Animal Conservation (2001) **4**, 357–367 © 2001 The Zoological Society of London Printed in the United Kingdom

New Chinese turtles: endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme electrophoresis and known-locality specimens

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(Received 11 December 2000; accepted 1 July 2001)

Abstract

Over the past 16 years, 13 new species of geoemydid turtles have been described from China. Ten of these new species are based on specimens purchased through the Hong Kong animal trade. Unfortunately, attempts by scientists to discover wild populations of some these newly described species have failed, raising questions about the legitimacy of the type localities and concerns over the validity of the species. Here the phylogenetic and taxonomic validity of two of these species is tested. Mitochondrial DNA haplotypes and allozyme genotypes of specimens matching the descriptions of *Mauremys iversoni* and *Cuora serrata* are compared to specimens of established species collected from known localities. The available evidence is consistent with the hypothesis that the specimens represent polyphyletic, intergeneric hybrids. The systematic status of all the new forms of turtles described from pet trade specimens are critical data for conservation efforts, particularly captive breeding.

INTRODUCTION

A major goal of systematic biology is to record the existence of species before they become extinct. It is of considerable importance that 13 new geoemydid (= batagurid; see Bour & Dubois, 1986; McCord *et al*., 2000) turtles, a diverse but highly endangered clade, have been described from China over the past 16 years (Table 1). The descriptions of these new species constitute a significant increase in known turtle diversity; fewer than 260 species of turtles were previously known worldwide (Ernst & Barbour, 1989; Iverson, 1992). These descriptions coincide with mounting international conservation efforts as herpetologists learn that all Asian turtles are threatened with extinction (Altherr & Freyer, 2000; van Dijk, Stuart & Rhodin, 2000). Recent economic reforms in China, combined with a long tradition of using turtles for medicinal purposes, have created a crisis for Asia's turtles (Behler, 1997). A recent symposium on the Asian turtle trade (van Dijk *et al*., 2000) implicates the demand of Chinese markets as the primary cause for the decimation of turtle populations throughout Asia. Given the intensity of this demand and the deep-rooted cultural basis of these needs, focused efforts to conserve Asia's diverse turtle fauna must be established rapidly. Because of their putative rarity, the newly described taxa have a high conservation priority by the National Environmental Protection Agency of the People's Republic of China (Wang, 1998).

Most of the recently described species are based on holotypes purchased through a single Hong Kong pet dealer (Yuk Wah 'Oscar' Shiu). This situation has resulted in substantial confusion, including the simultaneous description of the same species by separate authors, clouding the literature with names that were ultimately synonymized (see reviews in McCord & Iverson, 1991; Iverson, 1992). Efforts to rediscover several of these turtles at their alleged type localities have failed (de Bruin & Artner, 1999; Fritz & Obst, 1999), and some authors have suggested that some localities were mistaken or even falsified (McCord & Iverson, 1991; Fritz & Obst, 1999; Parham & Li, 1999). Furthermore, many of the recently described species possess a combination of morphological features that characterize known turtles into separate genera. Because some distantly related turtles can hybridize (reviewed in Fritz & Baur, 1995; Fritz, 1995), several investigators have raised the possibility that some of these new species represent intergeneric hybrids (Artner, Becker & Jost, 1998; Fritz &

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Table 1. Thirteen new species of geoemydid turtles described from China

1 *Cuora pani* Song 1984. Known from two localities in northern China (Parham & Li, 1999).
2 *Cuora* '*chriskarannarum*' Ernst & McCord 1987. Synonymous with *C. pani* (McCord & Iverson, 1991). The type locality in southern China may be fabricated (Parham & Li, 1999).
3 *Cuora aurocapitata* Luo & Zong 1988. Mistaken for *C. pani* by Ernst & McCord (1987). Known only from Anhui Province.
4 *Cuora mccordi* Ernst 1988. Known only from the pet trade and markets.
5 *Mauremys iversoni* Pritchard & McCord 1991. Known only from the pet trade and markets. Described from two distant localities in Fujian and Guizhou provinces. Wink *et al*. (2001) suggested that this turtle is the result of an ancient hybridization event.
6 *Cuora zhoui* Zhao, Zhou & Ye 1990. Described from specimens bought in a market in Guanxi Province. Known only from the pet trade and markets.
7 *Cuora* '*pallidicephala*' McCord & Iverson 1991. Synonymous with *C. zhoui*, but described from pet trade specimens supposedly from Yunnan Province.
8 *Ocadia philippeni* McCord & Iverson 1992. Known only from type description. It has some characters of *Cuora trifasciata* and could be a hybrid.
9 *Sacalia pseudocellata* Iverson & McCord 1992. Known only from type description. It has some characters of *Cuora trifasciata* and could be a hybrid.
10 '*Clemmys guangxiensis*' Qin 1992. A composite taxon. The type series includes *Mauremys mutica* and *M. iversoni* (Iverson & McCord, 1994).
11 *Cuora serrata* Iverson & McCord 1992. Originally described as a subspecies of *C. galbinifrons*, but later elevated to full species (Fritz & Obst, 1997). Known only from the pet trade.
12 *Ocadia glyphistoma* McCord & Iverson 1994. Known only from the type description.
13 *Mauremys pritchardi* McCord 1997. Known only from the pet trade. This species is a hybrid of *Chinemys reevesii* and *Mauremys mutica* (Artner *et al*., 1998; Wink *et al*., 2001).

Obst, 1998; Lau & Shi, 2000). Wink, Guiking & Fritz (2001) used a combination of molecular methods to conclude that at least two of the newly described species, *Mauremys pritchardi* McCord 1997 and *Mauremys iversoni* Pritchard & McCord 1991, have hybrid origins. However, questions about the details of these origins remain.

