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Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*)

Bryan L. Stuart[a,b,*] and James Ford Parham[c,d]

[a] *Field Museum, Department of Zoology, Division of Amphibians and Reptiles, 1400 S. Lake Shore Drive, Chicago, IL 60605-2496, USA*
[b] *Department of Biological Sciences (M/C 066), University of Illinois at Chicago, 845 W. Taylor, Chicago, IL 60607-7060, USA*
[c] *Department of Integrative Biology, University of California, Berkeley, CA 94720-3140, USA*
[d] *University of California Museum of Paleontology, Berkeley, CA 94720, USA*

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Abstract

The Indochinese box turtle *Cuora galbinifrons* is a polytypic, critically endangered species from Vietnam, Laos, and Hainan Island, China. We analyze up to 1790 bp of mitochondrial DNA under maximum parsimony and maximum likelihood criteria to test if the five historically recognized subspecies represent evolutionary lineages, and to elucidate the relationship of *C. galbinifrons* to other *Cuora*. *C. galbinifrons* is composed of three major mitochondrial DNA clades corresponding to the three subspecies *galbinifrons*, *bourreti*, and *picturata*. These three lineages are also morphologically diagnosable, and consequently we recommend elevating each to full species. *Cuora galbinifrons hainanensis* nests within the *galbinifrons* clade, and we retain it as a synonym of *galbinifrons*, as supported by morphology. *Cuora* "*serrata*" is known to be a hybrid of male *Cuora mouhotii* and female *C. galbinifrons*, and our findings show that *C.* "*serrata*" originates from both female *galbinifrons* and *bourreti*. Little or no mitochondrial DNA variation was found among the morphologically distinct species *Cuora aurocapitata*, *Cuora pani*, and *Cuora trifasciata*, for which hypotheses are proposed. Recognizing *galbinifrons*, *bourreti*, and *picturata* as separate species has consequences for ongoing ex situ captive breeding programs and prioritization of in situ conservation activities, particularly in Vietnam.

1. Introduction

The Indochinese box turtle *Cuora galbinifrons* (Bourret, 1939) is a terrestrial geoemydid turtle found in forested uplands of Vietnam, Laos, and Hainan Island, China. The natural history of *C. galbinifrons* is poorly known, as only a few specimens have been observed or collected in the field by biologists. However, threats to the species are well known. *C. galbinifrons* is heavily hunted throughout its range for food, the international pet trade, and especially traditional medicine purposes (Fiebig and Lehr, 2000; Hendrie, 2000; Lehr, 1997; Stuart and Timmins, 2000; van Dijk et al., 2000), and suffers from habitat loss by deforestation (Fiebig and Lehr, 2000; Hendrie, 2000). Because of the over-exploitation, *C. galbinifrons* was listed as Critically Endangered in the 2002 IUCN Red List of Threatened Species, which means the taxon "is facing an extremely high risk of extinction in the wild in the immediate future" (IUCN, 2002).

Cuora galbinifrons is morphologically variable and five subspecies have been named. Distributions of the subspecies are uncertain, as most records have been acquired from hunters, wildlife traders, or markets (e.g., Iverson and McCord, 1992; Lehr et al., 1998a) along the trade route that moves from Laos and Vietnam northward into China (Fiebig and Lehr, 2000; Stuart et al., 2000). *Cuora galbinifrons galbinifrons* (Bourret, 1939) is known from northern Vietnam (Bourret, 1939; Obst and Reimann, 1994; this study) and eastern-central Laos (this study). *Cuora galbinifrons hainanensis* (Li, 1958) is known from Hainan Island, China (Li, 1958); a report of its occurrence in Guangxi Province, China (Liu and Zhang, 1987) is based only on animals in trade. *Cuora galbinifrons serrata* Iverson and McCord, 1992 is known only from animals in trade that were claimed to have

* Corresponding author. Fax: 1-312-665-7697.
E-mail addresses: bstuart@fieldmuseum.org (B.L. Stuart), parham@socrates.berkeley.edu (J.F. Parham).

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originated from Hainan Island, China (Iverson and McCord, 1992) and Vietnam (de Bruin and Artner, 1999; Fritz and Obst, 1998), but these localities are now suspected to be mistaken or falsified (Dalton, 2003; de Bruin and Artner, 1999; Parham et al., 2001). *Cuora galbinifrons bourreti* (Obst and Reimann, 1994) is known from central Vietnam, but the describing authors extended its range to northeastern Cambodia and Laos solely based on the claims of animal dealers (Obst and Reimann, 1994). *Cuora galbinifrons picturata* (Lehr et al., 1998b) is known only from markets in southern Vietnam, but the describing authors extended its range to Cambodia without justification (Lehr et al., 1998a).

Two of these subspecies, *hainanensis* and *serrata*, are not recognized in recent taxonomic treatments (Fritz et al., 2002; Lehr et al., 1998a). *C. g. hainanensis* was elevated to full species status (Koshikawa, 1982; Sichuan Biological Research Institute and Beijing Institute of Zoology, 1975), but later treated as a junior synonym of *C. g. galbinifrons* (Lehr et al., 1998b; Obst and Reimann, 1994). *C. g. serrata* was also elevated to full species status (Fritz and Obst, 1997), but recently was inferred to be a hybrid between *C. galbinifrons* and *Cuora mouhotii* based on morphology, mitochondrial DNA, and allozymes (Parham et al., 2001). Consequently, we use the name "*serrata*" in quotations, following convention in Parham et al. (2001). The nomenclatural history of *C. galbinifrons* is presented in more detail in Fritz et al. (2002). The three currently recognized subspecies *galbinifrons*, *bourreti*, and *picturata* are distinguished by the coloration and shape of the shell, and the coloration of soft body parts (Fritz et al., 2002; Lehr et al., 1998a; Obst and Reimann, 1994).

The validity of the subspecies taxon rank has been controversial for many years. Critics have argued that subspecies represent arbitrary sections of clines, artificial subdivisions of species that are used as tools of convenience for museum workers, incipient species that may ultimately evolve into full species, or independently evolving lineages worthy of full species status (reviewed by Burbrink et al., 2000). Subspecies designations may mask evolutionary lineages by subsuming them under a single species name, a particular concern for threatened lineages that deserve independent conservation attention.

Parham et al. (2001) noted considerable mitochondrial DNA sequence variation among six samples of *C. galbinifrons* (including *C. "serrata"*), and suggested that *C. galbinifrons* might comprise more than one species. Given these preliminary findings on mitochondrial DNA variation, and the known morphological variation, we hypothesized that more diversity exists in *C. galbinifrons* than is presently recognized. If true, this conclusion would have obvious conservation implications.

