MORPHOLOGICAL AND GENETIC VARIATION IN THREE POPULATIONS OF *Hoplobatrachus tigerinus* FROM BANGLADESH

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ABSTRACT

In order to investigate the morphological and genetic variation of Indian bullfrog *Hoplobatrachus tigerinus*, live samples were collected from three populations located in Khulna (southern part), Char Nilokhia and Netrokona (central part) in Bangladesh. The proportions of head length and head width (HL : HW), eye length and tympanum diameter (EL:TD), head length and snout length (HL : SL), internarial space and interorbital distance (IN:IOD) and internarial space and distance from nostril to tip of snout (IN:NS) of the Khulna population were significantly higher ($p<0.05$) than those of the other two populations. For allozyme study, 4 enzymes were screened, where 5 presumptive scorable loci were identified. The highest values of mean proportion of polymorphic loci (<0.95), mean number of alleles per locus and mean number of heterozygous loci per individual were observed in the Khulna population (60%, 1.60 and 14.0% respectively). The pairwise genetic differentiation (F_{ST} and Nei's D) was the highest (0.279 and 0.157, respectively) between the Khulna and Netrokona populations. The UPGMA dendrogram showed two clusters i.e. the Char Nilokhia and Netrokona populations formed one cluster, whereas the Khulna population alone made another cluster. These results suggested that the Khulna population diverged morphologically and genetically from the other two populations probably due to its geographical distance. Further extensive study will be necessary for elucidating the whole aspects of differentiation in *H. tigerinus* from Bangladesh.

Key words : Morphology, Allozyme analysis, Geographical distance, *H. tigerinus*

INTRODUCTION

Bangladesh is enriched with aquatic biodiversity (DoF, 2007), and contains a number of diverse ecosystems supporting roughly 113 species of mammals, 628 species of birds, 126 species of reptiles, 22 species of amphibians, 708 species of freshwater and marine fish, about 400 species of mollusks, and 140 species of bees and wasps, but many of them are on the nation's list of threatened animals (Reza, 2007). Among these amphibian species, *Hoplobatrachus tigerinus* (previously called it *Rana tigerina*) was an important group, and it is available in many Asian countries such as India, Pakistan, Nepal, Bhutan, Myanmar,

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Thailand, Malaysia and Indonesia (Frost, 2007). Once *H. tigerinus* species was available in the diversified water bodies of Bangladesh. Its leg is an important delicious food item to many people especially tribal people in Europe and Asia. Some people of Bangladesh used to catch and export frog legs to abroad, but indiscriminate harvesting of frogs had brought a bad impact on the natural ecosystem which pursued the government of Bangladesh to impose a ban on this frog collection, harvesting and processing. It plays an important role in controlling ecosystem, so, it is important to protect the species. Thus, morphological and genetic variation of different natural stocks of the population must be studied. In India, the adverse impact of uncontrolled harvesting of frogs, specifically *H. tigerinus*, from rice-fields and wet-lands, where they protect crops by devouring damaging insects, led to a ban on collecting frogs during the breeding season (Husain and Rahman, 1978).

Research work on frogs of Bangladesh has started in last decade (Islam, 2000 ; Khan *et al.*, 2002; Islam *et al.*, 2008a, b; Alam *et al.*, 2008). The culture, breeding and genetics of frogs have been observed in many frog species. Using allozyme technique, many studies on anuran distributed in Japan and adjacent countries have been reported by Nishioka and her collaborators. The genetic differences in *R. limnocharis* (= *F. limnocharis*) (Nishioka and Sumida, 1990), *Hyla japonica* (Nishioka *et al.*, 1990), *R. japonica* (Sumida and Nishioka, 1994), *R. nigromaculata* and *R. brevipoda* (Nishioka *et al.*, 1992), and *R. rugosa* (Nishioka *et al.*, 1993) have been reported by allozyme techniques. The purpose of this study was to identify the morphological and genetic variation of this particular amphibian species (*H. tigerinus*) in three regions viz. Khulna (southern part), Char Nilokhia and Netrokona (central part) of Bangladesh by allozyme analysis.

