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Genetic Diversity Analysis of *Labeo rohita* (Hamilton, 1822) From Hatchery and Dhaura Reservoir of Uttarakhand by Using Microsatellite Markers

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A B S T R A C T

Labeo rohita, popularly known as rohu is a widely cultured species in the whole Indian subcontinent. Knowledge of the genetic diversity of this species is important to support management and conservation programs which will subsequently help in sustainable production of this species. DNA markers, mostly microsatellite markers are excellent tool to evaluate genetic variation of populations. The present study deals with genetic diversity analysis of *Labeo rohita* collected from hatchery and Dhaura reservoir of Uttarakhand through microsatellite marker. Total 20 microsatellite primers were designed by using software Primer-BLAST and Primer-3. A total of 12 microsatellite loci were successfully amplified. After performing native PAGE using amplified 50 DNA samples each, POP GENE Version 1.32 was used to calculate microsatellite variation. The average expected Nei's genetic diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from Dhaura reservoir. The observed and expected heterozygosity ranged from 0.2237 to 0.3326 and 0.2786 to 0.3763 respectively for *Labeo rohita* from hatchery. The mean value of observed heterozygosity was 0.2864 and that of expected heterozygosity was 0.3238. Mean Fis values were found to be 0.193 at all loci in hatchery and 0.169 at all loci in Dhaura reservoir. The observed and expected heterozygosity ranged from 0.4010 to 0.4612 and 0.4217 to 0.4985 respectively for *Labeo rohita* from Dhaura reservoir with mean value of observed heterozygosity was 0.4226 and expected heterozygosity was 0.4716. Mean values for Shannon's information index for all microsatellite loci were 1.1091 for hatchery and 1.1545 for Dhaura reservoir population. Genetic diversity analyses revealed substantial changes in genetic variation and significant genetic differentiation between the wild and hatchery-produced populations of *L. rohita*. These results indicate that genetic drift may have negative effects on the reproductive capacity of the stock, because genetic factors are important in the production of high quality seed. A wide geographical location, different hydro-biological conditions, different habitat and no connectivity between these two water resources and low or absence of gene flow between the populations may be the possible reasons to make reservoir and hatchery populations differentiated.

Introduction

Molecular markers find application in aquaculture to assess loss of genetic variation in hatcheries through, comparison of variation estimates between hatchery stocks and wild counterparts. The information is useful obtained in monitoring farmed stocks against

inbreeding loss and to plan genetic up gradation programmes. Molecular markers have proven to be an exceptional indicator of genetic variation within and between populations of many fishery animals (Choi and Kim, 2012; Lee and Hur, 2012). Among the available genetic markers, microsatellites are recognized as an essential tool in population studies (Han *et al.,* 2012; Kim *et al.,* 2013).

All wild-unstocked samples were highly differentiated populations and significantly different from each other and from hatchery samples.Use of DNA markers in population genetic studies of rohu is limited to allozyme (Rana *et al.,* 2004) and mtDNA (Luhariya *et al.,* 2012).

Microsatellite markers have been developed for selected Indian fish species such as rohu (Das *et al.,* 2005; Patel *et al.,* 2009), catla (McConnell *et al.,* 2001), chitala (Punia *et al.,* 2006) and mrigala (Lal *et al.,* 2011). Knowledge of genetic diversity in Indian major carps is considered significant for planning conservation of wild populations (Penman *et al.,* 2005 and Salgueiro *et al.,* 2003) which are facing multiple threats and consequently decline of populations. Wild populations of these carps also face the risk of genetic erosion in their native distribution.

Molecular genetic diversity in fish has been reported to be associated with life history traits that reflect habitat types (DeWoody and Avise, 2000); therefore, it is necessary to investigate genetic variability in the wild and hatchery-produced populations of *L. rohita* to accumulate significant scientific data fundamental to the success of aquaculture development strategies.

The aim of the present study was to assess genetic variation among hatchery stock and reservoir populations of *L. rohita* using microsatellite DNA markers.

