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Identification of Polymorphic Allozyme Markers for Assessing Genetic Variability in Golden Mahseer, *Tor putitora*

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Screening of thirty-two allozyme loci in *Tor putitora* yielded ten polymorphic loci, useful in determining genetic divergence in natural populations of the species. Samples from three different Indian rivers *viz.* the Satluj, Ganga and Garua were studied with the identified allozyme loci, to assess their suitability for genetic diversity analysis in *T. putitora.* The observed heterozygosity per locus varied from 0.0840 (Satluj) to 0.1245 (Ganga). The significant genotype heterogeneity was observed between the sample sets ($P<0.05$). The identified loci exhibited significant potential to determine population structure of *T. putitora* across its natural range of distribution.

Key words: allozyme loci, genetic divergence, *Tor putitora*, Indian rivers

The Golden Mahseer, *Tor putitora* (Family Cyprinidae) is a fascinating game and delicious food fish of India. The fish inhabits cold-water streams and is distributed widely from Bangladesh, Nepal, India up to Pakistan and Afghanistan (Jayaram, 1999). *T. putitora* is reported to grow over 50 kg in size and is a strong and hardy fish (Froese & Pauly, 2003). In India, it provides main fishery resources of Shiwalik Himalayas. Currently, abundance of the fish is declining in certain ranges of its natural distribution, stated to be the response of over exploitation and habitat alterations (Chonder, 1999). To save this important fishery resource, effective conservation and propagation assisted rehabilitation strategies need to be planned. However, scientific formulation of rehabilitation strategy requires information on stock structure and genetic variation of *T. putitora,* across its distribution range. Rehabilitation programs without stock structure data can result in mixing of stocks and ultimately loss of natural genetic variation. Genetic variation is an important feature of a population, both for short term fitness of individuals and the long term survival of the population through allowing adaptation to changing environmental conditions (Ferguson *et al.,* 1995). There is no recorded information on any class of genetic markers studies in genus *Tor* and the data is limited to karyological studies (Khuda-Baksh, 1982; Lakra, 1996; Kushwaha *et al.,* 2001).

Detection of variation through electrophoresis is a proven tool to determine population structure of several fish species (Jerry, 1997; Cashilo & McAndrews, 1998; Lal *et al.,* 2004; Salini *et al.,* 2004). This provides an independent estimate of levels of variation within a population without an extensive morphological and quantitative survey (Menezes, 1993). The objective of the present

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study is to identify polymorphic allozyme loci that are suitable for determining intraspecific genetic divergence in natural populations of *T. putitora*.

Materials and Methods

The *T. putitora* specimens were collected through commercial catches, from three rivers, Satluj (Nangal 31° 23'N, 75 ° 30' E, n=26), Ganga (Ajetpur 29° 58'N, 78 ° 10' E, n=30) and Garua (Katerniaghat 32°19', 75 ° 30' E, n=28). The riverine locations were chosen to cover geographically isolated populations. The river Satluj is part of the Indus River System while Ganga and Garua belong to the Ganges (ECAFE, 1966). The liver and muscle samples were excised from the fish specimens at site, immediately frozen and transported and stored in liquid nitrogen (196°C) till analysis.

Table 1 depicts the parameters optimised for allozyme electrophoresis in *T. putitora*. Muscle samples did not provide any additional loci or better resolution of any locus in comparison to that in liver. Therefore, liver was chosen as the optimum tissue for further analysis. Frozen liver sample (60-80 mg) was mildly crushed and homogenized @ 330 mg/ml in extraction buffer (0.17M Sucrose, 0.2M EDTA, 0.2M Tris-HCl, pH 7.0). Homogenates were centrifuged (SIGMA Laborzentrifugen, GmbH, Osterode) at 10,000 rpm for 1 hour at 4°C. The supernatant was subsequently re-centrifuged for 20 minutes (4°C). Polyacrylamide gels of size 10 cm x 8 cm were used for all the enzymes except esterase, which was resolved on 10 cm x 12 cm size gels. Allelic variation was investigated by running the sample extract (1-3 ml) on 7 and 8% polyacrylamide gel in TBE (D) buffer (Table 1) at a constant voltage (150 V) at 4°C in a cooling chamber. Optimized sample supernatant volume and electrophoresis running time for each enzyme systems are illustrated in Table 2. Allozyme patterns were visualized by histochemical staining (Whitmore, 1990; Gopalakrishnan *et al.*, 1997) and nomenclature of protein coding loci and alleles were based on the recommendations of Shaklee *et al.* (1990). The most common allele was designated as 100 and the other alleles were assigned values as per mobility relative to that of most common allele. *T. putitora* has been reported as a natural tetraploid on the basis of karyotyping (Nagpure *et al.*, 2002). As reported in fishes, the tetraploidization event is ancestral and the initial tetraploidy ultimately evolved, gene by gene, towards functional diploidy where all the chromosomes started working as pairs (Tsigenopoulos *et al.*, 1999). Hence,

