CHAPTER-1
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The turtle and tortoise of east and northeast (NE) India were acquired as subject specifically for the current research work. Various morphometrics data and field record were collected from wild as well as markets of different geographical location of east and NE India. The Institutional ethics committee and Department of Forest, Govt. of India, has allowed us to carry out this scientific research and collection of samples. All the critical examinations and conventional molecular characterization through species level DNA marker of the collected specimens and data analysis (insilico) was done in the Department of Biotechnology, Assam University. The traditional taxonomic method of species identification through morphological characters sometimes fails to detect the actual Chelonian species (Gray 1864; Jerdon 1870; Ernst and Barbour 1998; Adler 2007). Therefore, to facilitate turtle and tortoise conservation in east and NE India the species specific life history traits are to be understood to generate information on their biology and ecology (Bhupathy et al. 1992; Moll 1984; Moll and Iverson 2008; Whitaker 2012).

Therefore, there is an urgent necessity to adopt a rationalized molecular approach for accurate species identification (Crumly 1984; Al-Mohanna and George 2010). DNA barcoding is the widely used recent technique of species identification and is rapid and accurate (Herbert et al. 2003 a, b; Herbert et al. 2004 a, b). Its technique is based on the concept that sequences of a partial (~650 bp) mitochondrial COI show more similarity/ less divergence with same species available in databases (Naro-Maciel et al. 2008, 2010; Reid et al. 2011). This advantage provided by the similarity search in various databases may be accessed to detect the specimen from any of its body parts or at any life stage. Furthermore, DNA sequence based analysis has been proven potential in the forensic investigation of wildlife (Dawnay et al. 2007; Nelson et al. 2007). Therefore, in the present study we have surveyed the turtle and tortoise in east and NE India and identified the species accurately through DNA barcoding technique. These approaches can facilitate protection from incriminate hunting and poaching by tracing the unknown samples and conservation of the turtle species in the known distribution area.
1.1 Common lineaments of Turtle and Tortoise:

Turtle, tortoise and terrapins are reptiles of the order ‘Testudines’ (Superorder-Chelonia) and are characterised by a particular bony or cartilaginous shell that acts as a shield (Annandale 1915). These groups sometimes refer to the Testudines as a whole and include both extant (living) and extinct species (Gaffney 1975 a). Testudines have survived on earth since the existence of dinosaurs and are known as the oldest animal of all surviving reptiles (Chun et al. 2008). The Testudines shell is unique that protect them over 220 million years of altering climates and despite the evolution of vertebrate predators (Barth et al. 2004). As similar to other known reptiles, Testudines are ectotherms and their internal temperature changes along with the atmosphere, commonly called as cold-blooded animal (Biswa and Acharjyo 1984). Chelonians breathe air and do not lay eggs underwater, although many species survive in or around water similar to other amniotes (reptiles, dinosaurs, birds, and mammals) (Rieppel 2000; Hedges 2012). The largest turtle groups are aquatic; some are aquatic or terrestrial but tortoise are fully terrestrial in wild. The classification of Testudines has been a controversial issue for several years and after many scientific contributions the exact systematics of these reptiles are still intuitive (Turtle Taxonomy Working Group 2009, 2010, 2011, 2012).

Scientific classification:

Kingdom: Animalia
Phylum: Chordata
Class: Reptilia
Order: Testudines

Complete Lineage:

Cellular organisms; Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Deuterostomia; Chordata; Craniata; Vertebrata; Gnathostomata; Teleostomi; Euteleostomi; Sarcopterygii; Tetrapoda; Amniota; Sauropsida; Testudines; Cryptodira;

- Trionychoidea; Trionychidae (Softshell turtle)
- Testudinoidea; Geoemydidae (Hardshell turtle)
- Testudinoidea; Testudinidae (Tortoise)
- Chelonioidea (Sea turtle)

- Cheloniidae
- Dermochelyidae
Turtle and tortoise are characterized by a shell that completely encloses both of the limb girdles. The shell morphology is very unique and mysterious for all the extant Testudines species (Pritchard 1979). The carapace and plastron colour variation sometimes looks alike in their different life stages. All living Testudines members are divided into two suborders based on their unique neck morphology. One group can pull their neck inside the shell between the shoulders; hence the name Cryptodira, which implies ‘hidden neck’ and the other group, can keep their neck beside the shoulder; namely Pleurodira, the ‘side-necked’ turtle. Many representatives of Cryptodires species have their foot marks in most Southeast Asian countries. This group of animals is found in both aquatic and terrestrial environment and this unique morphology invariably expressed due to their different ecological substances (Smith 1931; Pritchard 1967).

