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Heterosis Analysis in Strain-Crossed Hybrid Rohu (*Labeo Rohita*) through Microsatellite DNA Variability Assay

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Abstract

Labeo rohita is an important commercial aquaculture species in Bangladesh, but it needs improved growth performance to meet the increasing demand for fish. Genetic improvement through inter-strain crossing, generating heterosis, is quicker than a selection strategy. Therefore, crosses were made between two strains of rohu: Padma River strain and a hatchery strain. Growth performance analyis showed the highest growth rate in the Padma × Padma cross followed by one of the reciprocal crosses Hatchery♂ × Padma♀ (hybrid). The lowest growth rate was observed in Hatchery × Hatchery cross. DNA variability study using three microsatellite markers showed polymorphism (*P95*) in these crosses. Variations were observed in number and frequencies of alleles and mean heterozygosity in different loci; the mean number of alleles varied from 4.667±0.882, 3.333±1.333 and 2.667±1.202 in Padma, hybrid and Hatchery strains respectively. Relatively high levels of gene flow and low Strain differentiation (F_{ST}) values were found between the Padma and hybrid strains. The UPGMA dendrogram based on genetic distance placed the Padma population in one cluster and the hybrid and Hatchery populations in another. The results revealed that there was relatively low level of genetic variability in the riverine and hatchery reared populations of *L. rohita* in Bangladesh.

Key words: Carp, rohu, strain-crossing, hybrid, heterosis, microsatellite

Introduction

Fish and Fisheries have importance in the economy of Bangladesh in terms of income, nutrition, employment and foreign exchange earnings. In recent years in the face of an increased gap between supply and demand there have been efforts to increase fish production: approaches have been taken include conserving genetic diversity in natural fish populations, which is eroding very fast due to anthropogenic stresses, and enhancing aquaculture productivity. The seed quality of hatchery produced cultured carp is declining because of insufficiently careful fish breeding, without appropriate genetic norms and principles; inbreeding and negative selection are frequent problems associated with carp seed production. Shah (2004) obtained an strong evidence of reduced genetic

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variation in the hatchery strains of rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) in Bangladesh, which he attributed to inbreeding, and regarded the strains having attained significant divergence from their counterpart natural populations in river.

Local strains have been used as brood stocks to produce seeds by the of private hatcheries in Bangladesh. Although the history of aquaculture is long, but the local stocks have not been studied to determine the genetic structure; the inbreeding level of the populations remained undetermined to date. Though there are years of domestication history of carp in Bangladesh, genetic diversity within and among different populations remains unknown. This suggests it is worth cross breeding hatchery rohu with the riverine strain and looking for possible heterosis. In hybridization study, growth comparison of the reciprocal hybrids is important because they are seldom the same (Tave, Jayaprakash, & Smitherman, 2007).

Various genetic studies have been carried out in *L. rohita*, including DNA fingerprinting by using a sex specific satelite DNA [Bkm 2(8)] and a minisatelite DNA [M13] probes, (Majumdar, Ravinder, & Nasaruddin, 1997), detection of hybrids in the three Indian major carp (catla, rohu and mrigal) using ribosomal DNA RFLP with *Xenopus laevis* rDNA as a probe (Padhi & Mandal, 1997), RAPD analysis (Barman et al., 2003; Islam & Alam, 2004) and allozyme analysis (Alam, Akanda, Khan, & Alam, 2002; Khan, Rahman, A1am, & Bhuiyan, 2006). Previous studies involving allozyme and RAPD analysis revealed relatively low levels of intra and inter-population genetic variation in the three river populations of rohu and catla (Alam et al., 2002; Islam & Alam, 2004; Alam & Islam, 2005; Islam, Ahmed, Azam, & Alam, 2005) and a wide spread hybridization practices in the hatchery stocks among the three Indian major carp species (Simonsen, Hansen, Mensberg, Serder, & Alam, 2005). In contrast, microsatellites are codominant markers, highly polymorphic, easily typed, and Mendelian inherited which facilitate for population structure study, pedigree analysis as well as detecting differences among closely related species (Abdul-Muneer, 2014). Microsatellite markers have been found to be effective in detecting high levels of polymorphism and rare alleles as well as population structure analysis of three Indian major caprs (Naish & Skibinski, 1998; Das, Baret, Meher, Ray, & Majumdar, 2005). These markers are now widely used for the determination of genetic variation in wild and cultured fish populations (Was & Wenne, 2002).

