CHAPTER-VIII

GENERAL DISCUSSION
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Genetic tools and datasets have been applied to problems at the intraspecific level which may represent the most important data for the recognition of intraspecific variation. For example, phylogeographic studies, employing mitochondrial DNA (mtDNA) and/or nuclear DNA (nDNA) sequences from geographically defined population can identify geographically restricted lineages that become candidates for subspecific recognition (Lenk et al., 1999; Engstrom et al., 2002; Starkey et al., 2003; Fritz et al., 2005; Spinks and Shaffer, 2005; Gaur et al., 2006).

Use of ND4 and R35 along with other genes to reconstruct the phylogeny of Asian box turtle (Cuora species) and to assess genetic diversity and species boundaries for various problematic taxa have already been accepted (Spinks and Shaffer, 2007).

Recently, Praschag et al. (2009) attempted to address the phylogenetic position of C. gemeli using sequence data of the mitochondrial cyt b gene as well as of three nuclear DNA fragments (C-mos, Rag2 genes, intron 1 of R35 gene) and confirm its genetic distinctiveness. According to their new records, the Naga Hills and the Arakan Mountains could constitute the geographical divide between C. gemeli and C. fusca. Morphologically, C. gemeli resembles other dark-bellied Cyclemys species and determination by external morphology alone is quite difficult. More recently, Praschag et al. (2011) used 599 bp partial ND4 gene and the DNA coding for the tRNAs along with 12S rRNA and cyt b and they established the genetic divergence and degree of differentiation among L. p. punctata, L. p. andersoni and L. p. vittata.

8.1 Systematics and Biogeography of Pangshura sylhetensis:

The present study on both live and museum specimens revealed that male and female individuals of all the four species of Pangshura can be identified by sexual dimorphism. The frequency of the morphometric measurements among and within Pangshura species reveals distinct sexual dimorphism in each species of Pangshura is well observed. Males are always smaller in size than the females. P. sylhetensis is strongly sexually dimorphic, with female’s carapace twice as large as males (Praschag et al., 2007; Das et al., 2010)
The present observations have revealed that size of *P. sylhetensis* seems to be larger (carapace length 18.5 cm) than that reported by Das *et al.* (2010). The present study shows that the female individuals from the temple tank of Hajo, Kamrup district, Assam having carapace length (CL) of 20.5 cm, carapace width (CW) 14.1 cm, plastron length (PL) 16.9 cm, plastron width (PW) 7.4 cm and shell height (SH) 8.3 cm with weight of 1200g. The present study has been able to present a diagrammatic representation of *P. sylhetensis* with plastral formula modified after Gunther (1864).

The present field survey has identified that Nameri National Park and Kaziranga National Park are the potential habitat of *P. sylhetensis* (Baruah *et al.*, 2010). The survey records of the year, 2009 showed the increased numbers of *P. sylhetensis* in the Jia Bharali River (Nameri National Park), Biswanath Ghat, Gomirighat and Kuruwa Ghat areas, probably due to the habitat conservation in protected areas (Sarma *et al.*, 2009; Baruah *et al.*, 2011a).

### 8.2 ISSR marker for *Pangshura*:

In the present study, each species of *Pangshura* is considered as a single population and the genetic distances among the four populations (among the four *Pangshura* species) ranged from 0.14 to 0.23. The shortest distance (0.14) existed between the populations of *P. smithii* and *P. tentoria*. The longest genetic distance (0.23) existed between the populations of *P. smithii* and *P. tecta* (Table 4.7).

Out of the 145 polymorphic bands in the ISSR amplicons, *P. sylhetensis* has 22 (highest) numbers of unique polymorphic bands followed by 20 in *P. tentoria tentoria*, 18 in *P. tentoria circumdata*, 15 each in *P. smithii smithii* and *P. tentoria flaviventer*, 11 in *P. tecta* and 7 in *P. smithii pallidipes*.

The Neighbor-Joining consensus tree from the phenetic study revealed that diversity existed among the *P. sylhetensis* samples. *P. sylhetensis* from Biswanath ghat (T 28) and Jia Bharali (T 8) were clustered together with 83% bootstrap support. However, *P. sylhetensis* of Buxa Wildlife Sanctuary, West Bengal (T 3) showed distinction from the Assam population of the same species and formed a distinct clade within the *P. sylhetensis* group with 81%, bootstrap separation.
In the *P. tecta* of the Khusiara River (Assam-Bangladesh Border) formed a monophyletic branch at 100% boot strap separation, while the Hajo and Gomerighat samples were grouped together with 100% bootstrap support. This separation indicates higher genetic variation among the geographically isolated populations of *P. tecta*.

