

## Enzyme polymorphism in Indian freshwater soft shell turtle *Lissemys punctata*

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**Abstract.** Genetic diversity among four geographically isolated populations of an Indian freshwater turtle, *Lissemys punctata*, was studied using seven metabolically important isozyme/allozymes as genetic markers. A total of twenty-three alleles at fourteen protein-coding loci were identified, six of these loci were monomorphic and the remaining eight were polymorphic. The result suggests that the geographic populations of *L. punctata* sampled can be characterized by a total genetic diversity of 0.180 (mean *HT*) with an average of 1.64 alleles per locus. The average proportion of polymorphic loci per population was estimated to be 1.75. The UPGMA tree of genetic relationship indicated significant differentiation among populations. The results also showed that the geographical and genetic distances are not correlated in these populations of *L. punctata*.

**Keywords.** Isozyme, Allozyme, Genetic diversity, UPGMA, *Lissemys punctata*.

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### INTRODUCTION

Protein based markers, such as isozyme and allozyme, are the potential tools for indirect assessment of genetic diversity in natural populations (Lewontin and Hubby, 1966). The information obtained from them can serve as probes to detect past, present and possibly future population declines (status), the level of inbreeding and their evolutionary history. Natural populations of threatened species are managed to conserve genetic diversity, enhance individual fitness and maintain the evolutionary potential for future adaptations (Awise and Saunders, 1984; Verspoor and Hammart, 1991; Carmichael et al., 1992; Parham et al., 2001). The amount and distribution of genetic variations within and between populations can provide relatively quick and indirect estimate of migration, population subdivisions, and isolation, which can define the appropriate scale for short- and long-term management of wild species in their natural habitat. The estimator most frequently uses the amount of heterozygosity to measure the population-wide genetic diversity. Individual heterozygosity describes the proportion of heterozygous loci within the genome of a single individual whereas the average heterozygosity reflects the proportion of heterozygous individuals within a population measured across several loci (Hartl and Clark, 1997). It is

commonly thought that populations that are declining as a consequence of human harvesting and habitat destruction suffer, from low level of genetic variations, which could inhibit their ability to adapt to changing environmental conditions (Sattler and Ries, 1995). In addition, studies have shown that single (Nei et al., 1975) and multiple population bottlenecks (Motro and Thomson, 1982) can significantly decrease genetic heterozygosity values, and populations that have passed through bottlenecks have shown greatly reduced levels of genetic variations (Bonnell and Selander, 1974; O'Brien et al., 1985). However, some species that have been close to extinction did not show reduced genetic variations (Dinerstein and McCracken, 1990), while in other species, such as bandicoot, populations that showed reduced variability have not passed through bottlenecks (Sherwin et al., 1991).

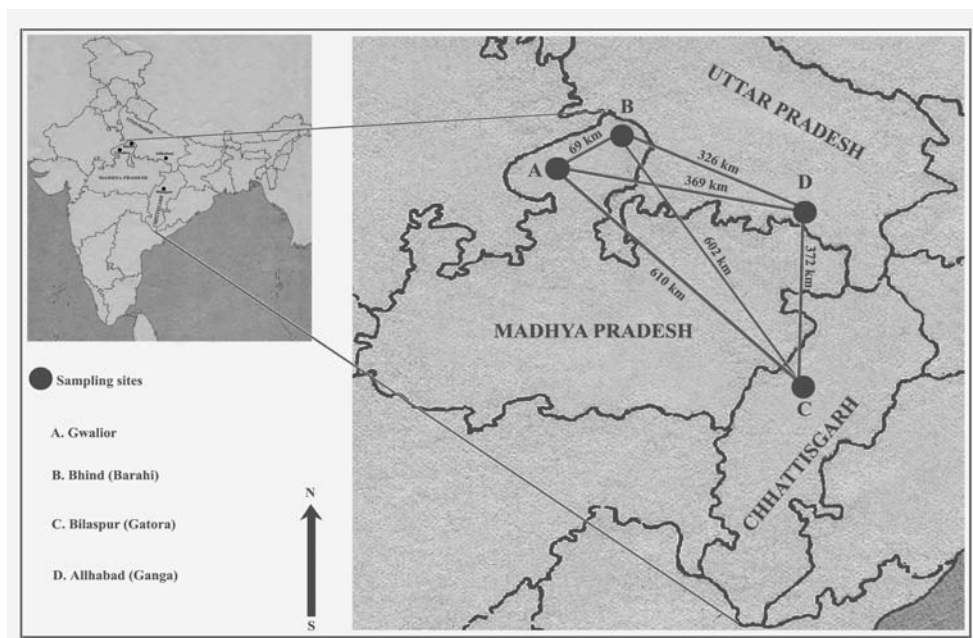
Genetic studies can increase knowledge about the factors that may affect biodiversity, specifically in relation to the loss of genetic diversity and the implications this could have on the persistence of species in their natural habitat. The maintenance of genetic diversity is important because it represents the evolutionary potential of a species (Frankham, 1995).

