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Genetic variation and differentiation of wild stocks of critically endangered *Puntius sarana* (Hamilton) and their F_1 crossbreed through allozyme electrophoresis

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Abstract

The lower genetic variation and differentiation in wild parental stocks of critically endangered *Puntius sarana* and slight genetic improvement in their F_1 crossbreed were inferred by horizontal starch gel electrophoresis with nine allozyme markers. Out of fifteen loci, four loci (*EST-1**, *GPI-2**, *G3PDH-2**, *PGM**) were polymorphic in F_1 crossbreed progeny and in maternal parent, and only two loci (*EST-1**, *G3PDH-2**) in paternal parent. A rare allele **c* at locus *EST-1**, the highest mean number of polymorphic loci (26.6), mean number of alleles per locus (1.33) and observed heterozygosity (0.066) were observed in F_1 generation. The higher fixation index (F_{IS}) value indicated heterozygote deficiency in parental stocks, and the negative F_{IS} value at locus *GPI-2** and *PGM** indicated excess of heterozygote in F_1 crossbreed progeny. The overall genetic differentiation (F_{ST}), gene flow (N_m), maximum genetic distance (D) were 0.0690, 3.3755 and 0.0183 respectively, showed very low genetic differentiation among them. Crossbreeding of geographically isolated endemic wild stocks of critically endangered *P. sarana* could be potential for the revival of the species from the being extinction.

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Introduction

The olive barb, *Puntius sarana* (Hamilton, 1822) is locally known as deshi sarputi or puda that belonging to the Cyprinidae family. This is an indigenous food fish in Southeast Asia including Bangladesh. Once it was one of the most abundant fish species in the rivers and natural depression of Bangladesh (Gupta, 1980; Rahman, 1989). It was also reported in India, (Menon, 1999), Nepal (Shrestha, 1994), Sri Lanka (Pethiyagoda, 1991), Myanmar (Doi, 1997), Thailand (Sidthimunka, 1970), Afghanistan and Bhutan (Talwar and Jhingran, 1991). The distribution and abundance of this species has greatly reduced in the open waterboides and natural depression of Bangladesh and identified as a critically endangered fish species (IUCN, 2000). The endemic situation of this species mainly in few open waterbodies of the country including Mymensingh and Sylhet division (Parvez *et al*., 2006), Rajshahi (Hossain *et al*., 2009) and Dinajpur districts (Parvez *et al.,* 2012) were reported.

Some sorts of research initiatives were taken to protect this species from the being extinction and to introduce in the aquaculture of Bangladesh. The research works so far have been completed include the distribution of this species (Parvez *et al*., 2006), artificial breeding (Chakrabortey *et al*., 2002; Akhteruzzaman *et al*., 1992), embryonic and larval development (Chakrabortey *et al*., 2003), larval rearing (Parvez and Khan, 2005), the culture potential of this species under semi-intensive culture system (Chakraborty *et al*., 2005), cross-breeding between two different wilds stocks of *P. sarana* (Parvez *et al*., 2010). In one of our previous studies, we observed that most of the hatcheries involved in *P. sarana* seed production collected broods from single sources either from Kangsha river of Netrokona district or from haor region of Sylhet division (Parvez *et al*., 2006). The use of brood fish from single source may be concern with the lower genetic variability of critically endangered *P. sarana* broodstock which may be subjected to lower growth and lower survival rate of produced seed. The essentiality for maintaining a sufficient amount of genetic variation for the long term survival, adaptability, growth and survival of produced spawns is described by Leberg (1990). Later on, with the view to increase genetic diversity, we conducted intra and inter breeding programme within and between geographically separated two wild stocks of *P. sarana* stock. The F_1 crossbreed obtained from the intra breeding of two separate *P. sarana* population showed higher growth and survival rate compared to two other F_1 generation obtained from the inter breeding programme (Parvez *et al*., 2010). The higher growth and survival of F_1 cross-breed from the intra breeding program may be due to the increased genetic variability's and differentiation in compared to their wild parental stocks. This current study current study was aimed to test the hypothesis whether the genetic variability and differentiation in F_1 cross-breed increased or decreased in compare to two wild parental stocks of *P. sarana* using horizontal starch gel electrophoresis.

