

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

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***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, 2003a; 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, 2003a-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 critically endangered but which have not been documented in the wild (*C. mccordi* and *C. zhoui*).

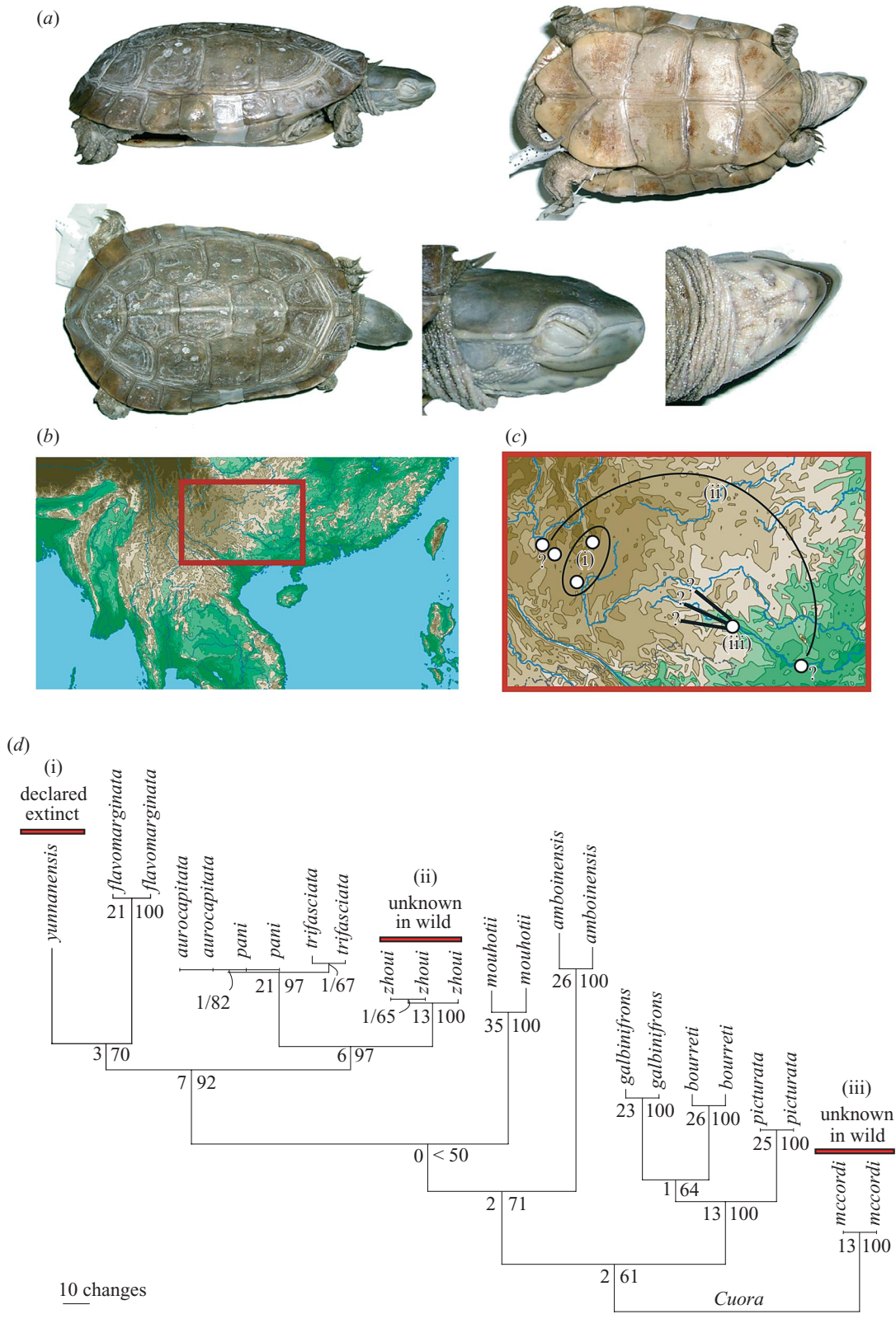


Figure 1. (a) The Yunnan box turtle, *Cuora yunnanensis*, specimen (MNHN 1907.10) sequenced in this study showing the diagnostic head stripes and mottled neck. (b) Map showing the region of Yunnan, China, where poorly known box turtles were reportedly collected. (c)(i) Reported localities for *C. yunnanensis*. (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).