To our knowledge, there are very few studies in chelonian, which have investigated the relationship between population structures, sex, age, geographical localities and species-specific heterozygosity values (but see, Smith et al., 1977; Seidel and Lucchino, 1981; Nevo et al., 1984; Bonhomme et al., 1987; Georges and Adams, 1992). In addition, multi-locus studies in turtles carried out earlier also used electrophoretic techniques but without estimating heterozygosity (Vogt and McCoy, 1980; Seidel and Lucchino, 1981; Sites et al., 1981; Derr et al., 1987; Seidel and Adkins, 1987). The present study was directed to understand the genetic characteristics of four geographically isolated populations of *Lissemys punctata* inhabiting different rivers and wetlands of Northern and Central India. In this study we used the polyacrylamide gel electrophoretic method to estimate the level of heterozygosity in seven metabolically important isozymes/allozymes and compared them with other chelonian species populations as an evidence of genetic divergence or the level of heterozygosity, which may be expected for the geographically distinct natural populations.

## MATERIALS AND METHODS

### *Geographical localities and sampling*

Adult individuals of freshwater soft shell turtle *L. punctata* were collected from various aquatic bodies distant to four geographical localities in India, viz., Gwalior and Chambal (Madhya Pradesh), Bilaspur (Chhattisgarh) and Allahabad (Uttar Pradesh), during a period of five years (2002-2007). Geographical distances between population sites were taken as Euclidean distance and calculated in Google Earth (Version 4.3.7284.3916 beta) using the line function (Fig. 1). In Gwalior region (26°13'31"N, 78°11'25"E), adult turtles were obtained from the local fish markets, collected from nearby water bodies (n = 30). Tissues from the turtles sacrificed in the fish markets were also collected for isoform studies. From Chambal River (26°41'39"N, 78°56'24"E), turtles (n = 30) were collected in a 5 km stretch near Barhai, District Bhind. From Chhattisgarh State, adult individuals (n = 30) were collected from local fish markets in Bilaspur (22°03'13"N, 81°13'35"E). From Ganga River (25°31'21"N, 81°39'40"E), a total of 30 individuals were obtained from the local fishermen at Niva village (Mallahi Tolla) near Allahabad, Uttar Pradesh. About 1 ml blood was collected from the femo-



**Fig. 1.** Geographic locations and distance between the sampling sites in India.

ral vein of each individual (Rohilla et al., 2006, Rohilla and Tiwari, 2008). All the individuals used for blood collection were active and were left to their respective habitats after blood collection.

#### *Enzyme extraction and electrophoresis*

Total protein was extracted from RBCs as described by Brewer (1970). For the study of tissue-specific isoforms, approximately 100 mg tissue was homogenized in 500  $\mu$ l ice-cold extraction buffer (50mM Tris-HCl, pH 7.2, 50% Sucrose and 10mM EDTA) in a microfuge tube using a sterilized glass pestle. The homogenate was centrifuged at 10000 rpm for 30 minutes and the middle layer was transferred to a separate microfuge tube. The middle layer was again centrifuged at 10000 rpm for 30 minutes and finally the supernatant was collected. Protein estimation was done by dye binding assay method (Bradford, 1976). Equal amount of protein samples were loaded on native PAGE in the form of a continuous vertical slab gel (5-7% Acrylamide) and electrophoresed at 140 V in 1X TBE at 4°C to resolve all the isoforms of an enzyme for complete visualization. After electrophoresis the gel was transferred to enzyme staining recipes (Shaw and Prasad, 1970).

