Molecular Systematics of Old World Stripe-Necked Turtles (Testudines: *Mauremys*)

CHRIS R. FELDMAN^{1,*} AND JAMES F. PARHAM^{2,3}

 ¹Department of Biology, Utah State University, Logan, UT, 84322-5305, USA, * Corresponding Author E-mail: elgaria@biology.usu.edu.
 ²Evolutionary Genomics Department, Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA, 94598, USA.

³University of California Museum of Paleontology, University of California, Berkeley, CA, 94720-3140, USA, E-mail: parham@socrates.berkeley.edu

Abstract. - Nine extant species of *Mauremys* (including *Ocadia* and *Chinemys*) represent a geographically widespread yet morphologically and ecologically conservative group of batagurid turtles. Here we examine the evolutionary relationships of *Mauremys* using 1539 base pairs of mitochondrial DNA encoding portions of COI, ND4, and three adjacent tRNA genes. These data contain 246 parsimony informative characters that we use to erect hypotheses of relationships for *Mauremys*. Both maximum parsimony and Bayesian methods suggest that *Mauremys japonica*, *M. sinensis*, *M. nigricans*, and *M. reevesii* form a well-supported monophyletic clade, as do *M. mutica* and *M. annamensis*. Furthermore, our analyses show that *M. mutica* is paraphyletic with respect to *M. annamensis*. The western taxa *M. leprosa*, *M. caspica*, and *M. rivulata* remain problematic and do not form a monophyletic group sister to the Asian taxa. Nevertheless, an east-west biogeographic hypothesis cannot be discounted with our molecular genetic data.

Key words. - Turtles, Bataguridae, Mauremys, molecular phylogenetics, mitochondrial DNA

Introduction

The Old World turtle genus *Mauremys* is represented by morphologically and ecologically conservative species that are diagnosed by a rigid plastron and a striped head and neck. These semi-aquatic, batagurid (= geoemydid, see Joyce et al., in press) turtles occupy lotic and lentic environments in both forested and arid habitats throughout Asia and the Mediterranean.

The genus contains some of the most commercially important freshwater turtles in Asia. For example, *M. mutica* is one of the most commonly reared and highly traded chelonians in Asia (Lau and Shi, 2000). Other *Mauremys* species have been at the center of a conservation and systematics controversy. In fact, two newly described *Mauremys* may be polyphyletic hybrids (Parham et al., 2001; Wink et al., 2001; Spinks et al., 2004).

Given the mounting conservation interest in the turtle fauna of Asia (van Dijk et al., 2000), understanding the extant diversity and phylogenetic relationships among the Bataguridae are areas of active research (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004). The genus *Mauremys* has received particular attention because of this recent conservation crisis and taxonomic confusion. The first examination of evolutionary relationships within *Mauremys* was a morphological treatment of the genus based on shell and scute measurements (Iverson and McCord, 1994). Consistent with the disjunct distribution of *Mauremys*, Iverson and McCord (1994) suggested that East Asian taxa form a monophyletic group, sister to a Mediterranean and Middle Eastern clade. A subsequent study used 12S and 16S ribosomal genes to resolve the phylogenetic relationships among species of Mauremys (Honda et al., 2002a). In contrast to the east-west hypothesis of Iverson and McCord (1994), Honda et al. (2002a) suggested that the deepest phylogenetic splits within Mauremys occur between Asian taxa. The ribosomal mtDNA data also cast doubt on the monophyly of traditional Mauremys by including the east Asian species, Chinemys reevesii, as the sister taxon to M. *japonica*. Two recent studies examined more extensive sequence data, predominantly cyt b mtDNA, as well as a more comprehensive sampling of batagurids (Barth et al., 2004; Spinks et al., 2004). Both studies firmly established the placement of Mauremys within the Bataguridae and show that the Chinemys and Ocadia are phylogenetically nested within *Mauremys* (Barth et al., 2004; Spinks et al., 2004). Barth et al. (2004) offer two possible solutions to reconcile the paraphyly of Mauremys: 1) split the species of Mauremys into four genera; 2) lump Chinemys and Ocadia into an expanded Mauremys. While Barth et al. (2004) refrain from a taxonomic decision, Spinks et al. (2004) adopt an expanded Mauremys. We also endorse an inclusive Mauremys

because we consider expanding genera to well-supported clades of species functionally preferable to proliferating monotypic genera based on subjective, typological ideas of uniqueness (Feldman and Parham, 2002; Parham and Feldman, 2002; Spinks et al., 2004).

Our objective here is to provide an independent estimate of *Mauremys* phylogeny using different molecular markers from other recent systematic investigations and separate museum voucher specimens (Barth et al., 2004; Spinks et al., 2004). We hope that our data help resolve areas of uncertainty in the emerging consensus on *Mauremys* systematics. In addition, our study will add to the growing body of information on the evolutionary history and diversity of Asia's threatened batagurid fauna (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004).

