INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 10, p. 57-63, 2015

RESEARCH PAPER OPEN ACCESS

Cytochrome b gene based phylogenetic studies of pangshura smithii from Pakistan

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Key words: Cyt-b, *Pangshura smithii*, Molecular Characterization, Pakistan.

http://dx.doi.org/10.12692/ijb/6.10.57-63 Article published on May 20, 2015

Abstract

DNA-based characterization and identification of fresh water turtle species for phylogenetic analysis as well as forensic identification is widely being carried out with the help of polymerase chain reaction (PCR) with DNA sequencing method. In the present study, *Pangshura smithii* found in Rawalpindi – Islamabad area were identified and characterized by using PCR method. *P. smithii* form a sub-cluster with five sequences derived from GenBank (AM495328, AM495288, 495289, AY434589, JN232521) with more difference than other sequences. *P. smithii* make cluster with two sequences AM495288 and AY434589 to form a single group with bootstrap value of 73%. Highest identity percentage was found to be related with AM495288. The results indicated the use of molecular techniques to be an efficient and more reliable to identify fresh water turtle species in general and endangered species in particular and to keep in their proper taxonomic position.

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Introduction

Fresh water turtles, "Testudines" occur in six genera and eight species in Pakistan. Family *Emididae* consists of four species of hard - shelled turtles; spotted pond turtle (*Geoclemys hamiltoni*), crowned river turtle (*Hardella thurjii*), brown river turtle (*Kachuga smithii*) and Indian roofed turtle (*Kachuga tecta tecta*). These are distributed in Pakistan, India, Bangladesh, Nepal, Burma, Thailand, Vietnam, Malaysia and Sumatra (Moll, 1987). Molecular methods are acquiescent in the resolution of taxonomic relationship and identifying units for conservation which helped in uncovering diversity in taxa that left ambiguous from morphometry. These genetic data related to phylogenies is helpful in species delimitation and lineages divergence within species and their evolutionary relationships (Nei and Kumar, 2000; Iverson *et al.*, 2007).

Advancement in molecular methods and techniques is beneficial in resolution of the general issues in turtle biology and conservation. It will increase effectiveness on DNA finger printing and SNP applications (McGaugh *et al.*, 2007). The main source of molecular characterization for phylogenetic inferences is the DNA sequencing of mitochondrial DNA and nuclear DNA. Optimal models of DNA evolution can be estimated for a set of sequences and incorporated into phylogenetic methods such as maximum likelihood and Bayesian analysis (Shaffer *et al.*, 2008). The mtDNA sequences can be used to improve the phylogenetic resolution and taxonomic position of the turtles without any ambiguity (Guillon *et al.*, 2012).

Zhang *et al.* (2009) viewed that the complete sequences of the mitochondrial DNA (mtDNA) control region (CR) of *Cistoclemmys flavomarginata*, *Cistoclemmys galbinifrons*, *Cuora aurocapitata* and *Cyclemys atripons*, were amplified by long-polymerase chain reaction (Long-PCR). The lengths were 1207 bp, 1722 bp, 1379 bp and 980 bp, respectively. Combining with the CR sequence of *Pyxidea mouhotii* (DQ659152), they compared the CR structure, and identified three functional domains (TAS, CD and CSB) in which the conservation sequences (TAS, CSB-F, CSB-1, CSB-2 and CSB-3) were also successfully identified according to their homology to those of other turtles. These 5 turtles have the identical CSB-2 and CSB-3 sequences, and 4 of them have the same CSB-1 sequence while there is one base transversion (T_A) in Cy. atripons. They analyzed the variable number of tandem repeat (VNTR) sequences or microsatellites at the 3' end of CR. The motifs of tandem repeats (7 types) are made up of 2-8 nucleotides, and the copy numbers are from 4 to 48. All of the 5 turtles except Cy. atripons have the "TATTATAT" repeats and are ended by TA. The results of CR structure analysis displayed that the Cuora, Cistoclemmys and Pyxidea have many similarities, but differ from Cyclemys. With Indotestudo elongate (DQ080043) and Indotestudo forstenii (DQ080044) as outgroups, using the CR sequences (1123bp) excluded the microsatellites at the 3' end of CR, they constructed the molecular phylogenetic trees using the MP, ML and BI methods. The results showed that there was a strong support to the monophyly of the Cuora group consisting of Cuora, Cistoclemmys and Pyxidea, which has a close relationship with Mauremys and Chinemys but far from Cyclemys, which are consistent with the analysis of the CR structure of the 5 turtles. Present study aimed to characterize fresh water turtles of Rawalpindi Islamabad on the basis of Cyt-b gene that will help scientist about actual picture of diversity and identifications of turtles and they make strategies for their preservation.