Materials and methods

The fish stock

Fish tissue samples for allozyme electrophoresis were collected from the stocks maintained by Parvez *et al.,* (2010) in rectangular sized earthen ponds of Field Complex, Bangladesh Agricultural University, Mymensingh, Bangladesh. In brief, two wild stocks *P. sarana* were collected from the Kangsha river of Netrokona district and Sukhair haor (natural depression) of Sunamganj district of Bangladesh. The broods were reared in rectangular size pond for a period of 6 months. Three breeding line were developed where in line-1 both male and female *P. sarana* broods from Sukhair stock, in line-2 female from Sukhair stock and male from Kangsha stock and in line-3 both female and male brood were breed form Kangsha stock. The F_1 crossbreed of line-1, line-2 and line-3 were named as SS, SK and KK respectively and reared in earthen ponds of the same field complex for a period of 4 month under same feeding regime (Parvez *et al*., 2010). As the SK line showed higher growth and survival rate, we selected the SK line and their parents as the samples for the allozyme electrophoresis.

Collection and preservation of fish tissue

Muscle samples from 30 individual of SK line and their parental stocks (female from Sukhair stock and male from Kangskha stock) were collected by using sterilized scalpel and scissors, and immediately after collection were stored in -80°C for electrophoresis.

Horizontal starch gel electrophoresis

Horizontal starch gel electrophoresis technique described by Shaw and Prasad (1970) was used in this study. In brief, the inoculation of protein extracts from collected muscle were done by using paper wicks, followed by loading the inoculated wicks in between two pieces of gel from left to right serially. Ethylene blue containing two wicks were placed as dye marker at both end of the gel to determine the rate of protein migration. The gel was placed in the electrophoresis chamber and electricity was run from power supply at 100V (EPS 301, Sweden) for 30 minutes and at 150V for 5-6 hrs after removing the wicks.

Staining gene with allozyme markers and scoring of alleles

After running, the gel was sliced horizontally into 1.0 mm sized five sections. The sliced gels were stained with 10 different enzymes. The enzymes analyzed, E.C. numbers, abbreviation, enzyme patterns and tissue type are presented in Table 1. The components of staining solution with some co-factors that were used in this experiment are listed in Table 2. All staining were routinely accomplished in an oven set at 45-55°C. Some of the staining (*EST*) was accomplished in the dark, due to the presence of light sensitive components in the enzyme staining reactions. Loci and alleles were designated following the nomenclature system of Shaklee *et al.*, (1990). The individual fish genotype at each allozyme locus was determined to assess the genetic variation between parents and offspring. A locus was considered polymorphic if the frequency of the most common allele was ≤0.99 (Hartl and Clark, 1997).

Data analysis

Allelic frequencies, polymorphic loci (*P*), and fixation index (F_{IS}) were estimated using GeneAlEx version 6.0 (Peakall and Smouse, 2001). The deviations of expected genotypes from the observed genotypes were calculated according to Hardy-Weinberg equilibrium. The mean number of polymorphic loci per population, the mean number of alleles per locus and the heterozygosities (observed, H_o and expected, H_e) were estimated with the help of POPGENE version 1.31 computer package programs (Yeh *et al.*, 1999). The genetic differentiation (F_{ST}) and gene flow (N_m) among populations were also estimated by using GeneAlEx computer program version 6.0. Genetic distance (*D*) was measured according to Nei (1972) with the help of POPGENE, (version 1.31) and G-Stat, (version 3.1) (Siegismund, 1995).

Results

To reveal the genetic diversities and differentiation among wild parental stocks of *P. sarana* and their F_1 generation, we checked for 10 enzyme systems. Except *G6PDH*, all other enzyme systems produced detectable loci; 15 presumptive loci with a total of 20 alleles were detected.

