African Journal of Biotechnology Vol. 12(3), pp. 238-248, 16 January, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1273
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Inter-simple sequence repeat (ISSR) polymorphism-based analysis of diversity in the freshwater turtle genus *Pangshura*

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Accepted 10 October, 2012

Assessment of genetic variability among the freshwater turtles is critical to the development of effective conservation strategies. In the present study, analysis of phenetic relationship of the genus *Pangshura* was studied using inter simple sequence repeat (ISSR) markers to see the concordance of currently available morphological data with genetic data along with spatial pattern of distribution. Ten microsatellite-based primers amplified 156 ISSR markers among 36 individuals belonging to four turtle species *Pangshura sylhetensis, Pangshura tecta, Pangshura simithii* and *Pangshura tentoria*, collected from certain localities of Assam, West Bengal and Uttar Pradesh of India. Estimations of Nei's genetic diversity (h), Shannon's Index (I) and Total genetic diversity (H_t) reveal the existence of greater genetic diversity in *P. tentoria* and *P. sylhetensis* than in the other two species. However, low values of gene flow (Nm) and of within sample diversity (H_s) indicate prevalence of inbreeding in these species groups. The coefficients of differentiation (GST) values divulge that divergence started in the *Pangshura* species. Projections on the principal components analysis (PCA) plot reflect the distinct genetic identity of these four species. The consensus Neighbour Joining dendogram depicts *P. sylhetensis*, *P. tecta*, and *P. tentoria* as genetically closer to each other than to *P. smithii*.

Key words: Differentiation, genetic diversity, inter simple sequence repeat (ISSR), *Pangshura*, turtle.

INTRODUCTION

The northern region of India, enriched with wide diversity of freshwater turtles has been designated as priority area of turtle conservation (Shrestha, 2001; Buhlmann et al., 2009). The family Geoemydidae to which the genus *Pangshura* belongs comprises of many of the highly endangered species (Van Dijk et al., 2000; IUCN, 2011). Most of the Geoemydid turtles are freshwater species and occupy a wide range of habitats, from highly aquatic (*Pangshura*) to terrestrial (*Geoemyda*). The genus *Pangshura* comprises small-sized turtles, having maximum shell length of 20 to 26.5 cm. The species that come under this genus are *Pangshura tecta, Pangshura sylhetensis, Pangshura tentoria* and *Pangshura smithii* (Das, 2001). Further, these species are fragmented to sub-species like *Pangshura tentoria, Pangshura tentoria circumdata, Pangshura tentoria flaviventer, Pangshura smithii* and *Pangshura smithii pallidipes.* Recent field studies indicate the decline in numbers of these freshwater turtles in the Brahmaputra and Ganga river basins (Baruah, 2010; Baruah et al., 2010; Choudhury et al., 2000). The aim of the present study was the assessment of the genetic diversity based on the polymorphism in inter simple sequence repeat (ISSR) markers (Zietkiewicz et al., 1994) among the freshwater turtles of the genus *Pangshura* from northeast India, as information regarding morphological and genetic identification of the four species under this genus is lacking.

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Genetic data have been used in turtle conservation to evaluate the genetic variability within and among population (Janzen et al., 1997; Souza et al., 2002; Schwartz and Karl, 2005) to recognize the existence of cryptic taxa (Russello et al., 2005), and to reveal migratory patterns (Bowen and Avise, 1996). Similarly, microsatellites have been used to evaluate the genetic consequences of population bottlenecks (Kuo and Janzen, 2004; Waldick et al., 2002), population sizes, migration rates (Nichols and Freeman, 2004), natal dispersal (Berry et al., 2002; 2004), hybridization (Burns et al., 2003), diversity (Fritz et al., 2008) and also used in wildlife forensics (Avise, 2004).

DNA-based markers have gained popularity in recent years in the assessment of genetic relationship among species. Of these markers, the ISSR markers (Zietkiewicz et al., 1994) are often used in the phenetic studies (Gupta et al., 1994; Wu et al., 1994; Marmi et al., 2006; Fritz et al., 2008; Guicking et al., 2009). These markers being polymorphic (Bornet and Branchard, 2001) and ubiquitous in the genome (Tautz and Renz, 1984), have the advantages of Simple Sequence Repeat (SSR) markers, while bypassing the major obstacle to the development of SSR markers, that is the need to know the flanking sequences. Generally, the ISSRs are scored as dominant markers which are inherited in Mendelian fashion (Ratnaparkhe et al., 1998). The SSR regions scattered evenly throughout the genome (Condit and Hubbell, 1991) and yielding a large number of polymorphic bands, which are interpreted as band present or band absent (Tsumura et al., 1996). The absence of band signifies primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe et al., 1998).

Friz et al. (2005, 2008) examined the evolutionary relationships of five species of the genus *Testudo* and *Cyclemys* diversity using the data generated from the inter simple sequence repeat- polymerase chain reaction (ISSR-PCR) genomic nuclear fingerprints. Hence, the ISSR markers are suitable for use in species distinction where extensive information on DNA sequences is not readily available (Meloni et al., 2006). We report here the findings of our ISSR polymorphism-based study on the genetic diversity and phenetic relationship of all the four species and the sub-species within the Indian freshwater turtle genus *Pangshura*.

MATERIALS AND METHODS

Study area

The field work and sample collection were carried out during January, 2008 to March, 2011 in different parts of Assam (including bordering areas of Bangladesh and Bhutan), West Bengal, Uttar Pradesh of India (Table 1, Figure 1). Almost all the target species and their phenotypic variations were captured and used for the analysis of molecular data. Taxonomy and nomenclature were followed after Das (1995, 2002) and Praschag et al. (2007).

