RESEARCH ARTICLE

# Genetic diversity of rohu, *Labeo rohita* (Hamilton, 1822) from Chenab River and its reservoirs

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***Labeo rohita*, the major carp (common name "Rohu") is a member of the *Cyprinidae* family and is among the ten most economically valuable aquaculture species worldwide. It is extensively spread throughout Pakistan's riverine systems. In Pakistan, like several other species of aquaculture, Rohu aquaculture has been facing problems regarding seed quality as well as inadequate genetic management of its brood stock. Moreover, in Pakistan, there is relatively less genetic information available on this species. The population of *L. rohita* in Punjab's riverine system has been endangered due to a few causes and genetic diversity and has declined mostly because of human interruptions causing major losses of fish population. Therefore, genetic monitoring of any species is essential not only for understanding its genetic changes but also for its proper conservational strategies. Various molecular techniques are available for complete analysis of genetic variations, but simple sequence repeat (SSR) markers have been proved the best tool from last few decades. This study involves 12 SSR markers for the assessment of genetic variation in 18 *Labeo rohita* (Hamilton, 1822) individuals which were collected from Head Muhammad Wala, Muzaffargarh, and Khangarh regions of Chenab River, Pakistan. After the extraction of DNA from fish muscles, the polymerase chain reaction (PCR) was done for amplification of target loci and products were resolved by 30% polyacrylamide gel. Results showed that the mean value of polymorphism for all markers was 78.63%. Out of 543 loci, 427 were found polymorphic in all samples. Allele number fluctuated from 2–7 while allelic frequency was 0.3333-1.0000. Gene diversities range from 0.0000 to 0.7901 and polymorphism information content (PIC) value was 0.0994 to 0.7618, which suggest that genetic structure of *L. rohita* has moderate to high variations. Values of Nei's genetic distance were 0.6667 and 0.7500 while Jaccard's similarity coefficient was 0.7500 and 0.9167, suggesting that genetic makeup of different *L. rohita* individuals of Chenab River differ from each other. This difference may be due to anthropogenic interventions, reproductive isolations, and environmental factors. Phylogenetic tree based on un-weighed pair group method with arithmetic mean (UPGMA) demonstrated that samples of one region showed similar genetic information, but genetic differences were more with the fish of other regions. It indicated that geographic isolation and different aquatic conditions influenced organism genetics.***

**Keywords:** *Labeo rohita*; simple sequence repeat markers; genetic diversity; polymorphism; Chenab.

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## Introduction

Among all the aquatic animals and plants, fish is the best source of food for human beings. Fish meat is a major source of dietary protein and poly

unsaturated fatty acids [1]. It is a rich source of iodine, carrier of a good amount of minerals, vitamins A and D, but low-fat content. Human can digest fish meat very easily. The fish population is a renewable source of nutrition and income for a country if it is exploited in a well-planned manner [2]. Economically, aquaculture practices provide investment and job opportunities to large number of interested people. Among the Indian major carps, rohu *Labeo rohita* (Hamilton, 1822) has become a popular table fish in South-East Asian countries like in Pakistan, Bangladesh, India, and Myanmar [3]. Due to its fast-growing nature and good taste, this species has the highest position in Bangladesh and Pakistan aquaculture practices [4]. *Labeo rohita* is among the top ten aquaculture species in the world [5]. Currently, public and private hatcheries provide 99% seed of *L. rohita* resulting in rapid expansion of *L. rohita* aquaculture [6]. In order to meet the requirements of fish meat for the increasing human population, it is necessary to establish good managemental programs, such as the estimation of genetic variability, morphological identification, and conservation.

In Pakistan, 60,470 hectares area is used for fish production, which earns 232.5 million US Dollars annually and contributes only 1% to the gross domestic product (GDP) of Pakistan [7, 8]. The major rivers in Pakistan are Indus, Chenab, Sutlej, Beas, Jhelum, and Ravi. All these rivers are the best homelands for Indian major and minor carps like Thaila, Rohu, Mrigala, Trout, Silver carp, and Grass carp, Khagga, Gulfam, Calbassu [9]. There are many genetic studies on fish, but unfortunately, genetic study on these species is not found in the literature from Pakistan. These fresh-water fish species are facing many anthropogenic and environmental problems that are reducing the population of these fishes [10]. Therefore, it is necessary to evaluate the genetics of these fishes, so that the important breeds can be conserved, and their population can be increased.