Table 1. Enzymes examined and tissue used for allozyme electrophoresis.

Enzymes	Enzyme abbreviations	Enzyme patterns	E.C. Number	Tissue
Alcohol dehydrogenase	ADH	Dimer	1.1.1.1	Muscle
Esterase	EST	Monomer	3.1.1.1	Muscle
Glycerol-3-phosphate dehydrogenase	G3PDH	Dimer	1.1.1.8	Muscle
Glucose -6-phosphate dehydrogenase*	G6PDH	Dimer	1.1.1.49	Muscle
Glucose-6- phosphate isomerase	GPI	Dimer	5.3.1.9	Muscle
Isocitrate dehydrogenase	IDHP	Dimer	1.1.1.42	Muscle
Lactate dehydrogenase	LDH	Tetramer	1.1.1.27	Muscle
Malate dehydrogenase	MDH	Dimer	1.1.1.37	Muscle
Phosphoglucomutase	PGM	Monomer	5.4.2.2	Muscle
Sorbitol dehydrogenase	SDH	Tetramer	1.1.1.4	Muscle

* G6PDH did not show clear resolution.

The allele frequencies and number of polymorphic loci in all three stocks of *P. sarana* are shown in Table 3. Four polymorphic loci (*EST-1**, *GPI-2**, *G3PDH-2** and *PGM**) were found in the maternal parent (Sukhair stocks) and in F_1 generation (SK line) but only 2 (*EST-1** and *G3PDH-2**) in paternal parent (Kangsha stock). Three alleles (**a,*b,*c*) were observed only in *EST-1**, 2 alleles (**a,*b*) were in *G3PDH-2**, *GPI-2** and in *PGM**; and only one allele (**a*) in rest of the loci (Table 3). The *EST-1** showed the rare allele **c* in very low frequency (0.033) only in F_1 generation. Based on Hardy-Weinberg equilibrium, the chi-square (χ^2) test was done in all the cases of polymorphic loci between observed and expected genotypes.

Table 2. Components of staining buffer used for allozyme electrophoresis.

Enzyme name	PMS (1mg)	NBT (10 mg)	MTT (10 mg)	Cofactor (20 mg)	Other components
ADH	*	*	-	NAD	10 ml Ethanol 0.05 M Tris-HCl*²(80 ml)
EST	-	-	-	-	0.1M Phosphate buffer*¹ (60 ml) 25 mg α-napthyl acetate 70 mg Fast blue BB salt
G6PDH	*	*	-	NAD	0.05 M Tris-HCl*²(80 ml) 100 mg DL- α-glycerophosphate (Na)
G3PDH	*	*	-	NAD	0.05 M Tris-HCl*²(80 ml) 100 mg DL- α-glycerophosphate (Na)
GPI	*	-	*	NADP	0.05 M Tris-HCl*² (80 ml) 100 mg Fructose-6-phosphate (Na) 110 µl Glucose-6-phosphate dehydrogenase 1 ml 1 M Magnesium chloride
IDHP	*	-	*	NADP	0.05 M Tris-HCl*² (80 ml) 60 mg DL-3-Na-Isocitrate 1 ml 1 M Magnesium chloride
LDH	*	*	-	NAD	0.05 M Tris-HCl*² (80 ml) 6 ml Sodium lactate
MDH	*	*	-	NAD	0.05 M Tris-HCl*² (80 ml) 6 ml Sodium malate
PGM	*	-	*	NADP	0.05 M Tris-HCl*²(80 ml) 150 mg Glucose-1-phosphate (Na) 70 µl Glucose-6-phosphate dehydrogenase 1 ml 1 M Magnesium chloride
SDH	*	-	*	NAD	0.05 M Tris-HCl*²(80 ml) 200 mg Sorbitol

*After use showed activity;

*¹ Phosphate buffer (pH 7.0): mixture of 0.2 M sodium dihydrogen phosphate and 0.2 M disodium hydrogen phosphate;

*² Tris HCl buffer (pH 8.7): Tris (hydroxymethyl) aminomethane, pH adjusted with 1N HCl

NAD: β-nicotinamide adenine dinucleotide, NADP: β-nicotinamide adenine, dinucleotide phosphate, sodium salt;

NBT: nitro blue tetrazolium; MTT: (3-[4, 5 dimethyl-2-thiazolyl]-2, 5 diphenyl tetrazolium bromide); PMS: phenazine methosulfate.