Table 1. Parameters optimized for electrophoresis in *Tor putitora*

Sl. No.	Parameters	Variation tested	Found optimum
1	Tissue	Liver, Muscle	Liver
2	Tissue (mg). extraction buffer* $(ml)^{-1}$	250mg.ml^{-1}, 330mg.ml^{-1} & 550mg.ml^{-1}	330mg.ml^{-1}
3	PAGE concentration	7, 8 and 9 %	8 %
4	Running buffer	TBE (D) *2, TCE (D) *3	TBE (D)
5	Extract volume loaded	1, 3 and 6 ml	Enzyme specific
6	Running time	45-150 min	Enzyme specific

*1-Extraction buffer- Liver- (0.17 M Sucrose, 0.2 M EDTA, 0.2 M Tris-HCl, pH 7.0) and Muscle- 10% Sucrose.
*2-TBE(D) - 500 mM Tris, 650 mM Boric acid, 16 mM EDTA, pH 8.0
*3-TCE(D) - 500 mM Tris, 650 mM Citrate, 16 mM EDTA, pH 8.0

Table 2. Enzyme E.C. numbers and scorable loci with tissue extract volume and running time of electrophoresis, optimized, in *Tor putitora*.

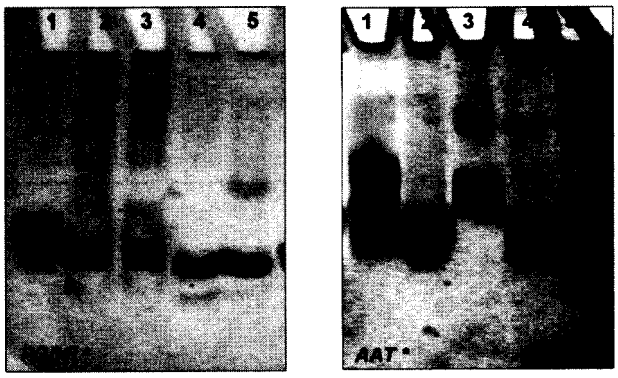
Enzyme system	E.C. Number	Loci	Tissue extract volume (µl)	Running time (min.)
α-Glycerophosphate dehydrogenase	1.1.1.8	*GPDH-1*,-2**	1	110
Acid phosphatase	3.1.3.2	*ACP**	3	75
Adenylate kinase	2.7.4.3	*AK**	3	100
Aspartate amino transferase	2.6.1.1	*AAT **	3	80
Creatine kinase	2.7.3.2	*CK**	3	120
Esterase	3.1.1.1	*EST -1*,-2*,-3*,-4**	1	135
Glucose dehydrogenase	1.1.1.47	*GLDH**	3	100
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6PDH**	1	120
Glutamate dehydrogenase	1.4.1.3	*GDH**	3	105
Lactate dehydrogenase	1.1.1.27	*LDH-1*,- 2*, -3*,-4*,-5**	3	145
Malate dehydrogenase	1.1.1.37	*MDH-1*,-2**	3	110
Malic enzyme	1.1.1.4	*ME**	3	110
Octonol dehydrogenase	1.1.1.73	*ODH**	3	105
Phosphogluco mutase	5.4.2.2	*PGM-1*,-2**	1	80
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH**	1	110
Superoxide dismutase	1.15.1.1	*SOD-1*,-2*,-3**	3	100
Xanthine dehydrogenase	1.1.1.204	*XDH**	3	135
Fumarase	4.2.1.2	*FH**	3	120
Isocitrate dehydrogenase	1.1.1.42	*ICDH**	3	90
Hexokinase	2.7.1.1	*HK**	3	120

the designation of loci and alleles was done as in diploid individuals.

