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STUDIES ON GENETIC VARIABILITY OF THREE POPULATIONS OF GOLDEN MAHSEER (*TOR PUTITORA*) USING MICROSATELLITE MARKERS

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The Golden mahseer (*Tor putitora*) is a prized species for both food and sport in several areas of upland Indian water bodies. But, due to, its reduction in population sizes in recent times, it needs attention for conservation. The present study was undertaken to assess the genetic variability of geographically isolated three populations using 6 highly polymorphic microsatellite markers. The number of alleles per locus ranged from 5 to 12 with an average frequency of 8.33. The mean expected heterozygosity was 0.78 and observed heterozygosity was 0.64. All the studied loci were polymorphic with mean PIC value of 0.75. It was observed that a considerable amount of genetic variation persists in these populations and there is no threat on these populations.

INTRODUCTION

The Golden mahseer (*Tor putitora)* is considered as flagship species among mahseer, which have been grouped under family Cyprinidae. Although the species is distributed through wide geographical regions of South-Asian countries in their mid-hills (Mohindra *et al.*, 2007; Naeem *et al*., 2011; Hussain, 2012), the continuous decline in population has resulted in their inclusion in IUCN (IUCN, 2012).The species is continuously declining due to deterioration of its natural habitat (Bhatt *et al*., 2004; Sharma, 2004; Khajuria *et al.,* 2013). Due to dam construction and irrational fishing in other lotic habitats, *T. putitora* is now mostly confined to lentic ecosystem where it may have less effective population size that ultimately result in inbreeding or decrease in genetic vigour of the species. Hence, the assessment of genetic variation is essential in further management plans for the conservation of this endangered species. Microsatellite marker is generally used for assessment of genetic status of a species other than assessments like classification and systematics (Amado *et al.,* 2011), parentage identification (Norris *et al.,* 2000), germplasm conservation (Fopp-Bayat and Ciereszko, 2012), bottleneck (Saha *et al.,* 2010) and population genetic analysis (Barat *et al.,* 2013; Sahoo *et al.,* 2013).

Microsatellite markers in combination with recent statistical approaches represent a useful tool for genetic characterization, which ultimately supports the stock management. Microsatellites are short repeat nucleotide sequence (di, tri, tetra, penta, hexa) arrayed in tandem. This codominant marker was found highly polymorphic and very useful for population genetic analysis in teleost fishes such as *T. putitora* (Mohindra *et al*., 2007; Sahoo *et al*., 2013),

Schizothorax richardsonii and *Gara gotyla* (Molecular Ecology Resources Primer Development Consortium *et al.,* 2012)

In order to gather information regarding genetic structure and relationship of golden mahseer populations, we assessed the genetic variation among three populations using microsatellite makers. These codominant genetic markers are one of the versatile marker systems to study intraspecific genetic variation and other major fundamental and applied fields in aquaculture (Lal *et al.,* 2004; Das *et al.,* 2005; Chistiakov *et al.,* 2006) as compared to other genetic markers.

MATERIALS AND METHODS

Sample collection, genomic DNA isolation and in vitro amplification

A total of 90 fin tissue samples of golden mahseer were collected from three different riverine populations (30 each) *viz.* River Jia Bhoreli, Bhalukpong, Assam (TBPA;27°02' N 92°35' E), River Satluj, Bhakara, Himachal Pradesh (TPBH;31°34' N 76°26' E) and River Beas, Jogindernagar, Himachal Pradesh (TPJH;31°24' N 76°26' E). The tissue samples were collected by non-invasive technique (Wasko *et al.,* 2003) and were immediately preserved in 70% ethanol (Merck Bioscience, Germany). Genomic DNA of the collected tissue samples was isolated following Phenol: chloroform: isoamyl alcohol protocol (Sambrook and Russell, 2001). Qualitative estimation of DNA was carried out in 0.8 % agarose gel electrophoresis and quantitative by taking the absorbance at $A_{260/280}$ using Nanodrop® (Thermo Fisher Sci., USA). Finally 28 samples from each population were selected for final assessment.