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*.

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- Barth, D., Bernhard, D., Guicking, D., Stöck, M. & Fritz, U. 2003 Is *Chinemys megalcephala* Fang, 1934 a valid species? New insights based on mitochondrial DNA sequences. *Salamandra* **38**, 233–244.
- Barth, D., Bernhard, D., Fritsch, G. & Fritz, U. 2004 The freshwater turtle genus *Mauremys* (Testudines, Geoemydidae)—a textbook example of an east-west disjunction or a taxonomic misconception? *Zool. Scripta* **33**, 213–221.
- Boulenger, G. A. 1905 Description of a new newt from Yunnan. *Proc. Zool. Soc. Lond.* **1905**, 277–278.
- Boulenger, G. A. 1906 Descriptions of new reptiles from Yunnan. *A. Mag. Nat. Hist.* **7**, 567–568.
- Diesmos, A. C., Gee, G. V. A., Diesmos, M. L., Brown, R. M., Widmann, P. J. & Dimalibot, J. C. 2004 Rediscovery of the Philippine forest turtle, *Heosemys leytensis* (Chelonia; Bataguridae), from Palawan Island, Philippines. *Asiat. Herpetol. Res.* **10**, 22–27.
- Fong, J. J., Parham, J. F. & Fu, J. 2002 A reassessment of the distribution of *Cuora flavomarginata* Gray 1863 on mainland China. *Russian J. Herpetol.* **9**, 9–14.
- Fritz, U. & Obst, F. J. 1998 Neue Schildkröten aus Südostasien. Teil I. Platysternidae und Bataguridae (*Cuora*). *Sauria* **20**, 9–22.
- Fritz, U. & Obst, F. J. 1999 Neue Schildkröten aus Südostasien. Teil II. Bataguridae (*Cyclemys*, *Heosemys*, *Mauremys*, *Ocadia*, *Pyxidea*, *Sacalia*) und Trionychidae. *Sauria* **21**, 11–26.
- Galgon, F. & Fritz, U. 2002 Captive bred hybrids between *Chinemys reevesii* (Gray 1831) and *Cuora amboinensis kamaroma* Rummeler & Fritz, 1991. *Herpetozoa* **15**, 137–148.
- IUCN 2003 Red list of threatened species. Available from <http://www.redlist.org> (downloaded 21 February 2004).
- IUCN/SSC Tortoise and Freshwater Turtle Specialist Group and Asian Turtle Trade Working Group 2000 Recommended Changes to 1996 IUCN Red List Status of Asian Turtle Species. In *Asian turtle trade: Proc. Workshop Conserv. and Trade of Freshwat. Turtles and Tortoises in Asia. Phnom Penh, Cambodia, 1–4 December 1999*. *Chel. Res. Monogr.* **2** (ed. P. P. van Dijk, B. L. Stuart & A. G. J. Rhodin) pp. 156–164. Lunenburg: Chelonian Research Foundation.
- Lau, M. & Shi, H. 2000 Conservation and trade of terrestrial and freshwater turtles and tortoises in the People's Republic of China. In *Asian turtle trade: Proc. Workshop Conserv. and Trade of Freshwat. Turtles and Tortoises in Asia. Phnom Penh, Cambodia, 1–4 December 1999*. *Chel. Res. Monogr.* **2** (ed. P. P. van Dijk, B. L. Stuart & A. G. J. Rhodin), pp. 30–38. Lunenburg: Chelonian Research Foundation.