The maternal stock collected from Sukhair haor of Sunamganj district showed significant variation in allele frequency of *EST-1*, G3PDH-1* and PGM** loci, where the paternal stock which was collected from the Kangsha river of Netrokona district showed significant variation only in allelic frequency of *G3PDH-1** locus, but in terms of allele frequency no significant variation was observed in F_1 generation (Table 4).

Table 3. Allele frequency at 15 presumptive loci of *P. sarana*.

		Allele Frequency		
Locus	Allele	Sunamganj	SK (Offspring)	Kangsha
*ADH-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*EST-1**	**a*	0.733	0.550	0.600
	**b*	0.267	0.417	0.400
	**c*	0.000	0.033	0.000
P		0.0001***	0.1004 NS	0.0108*
*EST-2**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*G3PDH-1**	**a*	0.517	0.367	0.333
	**b*	0.483	0.633	0.667
P		0.0001***	0.104 NS	0.0008**
*GPI-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*GPI-2**	**a*	0.650	0.917	1.000
	**b*	0.350	0.083	0.000
P		0.7226 NS	0.6585 NS	NS
*IDHP-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*IDHP-2**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*LDH-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*LDH-2**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*MDH-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*MDH-2**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*PGM**	**a*	0.717	0.933	1.000
	**b*	0.283	0.067	0.000
P		0.0001***	0.7367 NS	NS
*SDH-1**	**a*	1.000	1.000	1.000
P		NS	NS	NS
*SDH-2**	**a*	1.000	1.000	1.000
P		NS	NS	NS

P: Probability of chi-square value, *** significant level: $p<0.01$, ** significant level: $p<0.05$,* significant level: $p<0.10$; NS: Non-Significant.

The mean number of polymorphic loci was 26.6 both for maternal population and F_1 generation where the lowest (13.33) in paternal population (Table 5). The mean number of allele per locus was 1.33 in F_1 generation and 1.26 in both parental populations. The observed heterozygosity ranged from 0.017 in the paternal population to 0.066 in F_1 generation where the expected heterozygosity was 0.62 in paternal population and 0.085 in F_1 generation (Table 5). In each of the statistically significant locus, the fixation index (F_{IS}) was measured to estimate the deviation of random mating (heterozygote deficiency or excess). The negative sign (-) indicated the excess of heterzygote which was observed only in F_1 generation incase of *GPI-2** and *PGM** loci (Table 7). The F_{IS} value was very high in both parental populations which indicated significant heterozygote deficiency in parental population.

The genetic differentiation (F_{ST}) and the gene flow (N_m) in over all three populations are 0.0690 and 3.3755 respectively. In pair-wise analysis, highest F_{ST} *value* (0.87) was found in between maternal and paternal population and lowest value (0.53) was

found in between the pair of maternal population and F_1 generation (Table 6). The highest N_m value (4.467) was estimated in between the maternal population and F_1 generation and lowest value (2.624) in between maternal and paternal populations (Table 6).

Table 4. Analyzed sample size and chi-square (χ^2) test of fit to Hardy-Weinberg expectation.