MATERIALS AND METHODS

Live frogs of *H. tigerinus* were collected from three different geographical localities, i.e. Khulna, Char Nilokhia and Netrokona during the months of March to May, 2007. A total of 10 specimens were collected from each locality. The distance between Khulna and Char Nilokhia was 200 km, whereas Netrokona and Char Nilokhia was only 30 km. The muscle and liver samples were taken from each individual and stored at -21°C until use. Details of the sampling localities, number of specimen and date of collection are given in Table 1.

Table 1. Collecting locality, sample size and date of collection of three populations of *H. tigerinus*

Population No.	Locality	No. of individual	Date of collection
1	Khulna	10	March 13, 2007
2	Char Nilokhia	10	April 14, 2007
3	Netrokona	10	May 12, 2007

The morphometric data were recorded using normal centimeter scale, divider and forceps, recorded to the nearest centimeter. The morphometric characters viz., snout-vent length (SVL), hind limb length (HLL), head length (HL), head width (HW), thigh length (THIGHL), tibia length (TL), internarial space (IN), inter orbital distance (IOD), eye length (EL), tympanum diameter (TD), snout length (SL), distance from nostril to tip of snout (NS), distance from back of mandible to back of eye (MFE), distance from back of mandible to front of eye (MBE), snout tympanum length (STL), mouth angle snout length (MSL), nostril tympanum length (NTL), distance from front of eyes to nostril (EN), tympanum eye length (TEL), distance from back of mandible to nostril (MN), maximum width of upper eyelid (UEW), hand length (HAL), forelimb length (FLL), lower arm length (LAL), foot length (FOL), length of tarsus and foot (TFOL), third finger length (3FL), first finger length (1FL), fourth toe length (4TL), inner metatarsal tubercle length (IMTL) and inner toe length (ITL). The thirty-one morphometric characters were measured following the conventional methods described by Hubbs and Lagler (1958) with the help of a slide caliper.

In this experiment, horizontal starch gel electrophoresis was done following the methods by Shaw and Prasad (1970). Electrophoresis was conducted using amine-citrate buffer (C.A. 6) (Clayton and Tretaik, 1972). After electrophoresis, the gel slices (about 1 mm thickness) were histochemically stained for different enzymes as described in Aebersold *et al.* (1987). Non-parametric statistical analyses were used in all the comparisons (Zar, 1996). Differences in morphometric characters among populations were analyzed using the Kruskal-Wallis non-parametric analysis of variance (ANOVA). For instances, where significant differences between populations were detected, a non-parametric post hoc test (Zar, 1996) was conducted. 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 individual, mean proportion 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*) were examined according to Nei and Roychoudhury (1973). Genetic distance (*D*) value was calculated using Nei's formula (Nei, 1972).

RESULTS

Thirty one morphometric characters recorded from the samples of three populations of *H. tigerinus* are shown in Table 2. Average values of morphometric characters showed slight variation among three populations. Different proportions of morphometric characteristics (SVL : HLL, HL : HW, THIGH : TL, IN : IOD, EL : TD, HL : SL, IN : NS and MFE : MBE) of *H. tigerinus* are given in Table 3. The proportions of seven morphometric characters (HL : HW, THIGH : TL, IN : IOD, EL : TD, HL : SL, IN : NS and MFE : MBE) of all populations of *H. tigerinus* were significantly different (p<0.05) from each other. The proportion of head length-head width (HL : HW), eye length-tympanum diameter (EL :

TD), head length-snout length (HL : SL), internarial space-interorbital distance (IN : IOD) and internarial space-distance from nostril to tip of snout (IN : NS) of the Khulna population was significantly higher (p<0.05) than those of the rest two populations. Thigh length-tibia length (THIGH : TL) of the Char Nilokhia population was higher than those of the other populations.