Fish faunal diversity refers to the alleles or genotypes within the fish population or it may refer to the variety of fish species [11]. The genetic diversity of the natural fish population is considered to be proportional to the mutation and effective size of population [12]. There are many factors, such as mutation, selection, genetic drift and recombination, which are responsible for genetic diversity. A high level of genetic variations in fishes can provide valuable genetic resources to broaden the genotypes [13]. Genetic diversity helps to maintain and protect the species even in the changing environment from extinction [14]. Similarly, loss of genetic diversity results in many problems like resistance against diseases, fixation of genes, viability, and loss of fitness in term of fecundity, vigor, and even may lead to extinction of local population. Thus, knowledge of genetic diversity and genetic variation should be used for the setting of genetic enhancement programs, prevention of natural genetic resources, and management [1]. The basic purpose of molecular analysis is to determine the differences among the individuals in mitochondrial haplotype or nuclear alleles.

In case of fisheries, genetic variations can be determined by using molecular markers. These markers are associated with certain locations (locus) and used to find specific sequence in the pool of unknown genome. Due to mutations (insertion, deletion, duplication, inversion, and point mutation), if base composition of every individual at any single locus of the genome becomes different, it is called polymorphism. In the fields of biology and biotechnology, there are various techniques which can be used to find out the level of polymorphism among different individuals of the population [15]. However, polymerase chain reaction (PCR) based markers like amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and simple sequence repeat (SSR) are the most suitable for the determination of genetic diversity among the members of any specie.

Among all the molecular markers, SSR markers are considered as the best tool due to having numbers of good qualities such as hyper-variability, multi-allelic nature, reproducibility, tractability to automation, co-dominant nature, relative abundance, comprehensive genome coverage, suitability for high-throughput genotyping, and chromosome-fixed location [16]. SSR markers have been used effectively for the estimation of genetic diversity in *Labeo rohita* and some other fish species [1, 17-20]. This is the reason that this study selected SSR markers for the determination of variations among *Labeo rohita* individuals of different areas of river Chenab. The objective of this study is to estimate the level of genetic variations and genetic relationships among geographically far-off *Labeo rohita* populations.

## Material and methods

**Samples collection and genomic DNA extraction**

Eighteen (18) samples of *Labeo rohita* were collected from three different areas of the river Chenab (Muzaffargarh, Head Muhammad Wala, and Khangarh) with 6 samples from each area. Samples 1 to 6 (MG1-MG6) were collected from Muzaffargarh area, samples 7 to 12 (HM7-HM12) were collected from Head Muhammad Wala area, and samples 13 to 18 (KG13-KG18) were collected from Khangarh area. All collected samples were packed in zip lock plastic bags and kept in ice box for transportation. Upon reaching the laboratory, fish samples were kept at -80°C in freezer until further use. Lab work was performed in the Genome Mapping Laboratory of the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan. Fish muscle genomic DNA was extracted by using Natalia *et al*. method [21]. Briefly, a piece of muscle (50 mg) was excised below the dorsal fin and was preserved in urea buffer containing 10 mM Tris HCl (pH 7.5), 8 M Urea, 125 mM NaCl, 1% Sodium dodecyl sulfate, and 10 mM EDTA (Sigma-Aldrich Ptv. Ltd, Darmstadt, Germany). Proteinase K (175 ug/mL) was added and incubated at 37°C for 8 to 16 hours for sample digestion followed by phenol: chloroform extraction and ethanol (2X volumes in 0.3 M NaCl) precipitation. The precipitate was resuspended in TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA). The extracted DNA quality was checked by 0.8% agarose gel electrophoresis. DNA was quantified by using a PerkinElmer Spectrophotometer (PerkinElmer, Waltham, MA, USA). The DNA working concentration of 40-50 ng/µL was then prepared for 20 µL PCR reaction mixture.

