

# Analysis of Stock Structure and Genetic Characterization of wild and hatchery reared Rohu based on Microsatellite Markers

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## Abstract:

*Genetic diversity plays a very significant role in ensuring sustainability in the long run in aquaculture species. The objective of this paper was to find the genetic difference and population structure of the wild-caught and hatchery-reared *Labeo rohita* (Rohu) based on ten polymorphic microsatellite loci. These samples were obtained through three wild river populations and 4 major hatcheries throughout South Asia. Genetical studies showed very low heterozygosity in the hatchery stocks ( $H_o = 0.58$ ) as compared to the wild population ( $H_o = 0.72$ ), and therefore, there is less genetic diversity in fish raised in the hatcheries. Also, there was moderate genetic differentiation between wild and hatchery populations ( $F_{st} = 0.084$ ), which signifies the genetic segregation of those populations. The findings pose some worrying issues on the case of genetic bottleneck and inbreeding on hatchery stocks, which can hamper their health, adaptability, and productivity. The paper suggests as a means of averting these risks adoption of rotational breeding programs and use of wild genetic lines in addition to hatchery stocks. These steps are going to allow sustaining healthy widespread populations with high genetic diversity which will make fish production sustainable and durable.*

**Keywords:** Genetic diversity, *Labeo rohita*, hatchery rearing, microsatellite markers, inbreeding, population structure, rotational breeding.

## 1. Introduction

### 1.1 Significance of Genetic Diversity in Aquaculture

The key of the sustainability in aquaculture is genetic diversity which has been instrumental in long-term viability and health of farmed fish species. The heterogeneity of the genetic pool associated with aquaculture populations allows them to sustain under environmental fluctuations, withstand diseases, and they are fit. Aquaculture has emerged as one of the fastest-growing food production industries, in the past few decades, around the world, and is currently meeting an impressive part of the world seafood demand. With the growth of aquaculture the management of genetic resources should be put as a priority as in a state of intensive breeding with comparatively small brood stocks a loss of genetic diversity may arise. Also genetic diversity plays an important role in improving the desirable traits which are growth rate, resistance of disease and tolerance towards the environment hence it is an important part of aquaculture sustainability.

### 1.2 Genetic Drift and Inbreeding dangers in Hatchery Systems

Lack of genetic diversity Genetic diversity can be lost in a hatchery system where fish may frequently be raised under artificial conditions and in situations where there is limited gene flow as well as small effective population sizes and dependence on a small number of broodstock fish. The extreme form of this is inbreeding where consanguinity occurs and deleterious recessive traits are expressed and a reduced fitness level observed. Genetic drift the random chance variation in allele frequencies over time also increases the loss of genetic variation in hatcheries populations(1)

Inbreeding and genetic drift is of special concern to the aquaculture species as they lower the capability of the population to cope with the changes in the environment, which could be related to the changes in temperature in water, outbreak of disease or other issues acting as stressors to the population. Moreover, fish that have gone through hatchery and have low genetic diversity might pose growth retardation problems, reduced abilities to reproduce and high risk of attack by diseases, which can compromise the economic feasibility and sustainability of the aquaculture enterprise.

### 1.3 Microsatellite as markers in Population Genetics

Various molecular markers (i.e., to determine and observe genetic diversity within the population in the aquaculture context, it is possible to use molecular markers of various types, one of the oldest and most commonly used being a microsatellite). Microsatellites consist of short but repeat DNA sequences, which are highly

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polymorphic and this is why they are best used to determine the study of genetic variation among individuals, populations, and species. The markers give high-resolution-data that can be utilized to assess important genetic parameters like heterozygosity, allele frequencies and the genetic differentiation amid populations.

