzer (Applied Biosystems).

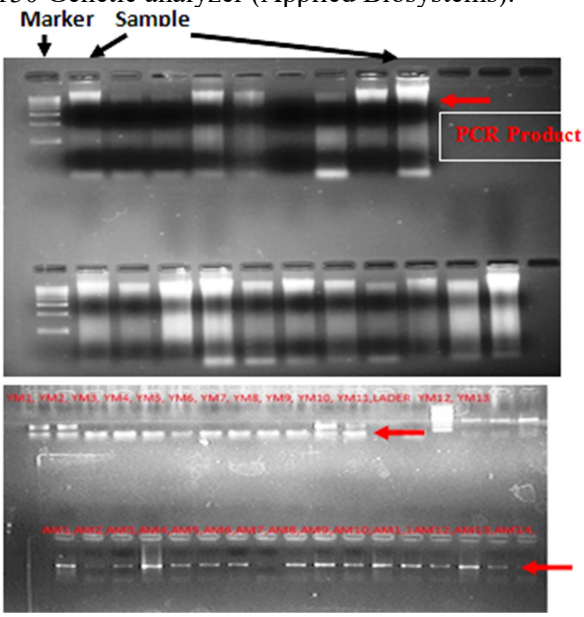

Fig. 2a. Cytochrome b mitochondrial DNA (sample loaded in Gel): Lane 1 is DNA marker:- Lane 2 onward Mastacembelus armatus samples from various location

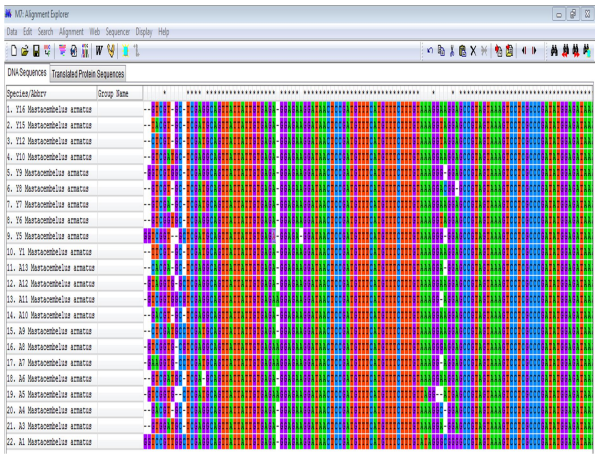

***Fig: 2b* Cytochrome b sequences were aligned by Cluster Omega using Bio Edit v5.0 Sequence editor**

Data analysis and Sequence alignments:

Phylogenetic comparison among the sample was carried out using MEGA 7.0.9 software. These sequence data was used to analyze population diversity. Two parameters of population diversity namely haplotype diversity (H) and nucleotide diversity (π) indices were tabulated by using DnaSP v5.10 (Librado and Rozas, 2009) and Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) software. The data generated through Arlequin software was further used to develop haplotype network using Network v4.6.1.1 software (Bandelt *et al*. 1999).

Results and Discussion

COI based identification:

A COI sequence of *Mastacembelus armatus* species were identified through COI based barcoding and identification technique, the sequence was then uploaded to the BOLD database/ NCBI with GenBank accession numbers: KC473940. The sequence was aligned using Clustal Omega and a sequence length of 651 bp was used for comparison. The sequence was checked for insertions, deletions, or stop codons before analysis. The COI data combined with the morphometric analysis enabled us to ascertain the fish species.

Phylogenetic and molecular evolutionary analysis:

Phylogenetic and molecular evolutionary analysis was conducted using MEGA 7.0.9 (Molecular Evolutionary Genetics Analysis) (Tamura *et al*. 2013). The sequencing data obtained for Cyto-b gene from the samples was analyzed using distance NJ (Neighbour-Joining) and Maximum Parsimony (MP) methods (Mount, 2008). Maximum parsimony is a robust and most commonly used method to construct Phylogenetic tree, whereas neighbor joining is a clustering algorithm-based phylogeny approach which construct tree by clustering haplotype based on genetic distance. The sampling error of NJ and MP trees was analyzed using bootstraps of 500 replicates. Kimura two-parameter model (Kimura, 1980) was used to check the pairwise sequence divergence among samples. Transitions / transversions in sequences were also checked. The analysis clearly shows that haplotypes were not localized to specific geographical location but were scattered to all locations (Fig. 3).

