THE ROYAL SOCIETY biology letters

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

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Recd 07.04.04; *Accptd* 17.05.04; *Published online* 20.07.04

***Cuora yunnanensis* is an extinct turtle known from 12 specimens collected from Yunnan, China, before 1908. We used ancient DNA methods to sequence 1723 base pairs of mitochondrial DNA from a museum specimen of *C. yunnanensis*. Unlike some rare 'species' recently described from the pet trade, *C. yunnanensis* represents a lineage that is distinct from other known turtles. Besides *C. yunnanensis*, two other valid species (*C. mccordi*, *C. zhoui*) are unknown in the wild but are supposedly from Yunnan. Intensive field surveys for surviving wild populations of these critically endangered species are urgently needed.**

Keywords: *Cuora yunnanensis*; ancient DNA; extinction; endangered species; China; turtle

1. INTRODUCTION

Asia's turtle fauna is highly threatened by over-harvesting for food, medicine and the international pet trade (van Dijk *et al.* 2000). Included in this threatened fauna is a suite of species known only by specimens obtained from Asian food markets, turtle farms and pet dealers: for example, *Cuora mccordi*, *C. serrata*, *C. zhoui*, *Mauremys iversoni*, *M. pritchardi*, *Ocadia philippeni*, *O. glyphistoma* and *O. pseudocellata* (Fritz & Obst 1998, 1999; Parham *et al.* 2001); or by very few specimens in the wild, for example, *C. pani*, *Heosemys depressa*, *H. leytensis* and *Leucocephalon yuwonoi* (Fritz & Obst 1998, 1999; Parham & Li 1999; Platt *et al.* 2001, 2003*a*; Diesmos *et al.* 2004).

Recent molecular studies have revealed that some of Asia's poorly known turtles represent hybrids of better-known species (Parham *et al.* 2001; Wink *et al.* 2001; Spinks *et al.* 2004), some share mitochondrial DNA (mtDNA) haplotypes suggesting introgression or recent divergences (Barth *et al.* 2003; Stuart & Parham 2004), and some represent ancient, independently evolving lineages (McCord *et al.* 2000; Barth *et al.* 2004; Spinks *et al.* 2004; Stuart & Parham 2004). At the same time, recent field efforts have located new populations of highly threatened Asian turtle species in the wild (Platt *et al.* 2001, 2003*a–c*; Diesmos *et al.* 2004). Clearly, molecular studies can play an important role in conservation policy by identifying distinct evolutionary lineages of turtles, and directing limited conservation resources towards finding and protecting these in the wild (van Dijk 2000; Parham *et al.* 2001).

One of Asia's least known turtle species, the Yunnan box turtle, *Cuora yunnanensis* (Boulenger 1906), is known from just 12 museum specimens. These were either purchased from natural history specimen dealers who obtained them from Yunnan, southern China, before 1908, or have no associated data (see electronic Appendix A). *Cuora yunnanensis* is now listed as extinct in the 2003 IUCN Red List of Threatened Species (IUCN 2003), meaning 'there is no reasonable doubt that the last individual has died'. The species was listed as extinct owing to a complete lack of verifiable records since those 12 specimens were collected (despite very high levels of turtle trade in Yunnan), and because one of its two known sites of occurrence has disappeared under the expanding city of Kunming (Zhao 1998; Lau & Shi 2000; IUCN/SSC Tortoise and Freshwater Turtle Specialist Group and Asian Turtle Trade Working Group 2000; IUCN 2003). The circumstances of the description of *C. yunnanensis* (purchased specimens and no field observations) resemble those of other recently described rare species that are probably hybrids of better-known species, including those currently classified in different genera (Parham *et al.* 2001; Wink *et al.* 2001; Spinks *et al.* 2004). These putative hybrid taxa are diagnosed by characters that are usually restricted to the parental species. The distinctly mottled neck of *C. yunnanensis* is also found in *Chinemys reevesii*, a species native to Yunnan (Zhao & Adler 1993) that is commonly reared in Chinese turtle farms and has been implicated in at least three other hybridizations, including one with a *Cuora* (Yasukawa *et al.* 1992; Wink *et al.* 2001; Galgon & Fritz 2002). If *C. yunnanensis* was a hybrid of *Ch. reevesii* and a species of *Cuora*, *C. yunnanensis* would share a mtDNA haplotype with the maternal species. However, the evolutionary distinctiveness of *C. yunnanensis* has not been tested by using molecular data because of a lack of fresh tissue samples.