Sample collection and DNA extraction

Thirty-six (36) *Pangshura* samples belonging to four species as presented in Table 1 were sampled by clipping off a tiny piece of the webbing of toes. Tissues were preserved in 95% ethanol and stored at –20°C until DNA extraction. DNA was extracted using the method of Ausubel et al. (1995) with minor modifications and stored in TE buffer at - 20°C until use.

Inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) amplification

Polymerase chain reactions (PCRs) were performed using ten ISSR primers (Table 2), after an initial screening of 19 primers. Primers P4 to P9 were based on turtle microsatellites reported by Aggarwal et al. (2004), Edwards et al. (2003) and Schwartz et al. (2003), with some modifications. Primers P1, P2, P3 and P10 were designed and used by Chen et al. (2009) in grass carp. PCRs were carried out in 20 µL of reaction volume containing 20 ng of genomic DNA, 1.0 U *Taq polymerase*, 200 µM dNTP, 1 µM primer, and 1X amplification buffer (containing 2 mM $MgCl_2$). The condition for amplification was an initial denaturation temperature 94°C for five min, followed by 35 cycles of 45 s at 94°C, then by 45 s at appropriate annealing temperature (Table 2) followed by 2 min at 72°C, and then by a final extension step for 7 min at 72°C. Amplicons were resolved at 100 V on 1.8% agarose gels containing 0.5 µg/ml ethidium bromide. Low range DNA ruler was used as size marker.

Data analysis

The molecular sizes of the amplicons were measured using the gel documentation software UVItec ver 12.8. Lanes representing the turtle samples were scored for the presence (1) or absence (0) of bands. POPGENE ver. 1.32 (Yeh and Boyle, 1997) was utilized to estimate genetic variability based on Nei's (1973) coefficient of gene differentiation (G_{ST}). The software was also used to calculate observed alleles, effective alleles, Shannon's information index (I), total genetic diversity (Ht), sample genetic diversity (Hs) and estimated gene flow (Nm).

The relatedness among turtle samples was analysed based on the Neighbour Joining algorithm (Saitou and Nei, 1987). The matrix of binary characters was bootstrapped using the software Phyltools (Buntjer, 2001). Re-sampling was done 1000 times using 35% bootstrap values. Phytools was then used to generate distance matrices based on Nei's coefficient (Nei and Li, 1979); S= 2a/ (a+b) + (a+c), where, a= band(s) shared by individual x and y; b= bands amplified in individual x but not in y; c= band(s) amplified in individual y but not in x. Distance was estimated as 1-S. The Consensus Neighbour Joining Tree was prepared using the software Phylip ver 3.1 (Felsenstein, 2004). Geneious Pro ver 4.8 (Drummond et al., 2010) was used to obtain a presentable consensus tree.

Principal components analysis (PCA) of the original binary matrix was also performed sequentially by using the modules of NTSYSpc ver 2.20r (Rohlf, 2005): stand, simint, eigen and project.

RESULTS

Inter-simple sequence repeat (ISSR) amplification

The primers generated 156 clear and distinguishable ISSR bands, where sizes ranged from 0.2 to 2.836 kb

Table 1. *Pangshura* samples used in the present studied.

Sample	Taxon	Place of collection	GPS Location
T52	*P. sylhetensis*	Kushiara River, Bhangabazar, Karimganj district, Assam	24°51'39.2238"N, 92°28'55.3865"E
T28	*P. sylhetensis*	Biswanath Ghat, Assam	26° 39´31.46˝ N, 93°10´18.91˝ E
T8	*P. sylhetensis*	Jia Bharali River (Nameri National Park), Assam	26°55'20.22"N, 92°50'27.12"E
C10	*P. sylhetensis*	Kuruwa Ghat , Darrang district, Assam	26°13'32.79"N, 91°46'39.74"E
T3	*P. sylhetensis*	Buxa Wildlife Sanctuary, West Bengal	26°50'17.05"N, 89°50'13.83"E
T7	*P. tecta*	Hajo, Kamrup district, Assam	26°14'41.1" N, 91°31'37.2'' E
C7	*P. tecta*	Gomrighat, Sonitpur district, Assam	26°44'47.93"N, 93°38'45.45"E
C4	*P. tecta*	Kushiara River, Karimganj district, India-Bangladesh border	24°52'37.53.41"N, 92°31'5.16.25"E
T2	*P. smithii*	Brahmaputra, Bhurhachapari Wildlife Sanctuary	26°33'12.7"N, 92°21'32.6"E
T50	*P. smithii*	Deeporbeel, Kamrup district, Assam	26°07'02"N 91°38'40"E
T1	*P. smithii*	Tengatoli char, Morigaon, Assam	26° 29' 016" N, 92° 20' 41.5" E
C12	*P. smithii*	Biswanath Ghat, Sonitpur district, Assam	26°46'30.74"N, 93°32'04.86"E
C15	*P. smithii pallidipes*	Ganga river, Farrukhabad, Uttar Pradesh	27° 12' 50.8" N, 79° 41' 34.6" E
T16	*P. tentoria circumdata*	Yamuna river, Etawah, Uttar Pradesh	26°45'13.44.53"N,79°0'28.20.74"E
T15	*P. tentoria circumdata*	Yamuna river, Uttar Pradesh	26°44'37.5749"N, 79°0'3.488"E
T12	*P. tentoria circumdata*	Ganga river, Farrukhabad, Uttar Pradesh	27° 14' 17.7" N, 79° 40' 27.1"E
T13	*P. tentoria circumdata*	Ganga river, Farrukhabad, Uttar Pradesh	27° 13' 20.4" N,79° 42' 51.2"E
T27	*P. tentoria flaviventer*	Lahorighat, Morigaon district, Assam	26° 27' 00.1" N, 92° 15' 22.2" E
T49	*P. tentoria*	Hajo, Kamrup, Assam	26 º 14'41.1''N, 91 º 31'37.2''E
T26	*P. tentoria*	Dimbur Char, Lahorighat, Morigaon district, Assam	26° 26' 31.5" N, 92° 16' 08.3" E