Materials and Methods

Collection of samples and isolation of genomic DNA

Kidney tissue samples were collected from each individual (n=50) of *L. rohita* from hatchery and Dhaura reservoir and stored at -86^0 c in deep freezer for further analysis. DNA was isolated from the dissected kidney tissue through DNA isolation kit purchased (BANGLORE GENEI). Total twenty microsatellite primers were designed by using software Primer-BLAST and Primer-3. To amplify the repeat regions, primers were designed using the web based tool Primer3 (http://primer3.sourceforge.net/)(Rozen and Skaletsky, 2000) to amplify a PCR product of approximately 120-150 bp, with an optimum Ta of 55°C and a minimum GC content of 40-70%. All the microsatellite primers were screened in 50 DNA samples of fishes from captivity and wild stock.

Amplification of microsatellite loci and analysis of microsatellite data

All the microsatellite primers were screened in each 50 DNA samples of fishes collected from hatchery and Dhaura reservoirs. A total of 12 microsatellite loci were successfully amplified and were produced clear and polymorphic bands from hatchery and reservoir populations of *L. rohita*. PCR amplification of microsatellite loci were performed in a 25 µl reaction mixture, which included 1X PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl), 0.2 mM of each dNTP, 2.0 mM of MgCl2, 5 p mol of each primer, 1.5 U Taq DNA polymerase and 25–50 ng of template DNA. Initial denaturation at 94 degree Celsius for 3 minutes followed by 30 cycles of 94 degree Celsius for 30 seconds, locus specific annealing temperatures for 60 seconds and 72 degree Celsius for 90 seconds and a final elongation of 1 cycle at 72 °C for 8

min and stored at 4 °C. Amplified products were mixed with 2 (µl) of gel loading dye and then separated on 6% denaturing poly acrylamide gel with 1x TBE on PAGE Gel along with standard marker Φ X 174/ Hinf I marker at constant power supply of 25 volts for 2 hrs. Polymorphic information content (PIC) of individual primer was estimated using the formula: PIC = 1- 1/n $\sum_{i=1}^{n} Pij$

Where Pij is the frequency of jth allele. After performing native PAGE using amplified 50 DNA samples each from both the populations, POP GENE Version 3.4 (Raymond and Rousset, 1998) was used to calculate Nei's observed heterozygosity (Ho), expected heterozygosity (He) and Fixation index (Fis). Nei's average expected gene diversity (Hi) was calculated from the banding pattern of every primer. Individual genotypes were scored using the GeneMapper (version 4.0; Applied Biosystems) with a size standard and an internal control for allele calling; each allele was coded according to its size in nucleotide base pairs (bp). A panel that included all of the alleles detected in the 50 individuals was created for each locus. Possible null alleles and genotyping errors caused by stuttering and/or large-allele dropout were tested using MICRO-CHECKER (1000 randomizations) (Van Oosterhout *et al.,* 2004). Scoring and human error were estimated by duplicate analyses. The polymorphic information content (PIC) calculated by using the CERVUS version 3.03 (Kalinowski *et al.,* 2007).

Results and Discussion

Primers amplification results of *Labeo rohita* collected from Dhaura reservoir

Twelve microsatellite primers were successfully amplified and showed polymorphism (Table 1). Total 65 numbers of alleles scored in Dhaura stock. Number of alleles per locus ranges from 4 to 7 with mean value of 5.41 per locus, a total of 6 SSR loci was scored by the primer PL-01. The product size ranged from 0.11 to 0.29 Kb and the PIC value and average expected gene diversity of the primer were 0.62 and 0.519 respectively. A total number of 5 SSR loci were scored by the primer PL-02 and three loci were polymorphic (Tables 2 and 5). The product size ranged from 0.13 Kb to 0.32 Kb and the PIC value and average expected gene diversity of the primer were 0.54 and 0.523 respectively. 4 SSR loci were scored for the primer PL-03 with product size ranged from 0.23-0.34 Kb and the PIC value and average expected gene diversity of the primer were 0.57 and 0.536 respectively. The total of 7 SSR loci was scored for the primer PL-08 (Tables 2 and 5). The product size ranged from 0.24 Kb to 0.48 Kb and the average expected gene diversity and PIC value of the primer were 0.59 and 0.549 respectively. Total numbers of 5 SSR loci were scored by the primer PL-10 and three loci were found to be polymorphic. The product size ranged from 0.19 Kb to 0.51 Kb and the average expected gene diversity and PIC value of the primer were 0.54 and 0.611 respectively (Tables 2 and 5). 7 SSR loci were scored by the primer PL-11 and the product size was 0.20-0.37 Kb. PIC value and the expected genetic diversity was 0.59 and 0.549 respectively. 6 SSR loci with product size ranged 0.23 Kb to 0.49 Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.61 and 0.602 respectively. 5 SSR loci were scored by the primer PL-14 and the average expected gene diversity and PIC value of the primer were 0.53 and 0.506 respectively and product size ranged from 0.14 to 0.33 kb (Tables 2 and 5). 5 SSR loci were scored by the primer PL-15 and the average expected gene diversity and PIC value of the primer were 0.53 and 0.625 respectively and product size ranged from 0.16 to 0.50 kb (Tables 2 and 5). 6 SSR loci

