CHAPTER-IX

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The turtle family Geoemydidae comprising mostly of freshwater turtles includes several highly endangered Southeast Asian turtle species. The genus Pangshura is characterized by more or less well defined sexual dimorphism. Understanding differentiation of these turtles at molecular level would significantly contribute to more powerful conservation strategy formulation. The genus Pangshura (Family-Geoemydidae) comprises small-sized turtles, having maximum shell length of 20 – 26.5 cm. The species that come under the genus are P.tecta, P. sylhetensis, P. tentoria and P. smithii. Further these species are fragmented to subspecies like P. tentoria tentoria, P. tentoria circumdata, P. tentoria flaviventer, P. smithii and P. smithii pallidipes. Our field studies indicate the decline in numbers of these fresh water turtles in Brahmaputra and Ganges river basins.

Recent advances in molecular biology, have given rise to development of the in silico methods for sequence analysis, protein structure prediction, functional annotation and evolutionary studies, which is being utilized in this study. In the present study, molecular characterization based on mitochondrial and nuclear genes along with protein sequence analysis from the Indian freshwater turtles of genus Pangshura has been performed. The study represents the first hand information generation that includes field data collection, sample collection and analysis including DNA isolation, PCR, microsatellite based ISSR marker development, DNA sequencing followed by application of in silico tools for sequence comparison, multi-gene phylogeny, RNA secondary structure prediction, 3D structure prediction of various proteins and functional annotation to predict the molecular genetic variation within the chelonian genus Pangshura as well as to identify their homology and evolutionary profile.

The present study has quantified the genetic variation within and among population of the Pangshura genus in two Indian River systems and their comparison. The present study adds a genetic dimension to the ongoing efforts to understand the ecology, demography and natural history of the endangered and endemic Assam Roofed turtle,
Pangshura sylhetensis (Jerdon, 1870). These actions are also supported under high Research priority in the 2006 "Indian Freshwater Turtles and Tortoises Action Plan".

A wide range of variation is indicated in the phenetic relationship among the Pangshura species viz. within P. tentoria and P. sylhetensis. Principal Components Analysis (PCA) projected P. sylhetensis to be more closely related to P. tecta than P. tentoria and P. smithii. PCA also exposed variations within the P. tentoria samples. The microsatellite loci provides useful marker for the assessment of genetic variability within the genus Pangshura and across populations of Pangshura species and other freshwater turtle species to understand their population structure, reproductive behaviour, phylogeography and species relationships, paving the way for their conservation and efficient management strategies.

Limited information is available on morphometric and current distribution range of the genus Pangshura. Therefore, in this study an attempt was made to reveal the distribution of Pangshura to define its biogeographical information as well as its deficiency of the size related data for identification of sex as well as age of the species. A total of 178 individuals including 80 numbers of Pangshura from Northeast India and Northern India along with 98 museum specimens were analyzed for size and sexual dimorphism. Distinct sexual dimorphism has been observed in our study. A diagrammatic model for identification of Pangshura has been predicted for the first time in this study.

Analysis of polymorphism in ISSR amplicons revealed diverse genetic relationship between the four species under the genus Pangshura. In a Neighbor-Joining dendrogram, based on Nei and Li’s distance matrices, the P. sylhetensis samples from different sites of Northeast India were clustered together with 81% bootstrap support. Principal Components Analysis (PCA) projected P. sylhetensis to be more closely related to P. tecta than P. tentoria and P. smithii. PCA also exposed variations within the P. tentoria samples.

Six (6), out of the ten (10) enzymes, produced species-specific RFLP patterns in the 4 Pangshura species. The sequence analysis indicated that within the genus Pangshura, there are minor sequence variations. Analysis of 16S rDNA sequence showed that there is minor variation in melting temperature. Minor structural changes have been
observed in some parts of 16S rRNA secondary structure with variation in the minimum folding energy.

All tree-building methods revealed that the genus *Pangshura* is a perfectly supported monophylum with bootstrap or posterior probability values of more than 90%. The present multi-gene phylogenetic study supports the fact that *P. smithii* and *P. tentoria* as sister species, followed by *P. tecta* and *P. sylhetensis* as their successive sister-taxa. Distinctness of the subspecies within *P. tentoria* is poorly supported by the evolutionary data. The *P. tecta* samples of Northern India and Northeast India have formed two separate clusters, which might be due to the existence of two separate subspecies of *P. tecta*, one from Assam and the other from the West Bengal and Uttar Pradesh. Minor phylogeographic variation was observed for *P. sylhetensis*.