(Table 2). The number of bands per primer ranged from 9 to 24 with a mean of 15.6 bands per primer. Primers 1, 4, 6 and 9 amplified monomorphic markers (Table 2). Primer 3 revealed three markers of 0.615, 0.666 and 0.722 kb sizes and Primer 5 revealed two markers of 0.52 and 0.592 kb sizes that were common to both the *P. smithii* subspecies (Figure 2). Primer 5 also amplified two markers of 0.541 and 0.949 kb sizes that were present in all the three *P. tentoria* subspecies.

The highest number of bands was generated from the primer P10. The highest percentage of polymorphism (100%) was generated from the primers P2, P3 and P5 (Table 2). The number of polymorphic bands produced per primer ranged between 6 (P9) to 23 (P10). Out of the 145 polymorphic bands, *P. sylhetensis* had 22 (highest) numbers of unique polymorphic bands followed by 20 in *P. tentoria*, 18 in *P. tentoria circumdata*, 15 each in *P. smithii* and *P. tentoria flaviventer*, 11 in *P. tecta* and 7 in *P. smithii pallidipes* (Table 3). The ISSR banding patterns were amplified by seven primers (Figure 2).

The band profiles generated by ISSR primers showed polymorphism among the *Pangshura* samples. The extent of polymorphism varied. The percentage polymorphic loci were calculated to be 38.59, 24.46, 27.72 and 47.83 for *P. sylhetensis*, *P. tecta*, *P. smithii* and *P. tentoria* respectively.

Genetic variability revealed through inter-simple sequence repeat (ISSR) markers

A summary of genetic variation statistics for all loci are presented in Table 4. Average numbers of alleles observed in *P. sylhetensis*, *P. tecta*, *P. smithii* and *P. tentoria* were 1.34 ± 0.49, 1.24±0.43, 1.26 ± 0.45 and 1.48 ± 0.5 respectively, while it was 2.0 ± 0.0 when all four *Pangshura* species were taken together (Table 5). The genetic diversity in the four *Pangshura* species is presented in Tables 5 and 6.

Phenetic relationship among the test species

Genetic similarity and distance values based on Nei's original (Nei, 1972) and unbiased (Nei, 1978) measures are presented in Table 6. In the present study, each species of *Pangshura* is considered as a single population and the genetic distances among the four

