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Cytochrome b gene based characterization of *Lissemys punctata*

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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, *Lissemys punctata* found in Rawalpindi – Islamabad area were identified and characterized by using PCR method. Lp|SamPK showed 100% genetic similarity with Gen Bank accession No. EF558363. The sister relationship of this clade with other clade consisting of six sequences downloaded from GenBank was weakly supported with a bootstrap of 60%. The maximum nucleotide genetic diversity was observed between Lp|SamPK and Genbank downloaded sequence (FR850644) which forms a clade with FR 850643, with less than 40% genetic diveristy to present studied fresh water turtle (Lp|SamPK). 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

Roman and Bowen (2000) conducted a research study on authenticity of meat of turtles in Florida and Louisiana. They used cytochrome b gene (256 bp) and control region (394 bp) of mitochondrial DNA for 36 turtle meat products which was supposed to contain Macrochelys temmimckii but molecular studies cleared that those mostly contain Chelydra serpentine and only fraction of Apalone species (softshell turtle) were found. This research recommended to check over- exploitation to decrease its effect on species.

Felsenstein (2003) reviewed that most of phylogenetic trees for different turtle groups have been constructed by DNA methodologies and techniques using molecular and morphological data (FitzSimmons and Hart 2007).

Randi (2003) reported that DNA based forensic techniques are used to watch illegal trade by confirming taxonomy and giving information on geographical origin of captured species. Usually species are identified on morphological basis. On the other hand, usually seizures contain fragments of carapace shell, eggshell, cooked meat, or powdered turtle shell, in which standard diagnostic characteristics are not apparent. Molecular techniques are best for forensics because degraded and treated specimens are identified and these methods can clarify and identify species and also their population and regional origins. With the help of genetic methods the products obtained from legal trade can be differenciated from poaching strategies, resolved the paternities and maternities of tag animals which is helpful for watching actions of licensed reptile breeders. The use of molecular methods in wildlife forensics is still in initial stages. Recently only a few molecular techniques are applied in problems of fresh water turtles and tortoises.

Krenz *et al.* (2005) while working on turtles concluded that with the help of DNA sequencing techniques and methods most of the phylogenetic issues among the families of turtles are resolved now but some controversial issues are still present among the families of turtles (Parham *et al.*, 2006a).

Materials and methods

Sampling and blood collection of lissemys punctata

Lissemys punctata were caught by cast net and identified on the basis of diagnostic morphological characters (Khan, 2006). As a first step the *L. punctata* were injected with 22-44 (mg/Kg) IM / SC dose of ketamine in their fore limb, hind limb or tail region to make them unconscious. The blood samples for DNA based taxonomy collected from adult *L. punctata* from jugular vein of neck and femoral vein of leg but in juvenile blood collected from dorsal cervical sinus because juveniles are too small to collect sufficient amount of blood (Wibbels *et al.*, 1998; Gregory and Gabriel, 2006; Rohilla and Tiwari, 2008). The weight of the body forces the fore limbs and neck stretched, after ketamine injection. In this position, the blood was collected in blood collecting vials coated with EDTA from jugular vein and femoral vein (Batra and Prakash, 1995). About 1-2 ml blood was withdrawn with the help of 5ml syringe equipped with 25G, 5/8 inch needle from the jugular and femoral vein in adult *L. punctata* and 0.2-0.3 ml blood was taken with the help of 1-mL insulin syringe equipped with 26G, 5/8 inch needle from dorsal cervical sinus of juveniles of *L. punctata* (Wibbels *et al.*, 1998; 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)

For the extraction of total genomic nucleic acid from *L. punctata*, the used methods included: i) Proteinase K Method as described by Sambrook and Russell (2001).

Proteinase K method

The genomic DNA was isolated from whole blood samples using Proteinase K (Invitrogen, Lot No. 1227566) Method as described by Sambrook and Russell (2001). About 200 µl blood samples from each *L. punctata* were collected in 2 ml capped microfuge tubes. One ml of extraction buffer with 200

µg proteinase K was immediately added to the blood samples, which was vigorously vortexed for few minutes incubated at 56ºC for overnight with occasional vortexting. About 0.6 ml chloroform was added after overnight incubation and then the sample was vortexed for 2 minutes and again kept at 65 ºC for 60 minutes, followed by centrifugation for 5 minutes at top speed at 4 ºC. The supernatant was added to a new microfuge tube and 0.8 ml isopropanol and 0.1 ml 3M Na-acetatae were added to precipitate the DNA and the microfuge tube was incubated at room temperature for 10-15 minutes. The pellet of DNA was obtained by following centrifugation of the tube at 14000 rpm for 4 minutes at 4 ºC. The 70% ethanol was used to washed DNA and finally resuspended and in 60-250 µl of nuclease H_2O. The DNA concentration was measured photometrically (Sambrook *et al.*, 2001). One µl of DNA was diluted in 99 µl of distilled water. The Optical Density was measured at 260 nm and 280 nm, using water as a blank.

