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MICROSATELLITE MARKERS REVEAL GENETIC DEGRADATION IN HATCHERY STOCKS OF *Labeo rohita*

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The genetic structure of *Labeo rohita* in hatchery stocks of the province Punjab was assessed by using five species-specific microsatellite markers. The samples of *L. rohita* were collected from six selected hatcheries. The genotypic data of each sample was generated through PCR amplification of target loci and allelic scoring by gel electrophoresis. The data was analyzed for calculation of various indices of genetic diversity. The polymorphism was observed for all alleles and allelic size fluctuated sample to sample over all the loci. We found low to moderate level of heterozygosity with the average values of H_o and H_e as 0.42-0.7 and 0.661-0.717 respectively. The inbreeding coefficient F_{IS} values showed the highest level of inbreeding in Mianchannun hatchery and lowest in Faisalabad hatchery. The analysis of F_{ST}, genetic distance, AMOVA and UPGMA dendrogram revealed genetic closeness between Faisalabad, Farooqabad and Mianchannun hatcheries. Knowledge on the genetic structure of *L. rohita* populations will be useful for effective broodstock management and conservation.
Keywords: Fish breeding, hatchery, carps, DNA markers, genetic diversity

INTRODUCTION

The genetic diversity of commercial fishes is devastated due to poor management of broodstock, inbreeding and lack of genetic characterization. Genetic conservation programs are carried out as an effort to recover depleted populations resulting from over-exploitation, environmental fluctuations and natural mortality. Genetic monitoring of a species is essential for the proper management of genetic variability and identification of population groups with independent evolutionary histories. The magnitude of genetic diversity has significant implications for the management and conservation of species.

Genetic diversity develops the potential in species and population to maintain and protect themselves against extinction (Ashley *et al*., 2003), as it provides the basis for survival in ever changing environment (Banerjee *et al.*, 2008). Loss of genetic diversity leads to the fixation of genes, loss of fitness in terms of vigor, fecundity, viability, resistance against diseases and ultimate extinction of local population.

Among the carps cultured in Asia, *Labeo rohita* production was ranked 7th (713267 tonnes) in 2003 (FAO, 2005). Rapid expansion of *L. rohita* aquaculture has been possible because of the availability of the hatchery produced seed. Currently about 99% of the *L. rohita* seed originates from private and public hatcheries (Islam and Alam, 2004). Rapid intensification of fry production in hatcheries however, has not been accompanied by genetic quality maintenance measures. In Pakistan, *L. rohita* aquaculture is suffering from poor genetic management of brood stock. Natural as well as farmed fish populations are facing genetic issues that are quite unique in fisheries management.

Anthropogenic intervention is largely responsible for such genetic issues, not only it altered the genetic diversity of fish stocks but also thousands of species and their spawns have been totally destroyed by disruption in breeding and nursing grounds, habitat destruction, overfishing, blockage of migratory routes and various other activities (Collares-pereira and Cowx, 2004).

On the other hand, indiscriminate genetic hybridization between introduced species, inbreeding due to small breeding population, negative selection, introduction of poor quality hatchery reared seed in wild, species migration in a new habitat and hydrological alteration are some other factors responsible for the loss of genetic diversity among species (Abbas *et al*., 2010). Unwanted hybridization of *L. rohita* with *C. catla* and *C. mrigala* has been reported as a major problem and partly assumed to be responsible for the decline in growth performance of these species (Simonsen *et al*., 2004). Introduction of hatchery reared poor quality *L. rohita* seed to the wild through escapes and government sponsored seed stocking program, often on a massive scale, are becoming a serious concern (Islam and Alam, 2004). It is therefore, imperative to understand the genetic composition of *L. rohita* stocks in order to ensure their proper management.

Large genetic changes due to inadvertent and unintentional selection occur as a part of routine operations in hatcheries such as choosing few but highly fertile females, non-random selection of brooders due to netting bias, replacing broodstock with few fingerlings when best ones has been sold or selecting large male for milt production etc. (Brown *et al*., 2005).

Population genetics studies such as identification of wild and hatchery stocks, detection of changes in genetic make-up of wild populations as a result of mixing of hatchery fish can only be performed with the aid of molecular markers. Moreover, it has become urgent to use marker technology to identify the inbreeding level of *L. rohita* stocks in hatcheries, estimate the relatedness between individuals and stocks for other hatchery management related issues. The importance of molecular markers, especially microsatellites, in genetic evaluation of wild and cultured species is universally acknowledged.