We surmounted this problem by using ancient DNA methods to sequence 1723 base pairs of mtDNA from a 1907 specimen of *C. yunnanensis* held in the Muséum National d'Histoire Naturelle in Paris (MNHN 1907.10). We present the first, to our knowledge, molecular phylogenetic analysis of the genus *Cuora* with complete taxon sampling. We use these molecular data to evaluate the evolutionary distinctiveness of *C. yunnanensis* and two other species of *Cuora*, reportedly from Yunnan, that are

DOI 10.1098/rsbl.2004.0217

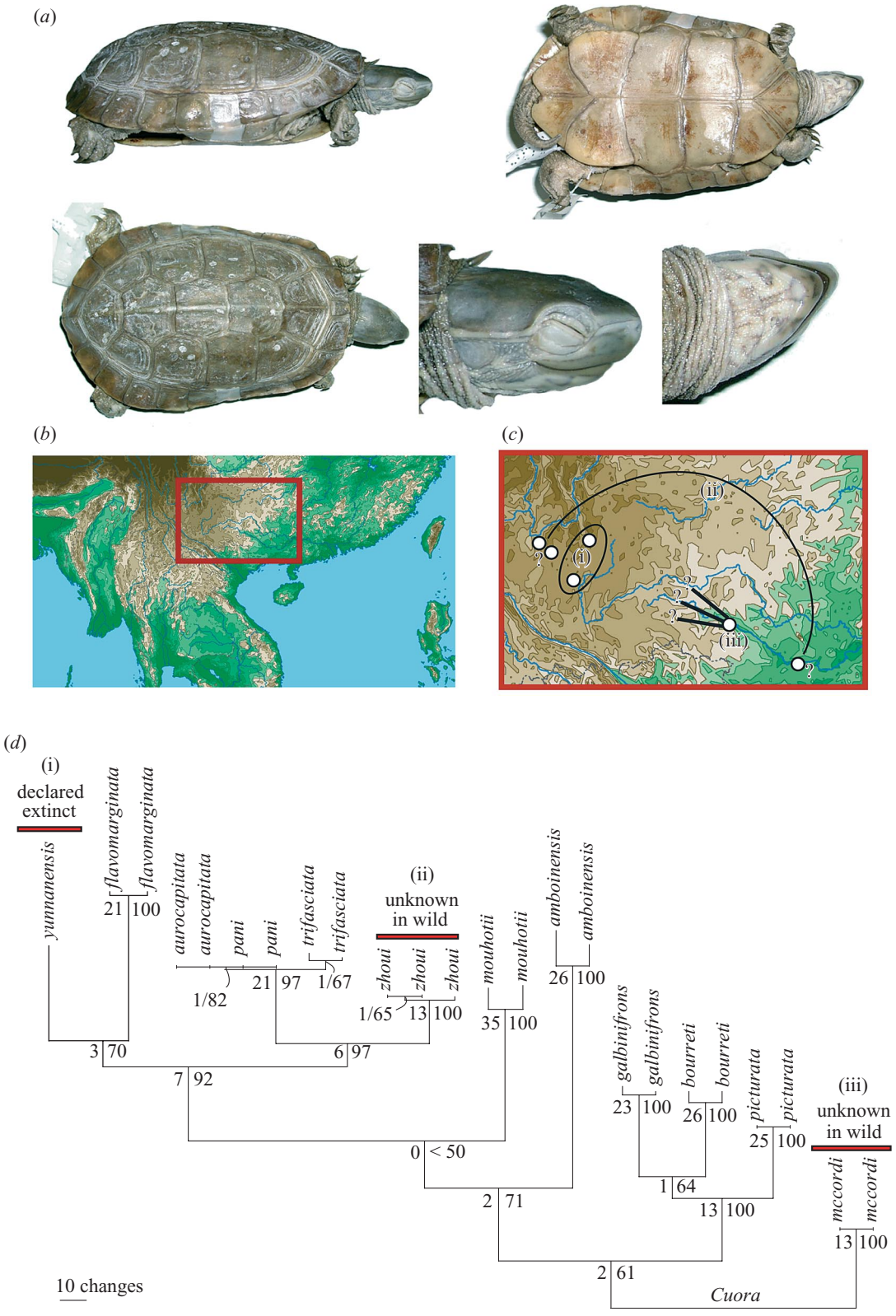

Figure 1. (*a*) The Yunnan box turtle, *Cuora yunnanensis*, specimen (MNHN 1907.10) sequenced in this study showing the diagnostic head stripes and mottled neck. (*b*) Map showing the region of Yunnan, China, where poorly known box turtles were reportedly collected. (*c*)(i) Reported localities for *C. yunnanensis*. (ii) Localities where *Cuora zhoui* specimens were reportedly purchased. (iii) Type locality for *Cuora mccordi*. Lines show that the actual locality may be further to the west according to McCord & Iverson (1991). (*d*) One of two equally most parsimonious trees obtained from maximum-parsimony analysis, and the single tree obtained from maximum-likelihood analysis, of an alignment containing 1790 bp of mtDNA. In the alternative equally most parsimonious tree, *C. mouhotii* is sister to *C. amboinensis*. Trees were rooted with *Chinemys reevesii*, *Ch. nigricans*, and *Mauremys mutica*. Numbers to the left of nodes are decay indices, and to the right of nodes are parsimony non-parametric bootstrap values. See electronic Appendix A for details of analysis. The reported localities of species (i), (ii) and (iii) are illustrated in (*c*).

critically endangered but which have not been documented in the wild (*C. mccordi* and *C. zhoui*).