Among the 3 subspecies of *P. tentoria*, the *P. tentoria circumdata* samples were collected from different areas of Ganga and Yamuna Rivers of Uttar Pradesh. The results of Principal Component Analysis (PCA) indicated differences among the subspecies of *P. tentoria* and *P. smithii*. PCA projected *P. sylhetensis* to be more closely related to *P. tecta* than *P. tentoria* and *P. smithii*. 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.

Genetic differentiation (*G_{ST}* value 0.93) and total genetic diversity (*H_d* 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_s*) and estimated gene flow (*N_m*).

Although few cross-species microsatellites can 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. Guicking *et al.* (2002) found that genomic fingerprinting via ISSR-PCR provided an insight into the history of *Cyclenys* in comparison to sequence data from cyt *b*, adding promise for this approach.

Beheregaray *et al.* (2003) used two different neutral genetic markers (nuclear microsatellites and mitochondrial DNA) to estimate levels of genetic variability within and among four island populations of Galapagos tortoises (*Geochelone nigra*). Microsatellites, with their faster rates of mutation, are extended to illuminate more contemporary situation compared to mtDNA (Avise *et al*., 1992).
In order to establish the variation in the genome of *Pangshura* species the ISSR has been appeared as powerful tool. These variations supports the validity of the morphologically weakly defined subspecies *P. tentoria tentoria* and *P. tentoria circumdata* as well as *P. smithii smithii* and *P. smithii pallidipes*. However, such observations were not true in establishing differences between two testudo species *Testudo marginata* and *Testudo wessingeri* at ISSR level. Morphologically *Testudo wessingeri* differs from *Testudo marginata* by its smaller size and colour pattern characteristics (Bour, 1996). The rationale behind these morphological differences without differing at genome level in this species is not clear (Perala, 2001a). But it is clear that certain animal species presented reduced body sizes in an environment with limited resources (*Emys orbicularis*) and suggested that *Testudo wessingeri* is not a distinct evolutionary lineage (Fritz, 2003).

The sequence information in the ISSR bands from the present study could be used to develop the species-specific SCAR markers. Furthermore, these bands confirm the presence of microsatellites, whose sequences are complimentary to that of the primers. These microsatellites could be used to develop STS (sequence tagged site) maps. The microsatellite loci, described here, provides potentially useful markers for the assessment of genetic variability within the genus *Pangshura* and across populations of *Pangshura species* and other freshwater turtle species, which perhaps could be used for conservation and efficient management strategies.

### 8.3 Multi-gene sequence analysis of *Pangshura*:

Six (6) out of the ten (10) enzymes produced species-specific patterns in the 4 species of *Pangshura*. Two restriction enzymes (*Fok* I and *Hae* III) recognized the highest proportion of species-specific sequences and generated the diagnostic Restriction Fragment Length Polymorphism (RFLP) pattern has been recorded as useful for differentiating the *Pangshura* species (autapomorphic restriction site) (Table 5.2-5.6). *Msp* I identified single, but species-specific restriction sites in the 16S rRNA gene of all the species analyzed, while *Hae* III recognized 3 restriction sites.
The present study find very clear signal that the 16S rRNA in *Pangshura* and *Batagur* have an evident evolutionary relationship, although it is not totally clear whether this was a relationship of homology or complementarities between the 5 prime and 3 prime halves of rRNA. Two coaxial interactions have been observed in all the three sub-species of *P. tentoria*. (i) between (8,376) and (377,410): $\Delta G = -2.1$ kcal/mol; (ii) between (413,441) and (442,513): $\Delta G = -3.3$ kcal/mol (Table 5.7).

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. The present analysis could be deployed in resolving the position and lineage of each species and subspecies of the genus *Pangshura* and this impression could be supported by the work of certain workers (Georges *et al.*, 1998; Noonan, 2000; Noonan and Chippindale, 2006).