It is unclear whether hybridization has played a role in the establishment of new species or whether the hybridization events are simply the result of an occasional breakdown of specific mate-recognition systems. Furthermore, it is uncertain whether the hybridization events are ancient or recent, or whether they occurred in wild populations or in captivity. None of these hypotheses excludes another, and multiple hybridization events (i.e., wild and ancient or recently in captivity) may have occurred. Conservation efforts have limited resources and so these hypotheses, with their bearing on the conservation value of the newly discovered turtles, must be tested expediently.

In light of this confusion, we set out to test the phylogenetic affinities and provenance of two of these newly described species, *Mauremys iversoni* and *Cuora serrata* Iverson & McCord 1992. We focused our efforts on *M. iversoni* and *C. serrata* for three reasons. First was the availability of material. Pet trade specimens are prohibitively expensive for scientific researchers and, because of their putative scarcity, known locality specimens of geoemydids (essential for understanding the provenance of mitochondrial lineages) are extremely rare in museum collections. As a result, our sample sizes are small, and we emphasize the need for further research and more resources to test hypotheses of species validity and provenance.

Second, both *M. iversoni* and *C. serrata* have unusually variable phenotypes. In fact, they express several morphological characters that are known in valid turtle species of separate genera. Even the type description of *Mauremys iversoni* (Pritchard & McCord, 1991) noted the extremely variable morphology and coloration of this species and that *M. iversoni* bears morphological characters previously diagnostic of *Cuora trifasciata*. Unlike other species of *Mauremys*, but like *C. trifasciata*, *M. iversoni* has a yellow head with dark postorbital stripes (Fig. 1) and a weakly tricarinate shell. Most specimens, including the holotype and the specimen examined here, have red or pink coloration on the underside and limbs.

Cuora serrata is diagnosed by a tricarinate shell that is dorsally flattened and has a posterior end that is visibly serrated (Fig. 2). These characters, in combination,

Fig. 1. Head coloration and patterning of *'Mauremys iversoni'* and putative parental species. (a) *Cuora trifasciata*, the dorsal surface of the head is yellow and the postorbital stripe is dark. (b) *'Mauremys iversoni'*, the dorsal surface of the head is yellow and the postorbital stripe is dark. (c) Vietnamese *Mauremys mutica*, the dorsal surface of the head is dark and the postorbital stripe is yellow.

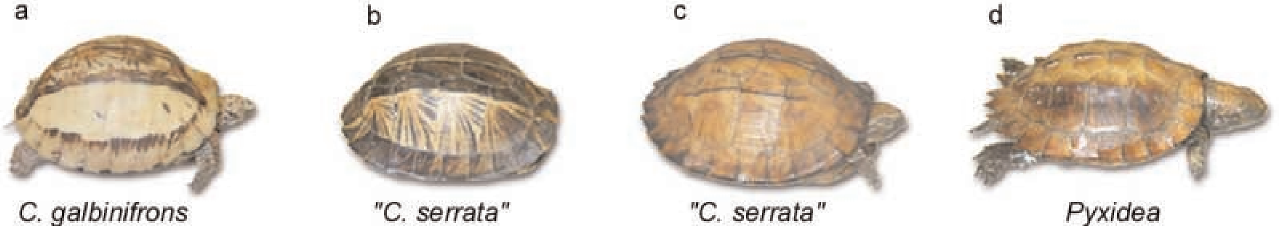

Fig. 2. Shell morphology and coloration of *'Cuora serrata'* and putative parental species. (a) *Cuora galbinifrons*, the shell is domed and unserrated, and has a dark medial stripe. (b) *Cuora*-like *'C. serrata'*, the shell is slightly flattened, slightly serrated, and has a dark medial stripe. (c) *Pyxidea*-like *'C. serrata'*, the shell is slightly flattened, slightly serrated and brown. (d) *Pyxidea*, the shell is flattened, serrated, and brown.

were previously diagnostic of *Pyxidea mouhotii* (Ernst & Barbour, 1989). Fritz & Obst (1998) noted that specimens of *C. serrata* are highly variable; some specimens resemble *C. galbinifrons*, while others are more similar to *P. mouhotii* (Fig. 2(b) & (c)). A high degree of morphological polymorphism within a turtle species is not uncommon, but it is unusual for different specimens of one species to resemble entirely different genera.

Third, there have been persisting rumours that *M. iversoni* has been artificially created in captivity by crossing *Mauremys mutica* and *C. trifasciata*. In fact, our sole specimen of this taxon was obtained from a Chinese turtle farm and was claimed to be a hybrid by its owner (a claim that we test below).

By testing the validity of the new forms, we hope to guide systematic efforts towards questions that are important for conservation. However, because there are fundamental differences between the traditional system of nomenclature and phylogenetic systematics, it is necessary to state explicitly our criteria of validity. For the purpose of this study, our operational definition of a species is provided by the phylogenetic species concept of Mishler & Theriot (2000). We chose this concept because it allows hybrid species to be considered valid. Therefore, if a new form represented an independent lineage that formed through a single, ancient hybridization event and all specimens represent a genetically cohesive unit (i.e., monophyletic mtDNA haplotypes), we would consider it a valid species. The International Commission on Zoological Nomenclature (ICZN; article 23h), on the other hand, explicitly states that names established for hybrids are not valid. Therefore, it is possible that a conceptually suitable species by our standards would not be recognized by the ICZN. Similarly, the ICZN does not require that species be monophyletic and could therefore recognize a species considered unacceptable by our species definition. Importantly, both the ICZN and the phylogenetic species concept would not consider taxa derived from multiple hybridization events to be valid.

