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Ecology and Genetic Identification of Freshwater Turtles in Pakistan

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ABSTRACT

***Background*:** The turtle population plays an important role in sustaining the water ecosystem by minimizing pollution from water. The identification and molecular investigation of freshwater fauna is essential for conservation of the species that are near to extinction. The quality of water, type of flora, fauna, and environmental condition are the major factors that directly affect the distribution of freshwater turtles. Two families including eight species of freshwater turtles are found in Pakistan. The Geoemydidae (*Geoclemys hamiltonii*, *Hardella thurjii*, *Pangshura smithii*, and *Pangshura tecta*) and Trionychidae (*Chitra indica*, *Nilssonia gangetica*, *Nilssonia hurum*, and *Lissemys punctata andersoni*). Studies on the species diversity and habitat of freshwater turtle have not been focused previously in the region. The present study was the first conducted to estimate the habitat and genetic diversity of freshwater turtles using 12S rRNA (ribosomal RNA) gene in Pakistan.

***Materials, Methods & Results*:** A total of 26 samples were collected from various localities using hand net, cast net, gills net, steel hooks, thick chemical wire, using chicken intestine and small fishes. The collected turtle specimens were morpho-taxonomically categorized into two genera, *Lissemys punctata andersoni* (n=13, 50%) and *Nilssonia gangetica* (n=13, 50%). The collected species showed an aggressive and active behavior in captivity during summer. Genomic DNA was extracted from collected specimens and used in PCR reaction by using specific primers for the amplification of short fragments of 12S rRNA gene. Analysis of generated sequences confirmed the existence of *L. p. andersoni* in the region. The generated sequences of *L. p. andersoni* correspond to Clad A and showed a close resemblance among different species of the genus *Lissemys*.

***Discussion*:** The climatic change such as temperature and rainfall have great effects on the occurrence of turtles. Habitat degradation occurred due to various factors such as draining wetlands, deforestation, converting clear water rivers to stagnant multi-purpose reservoirs and mortality on roads when turtles move around to feed. Current study concluded that the freshwater turtles *L. p. andersoni* and *N. gangetica* are interested in natural feeds. The analysis of 359 bp of 12S rRNA gene of the genus *Lissemys* turtles showed relationships of these turtles with cyclanorbines flap shell turtles, which agrees with previous reports. The African taxa are paraphyletic with respect to the Asian *Lissemys*. The ancestors of the extant genus cyclanorbines spread from North America to Asia [26]. It should be expected that each of the 3 taxa, *L. p. andersoni*, *L. p. punctata* and *L. scutata* represents a distinct genetic lineage. Present molecular investigation concluded that Clad A comprising *L. p. punctata*, *L. scutata*, *L. cylonensis* also include *L. p. andersoni* species. Clad B also contains one sequence from India, identified as *L. p. andersoni*. Their classification as conspecific evolutionary lineages are suggested by similar genetic divergences, the observation of mismatches between morphology (spotted vs. unspotted) and mitochondrial haplotypes in clades A and B. The clades A and B provides evidence for gene flow between the spotted subspecies *L. p. andersoni* and adjacent populations with unspotted flap shell turtles. This study is the first investigation about the habitat and of the endemic turtle species *L. p. andersoni* and *N. gangetica* in Pakistan. The genetic identification followed by phylogenetic analysis based on 12S rRNA partial genes revealed a closest similarity with the sequences generated for the same species from the neighboring countries. This study provided information to conduct further molecular studies that are essential to provide significant genetic data about turtle species.

Keywords: turtle, ecology, diversity, phylogeny, Pakistan.

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INTRODUCTION

Freshwater turtles in muddy environments, medium-size rivers, dams, and ponds are considered as water purifiers as it contributes to the reduction of algal bloom and are indicators of pollution [4,23,28,36]. Soft-shell turtles are a useful source of protein [11,17,37] and used for purifying blood and cure respiratory diseases and intestinal disorders [13,24]. Several factors such as habitat fragmentation, pollution, introduction of exotic species, hunting, and global climate change affect the biodiversity of turtle fauna [5,9,19,21,22]. The conservation and hatch and release programs of freshwater turtles was initiated in the last two decades for the purification of different freshwater bodies [16,25,31,32,37].

