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Genetic Variation of Three Populations of Indian Frog (*Hoplobatrachus tigerinus*) Revealed by Allozyme Marker

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ABSTRACT

The Indian bullfrog, *Hoplobatrachus tigerinus* plays a significant role in maintaining the natural balance in the ecosystems. It plays an important role in controlling the various agricultural pests because of its omnivorous feeding habit. The aim of the present study is to know the genetic variation of *H. tigerinus* in three natural habitats. Samples collected from three districts of Bangladesh were analyzed with five enzymes (MDH, LDH, GPI, PGM and EST) in CA 6.1 buffer system for their genetic variation. Four polymorphic loci (Mdh-1, Est-1, Gpi-1 and Pgm) were interpretable in muscle with starch gel electrophoresis. Among the 5 presumptive loci, the mean proportion of polymorphic loci was observed 80, 80 and 60% in Rangpur, Khulna and Mymensingh populations, respectively. The highest mean number of allele per locus and mean proportion of heterozygous loci per individual were observed in the Rangpur population. The average observed heterozygosity (H_o) was 0.163 and expected heterozygosity (H_e) was 0.469. In pair-wise analysis, comparatively higher N_m value (5.507) was estimated between the Rangpur and Khulna populations corresponding lower level of F_{ST} value (0.043). The UPGMA dendrogram showed two clusters among the three Indian bullfrog populations. Rangpur and Khulna populations formed one cluster while Mymensingh population formed another cluster. The Mymensingh population separated from Rangpur and Khulna by a genetic distance of 0.177 whereas, the Khulna population is different from the Rangpur population by the genetic distance of 0.052. The results suggested that the considerable genetic variation is maintained among the natural *H. tigerinus* populations.

Key words: Heterozygosity, gel electrophoresis, Indian bull frog, genetic variation, Bangladesh

INTRODUCTION

Bangladesh has a well-diversified landscape including wide flat land, marshy area, flood plain, dense forest, mangrove area and hilly region where there are different species of frogs everywhere (Reza, 2007). The Indian bullfrog, *Hoplobatrachus tigerinus*, Randiae, is widely distributed in South and South-East Asian countries such as Bangladesh, India, Pakistan, Nepal, Bhutan, Myanmar, Thailand, Malaysia and Indonesia (Husain and Rahman, 1978). The indiscriminate catch of frogs during breeding season is a common phenomena in Bangladesh. If this situation is going on, once frog population will vanish from Bangladesh and it will disturb the biological

balance and consequently lead to the ecological disaster. In these circumstances, it has been seriously thought to save the frogs from being extinction (Islam *et al*., 2012).

Hoplobatrachus tigerinus plays a significant role in maintaining the natural balance in the ecosystems. It also plays an important role in controlling the various agricultural pests because of its omnivorous feeding habit (mainly insectivorous). According to recent survey conducted by IUCN Bangladesh, *Hoplobatrachus tigerinus* was fairly common in Bangladesh and had a wide distribution mainly in irrigated, cultivated fields that create a new habitat for feeding, sheltering and breeding. But now-a-days the availability of frogs are going to be vulnerable or endangered due to different anthropological effects and environmental changes (Islam *et al*., 2012). So, it is urgently needed to know their genetic variation of different natural sources of frog population.

Allozyme electrophoresis is an effective tool for animal population variation studies (Khera *et al*., 2012; Nassiri *et al*., 2007; Aziz *et al*., 2011) and specially for frog farming management (Utter, 1991). Allozyme electrophoresis studies of anuran distributed in Japan and adjacent countries have been reported by Nishioka *et al*. (1993), Sumida and Nishioka (1994) and Khan *et al*. (2002). Until now, reliable information on the number of frog species and their genetic status in Bangladesh was unavailable, although very limited genetic analysis of Bangladeshi frogs was performed by Khan *et al*. (2002). The objective of the present study was to identify the genetic variation of this amphibian species (*Hoplobatrachus tigerinus*) in three different regions viz., Mymensingh, Rangpur and Khulna districts in Bangladesh using starch-gel electrophoresis.

MATERIALS AND METHODS

Sample collection and processing: The experimental frogs (*Hoplobatrachus tigerinus*) were collected from the three different locations (Mymensingh, Rangpur and Khulna districts) of the country. Major part of the work was accomplished in the "Fisheries Biology and Genetics Laboratory", Bangladesh Agricultural University, Mymensingh. A total of 20 specimens were collected from each region. The muscle and liver samples were taken from each individual and stored at -21°C until electrophoretic analysis. Details of the sampling localities, number of specimen and date of collection are given in Table 1.