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 " TGA TAT GAA AAA CCA TCG TTG), (SamCb3HR " GGC AAA TAG GAA RTA TCA TTC) 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 TAInsta 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 ampicilliin (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 DNA was purified by the miniprep method (Plasmid Miniprep Kit Cat. No. 208.05.02.05, Invitrogen, USA) and checked on a 1 % agarose gel.

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 *L. punctata* 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

A total of 20 specimens of fresh water turtles were captured from the study area which were morphologically recognized to belong to three species i.e., Lissemys punctata, Nilssonia gangetica and Pangshura smithii. These results are in agreement with Siddiq (2010) who reported similar three species from Rawalpindi- Islamabad area based on their morphological charatcers. On the other hand Azam *et al.* (2005) reported six species from Indus River while Akbar *et al.* (2006) discussed eight species from different barrages of Punjab.

Table 1. The *Lissemys punctata* with the highest identity compared with DNA sequences registered in GenBank.

Haplotype	Species with highest identity (Accession numbers)	Identity	Sample cases
1	*Lissemys punctata* (EF558363)	94%	Lp|Sam PK
2	*Lissemys punctata* (FR850626)	92%	=
3	*Lissemys punctata* (FR850622)	92%	=
4	*Lissemys punctata* (FR850623)	92%	=
5	*Lissemys punctata* (FR850621)	92%	=
6	*Lissemys punctata* (FR850616)	92%	=
7	*Lissemys punctata* (FR850630)	92%	=
8	*Lissemys punctata* (FR850635)	92%	=
9	*Lissemys punctata* (FR850636)	92%	=
10	*Lissemys punctata* (FR850625)	91%	=
11	*Lissemys punctata* (FR850631)	91%	=
12	*Lissemys punctata* (FR850642)	89%	=
13	*Lissemys punctata* (FR850643)	89%	=
14	*Lissemys punctata* (FR850632)	89%	=
15	*Lissemys punctata* (FR850633)	89%	=
16	*Lissemys punctata* (FR850644)	89%	=

Lissemys punctata (Indian mud turtle) has olive green carapace consisted of bright yellow round dashed spots which were scattered on the whole carapace. Plastron was of cream colour. Head and neck consisted of bright yellow spots. It consisted of long neck (Figure 1). These features are the same as discussed by Siddiq (2010).

Molecular characterization of captured fresh water turtles

Three individuals of *Lissemys punctata* were selected for molecular characterization. For this purpose two

DNA isolation protocols were adopted and blood samples were preserved in EDTA coated vials to prevent blood cloting and hinderance in 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).

Table 2. Sequence Identities (%) of Cytochrome b gene of *Lissemys punctata* (Lp|SamPK) from Pakistan and other countries.