Two families including 8 species of freshwater turtles are found in Pakistan [2,3,15,17,31]. The Geoemydidae (*Geoclemys hamiltonii*, *Hardella thurjii*, *Pangshura smithii*, and *Pangshura tecta*) and Trionychidae (*Chitra indica*, *Nilssonia gangetica*, *Nilssonia hurum*, and *Lissemys punctata andersoni*). *Lissemys* is unique among all turtles due the peripheral bone and flexible rubbery posterior shell margin [6].

Pakistan banned the export of wild mammals and reptiles in 1981 and struggled for conservation and protection of threatened species. However, illegal trade of turtles and their body parts have been regularly reported [1,15,16,18].

The harmful effects on freshwater turtles due to habitat destruction in Pakistan have been documented and mostly associated with anthropogenic activities [16,19,21,31]. Studies are scarce on the molecular identification and ecology of freshwater turtles in Pakistan. Therefore, present study aimed to investigate the ecology and molecularly identify these turtle species.

MATERIALS AND METHODS

Study area

The study was conducted in District Malakand namely Esaro Banda, Musamina, Roos Banda, Camp and Ghari Usmani Khel and specimens were collected during 2018-2019. A total of 5 sites were surveyed in the selected District. The Global Positioning System was used to obtain geographic coordinate data on a Microsoft Excel spreadsheet to create a map for the selected study areas using ArcGIS 10.3.1 (Figure 1).

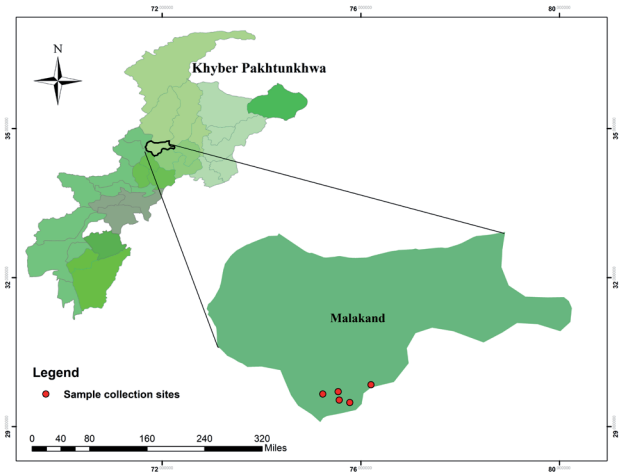

Figure 1. Map of Khyber Pakhtunkhwa (top), map of district Malakand, study area (bottom).

Capturing

The samples of freshwater turtles were captured by hand net, cast net, gills net, steel hooks, thick chemical wire, using chicken intestine and small fishes.

Identification of the turtle

The freshwater turtle species identification was based on standard published literature and keys [12,14,17,32]. The morphometry of turtles was carried out as described previously [33] based on carapace width (CW), carapace length (CL), plastron width (PW), plastron length (PL) and body weight (BW) [33].

Turtle habitat and population

Habitat preference of each freshwater turtle species was determined by field observation directly. Habitat types and water status in the targeted areas were recorded. The survey was conducted in morning time at 9 am to 2 pm and at nighttime 9 pm to 11 am in winter as well as in summer. Mostly *L. p. andersoni* and *N. gangetica* species come out of water for basking at 11 am to 1 pm in different targeted sites. To estimate the total population of turtles, the adopted method [27] was followed in which accurate determination of the species was done.

The water and air temperature were recorded through the use of a mercury thermometer which is essential for the conservation. To determine the physico-chemical parameters of water during field survey this data has been used to analyze the basking, ambient temperature and feeding activities. Physio-chemical parameters of water monitored each week for the period of one year in captivity.

Study of ecological in captivity

Culture conditions were created with use of tanks with following dimensions: total length (TL) 9, width 1.5 and height 1.2 m, in Abdul Wali khan University Mardan (AWKUM), KP, Pakistan. Cemented tank was made with suitable space inside the boundary of the tank which was used for the turtle feeding and breeding behavior and observed on daily bases (Table 1). Weekly physico-chemical parameters of water were analyzed in the analytical chemistry laboratory of AWKUM (Table 2). Average parameters were recorded, and the data was analyzed statistically using one-way ANOVA.

Blood sampling and DNA extraction

First the weight of the sample was determined and then 1 mL of i.m. ketamine (ketasol)[1] was injected and waited up to 10 min until complete anesthesia. Blood from the femoral vein through a 5 cc sterilized syringe was taken in an EDTA tube and stored at -20ºC.