#### *Statistical analysis*

Allele frequencies at each locus in the four populations of *L. punctata* were calculated by a simple genotyping method assuming that a protein with the same mobility is controlled by the same allele in different populations and that variations within species were controlled by two or more co-dominant alleles (Buth, 1990; Morizot and Schmidt, 1990). Allelic variants were designated accord-

ing to their relative mobilities. The most common allele was designated as 100 and the other allele given numbers that indicated their mobility relative to that of the common allele. Allele frequency was calculated using the formula:

$$\text{Allele frequency} = (2Ho + He)/2N$$

Where 'Ho' is the observed number of homozygotes for an allele at a given locus, 'He' is the number of observed heterozygotes, and 'N', is the number of individuals examined (Singh and Sharma, 1997). The observed individual heterozygosity/locus was calculated using the formula:

$$H = He/N$$

Where 'He', is the number of heterozygotes in a particular population and 'N' is the number of individuals examined (Singh and Sharma, 1997). The percent polymorphism was calculated as given by Singh and Sharma (1997). The genetic variations among populations were measured by Nei's (1972) method. The total genetic diversity, *DST*, was calculated by the formula:

$$DST = \text{total genetic diversity (HT)} - \text{Diversity within population (Hs)}$$

The Nei's coefficient of genetic diversity ( $G_{ST}$ ) was calculated using the formula of Nei (1973), as  $G_{ST} = DST/HT$ . The Nei's genetic identity (*I*) and genetic distance (*D*) were calculated as described by Nei (1972, 1973, 1978). The matrix of distances was converted into a dendrogram using the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

## RESULTS

### *Isozyme and allozyme polymorphisms at different enzyme coding loci*

Specific staining of various enzymes in five turtle tissues, viz., liver, heart, kidney, muscle, RBCs and plasma protein, isolated from 5-10 adult individuals, were carried out to visualize the tissue-specific isoforms and for selection of enzymes for further studies. To avoid animal killing, only RBCs and plasma proteins were used for detailed population-wide genetic analysis of the four geographic populations of *L. punctata*.

#### (A) Esterase (Est)

The pattern of esterase bands were visualized on the polyacrylamide gels (7%) in all tested tissues viz. muscle, liver, kidney, heart, RBCs and blood plasma. Esterase activity was not observed in RBCs. All other examined tissues showed almost similar pattern. A total of three bands, representing esterase activity, were observed. These bands were divided into two zones of activity, which could be related to two enzyme-coding loci, designated as Est-1 and Est-2. Esterase is a monomeric enzyme, made up of single polypeptide chain. Hence, in diploid organisms, co-dominant alleles code for two different characters (i.e., two allelomorphs). In turtle populations, the first zone of activity corresponds to Est-1 locus. A distinct band of enzyme was observed in the individuals of all four populations. This allele was designated as *Est-1\*100* and was monomorphic. The second locus Est-2

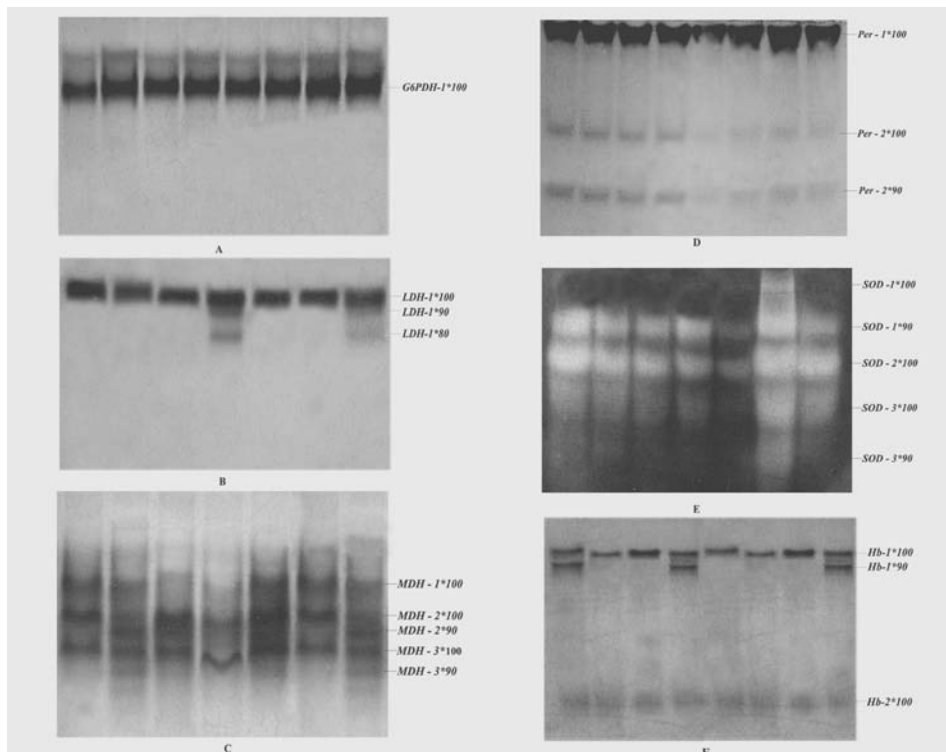
showed two zones of activity or a total of two bands, both were present in heterozygous individuals and designated as *Est-2\*100* and *Est-2\*90*. In homozygous individual only one band was observed, being either *Est-2\*100* or *Est-2\*90*.

