



Original article

Mitochondrial haplotype distribution and phylogenetic relationship of an endangered species Reeve's turtle (*Mauremys reevesii*) in East AsiaHong-Shik Oh^{a,†}, Seon-Mi Park^{a,†}, Sang-Hyun Han^{b,*}^a Faculty of Science Education, Jeju National University, Jeju, South Korea^b Educational Science Research Institute, Jeju National University, Jeju, South Korea

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ABSTRACT

This study was examined to reveal haplotype distribution and phylogenetic relationship using mitochondrial DNA *CYTB* gene sequences of Reeve's turtle (*Mauremys reevesii*) of East Asia. *CYTB* sequences of Reeve's turtles were divided into 6 haplotypes (Hap01–Hap06). Chinese turtles were found in Hap01, Hap02, Hap04, and Hap05, and Hap01 was the highest frequency of 85.0%. Korean Turtles were found in Hap01, Hap03, Hap04, and Hap05, and Hap03 was the highest frequency of 52.1%. Although there was no haplotype which includes only the *CYTB* sequence exclusive for Reeve's turtles of Korea, since no *CYTB* sequence of China was found in Hap03, it would be possible that Hap03 turtles of Korea are separated from those of China. The haplotypes of Reeve's turtles of East Asia were monophyletic, which indicated that they had been evolved from a single maternal lineage, but went through local evolution after geographical migration and isolation in East Asia.

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Introduction

Testudines consist of 14 families and 319 species in the world (Bickham et al 2007), and the Korean peninsula contains eight species. Among them the Reeves's turtle (*Mauremys reevesii*) is a Korean indigenous freshwater turtle which lives predominantly in unpolluted streams, rivers, and slow flowing freshwater (National Institute of Biological Resources 2011; National Institute of Environmental Research 2011; Song 2007). It is distributed in China, Japan, Taiwan, and the Korean Peninsula except in Jeju Island (Chen and Lue 2010; Lovich et al 1985; Song 2007; van Dijk et al 2012). The Reeve's turtle population has remarkably decreased due to habitat destruction, fragmentation, and water pollution by human activities in South Korea during industrial development period (Chen et al 2000; National Institute of Biological Resources 2011). They were further reduced from invasions of exotic species including Red-eared slider (*Trachemys scripta elegans*), which have been introduced for many purposes (National Institute of Biological Resources 2011; Oh and Hong 2007). Reeve's turtle was registered

as Natural Monument No. 453 and also enlisted as an Endangered Wild Species Class II in South Korea. In addition, this species is registered in the Red List of International Union for the Conservation of Nature (IUCN) and included in Appendix III in the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora list for restriction of international trade (CITES 2015; IUCN 2015).

Mitochondrial DNA (mtDNA) represents maternal inheritance, absence of recombination events, and a high mutation rate, so have been applied for genetic diversity. Particularly mtDNA *CYTB* has been mostly used for the analysis of phylogenetic relationships in many species (Birky et al 1989; Brown et al 1979; Clayton 1982; Lau et al 1998). The phylogenetic relationship among *Mauremys* species was firstly described by Honda et al (2002), the origin of Japanese populations of Reeve's turtle comparing with those of East Asia (Suzuki et al 2011). Natural hybridization among the *Mauremys* species were also examined using mtDNA polymorphisms in Japanese and Chinese turtle populations (Suzuki et al 2014; Xia et al 2011). Up to now just a few reports have been documented regarding the status of distribution and habitation characteristics, and restoration plans in the wild in South Korea (Chang et al 2012; Kim et al 2013; Song 2007). However, the genetic structure of Korean Reeve's turtle population and its evolutionary relationship among Asian populations still remains unclear. This study was carried out to compare the maternal lineages of Reeve's Turtle

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populations of East Asia to reveal a phylogenetic relationship using the polymorphisms of mtDNA *CYTB* gene sequences.

Materials and methods

Sample collection and DNA extraction

A total of 78 specimens of Reeve's turtle from South Korea and China were used for this study. One of those was collected from Gyeongsangnam-do, South Korea in June, 2013. Six specimens were obtained from the traditional market in Busan, South Korea. The other Korean turtles collected from the zoo and wild habitats, were kindly provided from the Korea National Park Research Institute, Wonju Korea and Kangwon National University, Chuncheon, South Korea. Tail tissues of Chinese turtles were provided from Northeast Forestry University and State Forestry Administration, People's Republic of China. Tissues and blood collections were subjected to DNA extraction, genomic DNA was extracted from blood and tissue using DNeasy blood & tissue kit (Qiagen GmbH, Hilden, Germany). The study was conducted in accordance (approval number 2015–0021) with recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by the Institutional Animal Care and Use Committee of the Jeju National University, Jeju, Republic of Korea.

