Indian J. Fish., ***53***(3) : 253-261, Jul.-Sep., 2006

Identification of polymorphic allozyme markers for population structure analysis in *Horabagrus brachysoma* (Gunther, 1864).

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ABSTRACT

Fourteen polymorphic allozyme loci were identified in yellow catfish, *Horabagrus brachysoma*. The genetic variation detected at each allozyme locus was assessed for samples collected from three rivers. The observed heterozygosities per locus ranged from 0.0286 to 0.4000. Significant genotype heterogeneity indicated that the samples are not drawn from same gene pool. The results suggest the potential of the identified loci to analyze stock structure of natural populations of *H. brachysoma*.

Introduction

Horabagrus brachysoma (Family: Bagridae) popularly called 'yellow catfish' or 'sun-catfish' is endemic to rivers originating from southern part of the Western Ghats. The Western Ghats is recognized as one of the thirty-four biodiversity rich hotspots of the world (Myers *et al*., 2000). *H. brachysoma*, with high food and ornamental value, has declined in abundance and now restricted to a few rivers *viz*. Nethravathi, Chalakkudy and Meenachil Rivers (Gopalakrishnan and Ponniah, 2000). The fish, though a freshwater inhabitant, is also reported from brackishwater, especially in the Vembanad lake, Kerala during south-west monsoon (Kurup and Samuel, 1982). Unnithan (2001) estimated annual landing of this species from Vembanad lake as 2150 kg (0.56% of the total fish landing from the lake). *H.brachysoma* is categorized as endangered, based on the IUCN (International Union for Conservation of Nature and natural resources) criteria due to restricted distribution, loss of habitat, over-exploitation, destructive fishing practices and trade (Anon., 1998). In view of the significance, *H. brachysoma* is a potential candidate

species for research on artificial propagation, for use in developing its aquaculture and rehabilitation of natural stocks. The present work is a part of integrated plan covering different aspects, including captive breeding, sperm cryopreservation protocols, documenting life history traits and information on genetic markers as well as stock structure. Identification of markers with scorable alleles is pre-requisite to generate stock structure data for any species (Ferguson *et al.*, 1995). There is no information available on any class of genetic markers in *H. brachysoma*. Allozyme loci are proven tools to determine population structure and estimate intra-population gene flow in natural fish populations. These have been consistently used to develop information on genetic structure of many fish species (Menezes, 1993, Gopalakrishnan *et al.*, 1997; Jerry, 1997; Van der Bank *et al.*, 1997; Cashihlo and McAndrew, 1998; Hawkins *et al.*, 2002; Lal *et al.*, 2004; Salini *et al.*, 2004). The present study analyses allozyme markers in *H. brachysoma*, to identify polymorphic loci and suitability of the identified polymorphic loci in analyzing population structure of the species.

Material and methods

Specimens of *H.brachysoma* were obtained through commercial catches from two rivers *viz.*, Chalakkudy (n=26) at Kanakkankadavu, Kerala ($10^0$08'N; $76^0$07'E) and Nethravathi (n=25) at Kankanadi, Karnataka ($12^0$52'N; $74^0$54'E). The liver samples were collected from the specimens and were immediately frozen in liquid nitrogen (-196^0C). The riverine locations were chosen to cover geographically isolated populations of *H. brachysoma*. The samples were transported to laboratory and stored at -80^0C until analysis. Frozen liver samples (approximately 100 mg) were homogenized in 250 mg/ml ice-cold extraction buffer (0.17 M sucrose, 0.2 M EDTA, 0.2 M Tris-HCl, pH 7.0). Homogenized samples were centrifuged for an hour at 12,000 rpm at 4^0C (Heraeus Biofuge-Stratos, Germany) and the supernatant was recentrifuged for 45 minutes. The enzyme systems were assessed using vertical native polyacrylamide gel electrophoresis (PAGE) (Amersham Biosciences Ltd.). Gels of 10 x 8 cm. size were used for all the enzymes. Investigations were done using 7.25% PAGE and electrophoresis was done using constant voltage (150V) at 4^0C using TBE buffer (90 mM Tris-borate and 2 m M EDTA, pH 8.0) system except in SOD, that was resolved in TG buffer (5 mM Tris-Cl and 0.038 M Glycine, pH 8.3) system. To increase the resolution of the bands, NAD or NADP was added in the gel mixture and in the upper tank of the electrophoresis apparatus based on the nature of the enzyme (Gopalakrishnan *et al.*, 1997). A total of 25 enzyme systems were examined (Table1). Visualization of different alleles of enzyme was done by histochemical staining following the procedures outlined by Whitmore (1990) and Shaw and Prasad (1970). Agar overlay method was used to detect zymograms in GPI and PGM. The gels were documented using Imagemaster 1D Elite gel documentation system (Amersham Biosciences, USA). Nomenclature of loci and alleles was followed as recommended by Shaklee *et al.* (1990). At all the loci, most common allele was assigned as 100. Alternate alleles were designated as per their mobility, in relation to the most common allele. Parameters of genetic variation like proportion of polymorphic loci ($P_{0.95}$ and $P_{0.99}$) in each population and heterozygosity at individual locus and

