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ALLOZYME BASED GENETIC VARIATION BETWEEN HATCHERY AND NATURAL POPULATIONS OF SAHAR (*TOR PUTITORA*)

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ABSTRACT

Sahar (*Tor putitora*) formed a substantial natural fishery in the major riverine and lacustrine ecosystem of Nepal. Biological diversity of this species is being threatened by various anthropogenic activities. In view of the conservational value and the aquaculture potential of *T. putitora*, significant development in artificial propagation of this species has been achieved. The successful hatchery production of *T. putitora* brought to the forefront problematic questions regarding genetic variation of the hatchery stocks. A study was, therefore, conducted to determine the genetic variability within and between hatchery stocks and their wild counterparts of *T. putitora* using allozyme markers.

Analyses of seven enzyme systems resuled in 11 loci being resolved from lake population and two consecutive generations of hatchery populations of *T. putitora*. Based on five polymorphic loci, all populations had percentage polymorphic loci 45.45. Significant reduction ($P<0.01$) in number of alleles per locus was evident in hatchery populations (1.45 ±0.181) compared to lake population (1.72 ±0.90). Loss of rear alleles, *EST-2*74*, *IDH*70* and *GDH*33* occurred in both of the hatchery populations which were present in wild counterparts- the lake population. All populations under study conform to the Hardy-Weinberg equilibrium at the 1% level. Although not significant ($P>0.05$), observed heterozygosity increased in first generation of hatchery population (H_o= 0.181 ±0.233) compared to natural population (H_o=0.179±0.221). The H_o of second generation of hatchery population was lowest (0.119 ±0.143) among the populations studied. Loss of rare alleles from the two generations of hatchery population, while these alleles were present in corresponding natural populations suggested the founders (20-30 individuals) of the hatchery populations probably represented bottlenecks to very small effective population size (N_e).

Keywords: alleles, allozyme marker, heterozygosity, natural fisheries, variability

INTRODUCTION

Fish of genus *Tor,* commonly referred to as the sahar or mahseers, are important to trans-himalayan mid hill regions for biodiversity reasons, and are also sought after as high-valued food and game fish (Ng 2004, Bista *et al.* 2002). Nepal harbours *T. tor* and *T. putitora* which are commonly known as golden mahseer. *T. putitora* is most prevalent and live in headwaters of major river systems of Nepal. This species of mahseer formed a substantial natural fishery in the major riverine and lacustrine ecosystem of the country (Gurung *et al.* 2003). Despite their importance, their biological diversity is being threatened by various anthropogenic such as urbanization, habitat fragmentation, over-exploitation and ecological alterations and physical changes in natural environment (Swar 2002, Gurung *et al.* 2003). These has resulted in depletion of natural stocks of *T. putitora* to such an extent that they have been identified as critically endangered species (Islam 2002).

In view of the conservational value and the aquaculture potential of *T. putitora*, there has been a concerted effort to artificially propagate this species. Since the *T. putitora* fetch a very high market price and is of high cultural value. Recognizing the importance of *T. putitora*, Nepal Agricultural Research Council (NARC), made a concerted attempts to evaluate its aquaculture potential, including captive breeding using long term pond reared broodstocks commencing in the period late 1980s. In early days of *T. putitora* domestication process, captive breeding of this species was based on wild caught, mature fish from lake and rivers (Gurung *et al.* 2002). Later in 1995, Fisheries Research Stations, Pokhara and Trishuli were able to captive breed *T. putitora* derived directly from progeny of the wild stocks. Recent hatchery productions of *T. putitora* are coming from the second generation of hatchery bred broodstocks. Hatchery produced offspring of this species are being used to stock enhancement in several natural water and to evaluate aquaculture potential in ponds.

The successful hatchery production of *T. putitora* brought to the forefront problematic questions regarding genetic variation of the hatchery stocks (broodstock). Long-term management of aquaculture production and conservation of this species would require information on levels of genetic variability within and among stocks which permits fish breeders to avoid potential detrimental effects of inbreeding and other genetic changes from one generation to another (Gjedrem 1992, Nguyen *et al.* 2005).