We used two complementary lines of evidence: biparentally inherited, recombinant proteins and maternally inherited, non-recombinant mtDNA. First, we surveyed four protein systems from specimens referrable to *M. iversoni* and *C. serrata* and compared them to specimens of well-established species (i.e., well known by herpetologists, found in the wild, and represented in museum collections) of their putative parental species to ascertain whether these species represent intergeneric hybrids. We also sequenced two protein-coding mitochondrial genes to determine the evolutionary relationships of the species in question. We then used both lines of evidence to test alternative hypotheses for the origins of *M. iversoni* and *C. serrata*. Some of the hypotheses for each of the new forms and the data that would support them are:

(1) The new form is a non-bisexual hybrid species with a single origin. There are several modes of reproduction of hybrid species (reviewed by Bullini, 1994) and consequently the expected mtDNA and allozyme electrophoresis results will depend on which mode is present in the new form as well as the antiquity of the hybrid lineage. In most vertebrate hybrid species, male hybrids are not fertile. Some female hybrids can reproduce. Under these circumstances, the female mates with a male of one of the parental species, the paternal genome is discarded, and the progeny are clones of the hybrid mother. If this is the case, all the specimens of the new form should share the genome of the female involved in the initial hybridization event. We would expect specimens to share nearly identical mtDNA and exhibit fixed heterozygosity for all loci that normally distinguish the parental species. If the lineage is ancient, in addition to sharing mtDNA haplotypes, we would expect specimens of the new form to share novel haplotypes. Polyphyletic mtDNA haplotypes would indicate that a single hybridization event is not sufficient to explain the new form.

(2) The new form is a valid bisexual, phylogenetic species. Under this hypothesis, a new form could still have a hybrid origin, but it would be ancient. We would expect specimens of the new form to share maternally inherited mtDNA haplotypes that evolved independently of other lineages. Only one case of a bisexual animal species that arose through hybridization is currently known (DeMarais, *et al.*, 1992). Polyphyletic mtDNA haplotypes would indicate that this hypothesis is not sufficient to explain the new form.

(3) The new form is a variant of a living species. Support for this hypothesis would include a phylogenetic position of specimens of a new form within the clade of a typical-looking species. In a

geographically isolated variety (or subspecies), the mtDNA of all the specimens of the new form would be similar. If the new form is polyphyletic, the allozymes should resemble closely related specimens of the typical-looking species.

(4) The new form is the result of a single, recent hybridization event. Support for this would include a low diversity of (or even identical) mtDNA haplotypes in all specimens of the new form. Furthermore, we would expect the new forms to be heterozygous for proteins that diagnose the parental species.

(5) The new form is the result of multiple, recent hybridization events. Support for this hypothesis would include a polyphyletic distribution of mtDNA haplotypes as well as heterozygous allozymes in loci that are fixed for the parental species.

Ultimately, a combination of hypotheses might be necessary to explain all specimens of the new forms. For example, in theory, some specimens attributed to *M. iversoni* may represent recent hybrids in captivity, whereas other specimens could be derived from wild populations that reflect an ancient hybrid origin. Our study is not capable of determining how many hybridization events account for all of the available specimens. However, we can test whether multiple hybridization events occurred in addition to the hypothesis that all of the specimens of these taxa represent valid species. This is important, especially for the captive-breeding effort, because specimens of the new species are only available through the animal trade and so have virtually unknown origins.

MATERIALS AND METHODS

We studied 22 turtles representing ten species acquired from the animal trade or museum collections (Table 2). Most of the specimens used in this study are from the frozen tissue collection of the Museum of Vertebrate Zoology. Species without locality data were obtained as whole frozen animals from the pet trade. These animals had died in captivity and were donated to the University of California. The species with localities in Zhejiang and Hainan provinces, China, were obtained by specimen exchange with Chinese universities as part of an ongoing collaboration between the Museum of Vertebrate Zoology, the Chinese Acaemy of Sciences, and Hainan Normal University. Specimens were captured by hand during herpetological surveys and euthanazed with a 60mg/cc nebutol injection following standard museum protocol as approved by the Office of Laboratory Animal Care of the University of California, Berkeley. Turtles of the genus *Cuora* have CITES II protection as of 7 July 2000. The specimen of *Cuora galbinifrons* (MVZ 230466) was obtained in 1999. All vouchered specimens are stored in the Museum of Vertebrate Zoology and Royal Ontario Museum and are available for study to qualified researchers. As mentioned previously, most of the newly described turtles from China are available only through the pet trade, where they can fetch high prices (often exceeding 1000 US dollars); hence our relatively low sample size. We verified the identity of our specimens through comparison to the type descriptions. Our specimens of *Cuora serrata* (Fig. 2) were ultimately obtained from Yuk Wah Shiu, the Hong Kong pet dealer who supplied the holotypes of *Cuora galbinifrons serrata* to Iverson & McCord (1992*b*). Vouchers for all of the sequenced specimens are available at the Museum of Vertebrate Zoology, University of California at Berkeley or the Royal Ontario Museum Toronto (Table 2).

We surveyed allozyme variation as a first test of phylogenetic affinity and possible hybrid status. We focused on the hybrid species and their possible parents. The potential parental species were determined by reference to Wink *et al*. (2001), our mtDNA results, and the morphology of the specimens themselves (showing mixed characters of two well-known species). Allozymes can be a useful tool for unravelling species identities in turtles and are generally concordant with morphological and biogeographic data (Seidel, Iverson & Atkins, 1986). We surveyed four protein systems (Table 3) from liver tissue and stained them using standard methods (Murphy *et al*., 1996). We resolved MDH (E.C. 1.1.1.37), and PEP-LA (E.C. 3.4.-.-.) on Tris-Citrate, pH 8.0 (Selander *et al*., 1971); 150 V for 6 hours and PGI (E.C. 5.3.1.9) on Lithium Hydroxide (Soltis *et al*., 1983); 250 V for 7 hours.