1.2 Ecology and life history:

All the extant turtle and tortoise inhabit a wide variety of environments; the open seas, tropical ridges and coastlines, saltwater, freshwater areas, and terrestrial biomes; including deserts, rainforests, mountains, and prairies (Talukdar 1979). This group of reptiles is generally herbivorous, omnivorous and occasionally carnivorous in nature (Ahmed et al. 2009). The majority of turtle are omnivorous, but certain land tortoise and sea turtle are strict herbivores. Although ectothermic, but many turtle bask in the sun to enhance their body temperature to an optimal point. Most turtle cannot be energetic during very hot or very cold periods of time. Therefore, hibernation in winter and aestivation in summer is common for members of this group. Being an amniotes, all turtle and tortoise hold the basic approaches of laying eggs in their land nests. It has been also reported that the sex of most turtle species is also influenced by environmental factors (Barman 1996).

1.3 Distribution pattern:

Currently, turtle and tortoise are represented by as many as 460 taxa (species and subspecies) found throughout the tropical and temperate regions of the world (The Reptile Database 2013). In Southeast Asian countries, the east and NE regions of India harboured abundant population of many turtle and tortoise species and had been regarded as a treasure trove of this particular reptile’s diversity.
Globally, 305 species within 12 families of extant turtle and tortoise are found in 7 major biogeographic regions of the globe (Buhlmann et al. 2009). At the beginning of the 21st century, turtle represent one of the most threatened groups of vertebrates, currently 228 recognized species have been included in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (IUCN 2012) and approximately 11% under Low Risk/ Near Threatened; 5% under Low Risk/ Least concern; 1% under Low Risk/ Conservation dependent; 12% under Least Concern; 4% under Near Threatened; 25% under Vulnerable; 14% under Critically Endangered; 19% under Endangered; 3% under Extinct and 5% under Data deficient status (Fig. 1). Among the total surviving turtle and tortoise in the world, 77 endemic species are found in Asian countries (Pakistan to Japan, including Indonesia and Philippine archipelagos; Oriental and Eastern Palearctic); among them more than 19 different species presently inhabited the Indian subcontinent (Fig. 2).

![IUCN status of the world’s Testudines species (IUCN-2012.2). Most of the species are included in Vulnerable or Endangered status.](image)

The eastern and NE region of India shares two biodiversity hotspots of the world: The Eastern Himalayan and The Indo-Barma (Ravindranath et al. 2011). The NE region is rich in biodiversity because this region sits at the biogeographical crossroads of two continental plates; many species are endemic and still many are
remained to be described from the region. At least 353 new species have been
discovered in the Eastern Himalayas, equating to an average of 35 new species finds
every year for the last 10 years (Traffic 2004). Altogether, 34 turtle and tortoise
occur in the Indian subcontinent and of these, Indotestudo travancorica, Nilssonia
leithii and Vijayachelys silvatica are strictly endemic to India. Table 1 shows a total
31 different Chelonians including marine species were found in east and NE regions
(Das 1991, 1995). Fig. 3 shows the known distribution of 18 Chelonian species in
east and NE India which also shares other regions and the range expansion of
Indotestudo forstenii remain inconclusive.

1.4 Importance in various arenas:

Testudines are the most important long living creature of the earth and play a vital
role in the eco-system (Reisz and Head 2008). This group is also very lucrative for
mankinds due to its various economic and therapeutic importances; although, this
activity creates additional pressure on the natural population.