Sample Size		Population		
		Sukhair	SK	Kangsha
		30	30	30
*EST-1**	χ^2	13.98*	6.24	6.40
	d.f.	1	3	1
*G3PGH-1**	χ^2	20.01*	2.70	31.30*
	d.f.	1	1	1
*GPI-2**	χ^2	0.1259	0.195	0
	d.f.	1	1	-
*PGM**	χ^2	11.222*	0.112	0
	d.f.	1	1	-

The genetic distance (*D*) among three populations ranged from 0.0010 to 0.0183 (Fig. 1). The minimum genetic distance (*D* = 0.0010) was observed in between paternal population and F_1 generations, while the maximum value (*D* = 0.0183) was found in between the maternal and paternal populations (Fig. 1).

Discussion

All the enzymes used in this study showed clear resolution except *G6PDH*. The failure of clear resolution with *G6PDH* might be due to buffer system, tissue and/or species specificity. The assumption agrees with that of Khan and Arai (2000) who also did not find clear band with this enzyme. Four polymorphic loci were found in the maternal parent (Sukhair stock) and in F_1 generation (SK line), and only two were found in paternal parent (Kangsha stock). Several studies reported *EST**, *G6PDH**, *GPI**, *LDH**, *PGM** and *SOD** as polymorphic loci that are pertinent to characterization of genetic structure in fish population (Salini *et al.*, 2004; Lal *et al.*, 2004).

Table 5. Genetic variabilities at 15 loci of *P. sarana*.

Parameter studied		Populations		
		Sukhair	SK	Kangsha
The mean proportion of polymorphic loci (%)		26.67	26.67	13.33
Mean number of allele per locus		1.266	1.333	1.26
Heterozygosity	H_o	0.055	0.066	0.017
	H_e	0.118	0.085	0.062

A rare allele **c* with a low frequency (0.033) were found in locus *EST-1** of F_1 crossbreed progeny that is similar to the study of Haniffa *et al.* (2007) where they found two rare alleles, *G6PDH-1*C* and *EST-4*C* in spotted Murrel of South Indian Tamirabarani and Kallada populations with low frequencies. Moreover, a rare allele *c** (0.05) in *PGM** in the natural population of *Labeo rohita* was also reported (Alam *et al.*, 2002). The observation of rare allele in our study represents the difference between parents and their F_1 crossbreed. The rare allele can be utilized as genetic markers for selection of a candidate stock for controlled breeding programme (Lester and Pante, 1992). The allele frequencies of maternal parent in four polymorphic loci were significantly different from the paternal parent and also from the F_1

crossbreed (Table 3). The variation in allele frequency in the population may be due to environmental factors such as temperature, alkalinity and pollution (Ponniah, 1989). The role of the temperature in maintaining alleles at different frequencies have been reported in natural population (Nyman and Shaw, 1971) and experimentally (Mitton and Koeh, 1975). The mean number of polymorphic loci per population in maternal population and in F_1 crossbreed was higher (26.67%) than the paternal population (13.33%) population (Table 5). Nevo *et al.,* (1984) estimated 15.2% polymorphic loci ($p \leq 0.95$) for polymorphism in fish is general. The mean number of allele per locus was highest (1.33) in F_1 crossbreed progeny compared to the parental stocks (1.26). The higher values of polymorphic loci and mean number of allele per locus in F_1 crossbreed indicate the increase of genetic diversity in the offspring than the paternal parents which may be due to the result of cross breeding between two different wild stocks of *P. sarana*.

Table 6. F_{st} and Gene flow *(Nm)* in pair-wise three *P. sarana* populations.

Populations	F_{ST}		Gene flow (*Nm)*	
	Pair-wise	Overall	Pair-wise	Overall
		Overall		
Maternal population - F_1 generation	0.053	0.0690	4.467	3.3755
F_1 generation – Paternal population	0.066		3.788	
Maternal population - Paternal population	0.087		2.624	

The best estimate of genetic variation in natural population is the mean observed heterozygosity (H_O) which varies non-randomly between loci, populations and species (Allendorf and Utter 1979). The heterozygosity value in F_1 generation and maternal population fall within the suitable range (0.05-0.07) reported by several authors (Kohlmann and Petra, 1999; Lal *et al*., 2004; Singh *et al*., 2004; Salini *et al*., 2004). For freshwater species with wide distribution higher observed heterozygosity in between 0.105-0.0135 were reported in *Cirrhinus mrigala* (Chauhan *et al*., 2007). The average heterozygosity (H_o or H_e) is considered as a good indicator of the genetic variability throughout the genome of the population (Leary and Booke, 1990; Allendorf and Ryman 1986). In both parental populations no excess of heterozygote loci was observed, only observed in *GPI-2** and *PGM** of F_1 crossbreed (Table-7).