To investigate the genetic variability, samples were genotyped for each allozyme locus. GENETIX ver.4.0 (Belkhir *et al.*, 1997) software was used to estimate the proportion of polymorphic loci ($P_{0.95}$ and $P_{0.99}$), heterozygosities at each locus and mean number of alleles per loci for each sample set (Table 3). The probability of conformity to Hardy-Weinberg expectations (Probability and score test) was estimated through GENEPOP 3.4 software (Raymond & Rousset, 1995a). Genetic homogeneity of the sample sets was determined through an exact test (G based test) that assumes random samples of genotypes. Genotypic homogeneity over all loci was estimated using GENEPOP 3.4 software.

Results and Discussion

Out of the twenty three enzyme systems investigated, twenty enzyme systems yielded a total of 32 consistently scorable loci (Table 2). Ten loci (31.2%) viz. *PGDH**, *AAT**, *GPDH-2**, *PGM-1**, *G6PDH**, *XDH**, *EST-1**, *-2**, *-3** and *-4** were found to be polymorphic (Fig. 1, 2 & 3). These loci exhibited polymorphism in samples from all three collection sites explored in this investigation. A locus was considered polymorphic, if the frequency of most common allele was < 0.99 (Hartl & Clark, 1997). Loci *AAT**, *EST -2**, *-3**, *& -4**, *XDH** and *G6PDH** were expressed by three alleles, while Loci *PGDH**, *PGM-1** and *GPDH-2** by two alleles each (Table 3). Locus *EST-1** was expressed by four alleles.

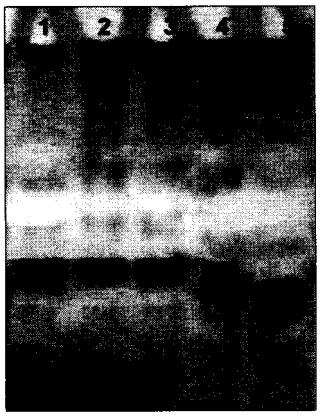


(a)

1	:	094/094
2, 3	:	094/100
4, 5	:	100/100

(b)

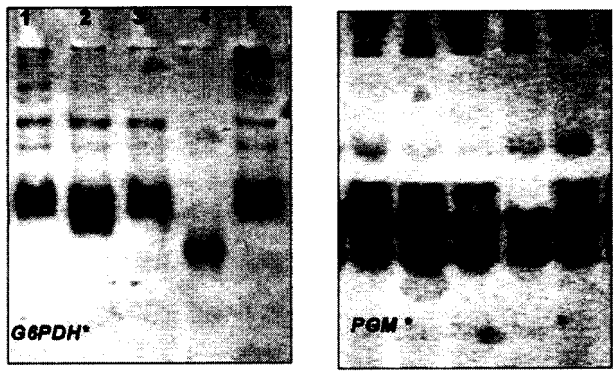
1	:	086/100
2, 4	:	100/100
3	:	086/086
5	:	100/114



(c)

1, 2, 3	:	100/100
4	:	100/104
5	:	104/104

Fig. 1. Pattern of Phosphogluconate dehydrogenase *(PGDH)*, Aspartate amino transferase *(AAT)* and α-Glycerophosphate dehydrogenase *(GPDH-2)* in *Tor putitora.*

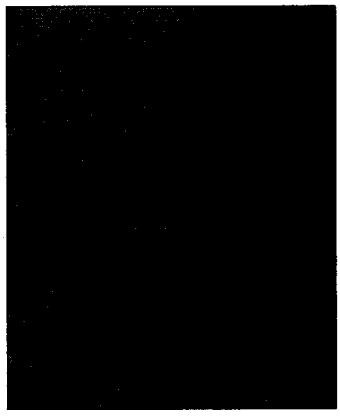


(a)

1, 3, 5	:	100/100
2	:	100/ 110
4	:	120/120

(b)

1, 2, 3, 5	:	072/100 (locus - 2)
4	:	100/100 (locus - 2)

