Journal of Asia-Pacific Biodiversity xxx (2018) 1–7

Contents lists available at ScienceDirect

Journal of Asia-Pacific Biodiversity

journal homepage: http://www.elsevier.com/locate/japb

Original Article

Analysis of genetic diversity in *CYTB* and control region sequences of *Melanochelys trijuga* (Schweigger, 1812) from Karnataka

Q1,12 R. Lalitha, Vidya R. Chandavar*

Molecular Endocrinology Laboratory, Department of Biotechnology, Yuvaraja's College, A Constituent Autonomous College of University of Mysore, Mysore 570005, India

Q 2 ARTICLE INFO

Article history:
Received 1 January 2018
Received in revised form
18 April 2018
Accepted 7 May 2018
Available online xxx

Keywords:
CYTB
Control region
Karnataka
Melanochelys trijuga
Transition mutation

ABSTRACT

Melanochelys trijuga (Cryptodira; Geoemydidae) indigenously called Indian black turtle or Indian pond terrapin is an endemic freshwater turtle species of Asia. This is the first report on mitochondrial marker of *M. trijuga* from this region. The current study aims to assess intraspecies nucleotide diversity in *CYTB* and control region (CR) sequences. The *CYTB* gene (1140 bp) analyzed revealed 4.47% variability with no *indels*, and CR (510 bp) region consist 13.5% variability with 22 *indels*. In *CYTB* specifically, pyrimidine transitions (Ts) (T↔C) exceeded purine Ts (A↔G). This resulted in synonymous substitutions of amino acids in the protein without altering its structure and function. The partial fragment of *M. trijuga* CR contained conserved sequence block units (CSB 1, 2, and 3) that are required for its regulatory function. This region being hypervariable and noncoding consists more of Ts than transversions. The nucleotide diversity (π) calculated for CR was greater than that of *CYTB*, which indicates CR is highly susceptible for mutation accumulation. The number haplotypes and haplotype diversity between the regions were poorly resolved, and probable reasons have been discussed. The mtDNA sequence data generated has its long-term application in the field of forensic, evolutionary and demographic studies.

Introduction

The family Geoemydidae (synonym: Bataguridae) represents the world's largest turtle family consisting most diversified group of turtles. It comprises 73 species belonging to 23 genera and 60 extant species (Sasaki et al 2006; Spinks et al 2004). *Melanochelys trijuga* (Schweigger 1812), commonly called Indian black turtle, is an abundant medium-sized geoemydid dwelling in freshwater bodies extending from Sri Lanka through India and Burma to Western Thailand (Das and Pritchard 1990). The species was reported from Indian subcontinent and is semi-aquatic in its habitat spending most of the time basking, omnivorous in its dietary habit, and a nonhibernating species (Das and Bhupathy 2009).

The survival status of *M. trijuga* as per Asian Turtle Trade Working Group (2000) states that the species is commercially exploited for food and used for wildlife smuggling in mass in various parts of India (Das and Bhupathy 2009). According to IUCN (2000) Red data list, the most severe cases have been reported from Bangladesh and Myanmar where it has been categorized as highly endangered and vulnerable species, respectively. Apart from these, there are unreported threats to the species reported because of fishing by local fisherman who accidently get these turtles caught in nets. They either sell these turtles in local fish markets or to toddy shops for high price to find their livelihood (Krishnakumar et al 2009).

Geoemydidae is one of the most vigorously investigated turtle family by researchers worldwide to understand interfamilial relationship both in its morphological and molecular perspectives (Sasaki et al 2006). Today, the use of different mitochondrial regions and complete mitogenome as markers are well-studied in various turtle groups. Intrafamilial relationship of Geoemydidae was successfully reported using mitochondrial cytochrome b (*CYTB*) and 12S and nuclear R35 gene sequence data in combination and separately (Spinks et al 2004). Phylogenetic relationship of *Cyclemys dentata* is reported using complete mitochondrial sequence (Huang et al 2015). The study was conducted on mitochondrial haplotype distribution and phylogenetic relationship using *CYTB* gene sequence in *Mauremys reevesii* (Oh et al 2017). A study on five Asian freshwater turtle species using complete control

* Corresponding author.
E-mail address: vidyachandavar@yahoo.co.uk (V.R. Chandavar).