A total of 13 morphologically identified samples of *L. p. andersoni* in the selected area were used for genomic DNA extraction through standard manual phenol chloroform method [30]. The concentration of DNA samples was quantified using a NanoDrop™ 2000/2000c Spectrophotometers[2] and stored at -20ºC until further processing. Partial fragment of 12S rRNA gene for *L. p. andersoni* was amplified by PCR (HT, ILF, UK) using specific primers. PCR reaction was performed in a total volume of 20 mL reaction mixture with composition of 1 mL of each forward (TACAAAAATATCCGCCAGAAAAC) and reverse primer (CTCAGGTC CGGTTTTAATTG), 2 mL of template DNA (100 ng), 4 mL of deionized water and 12 mL of master mix (DreamTaq PCR Master Mix [2X]2. The thermocycling conditions for reaction were optimized as 5 min at 95ºC for initial denaturation followed by 40 cycles of denaturation at 95ºC for 30 s annealing at 60ºC for 30 s, and extension at 72ºC for 1 min, and final extension for 10 min at 72°C. PCR products were confirmed by running 2% ethidium bromide-stained agarose gel with a 50 bp DNA marker[2]. The results were visualized using the GelDoc[3].

DNA purification and sequencing

The amplified PCR products were purified with the GeneClean II kit[4] according to the manufacturer's instructions. The purified PCR amplicons (13 PCR product samples) were sequenced unidirectionally by Macrogen[5]. The obtained sequences were trimmed using SeqMan V 5.0 (DNASTAR[6]) to remove poor quality sequences and further analyzed by using BioEdit Sequence Alignment Editor V. 7.0.5[7] [10] and subjected to BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) to collect the relevant sequences of closely related with the species of *L. p. andersoni* available in GenBank.

Phylogenetic analysis

The obtained trimmed sequences were aligned using ClustalW in BioEdit V 7.0.5[7] [10]. The phylogenetic tree for 12S rRNA sequences were generated based on the maximum likelihood method in MEGA-X[8], with 1000 bootstrapping [20].

Table 1. Feeding habit and behavior of freshwater turtles in captivity.

Species	Habitat	Food	Behavior
Lissemys punctate andersoni	Shallow pools, lakes, rivers, stream, ponds, rich flora, less fauna	carnivore/herbivore: *Anura* , *Blattodea*, *Hirudinea*, *Anisoptera*, and plants, *Mentha longifolia*, *arundo donax, Origanum vulgare* , *Cynodon dactylon* , *Convolvulus arvense*, *Desmostachya bipinnata* , *Channa gachua, Bufo surdus*, *Lumbricina*	Shy and aggressive
Nilssonia gangetica	River, lakes, permanent ponds, rick fauna and flora.	Omnivore: *Glyptothorax slocki*, *Tor putitora*, *Channa punctatus*, *Channa gachua, Bufo surdus*, *Lumbricina*, *Mastacembelus armatus* *Anas*, *Gallus gallus domesticus*, *Blattodea, Anisoptera* and plant like *Amaranthus viridis* , *Cynodon dactylon, Dalbergia sissoo*, *Desmostachya bipinnata*, *Ficus carica*, *Medicago minima*, *Tribulus terrestris*,	Very aggressive

Table 2. Physico-chemical parameters of water in freshwater turtles' captivity.

Time Duration	Conductivity (ms/cm)	Salinity (g/L)	pH	Resistivity	TDS (mg/L)	Water temp (˚C)
Initial First Week	3.01 ± .015	0.385 ± .0025	7.77 ± .020	1.57 ± .375	0.25 ± .020	22 ± 2.00
Final First Week	2.87 ± .020	0.185 ± .003	6.8 ± .100	1.58 ± .375	5.04 ± .020	25 ± 1.00
Initial Second Week	3.02 ± .020	0.384 ± .003	7.77 ± .020	1.58 ± .369	0.26 ± .020	23 ± 1.00
Final second Week	2.8 ± .100	0.177 ± .002	6.6 ± .200	4.92 ± 6.128	5.02 ± .010	26 ± 1.00
Initial third Week	2.8 ± .100	0.385 ± .002	7.76 ± .030	1.37 ± .020	5.02 ± .020	21 ± 2.00
Final third Week	2.8 ± .100	0.186 ± .002	6.5 ± .200	1.98 ± .015	5.02 ± .010	27 ± 2.00
Initial fourth Week	2.8 ± .100	0.385 ± .003	7.77 ± .020	1.58 ± .363	5.02 ± .02	23 ± 2.00
Final fourth Week	2.8 ± .000	0.176 ± .002	6.4 ± .200	1.97 ± .015	5.02 ± .020	25 ± 2.00