The overall Quality factors predicted by ERRAT verification programme for the predicted 3D structures of Cytochrome b, RAG2, and NADH4 are more than 95%. After successful verification of the coordinate files, the structures were deposited to PMDB Protein Model Database of University of Rome and available for further uses. Each 3D structure has been assigned a unique PMDB ID for the coordinate entry. Procheck verification proved that the models are of good quality as per the Ramachandran Plot.

In the present investigation, understanding of genetic relationships within the genus *Pangshura* has been presented and concluded.

1. The present study suggests that *P. smithii* and *P. tentoria* as sister species followed by *P. tecta* and *P. sylhetensis* as their successive sister-taxa.
2. Relation among five carapace and plastron measures indicated strongest sexual dimorphism in the shell (carapace) height/length relationship in the genus *Pangshura* with males are smaller than females.
3. The present study revealed that size of *Pangshura sylhetensis* may be up to 20.5 cm i.e. larger (carapace length 18.5 cm) than that reported by Das *et al.* (2010).
4. The ISSR studies indicated differences among the subspecies of *P. tentoria* and *P. smithii*. The *P. tecta* and the *P. sylhetensis* were closer to each other than either of them was to the *P. smithii*, which was well established by the cluster analysis and PCA results.
5. *P. sylhetensis* from West Bengal and Kushiara River bordering to Bangladesh are placed near to the *P. tecta* group in the PCA analysis, representing a close genetic relationship between the two species.

6. $G_{st}$ value (0.93) and $H_t$ value (0.13) in *P. sylhetensis* indicates good gene flow in the species. However, inbreeding possibility cannot be ignored within the *Pangshura* populations, as indicated by individual genetic diversity ($H_2$) and estimated gene flow ($N_m$).

7. *P. tecta* and *P. sylhetensis* represented higher genetic identity by having transition (A→G) at 212th position, deletion at 317th position. *P. tentoria* and *P. smithii* including *P. tentoria* hybrids exhibited genetic identiteties by sequence transition (T→C) at 248th position in the alignment. All the *P. tentoria* were identified by having a transversion (A→C) point at 505th position.

8. Multiple sequence alignment of 16s rRNA gene showed that there is difference between the Assam and West Bengal (WB) population of *P. sylhetensis*. Since there has been a deletion of ‘A’ at 58th position and transition of A→G at 202nd position in the *P. sylhetensis* group of West Bengal (*P. sylhetensis 16*).

9. The distinction between the two subspecies *P. s. smithii* and *P.s. pallidipes* could well be established by transition at 225th and 401st position (A→G) respectively.

10. ND4 and R35 gene have been able to establish variation among *P. t. tentoria*, *P. t. circumdata* and *P. t. flaviventer* as separate and distinct identity. Further, the 16S rRNA and R35 intron-1 showed genetic variation between the two subspecies *P. smithii smithii* and *P smithii pallidipes* of the species *P. smithii*.

11. The length of the restriction fragments demonstrated difference between the population of *P. sylhetensis* of Assam and West Bengal and also between the *P. tecta* of Assam and Uttar Pradesh. The population of *P. tecta* from Assam showed variation with that of *P. tecta*) of West Bengal in RF length at 34th position for ND4 gene.

12. Secondary structure of 16s rRNA structure has presented distinct variation in different taxon of this investigation. Three out of the four species viz., *P. sylhetensis*, *P. tecta* and *P. smithii* represented stem without hairpin, while the subspecies of the species *P. tentoria*, namely *P. tentoria tentoria* and *P. tentoria flaviventer* are found without dangling ‘A’ and ‘G’ giving distinct identity from that of the species *P. tecta*, *P. smithii* and *P. sylhetensis*. 
13. The AT rich (55.5%) RAG2 gene is only suggestive of the monophyletic origin of *Pangshura*, though such data are not readily available to support this proposition.

14. The present investigation provides a stable phylogenetic hypothesis for all *Pangshura* species, with *P. tentoria* and *P. smithii* being sister species and *P. tecta* and *P. sylhetensis* as their successive sister-taxa.

15. Distinctness within the three currently recognized subspecies of *P. tentoria* is not clearly visible by the evolutionary analysis.

16. *P. tecta* samples from Northeast India and the Northern India formed separate clusters indicating the possibility for the existence of two subspecies.

17. The predicted 3D structures can serve as a guide for the allocation of amino acid residues involved in each fold, which is important for further investigations on molecular mechanism of functions.

18. The molecular evolutionary analysis underline that detailed population genetics study on *P. tentoria* is in dire need for developing effective conservation strategies.

19. The evolutionary tree of Cytochrome *b*, Cytochrome *c* oxidase, NADH dehydrogenase 4, RAG2 supports the fact that *P. tecta*, *P. smithii* and *P. tentoria* are sister groups, while *P. sylhetensis* is their successive sister taxon. *Batagur dhongoka* is found to be an intermediate species of the two genera that belongs to genus *Batagur*.

