

Historical DNA from museum type specimens clarifies diversity of Asian leaf turtles (*Cyclemys*)

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Species boundaries in Asian leaf turtles of the genus *Cyclemys* are difficult to define on the basis of morphology, primarily because many populations exhibit considerable ontogenetic variation in shell and head coloration. Two recent molecular phylogenetic hypotheses of *Cyclemys* species relationships, based largely on market and pet-trade samples of uncertain provenance, were highly incongruent. We used historical DNA methods to sequence fragments of the mitochondrial cytochrome *b* gene from eight type specimens of *Cyclemys* (including one collected by Alfred Russel Wallace), and phylogenetically placed these type sequences into the context of published cytochrome *b* variation. Our phylogenetic hypothesis supports the recognition of four named species (*Cyclemys atripons*, *Cyclemys dentata*, *Cyclemys oldhamii*, and *C. pulchristriata*), as well as a fifth species of unknown geographical provenance obtained from the Hong Kong pet trade. The type sequences show that previous molecular phylogenetic studies were hampered by misidentifications, supporting the notion that *Cyclemys* of unknown provenance are not reliably identified to species solely on the basis of morphology. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **94**, 131–141.

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INTRODUCTION

Phylogenetic analyses of DNA sequence data are now regularly incorporated into taxonomic studies, and are especially useful in cases when species boundaries and evolutionary relationships are obscured by a high degree of morphological homoplasy. The most accurate means of matching DNA sequences to Linnaean names entail obtaining DNA sequence data from the type specimens on which the original descriptions of species were based (Tautz *et al.*, 2003; Austin & Melville, 2006). However, most existing type specimens were collected prior to the molecular revolution in systematic biology, and so lack associated tissue samples that were specially preserved for genetic analysis. Methods to retrieve DNA from degraded samples remain challenging (Handt *et al.*, 1994; Wayne, Leonard & Cooper, 1999; Cooper & Poinar, 2000; Hofreiter *et al.*, 2001), but have transformed traditional museum specimens

into valuable sources of genetic material for systematic studies on rare and extinct populations and species (e.g. Roy *et al.*, 1994; Sorenson *et al.*, 1999; Austin & Arnold, 2001; Payne & Sorenson, 2002; Austin, Arnold & Jones, 2004; Kearney & Stuart, 2004; Parham *et al.*, 2004; Wisely, Maldonado, & Fleischer, 2004; Fuchs, Fjeldså & Pasquet, 2005; Asher & Hofreiter, 2006; Fleischer *et al.*, 2006; Kohlsdorf & Wagner, 2006). Relatively few systematic studies have attempted to obtain DNA from old type specimens (e.g. Krings *et al.*, 1997; Amato *et al.*, 1999; Hughey, Silva & Hommersand, 2001; Austin, Arnold & Bour, 2002; Austin, Arnold & Bour, 2003; Hassanin & Ropiquet, 2004; Johnson, Watson & Mindell, 2005; Stuart, Inger & Voris, 2006a).

Asian leaf turtles of the genus *Cyclemys* provide an excellent example of a taxonomic group in which species boundaries and their evolutionary relationships are obscured by a high degree of morphological homoplasy. Species of *Cyclemys* are primarily diagnosed by shell and head coloration, but many

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populations exhibit considerable ontogenetic variation in these characters (Fritz, Gaulke & Lehr, 1997; Fritz & Ziegler, 1999). Furthermore, some species of *Cyclemys* were originally described from juvenile specimens, and others were described from adult specimens (Fritz *et al.*, 1997; Iverson & McCord, 1997), making comparisons difficult. As a result, species boundaries in *Cyclemys* are contentious and notoriously difficult to define, and the genus has a complicated taxonomic history.

The genus *Cyclemys* is widely distributed from north-eastern India to the Philippines (Fritz *et al.*, 1997; Iverson & McCord, 1997; Fritz & Ziegler, 1999). Historically, only one species, *Cyclemys dentata* (Gray, 1831), or sometimes two species, *C. dentata* and *Cyclemys tcheponensis* (Bourret, 1939) were thought to encompass this vast range. In 1997, a taxonomic revision (Fritz *et al.*, 1997) and a new species description (Iverson & McCord, 1997) brought the number of recognized species to five (Artner, 1998; Fritz & Ziegler, 1999): *Cyclemys atripons* Iverson & McCord, 1997, with a type locality of Krat, south-eastern Thailand; *Cyclemys dentata* (Gray, 1831), with a type locality of Java, Indonesia; *Cyclemys oldhamii* Gray, 1863, with a type locality of Mergui, Myanmar; *Cyclemys pulchristriata* Fritz, Gaulke & Lehr, 1997, with a type locality of Phuc-Son in central Vietnam; and *Cyclemys tcheponensis* (Bourret, 1939), with a type locality of Haute Sé-Bang-Hien on the border of southern Laos and central Vietnam. In the most recent taxonomic treatment, Guicking *et al.* (2002) resurrected *Cyclemys dhor shanensis* Annandale, 1918 out of synonymy with *C. oldhamii*, elevated *shanensis* to full species status, referred populations from Myanmar and Thailand to the subspecies *Cyclemys shanensis shanensis* Annandale, 1918, and referred *C. tcheponensis* to the subspecies *Cyclemys shanensis tcheponensis* (Bourret, 1939). Four additional described species of *Cyclemys* are currently considered to be junior synonyms of *C. dentata* or *C. s. tcheponensis* (reviewed in Fritz *et al.*, 1997). Specifically, *Cyclemys orbiculata* Bell, 1834 and *Cyclemys bellii* Gray, 1863, with uncertain type localities, and *Cyclemys ovata* Gray, 1863, with a type locality of Sarawak are treated as junior synonyms of *C. dentata*, and *Cyclemys tiannanensis* Kou, 1989, with a type locality of Yunnan, China, is treated as a junior synonym of *C. s. tcheponensis*.