Materials and methods

Taxon sampling and laboratory protocols. - We obtained liver tissue from 17 museum specimens representing nine currently recognized species of Mauremys and three species of *Cuora* (Appendix 1). The nine species of Mauremys used in our study include: M. annamensis, M. caspica, M. japonica, M. leprosa, M. mutica, M. nigricans, M. reevesii, M. rivulata, and M. sinensis. We do not consider "M. iversoni", "M. pritchardi", "O. glyphistoma" or "O. philippeni" to be valid taxa because specimens matching these species (all described from the pet trade) are likely hybrids (Parham et al., 2001; Wink et al., 2001; Spinks et al., 2004). In addition, we also excluded "M. megalocephala", which is probably a diet-induced variant of M. reevesii (Iverson et al., 1989; Barth et al., 2002). However, we do include a "M. iversoni"-like hybrid specimen described in Parham and Shi (2001) because mtDNA from this hybrid specimen is demonstrably Mauremys (Parham et al., 2001). All vouchers correspond to well-documented reference material and original species descriptions.

We isolated genomic DNA from tissue samples by standard proteinase K digestion and phenol/chloroform purification (Maniatis et al., 1982). We amplified 700 bp of mtDNA encoding a section of COI via PCR (Saiki et al., 1988) using primers HCO-2193 and LCO-1490 (Folmer et al., 1994). We amplified an additional 900 bp region of mtDNA encoding a portion of ND4 and flanking tRNA histidine (tRNA^{his}), serine (tRNA^{ser}), and part of leucine (tRNA^{leu}) using primers ND4 and Leu (Arevalo et al., 1994). We used the following thermal cycle parameters for 50µl amplification reactions: 35 cycles of 1min denature at 94°C, 1min anneal at 45°C (COI) or 52°C (ND4), and 2min extension at 72°C. We purified PCR products using the Wizard Prep Mini Column Purification Kit (Promega, Inc.) and used purified template in 10µl dideoxy chain-termination reactions (Sanger et al., 1977) using ABI Big Dye chemistry (Applied Biosystems, Inc.) and the primers listed above. Following an isopropanol/ethanol precipitation, we ran cycle-sequenced products on a 4.8% Page Plus (Ameresco) acrylamide gel using an ABI 377 automated sequencer (Applied Biosystems, Inc.). We sequenced all samples in both directions.

Sequence analyses. - We aligned DNA sequences with the program SequencherTM 4.1 (Gene Codes Corp.), and translated protein coding nucleotide sequences into amino acid sequences using MacClade 4.0 (Maddison and Maddison, 2000). We identified tRNA genes by manually reconstructing their secondary structures using the criteria of Kumazawa and Nishida (1993). We deposited all mitochondrial DNA sequences in GenBank (Appendix 1).

We performed a partition homogeneity test (PH), similar to the incongruence length differences test (ILD; Farris et al., 1994), to determine whether the ND4 and COI data could be combined. We used PAUP* 4.0b10 (Swofford, 2002) to generate a null distribution of length differences using 1000 same-sized, randomly generated partitions from the original data with replacement.

To evaluate base substitution saturation at first, second, and third codon positions, we plotted the uncorrected percent sequence divergence of transitions and transversions versus the corrected maximum likelihood estimates of divergence for each codon position.

Phylogenetic analyses. - We used maximum parsimony (MP; Farris, 1983) and maximum likelihood-based Bayesian (Larget and Simon, 1999) phylogenetic methods to infer evolutionary relationships among batagurid species. We conducted MP analyses in PAUP* and Bayesian analyses with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). We polarized the phylogeny via outgroup comparison (Maddison et al., 1984) using the Asian box turtles *Cuora mouhotii, Cuora picturata*, and *Cuora trifasciata*. Other molecular phylogenetic studies suggest these turtles are appropriate outgroup taxa (Wu et al., 1999; Honda et al., 2002b; Barth et al., 2004; Spinks et al., 2004).

We executed MP analyses with the branch-andbound search algorithm (Hendy and Penny, 1982) using equally weighted, unordered characters. To assess nodal support, we used the bootstrap resampling method (BP; Felsenstein, 1985) employing 1000 pseudoreplicates of branch-and-bound searches in PAUP*. Additionally, we calculated branch support (DI; Bremer, 1994) for all nodes using the program Tree Rot 2c (Sorenson, 1999).