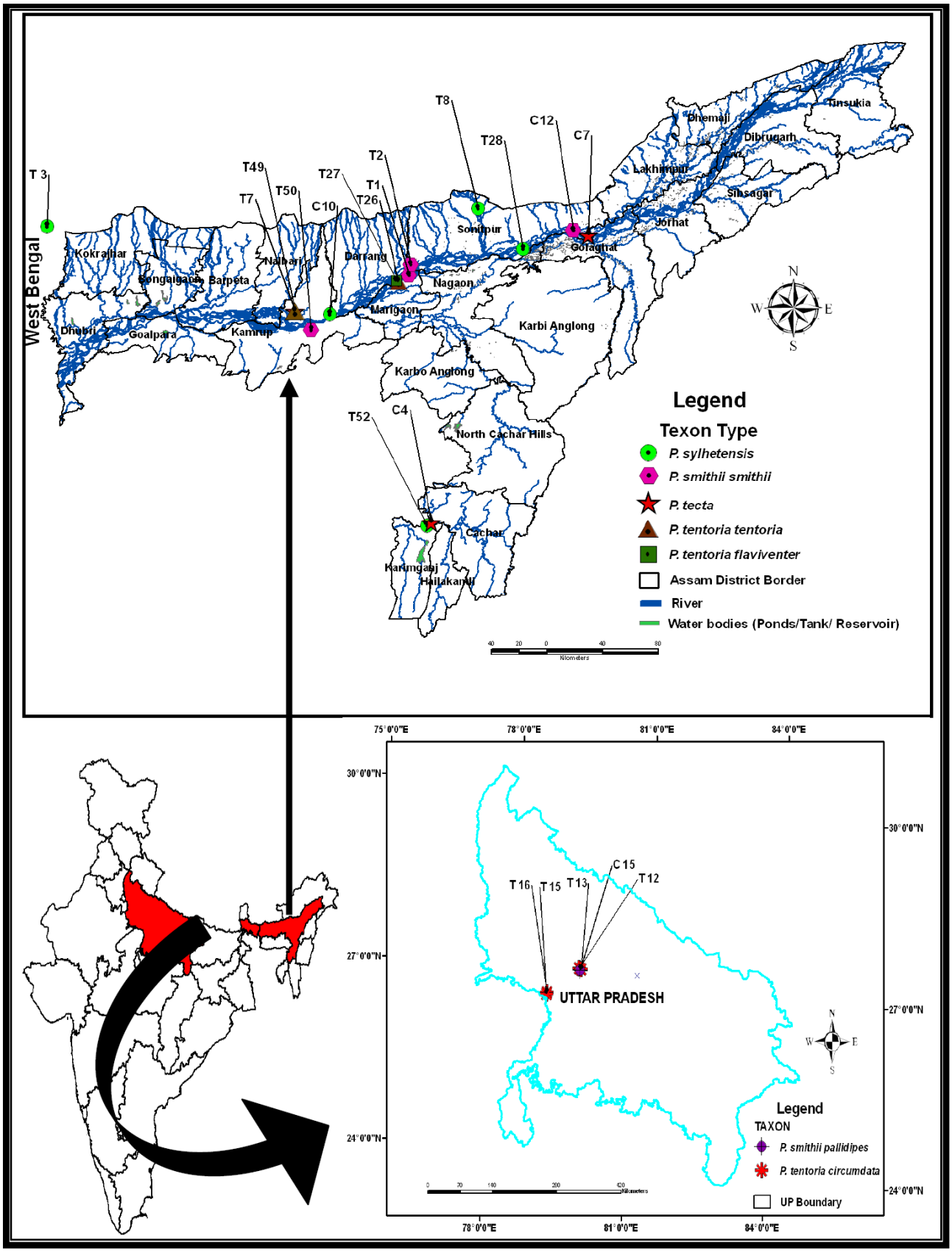

Figure 1. Map showing the location of sample collection sites in Assam (Including Assam-Bangladesh Border, Assam-Bhutan Border and part of West Bengal) and Uttar Pradesh.

Table 2. ISSR amplicons.

Primer	Primer sequence (5^{l}–3^{l})	Tm (°C)	Total bands	Polymorphism %	Amplicon size range (kb)
P1	5'(GA)8YT 3'	48	19	94.74	0.2 – 1.693
P2	5'(GATA)2$(GACA)_2$3'	46	22	100	0.4 – 2.836
P3	5'(ATG)6 3'	52	16	100	0.3 – 1.279
P4	5'(TC)8YG3'	52	21	95.24	0.255 – 01.182
P5	5' (TC)8HT 3'	50	15	100	0.508 – 1.152
P6	5' (TC)8BG 3'	52	11	90.91	0.353 – 1.15
P7	5' (TG)8RT 3'	50	9	100	0.409 – 1.083
P8	5' YG(GT)8 3'	52	10	100	0.574 – 0.991
P9	5' HR((TG)8 3'	50	9	77.78	0.611 – 1.091
P10	5'(AG)8YT 3'	47	24	100	0.227 – 1.231
Pooled values			156	100	

H= non – G; V= non – T; B= non – A; Y= pyrimidine; R= purine.

Pangshura species ranged from 0.14 to 0.23. The shortest distance (0.14) existed between *P. smithii* and *P. tentoria.* The longest genetic distance (0.23) existed between *P. smithii* and *P. tecta.* The genetic identity value ranged from 0.79 to 0.87 (Table 7).

The Neighbor-Joining (NJ) consensus tree is depicted in Figure 3. The species were grouped into three clusters. Cluster I comprised of *P. tecta* and *P. sylhetensis*. Topology of the cluster indicates that genetic diversity exists between the two species.

Both the species formed two distinct sub-clusters with 81 and 100% bootstrap support for *P. tecta* and *P. sylhetensis* respectively. Diversity existed among the *P. sylhetensis* samples. *P. sylhetensis* from Bishwanathgath (T28) and Jia Bharali (T8) from the state of Assam were clustered together with 83% bootstrap support. The *P. sylhetensis* samples from Kuruwaghat (C10) of northern Assam and Kushiara River (T 52) bordering Bangladesh were in the same cluster. However, *P. sylhetensis* of Buxa Wildlife Sanctuary (T3) of West Bengal formed a distinct clade within the *P. sylhetensis* group with 81%, bootstrap separation. In the *P. tecta* sub-cluster of Hajo (T7) and Gomerighat (C7), samples were grouped together with 100% bootstrap support, while the Kushiara river sample (C4) formed a monophyletic branch at 100% bootstrap separation.

All the three currently recognized subspecies of *P. tentoria* were clubbed in the clusters II with bootstrap separation 64%. *P. tentoria* sample collected from Hajo (T49) was on a monophyletic branch of the clade. *P. tentoria* (T 26) and *P. tentoria flaviventer* (T27) samples from Lahorighat were clustered together with 78% bootstrap support. Samples of *P. tentoria circumdata* collected from the Ganga (T12, T13) and Yamuna rivers (T15, T16) at different sites of Uttar Pradesh (Table 1) reflected their geographical distance from the *Pangshura* samples of Assam. One of the *P. tentoria circumdata* samples (T13) collected from the Ganga River at Farrukhabad, Uttar Pradesh was on a monophyletic branch (Figure 3).

Two subspecies of *P. smithii* were clustered together in cluster III. The *P. smithii* samples from Depor beel (T50) and Tengatoli (T1) were closer than the other samples. The *P. smithii* samples collected from Burachapari (T2) and Biswanathghat (C12) formed monophyletic branches. On the other hand, the samples of *P. smithii pallidipes* from Farrukhabad, Uttar Pradesh also formed monophyletic branch with 53% bootstrap separation.

The genetic interrelationships among *Pangshura* samples were projected on the PCA plot (Figure 4) and those depicted by the consensus NJ tree were similar; but were better represented on the plot. The subspecies of *P. tentoria* and that of *P. simithii* were well-separated by the first and the second principal components, however, their phenetic relationship with the subspecies of *P. tecta*, and *P. sylhetensis* was less defined. The subspecies of *P. tecta* and those of *P. sylhetensis* are projected as being closer to one another than they are either to the subspecies of *P. tentoria* or *P. smithii*.

DISCUSSION

Amplification of a large number of polymorphic ISSR bands (77.78 to 100%) indicates the existence of genetic diversity in the four *Pangshura* species. The average values of the observed and effective alleles, percentage polymorphic loci, Nei's gene diversity (h), Shannon's information index (I), total genetic diversity (H_t) (Table 5) all reveal that the highest polymorphism is present in *P. tentoria* followed by *P. sylhetensis*.