The application of biotechnology and genetic engineering to aquaculture and fisheries is a relatively recent practice as compared to plant and animal science. Nevertheless, biotechnology has played a pivotal role in aquaculture, genetic improvement and conservation of several fish species, especially the salmonids, tilapias and carps. In the present study, the intra-specific hybridization technique was used with the intention of genetic improvement (hybrid vigour) in rohu through strain crossing and assessment of heterozygosity through genomic DNA microsatellite assay.

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Materials and Methods

Experimental Design

The experiment was designed into three different phases; production of hybrids by crossing within two strains of Rohu in Basepara Fish Seed Multiplication and Extension Farm of Department of Fisheries in Jessore, 18 weeks rearing the spawn in nursery ponds in Khulna University campus and molecular analysis of heterosis by DNA microsatellite markers in Molecular Biology Laboratory at Khulna University and Fish Biology and Genetics laboratory at Bangladesh Agricultural University, Mymensingh.

Induced breeding was done by using Ovulin as a systhetic analoug of reproductive hormon (ZDHF PHARMA,OEM). Hormone was given to the brood fish at a rate of $1cm^3$/6 kg fish. Spawners were obtained ready for spawning within a few hours after injection**.** From two strains, two reciprocal hybrid crosses and two controls with the single strains were performed. The inseminated eggs were then transferred into nine incubation tanks provided with continuous water flow. The spent brood fishes were tagged intra-peritoneally with digital tags and released back to the ponds of Bangladesh Fisheries esearch Institute (BFRI), Jessore.

Stocking and Rearing of Fish

The fish were reared in the experimental ponds of Khulna University campus. Fry were reared for eighteen weeks in the ponds. The feed was given four times in a day. At the first week 100 g feed per 2 decimal pond was given. Then after every 5 days the amount was increased by 5 g. Three types of feed were given: Finisher feed, mega feed and floating pellets (Mustard oil cake, rice polish, wheat bran, fish meal, corn flour, vitamin mixture and pellet binder). Sampling of pure breed and reciprocal hybrids were carried out fortnightly for 16 weeks and heterosis was calculated using the following formula (Tave, 1993) :

$$H = \frac{(\text{Mean reciprocal F1 hybrids} - \text{Mean parents}) \times 100}{\text{Mean parents}}$$

Collection of Samples for DNA Analysis

Ten fishes from each of the crosses (Padma × Padma; Hatchery♂ × Padma♀; Hatchery × Hatchery) were collected from the ponds. Approximately 25- 50 mg of caudal fin tissue was clipped from each individual. The fin clip was cut into small pieces with sterile scissors and kept in 1.5 ml Ependorf tubes on ice and then stored in -20^0C until analysis.

Extraction of Genomic DNA

Genomic DNA was extracted from the fin tissue using *AccuPrep*® Genomic DNA extraction kit (Bioneer, Korea) following the instructions provided by the company. Extracted DNA was dissolved in 30µl elution buffer.

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Determination of DNA Concentration and Purity

The concentration and purity of extracted DNA samples were determined from absorbance at A_{260} and A_{280} (absorbance at 260 nm and 280 nm) values using a spectrophotometer against elution buffer as blank. Each DNA-sample-containing cuvette was washed properly before loading the next sample. The protocol used in this experiment was designed for a double-beam spectrophotometer (Dharmanandan Techno Projects Pvt. Ltd.).

Primer Selection

Three primer pairs, Lr3, Lr21 and Lr23, were used for polymerase chain reaction (PCR) amplification of microsatellite markers for *L. rohita* in this study (Table 1). These primers have been used in other strains of *L. rohita* (Alam, Jahan, Hossain, & Islam, 2009).