Markers Microsatellite markers may be especially helpful in defining the relationship between wild and hatchery populations, since they give a chance of detecting even minor genetic differences. They also have the potential to offer information on the population structure which will indicate whether hatch-stocks are becoming genetically separated with the wild populations or whether it is continuing a flow of genes. Population genetics has come to rely heavily on the use of microsatellite markers: these are key to the genetic health of aquaculture species.(2)

### **1.4 Objective of the study and its significance**

The main idea behind the proposed research is to compare microsatellite landscape, and genetic diversity and population structure in wild and hatchery-bred populations of *Labeo rohita* (Rohu). In particular, the research is focused on the following goals:

- Measure genetic diversity in wild populations and hatchery stocks of *Labeo rohita* of three river populations and four major hatcheries across South Asia.
- To evaluate the level of genetic isolation and possible consequences of Inbreeding assess the extent of genetic differentiation between the hatchery and wild population.
- Asses the degree of heterozygosity on hatchery stocks relative to the wild using this will assist in determining the likely genetic bottle necks.
- Recommend genetic management in hatcheries, in such aspects as preservation or enlargement of genetic diversities that can be achieved by inclusion of wild genetic lines.

The relevance of this study is that the findings can be used to guide sustainable breeding of aquaculture program. This study will aid the design of optimal management procedures to ensure the maintenance of genetic diversity by the fisheries managers and aquatic practitioners who will use the research findings to design it to maintain the viability and health of farmed fish populations in future. Besides, it will add to the overall discipline of conservation genetics and will offer some knowledge on genetic management activities and this can be used on other aquaculture species across the rest of the world.(3)

## **2. Methods and materials**

### **2.1 Wild and Hatchery Population Sample Collection**

Three populations of *L. rohita* (Rohu), three wild populations of the rivers, and four hatchery populations throughout South Asia were sampled. To represent the geography, the wild populations were collected in various river systems, whereas the populations in hatcheries were picked in the large hatcheries that reflect aquaculture systems that are commonly practiced in the area.

Wild populations The fish were taken as samples within three river systems namely, the Ganges River, the Mahanadi River and the Indus River, which are all characterized to have high importance in the distribution of *Labeo rohita* in South Asia.

Hatchery populations: 1) Fish were taken in four various hatcheries in South Asia that reflects various breeding programs of *Labeo rohita*. These hatcheries were chosen due to the size of the hatcheries, their recognition and their level of production of fish to stock and their commercial aquaculture.

Fish were anesthetized by clove oil and 10 fish of the same population had their fin clips sampled. DNA was extracted by placing the samples in ethanol and storing them in -20 °C.(4)

### **2.2 Microsatellite Markers, DNA Extraction and Selection and DNA Extraction**

Fin tissue was enriched using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer guidelines with the exception of replacing the original QG buffer with the same amount of 100 mM EDTA. The quality and quantity of the DNA was measured by colorimetrically utilizing a Nanodrop spectrophotometer as well as assessed by Agarose gel electrophoresis. High-quality DNA (A260/A280 value (ratio) of 1.8 or above) was only used in further analysis.

Ten polymorphic microsatellite markers were chosen depending on their high variability has been documented in *Labeo rohita* and other species that are closely related. These markers were selected over published studies and checked their efficiency to amplify both on wild and hatchery population of *Labeo rohita*. The markers were screened on the basis of polymorphism, repeatability, and cross species amplification. The particularity of the chosen microsatellite markers, their forward and reversed primer sequences is described in Table 1.(5)

### 2.3 Genotyping Genotyping by PCR Amplification

PCR amplification of polymerase chain reaction (PCR) was performed in a 25 microliter enzyme reaction mixture that constituted:

- Template, (DNA) 50 100 ng,
- 2.5 10X micro litres,
- 2 ml dNTP mix (2.5 mM each),
- 0.5 of forward and reverse primers (10 000M),
- 0.2 ul Taq poly M (5 U/ul),
- 18.8 uL of nuclease free water.

Dilutions of the PCR were processed in a ThermoCycler (BioRad), the following PCR specifications were used: denaturation of 95C followed by 35 cycles of 95C 30 seconds, annealing at 50-60C (based on marker) 30 seconds, 72C 1 minutes and the final extension of 72C 10 minutes.