Here we use mitochondrial DNA sequence data to test if the five named forms of *C. galbinifrons* represent evolutionary lineages. We use the same genes studied in Parham et al. (2001), but with an additional 188–254 bp. Because the sister taxon of *C. galbinifrons* has been hypothesized to be a clade containing most other *Cuora* species (Honda et al., 2002), we include samples in our phylogenetic analyses of all but one of the nine currently recognized, extant species of *Cuora*. All nine species are restricted to China, Vietnam, and Laos, except *Cuora amboinensis*, which is distributed throughout much of Southeast Asia (IUCN, 2002). Because of overexploitation and habitat loss, six of these species are listed as Critically Endangered (*aurocapitata*, *galbinifrons*, *mccordi*, *pani*, *trifasciata*, and *zhoui*), two as Endangered (*flavomarginata* and *mouhotii*), and one as Vulnerable (*amboinensis*) in the 2002 IUCN Red List of Threatened Species (IUCN, 2002). We discuss our phylogenetic findings with a view toward a taxonomy that reflects evolutionary history in these highly threatened turtles.

2. Materials and methods

2.1. Taxon sampling

In 1998, one specimen from Laos and two specimens from Vietnam of *C. g. galbinifrons* were collected in the field by BLS and shortly preserved in 10% buffered formalin after fixing leg muscle from each in 95% ethanol. The tissue samples and specimens were later deposited at Field Museum of Natural History, Chicago (FMNH 255694-95, 256544), and specimens were transferred to 70% ethanol upon arrival there. To our knowledge, these three samples represent the only available field-collected tissues of *C. galbinifrons*. Additionally, we sequenced tissue samples and examined voucher specimens from the holdings of the Field Museum of Natural History, Museum of Vertebrate Zoology (Berkeley), Royal Ontario Museum (Toronto), and Yale Peabody Museum (New Haven). Most of these originated from the food and medicine trade in Asia, or from the pet trade (Appendix A), an unfortunate necessity given the rarity of field-collected tissues of *Mauremys*, *Chinemys*, and *Cuora*. Specimens of *C. galbinifrons* were identified to subspecies prior to sequencing using the morphological characters provided by Iverson and McCord (1992) and Lehr et al. (1998a).

The only published phylogeny of *Cuora* failed to resolve the sister clade to *C. galbinifrons* (Honda et al., 2002). Therefore, we either sequenced or downloaded from GenBank homologous sequences (available from Parham et al., 2001) of every currently recognized species of *Cuora* except *Cuora yunnanensis* and *Cuora zhoui* (Appendix A). *C. yunnanensis* is considered extinct (IUCN, 2002) and *C. zhoui* is known only from the pet

trade, where it is extremely rare and expensive. Using different methods of analysis, Honda et al. (2002) consistently hypothesized a clade containing the genera *Mauremys* and *Chinemys* to be sister to a monophyletic *Cuora* clade, so we rooted trees in our study with sequences of *Mauremys mutica*, *Chinemys nigricans*, and *Chinemys reevesii* (Appendix A).

2.2. DNA amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved muscle using PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems). Primers for amplifying and sequencing mitochondrial DNA were designed from *Chrysemys picta* sequences in GenBank and are listed in Table 1. An 831–897 bp piece of mtDNA that encodes part of the cytochrome oxidase subunit I (COI) gene was amplified by PCR (94 °C 45 s, 56–58 °C 30 s, and 72 °C 1 min 20 s) for 35 cycles using the light-strand primers L-turtCOI or L-turtCOIc, and the heavy-strand primers H-turtCOI, H-turtCOIb, or H-turtCOIc. Generally the best results were obtained when amplifying and sequencing with the primer pair L-turtCOIc/H-turtCOIc. 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 °C 45 s, 56–62 °C 30 s, 72 °C 1 min 20 s) for 35 cycles using the primers L-ND4 and H-Leu. 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 bands using GELase (Epicentre Technologies). PCR products were sequenced in both directions by direct double strand cycle sequencing using Big Dye version 3 chemistry (Perkin–Elmer). The amplifying primers and the internal primers L-ND4int, H-ND4int, L-COIint, and H-COIint were used in the sequencing reactions. Cycle sequencing products were precipitated with ethanol and 3 M sodium acetate and sequenced with a Prism 3100 Genetic Analyzer (ABI). Protein-coding regions were translated into amino acids and sequences were edited and aligned with Sequencher version 4.1 (Genecodes).

More than one copy of the fragment containing part of the ND4 gene, the tRNAs His and Ser, and part of the tRNA Leu (hereafter 'ND4 region') was consistently amplified in the sample of *Cuora flavomarginata*. Consequently, DNA was amplified from this individual using the primer pairs L-ND4/H-Leu and L-ND4c/H-Leu2 under the PCR conditions described above, and the PCR products were cloned with a TOPO TA Cloning Kit (Invitrogen). Fifteen colonies from each of the two plates were re-amplified, but sequenced only with the heavy strand amplifying primer (ca. 400 bp) to minimize expense. Colonies that yielded fragments appearing to be authentic mitochondrial DNA were then completely sequenced using the two amplifying and two internal primers.

2.3. Phylogenetic analyses

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 heuristic search algorithm and TBR branch swapping. The analysis was unweighted because third codon position transitions of both COI and ND4 accumulated in linear fashion and showed no sign of an asymptote when numbers of pairwise differences were plotted against Kimura 2-parameter DNA divergences, indicating that the data set was not saturated. Maximum parsimony analyses were performed with COI alone, ND4 alone, and all sequences combined (COI + ND4 +

Table 1
Oligonucleotide primers used to amplify and sequence turtle mtDNA in this study

Primer	Product	Sequence
L-ND4	ND4, tRNAs His, Ser, Leu	5′-GTAGAAGCCCCAATCGCAG-3′
L-ND4c	ND4, tRNAs His, Ser, Leu	5′-CCAATCGCAGGATCAATAATC-3′
H-Leu	ND4, tRNAs His, Ser, Leu	5′-ATTACTTTTACTTGGATTTGCACCA-3′
H-Leu2	ND4, tRNAs His, Ser, Leu	5′-ATTTGCACCAAGGGTTAATGG-3′
L-ND4int	ND4, tRNAs His, Ser, Leu	5′-ACCCATACACGAGAACATCTACT-3′
H-ND4int	ND4, tRNAs His, Ser, Leu	5′-GGTTAGCTCTCCTATTAGGTTGAT-3′
L-turtCOI	COI	5′-ACTCAGCCATCTTACCTGTGATT-3′
L-turtCOIc	COI	5′-TACCTGTGATTTTAACCCGTTGAT-3′
H-turtCOI	COI	5′-CCCATACGATGAAGCCTAAGAA-3′
H-turtCOIb	COI	5′-GTTGCAGATGTAAAATAGGCTCG-3′
H-turtCOIc	COI	5′-TGGTGGGCTCATACAATAAAGC-3′
L-COIint	COI	5′-TGATCAGTACTTATCACAGCCG-3′
H-COIint	COI	5′-TAGTTAGGTCTACAGAGGCGC-3′

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

tRNAs). Nodal support was evaluated with 1000 non-parametric bootstrapping pseudoreplications (Felsenstein, 1985), and decay indices (Bremer, 1994) were calculated using a PAUP command file generated by MacClade 4.03 (Maddison and Maddison, 2001).