Microsatellite DNA is reliable marker due to unique features such as high rate of mutation (10^{-2} per generation), abundant distribution in coding as well as in non-coding region of DNA and fast detection protocol. These markers could be utilized in aquaculture for individual identification, parentage identification, broodstock management, marker assisted breeding programs, studies on population differentiation and to construct genetic linkage maps (Chistiakov *et al*., 2006). Microsatellites are now actively used to gauge mutation, even in closely related species and proved to be very useful in revealing information about allele frequency, heterozygosity, population differentiation, inbreeding co-efficient, gene flow, linkage disequilibrium and other parameters that are crucial measures of genetic diversity and population genetics (Liu and Cordes, 2004).

The present study was conducted to assess the genetic structure of *L. rohita* in selected hatcheries of Punjab using species-specific microsatellite markers. The findings of the present study are of great value for maintaining the genetic integrity and effective broodstock management.

MATERIALS AND METHODS

***Sampling and DNA isolation*:** The samples were collected from six selected hatcheries of Punjab including Faisalabad Hatchery (FSD), Farooqabad Nursery (FQD), Sargodha Nursery (SGD), Mainchannu Hatchery (MCH), Sahiwal Hatchery (SWH) and Kamalia Hatchery (KAH) (Fig. 1). The populations were named after the initial letters of sampling localities. Total 123 samples of *L. rohita* including fry and fingerling were randomly collected from sample hatcheries. Dorsal muscle tissue was removed and immediately frozen for subsequent DNA extraction following the method of Yue and Orban (2005). The isolated DNA was tested for quality and quantity by agarose gel electrophoresis and spectrophotometer respectively. The concentration of DNA was made uniform by dilution to the required level.

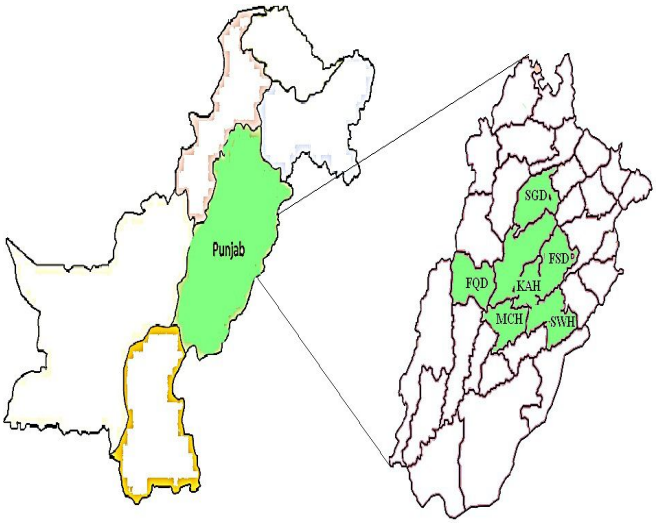

Figure 1. Map of the Punjab showing the distribution of sampling sites of *L. rohita*

***PCR amplification of microsatellite loci*:** Total five species-specific microsatellite loci (Lr1, Lr10, Lr12, Lr14b and Lr21)

Table 1. Characteristics of *L. rohita* specific microsatellite loci (Das *et al*., 2005)

Locus	Repeat unit	Primer sequence (52-32)	GenBank Accession No.	Allele size (bp)	T_a (°C)	N
Lr1	$(TG)_{14}$	F-GACCCTTAACCCTTGACCTT R-TGGGATAATGCAGGGAAAAC	AJ507518	171	58	2
Lr10	$(CA)_{13}$	F-GATCTTCAGCGCCAGCGTG R-GAGGACCTGCCCAGCATG	AJ507523	250	60	4
Lr12	$(CA)_{13}$	F-CACCGCTGCTGTCCATCA R-AGGTCGGCCAGATACACG	AJ507524	161	58	4
Lr14b	$(CA)_{12}$	F-TCACATGGGAACAACAAACC R-CCGCCGCTTACCCATCAC	AJ831434	172	58	5
Lr21	$(CA)_{11}$	F-GATCAGAGGGTCAATGTGG R-CAGCAGAGTACTATGGAAGA	AJ831436	148	58	6