20. Intergrades between the subspecies *P. tentoria* and *circumdata* have been obtained from the Ganga River. Das (1995) reported the possibility of a fourth subspecies of *P. tentoria* from in the Brahmaputra in Assam and Bangladesh. This subspecies intergrades with *flaviventer*, as examples from Bangladesh show loss of the plastral pattern to various degrees. This present study recorded it as a hybrid between *P. tentoria tentoria* and *P. tentoria flaviventer*.

21. The R35 gene Intron-1 has been able to show the distinction between the two population group of *P. sylhetensis* and *P. tecta* of Assam and West Bengal as well as Assam and Uttar Pradesh. Similarly, *in silico* restriction digestion profile of a wet lab sequence showed the variation between the population of *P. sylhetensis* and *P. tecta*.

22. Posterior probability support of near (100%) and distinct from *Batagur* strongly support the recognition of *Pangshura* as distinct and monophyletic genus.
23. The present study revealed that 11 pairs of marginal shield are present in *P. tecta*, *P. tentoria* and *P. smithii*. However, *P. sylhetensis* posses 13 pairs of marginal shields with strongly serrated posterior margin in the carapace.

24. The molecular method developed in the present study is simple, rapid, reliable and reproducible; hence could be routinely applied for species identification; essential for conservation and management of endangered chelonian species.

**Outcome of the present study:**
The present study has lot of implication for biodiversity documentation.

1. The present study explored the biogeography, natural history and evaluated the comparative genetic structure and distribution patterns of the populations of *Pangshura* species in Northeast and Northern India.

2. The present study for the first time diagrametically represented the plastral formula of the *Pangshura* genus.

3. The present study has been able to highlight the variation among the subspecies of *P. tentoria* and *P. smithii* through the sequence analysis of 7 taxon of the *Pangshura* genus.

4. The nucleotide and protein sequence data generated in the present study have been submitted to GENBANK, public domain to enhance global comparison on chelonian systematics.

5. Study clarified the taxonomic uncertainties of the 3 subspecies of *P. tentoria* and 2 subspecies of *P. smithii*.

6. Introduction of sequence analysis in the genetic identification of freshwater turtle species is not known and this would be the first report of its kind.

7. The present study successfully used non-invasive methods for species identification along with modern bioinformatics tools for molecular characterization could be used as an additional method for identification of species as well as for identification of unknown samples with unusual appearances and could be made available for the identification of confiscated specimens

8. The microsatellite loci described here provide potentially useful markers for the assessment of genetic variability within the genus *Pangshura* and across populations of *Pangshura species* and other freshwater turtle species to understand their population structure, reproductive behaviour, phylogeography and species relationships, paving the way for their conservation and efficient management strategies.
9. Phenetic relationship within the genus *Pangshura* using ISSR markers would be useful in the formulation of effective conservation strategies.

10. Two restriction enzymes (*Fok* I and *Hae* III) recognized the highest proportion of species-specific sequences and generated the diagnostic RFLP pattern most useful for differentiating the *Pangshura* species.

11. Sequence analysis of these genes 16S rRNA, ND4, RAG2 and R35 Intron-1 might be considered to be effective in establishing the closeness of *P. tecta* and *P. sylhetensis*. But in the present study the gene RAG 2 and R35 intron gene has been able to eradicate the conflict (Praschag *et al.*, 2009) among the subspecies of *P. tecta*.

12. The multi-gene phylogeny underline that the understanding phylogeny and diversity along with the degree of hybridization of *Pangshura* needs further phylogeographic sampling throughout the distribution range in the Indian subcontinent for developing effective conservation strategies.

13. Result of this R35 has been shown to be an excellent candidate as marker in establishing the sub species. However, RAG2 gene is not suggestive of establishing the intraspecific relationship in the genus Pangshura.

14. The computational models generated from the present study have been deposited to the Protein Model Database (PMDB) and each of them has been assigned a unique PMDB ID. The models can be downloaded for further understanding of various functional and evolutionary aspects.

Turtles are disappearing from the planet faster than any other group of animal. Today, nearly 50% of turtle species are identified as threatened with extinction. It has been noted that *Pangshura* population has been declining very fast. Insight into the genetic diversity- phenetic relationship, restriction mapping, and sequence analysis will certainly allow in formulation of conservation strategies. However, it's not too late for our turtle heritage to be salvaged. Turtle conservation groups in partnership with Partners in Amphibian and Reptile Conservation (PARC) are designating 2011 as the 'Year of the Turtle' for raising awareness of the issues surrounding turtles to work together to address issues and to help ensure long-term survival of turtle species and populations.