Despite the uncertainties in the number of taxa (species and subspecies), *Cyclemys* are readily classified into two distinct morphological groups on the basis of plastron (ventral part of the shell) coloration: *C. oldhamii*, *C. s. shanensis*, and *C. s. tcheponensis* have mostly dark brown or black plastra, whereas *C. atripons*, *C. dentata*, and *C. pulchristriata* have mostly yellow plastra (Fritz *et al.*, 1997; Guicking

et al., 2002). Within the dark-plastron group, only adults of *C. oldhamii* and *C. s. shanensis* are reliably distinguished from *C. s. tcheponensis*, as *C. oldhamii* and *C. s. shanensis* lose head and neck stripes during ontogeny, but *C. s. tcheponensis* does not (Fritz *et al.*, 1997; Guicking *et al.*, 2002). No morphological characters have been reported that can reliably distinguish *C. oldhamii* from *C. s. shanensis*. Within the yellow plastron group, *C. atripons* and *C. pulchristriata* have immaculate throats, but *C. dentata* has a striped throat (Fritz *et al.*, 1997; Iverson & McCord, 1997). No morphological characters have been reported that can reliably distinguish *C. atripons* from *C. pulchristriata*. Fritz *et al.* (2001) proposed that the number of ventral neck stripes differed between *C. atripons* and *C. pulchristriata*, but the range of neck stripes reported by Stuart & Platt (2004) in just two turtles collected together in south-western Cambodia (near to the type locality of *C. atripons*) exceeded the range reported for both species by Fritz *et al.* (2001). Because of their morphological similarity, some authors have suggested that *C. atripons* and *C. pulchristriata* may be conspecific (Fritz & Obst, 1999; Iverson in Guicking *et al.*, 2002).

Defining species boundaries in *Cyclemys* is more than academic. Asian turtles are overexploited for food, for traditional medicine, and for pets (van Dijk, Stuart & Rhodin, 2000), and now more than half of the Asian species are listed as endangered or critically endangered (IUCN, 2004). The conservation status of *Cyclemys* has been evaluated as a single, widespread taxon, *C. dentata*, owing to the uncertainty in the limits and geographical distributions of the other named species (IUCN/SSC Tortoise and Freshwater Turtle Specialist Group and Asian Turtle Trade Working Group, 2000). However, the genus *Cyclemys* contains multiple, highly divergent mitochondrial DNA lineages (Guicking *et al.*, 2002; Spinks *et al.*, 2004), each of which implicitly have smaller geographical ranges that are more vulnerable to extinction than a single evolutionary lineage of *Cyclemys*. A more realistic assessment of species boundaries in *Cyclemys* will allow for a more accurate, and probably direr, assessment of the conservation status of these turtles.

An independent molecular dataset is highly desirable given the uncertainties in defining species of *Cyclemys* solely on the basis of morphology. Two previous hypotheses of phylogenetic relationships of *Cyclemys* species have been proposed, both based on the mitochondrial cytochrome *b* (*cyt b*) gene. Guicking *et al.* (2002) found samples of *C. oldhamii*, *C. atripons*, and *C. pulchristriata* to be paraphyletic, and interpreted a lineage morphologically resembling *C. oldhamii*, and a lineage morphologically resembling *C. atripons* and *C. pulchristriata*, to be cryptic,

undescribed species. Spinks *et al.* (2004) included one sample of each of three species, and found *C. atripons* to be the sister taxon to a clade containing *C. dentata* and *C. tcheponensis*, with *C. dentata* and *C. tcheponensis* separated by a very short branch. This hypothesis implies that species with yellow plastra do not represent a monophyletic group. However, these studies relied entirely (Spinks *et al.*, 2004) or mostly (Guicking *et al.*, 2002) on pet-trade and market samples of uncertain provenance. For example, Guicking *et al.* (2002) assigned the names *C. atripons* and *C. pulchristriata* to samples depending on whether they were obtained from Cambodian markets (*C. atripons*) or Vietnamese markets (*C. pulchristriata*), despite the fact that turtles collected in Cambodia are mass exported to Vietnam (Stuart *et al.*, 2000). Pet-trade and market samples do have utility in phylogenetic studies, but with serious limitations (Parham *et al.*, 2001, 2004; Stuart & Parham, 2004). The uncertainties of identifying *Cyclemys* species based on morphology makes the limitations of using market and pet-trade samples even more severe, and the hypotheses of species boundaries and relationships of *Cyclemys* proposed in both studies need to be interpreted with caution. Clearly, DNA sequences from type specimens of *Cyclemys* would help resolve taxonomic uncertainty.