Gel electrophoresis: This experiment was performed by using allozyme markers through horizontal starch gel electrophoresis method followed by Shaw and Prasad (1970). The enzymes analyzed, E.C. numbers, abbreviation of enzymes and the buffer system used for horizontal starch-gel electrophoresis are shown in Table 2. Electrophoresis was conducted using amine-citrate buffer (CA 6) (Clayton and Tretiak, 1972). After electrophoresis, the gel slices (about 1 mm thickness) were histochemically stained for different enzymes as described by Aebersold *et al*. (1987).

Allelic frequencies were calculated directly from observed genotypes. The distribution of observed genotypes was calculated from the Hardy-Weinberg equilibrium using a chi-square (χ^2) test for allozyme system. When the most common allele existed in a frequency of less than 0.95 at a given locus, it was regarded as polymorphic. The mean proportion of heterozygous loci per

Table 1: Sources, number of specimens and date of collection of *Hoplobatrachus tigerinus* populations

Sample No.	Population	GPS	No. of frog	Date of collection
1	Mymensingh	24°38'3"N90°16'4"E	20	September 27, 2006
2	Rangpur	25°44'N 89°15'E	20	October 12, 2006
3	Khulna	22°49'0"N 89°33'0"E	20	October 22, 2006

Table 2: Five enzymes examined and showed clear banding using muscle tissue

Enzymes	Locus	Enzyme patterns	E.C. No.
Esterase (EST)	Est-1	Monomer	3.1.1.1
Glucose-6-phosphate isomerase (GPI)	Gpi-1	Dimer	5.3.1.9
	Gpi-2		
Lactate dehydrogenase (LDH)	Ldh-1	Tetramer	1.1.1.27
	Ldh-2		
Malate dehydrogenase (MDH)	Mdh-1	Dimer	1.1.1.37
	Mdh-2		
Phosphoglucomutase (PGM)	Pgm	Monomer	5.4.2.2

individual, mean proportions of polymorphic loci per population and average number of alleles per locus were calculated so as to show the extent of genetic variability for each population (Lewontin, 1974). Expected heterozygosity (He) and observed heterozygosity (Ho) was examined according to Nei and Roychoudhury (1973). Genetic distance (D) value was calculated by using Nei's formula (Nei, 1972). Statistical analysis was performed by using SPSS (v.11).

RESULTS AND DISCUSSION

Allele frequencies were calculated directly from observed genotypes at five loci in 60 samples of three frog populations (Table 3). Allele frequencies at 5 presumptive loci of *H. tigerinus* populations in three populations were significant ($p<0.01$) except Est-1. Among the five loci, two populations showed four (Mdh-1, Gpi-1, Pgm and Est-1) polymorphic loci whereas Mymensingh population showed three (Mdh-1, Pgm and Est-1) polymorphic loci. In the present study, the polymorphic loci of all populations had three common alleles a, b and c. The Ldh-1 locus was monomorphic with the allelic frequency of a = 1.00 in all the populations.

The mean proportion of polymorphic loci in Rangpur, Khulna and Mymensingh populations were 80, 80 and 60%, respectively (Table 4). In this study the observed proportion of polymorphic loci per population ranged from 60 to 80% (average 73.33%) which was higher than that obtained (55.2%) by Nishioka *et al.* (1987) for *Hoplobatrachus tigerinus* and that obtained (41.8%) by Sumida and Nishioka (1994) for *R. japonica*. Nishioka *et al.* (1993) found that the mean proportion of polymorphic loci in 40 populations of *R. rugosa* was 31.1% which was lower than that obtained (73.33%) for *Hoplobatrachus tigerinus* in the present study. Therefore the studied *Hoplobatrachus tigerinus* populations showed a higher level of polymorphism in comparison with the above mentioned frog species.

The mean proportion of heterozygous loci per individuals for all populations was 16.0% in average and ranged from 1.782 (Khulna) to 1.623 (Mymensingh). In the present study, the mean proportion of heterozygous loci per individual ranged from 12 to 20% (average 16%) was similar to the results of 16.1% obtained by Nishioka *et al.* (1987) for *R. tagoi*. In this study the mean proportion of heterozygous loci per individual (16%) was much higher than that obtained (14.4%) by Sumida and Nishioka (1996) for *R. ornativentris* and that obtained (11.3%) by Sumida and Nishioka (1994) for *R. japonica*. Again this value (16%) is lower than that obtained (17.8%) by Nishioka *et al.* (1993) for *R. rugosa*.