For maximum likelihood analyses, four identical haplotypes in the data set were removed to minimize computing requirements. Specifically, *Cuora aurocapitata* and *Cuora pani*, and four *C. g. picturata*, were collapsed into single terminal taxa. The model of sequence evolution that best described the data set was inferred using Modeltest 3.06 (Posada and Crandall, 1998). The model HKY + I + G was selected, with ti/tv ratio = 11.2036, proportion of invariable sites = 0.5659, gamma distribution shape parameter = 1.1228, and base frequencies as A = 0.3162, C = 0.2546, G = 0.1491, and T = 0.2801. Maximum likelihood analyses were performed with 100 random addition replicates with stepwise addition of taxa using the heuristic search algorithm and TBR branch swapping, and nodal support was evaluated with 100 non-parametric bootstrapping pseudoreplications.

3. Results

3.1. Sequences

Sequences of both COI and ND4 region were obtained for all taxa, except COI from one sample of *C. amboinensis* (sample 9). Sequencing products had single peaks in the chromatograms and protein-coding regions always translated into amino acids, except for the ND4 region in *C. flavomarginata* (see below). The only insertion–deletion event in the data set was an additional A at the 5′ end of the tRNA His in one *C. amboinensis* (sample 8). The frequencies of A, C, G, and T in the COI gene were 0.2667, 0.2572, 0.1787, and 0.2974, in the ND4 gene were 0.3455, 0.2662, 0.1298, and 0.2585, and in the tRNAs adjacent to ND4 were 0.3594, 0.2323, 0.1459, and 0.2624, respectively. The bias against *G* in the data set is consistent with mitochondrial DNA in other vertebrates (Kocher et al., 1989). Because the data set had single peaks in the chromatograms, coded for protein where it should, lacked insertion–deletions in coding regions, and was biased against *G*, we believe that authentic mitochondrial DNA product was obtained.

The 30 clones of the ND4 region from the single individual of *C. flavomarginata* yielded two different sequences. The first sequence was an 892-bp fragment that correctly coded for protein in the ND4 gene, but was ambiguous at two positions. We consider this piece to be authentic mitochondrial DNA and attribute the two ambiguities to Taq Polymerase error. This sequence was used to represent *C. flavomarginata* ND4 region in the phylogenetic analyses. The second sequence was represented only by part (398 bp) of a correctly sized (ca. 890 bp) PCR amplification product because it was sequenced only with the heavy strand amplifying primer. This fragment had two adjacent basepairs (AC) deleted in the ND4 gene, did not code for protein, and was ambiguous at four positions. We consider this piece to be a non-coding nuclear mitochondrial pseudogene, or *numt* (reviewed by Bensasson et al., 2001; Zhang and Hewitt, 1996). This appears to be the first *numt* reported in turtles, as none were cited in a recent review of *numt* incidence in animals (Bensasson et al., 2001), in the pseudogene database (http://www.pseudogene.net), or available on GenBank at the time of writing. An ND4 *numt* is known in humans (Zhang and Hewitt, 1996).

3.2. Phylogenetic analyses

3.2.1. Maximum parsimony

The total data set contained 310 (17.32% of 1790 bp) parsimony-informative sites. Specifically, COI had 133 (14.83% of 897 bases), the ND4 gene had 156 (22.45% of 695 bp), and the tRNAs adjacent to the ND4 gene had 21 (10.61% of 198 bp) parsimony-informative sites.

Maximum parsimony analysis of COI alone resulted in >450,000 equally most parsimonious trees of length 334 steps (Consistency Index = 0.64, Retention Index = 0.85), and analysis of ND4 alone resulted in two equally most parsimonious trees of length 323 steps (Consistency Index = 0.59, Retention Index = 0.87; trees not shown). The two ND4 trees differed only in the relationship of *Cuora trifasciata* to *C. aurocapitata* and *C. pani*; in one tree this relationship was unresolved, and in the other tree one individual of *trifasciata* formed a clade with *aurocapitata* and *pani*. The COI and ND4 trees both recovered the same clades of *galbinifrons*, but differed in the placement of *C. amboinensis*, *Cuora mccordi*, and *C. mouhotii*. In a strict consensus of the COI trees, *mouhotii* was sister to all *Cuora*, *amboinensis* was sister to all *Cuora* except *mouhotii*, and *mccordi* was sister to the *galbinifrons* clade. In the ND4 trees, *amboinensis* and *mouhotii* were sister taxa, and *mccordi* was sister to all other *Cuora*.

Our preferred approach is to combine the mitochondrial DNA sequences into a single data set for phylogenetic analysis. Mitochondrial DNA is usually non-recombinant (Avise et al., 1987), so different mitochondrial genes belonging to the same individual share a common history of inheritance and do not form linkage partitions (Slowinski and Page, 1999). Also, combining the data set maximizes the amount of character information and can increase resolution, support, and accuracy (de Queiroz et al., 2002; Wiens, 1998). Maximum parsimony analysis of all sequences combined resulted in six equally most parsimonious trees of length 727 steps (Consistency Index = 0.65, Retention Index = 0.85). The six trees were highly consistent, differ-

ing only in the arrangement of subclades within the B clade of *C. galbinifrons*. The six trees obtained from combined sequences will be used in subsequent discussion of the maximum parsimony analysis; one of these six trees is presented in Fig. 1.

The genus *Cuora* was monophyletic relative to *Mauremys* and *Chinemys* with a bootstrap value of 100% and decay index of 26. The species *mccordi* was sister to all other *Cuora*, but only with a bootstrap value of 65% and decay index of 4. The species *amboinensis* and *mouhotii*, which were represented by more than one sample, were each monophyletic and supported with bootstrap values of 100% and decay indices of 25 and 37, respectively. The sister relationship of *amboinensis* and *mouhotii* was weakly supported with a bootstrap value of 54% and decay index of 2. The three species *aurocapitata*, *pani*, and *trifasciata* together formed a clade with a bootstrap value of 100% and decay index of 27, and were supported as the sister clade to *flavomarginata* with a bootstrap of 84% and decay index of 7. The genetic diversity within the *aurocapitata/pani/trifasciata* clade was extremely low. The samples of *aurocapitata* and *pani* were genetically identical, and the clade had a maximum uncorrected pairwise distance across all sequences of 0.45%. The species *flavomarginata* and *galbinifrons* have sometimes been classified together in the genus *Cistoclemmys* Gray 1863 (reviewed by Honda et al., 2002), but *flavomarginata* and *galbinifrons* were not recovered as sister taxa exclusive of other species. To test whether a polyphyletic *Cistoclemmys* was statistically different from a monophyletic *Cistoclemmys* given our data, the maximum parsimony searches were constrained to recover only those trees that produced a monophyletic *Cistoclemmys*. The 10 shortest trees generated by the constrained search were 737 steps long, 10 steps longer than the six most parsimonious unconstrained estimates of phylogeny. A comparison of the constrained versus unconstrained phylogenies in PAUP* using a one-tailed Wilcoxon signed-ranks test (Templeton, 1983) found that the two hypotheses were incompatible ($p < 0.0001$), and a monophyletic *Cistoclemmys* was not supported.