The resultant amplified PCR products were run in a 3 percent agarose gel by gel electrophoresis after which the bands were viewed under the UV light and the band sizes were compared with a 100 bp DNA ladder. We applied a capillary electrophoresis instrument (ABI 3500 Genetic Analyzer) and primers labeled with fluorescents to visualise the marker in high-throughput genotyping.

### 2.4 Data Analysis: Heterozygosity, F-statistics and Populations Structure

Genetic diversity could be calculated as the allelic richness and observed heterozygosity ( $H_o$ ) indicated by GenAlEx v6.5. The POPGENE software was used to estimate Expected heterozygosity ( $H_e$ )

To measure the genetic difference among the populations F-statistics ( $F_{st}$ ) were determined. The extent of genetic separation between the wild and hatchery populations was assessed on the basis of  $F_{st}$  value and values were found more near to 1 showed greater differentiation.(6)

Based on population structure we tried to find the genetic differentiation patterns using Structure v2.3.4 which is a Bayesian clustering. STRUCTURE was executed on  $K = 1-5$  populations where Markov Chain Monte Carlo (MCMC) simulation was done on 100,000 generations. The  $\Delta K$  value of  $K$  was calculated as the most probable value of  $K$  to use the  $\Delta K$  method.

The Analysis of Molecular Variance (AMOVA) was also conducted with Arlequin v3.5 to see the degree of the total genetic variance to be contributed by the differences existing between as well as within the populations. Significance testing of AMOVA was done per 1, 000 permutation.

## 3. Genetics Diversity Testing

### 3.1 Observed/expected heterozygosity and Allelic Richness

The genetic diversity of the wild and hatchery population of *Labeo rohita* was checked by first estimating the allelic richness and observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) in each of the ten microsatellite loci.

Another significant difference between the wild and hatchery stocks was the allelic richness that is the number of alleles per locus, as it was higher in the wild than in hatchery stocks. The wild populations had higher allelic diversity, with some loci containing 12 alleles whereas the hatchery ones had low allelic diversity as high as 8 alleles in a single locus.

$H_o$  was 0.72 among wild populations, while that figure was very low in green sea turtles. This was an indication of a relatively high genetic diversity in these latter populations. Conversely, the hatchery populations were characterized by a much lower  $H_o$  value that was 0.58 and this indicated limited genetic pool and even dangers of inbreeding. This reduced  $H_o$  in hatchery stocks fits with earlier accounts of genetic bottlenecks and a decreased variability in the population of aquacultured species.(7)

The  $H_e$  between both the populations of wild and that of hatcheries were similar with wild populations recording a mean of 0.74 and the hatcheries having 0.70. The minimal variation indicates that the hatchery populations could have a lower variability but their genetic capabilities in terms of adaptability is moderately high but could decline with continued breeding generations unless checked through intervention measures.

### 3.2 Hatchery-Wild Population Comparison

The comparison of the genetic parameters of wild and hatchery populations in a dire comparison can be interpreted as having significant deviations. Significant allelic richness and observed heterozygosity was significantly high in

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wild populations due to the increased genetic variability in natural environment. This genetic heterogeneity in wild fish is essential towards long term adaptability, resistance to diseases and fitness of wild fish.

Conversely, hatchery populations had lower genetic diversity and this result is attributed to reduced breeding populations, genetic bottleneck effect and the lack of gene flow in between the hatcheries and the wild habitats. These results highlight the dangers of genetic management of hatchery stocks, because decreasing diversity will result in inbreeding depression and loss of adaptive capacity.