We then isolated genomic DNA using standard CTAB/phenol/chloroform extraction protocols (Maniatis, Fristch & Sambrook, 1982). We amplified a 700bp region of the mitochondrial genome that encodes part of the COI gene using polymerase chain reaction (PCR; Saiki *et al*., 1988) with the primers HCO-2193 and LCO-1490 (Folmer *et al*., 1994). We amplified an additional 900bp region of mtDNA that encodes a portion of the ND4 gene and the flanking tRNAs histidine, serine and leucine, using the primers ND4 and Leu (Arevalo, Davis & Sites, 1994). We sequenced all PCR products in both directions by direct double strand cycle sequencing using the above primers and the ABI® Big Dye cycle sequencing kit. We ran cycle-sequenced products on a 4% acrylamide gel using an ABI® Prism 377 automated DNA sequencer and we aligned the DNA fragments by eye on ABI® Sequence Navigator. We deposited all the mtDNA sequences in GenBank (http://www.ncbi.nlm.nih.gov Accession numbers AF263404 to AF263431).

We used the partition homogeneity test (PH) in PAUP* 4.0b8a (Swofford, 1998) to test for significant incongruence between the COI and ND4 data sets. Although ND4 and COI accumulate mutations at different rates, the PH test detected no significant incongruence ($P > 0.05$) between the two data partitions (Bull *et al*., 1993). Thus, we combined and analyzed the COI and ND4 data using two methods of phylogenetic reconstruction to infer the evolutionary relationships of mtDNA lineages: maximum parsimony (MP; Swofford *et al*., 1996) and maximum likelihood (ML; Felsenstein, 1981). We chose MP because MP is one of the simplest

Table 2. Voucher information for the specimens used in this study. The outgroup *Chrysemys picta* sequences are from Genbank (Mindell *et al*., 1999). MVZ = Museum of Vertebrate Zoology, Berkeley; ROM = Royal Ontario Museum, Toronto.

Species	Museum Number	Locality	ND4	COI
1 *Chinemys nigricans*	MVZ 130463	no locality data	yes	yes
2 *Chinemys reevesii*	MVZ 230533	no locality data	yes	yes
3 *Cuora amboinensis*	MVZ 230509	no locality data	yes	no
4 *Cuora galbinifrons*	MVZ 230534	no locality data	yes	no
5 *Cuora galbinifrons*	MVZ 230466	Hainan Province, China	yes	yes
6 *Cuora galbinifrons*	ROM 30062	Cat Tien, Dong Nai Province, Vietnam	yes	yes
7 *Cuora serrata*	MVZ 230629	no locality data	yes	yes
8 *Cuora serrata*	MVZ 230511	no locality data	yes	yes
9 *Cuora serrata*	MVZ 230628	no locality data	yes	yes
10 *Cuora trifasciata*	MVZ 230467	no locality data	yes	yes
11 *Cuora trifasciata*	MVZ 230636	no locality data	yes	yes
12 *Mauremys iversoni*	MVZ 230475	Turtle farm, Hainan Province, China	yes	yes
13 *Mauremys mutica*	MVZ 230487	no locality data	yes	no
14 *Mauremys mutica*	ROM 25613	Yen Bai, Yen Bai Province, Vietnam	yes	yes
15 *Mauremys mutica*	ROM 25614	Yen Bai, Yen Bai Province, Vietnam	yes	yes
16 *Mauremys mutica*	MVZ 230476	Zhoushan Island, Zhejiang Province China	yes	yes
17 *Pyxidea mouhotii*	MVZ 230481	Hainan Province, China	yes	no
18 *Pyxidea mouhotii*	MVZ 230480	no locality data	yes	no
19 *Pyxidea mouhotii*	MVZ 230482	Hainan Province, China	yes	yes
20 *Pyxidea mouhotii*	ROM 35002	Tam Dao, Bac Thai Province, Vietnam	yes	yes
21 *Pyxidea mouhotii*	ROM 35003	Tam Dao, Bac Thai Province, Vietnam	yes	yes
22 *Chrysemys picta*	N/A	N/A	yes	yes

Table 3. Genotype frequencies for putative hybrids and suspected parents.

Locus	Genotype	*P. mouhotii* *n* = 8	***C. serrata*** *n* = 2	*C. galbinifrons* *n* = 3	*C. trifasciata* *n* = 2	***M. iversoni*** *n* = 1	*M. mutica* *n* = 6
MDH-1	(AA)	0.94	1.00	1.00	**1.00**		
	(**AB**)				**1.00**		
	(AC)	0.06					
	(BB)					**1.00**	
	(BC)						
	(CC)						
MDH-2	(AA)	1.00	1.00	1.00	1.00	1.00	1.00
PEP-LA	(AA)	1.00	1.00	1.00	**1.00**		
	(**AB**)				**1.00**		
	(BB)					**1.00**	
PGI	(AA)			**1.00**			
	(AB)						
	(AC)						
	(**AD**)		**1.00**				
	(BB)				1.00	1.00	1.00
	(BC)						
	(BD)						
	(CC)	**0.25**					
	(DD)	**0.75**					

and most widely used phylogenetic methods (Swofford *et al*., 1996). We also used ML because ML has been shown to be more consistent than MP when dealing with molecular sequence data (Hasegawa & Fujiwara, 1993; Huelsenbeck, 1995; but see Farris, 1999). Moreover, ML can more accurately estimate branch lengths (Huelsenbeck & Rannala, 1997). We conducted all phylogenetic analyses in PAUP* and we coded insertions and deletions in the tRNAs as additional character states. We polarized the data using published sequences of the painted turtle, *Chrysemys picta* (Mindell *et al*., 1999). We executed MP analyses with the branch-and-bound search algorithm using equally weighted characters. We assessed the robustness of individual nodes using the bootstrap resampling method (Felsenstein, 1985) by employing 10,000 replicates of closest searches in PAUP*. Additionally, we calculated branch support (Bremer, 1994) for internal nodes using the program TreeRot 2 (Sorenson, 1999). We used the phylogenetic hypotheses generated by MP as starting trees for ML searches. We first used a hierarchical likelihood ratio test (Felsenstein, 1993) in the program Modeltest 3.0 (Posada & Crandall, 1998), and determined that the HKY + Γ model of DNA evolution (Hasegawa, Kishino & Yano, 1985; Yang, 1994*a*, Yang, 1994*b*) best fit the sequence data. We then reconstructed haplotype relationships using this model of nucleotide substitution and the empirical base frequencies.