**Simple sequence repeats (SSR) primers and amplification**

Twelve pairs of SSR marker primers including Clone R-26F and Clone R-21F [22], Clone Lr33, Clone Lr30, Clone Lr36, Clone22, CloneLr38, Clone Lr32, Clone Lr44, Clone Lr31 [23], MFW11, and MFW1 [24] were used in this study for the analysis of genetic diversity of 18 *Labeo rohirta* samples (Table 1). PCR was performed by using Thermo Fisher Scientific PCR kit (Thermo Fisher Scientific, Waltham, MA USA). The total volume of PCR reaction was 20 µL with 2 µL DNA template (40 ng/uL), 2.5 µL 10X thermopol buffer, 2.5 µL dNTPs, 1 µL forward and 1uL reverse primers, 0.25 uL Taq DNA polymerase, and 10.75 µL PCR water. PCR was performed by using an Applied Biosystems GeneAmp 9700 PCR System (Thermo Fisher Scientific, Waltham, MA USA) with the following program: 1 cycle of initial denaturation at 95°C for 5 mins; 35 cycles of denaturation at 94°C for 1 min, annealing at 55-62°C (vary according to each primer) for 1 min, initial extension at 72°C for 2 mins; and final extension at 72°C for 10 mins. 30% polyacrylamide gel electrophoresis (PAGE) was performed for the detection and separation of the bands of PCR amplified products by using a horizontal electrophoretic apparatus (CBS scientific, San Diego, CA, USA). Gel was then passed through silver staining, which involves three steps including fixation, staining, and image developing. The fixation was done by immersing the gel in the solution containing 10% acetic acid, 70% ethanol, and 50% water for 30 mins. Staining of gel was performed by immersing the gel in 0.1% silver nitrate for 30 mins. Image of the gel

**Table 1.** Information of SSR primers.

| No. | Primer | Accession No. | Sequence (5'—3') | Annealing Temp. | GC Contents | Product size |
|---|---|---|---|---|---|---|
| 1 | Clone R-26F | AJ831439 | F: GCC ACC TGG ACG CTT TGA<br>R: GCT CTG TGC AAA GCC AGC | 58.40°C | 61.11%<br>61.11% | 272 bp |
| 2 | Clone Lr33 | AM269523 | F: CTT GCC GCT GTC TTT CGC<br>R: GCC ACT GTT TAG CTT CAC AGG | 59.85°C | 61.11%<br>52.38% | 114 bp |
| 3 | Clone Lr30 | AM231179 | F: CAT ACA CGC CGA CCT CCC<br>R: CCA GGC CTC TGT GCT TCC | 60.70°C | 66.67%<br>66.67% | 117 bp |
| 4 | Clone Lr36 | AM269526 | F: AGC GTG TCT GAT GTG TGA AAG G<br>R: TCA GAT GCC TCC TGC ATT CTG | 60.00°C | 50.00 %<br>52.38% | 181 bp |
| 5 | MFW11 | AY288921 | F: GTG AGC TGT CCT GGC CTG<br>R: GCC AAG CTT GCA TGC CTG | 59.55°C | 66.67%<br>61.11% | 133 bp |
| 6 | Clone22 | AM285342 | F: TCT GTG TGT GTG TGT GCG<br>R: ATG TGG AGG AAT GCC GGC | 57.25°C | 55.56%<br>61.11% | 388 bp |
| 7 | CloneLr38 | AM269528 | F: AGC TGT GCG ATT GCC CAT<br>R: GGT TTG GAA GCG CTC CCA | 57.25°C | 55.56%<br>61.11% | 130 bp |
| 8 | Clone R-21F | AJ831436 | F: GGT CAA TGT GGC TGA AAG GC<br>R: GGG GCT TCT CTG TCC GTG | 60.60°C | 55.00%<br>66.67% | 280 bp |
| 9 | Clone Lr32 | AM231181 | F: GGC TCT CAG AAG ACC AGC G<br>R: TCC CCT GCC GTT CTC TGA | 60.05°C | 63.16%<br>61.11% | 295 bp |
| 10 | Clone Lr44 | AM269534 | F: ATGTCCTCCCACCCCCAG<br>R: AAGAGCATCATGGCATTGACT | 57.60°C | 52.63%<br>55.00% | 277 bp |
| 11 | MFW1 | AY291594 | F: AGC CTG CTC TGC ATG TGA A<br>R: TCATGACAATGCAGCCTCTGT | 58.35°C | 52.63%<br>47.62% | 142 bp |
| 12 | Clone Lr31 | AM231180 | F: TCC CTC CCA CTC TGC CAG<br>R: GCG GTC TGT GGT GAG TCA | 59.55°C | 66.67%<br>61.11% | 250 bp |