In an effort to elucidate species boundaries in *Cyclemys*, we used historical (or 'ancient') DNA methods to sequence fragments of the mitochondrial *cyt b* gene from museum type specimens, and phylogenetically placed these type sequences into the context of published *cyt b* variation. Although mitochondrial gene trees may not always represent species trees owing to introgression or lineage sorting of ancestral polymorphism (Moore, 1995; Maddison, 1997), an accurate matching of Linnaean names to major mitochondrial lineages provides an evidence-based hypothesis of species boundaries that is superior to the current state of taxonomic upheaval in *Cyclemys*. Furthermore, the hypothesis that major mitochondrial DNA lineages of *Cyclemys* represent species is more likely to overestimate diversity than to underestimate diversity, a source of error that we are willing to assume in this group of threatened turtles.

MATERIAL AND METHODS

EXTRACTION, AMPLIFICATION, AND SEQUENCING OF HISTORICAL DNA FROM MUSEUM SPECIMENS

Small pieces of bone, dried muscle, or connective tissue were removed from the inside of seven dried (stuffed or shell) type specimens of *C. atripons*, *C. bellii*, *C. oldhamii*, *C. orbiculata*, and *C. ovata*, and

a small piece of skin, muscle, or femur bone was removed from the leg of seven alcoholic type specimens of *C. dentata* and *C. pulchristriata* (Table 1). DNA was extracted following the method described by Kearney & Stuart (2004). Three fragments of 144, 179, and 168 nucleotide basepairs (bp; after primer sequences were trimmed) of mitochondrial DNA encoding part of the *cyt b* gene were amplified by polymerase chain reaction (PCR; 94 °C 45 s, 48 °C 30 s, 72 °C 1 min) for 40 cycles using the primer pairs L-350cytb/H-500cytb, L-430cytb/H-615cytb, and L-560cytb/H-730cytb, respectively (Table 2). Primers were designed so that the three fragments overlapped by 64 and 51 bp after primer sequences were trimmed. This measure ensured that fragments of contaminant DNA were not concatenated into chimeric sequences that might be erroneously judged to be authentic (Olson & Hassanin, 2003). The 25- μ L PCR reactions utilized 4 μ L of bovine serum albumin (BSA; New England BioLabs), to prevent PCR inhibitors, a relatively large volume (4 μ L) of DNA template to overcome low extraction yield, the high-quality Taq polymerase AmpliTaq Gold (Roche), and extra cycles (40 total) of the PCR reaction. All PCR reactions contained a positive control (described below) and a negative control (all reagents except the DNA template). PCR products were electrophoresed in a 1% low-melt agarose TALE gel stained with ethidium bromide and visualized under ultraviolet light. The bands containing DNA were excised, and agarose was digested from the bands using GELase (Epicentre Technologies). PCR products were sequenced in both directions by direct double-strand cycle sequencing using Big Dye version 3 (Perkin Elmer). The amplifying primers were used in the sequencing reactions. Cycle-sequencing products were precipitated with ethanol, 3 M sodium acetate, and 125 mM EDTA, and were sequenced with a Prism 3100 Genetic Analyser (ABI). Sequences were edited using SEQUENCHER v.4.1 (Genecodes). The type sequences were deposited in GenBank under accession numbers DQ444270–DQ444275 and EF183501–EF183502.

EXTRACTION OF DNA FROM FRESH TISSUE

Total genomic DNA was extracted from fresh tissue of a *C. pulchristriata* that was field-collected in eastern Cambodia by B. L. Stuart (Table 1) using the PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc.). This sample represented the only fresh tissue of *Cyclemys* that had previously been extracted in the molecular laboratory where the historical DNA extractions and amplifications were performed. The extraction was amplified as a positive control in PCR reactions of historical DNA, and was

Table 1. Sources of mitochondrial (mt) DNA of *Cyclemys* turtles used in this study