Materials and methods

Sampling and blood collection of pangshura smithii

Pangshura smithii were caught by cast net and identified on the basis of diagnostic morphological characters (Khan, 2006) then injected with ketamine @ 22-44 (mg/Kg) in their fore limb. The blood samples for DNA based taxonomy was collected from jugular vein of neck and femoral vein of leg but in juvenile blood collected from dorsal cervical sinus (Wibbels *et al.*, 1998; Gregory and Gabriel, 2006; Rohilla and Tiwari, 2008) and preserved in EDTA containing vials to prevent clotting and stored at –

20°C for later use (Rohilla and Tiwari, 2008).

Extraction of total nucleic acid (DNA)

Total genomic nucleic acid was extracted by using Proteinase K Method as described by Sambrook and Russell (2001).

PCR Amplification and purification

A 50 µl PCR reaction mixture contained µl genomic DNA template, 5 µl of 10 x Taq reaction buffers (10 mM Tris – HCl, pH 8.8, 10 mM KCl), 3 µl of MgCl2, 0.5 µl Taq polymerase (Invitrogen Cat. No.10342-020), 5 µl dNTPs (2 mM) (Invitrogen, Cat. No. 10297-018), 2.5 µl (20 pmol) of each primer (SamGlu-LF "TGATATGAAAAACCATCGTTG"), (SamCb3HR "GGCAAATAGGAARTATCATTC") and final volume was dome with nuclease free water (Invitrogen Lot. No.1147267). The reaction conditions were initial denaturation at 95°C for 5 min, followed by 30 thresh hold cycles of denature (94°C for 50 Sec), annealing (50°C for 50 second) and extension (72°C for 1 mint) and final extension was performed at 72°C for 10 mint (Jiang *et al.*, 2011). Amplified PCR products were checked for correctness size by electrophoresis on 1.5 % (w/v) agarose gel stained with Ethidium Bromide and were visualized and photographed under UV light using gel documentation system (GenoSens, Model 1510). The PCR products were then purified using a PureLink® PCR Purification Kit according to the manufacturer's protocol (Invitrogen, Life Technologies, USA).

Ligation and cloning of cytochrome b gene

All the amplified and purified PCR fragments of cytochrome b gene were directly ligated into the pTZ57R/T vector using TA cloning kit (Thermo Scientific). Electrocompetent cells of Escherichia coli strain XLI-Blue were prepared. Around 50 µl of E. coli cells were mixed with 2 µl of ligation mixture in a new microfuge tube. The cell suspension was placed between electrodes of a pre-cooled electroporation cuvette. The cuvette was dried with tissue paper and inserted into an electroporator. A pulse of 2500 V was given and immediately 350-500 µl of LB medium was transferred in to the cuvette, and the cells were transferred to a 1.5 ml microfuge tube and kept at 37OC for 1hr. About 50 µl of this culture was spread on solid LB medium having 1 µg/ml ampicillin (appropriate for pTZ57R/T vector) and the plates kept at 37OC overnight. After 24 hr incubation, colonies were transferred into 5 ml LB medium containing ampicillin (100 µg/ml) in glass universal tube. Cultures were kept at 37 OC for 24 hr with shaking at 225 rpm.