We performed Bayesian analyses to estimate branch lengths and search for additional tree topologies. To

determine the most appropriate model of DNA substitution for reconstructing Mauremys relationships under the Bayesian method, we executed hierarchical likelihood ratio tests (LRT; Felsenstein, 1993; Goldman, 1993; Yang, 1996) in the program Modeltest 3.06 (Posada and Crandall, 1998). Because MrBayes 3.0b4 can perform singular phylogenetic analyses using different models of evolution, we performed two separate LRTs on the two mtDNA regions. The model of nucleotide substitution that best fit the COI data was the HKY model (Hasegawa et al., 1985) in conjunction with Γ (Yang, 1994a; 1994b), and I (Gu et al., 1995), while the slightly less complex HKY + Γ model of DNA evolution best fit the ND4 data. We then performed Bayesian tree searches, allowing separate parameter estimates under the two models of DNA substitution for the COI and ND4 data partitions. We did not specify a topology or nucleotide substitution model parameters a priori. We ran Bayesian analyses for 3 x 10⁶ generations using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm with four heated Markov chains per generation, sampling trees every 100 generations. To determine when the Markov chains had converged on stable likelihood values, we plotted the -lnl scores against the number of generations (Huelsenbeck and Ronquist, 2001). We then computed a 50 % majority rule consensus tree after excluding those trees sampled prior to the stable equilibrium. Nodal support is given by the frequency of the recovered clade, which corresponds to the posterior probability of that clade under the given models of sequence evolution (PP; Rannala and Yang, 1996; Huelsenbeck and Ronquist, 2001). Lastly, we performed three Bayesian runs to be sure that independent analyses converged on similar loglikelihood scores (Leaché and Reeder, 2002).

Results

Genetic variation. - Sequences from the protein coding regions appear functional and there are no gene rearrangements in the data (Kumazawa and Nishida, 1995; Kumazawa et al., 1996; Macey and Verma, 1997; Macey et al., 1997). However, ND4 in the batagurids studied here appears truncated relative to that of emydid turtles, which have three additional residues: Phenylalanine, Tyrosine, and Cysteine (Feldman and Parham, 2002). Instead, these batagurids possess a stop codon, followed by a 12 bp stretch of highly polymorphic DNA between ND4 and tRNA^{his}. Additionally, tRNA^{ser} has a short D-stem, instead of a D-arm replacement loop like that of most metazoan taxa (Kumazawa and Nishida, 1993). This unusual tRNA condition is also seen in emydid turtles (Feldman and Parham, 2002).

The PH test shows that length difference between

the sum of the COI and ND4 trees and the combined COI and ND4 trees is not significantly different from the randomly generated test statistic (P = 0.93). Therefore, we combined the aligned DNA sequences for subsequent phylogenetic analyses.

Of the 1539 aligned nucleotides, 369 are variable and 246 are parsimony informative. Among ingroup taxa, 289 sites are variable and 205 parsimony informative. Of the 369 variable characters, 60 occur at 1st codon positions, 15 at 2nd positions, 261 at 3rd positions, and 33 in tRNAs. The scatter diagrams are linear and show no evidence of multiple hit problems for transitions or transversions (data not shown).

Phylogenetic relationships. - The branch-and-bound equally weighted MP analysis produces a single most parsimonious tree (L = 661; CI = 0.626; RI = 0.683) that is consistent with the model-based Bayesian analyses (Fig. 1). All three Bayesian analyses converge on the same topology and nearly identical mean log-likelihood values, parameter estimates, and nodal support. Thus we simply present results from the final search. The partitioned HKY + Γ + I and HKY + Γ Bayesian analysis (3 x 10⁶ generations) attains stable log-likelihood values within the first 15,000 generations, but we were conservative and discarded the first 20,000 generations. Because we sampled trees every 100 generations, we discarded the first 200 trees and retained 29,800 Bayesian trees, which we used to generate a 50% majority rule tree, and for which consensus values represent a group's posterior probability (Huelsenbeck and Ronquist, 2001). The summary topology of the nearly 30,000 Bayesian trees (mean -lnl = 5205.5110, σ^2 = 24.5038; mean ti/tv (COI) = 10.8360; $\sigma^2 = 10.6335$; mean α (COI) = 0.5479, σ^2 = 0.8874; mean P_{invar}(COI) = 0.4163, σ^2 = 0.0291; mean ti/tv (ND4) = 12.3499; σ^2 = 9.0505; mean α (ND4) = 0.2431, σ^2 = 0.0009) differs from the MP tree in the placement of only one taxon (Fig. 1).

In both analyses, species of *Cuora* unambiguously group to the exclusion of *Mauremys*, (BP = 100%; DI = 19; PP = 100%). *Mauremys japonica* is a member of a clade containing *M. nigricans*, *M. reevesii* and *M. sinensis* (BP = 100%; DI = 13; PP = 100%), yet relationships among these taxa are not well resolved, as indicated by the low nodal support and conflict between MP and Bayesian reconstructions. The MP tree places *M. sinensis* sister to a group linking *M. japonica*, *M. nigricans*, and *M. reevesii* (DI = 1), wherein *M. nigricans* and *M. reevesii* form an additional clade (BP = 86%; DI = 5). Alternatively, the Bayesian tree connects *M. japonica* to *M. sinensis* (PP = 59%), sister to the *M. nigricans* the *M. reevesii* clade (PP = 99%). The *M. japonica*, *M. nigricans*, *M. reevesii*, and *M. sinensis* clade is sister to a



ss above the diagonal indicate uncorrected pairwise differences (%) while	
a. Note	
fauremys and related taxa	ences (%).
ble 1. Pairwise comparisons of mtDNA sequences among Ma	ose below the diagonal denote $HKY + \Gamma + I$ sequence diverger
Tał	tho