TABLE 1: *The names of enzyme loci, enzyme commission (E.C.) number and observed alleles for allozyme analysis in* Horabagrus brachysoma. *The enzymes mark 'ns' did not yield any scorable activity.*

Enzyme Loci	E.C. number	Locus	Alleles
Acid phosphatase	3.1.3.2	*ACP**	ns
Adenylate kinase	2.7.4.3	*AK**	ns
Alcohol dehydrogenase	1.1.1.1	*ADH**	ns
Alkaline phosphate	3.1.3.1	*ALP**	ns
Aspartate amino transferase	2.6.1.1	*AAT*-1*	100
		AAT-2*	100,117,126
Creatine kinase	2.7.3.2	*CK**	ns
Esterase		*EST-1**	083, 100
		*EST-2**	100,106
	3.1.1.1	*EST-3**	095,100
		*EST-4**	100
		*EST-5**	100
Fumerase	4.2.1.2	*FUM**	ns
Glutamate dehydrogenase	1.4.1.3	*GDH**	ns
Glucose-6-phosphate dehydrogenase	1.1.1.49	G_6PDH*	086,100
Glucose phosphate isomerase	5.3.1.9	*GPI-1**	100
		*GP1-2**	096,100
Glucose dehydrogenase	1.1.1.47	*GLDH**	080,089,100,117
α-Glycerophosphate dehydrogenase	1.1.1.8	αG_3PDH*	088, 100
Glyceraldehyde-3-Phosphate dehydrogenase	1.2.1.12	*GAPDH –1**	100
		*GAPDH –2**	100
Hexokinase	2.7.1.1	*HK**	ns
Isocitrate dehydrogenase	1.1.1.42	*ICDH**	ns
Lactate dehydrogenase	1.1.1.27	*LDH-1**	100
		*LDH-2**	100,112,134
Malate dehydrogenase	1.1.1.37	*MDH**	086,100
Malic enzyme	1.1.1.40	*ME**	100
Octonol dehydrogenase	1.1.1.73	*ODH-1**	100
		*ODH-2**	091,100
		*ODH-3**	100
Phosphogluconate dehydrogenase	1.1.1.44	*6PGDH**	ns
Phosphogluco mutase	5.4.2.2	*PGM**	093,100
Pyruvate kinase	2.7.1.40	*PK**	ns
Superoxide dismutase	1.15.1.1	*SOD**	093,100
Xanthine dehydrogenase	1.1.1.204	*XDH**	093, 100

mean over all loci for each population were calculated with GENETIX (version 4.0, Belkhir *et al*., 1997). GENEPOP version 3.4 (Raymond and Rousset, 1995a) was used to assess conformity of allele frequencies to that expected under Hardy- Weinberg equilibrium and genetic differentiation. Genetic homogeneity of the sample sets was determined through an exact test (G based test) that assumes random samples of genotypes (Genepop version 3.4, Genotype differentiation test, Raymond and Rousset, 1995a). This test was performed on genotype tables and possible non- independence of alleles with in genotypes will not affect test validity (Raymond and Rousset, 1995b; Goudet *et al*., 1996).

TABLE 2 : *Parameters of genetic variation for two riverine populations at each allozyme locus in* Horabagrus brachysoma.