was developed by placing the gel in developing solution containing 30 g/L of anhydrous-potassium carbonate, 37% formaldehyde, 10% thiosulfate solution. After the completion of silver staining, the image of the gel was observed under the Ultraviolet Trans illuminator. Different types of bands were visible on the gel and were scored by applying the software for further analysis.

**Scoring of Gel**

Scoring of gel was done manually by using code 1 for the presence of band in the image of the gel and code 0 for the absence of band. The scoring results were recorded at the bottom of the PAGE gel pictures as a spreadsheet. The number of rows indicates the number of alleles of each primer. Different alleles of each primer were represented by lowercase alphabets (a, b, c, and d) with "a" represented the lower band of each row on the gel, and "b", "c", "d" represented the upper bands, respectively.

**Analysis of data**

After scoring and making a spreadsheet manually, polymorphism percentage, number of effective alleles (NE), and Shannon information index (I) were calculated for the formation of genetic profile of all the *Labeo rohita* samples from different areas of river Chenab. Analogous of heterozygosity which is called Nei's genetic diversity (HE) was also calculated for the measurement of variations of genetic among randomly mating populations. PIC values were determined by using the formulation:

$$PIC_i = 1 - \Sigma_{j=1}^{n} (PIJ)^2$$

where i indicates marker, n and PIJ represent the number and frequency of alleles for that marker,


| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| b | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| c | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| d | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

**Figure 1.** Gel scoring method.

respectively. POPGENE version 1.32 (Developed by Francis Yeh, Edmonton, Alberta, Canada) was used for the calculation of Nei's genetic distance and genetic relatedness among the *L. rohita* samples. Cluster analysis was done by Un-weighed Pair Group Method with Arithmetic Mean (UPGMA) [25] and dendrogram was constructed with the help of MEGA6 software [26] based on Nei's pair-wise genetic distance.

## Results

SSR markers were used in this study in order to assess the genetic relationships among 18 *Labeo rohita* samples. PCR amplification produced 43 alleles with 543 loci by 11 SSR primers (Figure 1). Out of 543 loci, 427 were polymorphic in all samples. Therefore, the average polymorphism of all primers was obtained as 78.63%. Percentage for polymorphism along with total and polymorphic loci number for each primer is shown in Table 2. Six out of successfully amplified 12 paired primers showed 100% polymorphism.

Allele number fluctuated from 1.000 (Clone R-26F, MFW11) to 7.000 (Clone Lr 33) with the average of 3.58 and allelic frequency ranged from 0.3333 (Clone Lr 44, Clone Lr 33, Clone Lr38) to 1.0000 (Clone Lr 30, Clone Lr 32) with the average of 0.6389. Gene diversity ranged from 0.1049 (Clone R-21 F) to 0 .7901 (Clone Lr 33) with the average of 0.5624 and PIC value was 0.0994 (Clone R-21 F) to 0.7618 (Clone Lr 33) with the average of 0.4988. All these values suggested that genetic structure of *Labeo rohita* showed moderate to high variations. Mostly, Nei's genetic distance among the different populations of different areas of river Chenab showed values of 0.6667 and 0.7500, while Jaccard's similarity coefficient showed 0.7500 and 0.9167. All 18 samples of *L. rohita* made three clusters on