Species	Museum voucher	Type status	Year collected	Condition	Locality	mtDNA sequence
<i>atrionis</i>	USNM 81865	Holotype	1929	Dried stuffed specimen	Kao Kuap, Trat, Thailand	GenBank DQ444271
<i>atrionis</i>	USNM 53423	Paratype	1914	Dried shell	Koh Chang, Trat, Thailand	GenBank DQ444270
<i>bellii</i>	OUMNH 8513	Holotype*	By 1834†	Dried shell	Unknown	GenBank EF183501
<i>dentata</i>	BMNH 1946.1.22.62	Lectotype	By 1828‡	Alcoholic specimen	Java, Indonesia	GenBank DQ444272
<i>oldhamii</i>	BMNH 1947.3.5.63	Lectotype	By 1856§	Dried shell	Mergui, Myanmar	GenBank DQ444274
<i>oldhamii</i>	BMNH 1947.3.4.26	Paralectotype	By 1862¶	Dried stuffed specimen	Lao Mountains, Thailand	GenBank DQ444273
<i>orbiculata</i>	OUMNH 8512	Syntype	By 1834**	Dried shell	Unknown	GenBank EF183502
<i>ovata</i>	BMNH 1863.6.21.1	Holotype	By 1863**	Dried shell	Sarawak, Malaysia††	GenBank DQ444275
<i>pulchriatriata</i>	NMW 29525:4	Holotype	By 1901‡‡	Alcoholic specimen	Phuc-Son, Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	NMW 29525:2	Paratype	By 1901‡‡	Alcoholic specimen	Phuc-Son, Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	NMW 29525:3	Paratype	By 1901‡‡	Alcoholic specimen	Phuc-Son, Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	NMW 29525:5	Paratype	By 1901‡‡	Alcoholic specimen	Phuc-Son, Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	SMF 7667	Paratype	By 1901‡‡	Alcoholic specimen	Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	ZMH R00292	Paratype	By 1901‡‡	Alcoholic specimen	Annam, Vietnam	Failed to amplify
<i>pulchriatriata</i>	FMNH 259050	None	2000	Fresh tissue	Mondolkiri Prov., Cambodia	GenBank DQ444276

Museum abbreviations: BMNH, The Natural History Museum, London; FMNH, The Field Museum; NMW, Naturhistorisches Museum Wien; OUMNH, Oxford University Museum of Natural History; SMF, Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt; USNM, National Museum of Natural History, Smithsonian Institution; ZMH, Zoologisches Museum Hamburg.

*Das (2005) erroneously reported that OUMNH 8867 is the holotype of *C. bellii*. The second author examined OUMNH 8513 and it bears an old label stating that it is the holotype of *C. bellii*. OUMNH 8513 also matches the upper figure in plate 3, part 8 of Bell (1832–1836), on which Gray (1863) based his original description of *C. bellii*.

†This specimen is simultaneously a syntype of *C. orbiculata* Bell, 1834 and so must have been collected by that year.

‡The original BMNH catalogue number 1828.5.12.1 implies that this specimen has been in the museum since at least 1828.

§The original BMNH catalogue number 1856.5.6.1 implies that this specimen has been in the museum since at least 1856.

¶The original BMNH catalogue number 1862.8.18.20 implies that this specimen has been in the museum since at least 1862.

**Based on the year of the original species description.

††Collected by Alfred Russel Wallace.

‡‡Collected by Hans Fruhstorfer, a German-Swiss naturalist who traveled to Annam during a south-east Asian expedition from 1899 to 1901 (Lamas, 2005).

Table 2. Oligonucleotide primers used to amplify and sequence part of the mitochondrial cytochrome *b* gene from *Cyclemys* turtles

Primer	Sequence
L-350cytb	5'-TTTGAGGTGCCACCGTCATC-3'
H-500cytb	5'-TCGTGGAGGAAGATTAAGTGTAC-3'
L-430cytb	5'-GGGATTCTCAGTAGACAATGC-3'
H-615cytb	5'-TTAGTAGGAGGGTGAGTATTAGGA-3'
L-560cytb	5'-CAGGATTAACCTCAAACGCTG-3'
H-730cytb	5'-GCATAGGCGAATAGGAAGTATC-3'

'L' and 'H' refer to light and heavy strands, respectively.

sequenced as described above. This sequence was deposited in GenBank under accession number DQ444276.

ALIGNMENT

In addition to the eight type specimens and one positive control tissue sequenced in this study, *Cyclemys* *cyt b* sequences were also obtained from three published sources. Thirty-nine sequences with a maximum length of 984 bp were obtained from Guicking *et al.* (2002), three sequences of 1140 bp (GenBank AY434577, AY434579, and AY434617) were obtained from Spinks *et al.* (2004), and one sequence of 1036 bp (GenBank AJ604513) was obtained from Schilde, Barth & Fritz (2004). Sequences from *Leucocephalon yuwonoi* (GenBank AY434608) and *Heosemys spinosa* (GenBank AY434578) were used as outgroups, based on the findings of Spinks *et al.* (2004). Sequences were aligned using SEQUENCHER v.4.1 (Genecodes).

PHYLOGENETIC ANALYSES

Phylogenies were reconstructed using maximum-parsimony and maximum-likelihood optimality criteria, and mixed-model Bayesian inference.