The observed heterozygosity (H_o) was 0.163 in average and ranged from 0.20 (Rangpur) to 0.11 (Mymensingh). The observed heterozygosity (H_o) obtained in the present study ranged from 0.11 to 0.20 (average 0.16) is higher than that reported (0.104) by Rafinski and Babik (2000) for *R. arvalis*. The present value (0.16) is much higher than that obtained (0.017) by Formas and Breva (2000) in case of *Batrachyla leptopus* and that obtained (0.002) by Sjogren (1991) for *R. lessonae*.

Table 3: Allele frequencies at 5 presumptive loci of *Hoplobatrachus tigerinus* populations

		Allele frequency		
Locus	Allele	Rangpur	Khulna	Mymensingh
Mdh-1	a	0.850	0.500	0.425
	b	0.125	0.500	0.150
	c	0.025	0.000	0.425
p-value		0.015**	0.005***	0.000***
Ldh-1	a	1.000	1.000	1.000
Gpi-1	a	0.425	0.500	1.000
	b	0.500	0.425	0.000
	c	0.075	0.075	0.000
p-value		0.012**	0.000***	NS
Pgm	a	0.550	0.650	0.550
	b	0.450	0.350	0.375
	c	0.000	0.000	0.075
p-value		0.000***	0.008***	0.000***
Est-1	a	0.575	0.675	0.875
	b	0.425	0.325	0.125
p-value		0.638ns	0.304ns	0.572ns

P: Probability of chi-square value, *,**,***Significant at $p<0.10$, $p<0.05$ and $p<0.01$, respectively ns: Non-significant

Table 4: Genetic variability at 5 loci of *Hoplobatrachus tigerinus* populations

		Mean number	Mean number of	The mean proportion	Heterozygosity		
Population	The mean proportion of polymorphic loci* (%)	of alleles (Na) per locus	effective alleles (Ne) per locus	of heterozygous loci per individual (%)	H_o	H_e	H_o/H_e
Rangpur	80	2.200	1.716	20.000	0.200	0.362	0.550
Khulna	80	2.000	1.782	16.000	0.180	0.392	0.460
Mymensingh	60	2.000	1.623	12.000	0.110	0.277	0.397
Average	73.33	2.067	1.707	16.000	0.163	0.344	0.469

*$p \leq 0.95$

The average expected heterozygosity (H_e) was 0.344 and ranged from 0.392 (Rangpur) to 0.277 (Mymensingh). The expected heterozygosity (H_e) obtained in the present study ranged from 0.277 to 0.392 (average 0.34) is higher than that obtained (0.156) by Rafinski and Babik (2000) for *R. arvalis*. The expected heterozygosity was obtained (0.275) by Khan *et al.* (2002) for *R. tigrina*. The present value (0.34) is much higher than that obtained (0.051) by Formas and Breva (2000) in case of *B. leptopus* and that obtained (0.0047) by Sjogren (1991) for *R. lessonae* (Table 4).

The higher observed and expected heterozygosity (H_o= 0.20 and H_e= 0.362) exhibited by Rangpur population indicated that the gene pool of the Rangpur population was maintained effectively. Among all the frog populations the H_o/H_e was higher in Rangpur and Khulna populations (0.55 and 0.46, respectively) and lowers in Mymensingh population (0.379).

The summary of the genetic differentiation (F_{ST}) and gene flow (N_m) are given in Table 5. Nei (1972) analysis of gene diversity within populations estimated the genetic differentiation (F_{ST}) and the gene flow (N_m) overall three populations are 0.128 and 1.699, respectively. In pair-wise analysis, comparatively higher N_m value (5.507) was estimated between the Rangpur and Khulna populations corresponding lower level of F_{ST} value (0.043).