The high G_{ST} values and very low Nm values (Table 5) for all species indicate that they are in a high state of differentiation; G_{ST} > 0.25 and Nm < 1.0 are threshold values beyond which significant population differentiation occurs (Kar et al., 2005). The PCA plot (Figure 4) also

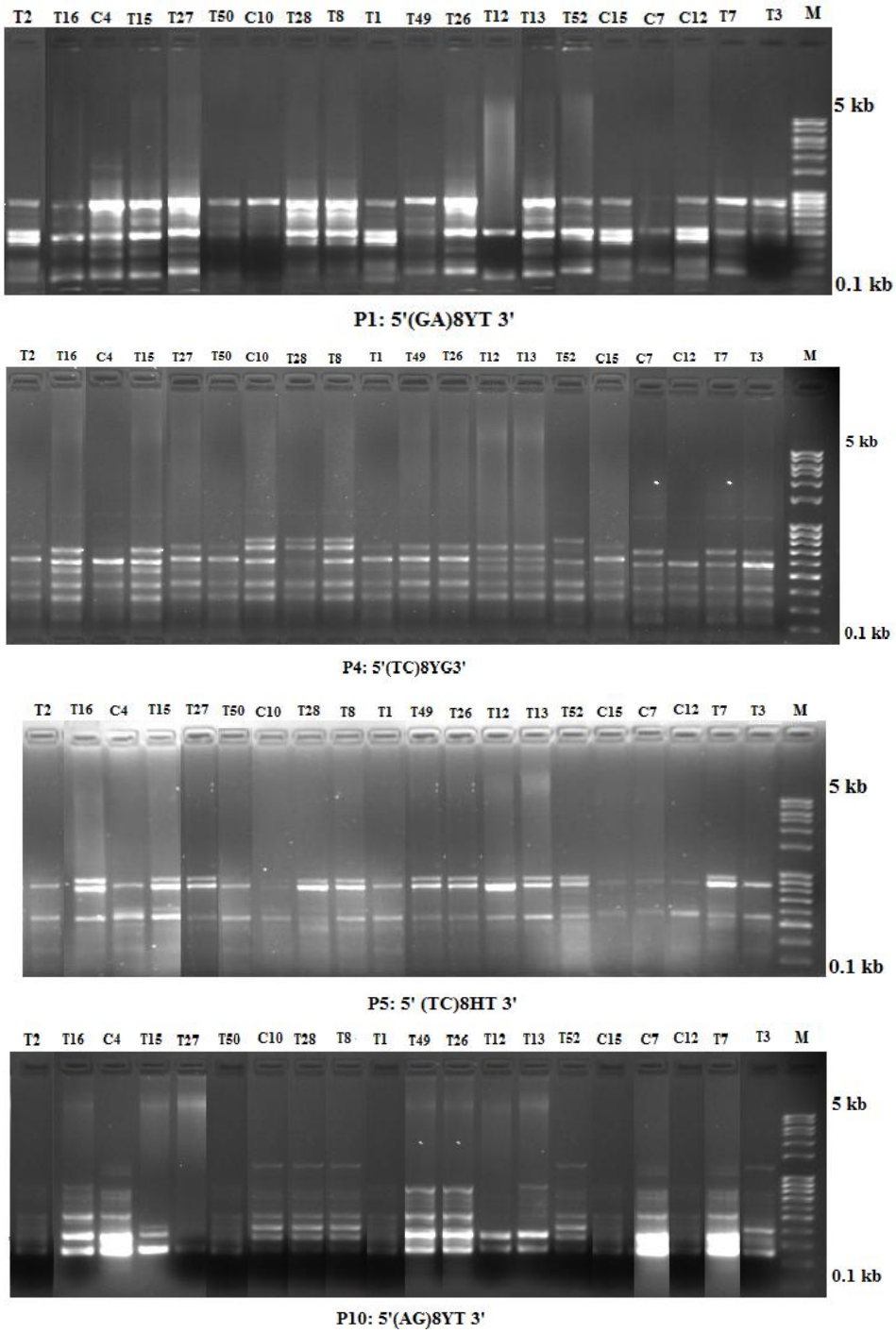

Figure 2. ISSR amplification profiles using primers P1, P4, P5 and P10, respectively. *Pangshura* samples with respective sample code are in lanes 1–20; M, 100 bp DNA marker (range 0.1 to 5 kb).

indicates the genetic distinctness of the four species. Differentiation might have occurred probably only due to genetic divergence through the evolutionary courses. The Neighbour Joining dendogram (Figure 3) presents the genetic interrelatedness of the *Pangshura* samples. The 100% bootstrap separation of *P. smithii* samples (cluster III) implies this species is more distant from the other two species. The G_{ST} values presented in Table 5 supports this result. Nei's measures of genetic identity and genetic distance (Table 7) are also in broad agreement of phenetic relationship that the NJ tree presents.

Of the primers, P1, P2, P3 and P10 were used earlier

Table 3. *Pangshura* species- specific unique ISSR bands.

Species	Number of unique bands	Unique Band size (kb)	Primer name
P. tecta	6	0.508 , 0.575	P5
		0.227, 0.663,0.779, 1.046	P10
P. sylhetensis	10	0.65	P1
		0.3, 0.565	P3
		0.566, 0.818, 0.975	P5
		0.348, 0.419,0.688, 1.231	P10
P. smithii	5	0.615, 0.666, 0.722	P3
		0.52, 0.592	P5
P.tentoria	2	0.541, 0.949	P5

Table 4. Observed and effective number of alleles in the four *Pangshura* species for all primers.