RESULTS

Allozyme data

We found three polymorphic loci out of the four protein systems we surveyed. MDH–1, PEP–LA and PGI showed variation below the species level, while locus

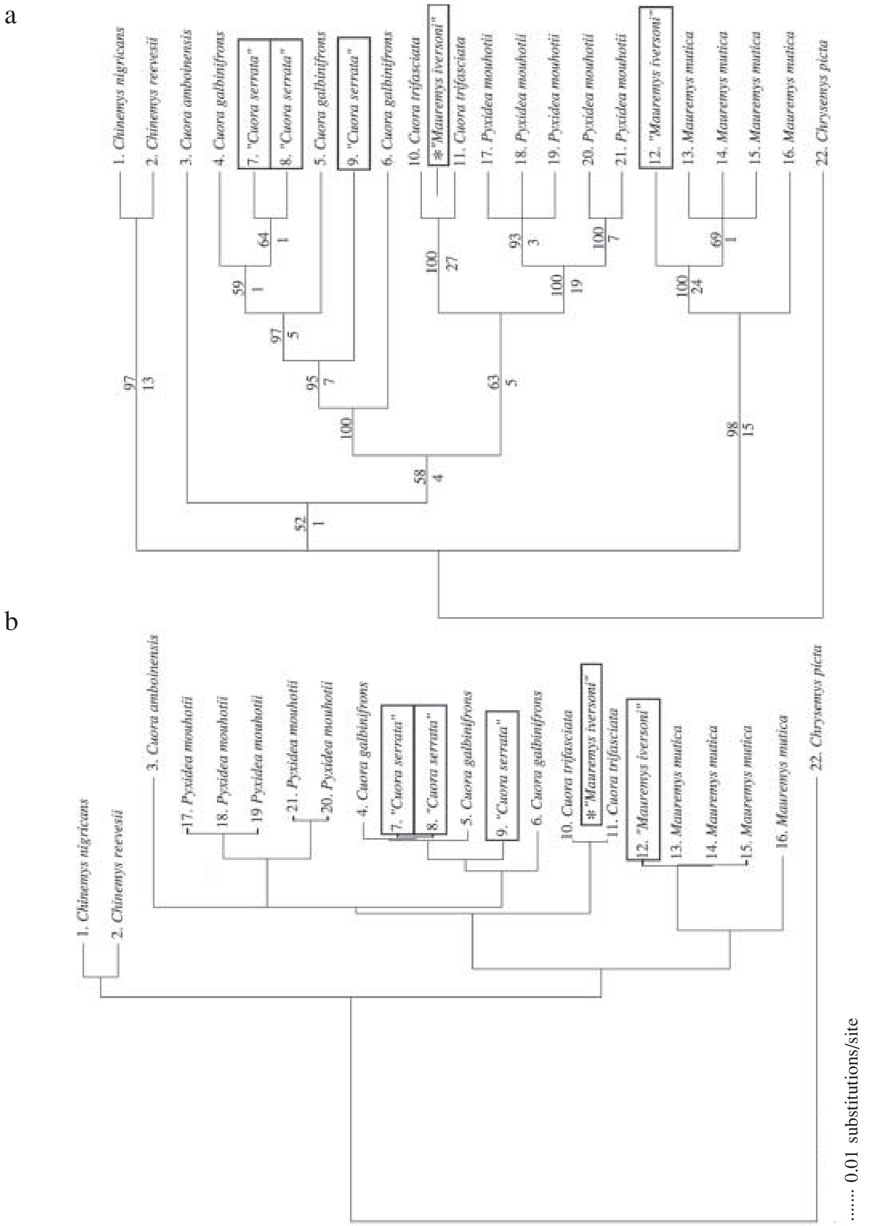

Fig. 3. Two phylogenetic hypotheses for the geoemydid turtles examined in this study. The numbers next to the names refer to the vouchers (Table 2). The suspected hybrids examined here are boxed. The '*M. iversoni*' indicated by "*" represents the phylogenetic placement according to Wink *et al.* (2001). (a) A consensus of the three shortest trees using maximum parsimony. Numbers above the branches represent bootstrap values (only values over 50% are shown), while numbers below the branches represent decay indices. (b) The maximum likelihood tree. The branch lengths are proportional to the ML genetic distances.

MDH–2 was not variable among the genera we examined (Table 3). Although, our sample sizes preclude us from determining whether differences between taxa are truly fixed, it is worth noting that genera and species can easily be distinguished from each other using these markers.

We found allelic differences at loci MDH–1 and PEP–LA that distinguish specimens of *Cuora trifasciata* from *Mauremys mutica*, the suspected parental species of *Mauremys iversoni* (Wink *et al.*, 2001). That the geographically and genetically divergent *M. mutica* specimens (Fig. 3(a) & (b)) possess the same allozyme genotype lends credence to our assertion that alleles at MDH–1 and PEP–LA are species specific. Our specimen of *M. iversoni* had heterozygous genotypes at both loci.

We also found allelic differences at PGI that distinguished our specimens of *Cuora galbinifrons* from *Pyxidea mouhotii*, the hypothesized parental species of *Cuora serrata*. Both *C. serrata* specimens examined here express a heterozygous allozyme genotype for this locus.