There have been documented cases in many fish species of genetic changes and loss of genetic variability in hatchery-reared stocks, such as in rainbow trout, *Oncorhynchus mykiss* (Koljonen 1986), guppy, Siamese fighting fish, *Betta splendens* (Meejui *et al.* 2005), Atlantic salmon, *Salmo salar* (Koljenon *et al.* 2002). Alteration of genetic diversity of wild counterparts would result due to interbreeding with escapees of hatchery-reared stocks (Cliford *et al.* 1998) or those used for restocking (Hinder *et al.* 1991). Genetic variation of hatchery populations of these species decreased due to small effective numbers of founders and/or genetic drift that occurred during the maintenance of the broodstock (Allendorf and Phelps 1980). Direct genetic interactions between wild and hatchery fish have been demonstrated in many studies (Simons *et al.* 2006). Hatchery-propagated Atlantic salmon were found to compete directly with native salmon for resources such as space, food or mates, alter predation regimes and transfer disease and parasites (Fleming *et al.* 2000). Such cases demonstrated problems that may rise in other species as well (Utter 2003).

Genetic variability is pivotal to maintaining the capability of restocked fish to adapt to a new or changing environment (Avise 1994). For conservation purpose, a successful restocking program depends largely on a broodstock management strategy that ensures maintenance of a wide gene pool (Nguyen *et al.* 2006). This minimize adverse effects on the genetic diversity of wild populations once stock enhancement commences, thereby helping to maintain the genetic integrity of the species under consideration (Vrijenhoek *et al.* 1985). The lack of genetic study to determine the genetic variability within and between hatchery stocks and their wild counterparts of *T. putitora* promoted the present study, the results of which may help in design of hatchery stock management strategy and subsequent application to future aquaculture production and genetic conservation strategies (Laikre *et al.* 1999). The objective of this study was to quantify genetic variation of lacustrine population and hatchery stocks of *T. putitora*

maintained at Fisheries Research Centre, Pokhara, Nepal, using allozymes. The data have been used to discuss the implication for management of this species including aquaculture and conservation developments.

MATERIALS AND METHODS

Collection of Fish Samples

Forty wild-fish specimens of *T. putitora* were collected using gill net from lake Phewa (423 ha, 28^0 13' N, 84^o 00' E). Live fish (40 individual/population) from the hatchery-reared two subsequent generations were taken from Fisheries Research Centre, Pokhara, where hatchery propagation of this species is being undertaken since 1995. Foundation stock of *T. putitora* directly served as parents for hatchery population I. Hatchery population II was consecutive generation of hatchery population I.

The live and gill net caught fish were subsequently sacrificed for muscle and liver tissues. The tissue samples were immediately frozen and transported to Biotechnology laboratory at Kathmandu. Frozen tissues were stored at -40 ^{o}c in the laboratory until electrophoresis.

Isozyme Analyses

Seven enzyme systems were analyzed following methods described by Morizot and Schmidt (1990) and Hara and Na-Nakorn (1996). Horizontal starch gel (11% w/v hydrolyzed potato starch) electrophoresis was operated in appropriate buffer systems. All electrophoresis runs were performed at 100 V and 40 mA for 16 hours. Tissue, enzyme systems, E.C. numbers, buffer system and the resolved loci are shown in Table 1. Chemical visualization followed Morizot and Schmidt (1990). Gene nomenclature suggested by Shaklee *et al*. (1990) was used.

Table 1. Names of enzyme systems, their E.C. number, tissue and buffer used, and the resolved loci.

Names of enzyme	E.C. number	Tissue	Loci
Isocitrate dehydrogenase (IDH)	E.C.1.1.1.42	L	*IDH**
Esterase (EST)	E.C.3.1.1.1	L	*EST-1**
			*EST-2**
Malate dehydrogenase (MDH)	E.C.1.1.1.37	M	*sMDH-1**
			*sMDH-2**
Malic enzyme (ME)	E.C.1.1.1.40	M	*ME-1**
			*ME-2**
Sorbitol dehydrogenase (SDH)	E.C.1.1.1.14	M	*SDH-1**
			*SDH-2**
Glucose-6-phosphate dehydrogenase (G6PD)	E.C.1.1.1.49	M	*G6PD**
Glucose dehydrogenase (GDH)	E.C.1.1.1.47	L	*GDH**

L= liver, M= muscle

Buffer: Tris-citrate pH 8.0

Data Analyses

Individual genotypes were used for the calculation of allele frequencies, percentage of polymorphic loci (a locus was considered polymorphic if the frequency of the most common allele was 0.95 or less), average and effective number allele per locus, observed and expected heterozygosity (Nei 1978). The calculations were performed by the POPGENE version 1.32 (Yeh and Boyle 1997). Differences in heterozygosity between populations were tested using independent sample comparisons (Archie 1985).