Analysis of Cytochrome b Sequence and genetic diversity:

Total 33 samples were used in the present study. A region of Cyto-b gene, which was 324 bp long, was amplified using PCR for *Mastacembelus armatus*. Both forward and reverse reads were obtained for each run and each sample was run two times to negate the sequencing errors. The amplified region was used to check the genetic variation among and within the geographically distinct populations of fish species. The sequence alignment was done using MEGA and Bio Edit v5.0 sequence editor. Sequences were analyzed using Arlequin software and the analysis revealed the presence of 06 haplotypes while the nucleotide diversity value ranged from 0.0172 to 0.0021 in *Mastacembelus armatus* (Table 1). Double population specific haplotype was observed in Yamuna and Asan. Data also found that these haplotypes were distributed across all sites.

Out of the 324 (bp) characters obtained 307 (96.03%) were constant (Invariable sites) and 13 (3.97%) were variable. The overall nucleotide base composition was as follows: A = 14.88 %, C = 31.23%, G = 27.60% and T = 26.29% and the C + G content were 58.83%.

Fst analysis:

The sequences were analyzed and compared using Arlequin software for genetic variations in *Mastacembelus armatus.* Comparison of data from study site before Asan dam and study site located at the merger point to Yamuna and Asan River, showed variations. For *Mastacembelus armatus*, the Fst value ranged from 0.099 (After Asan Barrage A3 Vs. Merge to Asan Y3) to 0.981 (Mirzapur A1 Vs. After Asan Barrage A3). Lower Fst values between Merge to Asan and Ponta Sahib indicate that these populations underwent some significant amounts of genetic exchange events (Table 2). Also, based on the AMOVA analysis, the majority of the variation was found to be within populations of this species (Table 3). Fst Analysis also revealed a negative low pair wise Fst value (-0.001258) between Kalsi (Y1) and after Asan Barrage (A3) which indicates that these populations underwent substantial genetic exchange events. This suggests that these locations were either connected at some point or the locations have regular floods and both these processes allowed mixing of populations.

The haplotype network of the samples from various locations was developed using Network v4.6.1.1 (Bandelt *et al.* 1999) software where the haplotypes pair wise differences were used to determine the number of mutational steps between haplotypes. Demographic expansion of *M. armatus* population in recent years at different sampling sites was checked using available molecular data. These tests were performed in Arlequin v3.1 using 1000 simulations under a selective model of neutrality and then the data was used to generate the haplotype network. This statistical test is designed to distinguish naturally evolving sequences (as naturally occurring mutation in sequences of specific genes and genome) from the changes in sequences that happen under non-neutral processes (including directional and balancing selection and demographic expansion or population contraction) (Ramos-Onsins and Rozas 2002, De Jong *et al*. 2011). The analysis suggests that these fishes from different locations were likely derived from different sources which have now merged and as they have genetic characters that are spread across all populations (Fig-3 & Fig. 4).

Table: 1 Cyto-b sequence divergence values for *Mastacembelus armatus* for populations from different sampling sites. Sequences were analyzed using Arlequin software to compute the number of haplotypes at each sampling site based on number of polymorphic sites in the sequences.

Fish populations	*Mastacembelus armatus*					
Parameters		**Sampling stations**				
	Mirzapur (A1)	Before Asan Barrage (A2)	After Asan Barrage (A3)	Kalsi (Y1)	Poanta Sahib (Y2)	Merge to Asan (Y3)
Number of Samples (n)	6	5	5	5	6	6
Number of Polymorphic sites (PS)	3	2	2	4	3	3
Number of Haplotypes (k)	1	1	1	1	1	1
Haplotype diversity (H)	1.0000	1.000	1.000	1.000	0.666	1.000
Nucleotide diversity (π)	0.0172	0.0102	0.0096	0.0086	0.0021	0.0096

Table 2: Population pair wise Fst comparisons for *Mastacembelus armatus* (below the diagonal) from different sampling sites.