PCR Amplification

After examination of the purity and concentrations of all the extracted DNA samples, concentrations of the extracted DNA samples were then adjusted 50 ng/µl . PCR reactions were performed for each DNA sample in a 20 µl reaction mixture containing 0.2 µl Taq DNA polymerase (5U/µl:, Bioneer, Korea), 4 µl (2.5 µM) oligonucleotide primer (forward) and 4 µl (2.5 µM) oligonucleotide primer (reverse), 0.5 µl of 10 mM dNTPs mixture (Bioneer, Korea), 2 µl of 10X reaction buffer (Bioneer, Korea), 2 µl of template DNA, 1.3 µl 25 mM $MgCl_2$ and 6 µl nuclease free deionized distilled water. DNA amplification was performed in a Thermal Cycler (C1000^{TM}, BIO-RAD, USA). The target DNA amplification process involved: 3 minutes initial denaturation at 94^{0}C followed by 35 cycles, each of 30 sec denaturation at 94 ^{0}C, 30 sec at the annealing temperatures (47-51^{0}C) and 1 min at 72^{0}C, ending with an additional 5 min at 72 ^{0}C for final elongation. Annealing temperatures used were 51^{0}C, 50^{0}C and 47^{0}C for Lr3, Lr21 and Lr23 respectively (Alam et al., 2009).

Electrophoretic Separation and Visualization of PCR Products

Each of the PCR-products (5µl) was electrophoresed on denaturing polyacrylamide gel (6%) containing 19:1 acrylamide: bis-acrylamide and 7 M urea. Electrophoresis was conducted using the SequiGen GT sequencing gel electrophoresis system (BIO-RAD Laboratories). The gel was pre-run at 120 W for 30 min followed by 60 W at 50 °C for the final run upon loading of denatured PCR products for a particular period of time according to the size of amplified fragments (generally 1 hour for 100 bp). A molecular weight marker DNA (100 bp DNA ladder) was loaded on either side of the gel. The DNA fragments were visualized after silver-staining and the staining protocol was followed by the instructions provided by Promega company.

Scoring and Analysis of Microeatellite Data

From the silver-stained plates the bands of particular alleles at the microsatellite loci were scored manually and the size of the bands were estimated using the DNAfrag (version 3.03) (Nash, 1991). Scoring of genotypes for a specific locus was performed on the basis of the number of alleles of a particular size present in that locus. For all the loci a single genotypic data matrix was constructed. Number and freequency of alleles were estimated by

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using the GenAlEx program version 6 (Peakall & Smouse, 2006). Alleles were deemed as present if they were found in one population and absent in the others. To test pair-wise homogeneity, as well as to calculate Nei's (1972) genetic distance (D) in different population pairs, expected and observed heterozygocities (He, Ho) were calculated by using G-stat program (Siegismund, 1995). POPGENE program version 1.31 was used for chi-square (X^2) test to estimate the fitness to Hardy-Weinberg proportions (H·W tests) with 1000 simulated samples (Yeh, Yang, & Boyle, 1999). Estimation of gene flow (N_m) was also done by using the same program. To test for differences (F_{ST} between populations; 1000 permutations) were estimated using the FSTAT program version 2.9.3 (Goudet, 2001). TREEVIEW program was used to draw the Unweighted Pair Group Method with Averages (UPGMA) dendrogram based on Nei's (1972) genetic distances (Page, 1996).

Results

Growth Performance

The growth analysis study revealed that the best growth was obtained in the intra-strain cross of Padma × Padma with a mean value being obtained at 39.95±3.1 g; whereas the growth was found to be 4.35±0.3 g for the other intra-strain cross Hatchery × Hatchery. One of the hybrid crosses Hatchery♂ × Padma♀ showed the better growth performance at 26.71±9.7 g of the two hybrid crosses (Table 2). The heterosis obtained in Hatchery♂ × Padma♀ was +0.2 and that in the reciprocal cross Padma♂× Hatchery♀ was -0.8. Due to the positive heterosis (+21%) in Hatchery♂ × Padma♀ therefore this hybrid was subjected to microsatellite analysis along with parental species.