Plasmid extraction

White colonies with the desired fragment were selected for following inoculation of LB broth (10 ml) with 10 µl ampicillin and incubated at 37 OC overnight. The plasmid was then purified from the culture using a Spin Column Plasmid Miniprep Kit as per manufacturer's instructions (NBSbio).

Digestion of Cyt-b gene with restriction enzymes and electrophoresis

The PCR products from individuals of freshwater turtle species from Rawalpindi- Islamabad area were digested with AluI, FokI, MspI, TaqI and HaeIII restriction endonucleases according to manufacturer's recommendations (Fermentas, Germany). The restriction fragments were separated on 2% Agarose gel by comparing with a standard molecular ladder 1kb/100-bp DNA ladder run in a parallel lane for size measurement. The gel was visualized and photographed.

Cyt-b Sequences/RFLP data and phylogenetic analyses

Cytochrome b gene sequences of *Pangshura smithii* from Rawalpindi-Islamabad area of Pakistan were sequenced by using ABI Big Dye Terminator sequencing kit (Eurofin). Alignments of sequences were done in CrustalW within BioEdit version 7.11 / MEGA (version 5.1) and compared against the GenBank database using BLAST were constructed on the basis of Nucleotides based genetic distance matrices computed with BioEdit version 7.2.1 (Hall, 1999) using default parameters were used to construct Maximum parsimony (MP) and Neighbour-Joining phylogenetic trees or / and diagnostic

restriction sites of Cyt-b RFLP data from under studied restriction enzymes were used to construct the UPGMA/ Neighbour-Joining tree with MEGA 5.1 program (Kumar *et al.*, 2004).

Results and discussion

Panghsura smithii (Brown river turtle) has olive carapace with three black or dark brown vertebral stripes in the centre of the carapace. On each plastral scute, a single large black spot present. Head was olive coloured having a reddish brown spot behind each eye. Neck and limbs consisted of yellowish spots (Figure 1). It consisted of long neck (Figure 1). These features are the same as discussed by Siddiq (2010).

Fig. 1. Specimen of *Pangshura smithii* captured from study area (A) Front view of carapace (B) Plastron.

Molecular characterization of captured fresh water turtles

For DNA isolation Proteinase K Method was adopted following PCR reactions. The isolated DNA was quantified by using spectrophotometer at 260 nm and 280 nm and a ratio between ~1.8-2.0 was accepted as "pure" for DNA and furthermore the quality of the DNAs was also checked by gel electrophoresis (Figure 2).

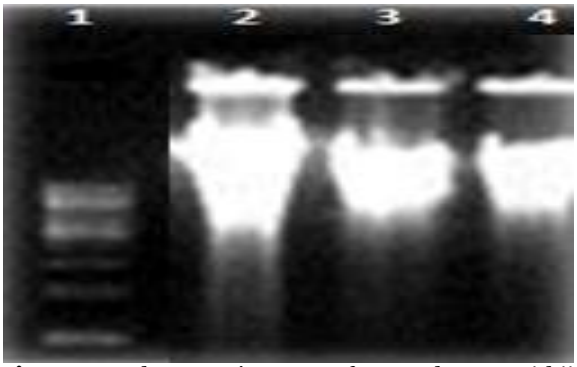

Fig. 2. Total Genomic DNA of *Pangshura smithii* with 1kb marker.

Amplification of cytochrome b gene of mitochondrial DNA

Successful PCR amplification followed by agarose gel electrophoresis revealed the cytochrome b gene bands for each turtle species with expected size of about ~900bp (Figure 3) with primer (SamGlu-LF " TGA TAT GAA AAA CCA TCG TTG), (SamCb3HR " GGC AAA TAG GAA RTA TCA TTC). These desired bands were further eluted. These results confirmed that all of the bands were of the partial cytochrome b gene fragments with the restriction sites for the desired selected restriction endonulease enzymes. These results are in consisteny with Rohilla *et al.* (2008) who reported the same size of amplified PCR products of cytochrome b gene from five Indian fresh water turtle species and this cyt b is also used to genetically differentiate northern and southern population. The present gel purified products of cytochrome b gene were furhter used for cloning and sequencing.