Locus	Population	Alleles	Hexp.	Hobs.	HW (p)	Genetic heterogeneity (p)
*AAT-2**	CH	3	0.4800	0.0800	<0.0000**	
	NT	3	0.5318	0.6538	0.0552	0.0178
*EST –1**	CH	1	0.0000	0.0000	—	
	NT	2	0.2604	0.0769	0.0035	<0.0001**
*EST –2**	CH	2	0.3432	0.1200	0.0037	
	NT	2	0.1738	0.1154	0.1901	0.2015
*EST-3**	CH	2	0.3200	0.4000	1.000	
	NT	2	0.0000	0.0000	—	0.0003**
*αG_3PDH**	CH	2	0.5000	0.3600	0.1327	
	NT	2	0.3550	0.1538	0.0087	0.0258*
*G_6PDH**	CH	2	0.3432	0.1200	0.0037	
	NT	2	0.4933	0.1154	0.0001**	0.0780
*GLDH**	CH	4	0.4224	0.4000	0.0172	
	NT	2	0.4734	0.3077	0.0686	<0.0001**
*GPI-2**	CH	2	0.3200	0.1600	0.0272	
	NT	2	0.3336	0.0385	<0.0001**	1.0000
*LDH-2**	CH	3	0.4864	0.0800	0.0104	0.0034**
	NT	2	0.0740	0.0000	0.0196	
*MDH**	CH	2	0.4712	0.1200	0.0002**	
	NT	2	0.3550	0.0769	0.0003	0.2678
*ODH-2**	CH	2	0.4872	0.2000	0.0033	
	NT	2	0.3107	0.0769	0.0009**	0.0028**
*PGM**	CH	2	0.3848	0.1200	0.0014**	
	NT	2	0.4260	0.3077	0.1419	0.7269
*SOD**	CH	2	0.4992	0.1600	0.0006**	
	NT	2	0.3750	0.2692	0.1494	0.0712
*XDH-1**	CH	3	0.4968	0.2800	0.0277	
	NT	3	0.5688	0.1923	<0.0001**	0.1543*
Mean over all loci	CH	1.60	0.2222	0.1040		
	NT	1.60	0.1892	0.0954	—	<0.0001**

* Significant at (P<0.05); ** Significant after critical significance level is adjusted for sequential Bonferroni correction (Lessios, 1992). CH – Chalakkudy River, Kerala; NT – Nethravathi River, Karnataka.

Results and discussion

Fourteen out of 25 enzymes were found to give scorable activity that provided 25 loci (Table 1). The name of enzyme loci, enzyme commission numbers and alleles observed are given in Table1. Eleven of twenty five loci were monomorphic represented by a single allele in all the studied populations. A locus was considered polymorphic, if the frequency of most common allele was ≤ 0.99 (Hartl and Clark, 1997). Fourteen loci (56%), *AAT-2**, *EST-1**, *EST-2**, *EST-3**, *αG_3PDH**, *G_6PDH**, *GLDH**, *GPI-2**, *LDH-2**, *MDH**, *ODH-2**, *PGM**, *SOD**, *XDH-1** were polymorphic (Table1). The banding pattern of heterozygous in polymorphic loci, confirmed to that expected as per the structure of the respective protein. Of the 14 polymorphic loci, *EST-1**, *EST-2**, *EST-3**, *G_6PDH**, *GPI-2**, *αG_3PDH**, *MDH**, *ODH-2**, *PGM** and *SOD** had

two alleles; *AAT-2**, *LDH-2** and *XDH-1** had three alleles and *GLDH** had four alleles. The zymograms of the studied proteins are shown in Fig. 1-14. Except *EST-1** and *EST-3**, all other

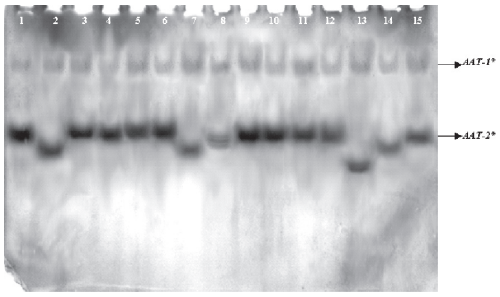

Fig. 1. Aspartate amino transferase (AAT) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

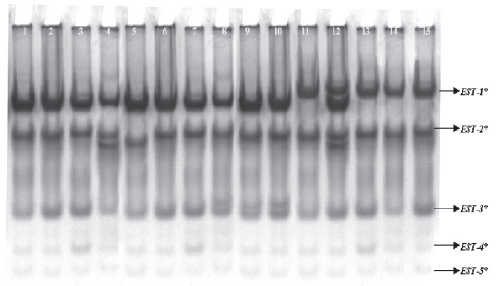

Fig. 2. Esterase (EST) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy river and 11-15 Nethravathi River.