Turtle is a popular symbol in mythology because of their longevity and
appearance. In ancient history, the Mesopotamian people believed that the turtle were
associated with one of their Gods called ‘Ea’, and in India the Hindu mythology one
avatar of ‘Vishnu’ is said to be the giant turtle ‘Kurma’. The turtle meat has been
considered as a high value because; its medical value is extremely high. Traditional
Chinese medicine (TCM) has used turtle for food and medicine for hundreds of
years. Nearly 15-20% of the Ayurvedic medicine is based on animal derived
substances in India (Gibbons et al. 2000). Most of the ethnic groups of east and NE
India are fond of turtle meat. The turtle blood is assumed to be a treatment for piles
and fistula and the meat is supposed to be a remedy for gout and arthritis, while the
carapace of the softshell turtle is also used as a medicine for skin disease, fever,
rheumatism, earache, sore throat, and swelling (Das et al. 2012 a, b). Turtle soup is
highly recommended as the best possible medicine to revive failing kidneys. Thus,
due to high demand of turtle for human use, the natural population of turtle are
facing severe threats from overexploitation. Therefore, in-situ and ex-situ
conservation of natural habitats as well as in the museum is urgent to protect this
nature’s informant of the late Triassic period (Bohm et al. 2012).
Phytoplankton flow patterns of India. This baseline map is from Fulthorpe et al. (2009).

Figure 2: Global patterns of species richness across all examined 305 terrestrial species (1000–45 and freshwater until 260). Scale of colour codes indicates the number of species for each area. The arrow shows the world’s greatest increase in the coverage of grassland species.
Figure 3: The small-scale maps showing the known distribution of certain Clethrionomys species of east and NE India. The grey colour in map denoted for the land tortoise, blue colour for softshell turtle and green colour for Hardshell turtle distribution.
Table 1: A list of the extant Chelonian species found in the Indian subcontinent.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Common Name</th>
<th>Scientific Name</th>
<th>CITES</th>
<th>IWPA</th>
<th>IUCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Indian softshell turtle</td>
<td>Nilssonia gangetica</td>
<td>Appendix I</td>
<td>Sch-I</td>
<td>VU</td>
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<tr>
<td>2</td>
<td>Black softshell turtle</td>
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<td>Sch-IV</td>
<td>Extinct in the wild</td>
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<td>Indian peacock turtle</td>
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<td>Sch-I</td>
<td>VU</td>
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<td>VU</td>
</tr>
<tr>
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<td>Narrow-headed softshell turtle</td>
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<td>Sch-II</td>
<td>EN</td>
</tr>
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<td>6</td>
<td>Indian flapshell turtle</td>
<td>Lissemys punctata</td>
<td>Appendix II</td>
<td>Sch-I</td>
<td>Low risk, Least concern</td>
</tr>
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<td>Malayan softshell turtle</td>
<td>Amyda cartilaginea</td>
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<td>Not listed</td>
<td>VU</td>
</tr>
<tr>
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<td>Sch-I</td>
<td>EN</td>
</tr>
<tr>
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<td>Malayan box turtle</td>
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<td>VU</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Keeled box turtle</td>
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<td>Appendix II</td>
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</tr>
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<td>Morenia petersi</td>
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<td>Not listed</td>
<td>VU</td>
</tr>
<tr>
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<td>Indian roofed turtle</td>
<td>Pangshura tecta</td>
<td>Appendix I</td>
<td>Sch-I</td>
<td>Low risk, Least concern</td>
</tr>
<tr>
<td>14</td>
<td>Assam roofed turtle</td>
<td>Pangshura sylhetensis</td>
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<td>Sch-I</td>
<td>EN</td>
</tr>
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<tr>
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<td>Sch-I</td>
<td>VU</td>
</tr>
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<td>Sch-I</td>
<td>Low risk, Near threatened</td>
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<tr>
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</tr>
<tr>
<td>19</td>
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<td>Sch-I</td>
<td>CR</td>
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<td>Sch-I</td>
<td>CR</td>
</tr>
<tr>
<td>21</td>
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<td>EN</td>
</tr>
<tr>
<td>No.</td>
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<td>Scientific Name</td>
<td>Status</td>
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<td>IWPA</td>
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<td>Sch-IV</td>
<td>VU</td>
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<td>Not listed</td>
<td>VU</td>
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<td>Sch-I</td>
<td>EN</td>
</tr>
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<td>Asian brown tortoise</td>
<td><em>Manouria emys</em></td>
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<td>Sch-IV</td>
<td>EN</td>
</tr>
<tr>
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<td>Elongated tortoise</td>
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<td>Sch-IV</td>
<td>EN</td>
</tr>
<tr>
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<td>Sch-IV</td>
<td>EN</td>
</tr>
<tr>
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<td>Travancore tortoise</td>
<td><em>Indostedus travancorica</em></td>
<td>Appendix II</td>
<td>Sch-IV</td>
<td>VU</td>
</tr>
<tr>
<td>26</td>
<td>Indian star tortoise</td>
<td><em>Geochelone elegans</em></td>
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<td>Sch-IV</td>
<td>VU</td>
</tr>
<tr>
<td>27</td>
<td>Loggerhead sea turtle</td>
<td><em>Caretta caretta</em></td>
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<td>-</td>
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</tr>
<tr>
<td>28</td>
<td>Olive ridley sea turtle</td>
<td><em>Lepidochelys olivacea</em></td>
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<td>-</td>
<td>VU</td>
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<tr>
<td>29</td>
<td>Green sea turtle</td>
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<td>Hawkshill sea turtle</td>
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<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>31</td>
<td>Leatherback turtle</td>
<td><em>Dermochelys coriacea</em></td>
<td>Appendix I</td>
<td>-</td>
<td>CR</td>
</tr>
</tbody>
</table>