				•													
	1	2	3	4	5	9	7	8	6	10	11	12	13	14	15	16	17
1 M. nigricans	ı	3.12	7.74	5.66	5.66	7.74	5.07	6.31	6.31	8.26	7.61	7.61	6.18	4.16	8.65	8.71	8.58
2 M. reevesii	3.68	,	7.74	5.33	5.33	7.74	4.75	6.50	6.50	8.06	7.61	7.61	6.18	4.42	8.65	8.39	8.32
3 M. annamensis	11.72	11.72	,	6.89	6.89	0.26	8.65	7.67	7.67	6.31	0.13	0.13	6.83	8.32	8.97	9.30	9.62
4 M. caspica (Iran)	7.56	7.01	9.75	ı	0	7.02	6.76	5.92	5.92	7.09	6.76	6.76	5.79	6.24	8.71	8.52	8.52
5 <i>M. caspica</i> (Bahrain)	7.56	7.01	9.75	0	ı	7.02	6.76	5.92	5.92	7.09	6.76	6.76	5.79	6.24	8.71	8.52	8.52
6 "M. iversoni"	11.71	11.71	0.26	10.00	10.00	ı	8.65	7.67	7.67	6.31	0.26	0.26	6.96	8.32	8.84	9.17	9.49
7 M. japonica	6.63	6.16	13.95	9.78	9.78	13.94	ı	8.00	8.00	8.58	8.52	8.52	7.48	4.94	10.14	9.75	9.36
8 <i>M. leprosa</i> (Spain)	8.92	9.28	11.59	7.98	7.98	11.59	12.66	ı	0	7.80	7.54	7.54	6.96	6.83	8.45	8.13	8.13
9 M. leprosa (Morocco)	8.92	9.28	11.59	7.98	7.98	11.59	12.66	0	,	7.80	7.54	7.54	6.96	6.83	8.45	8.13	8.13
10 M. mutica (China)	12.83	12.68	8.96	10.34	10.34	8.96	13.95	12.13	12.13	ı	6.18	6.18	7.54	7.41	9.69	9.49	9.49
11 M. mutica (Vietnam)	11.45	11.44	0.13	9.51	9.51	0.26	13.64	11.32	11.32	8.72	ı	0	6.70	8.19	8.84	9.17	9.49
12 M. mutica (Vietnam)	11.45	11.44	0.13	9.51	9.51	0.26	13.64	11.32	11.32	8.72	0	ı	6.70	8.19	8.84	9.17	9.49
13 M. rivulata	8.57	8.65	9.86	7.75	7.75	10.11	11.37	10.15	10.15	11.30	9.61	9.61	ı	6.05	8.32	8.00	8.32
14 M. sinensis	5.17	5.62	13.24	8.74	8.74	13.22	6.52	10.09	10.09	11.25	12.94	12.94	8.49	·	9.04	8.39	8.26
15 Cuora picturata	13.92	14.08	14.82	13.80	13.80	14.50	18.11	13.64	13.64	16.55	14.50	14.50	12.99	15.16	ı	6.57	7.35
16 Cuora trifasciata	14.29	13.67	15.88	13.56	13.56	15.54	17.35	13.10	13.10	16.33	15.54	15.54	12.48	13.81	9.26	·	6.63
17 Cuora mouhotii	14.33	13.85	16.64	13.90	13.90	16.31	16.55	13.42	13.42	16.55	16.31	16.31	13.51	13.85	11.02	9.72	ı

poorly supported M. mutica, M. annamensis, "M. iversoni", M. leprosa, and M. caspica assemblage (DI = 2; PP = 61%). Within this large group, M. mutica, M. annamensis and "M. iversoni" form a strongly supported clade (BP = 99; DI = 15; PP = 100%). In fact, inclusion of both M. annamensis and "M. iversoni" render M. mutica paraphyletic; two M. mutica (ROM 25613, 25614) are more closely related to M. annamensis and "M. iversoni" than they are to a Chinese M. mutica (MVZ 230476) (BP = 100%; DI = 36; PP = 100%). Within this "mutica complex", M. annamensis and "M. *iversoni*" are weakly allied (BP = 60%; DI = 1; PP = 86). This entire "mutica complex" is then sister to a weakly supported *M. leprosa* and *M. caspica* clade (DI = 1). Finally, both MP and Bayesian analyses suggest that M. rivulata is sister to a monophyletic clade containing the rest of Mauremys, but this phylogenetic arrangement receives almost no statistical support (DI = 2; PP = 60%).