Where F– forward; R – reverse; N- no. of alleles; T_a - primer specific annealing temperature.

developed by Das *et al*. (2005) were amplified through PCR (Table 1). The primers were synthesized by Penicon, USA. The PCR amplification was carried out in 20µL reaction mixture that contained template DNA (approximately 50ng), *Taq* polymerase (0.4µL), dNTPs (0.4µL) , 0.4µL of each primer and reaction buffer that include tris HCl, gelatin-0.01%, $MgCl_2$ and KCl (2.0µL) in a thermocycler. The denaturation was carried out at 94°C for 5min, 32 cycles of 1min at 94°C and elongation for 4min at 72°C.

***Gel electrophoresis*:** After amplification of microsatellite loci, 5µl of the PCR product was mixed with 1µl DNA loading dye. The mixture was loaded onto polyacrylamide gel for resolution at standard conditions. The bands in gel was be produced by silver staining and visualized in UV trans-illuminator for gel imaging. The bands were scored manually.

***Data analyses*:** The genotypic data of each locus was subjected to rigorous analysis to calculate allele frequency, population differentiation, heterozygosity, linkage disequilibrium, deviation from Hardy-Weinberg Equilibrium (HWE), and inbreeding coefficient. Software FSTAT Ver.2.9.3.2 (Goudet, 2002) was used to analyze allele frequency, heterozygosity allelic richness and inbreeding coefficient by the F-statistics. The program GENEPOP 3.3d (Raymond and Rousset, 1998) was used for testing the linkage disequilibrium and deviation from HWE. The population differentiation was determined and dendrogram constructed by the TFPGA (Weir and Cockerham, 1984). A multivariate ordination was conducted to visualize the genetic relationships among populations by principle component analysis using the software PCAGEN (Goudet, 2005).

RESULTS

***Genetic diversity*:** The microsatellite diversity indices for each population have been summarized in Table 2**.** All loci were found to be polymorphic, the maximum number of

Table 2 . Genetic diversity of six populations at five microsatellite loci of *L. rohita*

Population	parameters	Lr1	Lr10	Lr12	Lr14b	Lr21	Average
FSD	N_a	3	4	3	5	6	4.2
	A_r	3.00	4.00	3.00	5.00	6.00	4.20
	H_o	0.4500	0.3000	0.6000	0.8500	0.5000	0.54
	H_e	0.7641	0.7564	0.5949	0.7474	0.7269	0.7179
	F_{IS}	0.1299	0.5432	0.3501	0.0345	0.2774	0.2670
FQD	N_a	2	4	3	4	5	3.6
	A_r	2.00	4.00	3.00	4.00	5.00	3.60
	H_o	0.7500	0.7500	0.7000	0.7250	0.5750	0.7
	H_e	0.5481	0.6896	0.7785	0.7449	0.7054	0.69276
	F_{IS}	0.2110	0.0202	0.1786	0.1852	0.0832	0.1356
SGD	N_a	2	4	4	3	6	3.8
	A_r	2.00	4.00	4.00	3.00	6.00	3.80
	H_o	0.3500	0.5500	0.7500	0.8000	0.8000	0.65
	H_e	0.7859	0.5372	0.7436	0.7615	0.6808	0.7018
	F_{IS}	0.3857	0.1014	0.6894	0.2144	0.1745	0.3184
MCH	N_a	2	3	4	5	6	4.00
	A_r	2.00	3.00	4.00	5.00	6.00	4.00
	H_o	0.7750	0.5750	0.5500	0.7000	0.6250	0.645
	H_e	0.8009	0.7089	0.6835	0.6544	0.7313	0.7158
	F_{IS}	0.2411	0.5130	0.6529	0.4106	0.1252	0.3885
SWH	N_a	3	4	4	6	6	4.6
	A_r	3.00	4.00	4.00	6.00	6.00	4.6
	H_o	1.0000	0.7000	0.5500	0.8500	0.4500	0.71
	H_e	0.6205	0.5090	0.6449	0.5551	0.6705	0.60
	F_{IS}	0.5932	0.0345	0.1664	0.2945	0.1147	0.2406
KAH	N_a	2	4	3	5	6	4.00
	A_r	2.00	4.00	3.00	5.00	6.00	4.00
	H_o	0.5000	0.5500	0.1500	0.5000	0.4000	0.42
	H_e	0.7269	0.6372	0.6115	0.5731	0.5090	0.61154
	F_{IS}	0.3186	0.1348	0.2411	0.5130	0.6529	0.37208

alleles of all loci were 6, 5, 4, 3 and 2, respectively. The polymorphism was observed for all the loci and allelic size fluctuated from sample to sample over all the loci. Intra-population diversity was found only in single population while all other populations were found as genetically distinct.