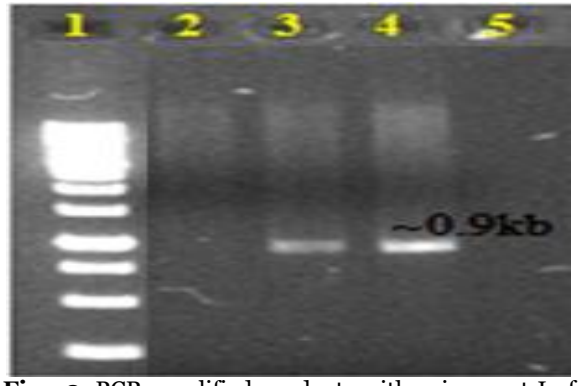

Fig. 3. PCR-amplified products with primer set I of cytochrome b gene of. Pangshura smithii.

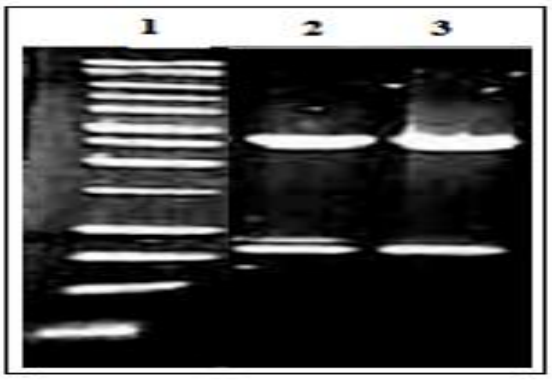

Fig. 4. Cloning of cytochrome b gene into pTZ57R/T vector. Lane 1: 1kb DNA ladder, Lanes 2-3: pTZ57R/T Digested with restricion enzyme to release cloned PCR products for *Pangshura smithii*.

Cloning of Cytochrome b gene

Successful ligation of amplified and gel purified Cyt b gene fragments was done and cloned into pTZ57R/T vector and subsequently transformed into XLI- Blue. The pTZ57R/T plasmids were digested with EcoRI enzyme to execute ligated cytochrome b gene. The agarose gel electrophoresis revealed the digestion products: bands for cytochrome b gene (about 0.9 kbp), linearized vector (about 0.3kbp) and bands for the intact plasmid (without digestion) as a control (Figure 4).

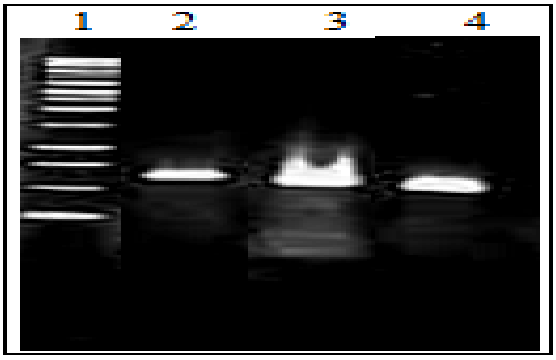

Fig. 5. Digestion of PCR amplified products (Cyt- b gene) with endonuclease restriction enzymes Lane 1: 1kb marker, Lane 2: *Pangshura smithii* digestion with AluI (Band size: 200, 650); Lane 3, Digestion of *Pangshura smithii* (690, 200bp) with TaqI, Lanes 4, also gave partial digestion with FokI for *Pangshura smithii*.