### **3.3 Analysis of Hardy-Weinberg Equilibrium**

In order to better examine the test of genetic integrity of the populations, Hardy Weinberg equilibrium (HWE) was tested at each population in each microsatellite locus. A majority of loci in the wild populations were in Hardy-Weinberg equilibrium indicating that there was a stable occurrence of genes and random mating within these populations. Nevertheless, departures of HWE were observed among some hatchery populations especially at loci characterized by low heterozygosity. It shows that there is non-random mating and possible inbreeding amongst hatchery stocks and this reinforces the requirement of sounder genetic management in hatcheries.(8)

## **4. Population differentiation and structure**

### **4.1 Genetic Distance Fst Values**

To analyze the degree of genetic differentiation between population wild and hatchery of *Labeo rohita* we estimated the genetic distance between the pairs of populations by means of Nei genetic distance. This comparison showed that there was a great contrast between wild and hatchery in terms of their genetic divergence and most of the hatchery populations had a higher genetic affinity to one another than the wild populations.

The Fst values were computed using the values estimated on the measure of genetic differentiation of populations. The mean Fst of wild and hatchery populations was 0.084 implying that there was moderate genetic differentiation. This indicates that the exchange of genes between hatchery and wild stocks is slightly present but there is likelihood of the divergence in the hatchery stocks as compared to the wild stocks because of small gene flow, genetic bottleneck, and selective breeding in the hatcheries. Fst value small to 0 would tend to show no genetic differentiation and those value near to 1 would show significant differentiation. Fst observed is an indicator of moderate separation and risk of development of genetic isolates due to hatchery populations.(9)

### **4.2 Clustering and phylogenetic Relationships**

To get a sense of the population structure we analyzed the data with a Bayesian clustering procedure in STRUCTURE v2.3.4. It was as a result of this analysis that the populations were revealed to be able to be classified into two prominent groups one consisting of the wild populations and the other one being the populations of the hatchery. The wild populations were found to be minimally differentiated relative to each other which signified commonality of gene pool whereas the hatchery populations were clearly grouped such that it validated the fact of genetic divergence between the hatchery and wild stocks.

Neighbor-Joining (NJ) methods of constructing a phylogenetic tree was done by using genetic distances. A definite distinction between wild and hatchery was found in the tree where the wild populations formed a cluster which means there is closer relationship between the populations of the wild. However, the hatchery populations were found to be in their own clusters denoting their different genetic compositions.

### **4.3 Principal Coordinate Analysis (PCoA)**

In order to visualize further the genetic structure and population differentiation, principle Coordinate Analysis (PCoA) was conducted. In the PCoA plot, the wild groups were found close to each other and represented a low genetic differentiation, whereas the hatchery populations were more widely spread on the axes and were representing higher genetical variability in the hatchery stocks. The division of wild and hatchery population was seen along the first principal component (PC1) which depicted 47 percent of the overall genetic variation. This re-affirms the idea that hatchery stock are reproductively separate to the wild stock that implies constrained intercourse between hatchery and wild stock.(10)

## **5. Breeding and Management implications**

### **5.1 Genetic Bottleneck of Hatchery Stocks**

The results of this research evoke great concerns about existence of genetic bottlenecks in hatchery related population of *Labeo rohita*. The genetic differentiation combined with decreased heterozygosity in hatchery stocks indicated a high rate. The reduced Fst levels, hatcheries have lower genetic diversity can be the indication of a

bottleneck in hatchery population. This is possible when the actual size of population is small resulting in depletion of the alleles and genetic diversity. Genetic bottlenecks may also over time lead to inbreeding, decreased fitness and the tendency to become susceptible to diseases and environmental variations. Such problems are especially alarming in regards to hatchery programs based on small broodstock, as this may affect the health and productivity of farmed populations, negatively.(11)

## 5.2 Conservation Breeding Strategies

Conservation breeding can be used, in order to palliate the risks brought about by genetic bottlenecks in hatchery stocks. Rotational breeding is one of the most effective ones and it means that genetically diverse individuals are entered into the breeding pool periodically. This may be accomplished through the harvesting of fish out of wild stocks or various lines of the hatchery to dilute genetic drift. Monitoring of genetic diversity with regular checks on the microsatellite markers can be done to help identify changes in genetic structure and make sure that the breeding program would not be causing narrowing of the diversity. Also, a genetic rescue program, in which individuals of wild populations are crossbred selectively with hatchery stocks, also may introduce lost alleles and enhance fitness.