**Table 2.** Results of all genetic parameters.

| Markers | Allele frequency | $N_E$ | $H_E$ | PIC | Number of loci | | Polymorphism |
|---|---|---|---|---|---|---|---|
| | | | | | Total | Polymorphic | |
| **Clone R-26 F** | 0.6667 | 2.0000 | 0.4444 | 0.3457 | 21 | 21 | 100% |
| **Clone Lr 33** | 0.3333 | 7.0000 | 0.7901 | 0.7618 | 36 | 36 | 100% |
| **Clone Lr 30** | 1.0000 | 3.0000 | 0.7272 | 0.6695 | 54 | 54 | 100% |
| **Clone Lr 36** | 0.6111 | 4.0000 | 0.5679 | 0.4889 | 66 | 12 | 18% |
| **MFW11** | 0.6667 | 2.0000 | 0.4012 | 0.3207 | 31 | 18 | 58.04% |
| **Clone 22** | 0.6667 | 3.0000 | 0.4444 | 0.3457 | 48 | 36 | 75% |
| **Clone Lr38** | 0.3333 | 3.0000 | 0.6667 | 0.5926 | 43 | 43 | 100% |
| **Clone R-21 F** | 0.9444 | 4.0000 | 0.1049 | 0.0994 | 37 | 36 | 97.29% |
| **Clone Lr 32** | 1.0000 | 4.0000 | 0.7216 | 0.6785 | 50 | 50 | 100% |
| **Clone Lr 44** | 0.3333 | 4.0000 | 0.7222 | 0.6695 | 60 | 48 | 80% |
| **MFW1** | 0.6667 | 4.0000 | 0.4444 | 0.3457 | 60 | 36 | 60% |
| **Clone Lr31** | 0.6111 | 3.0000 | 0.7099 | 0.6681 | 37 | 37 | 100% |
| **Mean** | **0.6528** | **3.58** | **0.5624** | **0.4988** | **543** | **427** | **78.63%** |