Primer	Alleles in *P. sylhetensis*		Alleles in *P. tecta*		Alleles in *P. smithii*		Alleles in *P. tentoria*	
	Observed	Effective	Observed	Effective	Observed	Effective	Observed	Effective
P1	1.35 ± 0.5	1.21 ± 0.4	1.17 ± 0.4	1.1 ± 0.2	1.17 ± 0.4	1.1 ± 0.2	1.43 ± 0.5	1.22 ± 0.3
P2	1.3 ± 0.5	1.13 ± 0.3	1.28 ± 0.45	1.1 ± 0.2	1.28 ± 0.45	1.1 ± 0.24	1.48 ± 0.5	1.13 ± 0.2
P3	1.3 ± 0.48	1.17 ± 0.32	1.1 ± 0.23	1.0 ± 0.04	1.16 ± 0.37	1.09 ± 0.25	1.47 ± 0.5	1.25 ± 0.3
P4	1.1 ± 0.32	1.04 ± 0.15	1.03 ± 0.19	1.0 ± 0.03	1.24 ± 0.44	1.09 ± 0.21	1.48 ± 0.51	1.17 ± 0.21
P5	1.5 ± 0.51	1.31 ± 0.39	1.5 ± 0.51	1.26 ± 0.3	1.7 ± 0.38	1.1 ± 0.28	1.33 ± 0.48	1.2 ± 0.18
P6	1.11 ± 0.33	1.06 ± 0.24	1.11± 0.33	1.08 ± 0.25	1.18 ± 0.39	1.08 ± 0.18	1.18 ± 0.39	1.05 ± 0.13
P7	1.33 ± 0.49	1.23 ± 0.36	1.08 ± 0.29	1.04 ± 0.12	1.42 ± 0.51	1.29 ± 0.4	1.67 ± 0.49	1.28 ± 0.32
P8	1.78 ± 0.44	1.38 ± 0.37	1.56 ± 0.53	1.35 ± 0.39	1.55 ± 0.53	1.38 ± 0.46	1.78 ± 0.44	1.31 ± 0.26
P9	1.98 ± 0.3	1.47 ± 0.31	1.36 ± 0.5	1.29 ± 0.44	1.64 ± 0.5	1.41 ± 0.44	1.64 ± 0.5	1.49 ± 0.48
P10	1.82 ± 0.4	1.44 ± 0.37	1.64 ± 0.5	1.35 ± 0.36	1.45 ± 0.52	1.25 ± 0.37	1.74 ± 0.47	1.43 ± 0.42

Table 5. Genetic parameters for the four species of genus *Pangshura.*

Genetic parameter	*P. sylhetensis*	*P. tecta*	*P. smithii*	*P. tentoria*	Overall
Percentage polymorphic loci	38.59	24.46	27.72	47.83	100
Number of observed alleles: na	1.34 ± 0.49	1.24 ± 0.43	1.26 ± 0.45	1.48 ± 0.5	2.0 ± 0.0
Number of effective alleles: ne	1.2 ± 0.33	1.13 ± 0.26	1.15 ± 0.29	1.22 ± 0.31	1.34 ± 0.3
Nei's genetic diversity: h	0.12 ± 0.12	0.08 ± 0.15	0.09 ± 0.16	0.14 ± 0.17	0.22 ± 0.16
Shannon's information index: I	0.19 ± 0.26	0.12 ± 0.22	0.13 ± 0.24	0.22 ± 0.25	0.36 ± 0.21
Total genetic diversity (H_t)	0.13 ± 0.03	0.1±0.03	0.09 ± 0.03	0.16 ± 0.04	0.22 ± 0.03
Sample genetic diversity (H_s)	0.01 ± 0.01	0.01±0.01	0.004 ± 0.04	0.03 ± 0.03	0.11 ± 0.01
Estimated gene flow (N_m)	0.04	0.05	0.02	0.09	0.46
Gene differentiation (G_{ST})	0.93	0.91	0.96	0.84	0.52

in grass carp (Chen et al., 2009); P10 showed better cross-species amplification than the others. The Nei's F-statistics and Shannon's information index values obtained due to P10 were comparable to the values obtained due to the primers P4, P5, P6, P7, P8 and P9 (Table 6) that were designed and slightly modified by us

Table 6. Nei's F-statistics and Shannon's information index in *Pangshura* species for all ISSR primers.

Primer	Overall gene diversity (H_t)	Within sample gene diversity (H_s)	Gene differentiation (G_{ST})	Gene diversity (h)	Shannon's information index (I)
P1	0.16	0.09	0.42	0.12	0.18
P2	0.15	0.08	0.46	0.15	0.27
P3	0.24	0.08	0.66	0.16	0.25
P4	0.19	0.05	0.74	0.19	0.31
P5	0.22	0.13	0.42	0.22	0.35
P6	0.29	0.04	0.85	0.28	0.44
P7	0.24	0.13	0.48	0.24	0.39
P8	0.36	0.21	0.39	0.34	0.52
P9	0.32	0.23	0.27	0.31	0.48
P10	0.29	0.22	0.24	0.28	0.43

Table 7. Nei's original (1972) and unbiased (1978) measures of genetic identity and genetic distance among the four *Pangshura* species.

***Pangshura* species**	***P. sylhetensis***	***P.tecta***	***P. smithii***	***P. tentoria***
P. sylhetensis	-	0.79 (0.8)	0.82 (0.83)	0.84 (0.85)
P.tecta	0.23 (0.22)	-	0.79 (0.79)	0.84 (0.84)
P. smithii	0.19 (0.19)	0.23 (0.23)	-	0.87 (0.88)
P. tentoria	0.17 (0.17)	0.18 (0.17)	0.14 (0.13)	-

Genetic identity (above diagonal) and genetic distance (below diagonal); figures in parentheses indicate the unbiased measures values.

based on earlier reports on turtle microsatellites by Aggarwal et al. (2004), Edwards et al. (2003) and Schwartz et al. (2003).

