ELECTRONIC APPENDIX

This is the Electronic Appendix to the article

Evolutionary distinctiveness of the extinct Yunnan box turtle (*Cuora yunnanensis*) revealed by DNA from an old museum specimen

by

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ELECTRONIC APPENDIX A

Voucher Information

A piece of leg muscle was excised from an ethanol-stored (but possibly formalin-fixed) museum specimen of *Cuora yunnanensis* held in the Muséum National d'Histoire Naturelle, Paris (MNHN 1907.10). This specimen agrees with the original description of *C. yunnanensis* (Boulenger 1906) and published images of *C. yunnanensis* (e.g., Ernst & Barbour 1989; McCord & Iverson 1991; Zhou & Zhou 1992; Zhao & Adler 1993) in having the mottled neck, distinctive head stripes, and other aspects of its coloration that distinguish it from all other known species (see McCord & Iverson 1991). Furthermore, MNHN 1907.10 was collected from Yunnan-Fu, the terra typica of *C. yunnanensis*. It was received by the Paris Museum in 1907 from W. F. H. Rosenberg, a collector and dealer of natural history collections. Many of Rosenberg's other specimens from China were recorded as being from "Yunnan-fu" and "Tongchuan-Fu", the same two localities given by Boulenger (1906) in the original description of *C. yunnanensis*. It is possible that Rosenberg also obtained his Yunnan specimens from John Graham and Rev. F. J. Dymond, the collectors that supplied Boulenger with the type series of the species.

The other 11 known specimens of *C. yunnanensis* consist of the following: (1-6) six specimens in the type series at the British Museum of Natural History, BMNH 1946.1.22.97-99; 1946.1.23.1-3; (7-8) two specimens in The Natural Museum of Vienna, NMW 29936:1-2; (9-11) three uncataloged specimens in The Institute of Zoology, Chinese Academy of Sciences, Beijing (Jinzhong Fu pers. comm. to JFP). Two of these are whole preserved specimens (field numbers 374 and 394), and one consists only of a skull and plastron after being dissected for an anatomy course. Two photographs of the largest specimen (field number 394; 16.5 cm carapace length) are figured in Zhou & Zhou (1992).

Other samples used in the study, including outgroups, correspond to those listed in Stuart & Parham (2004). New sequences generated in this study, and sequences of species used in analyses here that were represented by multiple samples in Stuart & Parham (2004), are listed in Table 1.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from muscle stored in 95% ethanol or blood stored in buffer (10% EDTA, 0.5% sodium fluoride, 0.5% thymol, 1% tris at pH 7.0) from samples other than *C. yunnanensis* using PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc.). An 831 bp piece of mtDNA that encodes part of the cytochrome oxidase subunit I (COI) gene was amplified by PCR (94^{o}C 45s, 56^{o}C 30s, 72^{o}C 1 min) for 35 cycles using the primers L-turtCOIc and H-turtCOIc (Stuart & Parham 2004; Table 2). An 892 bp piece of mtDNA that encodes part of the NADH dehydrogenase subunit 4 (ND4) gene, the complete tRNAs histidine (His) and serine (Ser), and part of the tRNA leucine (Leu) was amplified by the polymerase chain reaction (PCR; 94^{o}C 45s, 62^{o}C 30s, 72^{o}C 1 min) for 35 cycles using the primers L-ND4 and H-Leu (Stuart & Parham 2004; Table 2). PCR products were visualized, sequenced using the amplifying primers and the internal primers L-COIint and H-COIint or L-ND4int and H-ND4int, edited, and translated, as described by Stuart & Parham (2004).