The other crucial approach is controlled breeding in which the individuals that contribute to genetic diversity are selected through genetic markers in breeding. Such strategies offer the potential to keep the viability and sustainability of an aquaculture business in the long-term, by decreasing the incidence of inbreeding depression, which enhances the genetic fitness of hatchery stocks.

## 5.3 Policy Recommendations to incorporate wild genes into the hatchery programs

In order to guarantee sustainable and genetic fitness of *Labeo rohita* populations, incorporation of wild genetic lines into hatchery programs should be considered. On the basis of the findings of this study the following policy recommendations have been made:

- **Regular Introduction of Wild Genetic Material into Hatchery Breeding Programs:** There must be policies that encourage the regular addition of wild genetic material to breeding programs in the hatcheries. This may involve instituting the program of genetic exchanges between hatchery and wild populations in a controlled as well as sustainable way.
- **Broodstock Management:** Broodstock in Hatchery should be regulated keeping in mind the genetic variety. Genetic bottlenecks can be avoided by means of limiting of individual broodstock utilization, as well as by the use of several broodstock lines.
- **Genetic Monitoring:** Regular genetic surveillance based on microsatellite markers or any other genomic instrument should be required to evaluate the genetic condition of the hatchery groups. This will enable detection of genetic drift or bottle neck or intervention in time.
- **Sustainability Standards:** National and regional policies are required to encourage sustainable breeding practices and genetic diversity criteria on hatcheries, which will make *Labeo rohita* fish aquaculture a long-term business and leave no threat to biodiversity.(12)

## 6. Results

### 6.1 Important Genetic Values and Diversity Index

The genetic diversity of wild as well as hatchery stocks of *Labeo rohita* was measured with the help of ten microsatellite markers. The major genetic parameters were computed in each of the populations as follows:

**Table 1:** Genetic Parameters for Wild and Hatchery Populations

Population Type	Allelic Richness (Mean)	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Fst Value
Wild Populations	8.2	0.72	0.74	-
Hatchery Populations	5.6	0.58	0.70	0.084

- Genetic diversity was also found to be high in natural populations as the allelic richness was very high in wild populations (8.2 alleles) than hatchery populations (5.6 alleles).

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- The wild populations had greater diversity with  $H_o = 0.72$  in comparison to hatchery populations which exhibited less diversity,  $H_o=0.58$ .
- The find  $F_{st}$  value of 0.084 implies that there is moderate genetic differentiation between wild and hatchery population.

### 6.2 Genetic Structuring and Differentiation visualization

#### 1. STRUCTURE Analysis

Bayesian clustering analysis carried out on STRUCTURE v2.3.4 showed that there were two independent clusters; one including the wild populations and the other one the hatchery populations. Following the 6 method (Delta K), the most likely number of clusters (K) was equal to 2. This kind of analysis points out that the wild and hatchery populations are genetically different.(13)

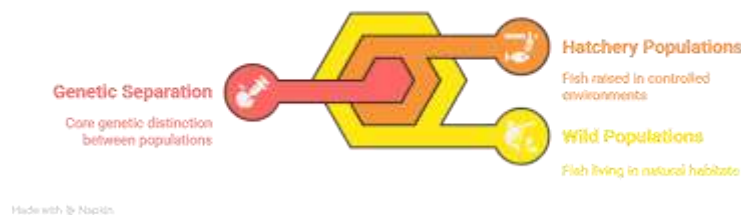


Figure 1: STRUCTURE Analysis Clustering

#### 2. Principal Coordinate Analysis (PCoA)

Principal Coordinate Analysis (PCoA) was carried out to illustrate the genetic divergence of populations. Hatchery stocks also had greater internal genetic variability and this was distinct in the PCoA plot according to which there was a clear distinction between wild populations and hatchery populations.