***Genetic variation and inbreeding co-efficient*:** We observed low to moderate level of heterozygosity at all the loci. The average values of observed and expected heterozygosity were, H_o =0.42-0.7 and H_e = 0.717-0.661 respectively. The locus Lr12 was found genetically maximum polymorphic while the Lr10 and Lr21 were less polymorphic as compared to the other loci. The population FQD showed highest genetic variability and KAH has the lowest. The average value of F_{IS} ranged from 0.1356 to 0.3885, the highest rate of inbreeding was observed in MCH population and lowest in FQD population.

***Deviation from HWE*:** The significant deviations from *HWE* were observed in 21 out of 30 tests. The deviations from *HWE* were hi ghly significant ($p<0.001$) at locus Lr12 in FSD stock and at locus Lr10 in MCH stock. The locus Lr1 in SGD and MCH stock, locus Lr10 in SWH, FQD stock, locus Lr12 in MCH, locus Lr14b in FSD and KAH, locus Lr21 in FQD stock were found to be non-significant.

***Genetic differentiation and inter-population genetic structure*:** The genetic differentiation was analyzed by pairwise comparison of each population as given in the table II and found to be significant. The values of F_{ST} indicate that all populations are not homogenous. The mean value of F_{ST} among all the populations range from 0.0091 to 0.3537.The analysis demonstrated the gene flow (N_m) is minimum between MCH and FQD while maximum between MCH and KAH populations. The highest value of F_{ST} indicated that two populations MCH and KAH are remarkably isolated from one another and do not share the alleles (F_{ST} = 0.3537) while the least value between FSD and MCH populations (F_{ST} = 0.0714), indicated gene flow between the population pair.

***Genetic distance*:** The genetic distance (D) was measured on TFPGA by all population's comparison, based on the allelic frequencies of loci. The value of population genetic identity were 0.8554, 0.8603, 0.8784, 0.7982 and 0.7994 for FSD-FQD, FSD-SGD, FSD-MCH, FSD-SWH, FSD-KAH population pairs and other genetic identity for all population comparisons are given in the Table 3. The genetic distance was 0.1562, 0.1504, 0.1296, 0.2253 and 0.2239 between FSD-FQD, FSD-SGD, FSD-MCH, FSD-KAH population pairs. The largest value of genetic distance was found between MCH-KAH population pair (D = 0.3927) whereas smallest value was measured between SGD-KAH (D = 0.1019).

***AMOVA analysis*:** The analysis of molecular variance conducted on ARLEQUIN (ver. 2.000) software, described the total diversity among populations. AMOVA analysis show that 16.19% variation exists among groups. The results proved that the majority of variations was from the intra population diversity and 74.81% was from the inter population diversity (Table 4).

***UPGMA dendrogram analysis*:** Analysis of genetic relatedness among all populations was estimated by constructing UPGMA dendrogram, based on the Nei's genetic distance (1972). This analysis suggest the segregation of three populations SGD, KAH and SWH from the other three populations; FSD, MCH and FDQ. The dendrogram based two major clusters are depicted in the Fig. 2. The figure shows that the two clusters are formed, each consists of three populations while the second clusters were further divided, each into two sub-clusters. In the first sub-cluster, the SWH population was alone in one cluster whereas SGD and KAH populations were in other cluster. The second sub-cluster consisted FQD population alone where as FSD and MCH in other cluster.