The RNA secondary structure can be determined in the absence of a crystal structure through comparative sequence analysis (Pace *et al.*, 1999), in which a large number of sequences are aligned to reveal the common base pairing pattern. Alternatively, when only one sequence is available, free energy minimization can be used to predict the secondary structure (Hofacker, 2003; Mathews *et al.*, 2004). The present study used the energy minimization method for the prediction on 16S rRNA secondary structure. Free energy minimization has been benchmarked as high as 73% accurate on average for predicting known base pairs for a diverse set of sequences in domains of fewer than 800 nucleotides (Mathews *et al.*, 1999a, b, 2004), but the average accuracy has also been reported as 56% for a different benchmark set of sequences with known structures (Dowell and Eddy, 2004). Several methods have been developed to predict suboptimal secondary structures, i.e. structures with free energy similar to the lowest free energy structure.

8. 4 Phylogenetic relationships among the *Pangshura* species:

The present investigation provides a stable phylogenetic relationship among the *Pangshura* species, with *P. tentoria* and *P. smithii* as sister species and *P. tecta* and *P. sylhetensis* as their successive sister-taxa.
Uncertainties have been appeared with respect to the phylogenetic position of the two sub-species of \textit{P. smithii}, yet the sequence analysis of \textit{P. t. tentoria} and \textit{P. t. circumdata} moderately supported clade under the species \textit{P. tentoria}. The present study supports that \textit{Batagur} is an outgroup and a distinctly separate genus from \textit{Pangshura}. The present study provides an indispensable groundwork for future molecular analysis at the protein level. The choice of molecular data is crucial for phylogenetic analysis and molecular studies can now be tailored specifically for particular phylogenetic groups and/or questions (Lamb and Lydeard, 1994).

The pale-coloured \textit{P. tentoria} from the Ghagra River (Uttar Pradesh, India) was assigned to an intergrade population between \textit{P. t. flaviventer} and \textit{P. t. circumdata} due to its pink shell and head markings, however, \textit{P. smithii} and \textit{P. tentoria} live in this river syntopically (Moll, 1987), Praschag et al. (2007a) reported shared haplotypes in \textit{P. smithii} and \textit{P. tentoria} with the suggestion that interspecific hybridization may happen in syntopically occurring \textit{P. smithii} and \textit{P. tentoria}, but the pale colouration of \textit{P. tentoria} in the Ghagra River may rather be the result of hybridization with the pale coloured \textit{P. smithii pallidipes}, not of subspecific intergradations with pale coloured \textit{P. t. flaviventer}.

Le et al. (2007) analyzed mtDNA and nuDNA sequence data to clarify fundamental phylogenetic relationships in turtles based on examination of 27 specimens of \textit{Batagur}, \textit{Callagur}, and \textit{Kachuga} and they proposed that the five species of the three genera \textit{Batagur}, \textit{Callagur}, and \textit{Kachuga} (=\textit{Pangshura}) are placed in the genus \textit{Batagur}.

However, Spinks et al. (2004) proposed a relationship in the \textit{Pangshura} species based on incomplete taxon sampling using \textit{cyt b}, 12S rRNA gene and R35 gene intron. Therefore, the present findings have been considered as the benchmark in the establishment of \textit{Pangshura} relationship due to complete taxon sampling. Moreover, the present multi-gene phylogeny for the first time reveals the distinctness of the northern and northeastern population of \textit{P. tecta}, which might be due to the existence of two subspecies in \textit{P. tecta}. 

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In an ongoing effort to construct phylogeny of the Indian freshwater turtles and tortoises (Baruah et al., 2011, In preparation), based on a complete taxon sampling of all species and subspecies of Pangshura along with other Indian freshwater turtles corroborate that Pangshura is monophyletic (bootstrap and posterior probability support of 100%) and distinct from Kachuga, and thus support the recognition of Pangshura as distinct genus.

8.5 *In silico* analysis and protein structure prediction of the genus Pangshura:

The molecular evolutionary analysis of Cytochrome b, Cytochrome c oxidase-I, NADH-dehydrogenase 4, RAG1 and RAG2 proteins in this investigation provides a stable phylogenetic hypothesis for all Pangshura species, with the suggestion that *P.smithii* and *P.tentoria* as sister species followed by *P.tecta* and *P.sylhetensis* as their successive sister-taxa. The present study clearly supports that *Batagur* is a distinctly separate genus from Pangshura. The tertiary structure of the proteins from the present study clarify that within the genus Pangshura, the species *P. tecta* and *P. sylhetensis* are the most closely related followed by *P. smithii* and *P. tentoria* as their successive sister taxa.