The PCA result reveals that a close relation within the respective *P. tentoria* could not be expected. From the *P. sylhetensis* group, samples from West Bengal and Kushiara River bordering to Bangladesh are placed near to the *P. tecta* group, representing a close genetic relationship between the two species (Figure 4). The analysis of species specific unique ISSR amplicons of the present study (Table 2) along with the genetic parameters (Table 5), the correlation of PCA plot and neighbour joining tree reveal that the genetic diversity among the *Pangshura* could be well inferred.

Praschag et al. (2007) sampled *P. sylhetensis* and *P. tentoria* from Assam (Northeast India) to construct phylogeny of endangered Southeast Asian turtles including *Pangshura*. However, their study was based on patchy taxon sampling and could not establish any distinctness between the subspecies of *P. smithii* and *P. tentoria* with the help of cyt *b* gene dataset. The present study has established that in order to establish the variation in the genome of *Pangshura* species, ISSR appear as powerful tool. These variations support the validity of the morphologically weakly defined subspecies *P. tentoria* and *P. tentoria circumdata* as well as *P. smithii* and *P. smithii pallidipes*. Although few cross-species microsatellites could be used for genetic diversity of *Pangshura*, the availability of species-specific markers is highly desirable for population structure assessments. These microsatellites thus provide efficient genetic markers to understand the population structure, phylogeography and species relationships of *Pangshura* and other freshwater turtle species.

Conclusion

Our study reveals the existence of a narrow genetic base for all the four turtle species, which we attribute to inbreeding. It is necessary to formulate conservation strategies in terms of protection of the habitat sites to minimize the reduction of gene pool, which might be the probable cause of inbreeding.

In-situ egg hatching of eggs followed by headstarting of those species can also be undertaken as a part of conservation strategy. Future work should focus on finer scale genetic analyses in order to gain a better baseline