A node supporting the monophyly of all forms of *C. galbinifrons* was recovered with a bootstrap value of 100% and decay index of 13. Three major subclades were recovered within *C. galbinifrons*, each supported with a boostrap value of 100% and decay indices of 16–22. The first of these three clades (A) was represented by samples of *C. g. galbinifrons* and two of the five samples of "*serrata*," the second clade (B) by samples of *C. g. bourreti* and three of the five samples of "*serrata*," and the third clade (C) entirely by samples of *C. g. picturata*. Genetic variation within each of the three clades was low, but variation among the three clades was high (Table 2). The relationship of the three *C. galbinifrons* clades to each other is less clear. The *picturata* clade was found to be sister to the *bourreti* and *galbinifrons* clades, but only with a bootstrap value of 64% and decay index of 1. To test whether the polyphyletic "*serrata*" found here was statistically different from a monophyletic "*serrata*" given our data, the maximum parsimony searches were constrained to recover only those trees that produced a monophyletic "*serrata*." The 44 shortest trees generated by the constrained search were 766 steps long, 39 steps longer than the six most parsimonious unconstrained estimates of phylogeny. A comparison of the constrained versus unconstrained phylogenies as described above found that the two hypotheses were incompatible ($p < 0.0001$), and a monophyletic "*serrata*" was not supported.

3.2.2. Maximum likelihood

Maximum likelihood analysis resulted in a single tree of likelihood score −ln 6040.70 (Fig. 2). This tree differed from the six maximum parsimony trees by not recovering *C. amboinensis* and *C. mouhotii* as sister taxa exclusive of other species, and by the arrangement of subclades within the B clade of *C. galbinifrons* (which differed among the six maximum parsimony trees).

4. Discussion

4.1. Cuora

The relationships of *amboinensis*, *mccordi*, and *mouhotii* to other *Cuora* were not well supported using either maximum parsimony or maximum likelihood criteria, probably due to conflict between the COI and ND4 data sets in the placement of these three taxa. COI alone under maximum parsimony hypothesized *mccordi* to be sister to the *galbinifrons* clade, but ND4 alone under maximum parsimony, and the total data set under both maximum parsimony and maximum likelihood, hypothesized *mccordi* to be sister to all other *Cuora*. *C. mccordi* is extremely rare and known only from the trade. It was originally described from specimens obtained by a Hong Kong animal dealer who first claimed their origin to be Guangxi Province, China (Ernst, 1988), but later to be Yunnan Province, China (McCord and Iverson, 1991). Many of the collecting localities provided by this Hong Kong animal dealer have since been found to be dubious (Dalton, 2003), leaving the origin and status in the wild of *C. mccordi* uncertain.

Our maximum parsimony tree is concordant with that of Honda et al. (2002) by hypothesizing a clade containing *amboinensis* and *mouhotii*. This is noteworthy because *amboinensis* is the type species of *Cuora* (Gray, 1855), and *mouhotii* is the type species of the monotypic genus *Pyxidea* (Gray, 1863). However, *amboinensis* and *mouhotii* represent the longest branches in our trees, and their grouping under maximum parsimony could

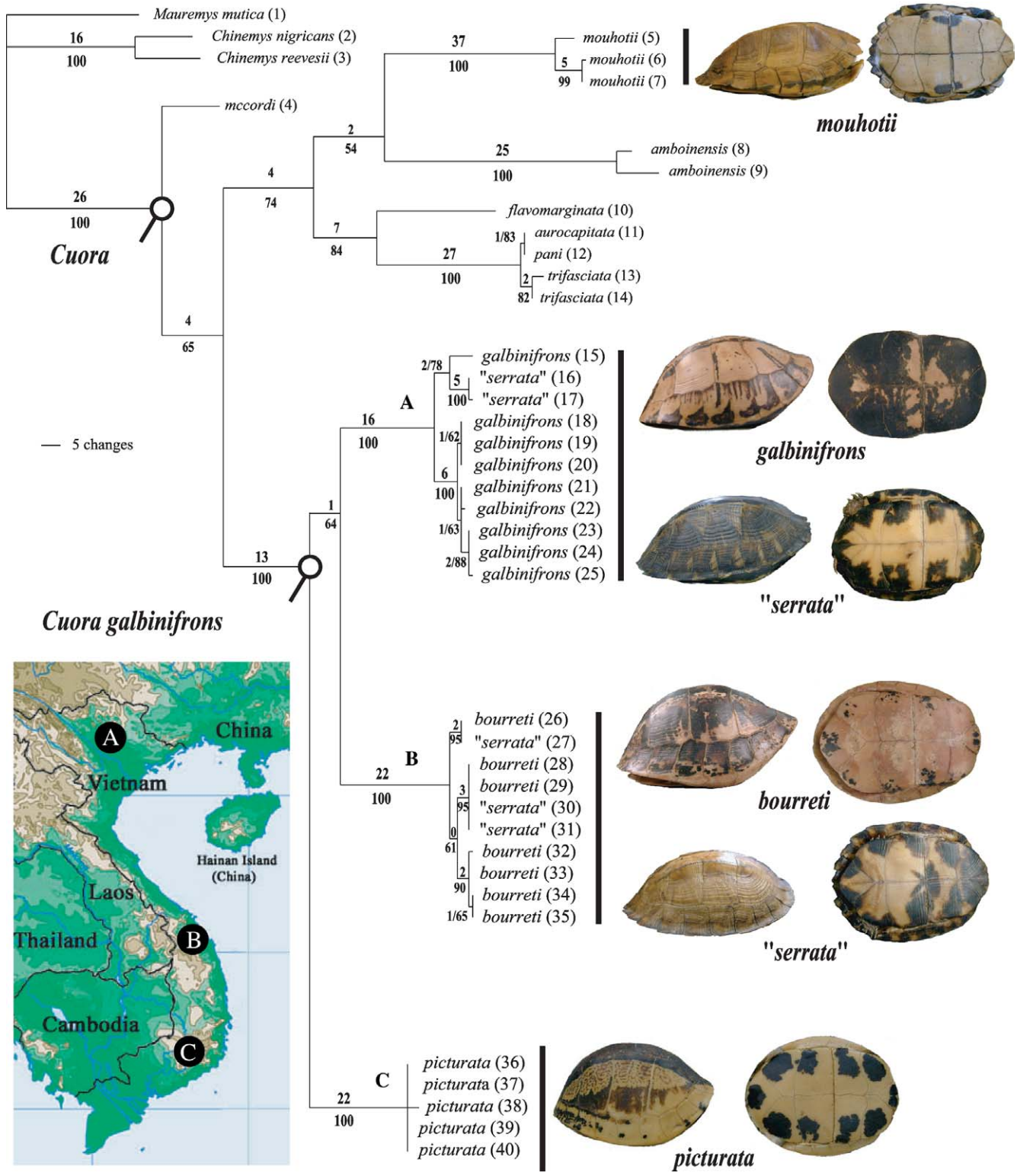

Fig. 1. One of the six equally most parsimonious trees recovered from all sequences combined using an unweighted maximum parsimony analysis. Branch lengths are proportional to the number of changes. The number above each stem is the decay index and the number below each stem is the bootstrap value. For some nodes these values are presented decay index/bootstrap for space reasons. The number following each taxon refers to the voucher for the sample (see Appendix A). Examples used in the study of *C. mouhotii*, *C.* "*serrata*," and the three *C. galbinifrons* lineages are illustrated. The type localities of the three *C. galbinifrons* lineages are labeled on the map.