Microsatellite loci identified by Sahoo *et al.* (2013) were used for the present study. Sahoo *et al.* (2013) have assessed two riverine populations using these markers which were collected from River Ravi, Anji (J&K) and River Kosi, Ramnagar (Uttarakhand) and here the study is extended to three more rivers; River Beas, River Satluj and River Jia Bhoreli. Eight microsatellite loci were amplified in a 10 µl reaction volume containing 50 ng of template DNA, 200 µM of each dNTP, 5 pM of each primer, 1.0 mM $MgCl_2$, 0.5 U of AmpliTaq® DNA Polymerase (Applied Biosystem, USA) and 1x PCR buffer II (Compatible with polymerase) using GeneAmp 9700 thermocycler (Applied Biosystem, USA). The reaction profile adopted for PCR was; initial denaturation at 94 °C (4 min) followed by denaturation at 94 °C (30 s); primer annealing (Ta) between 48–52 °C (30 s) (Table 1) and extension at 72 °C (60 s) iterated 34 times and a final extension at 72 °C (10 min) and decrease in temperature to 15 °C for storage. Amplified products were resolved through 6 % denaturing polyacrylamide gel with 7.5 M urea and 1×TBE buffer in a vertical gel electrophoresis (Hoeffer Inc., MA). For allele size estimation 200 ng of pBR322/Hae III (Thermo Fisher Sci., USA) standard DNA was also used in electrophoresis. Resolved alleles were visualized after staining with ethidium bromide and gels were digitized in AlphaImager® EP (Alpha Innotech, USA). Genotype of each locus was recorded from digitized images using AlphaView® software (Alpha Innotech, USA).

Table 1: Microsatellite loci used for the genetic assessment of three *T. putitora* populations.

Locus	Repeat motif	Primer Sequence (5'-3')	T_a (°C)	Size range (bp)	N_A	H_O	H_E	F_{st}	PIC
TPM01	$(GT)_{23}$	F: ACAAACTTCCAAGATGCG R: ACAGTCTTGTTTTGTGCTC	48	240-264	09	0.21	0.69	0.064	0.66
TPM02	$(CA)_{16}$	F: GGCCCAGATGAGAGAGAA R: ATCAGCCCTCTACAAACAA	50	126-146	11	0.98	0.88	0.039	0.86
TPM04	$(TAA)_4$	F: CTAGTAGGCTTGCTGCAATAG R: CGCGTTCAGTTTTAATTGTAG	48	128-158	05	0.67	0.78	0.104	0.74
TPM21a	$(CT)_7$/ $(CA)_{16}$	F: CCGTTCCATTCAGATGCC R: CGCTTGTGTCTTTGTGTGT	50	121-187	12	0.90	0.91	0.080	0.89
TPM11	$(CA)_{16}$	F: GTTGGAGAATGGCGTGTA R: AGGGGAAGAAGAGAGAAAA	50	122-144	05	0.63	0.70	0.129	0.66
TPM13	$(CA)_{18}$	F: TTAAGATAAACCCATTCGACA R: GAAGCTATTGTGTTTTTCACG	52	128-162	08	0.47	0.75	0.157	0.72
		Mean			**8.33**	**0.64**	**0.78**	**0.094**	**0.75**

N_A, mean number of alleles per locus; H_g expected heterozygosity; H_O, observed heterozygosity; Ta, annealing temperature; PIC, polymorphic information content.

Statistical Analysis of genetic data

a. Demographic traits of studied populations

Genetic polymorphism within 3 different populations were measured by using different parameters *viz.* number of alleles (Na), observed (Ho) and expected heterozygosity (He) and the effective number of alleles (Ne) using GDA v.1.1 (Lewis and Zaykin, 2002). The probability of null allele was calculated using Micro-Checker v.2.2 (Van-Oosterhout *et al.,* 2004). Polymorphism information content (PIC) of each locus was calculated using CERVUS v.3.0 (Kalinowski *et al.,* 2007). F_{is} (inbreeding coefficient) and F_{st} for each locus were calculated using FSTAT v.2.9 (Goudet, 1995). Genotypic linkage disequilibrium, deviation from Hardy-Weinberg equilibrium and population differentiation was computed using GENEPOP v.4.0 (Rousset, 2008).

b. Genetic structure analysis of different populations

Analysis of molecular variance (AMOVA) was computed to enumerate the differences among populations using ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). Genetic distance and genetic similarity index following Nei (1978) were also estimated using ARLEQUIN v.3.5 and data matrix was used to construct UPGMA dendrogram under MEGA v.5.05 (Tamura *et al.,* 2011).

Finally, we used two approaches to determine, which of the populations may have undergone significant size reduction. Firstly by depicting the extent of distortion of allele frequency mode shift (from typical 'L-shaped' distribution) and secondly by calculating the excess of observed heterozygosity using Wilcoxon signed rank test using BOTTLENECK v.1.2.02 (Piry *et al.,* 1999). Heterozygote excess was tested using two models of mutation-drift equilibrium *i.e.* infinite alleles model (I.A.M.) and stepwise mutation model (S.M.M.) using 1000 replicates of the dataset.