Maximum-parsimony analysis was performed using PAUP* 4.0b10 (Swofford, 2002). A heuristic search was performed with equal weighting of nucleotide substitutions, stepwise addition with 100 random-addition replicates, and TBR branch swapping was limited to 1000 trees per replicate. Nodal support was evaluated with 500 nonparametric bootstrap pseudoreplications (Felsenstein, 1985) using the heuristic search option, with TBR branch swapping limited to 10 000 000 rearrangements per replicate.

Maximum-likelihood analysis was performed using GARLI 0.95 (Zwickl, 2006). Five independent analyses were performed using the default settings, the GTR + I + G model, and estimated base frequencies A = 0.31052, C = 0.30726, G = 0.11636, T = 0.26586,

rate matrix A–C = 1.04241, A–G = 8.18342, A–T = 0.4883, C–G = 0.00549, C–T = 12.0303, proportion of invariable sites = 0.0458648, and gamma-distribution shape parameter = 0.283551. Nodal support was evaluated with 500 nonparametric bootstrap pseudoreplications.

Mixed-model Bayesian analysis was performed using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). The data were partitioned into first-codon, second-codon, and third-codon positions. The model of sequence evolution that best described the three data partitions was inferred using the Akaike Information Criterion, as implemented in MODELTEST 3.7 (Posada & Crandall, 1998). The selected models were K81uf + G for the first-codon position partition, TrN + I for the second-codon position partition, and GTR + G for the third-codon position partition. The K81uf and TrN models are not implemented in MrBayes 3.1, and so the next more complex model available in the program (GTR) was used for those partitions. Four independent Bayesian analyses were performed. In each analysis, four chains were run for 10 000 000 generations using the default priors, trees were sampled every 2000 generations, and the first 25% of trees were discarded as 'burn-in'. A 50% majority rule consensus of the sampled trees was constructed to calculate the posterior probabilities of the tree nodes. Trace plots of clade probabilities were viewed using AWTY (Wilgenbusch, Warren & Swofford, 2004).

RESULTS

HISTORICAL DNA SEQUENCES

All three overlapping *cyt b* fragments, consisting of a total of 376 bp, were obtained from the seven dried type specimens. Two overlapping *cyt b* fragments (from the primer pairs L-430cytb/H-615cytb and L-560cytb/H-730cytb), consisting of a total of 296 bp, were obtained from the alcoholic lectotype of *C. dentata*. None of the fragments were successfully amplified from the alcoholic type specimens of *C. pulchriata* (Table 1).

The electropherograms of the obtained sequences had strong signals with very little or no background noise. Identical sequences were obtained in all overlapping fragments. The eight type sequences were genetically distinct from the positive control that represented the only fresh tissue of *Cyclemys* previously extracted in the same laboratory. Consequently, the sequences of the eight type specimens used in the analyses here appear to be authentic.

PHYLOGENETIC RELATIONSHIPS

The alignment contained no insertion-deletions and was unambiguous. Of the 1140 aligned characters,

297 were variable and 187 were parsimony informative. Strict consensus of the saved 100 000 equally most parsimonious trees (tree length, $L = 435$; consistency index, $CI = 0.766$; retention index, $RI = 0.944$) showed that these trees differed only by the arrangement of individuals on short-terminal branches. The score of the best-likelihood tree ($-\ln L 3565.994676$) was within 0.043067 likelihood units of the best tree recovered in each of the other four runs, suggesting that the five runs had converged. The standard deviation of split frequencies among the four Bayesian runs was 0.004991, and trace plots of clade probabilities viewed using AWTY were relatively stationary. These two measures suggest that the four runs had converged, and that topologies were sampled in proportion to their true posterior probability distribution. The maximum-parsimony trees, maximum-likelihood tree, and Bayesian-consensus tree (Fig. 1) were highly consistent, and differed only by the arrangement of individuals on short-terminal branches.

Five major, well-supported clades were recovered (Clades A–E, Fig. 1). Clade A (Fig. 1) was sister to all other *Cyclemys*, and contained samples obtained from the Hong Kong pet trade (Guicking *et al.*, 2002). These were reported to have originated from Kachin, Myanmar, and were identified as an undescribed species morphologically resembling *C. oldhamii* (Guicking *et al.*, 2002). None of the type sequences that we obtained belong to this clade. Clade B (Fig. 1) contained samples identified as two subspecies of *C. shanensis* by Guicking *et al.* (2002), as *C. shanensis* by Schilde *et al.* (2004), as *C. dentata* and *C. tcheponensis* by Spinks *et al.* (2004), and the lectotype and paralectotype of *C. oldhamii*. Clade C (Fig. 1) contained samples identified as *C. atripons* and *C. pulchristriata* by Guicking *et al.* (2002), and the positive control sample of *C. pulchristriata* from eastern Cambodia. Clade D (Fig. 1) contained samples identified as *C. dentata* and *C. oldhamii* by Guicking *et al.* (2002), the lectotype of *C. dentata*, the syntype of *C. orbiculata*, the holotype of *C. bellii*, and the holotype of *C. ovata*. Clade E (Fig. 1) contained samples identified by Guicking *et al.* (2002) as an undescribed species morphologically resembling *C. atripons* and *C. pulchristriata*, and the holotype and paratype of *C. atripons*. Species with yellow plastra (Clades C–E, Fig. 1) form a well-supported monophyletic group, but species with dark plastra (Clades A–B, Fig. 1) are paraphyletic (Fig. 1).