- McCord, W. P. & Iverson, J. B. 1991 A new box turtle of the genus *Cuora* (Testudines: Emydidae) with taxonomic notes and key to the species. *Herpetologica* **47**, 407–420.
- McCord, W. P., Iverson, J. B., Spinks, P. Q. & Shaffer, H. B. 2000 A new genus of geoemydid turtle from Asia. *Hamadryad* **25**, 20–24.
- Parham, J. F. & Li, D. 1999 A new locality for *Cuora pani* Song 1984 with comments on its known range. *Asiat. Herpetol. Res.* **8**, 111–113.
- Parham, J. F., Simison, W. B., Kozak, K. H., Feldman, C. R. & Shi, H. 2001 New Chinese turtles: endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens. *Anim. Conserv.* **4**, 357–367.
- Platt, S. G., Lee, R. J. & Klemens, M. W. 2001 Notes on the distribution, life history, and exploitation of turtles in Sulawesi, Indonesia, with emphasis on *Indotestudo forstenii* and *Leucocephalon yuwonoi*. *Chel. Conserv. Biol.* **4**, 154–159.
- Platt, S. G., Win Ko Ko, Kalyar, Myo Myo, Lay Lay Khaing & Rainwater, T. R. 2003a Ecology and conservation status of the Arakan forest turtle, *Heosemys depressa*, in western Myanmar. *Chel. Conserv. Biol.* **4**, 678–682.
- Platt, S. G., Stuart, B. L., Heng, S., Long, K., Kalyar & Heng, K. 2003b Rediscovery of the critically endangered river terrapin, *Batagur baska*, in Cambodia, with notes on occurrence, reproduction, and conservation status. *Chel. Conserv. Biol.* **4**, 691–695.
- Platt, S. G., Win Ko Ko, Lay Lay Khaing, Khin Myo Myo, Thanda Swe, Tint Lwin & Rainwater, T. R. 2003c Population status and conservation of the critically endangered Burmese star tortoise *Geochelone platynota* in central Myanmar. *Oryx* **37**, 464–471.
- Spinks, P. Q., Shaffer, H. B., Iverson, J. B. & McCord, W. P. 2004 Phylogenetic hypotheses for the turtle family Geoemydidae. *Mol. Phylogenet. Evol.* **31**, 164–182.
- Stuart, B. L. & Parham, J. F. 2004 Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). *Mol. Phylogenet. Evol.* **30**, 164–177.
- Stuart, B. L. & Thorbjarnarson, J. 2003 Biological prioritization of Asian countries for turtle conservation. *Chel. Conserv. Biol.* **4**, 642–647.
- van Dijk, P. P. 2000 The status of turtles in Asia. In *Asian turtle trade: Proc. Workshop Conserv. and Trade of Freshwat. Turtles and Tortoises in Asia. Phnom Penh, Cambodia, 1–4 December 1999*. *Chel. Res. Monogr.* **2** (ed. P. P. van Dijk, B. L. Stuart & A. G. J. Rhodin), pp. 15–23. Lunenburg: Chelonian Research Foundation.
- van Dijk, P. P., Stuart, B. L. & Rhodin, A. G. J. (eds) 2000 *Asian turtle trade: Proc. Workshop Conserv. and Trade of Freshwat. Turtles and Tortoises in Asia—Chel. Res. Monogr.* **2**. Lunenburg: Chelonian Research Foundation.
- Wink, M., Guicking, D. & Fritz, U. 2001 Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard et McCord, 1991, and *Mauremys pritchardi* McCord, 1997 (Reptilia: Testudines: Bataguridae). *Zool. Abh. Mus. Tierkde. Dresden* **51**, 41–49.
- Yasukawa, Y., Kamezaki, N. & Ichikawa, N. 1992 On hybrids between *Mauremys japonica* and *Chinemys reevesii*. *Jpn J. Herpetol.* **14**, 206–207.
- Zhao, E. 1998 *China red data book of endangered animals: Amphibia and Reptilia*. New York: Science Press.
- Zhao, E. & Adler, K. 1993 *Herpetology of China*. Oxford, OH: Society for the Study of Amphibians and Reptiles.

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