were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were 0.478 and 0.56 respectively. Product size ranged from 0.19 to 0.41 kb. 4 polymorphic SSR loci were scored by the primer PL-17 and the average expected gene diversity and PIC value of the primer were 0.57 and 0.509 respectively and product size ranged from 0.17 to 0.38 kb (Tables 2 and 5). 6 SSR loci were scored by the primer PL-20 and the average expected gene diversity and PIC value of the primer were 0.55 and 0.517 respectively and product size ranged from 0.16 to 0.41 kb (Tables 2 and 5)

Primers amplification results of *Labeo rohita* collected from hatchery stock

Twelve microsatellite primers were successfully amplified and showed polymorphism (Table 1). Total 52 numbers of alleles scored in hatchery stock, number of alleles per locus ranges from 3 to 5 with mean value of 4.33 per locus. A total of 4 SSR loci were scored by the primer PL-01. The product size ranged from 0.11 Kb to 0.24 Kb and the PIC value and average expected gene diversity of the primer were 0.52 and 0.473 respectively. A total number of 3 SSR loci were scored by the primer PL-02 and all the loci were polymorphic (Tables 3 and 4). The product size ranged from 0.13 Kb to 0.31 Kb and the PIC value and average expected gene diversity of the primer were 0.48 and 0.528 respectively. The totals of 5 SSR loci were scored for the primer PL-03 with product size ranged from 0.20 to 0.43 Kb. and the PIC value and average expected gene diversity of the primer were 0.56 and 0.474 respectively. The total of 5 SSR loci was scored for the primer PL-08 (Tables 3 and 4). The product size ranged from 0.27 to 0.36 Kb and the average expected gene diversity and PIC value of the primer were 0.56 and 0.369 respectively. Total numbers of 4 SSR loci were scored by the primer PL-10. The product size ranged from 0.28 Kb to 0.53 Kb and the average expected gene diversity and PIC value of the primer were 0.52 and 0.418 respectively (Tables 3 and 4). 5 SSR loci were scored by the primer PL-11which and the product size was 0.30-0.44 Kb and the expected genetic diversity and PIC value of the primer 0.56 and 0.497 respectively (Table 3 and 4). 4 SSR loci with product size ranged 0.29 Kb to 0.47 Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.52 and 0.529 respectively. 5 SSR loci were scored by the primer PL-14 and the average expected gene diversity and PIC value of the primer were 0.54 and 0.452 respectively and product size ranged from 0.16 to 0.24 kb (Tables 3 and 4). 5 SSR loci were scored by the primer PL-15 and the average expected gene diversity and PIC value of the primer were 0.56 and 0.511 respectively and product size ranged from 0.19 to 0.43kb (Tables 3 and 4). 3 SSR loci were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were 0.328 and 0.48 respectively. Product size ranged from 0.15 to 0.40 kb. 4 SSR loci were scored by the primer PL-17 and the average expected gene diversity and PIC value of the primer were 0.52 and 0.439 respectively and product size ranged from 0.18 to 0.30 kb (Tables 3 and 4). 5 SSR loci were scored by the primer PL-20 and the average expected gene diversity and PIC value of the primer were 0.56 and 0.485 respectively and product size ranged from 0.21 to 0.34 kb (Tables 3 and 4).