Peer review under responsibility of National Science Museum of Korea (NSMK) and Korea National Arboretum (KNA).

https://doi.org/10.1016/j.japb.2018.05.001

Please cite this article in press as: Lalitha R, Chandavar VR, Analysis of genetic diversity in *CYTB* and control region sequences of *Melanochelys trijuga* (Schweigger, 1812) from Karnataka, Journal of Asia-Pacific Biodiversity (2018), https://doi.org/10.1016/j.japb.2018.05.001

region (CR or D-loop) sequences to understand their phylogenetic relationship was successful (Jiang et al 2011). The CR comparison study among four Hinged turtles (Geoemydidae) revealed monophyly of *Cuora* species, its close relationship with *Mauremys* and *Chinemy,* and its far relation with *Cyclemys* species (Zhang et al 2009). A phylogeography report on *Mauremys japonica* analyzing nucleotide variations in *CYTB* and CR found bottleneck effect caused because of low genetic diversity in their population (Suzuki and Hikida 2011).

The significance of mitochondrial study in turtles is extended to wildlife crime detection. The mtDNA haplotypes are used to link an individual to its geographical location or population during wildlife crime detection (Cooper and Cooper 2013). This also estimates the haplotype richness in the population of particular geographical location. *CYTB* nucleotide sequence consist species-specific variations that can be utilized for phylogenetic studies and for forensic identifications (Parson et al 2000). Partial *CYTB* gene sequence was used to identify turtle shells purchased commercially (Lee et al 2009). Today, we have around 31 complete mitochondrial genomic sequences from different Geoemydidae species at NCBI database for comparative and phylogenetic studies. The *M. trijuga* of the current study has only partial sequences for *CYTB*, 16S rRNA, and 12S rRNA regions of mitochondria. In the present study, we have used complete *CYTB* sequence and partial CR sequence to study genetic variations within the species belonging to the geographical region mentioned. The mitochondrial sequence data obtained from the study can be utilized in future for any evolutionary or phylogenetic studies or for molecular forensic identification of species.

Material and methods

Blood samples were collected from eight individuals (Sp1 to Sp8) of *M. trijuga* from the freshwater bodies—Belur kere (Latitude: 12.533026 and Longitude: 76.952450) and Sooley kere (latitude: 12.511484 and longitude: 76.991892) near Mysore, Karnataka, India. Collection of blood samples from *M. trijuga* was based on prior permission obtained from the Principal Chief Conservator of Forest (Wildlife), Bangalore, Karnataka, India vide Letter No. D/WL/CR/149/2010 and PS/WL/CR/21/2013. Only adults weighing 2–2.5 kg were selected for sample collection. Various blood cells of turtle are excellent source of genomic DNA or mtDNA. The method adopted for blood collection, animal handling, and genomic DNA extraction is explained and followed as in the study by Lalitha and Chandavar (2017, 2018).

Oligos to amplify respective regions from genomic DNA were newly designed using Primer3 (Utergasser et al 2012) online program. For *CYTB* gene amplification, oligos were designed based on reference sequence available at database, and for CR, it was based on the consensus region obtained from the alignment of CR regions of other database Geoemydidae species (Table 1). Polymerase chain reaction (PCR) cycling was performed in AB Veriti^{TM} 96-well thermal cycler using Qiagen Hotstart hifidelity PCR kit (Orbit Science, Bangalore, India). A total 50μL reaction consists 1x of 5x Hot Start High-Fidelity buffer, 1 μM of each primer, 2.5 units of Hot Start Hi-Fidelity DNA polymerase with 3 μL template and 26 μL of milliQ to make up the volume. The thermal profile included initial denaturation at 95°C for 1 minute followed by 45 cycles of denaturation at 95°C for 30 seconds, variable annealing temperature for 30 seconds to 1 minute, extension at 72°C for 2 minutes, final extension at 72°C for 10 minutes and final hold at 4°C.