Populations were tested for Hardy-Weinberg equilibrium using Markov chain method (demorization=1000, batches=20, iteration per batch=1000). Locus-wise F_{is} (Weir and Cockerham 1984) were calculated within each of the populations and significance level were Bonferoni corrected (Rice 1989). Wright's *F*-statistic approach (Wright 1951, 1978) and its exact test were calculated to test for genetic population structure. Population differentiation was tested using the Markov chain method. All these calculations were preformed by the GENEPOP version 3.3 (Raymond and Rousset 1995). The software TFPGA (Miller 1997) was used to calculate genetic distances (Nei 1972, 1978).

RESULTS AND DISCUSSION

Based on 5 polymorphic loci, the natural population had mean number of alleles per locus of 1.72 (±0.90), percentage polymorphic loci = 45.45, mean observed and expected heterozygosity (H_o, H_e) = 0.179 (±0.221) and 0.184 (± 0.196), respectively (Table 3 and 4).

Analyses of seven enzyme systems resuled in 11 loci being resolved from the two consecutive generations of hatchery populations of *T. putitora*. Five loci were polymorphic (a locus was considered polymorphic if a frequency of most common allele did not exceed 0.95- $P_{.95}$, *EST-2*, sMDH-1*, IDH*, GDH** and *MEP-1*.* Allele frequencies of the polymorphic loci are shown in Table 2.

Table 2. Allele frequencies of eleven polymorphic allozyme loci in three populations of *Tor putitora* from lake and hatchery of Pokhara Valley, Nepal.

Loci	**Allele frequencies**			
	Allele	Phewa Lake	Hatchery pop. I	Hatchery pop. II
*EST-2**	116	0.371	0.325	0.150
	100	0.611	0.675	0.850
	74	0.018	0	0
*IDH**	100	0.656	0.875	0.900
	83	0.281	0.125	0.100
	70	0.063	0	0
*GDH**	174	0.250	0.525	0.235
	100	0.694	0.475	0.765
	33	0.056	0	0

*sMDH-1**	100	0.333	0.625	0.792
	51	0.667	0.375	0.208
*MEP-1**	100	0.750	0.800	0.607
	39	0.250	0.200	0.393

The number of alleles per locus within hatchery populations was 1.45 (±0.52), percentage polymorphic loci was 45.45% (Table 3). Paired sample T-test revealed that both hatchery populations exhibit significantly lower (P<0.05) number of alleles per locus compared to that of natural population.

Loss of rear alleles, *EST-2*74*, *IDH*70* and *GDH*33* occurred in both of the hatchery populations compared to their wild counterparts - the lake population, although they were present at frequencies ≤0.05 in natural population with exception those at *IDH*70*. Given a frequency of lost alleles, the chance of drawing no individuals showing the rare alleles in a sample can be calculated as: frequency of alternate alleles $^{(2 \times \text{number of individual})}$ (Dillon and Manzi 1987). The chance of completely missing allele *EST-2*74* in the sample of 31 for both of the hatchery populations was 0.30. There is fair chance that this allele is present in hatchery populations, but was missed in the sample. However, the chance of completely missing allele *IDH*70* and *GDH*33* in the sample of hatchery populations was just 0.017 and 0.035, respectively. Thus it seems likely that the hatchery populations have indeed lost these alleles.

Effective number of alleles per locus (N_e) was calculated for each population thus diminishing the contribution of rare alleles. N_e decreased significantly (P<0.05) in subsequent generation of hatchery populations. Significant differences (P<0.05) were also observed in between N_e of natural population (1.37±0.44) and hatchery population I (1.31±0.4) and hatchery population II (1.23±0.31).

Table 3. Measures of genetic variability (number of alleles per locus ±SD in parentheses, effective number of alleles per locus ±SD in parentheses, percentage of polymorphic loci and mean sample size per locus at eleven loci) within hatchery and lake populations of *Tor putitora* in Pokhara Valley, Nepal.