(B) Glucose-6-phosphate dehydrogenase (G6PDH)

One broad band of activity was observed in all the tissues except blood plasma. High activity was observed in liver, heart, RBCs (blood) and kidney tissues, while it was found low in muscle sample. No allelic variation was observed at this locus, and was designated as *G6PDH-1\*100*. Thus, the enzyme (G6PDH) exhibited monomorphism (Fig. 2A).

(C) Lactate dehydrogenase (LDH)

The lactate dehydrogenase polymorphism was recorded on 6% polyacrylamide gel. A total of three bands were visualized in all the tissues and plasma of heterozygous individuals. Only one broad band was observed in homozygous individuals. The different allozymes



**Fig. 2.** Electrophoretic patterns of isozyme and allozyme in RBC samples of freshwater turtle *L. punctata* (n = 7/8). (A) Glucose-6 phosphate dehydrogenase (monomorphic), (B) Lactate dehydrogenase (polymorphic), (C) Malate dehydrogenase (polymorphic), (D) Peroxidase (monomorphic), (E) Superoxide dismutase (polymorphic) and (F) Hemoglobin (polymorphic).

of LDH, encoded by LDH-1 locus (homomeric), were designated as *LDH-1\*100*, *LDH-1\*90* and *LDH-1\*80*. High activity of LDH was visualized in heart, muscles, and RBCs samples but it was found low in liver, kidney and plasma (Fig. 2B).

#### (D) Malate dehydrogenase (MDH)

A total of five bands of MDH isozyme were observed, which could be divided into three zones based on their patterns of appearance on polyacrylamide gel. The first zone consisted only of a single band, observed in both heterozygote and homozygote individuals, encoded by MDH-1 locus and designated as *MDH-1\*100*, showed monomorphism. Other two loci, MDH-2 and MDH-3 were found polymorphic due to their co-dominant patterns in individuals of the four populations (Fig. 2C). At MDH-2 locus, two bands were observed in the second zone of activity, designated as *MDH-2\*100* and *MDH-2\*90* in heterozygotes and single band in the homozygote individuals. Similarly, MDH-3 locus encoded two bands *MDH-3\*100* and *MDH-3\*90*. In plasma, only one isoform, *MDH-1\*100*, could be recorded. Compared to other tissues, *MDH 1\*100* showed high activity in liver kidney and RBCs.

#### (E) Peroxidase (Per)

The pattern of Peroxidase enzyme was visualized on 7% polyacrylamide gel in all the tissues tested, viz., muscle, liver, kidney, heart RBC and blood plasma. A total of three bands, representing peroxidase activity were observed. These bands were divided into two zones of activity on the gel, which could be related to two enzyme-encoding loci. These loci were designated as Per-1 and Per-2 (Fig. 2D). The three isoforms of peroxidase encoded by two different loci were recorded with high activity in liver, heart, kidney, muscle and RBCs, but the blood plasma was found deficient for them. In turtle populations, the first zone of activity corresponding to the locus Per-1 revealed only one band, *Per-1\*100*. The Per-2 loci, showed two bands for the enzyme in all the individuals of the four geographic populations and were designated as *Per-2\*100* and *Per-2\*90*. Both the loci were found monomorphic due to absence of allelic variants in all the individuals of the four geographic populations.

#### (F) Superoxide dismutase (SOD)

The Superoxide dismutase was observed (on 6% polyacrylamide) polymorphic in the RBCs of all the individuals of the four geographic populations. A total of three loci SOD-1, SOD-2 and SOD-3 encoding five bands in heterozygotes were observed in three distinct zones of activity, and designated as *SOD-1\*100*, *SOD-2\*100*, *SOD-2\*90*, *SOD-3\*100* and *SOD-3\*90* (Fig. 2E). SOD-1 and SOD-3 showed polymorphism, while SOD-2 locus encoded only one band, both in homozygotes and heterozygotes.