Allelic and Genotypic Variation within Populations

Growth results of the above crosses were assessed and proved through looking at DNA variation using microsatellite markers. Three di-nucleotide microsatellite loci were analyzed to reveal genetic structure of these strains and all the loci were found polymorphic (*P95*) (a locus is termed as polymorphic when the frequency of the most common allele is less than or equal to 0.95) in all the three populations (Padma, Hatchery♂ × Padma♀ and Hatchery). The sizes of the alleles for all the loci in the three populations ranged from 152 to 192 bp (Table 3).

The strains were varied with respect to the number and frequencies of alleles, where the mean number of alleles varied between 4.667±0.882, 3.333±1.333 and 2.667±1.202 in Padma, Hatchery♂ × Padma♀and Hatchery respectively; the strains were also varied with regard to mean heterozygosity in different loci (Table 4).

The alleles 172 bp, 174 bp, 160 bp, 174 bp, 178 bp, 154 bp, 160 bp and 164 bp were considered as private alleles for the Padma population as they were present only in the Padma population at the frequency of 0.400, 0.050, 0.333, 0.111, 0.111, 0.063, 0.063 and 0.188 respectively. Moreover, allele 162 bp was private for the Hatchery♂ × Padma♀ population at the frequency of 0.500 and allele 178 bp was private for the hatchery population at the frequency of 0.400, as all these alleles were not present in the other populations (Table 3). The observed heterozygosity (H_o) values ranged from 0.00 to 1.00 while the expected heterozygosity (H_e) or gene diversity

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values ranged from 0.00 to 0.842 (Table 4). The $1\text{-}H_o/H_e$ values were positive in two loci (Lr21 and Lr23) and also negative in one locus (Lr3) in all the three populations (Table 4). Significant deviations from Hardy-Weinberg Equilibrium (HWE) were detected in 6 out of 9 tests (Table 4). These deviations were not systematic rather occurred at different loci for different populations. The Padma populations deviated in two loci, Hatchery♂ × Padma♀ populations deviated in three loci while the hatchery populations deviated in only one locus.

Inter population genetic structure and genetic distance pair-wise comparisons of different stocks of *L. rohita* using homogeneity tests are shown in Table 5. Of the nine tests, seven were found to be significant. From χ^2 values for test of Hardy-Weinberg expectations, the population of Hatchery♂ × Padma♀ was found to be significant at locus Lr3; likewise, at locus Lr21 Padma and Hatchery♂ × Padma♀ and at locus Lr23 Padma, hatchery & Hatchery♂ × Padma♀ were found to be significant ($P< 0.05$, $P< 0.01$, $P< 0.001$).

Population differentiation *(F_{ST})* values between Padma and Hatchery population was the highest (0.196) and found to be insignificant *($P < 0.05$)*; whereas the values between Hatchery and Hatchery♂ × Padma♀ was the lowest (0.118) and found to be insignificant (Table 6). Genetic distance value between the Padma and Hatchery populations was the highest (0.827) while that of the Hatchery♂ × Padma♀ and Hatchery was the lowest (0.247) (Table 6). Relatively high level of gene flow and low level of F_{ST} values were found between the Padma and Hatchery and Padma and Hatchery♂ × Padma♀ strain (Table 6).

Unweighed Pair Group Method with Averages (UPGMA) dendrogram studies based on genetic distance generated two clusters; one cluster was formed by the Padma population alone and the other cluster was formed by Hatchery♂ × Padma♀ and Hatchery population (Figure 1).