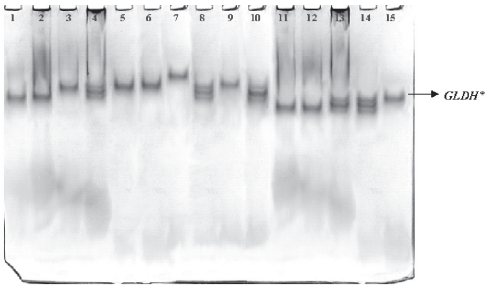

Fig. 3. Glucose dehydrogenase (GLDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy river and 11-15 Nethravathi River.

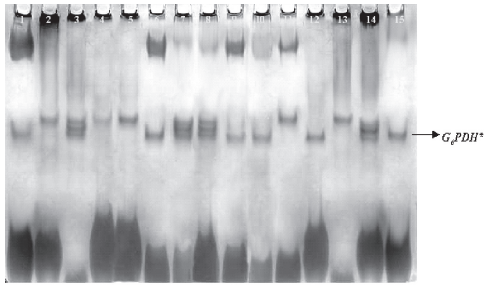

Fig. 4. Glucose 6-phosphate dehydrogenase (G6PDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

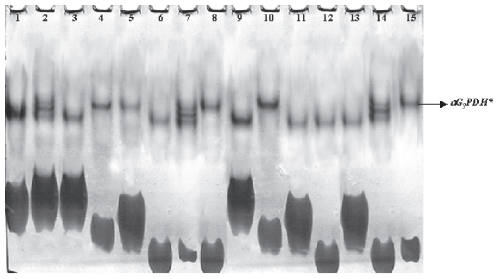

Fig. 5. -Glycerol 3-phosphate dehydrogenase (G3PDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

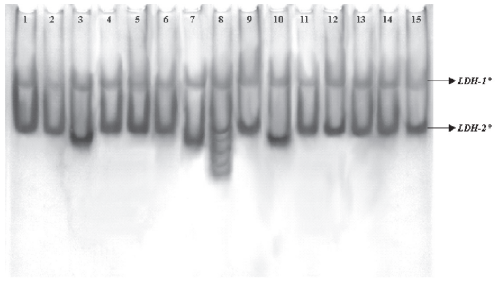

Fig. 6. Lactate dehydrogenase (LDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

polymorphic loci were polymorphic in all populations. *EST-1** locus was polymorphic only in Nethravathi population whereas, *EST-3** locus was polymorphic only in Chalakkudy

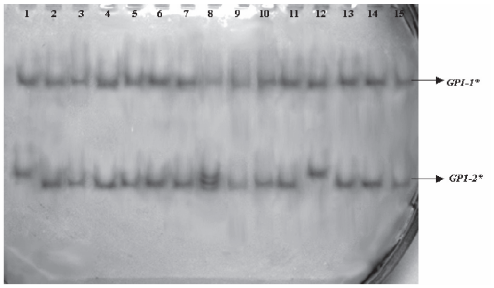

Fig. 7. Glucose phosphate isomerase (GPI) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

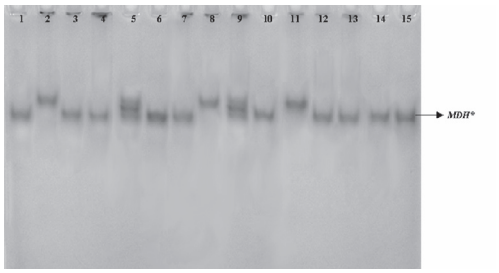

Fig. 8. Malate dehydrogenase (MDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

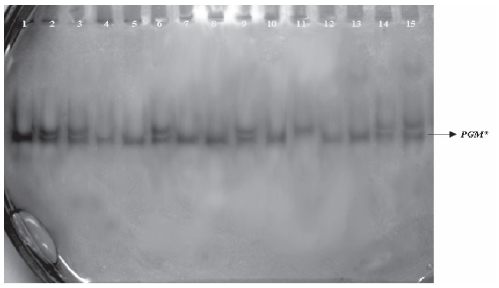

Fig. 9. Phosphoglucomutase (PGM) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

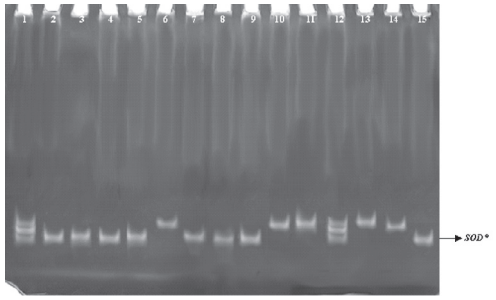

Fig. 10. Superoxide dismutase (SOD) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