#### (G) Hemoglobin (Hb)

The pattern of hemoglobin bands was also studied on polyacrylamide gels (5%) in all the five tissues (i.e., liver, kidney muscle, heart and RBCs). The highest activity of this protein was observed in RBC samples but was found poor in liver, muscle, heart and kidney.

A total of three bands, representing hemoglobin activity were observed. These bands were divided into two zones of activity on polyacrylamide gel, Hb-1 and Hb-2. Hb-1 locus, consisted of two bands, identified as the product of two alleles *Hb-1\*100* and *Hb-1\*90*. Hb-1 locus was observed to be polymorphic due to co-dominant pattern. The locus Hb-2 shared only one faint broad band and was observed in all the individuals of the four populations and designated as *Hb-2\*100*. It was, therefore, characterized as monomorphic in *L. punctata* (Fig. 2F).

#### *Genetic variations within and among the populations*

The genetic diversity within populations was estimated on the basis of the following parameters: (a) percentage of polymorphism, (b) average number of alleles per locus, (c) average heterozygosity/locus, (d) individual heterozygosity/locus and (e) observed heterozygosity/ locus. Values of these parameters of all four populations of *L. punctata* are given in Table 1. Allelic frequencies became the basis of all the above parameters, which were calculated from the patterns of protein bands observed on native polyacrylamide gels. The products of fourteen protein coding loci were detected, which provided interpretable results for population analysis. All polymorphic loci were noticed to have more than one allele. Out of fourteen protein-coding loci evaluated, only seven loci (Est-2, LDH-1, MDH-2, MDH-3, SOD-1, SOD-3, and Hb-1) were observed polymorphic. All polymorphic loci displayed allozyme-banding patterns consistent with that expected from the known quaternary structure of the proteins. Allele frequencies for polymorphic loci were calculated on the basis of gel interpretations (Fig. 3). A locus is considered to be polymorphic, if the frequency of most common allele is less than 0.999. The remaining seven loci (Est-1, G6PDH, MDH-1, SOD-2, PER-1, PER-2 and Hb-2) exhibited monomorphism as no allelic variations were recorded in them in all the four populations of *L. punctata*. Each of the four populations showed different numbers of hetero- and homozygous individuals for various enzyme-coding loci. Simultaneously, individual heterozygosity per locus for various polymorphic enzymes was also recorded separately across the four populations for comparison. The percent polymorphism observed in four populations was 50%, the average number of alleles per locus was recorded as 1.64

**Table 1.** Measures of genetic variations in populations of *L. punctata*.

Parameters of variation	Location			
	Gwalior	Chambal	Bilaspur	Allahabad
Percentage of polymorphic loci	50	50	50	50
Average proportion of polymorphic loci	1.75	1.75	1.75	1.75
Average number of alleles/locus	1.64	1.64	1.64	1.64
Observed heterozygosity/locus	0.242	0.291	0.250	0.225
Average heterozygosity/locus	0.252	0.214	0.220	0.234
Expected heterozygosity/locus	0.498	0.497	0.494	0.496

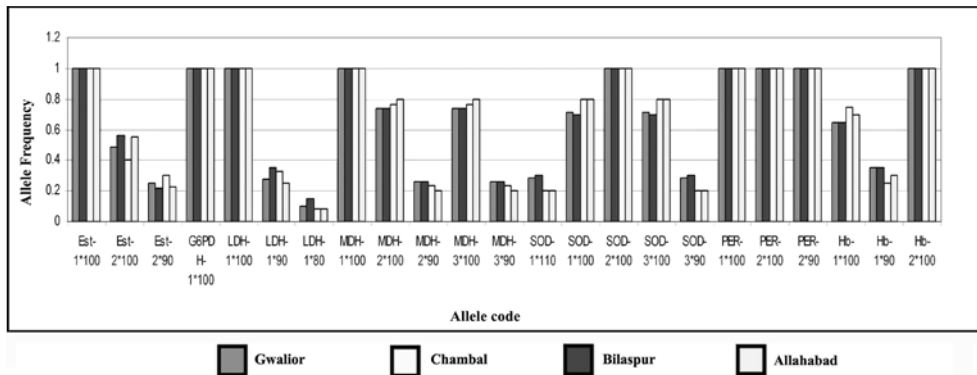


Fig. 3. Bar diagram showing distribution pattern of allele frequencies in four distantly located geographical populations of freshwater turtle *L. punctata*.