Mitochondrial data

The combined ND4 and COI mitochondrial data set contained 1535 aligned base pairs, 288 of which were phylogenetically informative. We were unable to sequence COI for five specimens (Table 2). However, this small proportion of missing data probably does not compromise the confidence or accuracy of our phylogenetic reconstructions (see Wiens & Reeder, 1995). The branch-and-bound equally weighted MP analysis produced three most parsimonious trees (bootstrap consensus in Fig. 3(a)), 767 steps in length (CI = 0.645; RI = 0.462). The multiple trees result from identical sequences in *M. mutica* and *P. mouhotii*. When identical sequences are deleted, a single topology is recovered. The ML HKY + Γ reconstruction yielded one tree ($\mathrm{LnL} = -5563.5477$; $\alpha = 0.1781$) similar to the three most parsimonious trees (Fig. 3(b)). In general, the turtle genera studied here form well-supported monophyletic clades. However, in both analyses the hinged genus *Cuora* is paraphyletic with respect to *Pyxidea* (Fig. 3). Our study was not designed to test the supra-generic relationships of geoemydids and so lacks several key taxa (*Ocadia*, other *Cuora* species) that would potentially resolve the phylogeny. Finally, mtDNA variation appears extensive within several geoemydid species, such as *C. galbinifrons*, *M. mutica* and *P. mouhotii*. We suggest that cryptic species may exist, but this would not alter our conclusions.

The MP and ML analyses of mitochondrial haplotypes placed *M. iversoni* within *M. mutica*, rendering *M. mutica* paraphyletic. In fact the mtDNA of our *M. iversoni* specimen is remarkably similar (4 base pairs; 0.2% sequence divergence) to *M. mutica* specimens collected from the wild in Vietnam, over 1400 kilometers from the type locality of *M. iversoni*, in eastern China (Pritchard & McCord, 1991; Fig. 4). By contrast, *M. mutica* from the type locality (Zhoushan Island) is morphologically similar to Vietnamese *M. mutica*, yet shows considerable sequence divergence (95 bp; 6.2% sequence divergence).

The mtDNA phylogeny shows that *C. serrata* is polyphyletic. Some *C. serrata* are more closely related to *C. galbinifrons* than they are to other *C. serrata*. It is important to note that both *Pyxidea*-like (MVZ 230628,9) and *Cuora*-like (MVZ 230511) *C. serrata* have mitochondrial DNA sequences that lie within the range of typical-looking *C. galbinifrons*. To test whether a polyphyletic *C. serrata* was statistically different from a monophyletic *C. serrata* given our data, we constrained the equally weighted, branch-and-bound MP searches to recover only those trees that produce a monophyletic *C. serrata*. The six shortest trees generated by that constraint search were 776 steps long, nine steps longer than the three most parsimonious unconstrained estimates of geoemydid phylogeny. A comparison of the constrained and unconstrained phylogenies in PAUP* using a one-tailed Wilcoxon signed-ranks test (Templeton, 1983) shows that the two hypotheses are incompatible (P = 0.0389) and a monophyletic *C. serrata* is unsupported.

Fig. 4. Map of southeast Asia showing localities mentioned in text.

DISCUSSION

Our allozyme and mitochondrial DNA data, together with morphological support and field information, provide compelling evidence that both *Mauremys iversoni* and *Cuora serrata* are of hybrid origin. Although we were only able to obtain one specimen of *M. iversoni*, the specimen studied by Wink *et al.* (2001) provides an independent assessment of this species. Phylogenetic analysis of the mitochondrial sequences shows our *M. iversoni* to be closely related to Vietnamese *Mauremys mutica*. Yet Wink *et al.* (2001) found their *M. iversoni* to be more closely related to *Cuora trifasciata* than to other *Mauremys* using mitochondrial cyt *b* sequences (Fig. 3). Consequently, *M. iversoni* is polyphyletic.

The mitochondrial evidence for a polyphyletic *C. serrata* is less clear because we lack an extra-generic maternal lineage for our *C. serrata* specimens. Based solely on the phylogenetic position of the *C. serrata* specimens within the *Cuora galbinifrons* complex, it is difficult to distinguish between the possibility of unsorted polymorphism and multiple origins (true polyphyly). Walker & Avise (1998) studied the phylogeography of 22 North American freshwater turtles and found two cases where the recognition of an established species (*Kinosternon baurii* and *Sternotherus depressus*) renders another established species paraphyletic. In these cases, peripheral isolates had evolved into new forms. However, they did not discover (and it is hard to imagine) two species that are paraphyletic with respect to one another, the case with the *C. serrata* and *C. galbinifrons* (Fig. 3). This pattern of mutual paraphyly is even more striking given the enormous sequence divergences (up to 5.0%) within the *C. galbinifrons-serrata* clade.

In order to place the genetic distances within *C. galbinifrons* into a broader context, we compared them with distances from another study on turtles (Dutton *et al.*, 1996) that used one of the same genes as we did (ND4). Since Bowen *et al.* (1992; 1998) demonstrated that mtDNA haplotypes of *Chelonia mydas* and *Lepidochelys* sp. from each of the two major oceanic basins (Indian-Pacific and Atlantic) have achieved reciprocal monophyly, the amount of genetic divergence between specimens from these basins should represent a minimum estimate for speciation (in the case of *Lepidochelys* sp.) or at least evolutionary distinctiveness (in the case of *Chelonia*). We recognize that there is not a direct correlation between genetic distance and evolutionary distinctiveness, but provide this comparison as a heuristic example. The uncorrected distance between intraspecific ND4 sequences for *C. mydas* from separate oceanic basins is only 2.2–2.7% while the distance between the endangered Kemp's ridley (*Lepidochelys kempii*) and its congener *L. olivacea* is only 1.5%. This is small compared to the 5.0% difference between ND4 sequences within *C. galbinifrons* and 3.7% within specimens of *C. serrata*. In addition to these large genetic distances, there is high bootstrap and decay index support for subclades within *C. galbinifrons-serrata* (Fig. 3). Consequently, we favour a polyphyletic *C. serrata* and predict that its origins are interspersed amongst separate lineages within a *C. galbinifrons* complex. Although the possibility that unsorted polymorphism is responsible for the polyphyletic mtDNA in our *C. serrata* is remote, this hypothesis should be tested against additional specimens of *C. galbinifrons* from known localities.