DNA extractions from leg muscle of the museum specimen of *C. yunnanensis* (MNHN 1907.10) were performed with UV-sterilized supplies inside a Purifier PCR Enclosure (Labconco) in a separate room from where fresh turtle tissues have been previously extracted and amplified. Two methods of DNA extractions were used for this sample. The first extraction method followed the DNeasy Tissue Kit (Qiagen) protocol for animal tissues, with these modifications: the tissue was digested in a 2 ml tube for 5 days with daily additions of 300 µg of proteinase-K, a second spin was added for 1 minute at full speed after discarding the Buffer AW2 flow-through fluid, and only 70 µl of Buffer AE was added to the DNeasy membrane rather than 100-400 µl, after which the membrane was incubated at room temperature for 5 min rather than 1 min before centrifuging. The second extraction method closely followed a protocol for extracting DNA from formalin-fixed tissues developed by Cathy Dayton of the U.S. Fish and Wildlife Service and made available to the public at http://www.public.iastate.edu/~curteck/Formalin_Fixed_DNA.htm. A piece of muscle about twice the size of a grain of rice was placed in three, 24-hour washes of 2 ml of 1X GTE buffer (Shedlock et al. 1997). The tissue was incubated at 55^{o}C for 5 days in a 2 ml tube containing 500 µl of PureGene Cell Lysis Solution (Gentra Systems, Inc.), 300 µg of proteinase-K, and 20 µl of 1mM DTT (dithiothreitol), with daily additions of 300 µg of proteinase-K added daily. The sample was placed on ice for 5 min before adding 200 µl of PureGene Protein Precipitation Solution (Gentra Systems, Inc.). The sample was inverted 50 times and centrifuged at 14,000 x g for 3 minutes. The supernatant was poured into a 1.5 ml tube containing 600 µl of cold 100% isopropanol and 3 µl of PureGene glycogen solution (Gentra Systems, Inc.), inverted 50 times, incubated at -20 ^{o}C for 48 h, and centrifuged at 14,000 g for 30 minutes at room temperature. The supernatant was discarded and the tube was washed with 200 µl of 70% ethanol, inverted 50 times, and centrifuged at 14,000 g for 3 minutes. The tube was drained on a paper towel and air-dried upside down for 6 h. The DNA was rehydrated with 40 µl of PureGene DNA Hydration Solution (Gentra Systems, Inc.) and incubated at room temperature for 12 h before storing at 4^{o}C.

Neither the 831 bp nor the 892 bp fragment could be amplified in a single piece from the extractions of *C. yunnanensis*, presumably because of DNA degradation. Consequently, the complete fragments were obtained from *C. yunnanensis* by amplifying and sequencing smaller fragments of 190-547 bp (not including primer sequences) at a time using primers obtained from Stuart & Parham (2004) or designed from *Cuora* sequences available in GenBank (Table 2). To avoid generating chimeric sequences during analysis, the primers were designed so that resulting DNA fragments overlapped by 36-155 bp after primer sequences were trimmed off. Fragments were amplified by PCR (94^{o}C 45s, 50-54^{o}C 30s, 72^{o}C 50s) for 40 cycles using AmpliTaq Gold (Roche) and adding 4 µl of purified, 10 mg/ml bovine serum albumin (BSA; New England BioLabs, Inc.) to 25 µl total PCR reactions. PCR products were visualized, sequenced using the amplifying primers, edited, and translated, as described by Stuart & Parham (2004).

Good quality mitochondrial DNA sequences were obtained from both extraction types of *C. yunnanensis* using all primer pairs listed in Table 2. Identical sequences were obtained in all overlapping fragments, and from both extraction types. Individual fragments and the concatenated sequence of *C. yunnanensis* were compared with all other sequences of geoemydid turtles obtained previously in our laboratory, and were found to

be unique. Consequently, we are confident that the sequence of *C. yunnanensis* used in analyses here is authentic and does not represent a contaminant or chimeric sequence.