***Principal component analysis*:** The *PCA* was carried to find the correlation matrix of the F_{IS} data, amongall the six populations (Fig. 3). The *PCA* analysis of the 7 significant

Table 3. Pairwise genetic differentiation (F_{ST}) (above diagonal) and genetic distance (below diagonal) among the *L. rohita* populations

	FSD	FQD	SGD	MCH	SWH	KAH
FSD	-	0.0938*	0.0910*	0.0714*	0.1574	0.1763
FQD	0.1562	-	0.2483	0.0957*	0.3101	0.2984
SGD	0.1504	0.3031	-	0.2845	0.1565	0.0618*
MCH	0.1296	0.1493	0.3353	-	0.2104	0.3537
SWH	0.2253	0.3735	0.2170	0.2698	-	0.2038
KAH	0.2239	0.3415	0.1019	0.3927	0.2525	-

Table 4. Hierarchal AMOVA analysis of populations

Source of variation	df	Sum of square	Variance component	% variation
Among groups	5	33.300	0.2693	16.19
Among populations within groups	19	42.961	1.2531	74.81
Total	119	346.267	2.0162	-

variables account 74.7% variances between KAH and FSD samples. The scatter diagram based on *PCA* analysis showed that KAH samples were genetically distinct from other samples whereas closeness were found between FQD and SWH. However, the obtained results were inconsistent with other analysis.

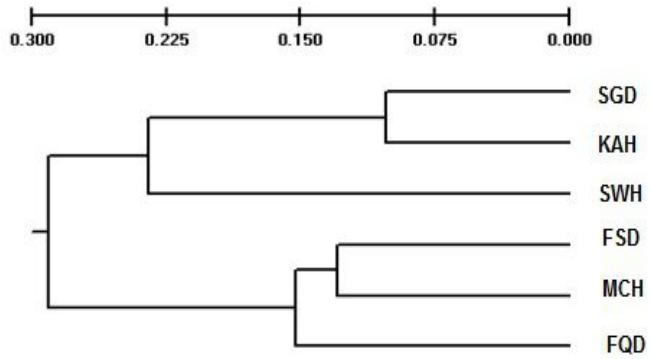

Figure 2. UPGMA dendrogram based on the Nei,s genetic between *L. rohita* populations, according to the microsatellite DNA analysis

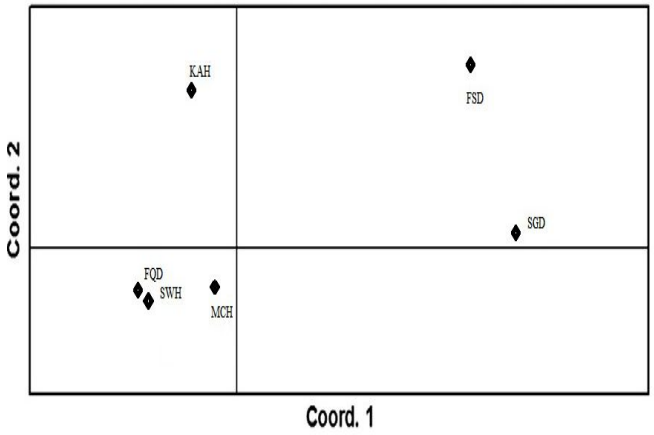

Figure 3. Principle component analysis of *L. rohita* samples by each sampling locality

DISCUSSION

The genetic diversity of hatchery stocks is devastated because of poor management and lack of genetic awareness among fish hatchery managers. It is important to know the level of genetic diversity in hatcheries and wild populations as effected by anthropogenic intervention. It is necessary not only for the conservation of wild stocks but also for the sustainability of aquaculture species. To address these problems, it is imperative to identify and disclose the problems and issues pertaining to genetic degradation of *L. rohita*.

The decline in fish yield due to genetic degradation has become a significant fisheries management problem that can potentially destroy the sustainability of the whole aquaculture business and the effects cannot be mitigated immediately. Awareness over genetic issues, in relation to artificial breeding is low in Pakistan. Various private and public fish hatcheries strive to protect the potential genetic degradation of cultured and endemic species through induced spawning technique (Bondad-Reantaso, 2007). However, due to lack of technical knowledge and awareness, negative selection, potential ecological consequences, widespread inbreeding depression and genetic introgression by hazardous hybridizations has continue to occur (Evans *et al*., 2004).

The present study was designed to assess the level of genetic diversity in selected hatcheries of *L. rohita* in Punjab as influenced by the conventional breeding practices. The poor genetic diversity reduces the population vigor to survive in an unfavorable environment. Various problems related to genetic diversity decline in hatcheries are inbreeding, limited number of broodstock, inadvertent selection of species, lack of genetic characterization, presence of null alleles, bottle neck effect and limited gene flow.