Microsatellite variation and gene diversity analysis

After performing native PAGE using amplified 50 DNA samples as above, POP GENE Version 1.32 was used to calculate Nei's observed heterozygosity, expected heterozygosity, Nei's genetic diversity and Fixation index (Fis). Average expected gene diversity was calculated from the banding pattern of every primer.

Table.1 Primer-BLAST designed microsatellite primers for *L. rohita*

Locus	Primer Sequence(5'-3')	Annealing Temp	Annealing Time
Lr-01	**F**-GAAAGCTGCTCGTCCTTGAA **R**-GAAAGCTGCTCGTCCTTGAA	53 ^{0}C	1min 30 sec
Lr-02	**F**-GGGTGTGGGAGAGAAAGAGAG **R**-GGAGTCTGACAAATGCAGCAAG	62 ^{0}C	1min 30 sec
Lr-03	**F**-TCTCAGTGGGTGTCATTACCTG **R**-CCCATCAAACCATCTCTCTAGC	52 ^{0}C	1min
Lr-08	**F**-CTGACACTCTTATCTCGCTGCC **R**-GACCTGAGCAAACAAACCTCAT	53 ^{0}C	1min 30 sec
Lr-10	**F**-TCTCTCTTTGTCTTTCCCCTTG **R**-CACAAGCCACTGTTTAGCTTCA	64 ^{0}C	1min
Lr-11	**F**-CAAATCTGTGAACATGCAAGC **R**-CCTAGTCCCACTCTAGTCAGCA	57 ^{0}C	1 min 30 sec
Lr-13	**F**-AGATAAGACCCTTCTTCCTCGG **R**-TTTATTAGGGAGCGTCGAGTG	62 ^{0}C	1min 30 sec
Lr-14	**F**-CTGTTGGTGACTGTAGGGTGAA **R**-GAGAACTCGGTTTGAACATGC	58 ^{0}C	1min
Lr-15	**F**-ACAGTAATCTTGTGTCTGTCTCTCTC **R**-GTCTAAACGTGTCTGAGCTGTG	55 ^{0}C	1 min 30 sec
Lr-16	**F**-TGAATGTTTCCAGTCACCACAT **R**-GTAATGCAGCGGAGAATAAACC	57 ^{0}C	1min
Lr-17	**F**-ACAATTCCTGTGTCAACTGTGC **R**-TACCGTCTCAGTCTCTTTTCGG	57 ^{0}C	1min 30 sec
Lr-20	**F**-ATAGTCGAAATTGGTCCTCTGC **R**- CAATACCATGACTGAAGTGCC	55 ^{0}C	1min 30 sec

Table.2 Screened primer amplification results of *Labeo rohita* collected from Dhaura

Locus	Amplified Product (Kb)	Number of alleles	(PIC)
PL- 01	0.11-0.29	6	0.62
PL-02	0.13-0.32	5	0.54
PL-03	0.23-0.34	4	0.57
PL-08	0.24-0.48	7	0.59
PL-10	0.19-0.51	5	0.54
PL-11	0.20-0.37	7	0.59
PL-13	0.23-0.49	6	0.61
PL-14	0.14-0.33	5	0.53
PL-15	0.16-0.50	5	0.53
PL-16	0.19-0.41	6	0.56
PL-17	0.17-0.38	4	0.57
PL-20	0.16-0.41	6	0.55

Table.3 Screened primer amplification results of *Labeo rohita* collected from hatchery

Locus	Amplified Product (Kb)	Number of alleles	PIC
PL- 01	0.11-0.24	4	0.52
PL-02	0.13-0.31	3	0.48
PL-03	0.20-0.33	5	0.56
PL-08	0.27-0.36	5	0.56
PL-10	0.28-0.53	4	0.52
PL-11	0.30-0.44	5	0.56
PL-13	0.29-0.47	4	0.52
PL-14	0.16-0.24	5	0.54
PL-15	0.19-0.43	5	0.56
PL-16	0.15-0.40	3	0.48
PL-17	0.18-0.30	4	0.52
PL-20	0.21-0.34	5	0.56