RESULTS

The census observed two species of turtle *L. p. andersoni* and *N. gangetica*. The targeted station was covered by grassy vegetation. It included such as mud content, hard soil, shallow water, and sandy rocky bank. All collected species belong to the family Trionychidae. The abundant population of *L. p. andersoni* was observed in river Camp (26.92%) while *L. p. andersoni* was absent in Musamina persh and *Ross* banda. The abundant population of *N. gangetica* was observed in Ross banda 23.07% and near camp and Ghari Usmanikhel were found free of *N. gangetica*. The locality of Esaro banda had both species (19.23%) where the area was covered by muddy soil and huge amounts of fauna and flora. During the survey, recorded water and air temperature was 30 - 43.88ºC, conductivity 720 - 824 (ms/cm), pH 7.6-8, resistivity 1.30 - 1.37, TDS 350 - 410 (mg/L) and salinity 0.35 - 0.42 g/L. The highest number of turtles were recorded in summer session.

The C/L, W, P/L, W and TL range of *L. p. andersoni* recorded 0.1217 - 0.383 m while the *N. gangetica* range 0.253 - 0.7114 m. The ratio of body weight *L. p. andersoni* 0.19 - 1.89 kg and *N. gangetica* 6.30 - 12.38 kg. In tank first The L. p. andersoni were consumed differently 4 animals and 9 plants. The *N. gangetica* were able to consume 12 animals and 6 plants. The *L. p. andersoni* showed shy and aggressive behavior while *N. gangetica* were extremely aggressive in behavior from April to September in cemented tanks especially in summer from 11am - 3pm (Table 1).

Habitat composition varied considerably among the targeted sites depending on the nature of banks and availability of fauna and flora. From June to August the turtle's habitat degraded heavy rainfall and major flood following dislodged and inundated most of our water level monitoring stations. Major sources of water come out from ground and also contaminated water with sewage coming from shop houses and institutes, water as polluted.

Sequence and phylogenetic analysis

Thirteen nucleotide sequences with 359 bp were obtained in this work and showed 100 % identity in nucleotide sequence. A total of 44 similar sequences of turtle 12S rRNA gene were downloaded from NCBI. Comparative analysis of a current sequence showed 99-100 % identity in nucleotide sequence, with 12S rRNA sequences reported from India (FR850504, MF041983, FR850529, FR850513, FR850522) and Sri Lanka (FR850544). Sequences generated in this study were deposited to the GenBank (MT000050). In phylogenetic analyses, the sequence from Pakistan was clustered with *L. p. andersoni* from India and Sri Lanka (Figure 2).

DISCUSSION

The present study investigated the ecology and molecular phylogeny of freshwater turtles in Pakistan. The turtles were reared in captivity for one year and the water parameters were checked thoroughly. Studies are conducted on ecology and genetic diversity of freshwater turtles while attempting to address strategies for turtle's conservation poses a problem. The Indian flap-shell turtle is an abundant species, due to its affections to stagnant waters of rivers, ponds, shallow streams, lakes, marshes and extends into the urban sewage system. The *N. gangetica* were abundant with a reasonable population in all surveyed sites [28,35,31]. As far as the present study reported that *L. p. andersoni* and *N. gangetica* are found in the drain system, stagnant water, canal, and stream at target sites.