Returning to the five hypotheses presented in the introduction, polyphyletic mtDNA haplotypes are not concordant with the hypotheses that the specimens of the new forms studied here represent valid phylogenetic species or are the result of a single recent hybridization event (hypotheses 1, 2 and 4). The polyphyletic mtDNA haplotypes, heterozygous allozyme genotypes, and intermediate morphology are not concordant with the hypothesis that the specimens represent variants of living species (hypothesis 3). All of the data are concordant with the hypothesis that the specimens resulted from multiple, recent hybridization events (hypothesis 5). However, we stress that we cannot falsify the hypotheses that some of the specimens do represent valid hybrid species or that some combination of the five hypotheses could explain our findings. Our data simply demonstrate that multiple explanations are necessary.

Given the polyphyletic mtDNA lineages, heterozygous allozyme genotypes and intermediate morphology, the most likely explanation for these data are multiple hybrid origins of *M. iversoni* and probably *C. serrata*. This hypothesis is consistent with all of the available data, including the morphology and their mysterious, recent appearance in the animal trade. It is worth noting that evidence showing that specimens of these new forms represent valid species is entirely lacking. Thus, these species apparently fail to meet the requirements of a valid phylogenetic species as well as the ICZN criteria. From now on, we shall refer to them as '*M. iversoni*' and '*C. serrata*', with both parts of the binomial in quotes. Although definitive evidence of an ancient monophyletic lineage that includes the name-bearing specimens (the holotypes) is not available, our taxonomic suggestion is for the specimens available in the animal trade. The holotypes still need to be tested.

Mauremys iversoni

A hybrid origin of '*Mauremys iversoni*' helps to reconcile much of the confusion surrounding the description of the type series. Although the type locality is designated as Fujian province, some of the type series is from Kweiying, Guizhou Province (Fig. 4). Furthermore, Qin (1992) included a '*M. iversoni*' specimen from Guangxi Province as part of his type series of '*Clemmys guangxiensis*' (Iverson & McCord, 1994). Pritchard & McCord (1991) went to great lengths to explain the biogeography of '*M. iversoni*' and suggested that the Guizhou locality is an error or the result of introduction by travelling Buddhists. The geographical inconsistencies are easily explained if '*M. iversoni*' is the result of multiple, independent hybridization events in nature or captivity.

Our results support the hypothesis that specimens of '*M. iversoni*' can be formed by the hybridization of *Mauremys mutica* and *Cuora trifasciata* in captivity. *Cuora trifasciata* is used in traditional Chinese medicine and *M. mutica* is a common food turtle (Karsen, Lau & Bogadek, 1986). In fact, our specimen of '*M. iversoni*' was found by two of us (JFP and HS) at a commercial facility that supplies the turtle trade in Tunchang County, Hainan Province, China (see Shi & Parham, 2001). The majority of geoemydids at the farm were *C. trifasciata* and *M. mutica*. When asked if the turtles ever hybridized, the owner produced several apparent hybrids of various sizes from juveniles to adults, indicating that hybridization was common. Later the owner admitted that the hybrids were produced intentionally and sold as counterfeit *C. trifasciata* to unsuspecting buyers (Shi & Parham, 2001; the "mock turtle syndrome" of Roman & Bowen, 2000).

Given the propensity of distantly related turtles to hybridize (Fritz, 1995; Fritz & Baur, 1995), the frequent crossing of commonly reared turtle species should not be surprising. Turtles endemic to different continents, separated by at least 30 million years, have produced offspring (Gonzalez, 1993). Since male *C. trifasciata* are known for their efforts to breed with females of other genera (Ernst & Barbour, 1989), they may be implicated in other instances of intergeneric hybridization besides '*M. iversoni*'. It may not be coincidental that some of the other newly described species from the pet trade (*Ocadia philippeni* McCord & Iverson 1992 and *Sacalia pseudocellata* Iverson & McCord 1992) can also be distinguished from their congeners by their resemblance to *C. trifasciata*.

Cuora serrata

Similarly, a hybrid origin of '*Cuora serrata*' resolves serious questions about its discovery. It is undeniable that new species of vertebrates continue to be discovered in southeast Asia, but not all new species have proven valid. As recently as 1992, a new large mammal, *Pseudoryx nghetinhensis* Dung *et al.* 1993, was discovered in the mountain forests of Vietnam and adjacent Laos. But about the same time, another enigmatic large mammal, *Pseudonovibos* Peter & Feiler 1994, was described from skeletal remains from the same region. Recently, Thomas, Seveau & Hassanin (2001) showed that specimens of '*Pseudonovibos*' are artificially modified domestic cows.

New discoveries of endangered geoemydids are indisputable. The discovery of the unusual *Leucocephalon yuwonoi* (McCord, Iverson & Boeadi 1995) from Sulawesi shows that new and valid forms of turtles can still be found. In China alone, at least two valid species of geoemydids (*Cuora pani* Song 1984 and *Cuora aurocapitata* Luo & Zong 1988) have only recently been discovered. The differences between these forms and '*C. serrata*' are that '*C. serrata*' is supposed to have a wide geographical distribution and '*C. serrata*' has never been documented in the wild.