Amino acid sequence																	
	Sam Pk	EF5 58363	FR8 50626	FR8 50622	FR8 50623	FR8 50621	FR8 50616	FR8 50630	FR8 50635	FR8 50636	FR8 50625	FR8 50631	FR8 50642	FR8 50643	FR8 50632	FR8 50633	FR8 50644
Lp|Sam PK		90.5	59.4	59.4	59.4	59.4	59.4	59.2	59.2	59.2	56.7	56.3	53.8	53.6	53.8	53.8	53.6
EF558363	93.8		63.9	63.9	63.9	63.9	63.9	63.6	63.6	63.6	60.9	60.5	57.9	57.6	57.9	57.9	57.6
FR850626	64.9	67.9		99.9	99.9	99.7	99.7	99.8	99.5	99.5	96.1	96.1	95.8	95.5	95.7	95.7	94.7
FR850622	64.9	67.9	99.9		100	100	99.7	99.7	99.7	99.4	99.4	95.1	94.9	91.8	91.2	91.8	91.8
FR850623	64.9	67.9	99.9	100		99.7	99.7	99.7	99.4	99.4	95.1	94.9	91.8	91.2	91.8	91.8	90.6
FR850621	64.9	67.9	99.7	99.8	99.8		99.4	99.4	99.1	99.1	94.9	94.6	91.5	90.9	91.5	91.5	90.3
FR850616	64.9	67.9	99.7	99.8	99.8	99.6		99.4	99.1	99.1	95.4	95.2	92.0	91.5	92.0	92.0	90.9
FR850630	64.8	67.8	99.8	99.9	99.9	99.7	99.7		99.1	99.1	94.9	94.6	91.5	90.9	91.5	91.5	90.3
FR850635	64.8	67.7	99.5	99.6	99.6	99.4	99.6	99.5		100	94.6	94.3	91.8	91.2	91.8	91.8	90.6
FR850636	64.8	67.7	99.5	99.6	99.6	99.4	99.6	99.5	100		94.6	94.3	91.8	91.2	91.8	91.8	90.6
FR850625	62.1	64.9	96.1	96.2	96.2	96.0	96.3	96.1	96.2	96.2		98.5	89.2	89.2	89.2	89.2	89.8
FR850631	62.2	65.0	96.1	96.2	96.2	96.0	96.3	96.1	96.2	96.2	99.0		89.5	89.5	89.5	89.5	90.0
FR850642	62.8	65.6	95.8	95.8	95.8	95.8	96.0	95.7	96.0	96.0	93.2	93.4		98.8	100	100	98.3
FR850643	62.7	65.5	95.5	95.5	95.5	95.5	95.7	95.4	95.7	95.7	93.2	93.3	99.4		98.8	98.8	99.4
FR850632	62.7	65.5	95.7	95.7	95.7	95.7	95.9	95.6	95.9	95.9	93.2	93.3	99.9	99.5		100	98.3
FR850633	62.7	65.5	95.7	95.7	95.7	95.7	95.9	95.6	95.9	95.9	93.2	93.3	99.9	99.5	100		98.3
FR850644	61.9	64.8	94.7	94.7	94.7	94.7	94.9	94.7	94.9	94.9	93.9	94.1	98.6	99.2	98.7	98.7	

Nucleotide sequence.

Amplification of cytochrome b gene of mitochondrial DNA

Mitochondrial genes in general and cytochrome b gene in particular are commonly used in turtles for population genetic and phylogenetic studies (Parham*et al.*, 2006). Successful PCR amplification followed by agarose gel electrophoresis revealed the cytochrome b gene bands for each turtle species with expected size of about ~900bp and 1100bp with primer set I and II, respectively (Figure 3). 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.

Cloning of cytochrome b gene.

The PCR amplified and gel purified Cyt b gene fragments were successfully ligated and cloned into pTZ57R/T vector and sebsequently transformed into XLI- Blue. The pTZ57R/T plasmids were digested with EcoRI enzyme to execute ligated cytochrome b gene. The agarsoe 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).

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). Moore *et al.* (2003) extracted DNA from turtle eggs and cooked meat used as diagnostic tool in producing RFLP patterns in the cyt b region of the mitochondrial DNA. According to them their method works well on DNA from any tissues and can be used /adopted as of the best option for wildlife law enforcement department to combat illegal take of endangered species.

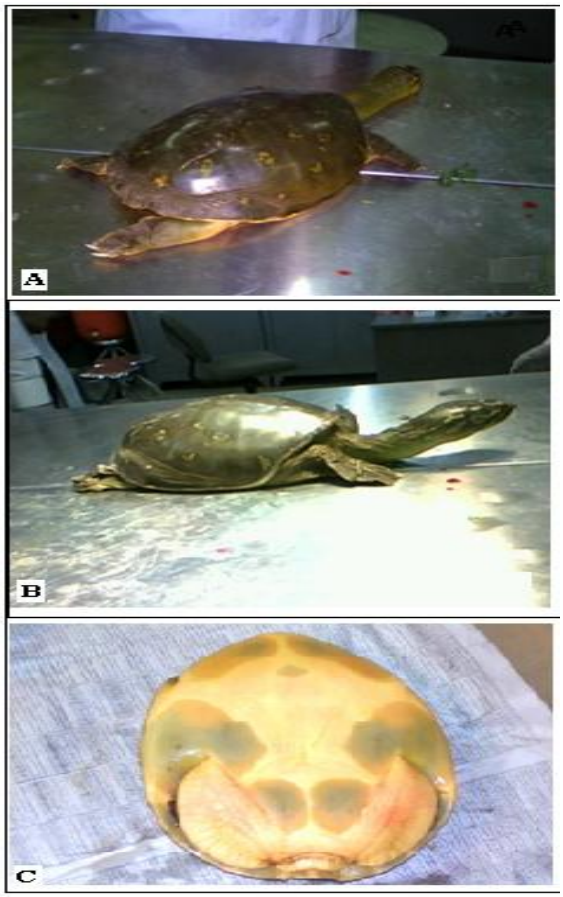

Fig. 1. Specimen of Lissemys punctata captured from study area.