Table 2. Mean measurement values of thirty-one morphometric characters in three populations of *H. tigerinus*. *n*=10 for each population.

Morphometric character	Khulna (Mean ± SD)	Char Nilokhia (Mean ± SD)	Netrokona (Mean ± SD)
SVL	10.59 ± 1.47	12.33 ± 0.79	11.55 ± 1.35
HL	4.68 ± 1.82	4.44 ± 0.26	4.26 ± 0.29
HW	4.14 ± 0.48	4.24 ± 0.58	4.53 ± 0.32
STL	2.81 ± 0.14	3.26 ± 0.21	3.00 ± 0.00
MSL	4.22 ± 1.95	3.76 ± 0.79	3.68 ± 0.11
NS	0.52 ± 0.11	0.72 ± 0.05	0.67 ± 0.05
SL	1.61 ± 0.36	1.94 ± 0.12	1.72 ± 0.09
NTL	1.75 ± 1.05	2.94 ± 0.25	2.73 ± 0.52
EN	0.96 ± 0.06	1.15 ± 0.16	1.01 ± 0.04
TEL	0.51 ± 0.05	0.52 ± 0.08	0.50 ± 0.00
MN	2.92 ± 1.06	3.65 ± 0.29	3.81 ± 0.33
MFE	2.67 ± 0.26	2.95 ± 0.15	2.96 ± 0.08
MBE	2.21 ± 0.22	2.08 ± 0.19	2.05 ± 0.08
IN	0.59 ± 0.05	0.52 ± 0.13	0.68 ± 0.04
EL	0.99 ± 0.03	1.02 ± 0.07	1.01 ± 0.04
IOD	0.63 ± 0.15	0.66 ± 0.14	1.05 ± 0.08
UEW	0.48 ± 0.31	0.67 ± 0.04	0.52 ± 0.04
HAL	2.03 ± 0.09	2.95 ± 0.94	2.18 ± 0.17
FLL	2.05 ± 0.15	2.59 ± 0.29	2.05 ± 0.15
LAL	2.42 ± 0.16	2.67 ± 0.24	2.28 ± 0.12
HLL	15.52 ± 2.80	18.41 ± 0.83	17.26 ± 1.43
THIGH	4.28 ± 0.78	5.22 ± 0.28	4.38 ± 0.44
TL	5.23 ± 0.61	6.06 ± 0.36	5.46 ± 0.44
TFOL	7.21 ± 0.46	8.03 ± 0.06	8.16 ± 0.51
3FL	1.10 ± 0.20	1.48 ± 0.12	1.35 ± 0.22
1FL	0.61 ± 0.16	1.00 ± 0.04	0.90 ± 0.22
4TL	3.13 ± 1.05	4.06 ± 0.67	4.00 ± 0.17
IMTL	0.50 ± 0.00	0.49 ± 0.02	0.50 ± 0.06
FOL	00	00	00

Table 3. Morphometric proportions in three populations of *H. tigerinus.* Values of the parameter in each column with different superscripts (a, b & c) differed significantly (p<0.05)

Population	SVL:HLL	HL:HW	THIGH:TL	IN:IOD	EL:TD	HL:SL	IN:NS	MFE:MBE
Char Nilokhia	0.670±0.01[a]	1.047±0.01[b]	0.861±0.01[a]	0.788±0.01[b]	1.109±0.01[c]	2.289±0.01[c]	0.722±0.01[c]	1.418±0.01[b]
Netrokona	0.669±0.01[a]	0.940±0.01[c]	0.802±0.01[b]	0.648±0.00[c]	1.247±0.01[b]	2.480±0.01[b]	1.010±0.01[b]	1.444±0.01[a]
Khulna	0.682±0.01[a]	1.130±0.01[a]	0.818±0.01[b]	0.937±0.00[a]	1.800±0.01[a]	2.900±0.01[a]	1.130±0.01[a]	1.208±0.01[c]