DISCUSSION

Our phylogenetic analysis of mitochondrial DNA sequences obtained from museum type specimens of *Cyclemys* greatly aids the resolution of species boundaries in the genus. Specifically, our phylogenetic

hypothesis supports the recognition of four named species (*C. atripons*, *C. dentata*, *C. oldhamii*, and *C. pulchristriata*), and a fifth species of unknown geographical origin. The substantial differences in identifications among Guicking *et al.* (2002), Spinks *et al.* (2004), and this study incorporating type sequences, support the notion that *Cyclemys* of unknown provenance are not reliably identified to species solely on the basis of morphology. The corrected taxonomy based on the type sequences (Fig. 1) shows that many samples were misidentified in previous studies (Guicking *et al.*, 2002; Spinks *et al.*, 2004). Unfortunately, the lack of voucher specimens associated with some (Guicking *et al.*, 2002) or all (Spinks *et al.*, 2004) of the genetic material analysed in those studies prevents re-evaluating identifications in the light of our new findings with type sequences.

The absence of substantial genetic variation among the types of *C. bellii*, *C. dentata*, *C. orbiculata*, and *C. ovata* corroborates the hypothesis based on morphology (Fritz *et al.*, 1997) that *C. bellii*, *C. orbiculata*, and *C. ovata* are junior synonyms of *C. dentata*, the oldest available name. Likewise, the absence of substantial genetic variation among the types of *C. oldhamii* and samples identified as *C. shanensis* (including topotypes of *C. s. shanensis* from Lake Inlé, Myanmar) and *C. tcheponensis* suggests that *C. shanensis* and *C. tcheponensis* are junior synonyms of *C. oldhamii*, the oldest available name. *Cyclemys oldhamii*, *C. shanensis*, and *C. tcheponensis* differ morphologically only by the retention or loss of head stripes with maturity (Fritz *et al.*, 1997; Guicking *et al.*, 2002), and adopting this taxonomy implies that the retention or loss of head stripes with maturity represents only geographical variation within a species.

Cyclemys atripons and *C. pulchristriata* are morphologically indistinguishable, but are paraphyletic with respect to *C. dentata*. Because we were unsuccessful in obtaining DNA from the type series of *C. pulchristriata* (Table 1), our identification of the *C. pulchristriata* clade is tentatively based on the assumption that the positive control sample belongs to this species. This sample was field-collected in hilly eastern Cambodia by B. L. Stuart, in the same Annamite (Truong Son) Mountain range that contains the type locality of *C. pulchristriata* in central Vietnam. Stuart & Platt (2004) tentatively referred this specimen to *C. atripons*, on the basis that most of the Cambodian *Cyclemys* specimens with yellow plastra they examined originated from the mountains of south-western Cambodia, close to the type locality of *C. atripons*, and that no morphological differences among the specimens were apparent. Stuart, Sok & Neang (2006b) identified additional specimens from eastern Cambodia as *C. atripons* to maintain consis-