Sequence analysis of cytochrome b gene and phylogenetic analysis

To assess more precisely the genetic diversity and similarity, sequences of cytochrome b gene for *L. punctata* was obtained. Mitochondrial DNA sequence approach is one of the best technique for phylogenetic analysis and many scientists/researchers sequenced different regions (CO1, Cyt b and ND4) of mitochondrial DNA for phylogenetic analysis of turtles (Stuart and Parham, 2004; Rohilla *et al.*, 2008). According to Iverson *et al.* (2007) the DNA based phylogenetic relationships among turtle families and most genera are also well-resolved and well-supported. Therefore they stressed that future work should be endeavoured to include the broadest taxonomic and geographic sampling possible (including type specimens) based on mtDNA and nDNA genes in order to maximize the understanding regarding the evolution of modern turtle diversity and resolving the tree of life for turtles. Phylogeny studies provide informative means for testing evolutionary hypotheses regarding genetic variation. Therefore the present study compared the partial sequences of cytochrome b gene of *L. punctata* with those closely related sequences available in the EMBL database that were retrieved from the NCBI Website(www.ncbi.nlm.nih.gov/).

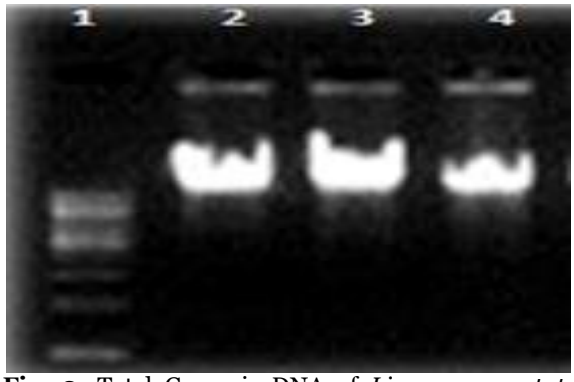

Fig. 2. Total Genomic DNA of *Lissemys punctate* with 1kb marker.

The obtained sequences of was first blasted and aligned by using the Multiple Alignments option available within BioEdit version 7.11 and sequences gaps of these sequences were completely eliminated. Our sequence of Lissemys punctate showed similarity more than 89% with 16 haplotypes retrieved from the EMBL databank (Table 1). It means that DNA based identification of species could be considered more reliable and authenticated methods and similar results regarding species identification of fragmented turtle shells by cyt b gene have been reported by Hsieh *et al.* (2008) but he also mentioned the

insufficient information of the EMBL databank and it illustrates the need ot expand database of turtles for species identification.

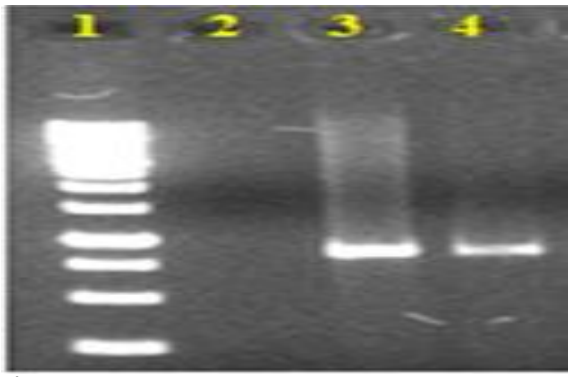

Fig. 3. PCR-amplified products with primer set I of cytochrome b gene of *Lissemys punctate*.