Table 2
Minimum and maximum uncorrected genetic divergences of individual genes and total sequences

Clade	A (*galbinifrons* + *"serrata"*), %	B (*bourreti* + *"serrata"*), %	C (*picturata*), %	All other *Cuora*
A (*galbinifrons* + *"serrata"*)				
COI	0.00–1.39			
ND4	0.00–2.06			
tRNAs	0.00–1.15			
Total	0.00–1.29			
B (*bourreti* + *"serrata"*)				
COI	2.84–3.84	0.00–0.48		
ND4	4.13–5.27	0.00–0.72		
tRNAs	0.50–1.82	0.00–1.00		
Total	3.53–3.96	0.00–0.58		
C (*picturata*)				
COI	2.59–3.43	3.37–3.97	0.00–0.40	
ND4	4.27–5.75	3.88–4.13	0.00	
tRNAs	2.01–3.08	3.01–4.26	0.00	
Total	3.39–4.23	3.60–4.04	0.00–0.19	
All other *Cuora*				
COI	5.33–9.11	5.29–9.59	4.45–9.09	0.00–9.17
ND4	5.91–10.07	5.47–9.35	6.19–9.44	0.00–10.50
tRNAs	2.40–5.56	4.02–5.55	4.24–7.07	0.00–5.57
Total	5.69–8.77	5.35–9.00	5.22–8.92	0.00–9.07

Clade letters refer to those used in Fig. 1. The lack of divergence in comparisons of all other *Cuora* to all other *Cuora* is a result of identical sequences in *C. aurocapitata* and *C. pani*.

be a misleading result from long-branch attraction (Felsenstein, 1978). These two species are not hypothesized to be sister taxa when analyzed under maximum likelihood, a method that is less sensitive to long-branch attraction (Huelsenbeck, 1997). Regardless of its sister taxon, *mouhotii* nests within a *Cuora* clade in all analyses in Honda et al. (2002) and in this study, except when the COI data set was analyzed alone under maximum parsimony. As a result of this nesting, Honda et al. (2002) recommended moving *mouhotii* to the genus *Cuora*, a taxonomy we have followed here. Fritz and Obst (1997) also questioned the generic separation of *mouhotii* from species of *Cuora*, based on morphology.

Further taxon sampling is not likely to resolve the relationships of *amboinensis*, *mccordi*, and *mouhotii* with other *Cuora* using molecular data, since all extant species of *Cuora* were included in this study except *zhoui*, a species that is very similar in 12S and 16SrRNA mitochondrial DNA sequence to *aurocapitata*, *pani*, and *trifasciata* (Honda et al., 2002). However, more sequence data, including nuclear markers, may improve the estimate of their relationships.

The species *flavomarginata* and *galbinifrons* have sometimes been classified together in the genus *Cistoclemmys* (Gray, 1863) (type species *flavomarginata*), although this arrangement has been historically contentious (reviewed by Honda et al., 2002). Neither our study nor Honda et al. (2002) recovered *flavomarginata* as the sister taxon to *galbinifrons* exclusive of other species, and the monophyly of these two species was statistically rejected. Owing to the non-monophyly, Honda et al. (2002) recommended abandoning the genus name *Cistoclemmys* and placing both *flavomarginata* and *galbinifrons* into the genus *Cuora*, a taxonomy we have followed here.

Our maximum parsimony and maximum likelihood trees are concordant with those of Honda et al. (2002) by hypothesizing *flavomarginata* as the sister taxon to a clade containing *aurocapitata*, *pani*, and *trifasciata*. The low genetic diversity found among the three species *aurocapitata*, *pani*, and *trifasciata*, including identical sequences in *aurocapitata* and *pani*, is intriguing. Similarly, Honda et al. (2002) found a close relationship among the four species *C. aurocapitata*, *C. pani*, *C. trifasciata*, and *C. zhoui* (not included in our study), and reported only 2–11 bp changes among these four taxa in an 882-bp fragment of the 12S and 16SrRNA mitochondrial genes. The three species *aurocapitata*, *pani*, and *trifasciata* share a common body plan of yellow heads and low carapacial profiles, but are morphologically diagnosable (McCord and Iverson, 1991). Specifically, *C. aurocapitata* has a lemon-yellow head, a brown carapace with reddish vertebrals, and black plastral markings that are usually triangular or streaked diagonal to the scale seams; *pani* has an olive to olive-yellow head lacking a black postorbital stripe, an olive-brown carapace with light brown vertebrals, and black plastral markings that are broad and restricted to the scale seams; *trifasciata* has a yellow crown with a distinct black postorbital stripe enclosing a brown or olive triangle behind the eye, a carapace with three black longitudinal stripes, and a plastron with a large central dark marking (McCord and Iverson, 1991). Despite these