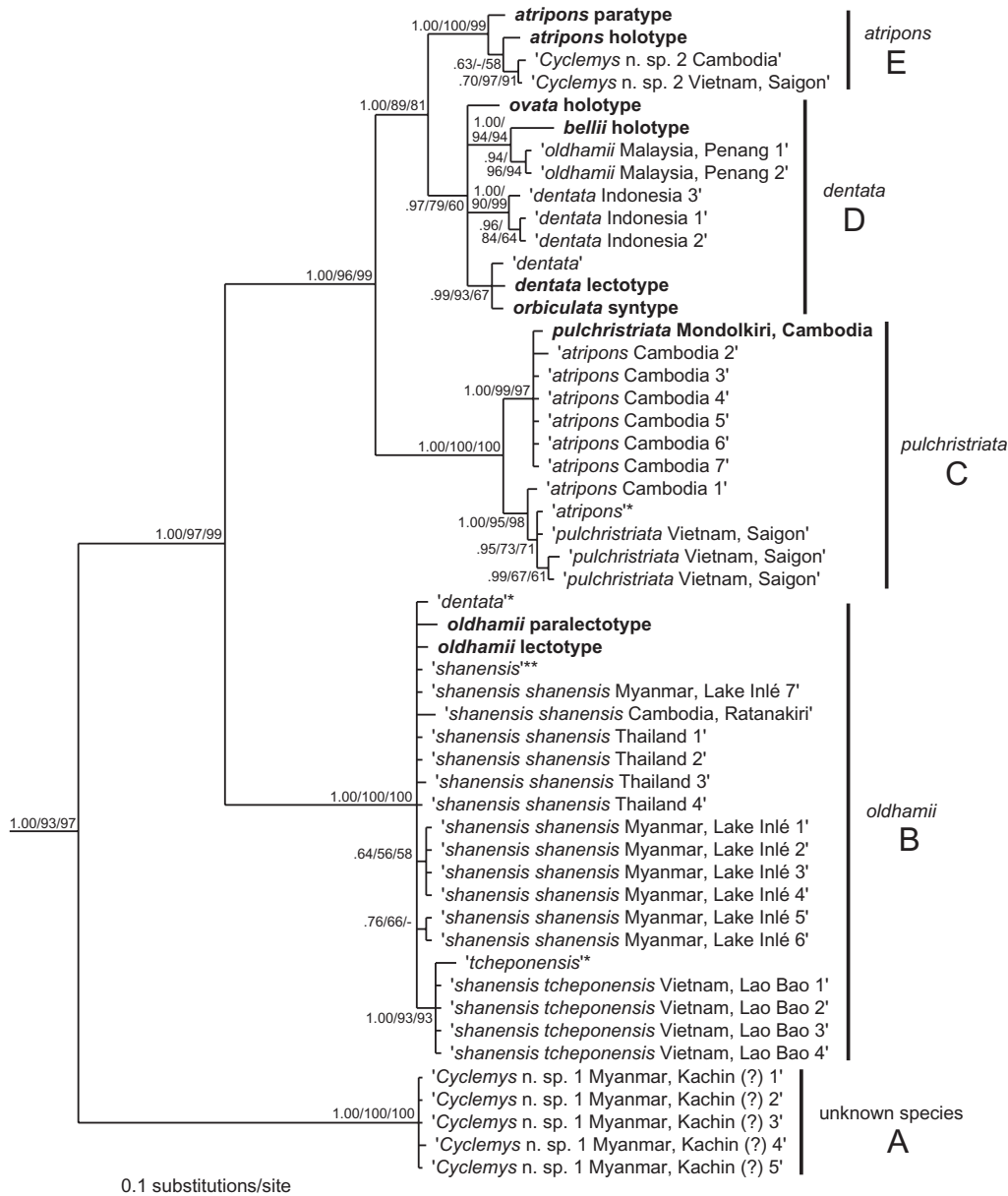


Figure 1. Fifty-percent majority-rule consensus phylogram resulting from mixed-model Bayesian analysis of the mitochondrial cytochrome *b* gene from Asian leaf turtles (*Cyclémyes*). Maximum-likelihood and maximum-parsimony analysis recovered the same topology, and differed only by the arrangement of individuals on short-terminal branches. Numbers next to nodes are Bayesian posterior probabilities/maximum-likelihood bootstrap values/parsimony bootstrap values. Haplotypes in boldface were sequenced in this study, haplotypes marked with an asterisk (*) were obtained from Spinks *et al.* (2004), the haplotype marked with two asterisks (**) was obtained from Schilde *et al.* (2004), and the remaining haplotypes were obtained from Guicking *et al.* (2002). Species identifications of haplotypes obtained from Guicking *et al.* (2002), Schilde *et al.* (2004), and Spinks *et al.* (2004) are enclosed in single quotations. Our recommended taxonomy is on the right.

tency with Stuart & Platt (2004). Sequences from type material of *C. pulchristriata* or from fresh topotypic samples are needed to identify the *C. pulchristriata* lineage with certainty. It is clear, however, from our results that the lineage interpreted by Guicking

et al. (2002) to be a cryptic, undescribed species morphologically resembling *C. atripons* and *C. pulchristriata* (*Cyclémyes* n. sp. 2') is *C. atripons*.

The lineage interpreted by Guicking *et al.* (2002) to be a cryptic, undescribed species morphologically