Polymerase Chain Reaction (PCR) amplification for mtDNA *CYTB*

The primers used for the amplification of the mtDNA *CYTB* fragment were designed from the reported complete mtDNA genome sequences for the Reeve's turtle (NCBI accession no. FJ469674). The primer sequences were as follows: NS_CYTB_F (5'-GAC TTT AAC CAA GAC CCG TGA TTT-3') and NS_CYTB_R (5'-AAG GTT TGG AGC CTT CTC TTT TGA-3'). PCR was conducted in 25 µL volumes, each containing 1× reaction buffer, 200µM of dNTPs, 2.5 units of *Taq* DNA polymerase (GenetBio, Daejeon, South Korea), 10mM of each primer, and ~100 ng of total DNA. Amplification was conducted using Mastercycler Gradient (Eppendorf, Hamburg, Germany) under the following conditions: an initial denaturation at 95°C for 3 minutes, followed by 40 cycles at 94°C for 1 minutes, 54°C for 1 minute and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR products were confirmed by electrophoresis on 1.5% agarose gels containing ethidium bromide, and further purified with a QiaexII Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

DNA sequencing and phylogenetic analysis

The purified PCR products were directly applied to DNA sequencing ABI 3130XL Genetic Analyzer (Applied Biosystems, CA, USA). Similarity searches were carried out to compare the newly determined sequences with those previously reported in the NCBI database using BAST search web program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). *CYTB* sequences newly determined in Reeve's turtles have been deposited in NCBI database under accession numbers KR921503–KR921580. We produced sequences from 78 Reeve's turtles in this study and collected an additional 148 sequences of Reeve's turtle previously reported from Japan, Taiwan, and China (Table 1). A multiple alignment of the *CYTB* sequences was performed using CLUSTAL W (Thompson et al 1994). The haplotype structure was estimated using DnaSP version 5 (Librado and Rozas 2009). Phylogenetic analysis was conducted using the MEGA 6.0 program (Tamura et al 2013). Genetic distances of all haplotypes were calculated based on Kimura's two-parameter method (Kimura 1980) and estimated transition/transversion ratio. After determining haplotypes, all unique haplotype sequences

Table 1. A list of sequence data used in the analysis of *CYTB* gene sequences.

Species	Country	Accession no.	Refs	
<i>Mauremysreevesii</i>	South Korea	KR921539	This study	
		–KR921580		
		KF732683,	Unpublished	
		KM289199,	observation	
		KM289198		
			AB562027	Suzuki et al 2011
			JQ798814	Unpublished
				observation
		China	KR921503	This study
			–KR921538	
			HQ442410	Xia et al 2011
			–HQ442412	
		Japan	AJ519497	Barth et al 2002
			AB571605	Suzuki et al 2011
			–AB571615,	
		AB561906		
		–AB562026		
		AB920782	Fujii et al 2014	
		–AB920783		
	Taiwan	AB562028	Suzuki et al 2011	
		FJ026830, FJ026852	Lee et al 2009	
		–FJ026854		
<i>Mauremys</i>		HQ442419	Xia et al 2011	
<i>annamensis</i>				
<i>Mauremys japonica</i>		HQ442418	Xia et al 2011	
<i>Mauremys mutica</i>		HQ442417	Xia et al 2011	
<i>Mauremys sinensis</i>		HQ442409	Xia et al 2011	
<i>Salalia bealei</i>		HQ442416	Xia et al 2011	

were phylogenetically analyzed with those from genus *Mauremys* species (*Mauremys japonica*, *Mauremys sinensis*, *Mauremys annamensis*, and *Mauremys mutica*) and that of *Salalia bealei* for out-group rooting. Reliability of neighbor-joining (NJ) tree (Saitou and Nei 1987) topology was assessed using the bootstrapping (1,000 repetition) method.

Results

Distribution of *CYTB* haplotypes

As a result of analyzing the haplotype by combining all *CYTB* sequences of Reeve's turtles reported in East Asia previously and sequences determined in this study, it was classified into six haplotypes (Hap01–Hap06) in all (Table 2). Hap03 was dominant including 128 (56.6%) sequences, and most of these sequences were identified as originating from the Reeve's turtle populations of Korea ($n = 24$) and Japan ($n = 103$). In addition to these, a Reeve's turtle of Taiwan sequence was discovered, but no *CYTB* sequence of population of China was found.

Figure 1 indicates biogeographical distribution of the haplotype of the mtDNA *CYTB* gene sequence of Reeve's turtle populations in East Asia. Hap01 was discovered in all Reeve's turtles of East Asian

Table 2. Distribution of *CYTB* haplotypes of *Mauremys reevesii* in East Asia.

Haplotype	Country				
	South Korea	China	Japan	Taiwan	Total
Hap01	20 (42.6)	34 (85.0)	28 (20.9)	3 (60.0)	85
Hap02		1 (2.5)			1
Hap03	24 (51.1)		103 (76.9)	1 (20.0)	128
Hap04	2 (4.2)	1 (2.5)		1 (20.0)	4
Hap05	1 (2.1)	4 (10.0)	2 (1.5)		7
Hap06			1 (0.7)		1
Total	47	40	134	5	226

Data are presented as n (%).

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