Population	No. samples per locus	No. alleles per locus (±SD)	Effective number of alleles per locus (±SD)	Polymorphic loci (%)#
Lake	25.5	1.72 (0.90)[a]	1.37 (0.44)[a]	45.45
Hatchery I	16.5	1.45 (0.52)[b]	1.31 (0.40)[b]	45.45
Hatchery II	14.5	1.45 (0.52)[b]	1.23 (0.31)[c]	45.45

Superscripted with the same letter in a column are not statistically different (P>0.05) between

populations (Archie 1985)

[#] 95% criterion

H_o was between 0.181 (±0.233) to 0.119 (±0.143) and H_e was between 0.176 (±0.216) to 0.145 (±0.181) for hatchery populations I and II, respectively (Table 4). There were no differences ($P<0.05$) in H_o between hatchery populations. However, lower H_o ($P<0.01$) was evident in hatchery population II when compared with the natural population. Independent sample comparison (Archie 1985) revealed that there is no significant different in observed and expected heterozygosity after bonferroni correction ($P>0.003$) within populations. All populations under study conform to the Hardy-Weinberg equilibrium at the 1% level after Bonferroni correction (Rice 1989).

Table 4. Measures of genetic variability (mean heterozygosity: observed and expected, fixation index (F_{is}), Hardy-Weinberg exact test value[*] (Bonferroni corrected; *P*=0.003) within hatchery and lake populations of *Tor putitora* in Pokhara Valley, Nepal.

Population	No. samples per locus	Average heterozygosity		F_{is}[æ]	P
		H_o (±SD)	H_e (±SD)[+]		
Lake	25.5	0.179 (0.221)[a]	0.184 (0.196)	0.02	0.4316
Hatchery I	16.5	0.181 (0.233)[ab]	0.176 (0.216)	0.02	0.6414
Hatchery II	14.5	0.119 (0.143)[b]	0.145 (0.181)	0.17	0.0111

[*] Markov chain method

[+] Nei's expected heterozygosity

[æ] $F_{is} = (H_e-H_o)/H_e$.

Superscripted with the same letter in a column are not statistically different ($P>0.05$) between populations (Archie 1985).

Most of the locus wise F_{is} values within each population (Table 5) were not different from 0 ($P>0.01$) after Bonferroni correction. Among the significant test lake population was at *GDH**, hatchery population I at *sMDH-1** and hatchery population II was at *MEP-1**. The F_{is} values for *sMDH-1** was relatively high in both wild and hatchery populations.

Table 5. Locus-wise F_{is} (Weir and Cockerham 1984) within each of 3 populations of *T. putitora* in Pokhara Valley, Nepal.

Locus	Phewa Lake	Hatchery Pop. I	Hatchery Pop. II
*EST-2**	-0.117	-0.000	-0.152
*IDH**	0.385	-0.077	-0.059
*GDH**	0.410[**]	-0.280	0.050

*sMDH-1**	0.270	0.486**	0.283
*MEP-1**	-0.318	-0.200	0.576**

* Statistically significant (P< 0.002-Bonferroni correction)

F_{st} (0.0529) did not show significantly population differentiation among the hatchery populations (Table 6). Pair-wise genetic differentiation was also not significant in all loci of the two hatchery populations. The genetic distance (Nei 1972, 1978) between the *T. putitora* populations are presented in Table 7. Genetic distance between the two hatchery populations was 0.0195 (Nei, 1972) and 0.0127 (Nei 1978), with greater distance of 0.0371 (Nei 1972) and 0.0308 (Nei 1978) between the wild population and hatchery population II, descendent of hatchery population I. However, the Nei's genetic distance ranged from 0.0127 to 0.0371 falls within the range of conspecific.

Table 6. Values for *F*-statistics of hatchery populations of *Tor putitora* in Pokhara valley, Nepal.

Locus	**F_{is}**	**F_{st}**
*EST-2**	-0.0556	0.0589*
*IDH**	-0.0684	-0.0520
*GDH**	-0.1537	0.1420
*sMDH-1**	0.4268	0.0159*
*MEP-1**	0.3307	0.0275*
Average	0.1212	0.0529
Jacknifing over loci (±SD)	0.1262 (0.1415)	0.0559 (0.0344)

* Statistically significant (P< 0.002-Bonferroni correction)

Table 7. Genetic distance for the three *Tor putitora* populations according to the methods of Nei (1972) below the diagonal and Nei (1978) above the diagonal.

Population	**Lake pop.**	**Hatchery pop. I**	**Hatchery pop II**
Phewa Lake	****	0.0149	0.0308
Hatchery pop. I	0.0217	****	0.0127
Hatchery pop II	0.0371	0.0195	****

In order to interpret the level of the genetic variation of the hatchery stocks, the data of their wild counterpart-the lake population was used as a baseline. Lake population has served the founding stock of the hatchery populations. However, the interpretation presented here should be taken with caution because of the small number of allozyme loci were used to describe

genetic variation of natural and hatchery populations of *T. putitora*.