Sampling stations	Mirzapur, (A1)	Before Asan Barrage(A2)	After Asan Barrage(A3)	Kalsi (Y1)	Poanta Sahib (Y2)	Merge to Asan (Y3)
Mirzapur, (A1)	0.00					
Before Asan Barrage(A2)	0.78378±0.0385	0.00				
After Asan Barrage(A3)	0.94595±0.0205	0.65766±0.0490	0.00			
Kalsi (Y1)	0.98198±0.0096	0.96396±0.0196	-0.001258	0.00		
Poanta sahib (Y2)	0.42342±0.0526	0.35135±0.0478	0.35135±0.0697	0.30631±0.0411	0.00	
Merge to Asan (Y3)	0.32432±0.0388	0.13514±0.0311	0.09910±0.0252	0.50450±0.0433	0.23423±0.0388	0.00

*significant p values (<0.05).

Table: 3 Analysis of molecular variance (AMOVA) analysis for *Mastacembelus armatus* based on mitochondrial Cytochrome b region. The data shows that the majority of the variations occur within populations for this species.

Source of variation	d.f.	Sum of components	Variance components	Percentage of variation
Among group	1	8.262	0.33129 Va	5.81
Within populations	16	90.167	5.63542 Vb	98.90
Total	17	98.429	5.96671	-

Va-Variation among group Vb-Variation within population
*Significant p values (<0.05), **Significant p values (<0.01).

Table: 4 A statistical Neutrality Test initially developed to analyze selective neutrality of mutations was implemented to test demographic expansion of *Mastacembelus armatus* in different Population. One site on the River Asan, site A1 (before dam) showed a negative Tajima D value. which implies that rare alleles are present at high frequency (excess of rare alleles)

	Mastacembelus armatus							
	Mirzapur, (A1)	Before Asan Barrage(A2)	After Asan Barrage(A3)	Kalsi (Y1)	Poanta Sahib (Y2)	Merge to Asan (Y3)	Mean	S.D.
Tajima's D	-0.11674	1.53598	0.69482	0.00000	0.00000	0.52223	0.43938	0.62770
Tajima's D p-value	0.60900	0.90100	0.79200	1.00000	1.00000	0.74200	0.84067	0.15504
Fu's FS	0.93592	0.52896	0.46229	1.33223	3.47266	0.01708	1.12485	1.23387
FSp-value	0.40400	0.35700	0.39700	0.51500	0.90700	0.31700	0.48283	0.21811

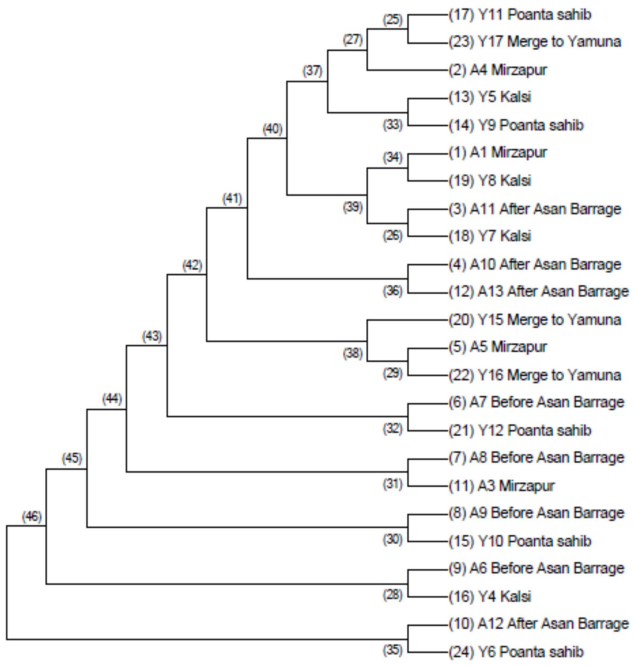

Fig: 3 Analysis of phylogenetic relationship between samples from various locations was carried out using Neighbor-Joining method (MEGA software, Bootstrap value obtained from 500 replicates). Analysis clearly shows that haplotypes were not localized to specific geographical location but were scattered to all locations.