and the average proportion of polymorphic loci per population was estimated to be 1.75. The observed heterozygosity values in pond populations from Gwalior and Bilaspur were recorded as 0.242 and 0.250. Similarly, in the river populations of Chambal and Ganga, these values were 0.291 and 0.225, respectively. The average heterozygosity per locus in all the four populations was recorded as 0.252, 0.291, 0.250 and 0.225 for Gwalior, Chambal, Bilaspur and Allahabad, respectively (Table 1). The Nei's coefficient of genetic diversity ( $G_{ST}$ ) is the quotient of genetic diversity between populations ( $D_{ST}$ ) and total genetic diversity ( $H_T$ ). The Nei's coefficient of genetic diversity ( $G_{ST}$ ) at all polymorphic loci is shown in Table 2. The  $G_{ST}$  values varied from 0.228 to 0.356 under present investigation, while the  $F_{IT}$  values or index of population differentiation ( $F$ -statistics) varied from 0.42 to 1.00 for different polymorphic loci. Significant departures ( $P < 0.001$ ) from the Hardy-Weinberg expectations were encountered at seven loci.

Table 2. Nei's coefficient of genetic diversity at all polymorphic loci.

Locus	Total genetic diversity ( $H_T$ )	Average heterozygosity within populations ( $H_S$ )	Heterozygosity between populations ( $D_{ST}$ )	Nei's Coefficient ( $G_{ST}$ )
Est-2	0.748	0.499	0.249	0.332
LDH-1	0.628	0.476	0.152	0.242
MDH-2	0.774	0.498	0.276	0.356
MDH-3	0.774	0.498	0.276	0.356
SOD-1	0.757	0.570	0.187	0.247
SOD-3	0.757	0.570	0.187	0.247
Hb-1	0.605	0.467	0.138	0.228
Average value	0.720	0.587	0.209	0.286

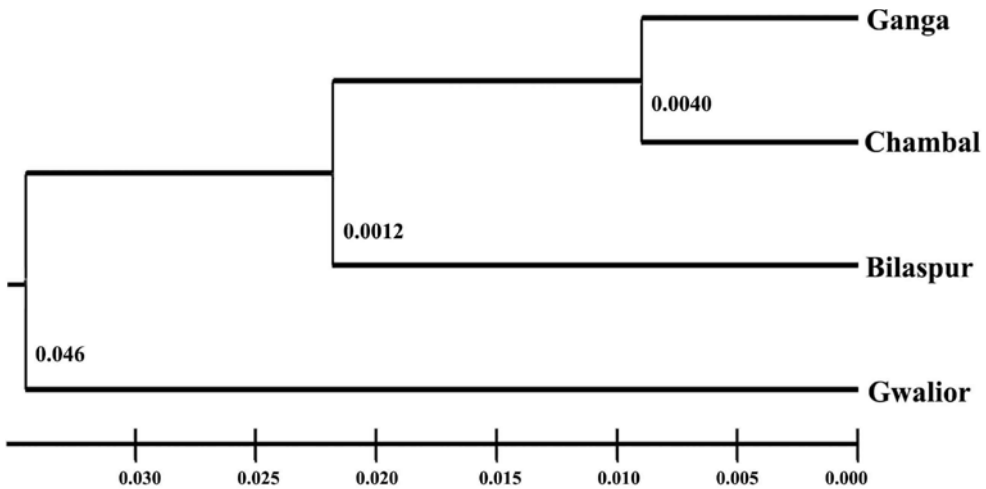


*Phylogenetic analysis*

Nei's genetic distance calculated for different pair-wise comparisons among four distantly located geographical populations of *L. punctata* showed lowest distance between Bilaspur (pond) and Ganga (River, Allahabad) populations ( $D = 0.0012$ ) and highest between Gwalior (pond) and Chambal population (River) ( $D = 0.100$ ) (Table 3). Similarly, Chambal and Ganga populations appeared more closer, while Bilaspur and Gwalior are nearest to each other (Fig. 4).