The PCR products were run on 2% LMA gel electrophoresis to check the amplification, and the molecular weight was assessed by running 50 bp DNA ladder (HiMedia, Mysore, India). PCR products were outsourced for further column purification of PCR amplicons and for bidirectional Sanger sequencing at Chromous Biotech Pvt. Ltd, Bangalore, India to obtain the sequences. The chromatogram peaks showing quality value (Phred quality score) of >20 was considered as good quality read with 99% accuracy in base calling. As suggested in literature, the use of genomic DNA extracted from blood samples of birds and reptiles for mitochondrial study would have chances of nuclear contamination of mtDNA (*numts*) (Grosso et al 2006). Few measures were taken to avoid amplifying or reporting such pseudogenes in the current study. Use of hot start PCR kit gives improved specificity, by avoiding nonspecific amplification, sensitivity, and good product yield (Birch et al 1996). Not using any universal primers (Grosso et al 2006) and used primers that could amplify large fragments (>150–200 bp in the current study) (Spinks and Shaffer 2007). Every chromatogram obtained was thoroughly examined for heterogeneity (double peaks or mixed peaks) that could arise because of contamination of sample or presence of pseudogene or both (Spinks and Shaffer 2007).

The sequences obtained were aligned using online program MUSCLE-EMBL (Edgar 2004) with default parameters. They were viewed and edited in BioEdit v7.2.5 (Hall 1999). The substitution analysis and polymorphism analysis were estimated using MEGA v6.0 (Tamura et al 2013) and DNaSP v6 software program (Librado and Rozas 2009), respectively. The *CYTB* and CR sequences of *M. trijuga* have been deposited in NCBI database under accession numbers MG640589 to MG640600.

Table 1. Primer pairs used for amplifying cytochrome b (*CYTB*) and control region (CR) of *M. trijuga*.

Oligo sequence (5′–3′)	Len	Tm (°C)	Ta (°C)	GC%	Hairpin (kcal/mol)	Self dimer (kcal/mol)	Cross dimer (kcal/mol)	Product size (bp)
***CYTB* primers**								
CBMt1F—GAGGACTTTACTACGGCTCA	20	58	55	50	No	−4.17	−5.85	158
CBMt1R—GTGGCTGAGAGTAAGTTGGT	20	58		50		No		
CBMt2F—CATCACCGGAATCTTCCTAGC	21	60	55	52	−4.64	−9.75	−9.98	250
CBMt2R—AAATGCGGTGGCTATGGTTA	20	60		45	No	No		
CBMt3F—TCTCAGCCACCCCTTACATC	20	60	57/58	55	No	No	−6.0	625
CBMt3R—TATAGGATGGAGGCCGTTTG	20	59		50		−9.28		
CBMt4F—GGCACCTGCTTAATCCTACAA	21	59	55/56	47	No	−4.85	−6.01	430
CBMt4R—AGGTAAAGAATCGGGTCAAGG	21	59		47		No		
CBMt5F—CACATCAAAACAACGCACAG	20	58	55/56	45	No	No	−11.06	201
CBMt5R—TTGTTTTCGATTAATCCTACAAGG	24	58		33	−0.64	−10.95		
CBMt6F—GACCCGTGATTTGAAAAACC	20	59	55/56	45	No	−4.89	−5.41	698
CBMt6R—TGATGGTGAAGGGGAGTAGG	20	59		55		No		
CBMt7F—CATTACTGGGCACCTGCTTA	20	58.8	55/56	50	No	−6.09	No	403
CBMt7R—GAGAATCCCCCTCAGATTCA	20	59		50	−3.47	−7.57		
CR primers								
CRMtF—CGAGAGATAAGCAACCCTTGT	21	56.4	54/55	47	−0.14	−4.9	−9.42	500–520
CRMtR—GGATTTAGGGGTTTGACGAG	20	56.3		50	No	No		

Please cite this article in press as: Lalitha R, Chandavar VR, Analysis of genetic diversity in *CYTB* and control region sequences of *Melanochelys trijuga* (Schweigger, 1812) from Karnataka, Journal of Asia-Pacific Biodiversity (2018), https://doi.org/10.1016/j.japb.2018.05.001