Inbreeding is major problem in hatcheries as observed by Ruzzante *et al*. (2001) in hatchery stocks of *Salmo trutta* who found loss of beneficial alleles and heterozygosity. Inbreeding occurs because of limited number of broodstock (small population size). When effective population size is small then hatchery operators use their limited broodstock to produce fish seed that reduce the viability of the succeeding progeny. In many cases, hatchery operators breed related stock due to unawareness about the genetic characterization of species which ultimately results in inbreeding and reduce survival rate. Lower effective population sizes or lower inter-population migration rates in the hatchery reared species predicts that populations of farm species are expected to be more prone to extinction than wild species and thus should be of particular conservation concern (Hansen *et al*., 2006). Same is true for selection and poor health of the brooders.

Microsatellite markers proved to be very powerful marker for determining genetic status of *L. rohita* in sample hatcheries. The data obtained by using microsatellite markers revealed low to moderate level of heterozygosity over all the loci. With the same markers, Alam *et al*. (2009) examined the genetic structure of *L. rohita* and found similar patterns of genetic polymorphism in the Halda, Jamuna and Padma populations. Similar conclusions were obtained for *C. carpio* by Zhou *et al*. (2004) using microsatellite markers. The number of alleles ranged from 2 to 6 which is consistent with the findings of Das *et al*. (2005) except for some populations. The allele size obtained for *L. rohita* in the present study for the loci Lr 1, Lr 10, Lr 12, Lr 14b and Lr

21 were 158-171, 240-251, 157-164, 167-172 and 141-147 respectively, with exception in some populations.

The average observed and expected heterozygosity were different for all populations, H_o =0.42-0.7 and H_e = 0.661-0.717 were observed. Locus Lr 12 was found genetically more polymorphic while Lr 10 and Lr 21 were less polymorphic as compared to other loci. The population FQD has highest genetic variability while KAH has the lowest. The average value of F_{IS} ranged from 0.1356 to 0.3885, the highest rate of inbreeding was observed in MCH population and lowest in FQD population. The F_{ST} analysis demonstrated minimum gene flow between MCH and FQD while maximum between MCH and KAH populations.

From *PCA* analysis, we examined genetic closeness between SWH and FQD samples while KAH were found to be genetically distinct from other samples (Fig. 3). The genetic distance measurement revealed large genetic isolation of MCH and KAH populations while small for SGD and KAH populations. Our results confirmed correlation between geographical distance and genetic distance as shortest genetic distance between SGD and KAH corresponded to shortest geographical isolation of both populations. UPGMA dendrogram analysis divided six populations in to two clusters (SGD, KAH, and SWH from FSD, MCH, and FQD). Our overall results examined the following percentage of genetic diversity in each population: 25.24% in KAH, 31.23% in SWH, 46.62% SGD, 48.4% in FSD, 52.55%in MCH and 62.39% FQD.

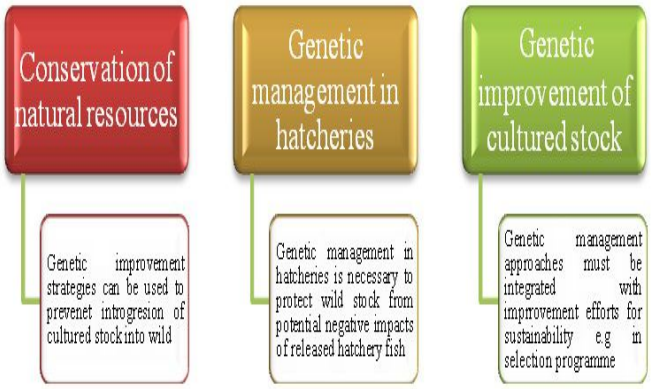

Figure 4. Three major management programs needed to conserve genetic diversity

In the present project, the microsatellite markers proved to be robust and reliable tools for evaluation of genetic diversity in *L. rohita*. The findings of present research endeavor would provide baseline information for gentic management of broodstock on fish hatchereis. It has been well recognized that keeping maximun number of brooders and avoidance from inbreeding are prerequisite for genetic sustainability of hatcheries. In order to minimize genetic deterioration of hatchery-produced seed, attension need to directed toward management programmes described in the Figure 4. The concordant progress in these areas will provide necessary protection of hatchery stock from genetic decline to ensure the preservation of genetic diversity and achieve the sustainable fishery stocks.

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