Discussion

Growth analysis showed that Padma♂ × Padma ♀ obtained highest growth (39.95±3.1g) followed by the Hatchery♂ × Padma ♀ (26.71±9.7g) and the poorest growth observed in Hatchery ♂ × Hatchery ♀ which is 4.35±0.3g. Poor growth performance of the hatchery-produced seed may be due to inbreeding pressure of limited number of brood stock in the hatchery. Similar results were obtained by Biswas, Shah, Takii, and Kumai (2008); Dunham, Smitherman, Goodman, and Kemp (1986); Islam and Shah (2007), where these authors found that fry from riverine sources showed better growth than the hatchery sources.

Heterosis analysis revealed that Hatchery♂ × Padma ♀ showed postive heterosis (+21%) whereas the reciprocal cross Padma♂ × Hatchery ♀ showed negative heterosis (-76%). The postive heterosis in Hatchery♂ × Padma ♀ may be due to the cytonuclear interaction as well as individual loci effects (Kimball, Cambell, & Lessin, 2008). Similar results were obtained where Hb♀ × Ca♂ reciprocal cross of *C. anguillaria* and *H. bidorsalis* show better growth performance than Hb♂ × Ca♀ reciprocal (Diyaware & Onyila, 2014), and also Ht♀ × Cl♂ reciprocal cross of *Clarias gariepinus* and *Heterobranchus longifilis* show better growth performance than Cl♀×Ht♂ but

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lower than the pure breeds (Ataguba, Annune, & Ogbe, 2010). Sheridan (1981) reported that negative heterosis in growth indicated that a negative interaction has occurred in the parental genes found at different loci of the inter-generic hybrid genome. The phenotypic variance of a quantitative trait is governed by genetic variance, environmental variance and genetic-environmental interaction variance (Tave, 1993).

Natural population growth and reproductive quality of the major carp species have been on the decline over the last three decades, due to reductions in population size. The population genetic structures of *L. rohita* populations in the Halda, the Jamuna and the Padma rivers, and selected hatcheries (Islam & Alam, 2004; Khan et al., 2006; Islam, Shah, Ibn Shams, Ali, & Rahi, 2015), *Catla catla* (Shah & Khan, 2008; Ali, Rahi, Islam, Shah, & Ibn Shams, 2015) have been studied by using RAPD and allozyme markers. Khan et al. (2006) using allozyme marker reported 27 % polymorphic loci in the three river population of *L. rohita*. Das et al. (2005) developed microsatellite markers obtained all the studied loci polymorphic in an Indian farmed population of *L. rohita*. Using four loci they obtained a total of 20 alleles (5 alleles in average) and Alam et al. (2009) obtained only 14 alleles, while we obtained 20 alleles by using three loci. The authors did not, however, study population level genetic variation of this important fish species through intra-strain crosses, and assessing hetetosis in relation to these markers. In the present study the observed average number of alleles was the lowest in the Hatchery population (2.667±1.202) and thus they have the highest number of null alleles (12). Loss of allelic variation has also been reported for the three river populations (the Padma, the Halda and the Jamuna) and one hatchery population of *Catla catla* by Alam and Islam (2005). Presence of non-parental band (162bp using Lr3 marker) in addition to parental bands might be due to formation of heteroduplex molecules by two allelic sequences of different amplified fragments (Hashemi, Mirmohammadi-Maibody, Nematzadeh, & Arzani, 2009). Several authors reported that non-parental band(s) is a useful feature and supplemental evidence for codominancy of the markers (Davis, Yu, Haigis, & Mcgowan, 1995; Wu, Wu, & Chung, 2002; Zheng et al., 2003; Huang, Tsai, & Sheu, 2000; Heckenberger et al., 2002). Hence, the presence of heteroduplex non-parental 162 bp allele may be an indicative feature for hybrid individuals.

On the other hand the highest average number of alleles was found in the Padma population (4.667±0.882) and it also had six null alleles. The study of Alam et al. (2009) indicates that the Bangladesh populations of *L. rohita* have lost a few alleles. But presence of a large number of null alleles in all the population in the present study also indicates loss of different alleles from the rivers as well as hatchery populations.