Phylogenetic Methods

Phylogenies were reconstructed using both maximum parsimony and maximum likelihood optimality criteria, as implemented in PAUP* 4.0b10 (Swofford 2002). Maximum parsimony analyses were performed treating transitions and transversions as equally weighted for 1000 random addition replicates with stepwise addition of taxa using the branch and bound search algorithm. Nodal support was evaluated with 500 non-parametric bootstrapping pseudoreplications (Felsenstein 1985) and decay indices (Bremer 1994). The latter were calculated using TREEROTV.2 (Sorenson 1999). Maximum likelihood analyses were performed with 150 random addition replicates with stepwise addition of taxa using the heuristic search algorithm and TBR branch swapping. The model of sequence evolution that best described the data set was inferred using Modeltest 3.06 (Posada & Crandall 1998). The model HKY +G was selected, with ti/tv ratio = 10.9965, proportion of invariable sites = 0, gamma distribution shape parameter = 0.1761, and base frequencies as A = 0.3203, C = 0.2537, G = 0.1503, and T = 0.2756.

Table 1. New sequences of mitochondrial DNA obtained in this study, and sequences of species used in analyses here that were represented by multiple samples in Stuart & Parham (2004). FMNH refers to Field Museum of Natural History (Chicago, USA), MNHN to Muséum National d'Histoire Naturelle (Paris, France), MTD T to Museum für Tierkunde (Dresden, Germany), and YPM R to Yale Peabody Museum (New Haven, USA).

Species	Voucher	GenBank Accession (COI / ND4 + His + Ser + Leu)
Cuora aurocapitata	MTD T 1076	AY590463 / AY572867
Cuora bourreti	FMNH 261574	AY357757 / AY364618
Cuora bourreti	FMNH 261577	AY357751 / AY364624
Cuora flavomarginata	MTD T 232	AY590459 / not sequenced
Cuora galbinifrons	FMNH 256544	AY357748 / AY364615
Cuora galbinifrons	FMNH 255694	AY357742 / AY364612
Cuora mccordi	MTD T 1083	AY590456 / not sequenced
Cuora pani	MVZ 230513	AY590457 / AY590461
Cuora picturata	FMNH 261575	AY357760 / AY364628
Cuora picturata	YPM R 11679	AY357745 / AY364630
Cuora zhoui	MTD T 949	AY590458 / AY590462
Cuora zhoui	MTD T 1074	AY593968 / AY572865
Cuora zhoui	MTD T 1075	AY593969 / AY572866
Cuora yunnanensis	MNHN 1907.10	AY590460 / AY572868

Table 2. Oligonucleotide primer sequences used to amplify and sequence mitochondrial DNA from a museum specimen of *Cuora yunnanensis*. 'L' and 'H' refer to light and heavy strands, respectively.

Primer	Sequence	Source
L-turtCOIc	5'-TACCTGTGATTTTAACCCGTTGAT-3'	Stuart & Parham (2004)
H-COIint	5'-TAGTTAGGTCTACAGAGGCGC-3'	Stuart & Parham (2004)
L-COIint	5'-TGATCAGTACTTATCACAGCCG-3'	Stuart & Parham (2004)
H-turtCOIc	5'-TGGTGGGCTCATACAATAAAGC-3'	Stuart & Parham (2004)
L-330COI	5'-TACTTTTACTCCTAGCCTCCTCAG-3'	This study
H-610COI	5'-GTATTTAGGTTTCGGTCAGTGAG-3'	This study
H-715COI	5'-GCCAAATCCTGGTAAGATTAAGAT-3'	This study
L-ND4	5'-GTAGAAGCCCCAATCGCAG-3'	Stuart & Parham (2004)
H-285ND4	5'-CTAGGCAGAAAAGTATTGATGATG-3'	This study
L-190ND4	5'-TCATTAATTGCTTATTCATCCGT-3'	This study
H-395ND4	5'-GGTCAGACTAGCTGAGAATCA-3'	This study
L-360ND4	5'-AGCCGAACACTACTTTTAGCTC-3'	This study
H-550ND4	5'-CTCATTGTGTAATGATTAGTATG-3'	This study
L-510ND4	5'-AATACTACAATCCTAATAACAGG-3'	This study
H-730ND4	5'-TTTAGAGCCACAGTCTAATG-3'	This study
L-660ND4	5'-CATATACTACCAATAGCACTGCT-3'	This study
H-Leu	5'-ATTACTTTTACTTGGATTTGCACCA-3'	Stuart & Parham (2004)

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