Table 7. Inbreeding co-efficient (*F*is) at 4 polymorphic loci for 3 populations of *P. sarana*.

Locus	Population		
	Sukhair	SK (offspring)	Kangsha
*EST-1**	0.6591	0.2986	0.4444
*G3PDH-2**	0.7998	0.2823	1.0000
*GPI-2**	0.0476	-0.0909	-
*PGM**	0.5896	-0.0714	-

The heterozygote deficiency indicated the deviation of random mating in wild population of *P. sarana* . Heterozygote deficiency has been reported quite commonly in many fish species (Engelbrecht *et al*., 1997; Steenkemp *et al*., 2001). Several hypotheses have been mentioned to explain homozygote excess in fishes including inbreeding, population admixture or the presence of non-expressed alleles (Appleyard *et al*., 2001; Ward *et al*., 2003).

Compared to several other studies, the co-efficient of gene differentiation (F_{ST}) in present study showed lower levels of genetic differentiation with an overall value 0.069 (Table 6). The F_{ST} value in two species of

Oreochromis were in a range of 0.501 to 0.598 (Appleyard and Mather, 2002) and a very high F_{ST} value (0.774) in loach (Khan and Arai, 2000) were reported. The lower F_{ST} value indicated the presence of subdivided population with a little genetic differentiation. The number of individuals that migrate from one subdivided population to another is high (N_m =3.375). This result was found to be similar with the findings of Islam *et al.*, (2004). The pair-wise population gene flow was higher (4.467) between the maternal population-F_1 crossbreed than all other pair. The genetic distance (*D*) among three populations was ranged from 0.0010 to 0.0183 which is much lower than the findings of several studies. The genetic distances in yellow catfish were ranged from 0.005 to 0.164 (Leesa-Nga-SN *et al.*, 2000). Nei (1972) found that in a variety of animals, *D* is approximately 1.0 for inter species comparisons, around 0.1 for subspecies, and 0.01 for local races. Considering from the above-mentioned criteria, the studied *P. sarana* population may be considered as local race.

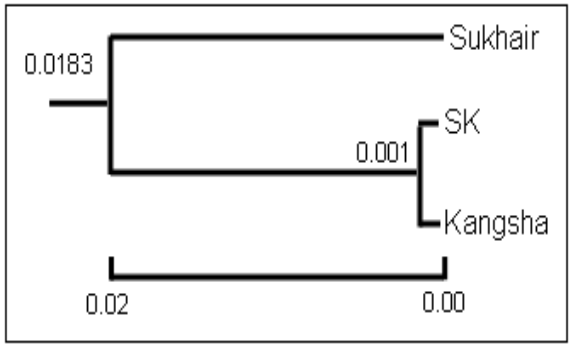

Fig. 1. Nei's (1972) UPGMA dendogram showing the genetic distance (*D*) among parents and their offspring.

In Conclusion, the wild stocks of the critically endangered *P. sarana* showed very lower levels of genetic diversity and differentiation. The crossbreeding between two wild stocks produced F_1 progeny that showed higher genetic variation. The higher genetic variation in SK population may be due to using of broods from two different sources. In the previous study we observed that this crossbreed progeny performed a higher survival and growth rate compared to two other intra-breed progeny. This higher growth and survival may be due to slight genetic improvement in SK population. For the conservation of this critically endangered fish species long term research initiatives with a massive collection of *P. sarana* wild stocks from all the reported region of the country is required. After collection, a well planned crossbreeding program needed to be design and implement.

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