Again in these same four loci the average observed heterozygosity and expected heterozygosity were 0.746 and 0.621 (Das et al., 2005) and 0.413 and 0.493 (Alam et al., 2009) in the Indian major carp populations. We observed that average observed heterozygosity and expected heterozygosity was lower than that of obtained in the study by Das et al. (2005) but higher than that of obtained by Alam et al. (2009). Moreover, heterozygosity in the hybrid population was also fairly good with the observed value of 0.542±0.292 and expected value of 0.534±0.176; that indicated positive heterosis through strains crossing of rohu.

A significant deficiency of heterozygotes was observed in six out of the nine H-W comparisons. Null alleles, alleles that are not amplified due to mutation in primer site may contribute to an excess of homozygotes and another possibility is the violations of the assumptions underlying the Hardy-Weinberg equilibrium relevant to natural populations (mutation, migration and selection) (Paetkau & Strobeck, 1995; Ferguson, 1995). Of the

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three microsatellite loci tested in this study the Padma populations deviated from Hardy-Weinberg proportions at two loci, the Hatchery♂ × Padma♀ populations deviated at three loci while the hatchery population deviated at only one locus (Table 4). The deviation from Hardy-Weinberg equilibrium at Lr21 and Lr23 in the Padma and Hatchery♂ × Padma♀ population were high ($P < 0.001$) and in the Hatchery population at Lr23 was low ($P < 0.05$) due to loss of heterozygosity perhaps as a result bottleneck effect. Reduction in allelic variation may also be explained by founder effect and genetic drift in the population resulting from a sudden decrease in population size (Ne) (Islam, Ahmed, Azam, & Alam, 2005).

A test for genetic differentiation was performed to test the hypothesis that the sample sets had genetic heterogeneity. The population pairs studied here showed a low levels of genetic differentiation. The differentiations (F_{ST}) between all the population-pairs were found to be insignificant. There is no physical connection between the Padma and hatchery populations. Therefore, naturally no mixing is possible between fishes of Padma with those of the hatchery. Similar to the present study, a low level of population differentiation (F_{ST})was also observed among populations and significant differentiation ($P < 0.05$) was evident only between the Halda riverine and hatchery populations in Bangladesh in *Catla catla* (Alam & Islam, 2005). Another recent study found that there is no genetic differentiation ($F_{ST} = 0.001$) between wild and hatchery population of Paciifc threadfin in Hawaii (Pan & Yang, 2010). Homogeneity tests for pair-wise comparisons of different stocks showed that the Hatchery - Hatchery♂ × Padma♀ populations are more homogeneous compared to the Padma-Hatchery♂ × Padma♀ and Padma-Hatchery population pairs (Table 5). Moreover, a relatively high level of gene flow and low level of genetic distance between the Padma and Hatchery population indicated that there might be a possibility of intermixing of fishes between these two interconnected populations (Table 6) due to escaping/releasing of hatchery bred fish into open water.

Though the three microsatellite loci revealed some degree of intra and inter-populaton genetic variation in one riverine, one hatchery and one hybrid of Hatchery♂ × Padma♀ populations of *L. rohita*, the overall genetic variation is not large enough reflecting that the populations need proper management. The initiatives that could be undertaken in favor of increasing population sizes includes restriction on fishing in major fish breeding ground especially in the breeding season, creation of fish sanctuary, banning on stocking of poor quality hatchery produced fry in open water bodies, increase public awareness, improvement of feeding and breeding ground by dredging of rivers etc. Moreover, to monitor the genetic condition effectively, large sample size covering other natural populations of this important freshwater fish species should be analyzed with more microsatellite DNA markers in future.