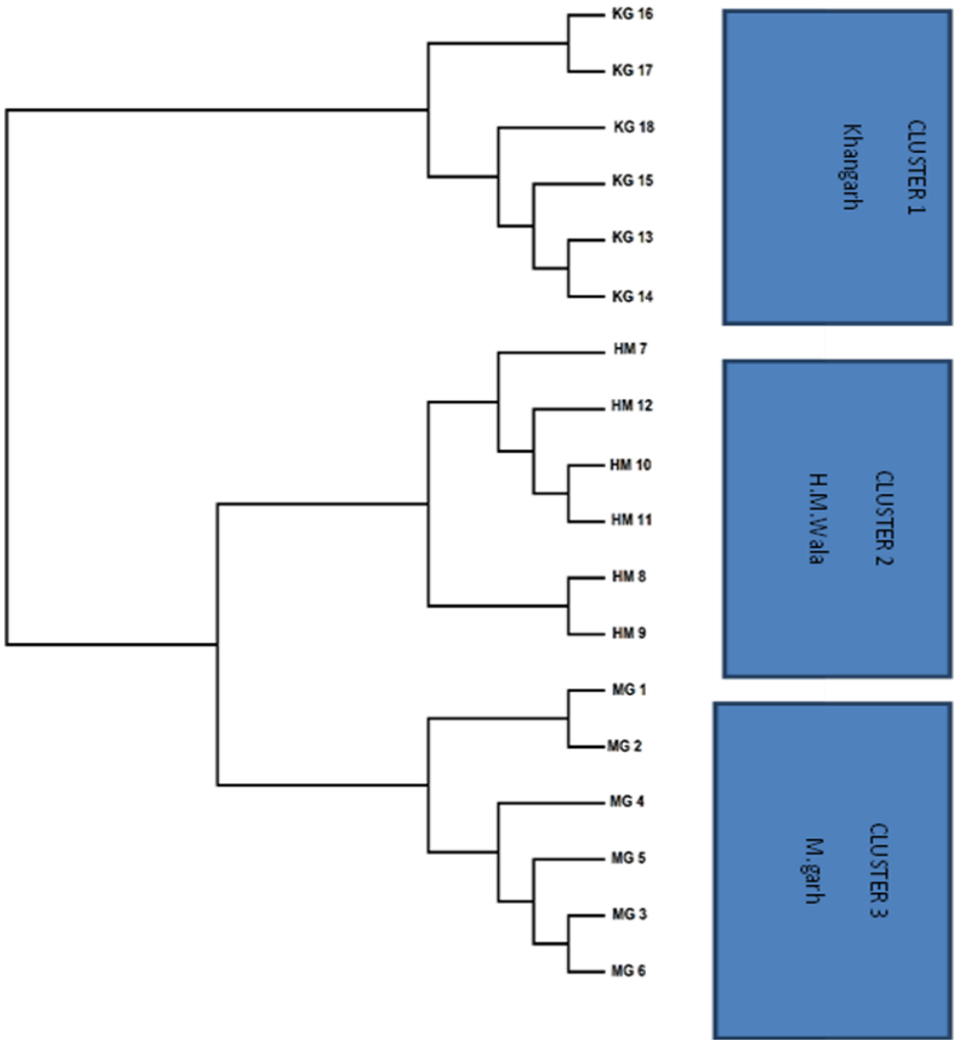


**Figure 2.** UPGAMA Dendrogram based on Nei's genetic distances.

Dendrogram namely C1, C2, and C3 for samples of Khangarh, Head Muhammad Wala, and Muzaffargarh, respectively (Figure 2).

## Discussion

Determination of genetic diversity is helpful for the development and conservation of species [27]. Based on various qualities like highly polymorphic, co-dominant, and reproducible nature of molecular markers, they are widely used in the genetic study of plant and animal species [28]. Large range of comparative allelic variations of germplasm can be detected easily with the help of these markers due to their multi-allelic nature [29].

In this study, 12 SSR markers were used for the estimation of genetic diversity among 18 *Labeo rohita* samples of different areas of river Chenab. All the SSR markers produced 543 clearly distinguishable bands. Out of 12 markers used, 6 were 100% polymorphic, but the average polymorphism shown by all markers obtained as 78.63%. Clone Lr33 showed the highest PIC value of 0.7618. According to the results of this study, all SSR primers successfully amplified DNA fragments from fish samples, which indicated that SSR markers are the best tool for genetic variation study.

In the previous study, twenty microsatellite markers were used by Danish and Singh [1] for the determination of genetic diversity of *Cyprinuscarpio var*. *Communis* and *Labeo rohita* (Hamilton, 1822). Observed heterozygosity in common carp was slightly high than *Labeo rohita*, which was possibly due to differentiated stocks, and observed heterozygosity slightly lower in Rohu than common carp was due to inbreed in successive generations. This result indicates that common carp is more diverse genetically than Rohu. In another study by the same authors on *Labeo rohita*, 20 microsatellite primers were used. The results of Nei's value, observed and expected heterozygosity, inbreeding coefficient (FIS), and Shannon's Information Index indicated that between wild and hatchery population of fishes, genetic variations and genetic differentiation existed. The possible reasons of this variation might be due to the absence of gene flow between populations, wide geographical locations, and difference in biological conditions of water bodies. The markers MFW1, MFW15, Cc7, and Cc8 were used by Paul *et al*. [30] for the determination of inter and intra population genetic variations of hatchery and wild populations of Indian major carp. The results confirmed that inter and intra population genetic variations existed in the study area of West Bengal. Species specific primers were used by Basharat *et al*. [31] for *Wallagoattu* which was collected from Jhelum River. The mean fixation index (FST) value was low (0.0248), which suggested small genetic differentiation. The average expected heterozygosity was 0.5363-0.5777 and the average observed heterozygosity value was 0.3450 to 0.4400. 81% variation was found within the population and 16% variations existed in between populations by Analysis of Molecular Variance (AMOVA). It is clear from the results of this study that level of genetic diversity in *Labeo rohita* is moderate to high. Therefore, we can conclude that Pakistan has the best genetic base of *Labeo rohita*. It is important to use the wild brooders of *Labeo rohita* in hatcheries and other commercial fish farms for breeding purpose to enhance the yield of *Labeo rohita*.

## Conclusion

Samples of three selected areas of river Chenab are genetically dissimilar from each other. But this genetic difference is not the same among all samples. Some of them are more genetically similar to each other as comparing to the other ones. It is very interesting that samples of the same region are genetically similar to each other and are placed in the same cluster on dendrogram, which indicates the genetic similarity vary from region to region. Knowledge of genetic variations and genetic similarities is helpful for selecting the breeders with desirable

genes. The SSR markers are the best tool for genetic study. Marker identification is strongly linked to the target genes. Genetic analysis of *Labeo rohita* may be hindered due to lack of sufficient informative markers. Therefore, there is a need to identify and develop a large number of high-quality and highly polymorphic SSR markers for the analysis of *Labeo rohita* genome.

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