Using diverse molecular markers, the present study has been able to generate a robust phylogeny with high statistical support values for all nodes, regardless of analysis methods employed, except for the uncertainty in the relationship between the two subspecies of *P. smithii*. The present analysis confirm that the sister relationship between *Batagur* and Pangshura although weakly supported in Diesmos et al. (2005). The results are highly consistent with earlier molecular genetics study involving 12S rRNA genes (Shaffer et al., 1997) that within *Batagur* and Pangshura, all species correspond with well-supported clades.

The structures of all the proteins were found to be statistically significant by the structure verification programs. The present analysis corroborate that the genus Pangshura is monophyletic. The modeling of proteins from the genus Pangshura gains importance for the structural biology and even to the conservation genetic research from several angles.
8.6 Genetic Issues in Freshwater Turtle and Tortoise Conservation:

Turtle geneticists began using molecular genetic techniques in the past primarily to study turtle systematics and the structure of chromosomes. It is now possible to estimate levels of gene flow using a diversity of markers, to estimate migration rates and identify potential migrants, and to determine whether there is sex-biased gene flow in a given group. Markers can be used to quantify the relatedness between the individuals within a population or sampling site and population boundaries.

Genetic diversity represents the raw material to facilitate adaptation to changing environmental conditions through natural selection. Hence, loss of genetic diversity can result in the loss of adaptive potential. Knowledge of genetics is increasingly recognized as a critical element of conservation biology (Moritz, 1994; Soltis and Gitzendanner, 1999). Molecular techniques and methods of statistical analysis derived from evolutionary theory can be used to estimate how genetic diversity is apportioned spatially, how rapidly diversity will be lost over time, to identify crucial forces (anthropogenic or otherwise) contributing to present and future loss of diversity, to gain insight into fundamental aspects of an organism’s biology and to provide informed guidance for conservation and management (Moritz, 1999; Reed and Frankham, 2003; DeYoung and Honeycutt, 2005; Whiteley et al., 2006). Despite the clear importance of genetics as a foundation for understanding turtle biology and directing turtle conservation actions, there is paucity of turtle genetic studies relative to many other taxa.

8.7 Future directions:

The present study provides a strong basis for further analysis concerning population structure and evolutionary relationships of different species of freshwater turtles in India. The study also shows that in silico RFLP can be used not only to distinguish Indian freshwater turtle species from each other, but also from marine turtles, showing different RFLP patterns with the same restriction enzyme (Moore et al., 2003). The study raises the possibility of existence of two sub-species in P. tecta.

The 3D structures generated under the present study can be helpful in structural biology for further investigations on allocation of amino acid residues in each fold,
prediction of active sites, molecular mechanism of function and structure based phylogeny.

Rapid application of new genetic tools and analytical techniques is apparent within the literature on turtles. Turtle geneticists began using molecular genetic techniques in the past primarily to study turtle systematics and the structure of chromosomes (FritzSimmons and Hart, 2007). Since these initial studies, the techniques and markers have improved, and we have now been able to provide better resolution to phylogenetic analysis and to discover aspects of turtle biology. It is now possible to estimate levels of gene flow using a diversity of markers, to estimate migration rates and identify potential migrants and to determine whether there is sex-biased gene flow. At least 42% of freshwater turtles and tortoises are facing a high risk of extinction, and there is a need to focus on intense conservation attention on these species.

The development of molecular tools for freshwater turtles and tortoises is not complete. Obviously there is great potential in exploring and applying entirely new molecular techniques, such as sequencing entire mitochondrial genomes (Parham et al., 2006a,b), development of additional informative nuclear markers (Fujita et al., 2004), or microarrays and beyond.

The emergence and development of DNA sequencing techniques and methods for the analysis of molecular and morphometric data (Felsenstein, 2003) has led to an exponential increase in the number of papers that have included phylogenetic trees for various turtle groups (FitzSimmons and Hart, 2007). On 31st October 2006, it was announced that the first full turtle genome project (for the painted turtle, Chrysemys picta) is moving forward (www.genome.gov/10002154). The genetics and genomics world continue to move at a decidedly non-turtle pace. It is expected that the present study will provide ideas and directions for how to use these amazing genetic resources to study, understand and save the turtles of the world.

As computational methods continue to improve, it may be possible to predict interactions between domains of RNA and proteins or other molecules. These types of correlation may be used to find previously unknown functional sites from genomic sequences. We anticipate that new RNA structures of functional importance will be identified in the years to come.

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