The results revealed a relatively low level of genetic variability in the river populations of *L. rohita* in Bangladesh. There are sporadic reports available from the farmers that the production performance of hatchery produced seed of this important Indian major carps species is declining rapidly in Bangladesh. Improvement efforts can be approached through genetic selection but that could be quite lengthy considering the longer generation time and thus hybridization of the strain/stocks that are presumably taxonomically differentiated could be a potential option for quick gain in higher production performance. The present trial on reciprocal inter-strain crossing of rohu among two strains, one from river, Padma and one from a hatchery in Jessore was successful in producing 21 % positive heterosis in one reciprocal cross (Hatchery♂ × Padma♀). Thus, together

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with seed production from each strain, intra-strain hybrid seed production can also be undertaken in future using brood fish stock with tag marks in Bangladesh.

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Table 1. Primers used in the experiment with their base sequence and number of bases with melting temperature (Tm).

Sequence Name	Base sequence	Number of bases
Lr3 Forward	ATCTGGCTGCCTATTCACC	$19/Tm_{51}$
Lr3 Reverse	CATCGGCGACTGCATTGGA	$19/Tm_{53}$
Lr21 Forward	GATCAGAGGGTCAATGTGG	$19/Tm_{51}$
Lr21 Reverse	CAGCAGAGTACTATGGAAGA	$20/Tm_{50}$
Lr23 Forward	CAAGGCCAAAAGTGTCCAT	$19/Tm_{49}$
Lr23 Reverse	AGGAAATTGGTAAAGTGTTTC	$21/Tm_{47}$

Table 2. Average weight (g) of hybrids and single strain crosses of rohu

No. of sampling	Padma ♂ × Padma♀	Hatchery♀ × Hatchery♂	Hatchery♂ × Padma♀	Padma♂ × Hatchery♀
1	0.02±0.0	0.08±0.0	0.01±0.0	0.05±0.0
2	0.28±0.0	0.19±0.0	0.07±0.0	0.10±0.0
3	0.60±0.0	0.37±0.0	0.27±0.0	0.20±0.1
4	7.90±0.6	0.55±0.0	5.82±2.5	0.67±0.1
5	13.96±0.7	0.66±0.1	8.59±2.7	2.25±1.2
6	24.82±1.1	1.57±0.1	17.77±6.4	3.17±2.0
7	34.39±0.6	3.21±0.2	20.20±7.1	3.52±2.2
8	39.95±3.1	4.35±0.3	26.71±9.7	5.41±2.3

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Table 3. The sizes and frequencies of alleles at three microsatellite loci in Padma, Padma × Hatchery and Hatchery populations of *L. rohita.*

Locus	Alleles (bp) across the loci/Number of samples (N)	Allele frequencies		
		Padma	Padma × Hatchery	Hatchery
Lr3	N	10	10	10
	152 bp	0.550	0.500	0.600
	162 bp	0.000	0.500	0.000
	172 bp	0.400	0.000	0.000
	174 bp	0.050	0.000	0.000
	176 bp	0.000	0.000	0.400
Lr21	N	9	8	10
	158 bp	0.222	0.875	1.000
	160 bp	0.333	0.000	0.000
	162 bp	0.222	0.125	0.000
	174 bp	0.111	0.000	0.000
	178 bp	0.111	0.000	0.000
Lr23	N	8	8	10
	154 bp	0.063	0.000	0.000
	160 bp	0.063	0.000	0.000
	164 bp	0.188	0.000	0.000
	166 bp	0.125	0.125	0.000
	168 bp	0.125	0.125	0.000
	170 bp	0.438	0.313	0.050
	176 bp	0.000	0.125	0.200
	178 bp	0.000	0.000	0.400
	182 bp	0.000	0.250	0.200
	192 bp	0.000	0.063	0.150

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Table 4. Allelic variations [N = Number of alleles, A_r = allelic richness, A_e = effective alleles, H_o = heterozygosity oberved, H_e = heterozygosity expected and χ^2 (Chi-square) values for test of Hardy-Weinberg expectations (degrees of freedom in parentheses)] at three microsatellite loci in Padma, Padma×Hatchery and Hatchery populations of *L. rohita*.