The enzyme analyzed, E.C. number, abbreviation of enzyme and the buffer system used for horizontal starch-gel electrophoresis are shown in Table 4. Allele frequencies were calculated directly from genotypes at 5 loci for each population. Among the five loci observed, three loci (*Gpi-2*, *Mdh-1*, and *Pgm*) were polymorphic, and the *Ldh-1* and *Ldh-2* loci were invariant. The *Gpi-2*, *Mdh-1* and *Pgm* loci showed allelic frequencies of $a = 0.250$ and $b = 0.750$, $a = 0.900$ and $b = 0.100$, and $a = 0.850$ and $b = 0.150$ for the Khulna population, $a = 0.700$ and $b = 0.300$, $a = 1.000$, $a = 0.800$ and $b = 0.200$ for the Char Nilokhin population, and $a = 1.000$, $a = 0.800$ and $b = 0.200$, and $a = 0.650$ and $b = 0.350$ for the Netrokona population, respectively (Table 5, Figs. 1 and 2). The chi-square test was done in all the cases of polymorphic loci between observed genotypes and expected genotypes, based on Hardy-Weinberg equilibrium. In case of three populations of *H. tigerinus*, the *Gpi-2* locus was shown to be significant ($p \le 0.01$) in fitness test of Hardy-Weinberg equilibrium. The observed number of alleles ($Na = 1.60$), the proportion of heterozygous loci per population (14.0%) and the proportion of polymorphic loci per population (60.0%) were higher in the Khulna population than those of the other two populations (Table 6). The fixation index (F_{ST}) and gene flow (N_m) are given in Table 7. The fixation index (F_{ST}) and the gene flow (N_m) overall three populations are 0.219 and 0.889, respectively. In the pair-wise analysis, the highest F_{ST} value and the lowest N_m value were estimated to be 0.279 and 0.647, respectively, between the Khulna and Netrokona populations (Table 7).

Table 4. Enzyme and buffer system used for allozyme analysis

Enzyme (Abbreviation)	Locus	Enzyme pattern	E.C. Number	Buffer system*
Glucose-6-phosphate isomerase (GPI)	*Gpi-2*	Dimer	5.3.1.9	C.A. 6
Lactate dehydrogenase (LDH)	*Ldh-1*	Tetramer	1.1.1.27	C.A. 6
	Ldh-2			
Malate dehydrogenase (MDH)	*Mdh-1*	Dimer	1.1.1.37	C.A. 6
Phosphoglucomutase (PGM)	*Pgm*	Monomer	5.4.2.2	C.A. 6

* C.A. 6 represents the amine-citrate pH 6 buffer

Table 5. Allele frequency at five presumptive loci of three *H. tigerinus* populations

Locus	Allele	Allele frequency		
		Khulna	Char Nilokhia	Netrokona
Gpi-2	*a*	0.250	0.700	1.000
	b	0.750	0.300	-
P		**0.009***	**0.001***	**-**
Ldh-1	*a*	1.000	1.000	1.000
Ldh-2	*a*	1.000	1.000	1.000
Mdh-1	*a*	0.900	1.000	0.800
	b	0.100	-	0.200
P		**0.808**[NS]	**-**	**0.146** [NS]
Pgm	*a*	0.850	0.800	0.650
	b	0.150	0.200	0.350
P		**0.655**[NS]	**0.500**[NS]	**0.880**[NS]

p : Probability of Chi-square value. * Significant level : $p \le 0.01$.   [NS] Non-significant $p \le 0.1$

Table 6. Genetic variabilities at 5 loci of three *H. tigerinus* populations

Population	Mean proportion of polymorphic loci * (%)	Mean number of alleles per locus (*Na*)	Mean proportion of heterozygous loci per individual (%)	Heterozygosity		
				H_o	H_e	H_o/H_e
Khulna	60.0	1.60	14.0	0.12	0.17	0.71
Netrokona	40.0	1.40	12.0	0.08	0.16	0.52
Char Nilokhia	40.0	1.40	14.0	0.140	0.16	0.88
Average	46.7	1.47	13.3	0.11	0.16	0.67