**Table 3.** Nei's genetic identity (I) (above diagonal) and genetic distance (D) (below diagonal).

Population Name	Gwalior	Chambal	Bilaspur	Allahabad
Gwalior	0.000	0.9042	0.9545	0.9386
Chambal	0.1000	0.000	0.9904	0.9960
Bilaspur	0.0466	0.0096	0.000	0.9988
Ganga	0.0633	0.0040	0.0012	0.000



**Fig. 4.** UPGMA clustering of genetic distance showing phylogenetic relationship of the four geographical populations of freshwater turtle *L. punctata* based on allozyme pattern.

DISCUSSION

The average genetic variability of individual turtles (*L. punctata*) from Gwalior, Chambal, Bilaspur and Ganga was found to be very high, similar to that observed for American freshwater turtle species *Trachemys scripta* (Nevo et al., 1984). The mean het-

erozygosity in reptiles is reported to be  $0.060 \pm 0.053$  (S.E.). The level of heterozygosity ( $H$ ) in turtles from several localities all around the world is reported to be quite variable, e.g., 0.080-0.138 in *Sternotherus odoratus* (Seidel and Lucchino, 1981), 0.019-0.022 in *Caretta caretta* and 0.000-0.135 in *Chelonia mydas* (Smith et al., 1977; Bonhomme et al., 1987). In *L. punctata*, the level of average heterozygosity observed in the present study was  $0.23 \pm 0.008$  (S.E.), suggesting that the populations of this species showing higher genetic variability are fitter to adapt to different habitats or sudden environmental changes than those showing lower variability (Gillespie and Guess, 1978; Lewontin et al., 1978; Nevo, 1983, 1988; Hedrick, 1986). Pemberton et al. (1988) and Teska et al. (1990) also, correlated the increased heterozygosity with adaptive fitness of individuals from natural populations. We assumed that presence of high heterozygosity in populations of *L. punctata* could likely be due to wide range of aquatic habitat conditions they are inhabiting (varying biotic and abiotic factors), influencing the animal differentially at various stages of its life cycle.

The heterozygosity is reported to vary with age (Tinkel and Selander, 1973; Chessser et al., 1982; Scribner et al., 1985; Al-Hassan et al., 1987), sex (Manlove et al., 1975), sex-ratio (Simanek, 1978) and or body size (Avisé and Smith, 1974; Smith and Chessser, 1981; Feder et al., 1984). Moreover, similar levels of heterogeneity within populations are normally not expected for long-lived vertebrate species, especially when high vagility and extensive interbreeding occur within the population. However, high level of genetic variations may be common within and among the populations of freshwater turtle species, *T. scripta* (Smith and Scribner, 1990) and *L. punctata* (this study). Environmental perturbations, which may promote large-scale dispersal, local selection or extreme fluctuations in population size, may prevent genetic equilibrium. Further, significant departures ( $P < 0.001$ ) from Hardy-Weinberg expectations were encountered at seven loci in *L. punctata*. A Hardy-Weinberg expectation is an important criterion for inferring the genetic nature of electrophoretic banding variants. Several factors may contribute to Hardy-Weinberg disequilibrium and the ideal Hardy-Weinberg populations actually do not exist in nature (Althukov, 1981). Estimates of genetic variability, e.g. percentage of polymorphic loci ( $P$ ) and heterozygosity ( $H$ ), are two useful parameters to employ when analyzing the genetic structure of populations. The long-term adaptability of populations, and hence species, is dependent upon sufficient amount of genetic variations, which enables them for continued adaptation to environmental and biotic challenges. As the ultimate goal of conservation is to maintain biological lineages over evolutionary time, a thorough understanding of the extent of genetic variation and diversity in populations is of critical importance in any conservation management plan. However, the reliability of percent polymorphism and heterozygosity are closely correlated with the effective population size, the degree of migration, variability of the environment and the number and choice of loci analysed (Simon and Archie, 1985; Kirpichnikov, 1992). The  $F_{IT}$  values indicated extensive genetic differentiation in the species at certain loci. These differences may have resulted from different selective forces acting on the gene pools of the species or from stochastic processes. This is the first electrophoretic study of enzymatic and non-enzymatic proteins, which assessed the genetic variations in the Indian freshwater turtle *L. punctata*. This study provides the first account of the genetic analysis of Indian freshwater turtle species forming a basis for future population genetic studies involving the species of *Lissemys* and other freshwater turtle species from India. The populations we analysed revealed that the observed high level of genetic variability may be

comparable to those of other endangered turtle species from the same or other river systems and hence, will be highly useful for better management and determination of conservation priorities based on genetic variability.

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