Table.4 Genetic Diversity of *L. rohita* from hatchery based on microsatellite markers

Locus	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Nei's genetic diversity (Hi)	Shanon's Information Index	Fixation Index Fis
PL- 01	0.2682	0.2885	0.473	1.1522	0.162
PL-02	0.2981	0.3042	0.528	1.1121	0.208
PL-03	0.3326	0.3763	0.474	1.0972	0.289
PL-08	0.2549	0.3119	0.369	1.1020	0.291
PL-10	0.2237	0.2786	0.418	1.0124	0.259
PL-11	0.2646	0.3127	0.497	1.2149	0.188
PL-13	0.3015	0.3269	0.529	1.0975	0.154
PL-14	0.2988	0.3420	0.452	1.0556	0.179
PL-15	0.3004	0.3119	0.511	1.1061	0.132
PL-16	0.2817	0.3438	0.328	1.2239	0.161
PL-17	0.3114	0.3329	0.439	1.1241	0.141
PL-20	0.3018	0.3569	0.485	1.0118	0.153
Mean	**0.2864**	**0.3238**	**0.4585**	**1.1091**	**0.193**

Table.5 Genetic diversity of *L. rohita* from Dhaura based on microsatellite markers

Locus	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Nei's genetic Diversity (Hi)	Shanon's Information Index	Fixation Index Fis
-0.147	0.4037	0.4764	0.519	1.2134	0.152
-0.198	0.4176	0.4875	0.523	1.2379	0.203
-0.186	0.4153	0.4548	0.536	1.1258	0.199
-0.191	0.4612	0.4985	0.549	1.2037	0.201
-0.188	0.4535	0.4956	0.611	1.1928	0.198
-0.181	0.4143	0.4652	0.549	1.1098	0.194
-0.114	0.4010	0.4789	0.602	1.1042	0.125
-0.122	0.4017	0.4217	0.506	1.1026	0.136
-0.154	0.4254	0.4547	0.514	1.1095	0.169
-0.132	0.4312	0.4674	0.478	1.1128	0.149
-0.126	0.4032	0.4765	0.509	1.1062	0.146
-0.141	0.4441	0.4828	0.517	1.1363	0.157
0.157	**0.4226**	**0.4716**	**0.534**	**1.1545**	**0.169**

The average expected Nei's genetic diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from Dhaura reservoir. 73.8 % polymorphism was shown by microsatellite marker in Dhaura reservoir population while 67.3% polymorphism in hatchery stock. The observed and expected heterozygosity ranged from 0.2237 to 0.3326 and 0.2786 to 0.3763 respectively for *Labeo rohita* from hatchery (Tables 4 and 5). The mean value of observed heterozygosity was 0.2864 and that of expected heterozygosity was 0.3238. Mean Fis values were found to be 0.193 at all loci in hatchery and 0.169 at all loci in Dhaura.

The observed and expected heterozygosity ranged from 0.4010 to 0.4612 and 0.4217 to 0.4985 respectively for *Labeo rohita* from Dhaura reservoir with mean value of observed heterozygosity was 0.4226 and expected heterozygosity was 0.4716. Mean values for Shannon's information index for all microsatellite loci were 1.1091 for hatchery population and 1.1545 for Dhaura reservoir population (Tables 4 and 5).

When the level of diversity in the hatchery-produced population was compared with that of the wild population, significant differences were noted in the average number of alleles per locus and the average expected heterozygosity (Wilcoxon signed-rank test; $P < 0.05$). Because the allele number is positively related to the sample size as well as to the mutation rates at the polymorphic loci, the number of alleles observed at all 12 loci in this study is related to the relatively small size of the samples examined (Liu *et al.,* 2009). Similar genetic variability has been reported for some other marine fish species (An *et al.*, 2011a; Wang *et al.*, 2011), suggesting that these polymorphic microsatellite loci were sufficient to reveal the intraspecific diversity among *Labeo rohita*. In hatchery strains, the probability of the loss of rare alleles is high (Hutchings and Fraser, 2008).The loss of alleles is more important than the change in allele frequencies, because the latter may

again change due to random drift, whereas a lost allele cannot be recovered, in which genetic factors are of vital importance for the production of high-quality seed. An obvious degeneration of characteristics has been reported in the cultured fish stock, where the cultured fish does not reach full size, although they mature at an earlier age and have reduced resistance against diseases (Fang *et al.,* 2000). Thus, the production of progeny should be based on well-organized brood stock management strategies.