Restriction fragment length polymorphism (RFLP)

All the DNA samples produced 900 bp bands of cyt-b gene fragment amplified using primer set I. Digestion of these bands with three enzymes AluI, Taq I and Fok I is shown in Figure 5 with their respective bands. These results are almost similar as reported by Rohilla *et al.* (2008) with exceptions where partial digestion is obtained with need to be repeated for further confirmation. Walker *et al.* (1998) have also reported considerable genetic variation for mitochondrial DNA haplotypes within each of several species of freshwater turtles in the south-eastern USA. Surveys of mtDNA restriction sites in several other terrestrial as well as aquatic turtles in south-eastern USA have also revealed modest to high levels of intraspecific varaitons and strong geographic partitioning of gene-tree branches (Osentoski and Lamb, 1995; Walker *et al.*, 1997, 1998).

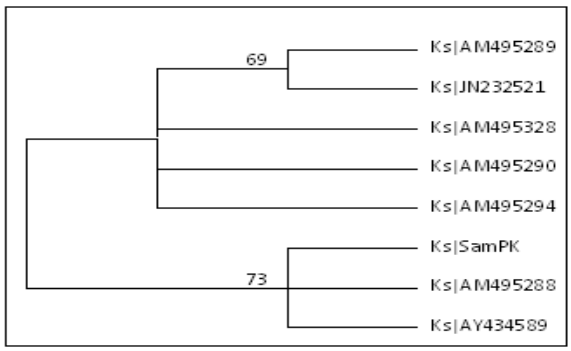

Fig. 6. Maximum Parsimony Phylogenetic tree of freshwater turtle P*angshura/Kachuga smithii* based on partial sequences of Cyt b gene of Ks|SamPK and 7 other species aligned using ClustalW with in BioEdit version 7.11 and tree was created using MEGA version v5.1. Values at the forks represent the percentage of times out of 1000 the grouping occurred after bootstrapping.

Sequence analysis of cytochrome b gene and phylogenetic analysis

Pangshura smithii form a sub-cluster with five sequences derived from GenBank (AM495328, AM495288, 495289, AY434589, JN232521) with more difference than other sequences. Four of these sequences originate from South Asia and the fifth is a sequence of unknown geographical provenance downloaded from GenBank (AY43600, Spinks *et al.*, 2004). Partial nucleotide sequences of cytochrome b gene of *Pangshura smithii* collected from study area were compared with seven other investigated sequences of *P. smithii* (available in GenBank) and highest identity percentage was found to be related with AM495288 (Table 1, 2). Pakistani based *P. smithii* was to be close relative to turltes whose accession numbers are shown in Figure 6. Phylogenetic analysis with partial cytochrome b gene sequences of representing *P. smithii* cluster together with two other sequences (AM495288 and AY434589) to form a single group with bootstrap value of 73% (Figure 6).

Phylogenetic analysis based on Cyt-b sequences did not reveal any intra-specific variation. Chiari *et al.* (2005) studied d Cyt-b gene of tortoise species *Pyxis arachnoi* to recover three distinct genetic subspecies regarding their geographic separation and plastron

differences. Sequence comparison showed that sequence of each species showed close relationship at interspecific level. These results are in an agreement with Rohilla *et al.* (2008) who reported the same feature from the estimated genetic distances of five freshwater turtles of both the soft-shell and hard-shell group, commonly found in the rivers and other water bodies in adjacent region of India. The hard shell species showed close relationship with Indian hard shell species. It means that sub-continent (including our sequence) based fresh water turtles are proved to highly distinct from Indonesian and Malaysian based same species. These results are agreed with Praschag *et al.* (2007) who reported a highly significant Cyt-b sequence distinction of Indonesian and Malaysian Batgur baska from the Sundrabans (India and Bangladesh), suggesting that a previously unidentified species is involved. Besides this, they (Praschag *et al.*, 2007) also reported shared haplotypes in *P. tentoria* and *P. smithii*, suggesting the unusual morphological characters of the Ghaghra River population of *P. tentoria* could be the result of interspecific hyberdization. The present study is also supported by the findings of Spinks *et al.* (2004) and Rohilla *et al.* (2008).

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