Common name, scientific name and species status of Indian turtle and tortoise species. CITES= Convention on International Trade in Endangered Species of Wild Fauna and Flora; IWPA= Indian Wildlife Protection Act, 1972; IUCN= International Union for Conservation of Nature and Natural Resources; Appendix I= Most threatened species (No trade allowed); Appendix II= Species likely to be threatened due to unregulated trade (Trade is allowed through licensing); Schedule I and II = Provide absolute protection-offences under these are prescribed the highest penalties; Schedule IV= Protected, but the penalties are much lower. VU= Vulnerable; EN= Endangered; CR= Critically Endangered.
1.5 Conservation initiatives prioritize to Chelonians:

Several international, national or regional conservation organizations viz., Turtle Survival Alliance (TSA), Indian Turtle Conservation Program (ITCP), Wildlife Institute of India (WII), International Union for Conservation of Nature/Species Survival Commission (IUCN/SSC), Tortoise and Freshwater Turtle Specialist Group, World Wildlife Fund (WWF) India, The Madras Crocodile Bank Trust and centre for Herpetology (MCBT), Wildlife Protection Society of India (WPSI), Indian Scientific and Industrial Research Organization (SIRO), Centre for Wildlife Research and Conservation Action (CWRCA), The Turtle Conservation & Research Programme (TCRP) and The Rufford Small Grants Foundation are concerned to protect wetlands, wild habitats and also attempting to regulate the trade of endangered turtle species. The gap of research in this particular field should be found out for better acquisition and strategic conservation (Gong et al. 2006; Das 2009 a).

The expiration of biodiversity especially Chelonians often reduces the productivity of ecosystems. While the extinction of species has always occurred as a natural phenomenon, the rapidity has accelerated as a result of human activity. Gradually, ecosystems are being fragmented or eliminated, and infinite species are in decline or already extinct from nature. The conservation of biological diversity is a common concern of humankind and is an integral part of the development process. It is important to study the ecosystems, species, and genetic resources from distant geographical location globally. Many Taxonomists, conservationist and biologists are presently involved to protect the biodiversity by utilizing traditional conservation efforts and also by the application of biotechnology for further exploration (Van Dijk et al. 2000).

1.6 Molecular markers in species identification:

Recent developments in biotechnology have opened the possibility of employing various types of molecular tools to identify and use genomic variation improvement of various organisms. The idea of using variations at the DNA level as first generation genetic markers started with the Restriction Fragment Length Polymorphism (RFLP) (Sambrook and Russel 2001). When the DNA of different
individuals is digested with restriction enzymes, differences in size of the resulting fragments of DNA can be visualized via Southern hybridization with labelled probe. The conventional hybridization based assay for detecting DNA level variations was replaced by the Polymerase Chain Reaction (PCR) based assay and it has been evolved to detect variations at the DNA level by specific primer for turtle and tortoise (Engstrom et al. 2007).