2. MATERIAL AND METHODS

We sequenced an 831 bp piece of mtDNA that encodes part of the cytochrome oxidase subunit I (COI) gene and 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) from a specimen of *C. yunnanensis* (MNHN 1907.10; figure 1) that was recently identified in the Muséum of National d'Histoire Naturelle in Paris (see electronic Appendix A for the history of the specimen). We also sequenced these mtDNA fragments from three specimens of *C. zhoui*, the only other *Cuora* species missing from the Stuart & Parham (2004) dataset, and one additional specimen each of *C. aurocapitata*, *C. flavomarginata*, *C. mccordi* and *C. pani*. (See electronic Appendix A for voucher information, GenBank accession numbers, sequencing protocols and methods of phylogenetic analyses.)

3. RESULTS

Parsimony and maximum-likelihood analyses recover the same hypothesis of phylogenetic relationships for *Cuora* (figure 1), except that one of the two equally most-parsimonious trees places *C. mouhotii* as sister to *C. amboinensis*. *Cuora yunnanensis* nests within the *Cuora* clade, and is moderately supported to be the sister taxon of *C. flavomarginata*. Together, *C. yunnanensis* and *C. flavomarginata* form a well-supported clade with *C. zhoui* and a '*C. trifasciata* complex' containing three (*trifasciata*, *pani* and *aurocapitata*) potentially conspecific or recently diverged taxa (see Stuart & Parham (2004) for discussion). The mtDNA of *C. yunnanensis* is distinct from sampled specimens of all other known species. *Cuora yunnanensis* has an uncorrected pairwise sequence divergence of 4.9–5.0% from its sister taxon *Cuora flavomarginata*, a high-domed, terrestrial *Cuora* from northeastern China (Fong *et al.* 2002), and an uncorrected pairwise sequence divergence of 3.7–4.4% from *C. zhoui* and the '*C. trifasciata* complex'.

4. DISCUSSION

The divergent mtDNA sequence obtained from the museum specimen implies that *C. yunnanensis* is not of recent hybrid origin, but rather represents a distinct evolutionary lineage. We included at least two individuals of every species of *Cuora* in our analysis, but we cannot rule out the possibility that *C. yunnanensis* matches a haplotype in an unknown distinct lineage that will be discovered by future sampling within the clade containing *C. flavomarginata*, *C. zhoui*, *C. aurocapitata*, *C. pani* and *C. trifasciata*.

China has the highest species richness, highest endemism and most threatened turtle fauna of any country in Asia (Stuart & Thorbjarnarson 2003), and *C. yunnanensis* represents the first recent documented loss to that fauna. The natural habitat of the high elevation (more than 2000 m) wetland 'Yunnan-Fu,' the type locality of *C. yunnanensis* and the extinct Kunming newt, *Cynops wolterstorffi* (Boulenger 1905; figure 1), has been destroyed by the expanding city of Kunming (Zhao 1998). The second locality for *C. yunnanensis* given by Boulenger (1906), 'Tongchuan-Fu' (= Dongchuan; figure 1), lies *ca.* 100 km north of Kunming. Lau & Shi (2000) reported that a suitable habitat for *C. yunnanensis* may still exist there, but no specimens have been found.

Two other species of *Cuora*, reportedly from Yunnan, *C. mccordi* and *C. zhoui*, also represent distinct lineages of *Cuora* (figure 1). Both are listed as critically endangered in the 2003 IUCN Red List of Threatened Species (IUCN 2003), meaning that they are 'facing an extremely high risk of extinction in the wild'. Unlike *C. yunnanensis*, living specimens of *C. mccordi* and C. *zhoui* exist in captivity and command very high prices in the international pet trade. Despite their commercial value, few wild-caught individuals of *C. mccordi* and *C. zhoui* have appeared in trade since their description (Lau & Shi 2000), suggesting that they have become commercially extinct (IUCN/SSC Tortoise and Freshwater Turtle Specialist Group and Asian Turtle Trade Working Group 2000). *Cuora mccordi* and *C. zhoui* were described from pet trade and market specimens, are unknown in the wild and have no reliable localities (figure 1). If these species are not located and protected, they will soon become extinct in the wild like *C. yunnanensis*.

Acknowledgements

Sequencing was conducted in The Field Museum's Pritzker Laboratory for Molecular Systematics and Evolution and operated with support from the Pritzker Foundation. Financial support to B.L.S. was provided by The John D. and Catherine T. MacArthur Foundation (with Harold Voris and Robert Inger). This is UCMP contribution 1853. This work is LBNL-54722 and was performed under the auspices of the US Department of Energy, Office of Biological and Environmental Research. Richard Gemel, Vienna Natural History Museum (NMW) provided data on specimens in his care. The authors thank Ted Papenfuss for his encouragement and comments. Phil Spinks and Tortsen Blanck are thanked for discussions and ideas. The first extraction protocol used for the sample of *C. yunnanensis* is based on a protocol provided to us by Kerri Dugan of the Counterterrorism and Forensic Science Research Unit at the Federal Bureau of Investigation.

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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

Parham *et al*.

Proc. R. Soc. B **271** (Suppl. 6), 391–394 (doi:10.1098/ rsbl.2004.0217)

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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