Discussion

Phylogenetic relationships. - Both MP and Bayesian phylogenetic methods show that *M. japonica* is a member of a clade containing M. nigricans, M. reevesii, and M. sinensis, exclusive of other Mauremys. The M. japonica, M. nigricans, M. reevesii, and M. sinensis clade is joined to a poorly supported M. mutica, M. annamensis, M. leprosa, and M. caspica assemblage. Within this grouping, M. mutica and M. annamensis form a solid clade, congruent with shell and scute data (Iverson and McCord, 1994), other molecular data (Barth et al., 2004; Spinks et al., 2004) but not 12S and 16S mtDNA data (Honda et al., 2002a). Our analyses further suggest that *M. mutica* is paraphyletic. Two *M.* mutica (ROM 25613, 25614) purchased in Vietnam are more closely related to M. annamensis than they are to topotypic M. mutica (MVZ 230476) from China. We tested the paraphyly of *M. mutica* by constraining the MP searches to recover only those trees that produce a monophyletic M. mutica. The shortest two trees generated by the constraint search are 697 steps long (CI =0.594; RI = 0.636), 36 steps longer than the unconstrained MP tree. The two-tailed Wilcoxon signed-ranks test (Templeton, 1983) fails to support (P < 0.0001) the monophyly of *M. mutica*. The *mutica* complex is linked to a tenuous *M. leprosa* and *M. caspica* group. Lastly, both MP and Bayesian phylogenetic analyses tentatively place M. rivulata sister to a monophyletic clade containing the remaining ingroup taxa.

Genetic Variation. - Our samples of *M. leprosa* from Spain and Morocco, and *M. caspica* from Iran and Bahrain, show no intraspecific haplotype diversity (Table 1), yet exhibit sizeable morphological variation (Busack and Ernst, 1980). This discrepancy between intraspecific mtDNA diversity and geographic variation seems to be common among turtles (e.g., Lenk et al., 1999; Starkey et al., 2003) and may be related to extensive phenotypic plasticity or the slow rate of molecular evolution in turtles (Avise et al., 1992; Lamb et al., 1994).

In contrast, most interspecific mtDNA variation appears extensive, with uncorrected sequence divergences higher than 8% between a number of ingroup taxa (Table 1). Additionally, the mitochondrial sequence divergences between M. rivulata and M. caspica (Table 1), formerly considered conspecifics (Fritz and Wischuf, 1997), are equivalent to or greater than the genetic distances observed between other congeneric emydid and batagurid turtles (e.g., Feldman and Parham, 2002; Starkey et al., 2003; Stuart and Parham, 2004). Hence, these mtDNA data, together with the differing shell morphologies, distinct color patterns, and unique habitat preferences of M. rivulata and M. caspica (Busack and Ernst, 1980), support the recent elevation of M. rivulata as a distinct evolutionary lineage independent of M. caspica (Fritz and Wischuf, 1997).

Mauremys annamensis, a robust batagurid endemic to central Vietnam, is characterized by extensive axillary buttresses, a massive bridge, a slightly tricarinate and high-domed shell, a vividly striped head and neck, and reverse sexual size-dimorphism (McDowell, 1964; Iverson and McCord, 1994). The taxon is so distinctive it was once placed into its own genus, Annamemys Bourret 1939. McDowell (1964) originally demonstrated that M. annamensis and M. mutica share a number of derived features and Iverson and McCord (1994) subsequently confirmed a close kinship between these taxa with shell measurements. Hence, the close relationship revealed by our mitochondrial genes is not novel. What is surprising, however, is that M. annamensis differs from Vietnamese M. mutica and our "M. iversoni"-like hybrid by only two transitions. Furthermore, this clade shows a roughly 6% uncorrected sequence divergence from topotypic M. mutica from Zoushan Island, Zheijung Province, eastern China. In contrast, distantly collected samples of *M. leprosa* and *M. caspica* show no such intraspecific mtDNA variation (Table 1). These data question our ideas of species limits within Mauremys. Is M. annamensis a distinct species? Does *M. mutica* represent multiple species?

Several potential hypotheses might account for these unexpected results. *M. annamensis* may simply represent a recent species, derived from *M. mutica*, or even a geographical variant of *M. mutica*. The dramatic morphological differences exhibited by *M. annamensis* could reflect intense selection and rapid phenotypic evolution while the minute mitochondrial divergences and paraphyly represent the nature of speciation and unsorted polymorphism. Alternatively, there may be historical or ongoing introgression between *M. annamensis* and Vietnamese *M. mutica*, perhaps facilitated by selection.

Two additional hypotheses involve the possibility of hybridization. While our specimen of *M. annamensis* conforms to the species description, it was acquired from a Chinese turtle farm (Appendix 1) where *M. annamensis* and *M. mutica* are reared together in large numbers (J.F. Parham, pers. obs.). Hence, our *M. annamensis* could be a captive hybrid between *M. annamensis* and *M. mutica*, though we find no morphological characters supporting this notion. Ideally, we would examine the morphology and compare the sequences of a wildcaught *M. annamensis* to our sample, but to our knowledge, no tissued, field-collected vouchers of *M. annamensis* exist in collections; all modern museum specimens of *M. annamensis* have been obtained from either animal markets or the pet trade.