The next generation of molecular markers responsible for various revolutions in the field of Molecular genetics revolution is micro satellites- arrays of tandemly repeated nucleotide DNA sequences used in Testudines (Schwartz et al. 2003; Alacs et al. 2009 b). But, recent developments in molecular biology have opened the possibility of employing various types of molecular markers to identify various organisms by using genomic variation; which expressed in morphometrics characters.

1.7 Inventorying of biodiversity through DNA markers:

Taxonomy is the field of biology which deals with the description, morphological identification, nomenclature and classification of biological organisms; although it is ineffective for clear distinction among the earth’s biota. The assessment of biodiversity in a given ecosystem depends on making detailed inventories of species and varieties. This is an astonishing task and according to estimates, out of the 10 million existing species, only 1.5 million have been described so far (Borisenko et al. 2008). With the advent of molecular genetics, data on biodiversity are being collected at an unprecedented rate by DNA sequencing. A global effort to survey genetically diverse organisms in the earth through mtDNA marker as well as nuclear DNA marker is underway (Arif and Khan 2009; Vamberger et al. 2011).

1.7.1 Mitochondrial DNA (mtDNA):

The significance of mtDNA data of any biological species is underlying a handy and authentic technique presently. The mitochondrion is the organelle contains its own DNA and encodes by 37 genes (Fig. 4). It is evident that the mitochondrial genome of animals is a better target of analysis than the nuclear genome because of its lack of introns and its limited exposure to recombination and haploid mode of inheritance.
The mtDNA has a relatively fast mutation rate, which results in a significant variation in gene sequences between species and carried comparatively small variation within Testudines species (Caccone et al. 1999; Praschag et al. 2007 a). The whole mitochondrial DNA with its different loci makes it a useful source of genetic information for scientists, involved in population genetics and evolutionary biology of any Cheloniens (Zardoya and Meyer 1998; Serb et al. 2001; Parham et al. 2006 a, b; Zhang et al. 2008; Drosopoulou et al. 2012). Although several loci (12S, 16S, cytb etc.) have been suggested, a 648 bp region of the cytochrome oxidase subunit I (COI) mitochondrial gene has provided strong species-level resolution ability for varied animal groups (Hsieh et al. 2008; Lee et al. 2009; Linacre 2012).

![Diagram of mitochondrial genome and relevant arrangement of genes.](image)

**Figure 4:** Diagram of mitochondrial genome and relevant arrangement of genes.

### 1.7.2 DNA barcoding as species level marker:

DNA barcoding for species identification is based on focusing amplification of only 648 bp of the mitochondrial COI gene near its 5′ end. Paul D. N. Hebert (Hebert et al. 2003 a, b), a professor at the University of Guelph, Ontario, Canada, first time brought this concept of DNA barcoding with an announcement that it would serve a basis for identification of entire global biological samples. The COI gene of mitochondria has two important advantages.
(i) Firstly, the universal primers for this gene are very robust, enabling recovery of its 5' end and (ii) Secondly, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene.

In common with other protein coding genes, its third-position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than other locus. In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species. This gene is more likely to provide deeper phylogenetic insights for the entire animal biodiversity (Desalle et al. 2005; Gregory 2005; Cywinska et al. 2006; Desalle 2006; Rubinoff et al. 2006; Bergsten et al. 2012). The global success of DNA barcoding led the enthusiasts to think for a gathering of large scale DNA barcode datas in databases from all over the world for every species that persist. This common campaign has been given the name ‘The Barcode of Life Project’. Several earth biotas have been included in every major initiative like: (i) Consortium for the Barcode of Life (CBOL) (ii) FISH-BOL, the Fish Barcode of Life etc. Although, the reptiles are not entrusted in any such major initiatives still date, but recently, an initiative namely the new barcoding campaign for cold-blooded amphibians and non-avian reptiles (COLD-CODE) are taken by the Kunming Institute of Zoology (KIZ) and funded by the Chinese Academy of Sciences and National Science Foundation of China. Similarly, these types of initiatives may help to know the exact biodiversity of herpetofauna in the earth. Compiling a public library of species specific sequences and their nucleotide attributes will make this new conception (Vilgalys 2003; Will et al. 2005; Yoo et al. 2006; Wong et al. 2009).