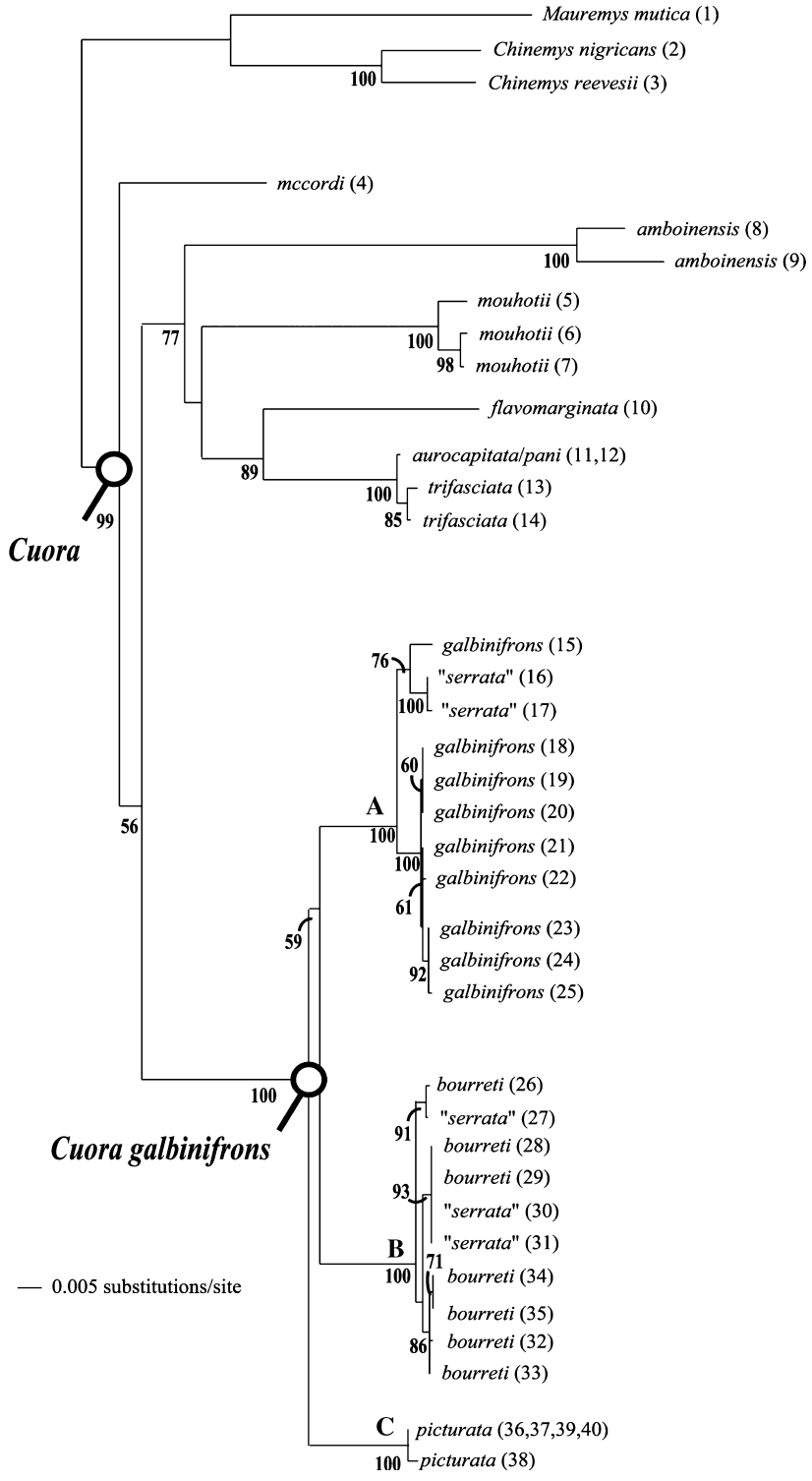

Fig. 2. The single most likely tree (−ln 6040.70) obtained from all sequences combined using a maximum likelihood analysis of the HKY + *I* + *G* model of sequence evolution. The number above each stem is the boostrap value and the number following each taxon refers to the voucher for the sample (see Appendix A).

color and pattern differences, McCord and Iverson (1991) reported specimens of *aurocapitata* and *pani* that were intermediate in plastral patterns, and failed to discriminate *aurocapitata* and *pani* using morphometric data. Consequently, McCord and Iverson (1991) suggested that *aurocapitata* and *pani* might be only subspecifically distinct, but withheld taxonomic judgment pending further study.

We present five possible explanations for the lack of divergence among *aurocapitata*, *pani*, and *trifasciata*. First, identical sequences in *aurocapitata* and *pani* could be a result of contamination of samples. However, we strongly discount this explanation, as the tissue samples in our study originated from separate sources and were sequenced about five months apart, with most of the other taxa in the study sequenced in the interim. Furthermore, Honda et al. (2002) independently reported very similar sequences among these taxa. Second, as proposed by McCord and Iverson (1991), *aurocapitata* and *pani* could be conspecific, with differences in color and pattern attributable to polymorphism or geographic variation. Unfortunately, the ranges of *aurocapitata* and *pani* are very uncertain (McCord and Iverson, 1991; Parham and Li, 1999). Third, these species may have diverged very recently (either all three species from each other, or *aurocapitata pani* from *trifasciata*, depending on the outcome of the second hypothesis), and strong selection has accelerated changes in morphology. Mutation rates in protein-coding or neutral genetic markers would not necessarily reflect morphological divergence under strong directional selection (Bowie, 2003). Parallel cases of considerable morphological divergence with little or no mitochondrial DNA divergence are reported between two lineages of *Nectarinia* sunbirds (Bowie, 2003) and among 12 species of *Graptemys* map turtles (Lamb et al., 1994). Honda et al. (2002) also proposed that *C. aurocapitata*, *C. pani*, *C. trifasciata*, and *C. zhoui* diverged relatively recently. Fourth, mitochondrial DNA of one of these species may have introgressed into populations of the other species at historical contact zones, and fixation occurred by selection or drift. Mitochondrial DNA introgression between allopatrically diverged taxa in zones of secondary contact, including the complete fixation of mitochondrial DNA haplotypes of one species found in another species, has been reported in lake fishes of Canada (Wilson and Bernatchez, 1998) and eastern Africa (Rüber et al., 2001). Fifth, one or more of these species could be hybrids from the same maternal species in captivity, resulting in similar or identical mitochondrial DNA sequences. Hybridization of geoemydid turtles, even among distantly related taxa, is well known (Dalton, 2003; Galgon and Fritz, 2002; Parham et al., 2001; Wink et al., 2001), and the high value of these turtles in the international pet trade would provide incentive for Chinese turtle farmers to produce them (see Parham and Shi, 2001). A hybrid origin could explain the specimens of *aurocapitata* and *pani* reported by McCord and Iverson (1991) that were intermediate in plastral patterns. However, unlike other candidate hybrid geoemydid turtles (Parham et al., 2001), both *pani* and *trifasciata* are reported from the wild (Lau et al., 2000; Pope, 1935; Song, 1984; Zhao, 2001), and a likely paternal species is unknown. All of our samples of these three rare species originated from the pet trade (Appendix A). The study of nuclear markers could test the latter two hypotheses. Clearly, further investigations into the molecular relationships of these taxa are warranted, particularly with the use of nuclear markers and, if they exist, tissue samples of known provenance.

4.2. *Cuora galbinifrons*

Cuora galbinifrons is composed of three major mitochondrial DNA clades. Each clade corresponds to one of the three currently recognized subspecies *galbinifrons*, *bourreti*, and *picturata*, except that samples of *C.* "*serrata*" are nested within the *galbinifrons* and *bourreti* clades. Specimens of *C. "serrata"* have been shown with morphology, mitochondrial DNA, and allozymes to be a hybrid between *C. galbinifrons* and *C. mouhotii* (Parham et al., 2001). Our study does not provide a new test of the hybrid hypothesis of *C.* "*serrata*" because we only examined mitochondrial DNA, which is maternally inherited (Avise et al., 1987). However, our findings of a polyphyletic *C.* "*serrata*" are consistent with the hybrid hypothesis of Parham et al. (2001). The five samples of *C.* "*serrata*" used in this study consisted of the same three samples used in Parham et al. (2001); (samples 16, 17, and 30), plus two additional samples (samples 27 and 31). All five samples have mitochondrial DNA of *C. galbinifrons* rather than of *C. mouhotii*. Accepting that they are hybrids, these five examples had *C. mouhotii* fathers and *C. galbinifrons* mothers because mitochondrial DNA is maternally inherited and usually non-recombinant (Avise et al., 1987). Furthermore, our data show that *C.* "*serrata*" can have either *C. g. galbinifrons* or *C. g. bourreti* mothers. It remains unknown whether *C.* "*serrata*" are the outcome of hybridization in captivity at turtle farms or in the wild (Parham et al., 2001). Following Parham et al. (2001), we do not recognize *C.* "*serrata*" to be a valid taxon, and consequently the three subspecies *galbinifrons*, *bourreti*, and *picturata* are rendered monophyletic, evolutionary lineages in our analyses.