* $p \le 0.95$

Table 7. Fixation index (F_{ST}) and gene flow (N_m) in three *H. tigerinus* populations

Pairwise	F_{ST}	N_m*
Khulna-Char Nilokhia	0.122	1.802
Char Nilokhia-Netrokona	0.092	2.484
Khulna-Netrokona	0.279	0. 647
Overall	0.219	0.890

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

Table 8. Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) estimated among three *H. tigerinus* populations based on 5 loci

Population	Khulna	Char Nilokhia	Netrokona
Khulna	—	0.94	0.854
Char Nilokhia	0.052	—	0.964
Netrokona	0.157	0.036	—

Mdh-1*: *aa *aa *ab *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *ab *ab *aa *bb
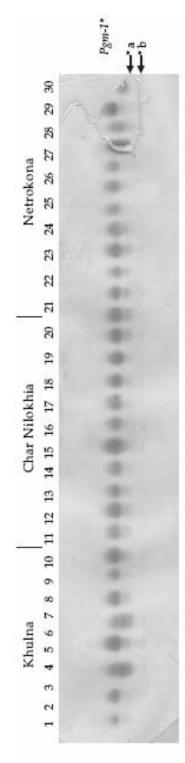
Fig. 1. Electrophoregram of enzyme malate dehydrogenase (MDH) of *Mdh-1** locus in three populations of *H. tigerinus.*

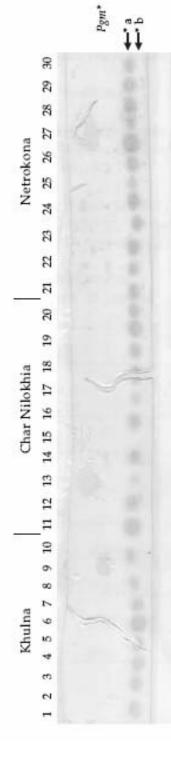*Pgm**: *aa *aa *aa *ab *aa *aa *ab *ab *ab *ab *aa *aa *aa *aa *aa *bb *aa *aa *aa *bb *ab *ab *ab

Fig. 2. Electrophoregram of enzyme (PGM) of *Pgm** locus in three populations of *H. tigerinus.*

Fig. 3. Nei's (1972) UPGMA dendrogram showing the genetic distance (*D*) among
three populations of *H. tigerinus*

The genetic distance (*D*) (Nei, 1972) varied from 0.036 to 0.157 among three populations.
The minimum value (0.036) was observed between the Char Nilokhia and Netrokona
populations, whereas the highest value (0.157) was observed between the Khulna and
Netrokona populations (Table 8, Fig. 3).

DISCUSSION

Mean snout-vent length of the species recorded as 12.33 cm in the Char Nilokhia
population, 11.55±1.35 cm in the Netrokona population, and 10.59±1.47 cm in the Khulna
population may be explained as the environmental effects. These may be due to the
influence of photoperiod, day-length and feeding schedule on tadpole growth and
development (Wright *et al.*, 1988). The longest head length was in the Khulna population
with the value of 4.68±1.82 cm, whereas the shortest was 4.26±0.29 cm in the Netrokona
population. Notwithstanding, these deviations were found due to the anthropological
and other genetic factors affected on their growth, which was supported by Hirata *et al.*
(1991). Bruneau and Magnin (1980) observed that *Rana catesbeiana* might attain a length of
10.50 cm and its growth depends on the temperature, availability of food and fecundity.