resembling *C. oldhamii* (*Cyclemys* n. sp. 1') does not match any of the type sequences obtained in this study, and may indeed represent an undescribed species. We did not attempt to sequence the type material of three described taxa of *Cyclemys* that are currently considered to be junior synonyms of other species, and which exhibit a dark plastron like that of '*Cyclemys* n. sp. 1': *C. tcheponensis*, *C. dhor shanensis*, and *C. tiannanensis*. The sequences obtained from Guicking *et al.* (2002) include samples from Lake Inlé, Myanmar, the type locality of *C. dhor shanensis* (referred to as *C. s. shanensis* in Guicking *et al.*, 2002) and from Lao Bao, Vietnam, near to the type locality of *C. tcheponensis* (referred to as *C. s. tcheponensis* in Guicking *et al.*, 2002), and so it is unlikely that '*Cyclemys* n. sp. 1' represents either of these two taxa. *Cyclemys tiannanensis* has not yet been characterized molecularly, and its relationship to '*Cyclemys* n. sp. 1' remains unevaluated. Unfortunately, the provenance of '*Cyclemys* n. sp. 1' is unknown. These samples were obtained from a Hong Kong pet dealer who reported that they originated from Kachin Province, Myanmar (Guicking *et al.*, 2002). During the past two decades, many newly described turtle species were based on specimens purchased from a Hong Kong pet dealer (Parham *et al.*, 2001; Dalton, 2003). Some of these newly described species have been shown to be hybrids of other, better known species (Parham *et al.*, 2001; Wink, Guicking & Fritz, 2001; Spinks *et al.*, 2004; Stuart & Parham, 2007), whereas others have been shown to represent distinct, evolutionary lineages that remain unknown in the wild (Parham *et al.*, 2004). Because the mitochondrial DNA of '*Cyclemys* n. sp. 1' does not match any other known taxon (except potentially *C. tiannanensis*), this distinct lineage is unlikely to be another example of a pet-trade species with a recent hybrid origin. In many cases, the provenance supplied by the Hong Kong pet dealer for specimens have been questioned (de Bruin & Artner, 1999; Parham & Li, 1999; Dalton, 2003; Shi *et al.*, 2005), underscoring the urgent need for scientific fieldwork.

Although we hypothesize that the major mitochondrial DNA lineages represent species of *Cyclemys*, one apparent example in our dataset of introgression between mitochondrial DNA lineages suggests that these lineages may be imperfect proxies for species. Mitochondrial DNA sequences obtained by Guicking *et al.* (2002) from two turtles with black plastra purchased in local trade at Penang, Peninsular Malaysia, and identified by those authors based on morphology as *C. oldhamii*, are deeply nested within a clade of turtles with yellow plastra referred here to *C. dentata* (Fig. 1). A nuclear DNA dataset (ISSR-PCR) presented by Guicking *et al.* (2002) on the same samples readily distinguished the Penang turtles from *C.*

dentata, supporting the hypothesis that the Penang samples are an example of mitochondrial DNA introgression. *Cyclemys oldhamii* and *C. dentata* occur in sympatry in Peninsular Malaysia (Fritz *et al.*, 1997), but owing to the trade origin of the Penang samples, the introgression may alternatively have occurred in captivity. Field sampling in Peninsular Malaysia should offer insight into this topic. A nuclear DNA dataset of the type sequences would also provide an independent estimate of relationships and species boundaries in *Cyclemys*. However, obtaining nuclear DNA sequences from degraded samples is more challenging than obtaining mitochondrial DNA sequences, probably because mitochondrial DNA occurs in higher copy number in the cell, and is therefore more likely to be retrieved (Hofreiter *et al.*, 2001; Huynen *et al.*, 2003; Isenberg, 2005).

Sampling genetic material from a museum specimen necessarily requires removing part of the specimen, and there is justifiably great concern that damage to the specimen be kept to a minimum, particularly with those specimens that are as irreplaceable as types (Graves & Braun, 1992; Mundy, Unitt & Woodruff, 1997; Rohland, Siedel & Hofreiter, 2004; Wisely *et al.*, 2004; Asher & Hofreiter, 2006). New protocols for obtaining DNA from museum specimens are commendably seeking to minimize such damage (Mundy *et al.*, 1997; Rohland *et al.*, 2004; Wisely *et al.*, 2004; Asher & Hofreiter, 2006), but still modify specimens in some manner. We submit that sampling genetic material from freshly collected specimens of turtles also requires destructive sampling by removing tissue (typically muscle or liver) from the carcass. As with freshly collected specimens, in this study we aimed to sample tissues that were subjectively deemed to have limited phylogenetic utility. These included small pieces of skin, muscle, bone, or connective tissue, usually in the form of unrecognizable organic material adhering to the inside of dried specimens. The most drastic sampling (and ironically the least successful) entailed removing a femur bone from a leg that was retracted within the shell of some alcoholic specimens. Efforts should continue to find new means to extract DNA with minimal damage to specimens, and curators should continue to critically review requests to destructively sample specimens in their care (Graves & Braun, 1992). However, our ability to sample type specimens of *Cyclemys* in this manner enabled us to reveal new phylogenetic characters from critically important specimens, including one notably collected by Alfred Russel Wallace.

The type sequences generated in this study provide useful reference points for future phylogenetic studies of *Cyclemys* because their species identifications are unambiguous. New efforts to resolve *Cyclemys* species

boundaries and relationships should be directed towards obtaining mitochondrial DNA sequences from *C. pulchrirostrata* and *C. tiannanensis* type material, obtaining nuclear DNA sequences from all type material, and especially, obtaining field-collected, vouchered, genetic material of known provenance. The genus *Cyclemys* contains multiple, distinct evolutionary lineages that each warrant recognition and protection, but an effective conservation strategy requires knowing where these lineages exist in nature.

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