The three subspecies *galbinifrons*, *bourreti*, and *picturata* are readily diagnosed by the coloration and shape of the shell, and coloration of soft body parts (Lehr et al., 1998a). Specifically, *galbinifrons* has a more elongate and flatter carapace than *bourreti* and *picturata*, no wide brown band on the pleural scales, and mostly or entirely black plastron and submarginals; *bourreti* has a wide brown band on the pleural scales closer to the vertebrals than in *picturata*, and a plastron with a black blotch on each scale; *picturata* has a higher domed carapace than *galbinifrons* and *bourreti*, light proximal marginals, a wide brown band on the pleural scales closer to the marginals than in *bourreti*, a plastron with black blotches, and reticulate olive or yellow lines on the head (Lehr et al., 1998a). Each also has a number of molecular synapomorphies.

Species concepts and the criteria used to implement them are contentious and widely debated in the literature (de Queiroz, 1998; Wheeler and Meier, 2000). We adopt the "diagnostic" version of the phylogenetic species concept (Cracraft, 1983; Nixon and Wheeler, 1990), but concur with Crowe (1999) that diagnosability in more than one independent line of evidence, such as morphology, molecules, ecology, physiology or behavior, is necessary and sufficient to define species. This modification by Crowe (1999) helps to avoid diagnosing evolutionary units on the basis of trivial characters, and ultimately recognizing "too many species," both of which are major criticisms of the phylogenetic species concept (McKitrick and Zink, 1988). Because the three subspecies *galbinifrons*, *bourreti*, and *picturata* are diagnosable with both morphological and molecular characters, we recommend recognizing each as full species.

We support the notion that designating a clade as a species is a hypothesis, rather than a conclusion. In this case, two independent lines of evidence, morphology and mitochondrial DNA, support recognizing *galbinifrons*, *bourreti*, and *picturata* as separate species, and no evidence suggests the contrary. We realize our sample sizes are relatively small, and that the unknown or uncertain provenance of the majority of samples prevents knowing how adequately these taxa have been sampled geographically. However, we also realize that only three field-collected tissues of the *galbinifrons* species group are currently available to the scientific community (all *galbinifrons* lineage and used here) and that field collections may not improve much in the near future given the worsening conservation status of these turtles (van Dijk et al., 2000). Consequently, we feel justified making these taxonomic recommendations under these sampling limitations.

Fritz et al. (2002) interpreted some trade specimens from near the inferred area of contact of *galbinifrons* and *bourreti* to represent intergrades, but we feel this does not preclude recognizing these lineages as separate species. Hybridization between sister clades in places of contact should not be surprising, especially given the willingness and ability of geoemydid turtles to reproduce with even distantly related taxa. In addition to the *C.* "*serrata*" example, hybrids are known from *C. trifasciata* and *M. mutica* (Parham et al., 2001; Wink et al., 2001), *Ch. reevesii* and *M. mutica* (Wink et al., 2001), *C. reevesii* and *C. amboinensis* (Galgon and Fritz, 2002), and *Mauremys annamensis* and *C. amboinensis* (Fritz and Mendau, 2002).

A sample of *galbinifrons* from Hainan Island, China (sample 15; Appendix A), formerly recognized as *C. g. hainanensis* or *C. hainanensis*, showed little genetic differentiation from known-locality samples of *galbinifrons* from Laos and Vietnam (samples 18, 19, and 23; Appendix A). Uncorrected divergence of total sequence of the Hainan sample to the Laos and Vietnam samples ranged from 1.17 to 1.31%. Similarly, Liu et al. (2000) reported a close genetic relationship of populations of the toad *Bufo melanostictus* between Hainan Island and northern Vietnam. Hainan Island has been repeatedly connected to Vietnam and southeastern China during the Pleistocene glacial periods that lowered sea levels and drained the Gulf of Tonkin, including a connection as recent as 17,000 years before present (Voris, 2000). The nesting of the Hainan sample within the *galbinifrons* A clade is concordant with the recommendation based on examination of morphology by Obst and Reimann (1994) and Lehr et al. (1998b) that *hainanensis* should be recognized as a junior synonym of *galbinifrons*.

It has been established that genetic divergence alone is not a sufficient criterion for defining species (Frost and Hillis, 1990; Wake and Schneider, 1998), but we provide this comparison to put the divergences among *galbinifrons*, *bourreti*, and *picturata* into perspective. The North American box turtles of the genus *Terrapene* are in many ways the ecological and morphological counterparts of the *C. galbinifrons* species group. Uncorrected pairwise divergences of the ND4 gene (excluding adjacent tRNAs) among the four distinct, well-established species *Terrapene carolina*, *Terrapene coahuila*, *Terrapene nelsoni*, and *Terrapene ornata* (Feldman and Parham, 2002) range from a minimum of 3.92% between *carolina* and *coahuila* to a maximum of 5.73% between *coahuila* and *ornata*. These values are nearly identical to the divergences of the ND4 gene among the *C. galbinifrons* group, which range from a minimum of 3.88% between *bourreti* and *picturata* to a maximum of 5.75% between *galbinifrons* and *picturata* (Table 2).

The factors responsible for diversification of the *C. galbinifrons* species group into three lineages over such a small geographic area remain unclear. The Annamite Mountains straddling the borders of Laos, Cambodia, and Vietnam form a nearly continuous chain through the ranges of these taxa, interrupted only by a few narrow, low passes that do not seem likely to be present-day dispersal barriers. However, the morphological and molecular distinctiveness of the *galbinifrons* species group infers that dispersal barriers have existed in the past. Two primate clades that also occur in upland evergreen forest of the Annamite Mountains share with the *C. galbinifrons* species group a distribution pattern of three subdivided biological units on a north–south axis. These include, from north to south respectively, the gibbons *Hylobates leucogenys*, *Hylobates siki*, and *Hylobates gabriellae* (Fooden, 1996; Groves, 2001), and the douc langurs *Pygathrix nemaeus*, *Pygathrix cinerea*, and *Pygathrix nigripes* (Groves, 2001; Nadler, 1997). In both the gibbons and the douc langurs, the southern species is sister to a clade containing the central + northern species (Garza and Woodruff, 1992; Roos and Nadler, 2001). Our findings in the *galbinifrons* species group are concordant with the gibbons and douc langurs by placing

the southern species (*picturata*) as sister to a clade containing the central (*bourreti*) + northern (*galbinifrons*) species, although the node illustrating this relationship is weakly supported with a decay index of 1 under maximum parsimony and bootstrap values of 64 and 59% under maximum parsimony and maximum likelihood, respectively (Figs. 1 and 2). Hybrid gibbons *siki* × *gabriellae* (Groves, 2001) and douc langurs *nemaeus* × *nigripes* (Nadler, 1997) have been reported from contact zones in the field, and hybrid *galbinifrons* x *bourreti* have been found in trade in the area where these taxa are suspected to come into contact (Fritz et al., 2002). Additional studies of upland taxa may show concordance with this emerging pattern of tripartite distinctiveness, albeit with some hybridization, in the Annamite Mountains.