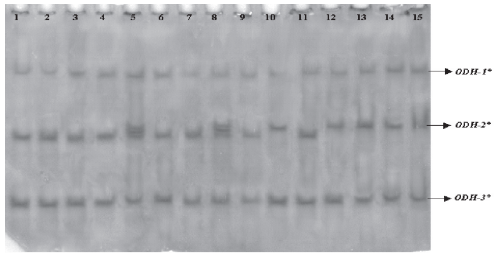

Fig. 11. Octanol dehydrogenase (ODH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

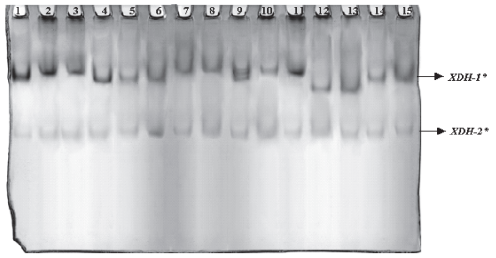

Fig. 12. Xanthine dehydrogenase (XDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

population. Parameters of genetic variation are given in Table 2. The proportion of polymorphic loci at $P_{0.99}$ criteria was 0.52 and the mean number of alleles per locus is 1.60, for both the sample sets. The observed mean heterozygosity values ranged from 0.09 to 0.10 (Table 2). These values were found to be marginally higher than the value described for teleost fishes (Nevo, 1978)

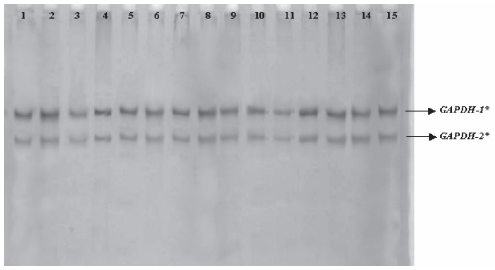

Fig. 13. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

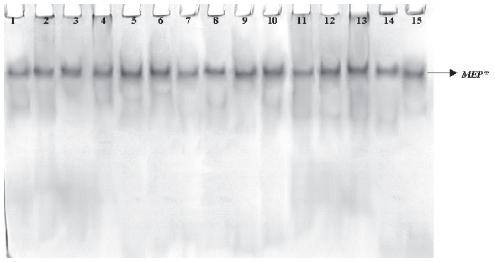

Fig. 14. Malic enzyme (MEP) pattern in *H. brachysoma*. Lanes 1-5 samples from Meenachil River, 6-10 Chalakkudy River and 11-15 Nethravathi River.

but are common. The probability test provided the evidence that the observed allele frequencies significantly ($P<0.05$) deviated from that expected under Hardy-Weinberg equilibrium. Deviation was observed at least in five to six loci for both the sample sets. Significant deficiency of heterozygote was observed at these loci. This indicates the possibility of genetic bottlenecks existing in the natural populations of *Horabagrus brachysoma*.

The genetic heterogeneity was tested based on the genotype rather than allele frequencies, in view of the observed non-conformity to Hardy-Weinberg expectations (Raymond and Rousset, 1995b; Goudet *et al.*, 1996). Significant heterogeneity ($P<0.05$) in genotype proportions was observed (Table 2), after the sequential Bonferroni correction was made to the probability levels. Highly significant probability ($P<0.0001$) over all the loci suggests that the three sample sets are not part of the same gene pool. Coefficient of genetic differentiation (Gst) calculated for sample size (Nei and Chesser, 1983), was found to be 0.1303 confirming the existence of population sub-structuring in *H.brachysoma* (Table 2).

In conclusion, the present study identified fourteen polymorphic allozyme loci, useful in determining genetic divergence in natural populations of *H. brachysoma*. Nevertheless, analysis of larger sample size, from other regions of distribution will provide better insights, not only for genetic divergence but also for pinpointing the genetic bottlenecks in the natural populations of the species.

Acknowledgment

The authors gratefully acknowledge the constant help rendered by Mr. Devassy, Fish Hobbyist at Kanakkan-kadavu, Kerala during collection of specimens.

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Date of Receipt : 03-12-04
Date of Acceptance : 16-09-05