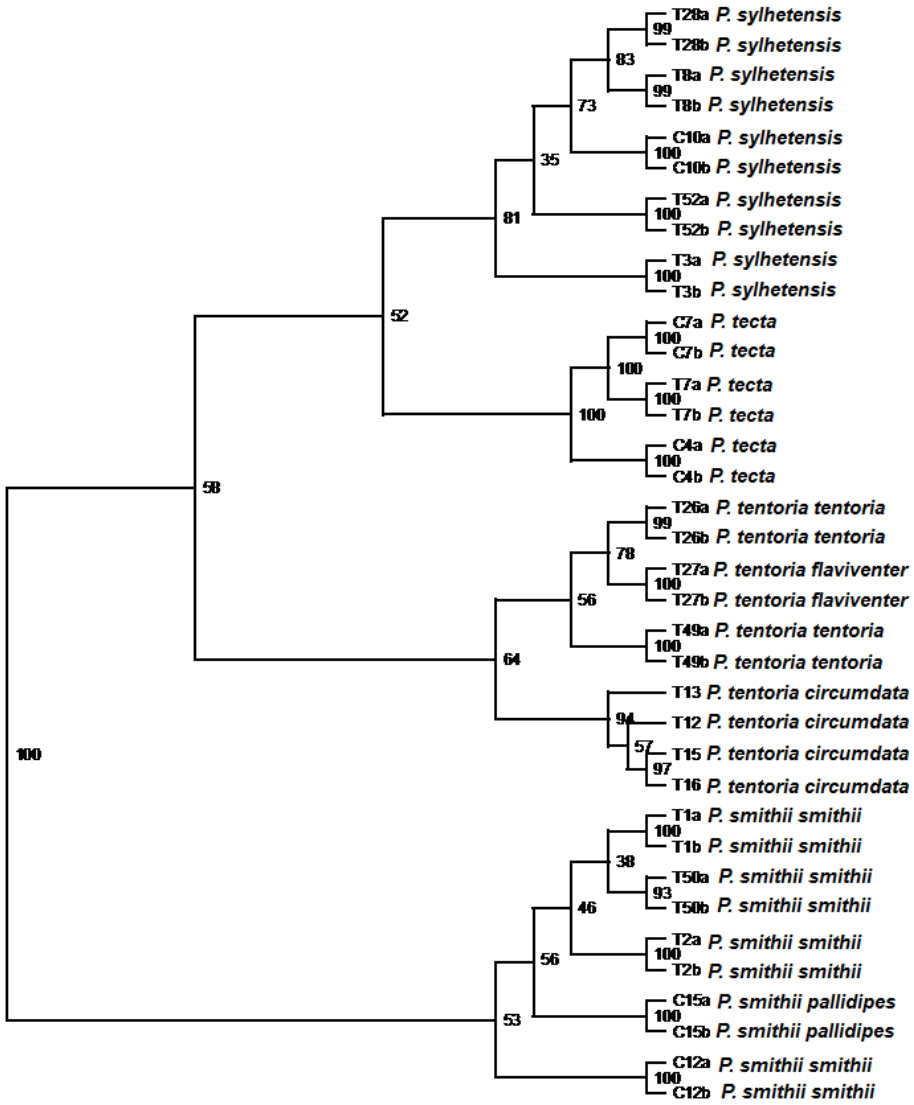

Figure 3. Neighbor-Joining Consensus Tree generated after bootstrap analysis of ISSR data. Bootstrap support values, in percentages, based on 1000 replications are presented. Clusters are indicated in roman numerals. [T28 (*P. sylhetensis*, Biswanathghat); T8 (*P. sylhetensis*, Jia Bharali river); C10 (*P. sylhetensis*, Kuruwa Ghat); T52 (*P. sylhetensis*, Kushiara river); T3 (*P. sylhetensis*, Bauxa WLS, West Bengal); C7 (*P. tecta*, Gomerighat; T7 (*P. tecta*, Hajo); C4 (*P. tecta*, Kushiara river); T26 (P. *tentoria* Lahorighat); T27 (P. *tentoria flaviventer*, Lahorighat); T49 (P. *tentoria*, Hajo); T13 (*P. tentoria circumdata*, Ganga river); T12 (*P. tentoria circumdata, Ganga river);* T15 (*P. tentoria circumdata*,Yamuna river); T16 (*P. tentoria circumdata*,Yamuna river); T1 *(P. smithii*, Morigaon); T50 (*P. smithii*, Deeporbeel); T2 (*P. smithii*, Burhachapari WLS); C15 (*P. smithii pallidipes*, Ganga river); C12 (*P. smithii*, Biswanathghat); 'a' and 'b' represents sample replicates of respective species from the same locality.]

understanding of population genetic structure of *Pangshura*.

ACKNOWLEDGEMENTS

The authors are thankful to the Ministry of Environment and Forests, Government of India and the state forest departments of Assam, West Bengal and Uttar Pradesh for necessary permission to carry out the research. The Principal, St. Anthony's College, Shillong, Meghalaya, India is acknowledged for providing the laboratory facilities at the Biotechnology department. The authors gratefully acknowledge the Department of Biotechnology,

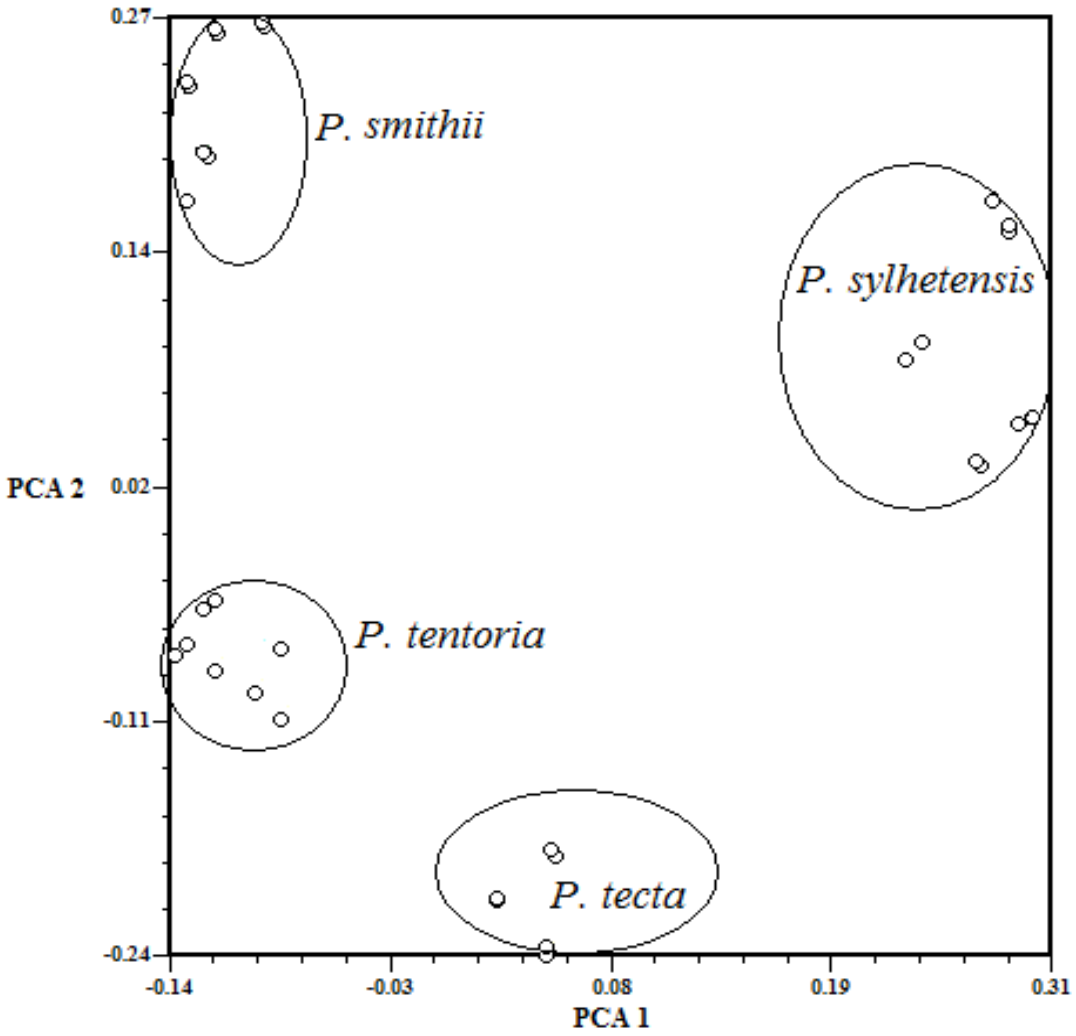

Figure 4. Plot of the first two principal components in Principal Components Analysis of ISSR data from *Pangshura species* mentioned in the plot. *P. sylhetensis*, T3, T8, T28, C10, T52; *P. tecta*, C4, C7, T7 C4; *P. tentoria,* T12, T13, T15, T16, T26, T27, T49; *P. smithii*, T1*,* T2, T50, C12, C15].

Government of India for the financial support to establish the Bioinformatics Infrastructure Facility (BIF). The authors are very grateful to Asutosh Triapthi of TSA-India for providing the samples.

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