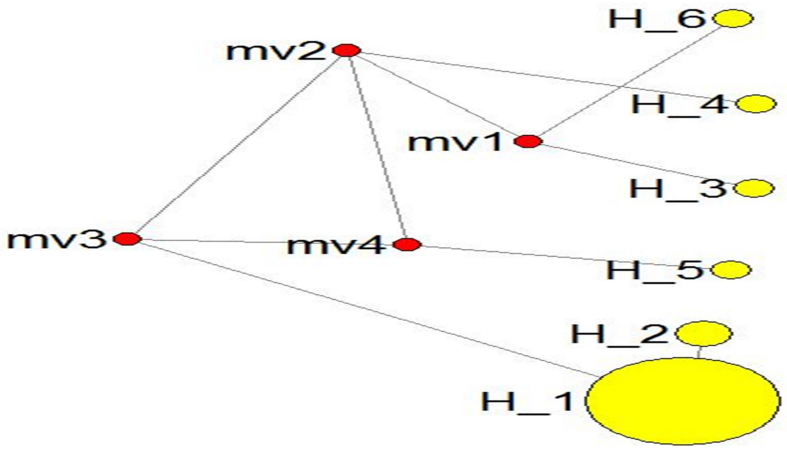

Fig: 4 Haplotype network of *Mastacembelus armatus* populations generated through throug Network v4.6.1.1 software. The network map strongly suggests that these fishes were likely derived from different sources and they have mixed haplotype distribution. The data suggests that the sampled fishes were likely derived from different sources which have now merged and they have common genetic characters that are spread across all populations.

The Cytochrome-b sequence data of *Mastacembelus armatus* were used in the present study and the fish samples were collected from river Yamuna and its tributary, river Asan, in Dehradun district. The specific 324 bp long region of Cytochrome-b was amplified using universal primers (Kocher, 1993). These primers have been used by other investigators also and they have been reported as useful tools in detecting intraspecific variation in several species. Most represented bases were in the following order; C<A<T<G in Cyto-b sequence of *Mastacembelus armatus***.** Nucleotide Sequences of Cytochrome-b *Mastacembelus armatus* have CG content as 58.83%. The 324 bp region of Cyto-b gene that we have used in our study is polymorphic and has been used successfully for intra specific genetic diversity analysis in various fish species, like *Salmotrutta* (Apostolidis *et al*. 1997), *Actinopterygii* (Buj *et al*. 2014) and *Latesca lcariferosteo brama belangeri.* AMOVA analysis revealed high within population variation in *Mastacembelus armatus* (98.90%) and low among group variation (5.81%). It is usually seen that the migrating fishes have a higher genetic divergence. Our analysis reveals that in case of *Mastacembelus armatus,* the genetic divergence level between populations was even higher than that reported for a migratory fish. Results of Fst analysis also support the presence of significant genetic difference between populations of river Yamuna between Kalsi (Y1) and after Asan Barrage (A3). A negative Fst value (of -0.001258) indicates that these populations underwent substantial genetic exchange events. The data suggests that it is likely that the populations being studied evolved after fragmentation from common ancestors and these ancestors spread to various locations due to anthropogenic pressure that was created during construction of Asan dam/barrage. Moreover, keeping the topography of the area in view of these study sites, it seems that at the location of merger point of river Asan onto river Yamuna, if the water levels exceed certain height, the area could have caused the populations to mix for a brief period resulting in distribution of haplotype uniformly in all sites. It is hence evident that populations of *Mastacembelus armatus* belong to common ancestral populations that are distributed uniformly.

Impact of habitat fragmentation by dams on genetic diversity has been well studied and most of the studies argue that the genetic diversity is reduced (Yamamoto *et al*, 2004, Morita *et. al*, 2009, Excoffier and Lischer 2010, Bouzat 2010, Fuller *et al*. 2015, Pavlova *et. al*, 2017, Barborossa *et. al*., 2020, Pereira *et. al*., 2020). However, authors have also reported that in some cases because of factors like permeability, the length of river that is fragmented and the ability of particular species to adapt fast, the dam related effect on genetic diversity can be counterbalanced (Reid *et. al*, 2008, McDermid *et. al*., 2014).

Conclusion

In our study, the River Yamuna (including its tributary river Asan) was selected as the study site because there has been extensive fragmentation of aquatic habitat in this region due to anthropogenic activity. The results clearly show that anthropogenic activity had almost no impact on genetic diversity and that habitat fragmentation due to dams has not led to segregation or genetic differences in population of *Mastacembelus armatus*. We hypothesize that the fish species inhabiting the unique Himalayan ranges have evolved plasticity of genome to accommodate drastic pressures of environment which might be due to several processes like flash flooding (either due to cloud bursts or due to lake formation after landslides in river valley's) or huge overflow of water during rainy seasons.

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