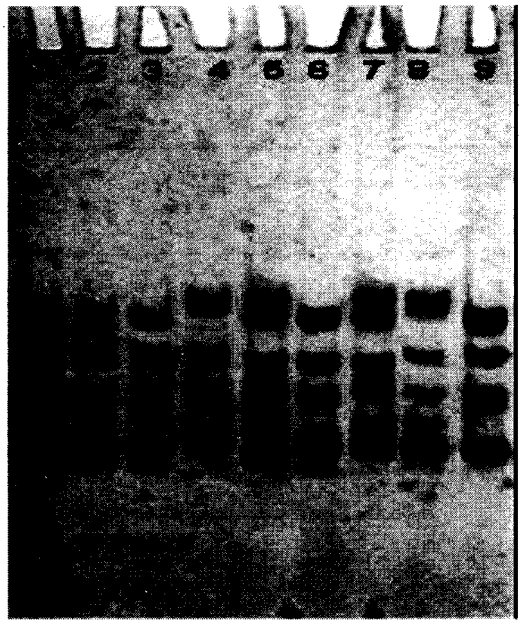


(c)

1, 5	:	100/100
2, 4	:	095/100
3	:	106/106

Fig. 2. Pattern of Glucose-6-phosphate dehydrogenase *(G6PDH)*, Phosphoglucomutase *(PGM)* and Xanthine dehydrogenase *(XDH)* in *Tor putitora.*

Parameters of genetic variation for samples from three collection sites, the Satluj, Ganga and Garua, are given in Table 3. The mean number of alleles per locus was found to be the highest in samples of Garua (1.50) and the lowest in that of Satluj (1.44). The observed heterozygosity per locus varied from 0.0840 (samples of Satluj) to 0.1245 (samples of Ganga). The H_{obs}. values were consistent with that reported for teleostean fish species (Nevo, 1978). Probability test was performed to assess the conformity of allele frequencies to that expected under Hardy-Weinberg (HW) equilibrium. A significant deviation from the HW expectations, after sequential Bonferroni adjustment ($P<0.0023$) of probability levels (Lessios, 1992), was observed at locus *EST-2** (in samples of Satluj and Ganga) and *EST-3** (samples of Satluj), at locus *G6PDH** (samples of Satluj and Garua), *PGM-1** (samples of Ganga and Garua), and *XDH** (samples of Ganga). Samples from a particular collection site that deviated significantly from Hardy-Weinberg (HW) equilibrium exhibited positive F_{is} values and



*Est-1**		*Est-2**	
1, 3, 6, 9	: 100/100	2, 4	: 100/103
2	: 096/102	3	: 098/103
4, 8	: 096/096	1, 5, 6, 7	: 103/103
5, 7	: 096/100	8, 9	: 100/100

*Est-3**		*Est-4**	
1	: 100/103	1, 3	: 103/103
2, 3, 4, 5, 8	: 100/100	2	: 100/103
6, 9	: 103/103	4, 6, 8, 9	: 100/100
7	: 095/100	5	: 098/103
		7	: 098/100

Fig. 3. Pattern of Esterases *(EST)* in *Tor putitora.*

a test of heterozygote deficiency confirmed that those samples had a significant deficit of heterozygotes.

Tests for allelic differentiation were performed to validate the null hypothesis that the samples from three rivers had homogenous allele frequencies. The genetic heterogeneity was tested on genotype data rather than allele frequencies (Raymond & Rousset, 1995b; Goudet *et al.,* 1996). The combined probability over all loci and sample sets was also found to be significant ($P<0.0001$), indicating the genetic heterogeneity between the samples from three rivers. The probability values depicted the significant heterogeneity ($P<0.05$) at seven loci, *EST-1*, -2*, -3* & -4*, G6PDH*, PGM-1* and *XDH** (Table 3). After adjusting significance level for sequential Bonferroni correction, loci *EST-1*, -2*, -4** and *PGM-1** had significant heterogeneity. The estimate of G_{st} for small sample size was 0.0505, and indicated the existence of moderate levels of genetic differentiation. Various estimates indicated that the different sample sets are not drawn from single panmictic population and distinct population sub-structuring in *Tor putitora* may be possible across its range of distribution.

Ten polymorphic allozyme loci identified in the current study exhibited adequate genetic variability. These loci clearly indicate significant potential to be used in determining intraspecific genetic divergence in *Tor putitora* across the range of natural distribution.

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