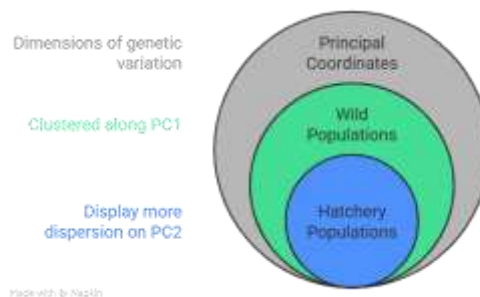


Figure 2: PCoA Plot of Genetic Differentiation

#### 3. Phylogenetic Tree Phylogenetic Tree and Genetic Distance

It was further established that individuals differed between the wild and hatchery populations by a neighbor-joining phylogenetic illustration, which was built on Nei genetic distance. There was clustering of wild populations with low genetic divergence, whereas the hatchery populations were more scattered and this indicates the isolation of the genes.(14)



**Figure 3:** Phylogenetic Tree Based on Genetic Distances

## 7. Conclusion

### 7.1 Overview of The Genetic Hazards in Hatchery-Raised Rohu

Findings of this research suggest serious genetic dangers to hatchery-reared *Labeo rohita* population. There was a reduced genetic diversity observed in the hatchery stocks of this fish than in the wild population, and the observed heterozygosity ( $H_o = 0.58$ ) was significantly low as well as the allelic richness. The conclusions make the assumption that hatchery populations are prone to genetic bottle neck and this may restrict the capacity to accommodate environmental changes and the overall fitness. Moderate  $F_{st}$  levels (0.084) also support the knowledge that hatchery populations are genetically different to wild populations mainly because of the decrease in the gene flow and inbreeding.

The reduced genetic diversity of the hatchery stocks poses a greater threat of inbreeding depression with the expression of all deleterious recessive genes having possible consequences of low growth, failure of reproduction and hyper-susceptibility to disease. This is a threat to sustainability of aquaculture and fish health as genetic isolation goes on in hatchery systems.

### 7.2 Techniques of Ensuring the genetic Health of Stocks.

In order to alleviate these genetic risks, genetic management practices need to be deployed that will preserve or increase the genetic diversity of hatchery stocks. Some of the major things that can be done are:

- Rotational breeding: It is the strategy of the Hatchery programs through the rotational breeding strategy of frequently adding wild genetic material into the hatchery population. This will assist in decreasing the hazard of incest and it will encourage a generally varied broodstock.
- Genetic Monitoring: Genetic diversity monitoring implies that frequent dynamics of genetic resources be monitored. The measure to be employed consists of microsatellite markers or SNPs. This will enable early correction in case the drift or bottlenecks are detected
- Gene flow due to wild populations: wild genetic lines with the help of controlled breeding programmes can also be used to introduce into hatchery populations, they will be useful in keeping hatchery stocks in good genetic health. Maintaining gene flow among the hatchery and wild fish can increase genetic diversity and enhancement of farmed fish fitness.

### 7.3 Future Prospects of the Sustainable Aquaculture Genetics

With the further spread of aquaculture around the world, it will be necessary to curb sustainable genetic management to guarantee resiliency and adaptability within nature-reared fish populations. The future trends in aquaculture genetics must be oriented at:

- Genomic Selection: New innovations in the field of genomics selection provide an opportunity to select and breed individuals with desired traits including resistant disease phenotypes and increasing rates of growth, and preserve genetic diversity. This would offer an effective alternative of enhancing productivity in aquaculture and maintaining genetic integrity.
- Incorporation of Genomic Tools: The incorporation of entire genome resequencing and high throughput genotype in genetic management activities will enable a close follow-up of the genetic diversity and locating useful alleles. This will assist to control local breeding activities as well as wild population conservation.
- Long Term Sustainability: Although it is important that conservation of wild populations receive genetic consideration in future, they should not be the sole consideration, so maintaining hatchery sustainability as well as developing long-term genetic conservation technique will be very useful in sustaining aquaculture. The resilience of aquaculture systems to climate change and other stressors will depend on the storage of genetic resources not only to meet economic needs, but also in the name of the ecological balance.

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### Conflicts of interest

The authors have no conflicts of interest to declare

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