Another possibility is that the Vietnamese *M. muti*ca could be hybrid offspring of female *M. annamensis* and male *M. mutica*, accounting for the scant mtDNA differences between Vietnamese *M. mutica* and *M.* annamensis and the sizeable divergences between these samples and topotypic *M. mutica*. Although the "Vietnamese *M. mutica*" are phenotypically similar to typical *M. mutica*, their darker coloration is evocative of *M. annamensis*. Both Barth et al. (2004) and Spinks et al. (2004) found substantial mitochondrial variation between *M. mutica* and *M. annamensis*, but we do not know the provenance or morphology of their samples.

The hybridization of batagurid turtles has lead to other cases of taxonomic confusion (Parham and Shi, 2001; Parham et al., 2001; Shi and Parham, 2001; Wink et al., 2001; Spinks et al., 2004) and cannot be discounted here. Unfortunately, our small sample size prohibits us from effectively evaluating these hypotheses. Clearly a more detailed genetic study is needed to unravel this problem. With our present knowledge, any change in conservation policies for *M. annamensis*, one of the world's most poorly known turtles, would be premature.

Biogeography. - The distribution of *Mauremys* is characterized by a major break between the Zagros Mountains of western Iran (easternmost *M. caspica*) and the Annamite Mountains of central Vietnam (range of *M. annamensis*). This disjunction includes the entire Indian subcontinent (home to a diverse, endemic batagurid fauna), and the inhospitable Tibetan plateau. We suggest that the collision of India into Asia may be the vicariant event responsible for the current distribution of *Mauremys*, as proposed for anguine lizards (Macey et al., 1999). Molecular data are ambiguous on this point.

Given that neither eastern nor western species assemblages appear monophyletic (though a Wilcoxon signed ranks test topology test cannot discount this hypothesis [P = 0.35]), the current divergences between the living species may have occurred before the development of the Indo-Tibetan gap. The collision and subsequent uplift of the Tibetan plateau took place in multiple stages between 50 and 10 MYBP (Shackleton and Chang, 1988; Dewey et al., 1989; Windley, 1988). Hervet (2004) attributed some Paleogene (>50 MYBP) European fossils to the stem of Mauremys, but did not investigate their relations to east Asian Mauremys. In addition to employing additional molecular markers to vouchered museum specimens, the integration of all extant Mauremys into analyses of morphological characters and fossil taxa will be necessary to unravel the historical biogeography of this clade of turtles.

Acknowledgments

We thank T. J. Papenfuss, C. Cicero, and D. B. Wake (MVZ), and R. W. Murphy (ROM) for kindly contributing specimens and tissues essential to this project. We are grateful to M. E. Pfrender and P. G. Wolf for generously providing laboratory space, and D. G. Mulcahy and W. B. Simison for much needed lab assistance. We appreciate helpful discussions about phylogenetic methods from E. M. O'Neill, A. D. Leaché, and F. T. Burbrink, and useful comments on this manuscript from M. D. Matocq, J. R. Mendelson III, E. D. Brodie Jr., H. B. Shaffer, and two anonymous reviewers. We also thank P. Q. Spinks and U. Fritz for sharing unpublished data. Finally, we thank K. Padian and T. J. Papenfuss for funding, space, advice, and encouragement. This is University of California Museum of Paleontology Contribution #1826 and LBNL #54656. This research was performed under the auspices of the U.S. Department of Energy, Office of Biological and Environmental Research.

References

- Arevalo, E., S. K. Davis and J. W. Sites. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. Systematic Biology 43: 387-418.
- Avise, J. C., B. W. Bowen, T. Lamb, A. B. Meylan and E. Bermingham. 1992. Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. Molecular Biology and Evolution 9: 457-473.

- Barth, D., D. Bernhard, D. Guicking, D. Stock and U. Fritz. 2002. Is *Chinemys megalocephala* Fang, 1934 a valid species? New insights based on mitochondrial DNA sequence data. Salamandra 38: 233-244.
- Barth, D., D. Bernhard, G. Fritzsch and U. Fritz. 2004.
 The freshwater turtle genus *Mauremys* (Testudines, Geoemydidae) a textbook example of an east-west disjunction or a taxonomic misconcept? Zoologica Scripta. In press.
- Bourret, R. 1939. Notes herpétologiques sur l'Indochine Française, XVIII. Reptiles et batraciens reçus au Laboratoire des Science Naturelles de l'Université au cours de l'annee 1939, Descriptions de quatre espécies et d'une variété nouvelles. Bulletin Général de l'Instruction Publique Hanoi 1939: 5-39.
- Bremer, K. 1994. Branch support and tree stability. Cladistics 10: 295-304.
- Busack, S. D. and C. H. Ernst. 1980. Variation in the Mediterranean populations of *Mauremys* Gray 1869 (Reptilia, Testudines, Emydidae). Annals of Carnegie Museum 49: 251-264.
- Dewey, J. F., S. Cande and W. C. Pittman III. 1989. Tectonic evolution of the India/Eurasia collision zone. Eclogae Geologicae Helveticae 82(3): 717-734.
- Farris, J. S. 1983. The logical basis of phylogenetic analysis. In N. Platnick and V. A. Funk (eds), The logical basis of phylogenetic analysis. Columbia University Press, New York, NY, pp. 7-36.
- Farris, J. S., M. Kallersjo, A. G. Kluge and C. Bult. 1994. Testing significance of incongruence. Cladistics 10: 315-319.
- Feldman, C. R. and J. F. Parham. 2002. Molecular phylogenetics of emydine turtles: taxonomic revision and the evolution of shell kinesis. Molecular Phylogenetics and Evolution 22: 388-398.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.
- Felsenstein, J. 1993. PHYLIP (Phylogentic Inference Package). Ver. 3.5c. Department of Genetics, University of Washington.

- Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.
- Fritz, U. and T. Wischuf. 1997. Zur systematic westasiatisch-südosteropäischer Bachschilkröten (Gattung *Mauremys*) (Reptilia: Testudines: Bataguridae).
 Zoologische Abhandlungen, Staatliches Museum für Tierkunde Dresden 49: 223-260.
- Goldman, N. 1993. Statistical tests of models of DNA substitution. Journal of Molecular Evolution 63: 182-198.
- Gu, X., Y. Fu and W.-H. Li. 1995. Maximum likelihood estimation of the heterogeneity of substitution rate among nucleotide sites. Molecular Biology and Evolution 12: 546-557.
- Hasegawa, M., K. Kishino and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 21: 160-174.
- Hendy, M. D. and D. Penny. 1982. Branch and bound algorithms to determine minimal evolutionary trees. Mathematical Biosciences 59: 277-290.
- Hervet, S. 2004. Systématique du groupe <<*Palaeochelys* sensu lato - *Mauremys>>* (Chelonii, Testudinoidea) du Tertiaire d'Europe occidentale: principaux résultas. Annales de Paleontologie 90: 13-78.
- Honda, M., Y. Yasukawa and H. Ota. 2002a. Phylogeny of Eurasian freshwater turtles of the genus *Mauremys* Gray 1869 (Testudines), with special reference to a close affinity of *Mauremys japonica* with *Chinemys reevesii*. Journal of Zoological Systematics and Evolutionary Research 40: 195-200.
- Honda, M., Y. Yuichirou, R. Hirayama and H. Ota.
 2002b. Phylogenetic relationships of the Asian box turtles of the genus *Cuora* sensu lato (Reptilia: Bataguridae) inferred from mitochondrial DNA sequences. Zoological Science 19: 1305-1312.
- Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

- Iverson, J. B., C. H. Ernst, S. Gotte and J. E. Lovich. 1989. The validity of *Chinemys megalocephala*. Copeia 1989: 494-498.
- Iverson, J. B. and W. P. McCord. 1994. Variation in East Asian turtles of the genus *Mauremys* (Bataguridae: Testudines). Journal of Herpetology 28: 178-187.
- Joyce, W. G., J. F. Parham, and J. Gauthier. In press. Developing a protocol for the conversion of rankbased taxon names to phylogenetically defined clade names, as exemplified by turtles. Journal of Paleontology. In press.
- Kumazawa, Y. and M. Nishida. 1993. Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. Journal of Molecular Evolution 37: 380-398.
- Kumazawa, Y. and M. Nishida. 1995. Variations in mitochondrial tRNA gene organization of reptiles as phylogenetic markers. Molecular Biology and Evolution 12: 759-772.
- Kumazawa, Y., H. Ota, M. Nishida and T. Ozawa. 1996. Gene rearrangements in snake mitochondrial genomes: highly concerted evolution of controlregion-like sequences duplicated and inserted into a tRNA gene center. Molecular Biology and Evolution 13: 1242–1254.
- Lamb, T., C. Lydeard, R. B. Walker and J. W. Gibbons. 1994. Molecular systematics of map turtles (*Graptemys*): A comparison of mitochondrial restriction site versus sequence data. Systematic Biology 43: 543-559.
- Larget, B. and D. L. Simon. 1999. Markov chain monte carlo algorithms for the bayesian analysis of phylogenetic trees. Molecular Biology and Evolution 16: 750–759.
- Lau, M. and H. Shi. 2000. Conservation and trade of terrestrial and freshwater turtles and tortoises in the People's Republic of China. Chelonian Research Monographs 2: 30-38.
- Leaché, A. D. and T. W. Reeder. 2002. Molecular systematics of the Eastern Fence Lizard (*Sceloporus undulatus*): A comparison of parsimony, likelihood, and Bayesian approaches. Systematic Biology 51: 44-68.