Considerable reduction of genic variability was found in the hatchery populations, as summarized in Table 3 and 4. The magnitude of reduction was 18.6% in the average number of alleles per locus, 7.8% in the average number of effective alleles (N_e) per locus in both of the hatchery populations, and 33.3% in the observed heterozygosity in hatchery population II relative to data from the natural population.

The loss of rare alleles is quite common in hatchery populations. It would occur initially because of a founder effect, the situation in which small numbers of brooders were taken from the natural population for domestication (Allendorf and Phelps 1980, Norris *et al.* 1999). The populations keep facing allele loss during domestication process due to inbreeding and or genetic drift (Fujio *et al.* 1999). The effect of allele loss have been clearly demonstrated in hatchery populations of fish and shellfish; for example, Atlantic salmon, *Salmo salar* (Koljenon *et al.* 2002);

Abalone, *H. rubra* (Evans *et al.* 2004) and hard calm, *Mercenaria marcenaria* (Dillon and Manzi 1987).

Observed heterozygosities (H_o mean=0.150; ranged between 0.181 and 0.119) observed in this study were quite high compared to H_o of hatchery populations of other species, based on same marker types (with large numbers), such as *Beta splendens* (H_o=0.099) (Meejui *et al.* 2005); black sea bream, *Acanthopagrus schlegeli* (H_o=0.048-0.052); *Oreochromis mossambicus* (H_o=0.073); common carp (H_o=0.074); guppy (H_o=0.054) (Macaranas and Fujio 1990). However, a drastic reduction in heterozygosity (33.3%) of hatchery population II relative to natural population was the evidence in the present study. Generally heterozygosity of hatchery populations tend to decline due to increased inbreeding rate which was the result of small effective population size (Falconer 1983).

With respect to low allelic diversity, founder effect could explain such a genetic change in hatchery populations and may result from the sampling variation when founders were taken from natural population, i.e., random genetic drift- the smaller founding population. Random genetic drift associated with selection and inbreeding has also been suggested as possible causes of genetic variability in hatchery populations (Allendorf and Phelps 1980). In the hatchery population I examined here, the effect of selection and inbreeding may be disregarded, since no intentional selection was applied to the stock and no evidence of inbreeding was observed as revealed by high heterozygosity, lack of population specific nature of locus-wise F_{is} and $(H_e-H_o)/H_e$ near equity. Thus the founder effect, i.e., the small number of parents having actually contributed to the reproduction of hatchery population I.

Low heterozygosity accompanying with low allelic diversity in hatchery population II might have been resulted from inbreeding. Evidence of inbreeding was observed in this population as revealed by an increase in homozygosity and the indicator of inbreeding ($(H_e-H_o)/H_e$) value. It is likely that number of effective parents (effective population size) were much smaller than the numbers of fish used to produce hatchery population II. It seems essential to make efforts to increase the number of fish which contribute to reproduction of subsequent generation. Kapuscinski and Jacobson (1979) recommended that inbreeding could be minimized if effective population size (N_e) exceeded 50 or by using a number of brooders at between 263 and 344

fish per generation (Tave 1986).

Loss of alleles is a major concern for sustainability of the stocks in the long term because rare alleles are frequently associated with fitness traits (Allendorf and Phelps 1980). Population bottlenecks (founding of a stock) often have a greater effect on allelic diversity than on heterozygosity. To help reduce the founder effect, including losses of rare alleles, Allendorf and Ryman (1987) suggested that a founding population of at least 25 females and 25 males is a reasonable absolute minimum and they urged that efforts be taken to equalize the contribution of all founders. However, the present captive stocks of *T. putitora* should be supplemented periodically with wild genetic material, provided appropriate wild stocks are available. Despite there is no ideal frequency and proportion of such infusions from the wild (Edds and Echelle 1989), Allendorf and Ryman (1987) conservatively stated that a 10% contribution of wild fish every second or third generation would be sufficient under most circumstances. In this connection, much smaller infusions on the order of one effectively breeding individual of *T. putitora* per generation can considerably reduce effects such as loss of alleles in founding populations and subsequent selection and genetic drift in captivity.

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