The reported distribution of '*C. serrata*' includes southern China and even Vietnam (Iverson & McCord, 1992*b*; Fritz & Obst, 1998; de Bruin & Artner, 1999; Yuk Wah Shiu, pers. comm. to JFP). It is extremely unlikely that a geographically widespread, large, distinct-looking, terrestrial turtle could escape over 200 years of scientific collection. People living in the type region, although familiar with *C. galbinifrons*, *Pyxidea mouhotii* and other turtles, do not recognize pictures of '*C. serrata*' (de Bruin & Artner, 1999). One of us (HS), a turtle specialist living and working near the type locality on Hainan Island for years, has never seen '*C. serrata*' in the wild or in the local markets. In fact, although new specimens of '*C. serrata*' are available through the pet trade every year, no scientist has ever found them in the wild. The only specimens in scientific collections are from the pet trade and these only began to appear within the past 15 years.

The exact origin of '*C. serrata*' remains unclear because, unlike '*M. iversoni*', its suspected parent species, *C. galbinifrons* and *P. mouhotii*, are not commonly bred in captivity. However, the inner workings of most Chinese turtle farms remain a mystery. *Cuora galbinifrons* and *P. mouhotii* do occur in turtle-breeding facilities (HS, personal observation), but it remains to be determined whether these turtles are bred and whether they are ever kept together. A second possibility is that, inasmuch as *C. galbinifrons* and *P. mouhotii* share a terrestrial ecology and overlap greatly in their geographical distribution (Ernst & Barbour, 1989; Iverson, 1992), the origin of '*C. serrata*' could be the relatively recent hybridization events occurring naturally, maybe in disturbed environments. A third possibility is that '*C. serrata*' was originally (perhaps inadvertently) manufactured by fanciers and is now demanded by dealers because individuals can sell for over 1000 US dollars. Testing these hypotheses should prove extremely difficult, and the possibility that multiple explanation are necessary to explain '*C. serrata*' cannot be discarded.

CONCLUSIONS

Whether the holotypes of the newly described turtles are based on natural or commercial hybrids remains to be determined, but we underscore the problems of describing species based on commercial specimens with locality data that cannot be verified and natural populations that cannot be found. The verification of the original localities of pet trade specimens is a daunting endeavour, but most systematists consider it necessary for scientific description. For example, Cann & Legler (1994) waited to catch their own specimens before describing a new turtle, *Elusor*, even though it had been known in the pet trade since 1961.

Despite some effort, many of the new forms have not been seen in the wild. In some cases, the local people who reportedly captured the holotypes, although they are familiar with native species, do not recognize the newly described forms (de Bruin & Artner, 1999; HS, personal observation). Thus, the type localities given by Yuk Wah Shiu have not been substantiated. The recent discovery of a breeding and ranching center in which large numbers of geoemydids, even hybrids, are bred and sold to the highest bidder (Shi & Parham, 2001) reveals how little we know about the source of pet trade specimens, including the holotypes provided by Yuk Wah Shiu. Because dealers are rewarded for finding new and interesting specimens with locality data, there is incentive to fabricate data (Parham & Li, 1999). Although the overlap of the science and the business of natural history specimens can yield positive results for systematics and conservation, all purchased specimens must be tested against independent lines of evidence or else be considered questionable.

On the other hand, history tells about an instance when a valid species of turtle was mistakenly hypothesized to be an intergeneric hybrid. The Kemp's ridley sea turtle (*Lepidochelys kempii*) was thought to have been a hybrid of the loggerhead (*Caretta*) and either the hawksbill (*Eretmochelys*) or green (*Chelonia*) sea turtles (see Carr, 1942). Today we know that *L. kempii* is a distinct and valid species (Bowen, Meylan & Avise, 1991) and arguably the most endangered sea turtle. We must be extremely careful not to condemn valid species to hybrid status without strong evidence. Furthermore, the role of hybridization as an evolutionary mode in turtles cannot be entirely discounted by our data. We agree with DeMarais *et al.* (1992) that, 'protection of distinct forms coupled with an active bias against suspected hybrids could prove detrimental to the entire complex'. However, the data to establish that some of the new forms represent hybrid lineages is entirely lacking and cannot be derived from pet trade specimens.

In order for herpetologists to unravel this problem it will be necessary: (1) to gather as much genetic data as possible from known locality specimens without endangering fragile populations and (2) to confirm data from purchased specimens by first-hand observations of turtles in the wild. Establishing better collaborations with Asian herpetologists, instead of relying on pet dealers, is an important prerequisite.

These caveats come at a time when the need for data on the actual diversity and distribution of all Asian turtles is desperate (Altherr & Freyer, 2000; van Dijk *et al.*, 2000). Some of the new turtles described from the pet trade have been implicated as species of special concern because they are so scarce (Stubbs, 1991; Altherr & Freyer, 2000). This has resulted in a heroic effort to save these species by zoos (e.g., the Münster Zoo and the Fort Worth Zoo) with the help of private individuals. In order to help conservation efforts focus their limited resources on valid species, the remainder of the new pet trade "species" (Table 1) should be investigated using genetic and biogeographic data as soon as possible.

Acknowledgements

We thank Ted Papenfuss for collecting the turtles in China. Jim Buskirk and Jim Barzyk provided the pet trade specimens. Javier Rodriguez-Robles, Robert Murphy, Ted Papenfuss, and Homan Faraji facilitated the use of museum specimens. Kevin Padian, Ted Papenfuss, Marjorie Matocq, Tonya Van Leuvan-Smith, Carl Gillies, David Wake, Javier Rodriguez-Robles, Peter Paul van Dijk, Kevin Omland, John Iverson and most anonymous reviewers of previous manuscripts provided helpful comments. This work was supported by National Science Foundation and Vice Chancellor's fellowships to JFP. The University of California Museum of Paleontology provided financial support to WBS. This is UCMP Contribution #1747.

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