Table 5: Pair-wise and overall population differentiations (F_{ST}) and gene flow (N_m) in three *R. tigrina* populations

	F_{ST}		N_m	
Populations	Pair-wise	Overall	Pair-wise	Overall
Rangpur-Khulna	0.043	0.128	5.507	1.699
Mymensingh-Rangpur	0.156		1.355	
Khulna-Mymensingh	0.116		1.897	

N_m: Gene flow estimated from $F_{ST} = 0.25 (1- F_{ST})/F_{ST}$

Table 6: Nei (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) estimated among 3 populations of *Hoplobatrachus tigerinus* based on 5 loci

Population	Rangpur	Khulna	Mymensingh
Rangpur	-	0.949	0.837
Khulna	0.052	-	0.877
Mymensingh	0.177	0.131	-

The co-efficient of gene differentiation (F_{ST}) in all three *Hoplobatrachus tigerinus* populations examined (Nei, 1972) for all loci was 0.128, indicated the presence of population with a slight genetic differentiation and the number of individuals that migrate from one population to another is high ($N_m = 1.699$). The pair-wise population gene flow was higher (5.507) between the Rangpur-Khulna populations than all other between population comparisons with corresponding lowest F_{ST} value of 0.043. The F_{ST} value (0.128) of *Hoplobatrachus tigerinus* populations as obtained in the present study is lower than that obtained (0.306) for *R. ornativentris* (Sumida and Nishioka, 1996) and that obtained (0.450) by Brieva and Formas (2001) for *B. taeniata*. The observed genetic distance differs between Mymensingh and other populations might be due to geographical isolation. The genetic distance (D) values among three populations ranged from 0.052 to 0.177. The minimum genetic distance (D = 0.052) was observed between Rangpur and Khulna populations, while the maximum value (D = 0.177) was found between the Mymensingh and Rangpur populations (Table 6).

The UPGMA dendrogram (Nei and Roychoudhury, 1973) constructed from Nei (1972) genetic distances is shown in Fig. 1. The UPGMA dendrogram showed two clusters among the three Indian bullfrog populations. Rangpur and Khulna populations formed one cluster while Mymensingh population formed another cluster. The dendrogram showed that the Mymensingh population separated from Rangpur and Khulna populations by a genetic distance of 0.177 where the Khulna population is different from the Rangpur population by the genetic distance of 0.052 (Table 6).

In the present study, the genetic distance (D) (Nei, 1972) varied from 0.052 to 0.177 (average 0.115) among three populations of *Hoplobatrachus tigerinus* was much lower than that in the same genus *R. narina* (D = 0.012 to 1.079, average 0.545), reported by Nishioka *et al.* (1987). In *R. japonica* the genetic distances between nine populations of the eastern group and sixteen populations of western group ranged from 0.099 to 0.239 with a mean of 0.157 (Sumida and Nishioka, 1994) were higher than that of the D-value (D = 0.115) of the present study. The minimum genetic distance (D = 0.052) was observed between Rangpur and Khulna populations, while the maximum value (D = 0.177) was found between the Mymensingh and Rangpur populations (Table 6). 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. Ayala (1975) reported

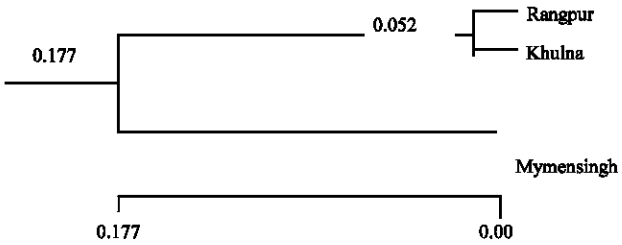

Fig. 1: Nei's UPGMA dendrogram showing the genetic distance (D) among three Indian bullfrog, *Hoplobatrachus tigerinus* populations

that the D-value between subspecies is approximately 0.20. Considering from the above-mentioned criteria, the studied *Hoplobatrachus tigerinus* populations may be categorized as local race or population.

CONCLUSION

The frog population collected from Rangpur showed comparatively higher mean proportion of polymorphic loci and mean proportion of heterozygous loci per individual, which was followed by Khulna population and lower values in Mymensingh population. The lower genetic variation in Mymensingh population may be due to mating of closely related individuals of same species i.e., inbreeding and/or small number of effective breeding population (Ne). So, the present study concludes that still there is considerable genetic variation among the natural *H. tigerinus* populations but this species are threatened in nature, their distribution is reduced day by day and become critically endangered in natural populations. Recently the USA based organization, save the frog expresses deep concern about conservation status of amphibians in Bangladesh and elsewhere in the World. They are no detail study on the genetic variation of other species as well in Bangladesh, so more investigations should be carried out on this line. As this specie has a significant ecological value, conservation measures should be taken.

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