Wang *et al.,* (2002) reported that the effects of inbreeding and genetic drift of hatchery operations contributed to the reduction of genetic diversity of natural stocks of salmonid species. Moreover, siltation since ages, withdrawal of water by constructing dam on main flow are reducing the population size and subsequently declining the genetic variability of the species. The presence of null alleles and/or the inability to separate closely sized alleles due to presence of stutter bands in the microsatellites used might lead to reducing measures of heterozygosity. Microsatellite loci generally show considerable evolutionary conservation, suggesting that primers developed for any one species may often be useful across a wide range of taxa.

However, one drawback of heterologous primers is that mutations in the flanking sequences, to which PCR primers are designed to anneal, can result in non-amplifying PCR null alleles (Hoffman and Amos, 2005; Selkoe and Toonen, 2006). Heterozygote deficiency can also reflect various biological processes such as inbreeding, Wahlund effects and selection (Van Oosterhout *et al.,* 2004). The protection of genetic characteristics of the cultured stock should be considered in artificial reproduction. In the wild population, heterozygote deficit can be explained by several factors, such as the presence of unrecognized null alleles, natural selection acting on genetic markers, mating among relatives, the reduction of heterozygosity in a population caused by a subpopulation structure known as the Wahlund's effect, or a combination of these factors. In hatchery populations, heterozygote deficiency is commonly caused by the limited number of founders, inbreeding, or both (Kohlmann *et al.,* 2005; An *et al.,* 2011b). This deficit may also be attributed to improper domestication processes occurring in the hatchery populations. The FST indicates the proportion of genetic variation that could be attributed to the genetic differentiation processes between the co-specifics from two localities (Coelho *et al.,* 1995). Since there is no physical connection between the hatchery and reservoir, naturally no mixing is possible between stocks and hence they are expected to exhibit high genetic differentiation. However, our results indicate a low level of genetic differentiation between populations with FST values ranging from 0.009 to 0.047. The sample size in the present study was 50 individuals in each population. Therefore, estimates of population differentiation obtained are unlikely to be confounded by small sample sizes. The overall FST for all samples combined was found to be 0.047. Thus, approximately 4.7 % of genetic variation was found to be caused by genetic differentiation in *L. rohita*, indicating low level of genetic differentiation. This pattern of variation corresponds to that obtained in other Indian freshwater fishes (Chaturvedi *et al.,* 2011; Gopalakrishnan *et al.,* 2009). A wide geographical location, different hydro-biological conditions, different habitat and no connectivity between these two water resources and low or absence of gene flow between the populations may be the possible reasons to make reservoir and hatchery populations differentiated. The significant differentiation between the 2 populations,

particularly in the number of private alleles is probably related to several factors such as habitat fragmentation, reduction in the effective number of contributing parents, and the effects of artificial selection on hatchery progeny. Hence, genetic drift has probably played an important role in the loss of genetic diversity and in the differentiation between wild and hatchery-produced populations. The genetic integrity of wild population should be protected from the impact of hatchery production through a carefully planned brood stock management strategy. Unknown and known genetic changes and the possible loss of genetic variation in the wild and hatchery-produced populations should be monitored by using molecular tools such as nuclear DNA markers.

In summary, genetic diversity analyses revealed substantial changes in genetic variation and significant genetic differentiation between the wild and hatchery-produced populations of *L. rohita*. These results indicate that genetic drift may have negative effects on the reproductive capacity of the stock, because genetic factors are important in the production of high quality seed. A wide geographical location, different hydro-biological conditions, different habitat and no connectivity between these two water resources and low or absence of gene flow between the populations may be the possible reasons to make reservoir and hatchery populations differentiated.

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