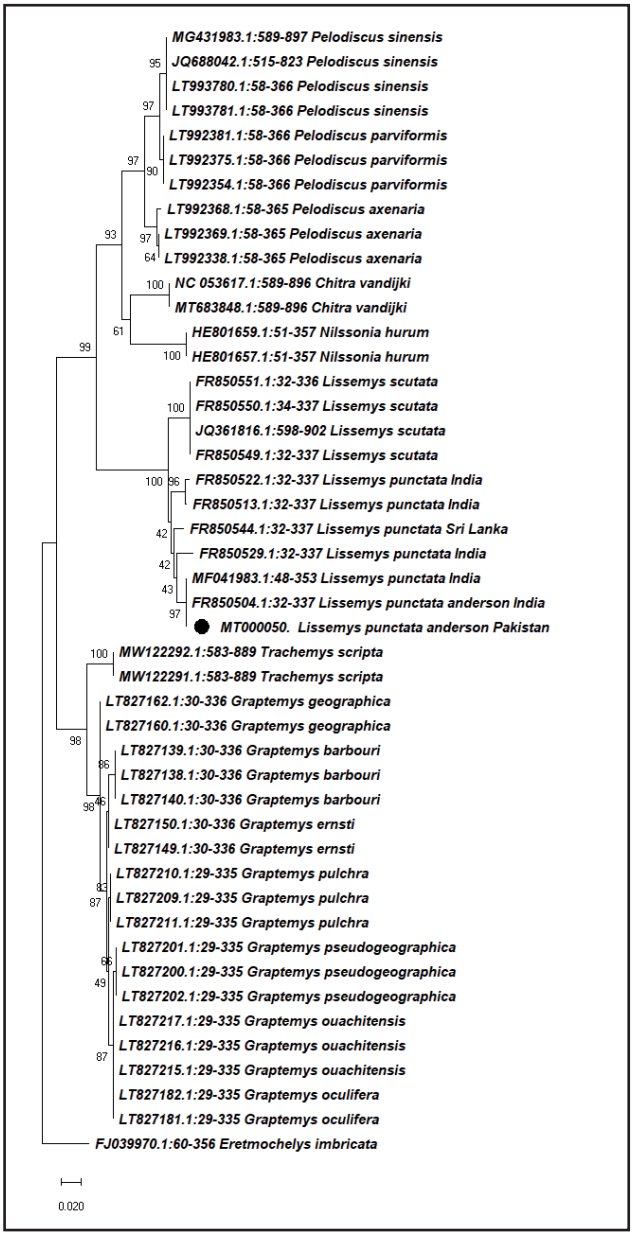

Figure 2. Maximum likelihood analyses inferred from 12S rRNA sequences from the genus *Lissemys* and *Eretmochelys imbricata* were used as an outgroup. GenBank accession numbers are followed by species name and location of collection. Support values (Bootstrapping values) were indicated at each node. The bar represents 0.020 substitutions per site. Obtained sequences has been represented by black dot.

The climatic change such as temperature and rainfall have great effects on the occurrence of turtles. Habitat degradation occurred due to various factors such as draining wetlands, deforestation, converting clear water rivers to stagnant multi-purpose reservoirs and mortality on roads when turtles move around to feed [34]. Food habitats will be helpful for potential farmers to manage their farms in the most effective and natural way [38]. Current study concluded that the freshwater turtles *L. p. andersoni* and *N. gangetica* are interested in natural feeds.

The analysis of 359 bp of 12S rRNA gene of the genus *Lissemys* turtles showed relationships of these turtles with cyclanorbines flap shell turtles, which agrees with previous reports [7,26]. The African taxa are paraphyletic with respect to the Asian *Lissemys*. The ancestors of the extant genus cyclanorbines spread from North America to Asia [26]. It should be expected that each of the 3 taxa, *L. p. andersoni*, *L. p. punctata* and *L. scutata* represents a distinct genetic lineage [3,6-8,26]. Present molecular investigation concluded that Clad A comprising *L. p. punctata*, *L. scutata*, *L. cylonensis* also include *L. p. andersoni* species. Clad B also contains one sequence from India, identified as *L. p. andersoni*. Their classification as conspecific evolutionary lineages are suggested by similar genetic divergences, the observation of mismatches between morphology (spotted vs. unspotted) and mitochondrial haplotypes in clades A and B. The clades A and B provides evidence for gene flow between the spotted subspecies *L. p. andersoni* and adjacent populations with unspotted flap shell turtles. This molecular investigation will contribute to a better understanding in the systematics and taxonomy of freshwater turtles.

CONCLUSION

This study explored the ecology, habitat and diversity of freshwater turtles at molecular level in Pakistan. Two species of turtle, *L. p. andersoni* and *N. gangetica* were reported from the selected region. It was the first study that molecularly characterized important turtle species, *L. p. andersoni*, in Pakistan. The phylogeny of 12S rRNA showed similarity with *L. p. andersoni* from other Asian regions. Finally, this study provided the foundation to conduct further molecular studies that are essential to explore the genetic diversity of turtle species.

MANUFACTURERS

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***Ethical approval*.** The present study was approved by the advance studies and research board of the Abdul Wali Khan University Mardan (Dir/A&R/AWKUM/2020.4871).

***Declaration of interest*.** The authors certify that they have no affiliations with, or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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