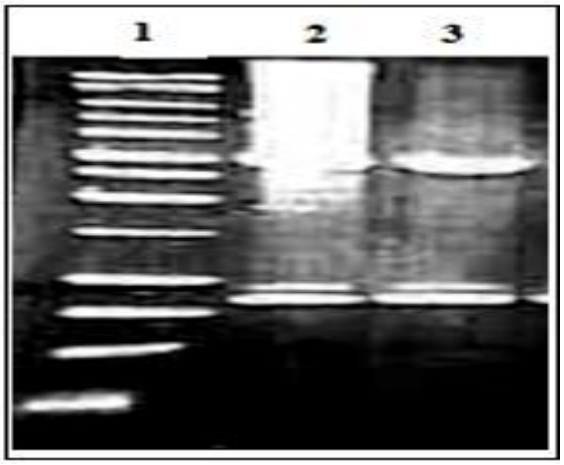

Fig. 4. Cloning of cytochrome b gene into pTZ57R/T vector. Lane 1: 1kb DNA ladder, Lanes 2-3: pTZ57R/T Digested with restricion enzyme to release cloned PCR products for *L. punctuate*.

The percentage difference/similarity between species was determined at nulceotide and amino acid level. *Lissemys punctata* showed nucleotide and amino acid similarity ranges from 61.9 to 93.8 % and 53.6 to 90.5, respectively, with highest similarity to the sequence EF558363 downloaded from Gen Bank (Table 2). This is a sequence of an Indian freshwater turtle of the same species. The genetic diversity within the Lp|SamPK and Rohilla *et al.* (2008) reported that sequence with Gen Bank accession No. EF558363 was extremely low and they both together formed a clade with a bootstrap 100%. The sister relationship of this clade with other clade consisting of six sequences (Accession numbers shown in Figure 6) downloaded from GenBank was weakly supported with a bootstrap of 60%. The maximum nucleotide genetic diversity was observed between Lp|SamPK and Genbank downloaded sequence (FR850644) which forms a clade with FR 850643 with less the 40% genetic diveristy to present studied fresh water turtle (Lp|SamPK). The amino acid identity to Lp|Sam PK remained between 53.6 to 90.5% with highest similarity with EF558363 (Table 2).

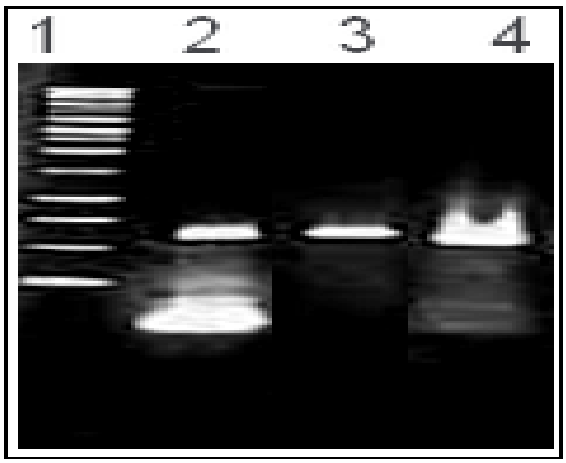

Fig. 5. Digestion of PCR amplified products (Cyt- b gene) with endonuclease restriction enzymes Lane 1: 1kb marker, Lane 2: *L. punctata* digestion with AluI (Band size: 200, 650); Lane 3, Digestion of *L. punctata* (690, 200bp) with TaqI, Lanes 4, also gave partial digestion with FokI for *L. punctata*.

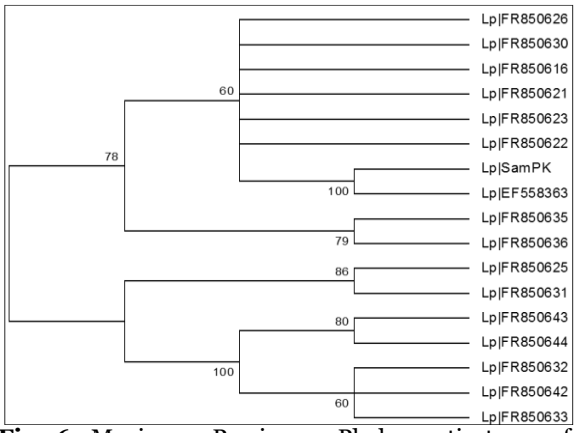

Fig. 6. Maximum Parsimony Phylogenetic tree of freshwater turtle *Lissemys punctata* based on partial sequences of Cyt b gene of Lp|SamPK and 16 other species aligned using ClustalW with in BioEdit version 7.11 and tree was created using MEGA version v5.1. Values at the forks represent the percentage of times out of 1000 the grouping occurred after bootstrapping.

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 agrement 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.

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