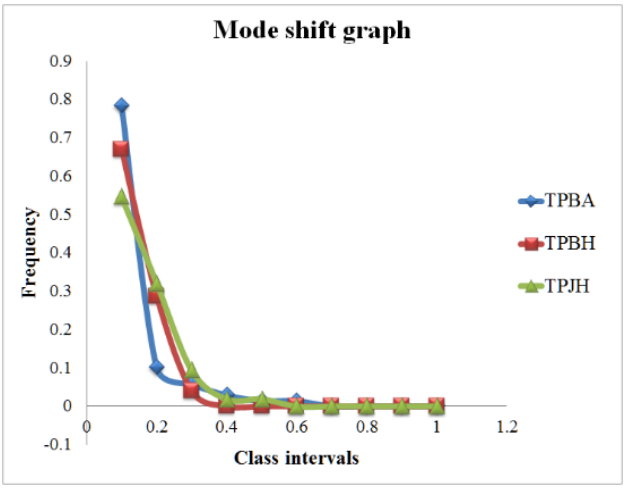

Fig. 1. UPGMA dendrogram constructed using Nei's Genetic distances among three population of *Tor putitora*.

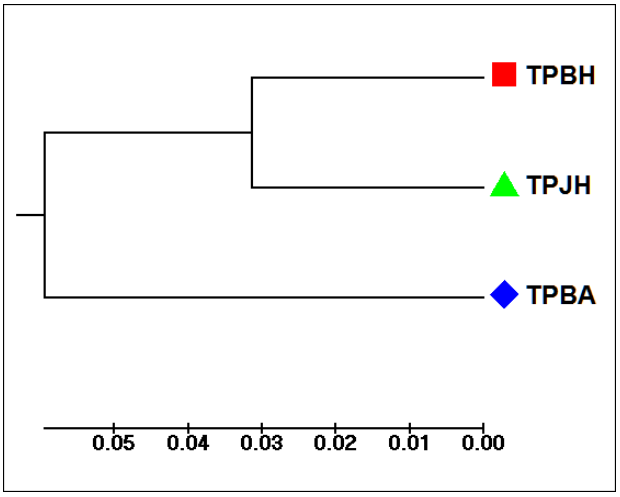

Fig. 2. Mode shift curve showing the normal 'L' shaped distribution in both models of mutation-drift equilibrium.

RESULTS AND DISCUSSION

The number of alleles per locus ranged from 5 to 12 with an average frequency of 8.33, which is comparable to that of Sahoo *et al.* (2013). Most of the primers revealed higher number of alleles (Na) in the present studies (table-1) as compared to earlier studies (Sahoo *et al.,* 2013). The expected heterozygosity ranged from 0.69 to 0.91 and observed heterozygosity from 0.21 to 0.98. This indicated a significant genetic variation in all the studied populations. Overall F_{ST} and F_{IS} value were found to be 0.094 and 0.363 ($P < 0.05$), respectively. Null alleles were observed in TPBA at locus TPM21a (Brookfield 1 freq. = 0.087) and in TPBH at locus TPM21a (Brookfield 1 freq. = 0.125) however the frequency of null allele was not significant. Null frequencies were significantly high for two loci TPM20a and TPM15b and hence removed from the analysis and the final analysis was carried out using data from 6 loci only. No linkage disequilibrium was detected for any pair wise combination of loci. All the loci were at Hardy–Weinberg equilibrium (HWE) and the probability was highly significant as estimated by Genepop v.4.0. Polymorphism Information Content (PIC) was estimated using Cervus 3.0 (Marshall *et al.,* 1998). PIC ranged from 0.66 to 0.89 and mean PIC value over all loci was found to be 0.75. There was no evidence for scoring error due to stuttering or large allele dropout.

Genetic relation among three populations is also studied based on Nei's genetic distance. The dendrogram constructed using UPGMA method in MEGA v.5.05 depicted that there was a significant genetic difference between TPBA and two other populations viz. TPBH TPJH. TPBH and TPJH are adjoined to each other. There is less geographical barriers and more chances of intermixing among these two population which may be the major reason behind their closeness among them. Seven mahseer populations have been studied using 2 different molecular markers by Sati *et al.* (2013), which indicated that the population of River Beas and River Satluj are closer to each other while the population of River Jia Bhoreli is quite distinct from other studied populations. They have hypothesized the role of geographical isolation of drainages for their significant differentiation. The present study supports the data as TPBA is quite distinct from TPBH and TPJH populations. The markers used in the present study (adopted from Sahoo *et al.,* 2013) have been used for population differentiation of two populations collected from River Ravi and River Kosi and revealed their ability for population genetic analysis. The markers are highly polymorphic with a mean PIC value of 0.75 (for the present study) and 0.68 (for 12 loci, Sahoo *et al.,* 2013) therefore all the studied loci are ideal for population analysis of mahseer species.

The bottleneck analysis using both the models of mutation-drift equilibrium *i.e.* infinite alleles model (I.A.M.) and stepwise mutation model (S.M.M.) indicated that there were very less likely chances of bottleneck among all the populations. The mode shift curve was typical 'L' shaped, also none of the loci showed heterozygote deficiency and hence there was a significant genetic vigour in all the studied populations.

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