1.8 Genomic traits, phylogenetics and molecular evolution:

Genetic variation is mostly considered as neutral but a single base change in and around a gene can affect its expression or the function of its protein products. Most alterations are deleterious and so are eventually eliminated through purifying selection within the nature. However, positive mutations can sweep through the population and become fixed, thus contributing to species differentiation (Janzen et al. 2005; Hajibabaei et al. 2006; Bhattacharjee et al. 2012 a, b).
Molecular phylogenetics, a graphical representation of evolutionary history applies an amalgamation of molecular and statistical techniques to infer evolutionary relationships among turtle and tortoise or genes (Cao et al. 2000; Barley et al. 2010). According to modern evolutionary theory, all organisms on earth have descended from a common ancestor, which means that any set of species, extant or extinct, is related. Within the past few decades, this field has been further stimulated and delineated for many complex organisms. As mounds of genomic data become publicly available, phylogenetic approaches serve diverse applications in biological fields especially in Testudines (Fujita et al. 2004; Chandler and Janzen 2009).

Molecular phylogenetics and dating of speciation events is one of the major objectives of evolutionary studies (Avise et al. 1992; Feldman and Parham 2002; Caccone et al. 2004). The molecular clock hypothesis has been used to estimate the time of occurrence of events called speciation or radiation. The evolutionary clock alone can only say the rate of evolution with time when merged with the fossil records, and allows the dating of branching events on phylogenetic trees. This technique is an important tool in molecular systematics, the use of genetic information to determine the correct scientific classification of organisms (Near et al. 2005).

1.9 Research gap in relevance to Testudines identification:

Over-exploitation and unregulated trade are the primary causes for sharp declines in many turtle species populations, especially from Asia (Van Dijk et al. 2000; Altherr and Freyer 2000; Fong et al. 2007). The continuity of such an ancient and iconic group is under concerted attack, and they have become prominent casualties of the alarming global biodiversity disaster. Without conducting conservation, a major portion of turtle diversity could be lost over the next century. Therefore, there is an urgent need to protect the turtle gene pools particularly in east and NE India before they reach the brink of extinction.

This dilemma often leads to Indian Testudines species identification and is a major drawback in turtle biodiversity research when traditional morphological methods are the basis. Accurate species level identification is strictly essential prior endeavour to the development of package of practices and conservation application
of any prioritized species (Bowen et al. 1993; Stoeckle 2003). In recent times, the
distribution patterns of many turtle and tortoise are also creating confusion (Das and
Gupta 2011). Since, the beginning of biotechnological methods, and principle being
gene sequencing, the changes and reshuffle in the four nucleotides set the backbone
of turtle and tortoise identification and their evolution (Engstrom and McCord 2002;
Austin et al. 2003; Alacs et al. 2009 a, b). Conservation biologists are also
couraged to consider using genetic data and concepts when developing
conservation strategies for turtle. Therefore, we have taken such objectives for the
present research, which will facilitate the east and NE Indian Chelonian conservation
genetics by proper identification through rationalized molecular markers based
approaches along with morphological studies (Kundu et al. 2013 a, b).

1.10 Objectives of the research:

Having known the lack of information in the global database on the Indian
Testudines species, the following objectives were set to explore and to bridge the gap
of knowledge:

1) A detailed study of the Testudines diversity of east and northeast India based
on morphological characteristics.

2) Development of species specific molecular marker as DNA barcode by
Sequencing of coxl gene of mitochondrial genome.

3) Assessment of haplotypes in turtle and tortoise species in east and northeast
India based on barcode region.

4) To establish the phylogenetic relationship within the order Testudines by
analyzing the barcode region of coxl gene.

5) Identification of characteristic amino acid variation within the Testudines
species under study.