Among five presumptive loci, only three loci (*Gpi-2*, *Mdh-1* and *Pgm*) were found to be
polymorphic in all populations. The polymorphic loci for all populations had two alleles
(*a* and *b*). Nishioka *et al.* (1993) found that the mean proportion of polymorphic loci in 40
populations of *R. rugosa* was 31.1%, which was much lower than the value (46.7%)
obtained in the present study. The *H. tigerinus* populations clearly showed a higher level
of polymorphism than the above-mentioned *R. rugosa* populations. The mean number of
alleles per locus ranged from 1.40 to 1.60 (average 1.47), which was a little bit higher than
the value (1.42) obtained by Nishioka *et al.* (1993) in *R. rugosa*. In contrast, this value
(1.47) was lower than that (2.00) obtained by Nishioka *et al.* (1987) in three genera
Buergeria, Rhacophorus and *Polypedates,* and by Khan *et al.* (2002) in *H. tigerinus*. These
variations might be due to different sampling size and the difference in the number of
loci observed. The mean proportion of heterozygous loci per individual ranged from
12.0% to 14.0% (average 13.3%), which was similar to those obtained by Sumida and
Nishikoka (1996). The mean proportion of polymorphic loci of *R. ornativentris* and *R.*

rugosa were 14.4% and 11.3%, respectively. The observed heterozygosity (H_o) ranged from 0.08 to 0.14 (average 0.11) was higher than that (0.10) reported by Raginski and Babik (2000) in *R. arvalis.* The expected heterozygosity (H_e) was found to be 0.16 in the present study, which was similar to those obtained by Rafiniski and Babik (2000) and Khan *et al.* (2002). However, the present value was much higher than that obtained by Formas and Breva (2000). The level of heterozygosity was related with the size of populations within a species, although higher heterozygosity (H_o) value is necessary for breeding program. The highest observed and expected heterozygosities (H_o = 0.14 and H_e = 0.17) were observed in the Char Nilokhia population and the Khulna population, respectively.

The co-efficient of gene differentiation (F_{ST}) in all three *H. tigerinus* populations were examined (Nei, 1972) and F_{ST} was found to be 0.219, which indicated that the population contained slight genetic differentiation, and that the number of migrated individuals from one population to another was high (N_m = 0.890). The overall F_{ST} value (0.219) obtained in *H. tigerinus* populations was lower than that (0.306) obtained in *R. ornativentris* populations (Sumida and Nishioka, 1996). The observed genetic distance between the Khulna population and the other populations might be due to the geographical distances. Based on Nei's (1972) genetic distance (*D*-value), the UPGMA dendrogram showed that the three populations could be grouped into two clusters as shown in the dendrogram (Fig. 3). First cluster contained the Khulna population alone, and separated from second one by *D* = 0.157, whereas the second cluster comprised of the Char Nilokhia and Netrokona populations. In the second cluster, the Char Nilokhia population differentiated from the Netrokona population by *D* = 0.036. Islam *et al.* (2008a, b) reported that the mean genetic distance between the medium and small types of the *Fejervarya limnocharis* complex was 0.782, and suggested that these two types are distinct species. The genetic distance (0.157) observed between two clusters (the Khulna population and the Char Nilokhia and Netrokona populations**)** was smaller than that between two types of the *Fejervary limnocharis* complex mentioned above. Among the *Dicroglossus* species from the Mediterranean area, genetic distances were reported to be 0.17 - 1.04 (Zangari *et al.*, 2006). These differentiations might be due to different species or geographical variations. The genetic distance (*D*) ranged from 0.036 to 0.157 among the three populations of *H. tigerinus.* The minimum genetic distance (0.036) was observed between the Char Nilokhia and Netrokona populations, while the maximum value (0.157) was found between the Khulna and Netrokona populations (Table 8). Nei (1972) stated that values of *D* for inter-species comparison, sub-species and local races are 1.0, 0.1 and 0.01, respectively, while Ayala (1975) proposed that the *D* value for sub-species is 0.20. The present findings assumed that *H. tigerinus* populations might be under local races. Extensive genetic divergence might establish reproductive isolation that reflects further accumulation of genetic differentiation at many loci over a long period of time (Ferguson, 2002; Sites and Marshall, 2003, 2004). However, further extensive research works on genetic variability of the frog populations of many localities will be necessary for elucidating the whole aspects of differentiation of *H. tigerinus* in Bangladesh using different molecular markers i.e. RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), microsatellite DNA markers etc.

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