Recognizing *galbinifrons*, *bourreti*, and *picturata* as full species has conservation consequences. First, ex situ captive breeding of turtles confiscated from the Asian food and medicine trade has recently become a major strategy for thwarting the extinction of certain endangered species (Hudson and Buhlmann, 2002; Turtle Conservation Fund, 2002). Our mitochondrial DNA data, in combination with morphology, lead us to hypothesize that *galbinifrons*, *bourreti*, and *picturata* represent independent evolutionary lineages. Captive breeding efforts should try to maintain the integrity of these evolutionary lineages by propagating them separately in captivity. Second, their recognition splits one species range into three, smaller species ranges, thereby increasing threat levels to each species from exploitation and habitat loss. Third, Stuart and Thorbjarnarson (2003) ranked Asian countries according to the diversity, endemism, and threat level of their turtle faunas, and found China and Vietnam to be the top two priority countries in Asia for turtle conservation activities. Recognizing *bourreti* and *picturata* as full species increases the number of turtle species endemic to Vietnam from one (*Mauremys annamensis*) to three, and consequently, makes turtle conservation needs in Vietnam even more acute. Although *bourreti* and *picturata* have been suspected to occur in neighboring Cambodia and Laos (Lehr et al., 1998a; Obst and Reimann, 1994), no record of either taxon from either country currently exists. Based on current information, the onus for conserving wild populations of *bourreti* and *picturata* lies on Vietnam, where turtle populations have been devastated from overexploitation for sale to China and habitat destruction (Hendrie, 2000).

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Appendix A

List of vouchers used in this study and GenBank accession numbers of sequences. FMNH = Field Museum of Natural History, Chicago; MVZ = Museum of Vertebrate Zoology, Berkeley; ROM = Royal Ontario Museum, Toronto; YPM R = Yale Peabody Museum, New Haven. Accession numbers are presented as COI/ND4 region.

(1) *M. mutica* ROM 25613 purchased from reptile trader in Yen Bai Prov., Vietnam (AF348261/AF348279). (2) *Ch. nigricans* MVZ 130463 pet trade

(AF348264/AF348289). (3) *Ch. reevesii* MVZ 230533 pet trade (AF348263/AF348288). (4-14) *Cuora*. (4) *mccordi* FMNH 261571 pet trade (AY357737/AY364608). (5) *mouhotii* MVZ 230482 purchased from hunter on slopes of Wuzhi Shan, Hainan Prov., China (AF348274/AF348287). (6) *mouhotii* ROM 35002 purchased from hunter in Bac Thai Prov., Vietnam (AF348272/AF348285). (7) *mouhotii* ROM 35003 purchased from hunter in Bac Thai Prov., Vietnam (AF348273/AF348286). (8) *amboinensis* FMNH 255262 purchased from hunter in Champasak Prov., Laos (AY357738/AY364609). (9) *amboinensis* MVZ 230509 pet trade (no COI/AF348267). (10) *flavomarginata* MVZ 230464 born in captivity from two adults field-collected in Anhui Prov., China (AY357739/AY364610); ND4 region *numt* (AY364632). (11) *aurocapitata* FMNH 261570 pet trade (AY357740/AY364606). (12) *pani* MVZ 230512 pet trade (AY357741/AY364607). (13) *trifasciata* MVZ 230636 pet trade (AF348270/AF348297). (14) *trifasciata* MVZ 230467 pet trade (AF348271/AF348296). (15-40) *C. galbinifrons* species group. (15) *galbinifrons* (formerly *hainanensis*) MVZ 230466 purchased from turtle trader in village on slopes of Dia Loushan, Hainan Prov., China (AF348266/AF348291). (16) "*serrata*" (*mouhotii* × *galbinifrons*) MVZ 230511 pet trade (AF348269/AF348294). (17) "*serrata*" (*mouhotii* × *galbinifrons*) MVZ 230629 pet trade (AF348268/AF348295). (18) *galbinifrons* FMNH 255694 field-collected in Nghe An Prov., Vietnam (AY357742/AY364612). (19) *galbinifrons* FMNH 255695 field-collected in Nghe An Prov., Vietnam (AY357743/AY364617). (20) *galbinifrons* FMNH 261580 pet trade (AY357744/AY364611). (21) *galbinifrons* YPM R 14078 pet trade (AY357746/AY364613). (22) *galbinifrons* YPM R 14079 pet trade (AY357747/AY364614). (23) *galbinifrons* FMNH 256544 field-collected in Khammouan Prov., Laos (AY357748/AY364615). (24) *galbinifrons* MVZ 230933 pet trade; incorrectly listed as MVZ 230544 in Parham et al. (2001) (AY357749/AF348290). (25) *galbinifrons* YPM R 14080 pet trade (AY357750/AY364616). (26) *bourreti* FMNH 261577 pet trade (AY357751/AY364624). (27) "*serrata*" (*mouhotii* × *bourreti*) FMNH 261572 pet trade (AY357752/AY364627). (28) *bourreti* YPM R 14074 pet trade (AY357753/AY364623). (29) *bourreti* FMNH 261578 pet trade (AY357754/AY364622). (30) "*serrata*" (*mouhotii* × *bourreti*) MVZ 230628 pet trade (AF348267/AY364626). (31) "*serrata*" (*mouhotii* × *bourreti*) FMNH 261573 pet trade (AY357755/AY364625). (32) *bourreti* YPM R 14076 pet trade (AY357756/AY364621). (33) *bourreti* FMNH 261574 pet trade (AY357757/AY364618). (34) *bourreti* YPM R 14075 pet trade (AY357758/AY364620). (35) *bourreti* FMNH 261579 pet trade (AY357759/AY364619). (36) *picturata* FMNH 261575 pet trade (AY357760/AY364628). (37) *picturata* FMNH 261576 pet trade (AY357761/AY364629). (38) *picturata* ROM 37067 purchased in Ho Chi Minh City market, Vietnam; incorrectly listed as ROM 30062 Cat Tien, Dong Nai Province, Vietnam, in Parham et al. (2001) (AF348265/AF348292). (39) *picturata* YPM R 11679 pet trade (AY357745/AY364630). (40) *picturata* YPM R 14077 pet trade (AY357762/AY364631).

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