Microsatellite loci	Parameters	Padma	Padma × Hatchery	Hatchery
Lr3	N	3	2	2
	A_r	2.800	2.000	2.000
	A_e	2.151	2.000	1.923
	H_o	0.900	1.000	0.800
	H_e	0.563	0.526	0.505
	χ^2 of H-W test	5.900^{NS} (3)	9.000**(1)	3.818^{NS}(1)
	1-H_o/H_e	-0.682	-1.000	-0.667
Lr21	N	5	2	1
	A_r	4.987	2.000	1.000
	A_e	4.263	1.280	1.000
	H_o	0000	000	0.000
	H_e	0.811	0.233	0.000
	χ^2 of H-W test	57.867***(10)	15.076***(1)	---
	1-H_o/H_e	1.000	1.000	1.000
Lr23	N	6	6	5
	A_r	6.000	6.000	4.796
	A_e	3.765	4.741	3.774
	H_o	0.250	0.667	0.400
	H_e	0.783	0.842	0.774
	χ^2 of H-W test	40.571***(15)	30.625***(15)	27.548**(10)
	1-H_o/H_e	0.660	0.208	0.456
Average number of alleles		4.667±0.882	3.333±1.333	2.667±1.202
Average A_e over loci		3.393±0.683	2.674±1.054	2.232±0.815
Average H_o over loci		0.383±0.268	0.542±0.292	0.400±0.231
Average H_e over loci		0.719±0.078	0.534±0.176	0.426±0.227
Polymorphism (P_{95})		100%	100%	66.67%

Statistically significant values are marked with asterisks. *P<0.05, **P<0.01, ***P<0.001 and NS = Not significant.

Table 5. Homogeneity between the samples of *L. rohita*. χ^2 (Chi-square) values followed by degrees of freedom in parentheses.

Populations	Loci	Padma×Hatchery	Hatchery
Padma	*Lr3*	19.048***(3)	17.043***(3)
	Lr21	16.160**(4)	24.630***(4)
	Lr23	12.333^{NS}(8)	32.456***(9)
Hatchery	*Lr3*	18.182***(2)	
	Lr21	2.647^{NS}(1)	
	Lr23	16.088*(6)	

Statistically significant values are marked with asterisks. *P<0.05, **P<0.01, ***P<0.001 and NS = Not significant.

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Table 6. Multilocus F_{st} (above diagonal) and Nei's (1972) genetic distance (below diagonal) and N_m values (below diagonal in parentheses) between pairs of three populations of *L. rohita* across all loci.

Populations	Padma	Padma×Hatchery	Hatchery
Padma	---	0.138	0.196
Padma×Hatchery	0.590 (1.563)	---	0.118
Hatchery	0.827 (1.024)	0.247 (1.863)	---

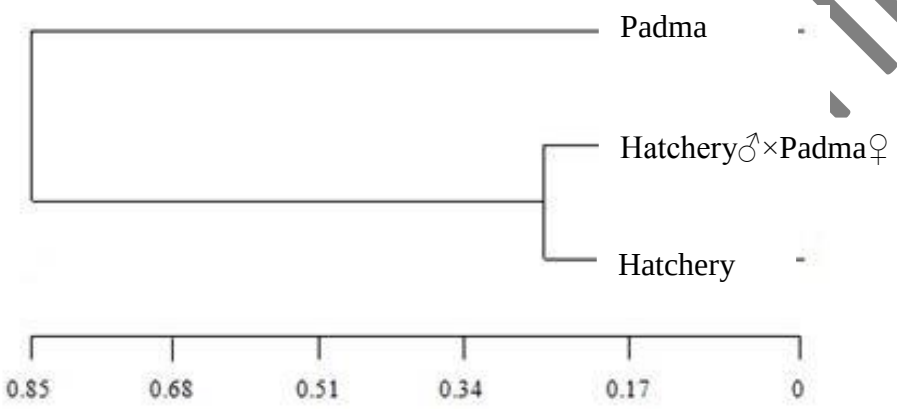

Figure 1. Dendrogram based on Nei's (1972) genetic distance summarizing the differentiation among Padma, Padma × Hatchery and Hatchery populations of *L. rohia*.