- Lenk, P., U. Fritz, U. Joger and M. Winks. 1999. Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). Molecular Ecology 8: 1911-1922.
- Macey, J. R., A. Larson, N. B. Ananjeva, Z. Fang and T. J. Papenfuss. 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. Molecular Biology and Evolution 14: 91-104.
- Macey, J. R., J. A. Schulte, II, A. Larson, B. S. Tuniyev, N. Orlov and T. J. Papenfuss. 1999. Molecular phylogenetics, tRNA evolution, and historical biogeography in anguid lizards and related taxonomic families. Molecular Phylogenetics and Evolution 12: 250-272.
- Macey, J. R. and A. Verma. 1997. Homology in phylo genetic analysis: alignment of transfer RNA genes and the phylogenetic position of snakes. Molecular Phylogenetics and Evolution 7: 272-279.
- Maddison, W. P., M. J. Donoghue and D. R. Maddison. 1984. Outgroup analysis and parsimony. Systematic Zoology 33: 83-103.
- Maddison, D. R. and W. P. Maddison. 2000. MacClade: Analysis of phylogeny and character evolution. Ver. 4.0. Sinauer.
- Maniatis, T., E. F. Fristch and J. Sambrook. 1982. Molecular cloning; a laboratory manual. Cold Spring Harbor Publication, Cold Spring Harbor, New York.
- McDowell, S. B. 1964. Partition of the genus *Clemmys* and related problems in the taxonomy of the aquatic Testudinidae. Proceedings of the Zoolological Society of London 143: 239-279.
- Parham, J. F. and C. R. Feldman. 2002. Generic revisions of emydine turtles. Turtle and Tortoise Newsletter 6: 30-32.
- Parham, J. F. and H. Shi. 2001. The discovery of *Mauremys iversoni*-like turtles at a turtle farm in Hainan Province, China: The counterfeit golden coin. Asiatic Herpetological Research 9: 71-77.
- Parham, J. F., W. B. Simison, K. H. Kozak, C. R. Feldman and H. Shi. 2001. New Chinese turtles: Endangered or invalid? A reassessment of two species using mitochondrial DNA, allozyme elec-

trophoresis, and known locality specimens. Animal Conservation 4: 357-367.

- Posada, D. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.
- Rannala, B. and Z. H. Yang. 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. Journal of Molecular Evolution 43: 304-311.
- Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. G. Higuchi, T. T. Horn, K. B. Mullis and H. A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.
- Sanger, F., S. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 74: 5463-5467.
- Shackleton, R. M. and C. Chang. 1988. Cenozoic uplift and deformation of the Tibetan Plateau: The geo morphological evidence. Philosophical Transactions of the Royal Society of London A 327: 365-377.
- Shi, H. and J. F. Parham. 2001. Preliminary observations of a large turtle farm in Hainan Province, People's Republic of China. Turtle and Tortoise Newsletter 3: 2-4.
- Sorenson, M. D. 1999. TreeRot. Ver. 2. Boston University.
- Spinks, P. Q., H. B. Shaffer, J. B. Iverson and W. P. McCord. 2004. Phylogenetics hypotheses for the turtle family Geoemydidae. Molecular Phylogenetics and Evolution. In press.
- Starkey, D. E., H. B. Shaffer, R. L. Burke, M. R. J. Forstner, J. B. Iverson, F. J. Janzen, A. G. J. Rhodin and G. R. Ultsch. 2003. Molecular systematics, phy logeography, and the effects of Pleistocene glaciation in the painted turtle (*Chrysemys picta*) complex. Evolution 57: 119–128.
- Stuart, B. L. and J. F. Parham. 2004. Molecular phylogeny of the critically endangered Indochinese box turtle *Cuora galbinifrons*. Molecular Phylogenetics and Evolution 31: 164-177.

- Swofford, D. L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Ver. 4.0b10. Sinauer Associates Inc.
- Templeton, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37: 221-244.
- van Dijk, P. P., B. L. Stuart and A. G. J. Rhodin. 2000. Asian Turtle Trade: Proceedings of a workshop on conservation and trade of freshwater turtles and tortoises in Asia. Chelonian Research Monographs 2: 164.
- Windley, B. F. 1988. Tectonic framework of the Himalaya, Karakorum and Tibet, and problems of their evolution. Cambridge University Press, Cambridge.
- Wink, M., D. Guicking and U. Fritz. 2001. Molecular evidence for hybrid origin of *Mauremys iversoni* Pritchard et McCord, 1991, and *Mauremys pritchardi* McCord, 1997 (Reptilia: Testudines: Bataguridae). Zoologische Abhandlungen, Staatliches Museum für Tierkunde Dresden 51: 41-49.
- Wu, P., K.-Y. Zhou and Q. Yang. 1999. Phylogeny of Asian freshwater and terrestrial turtles based on sequence analysis of 12S rRNA gene fragment. Acta Zoologica Sinica 45: 260-267.
- Yang, Z. 1994a. Estimating patterns of nucleotide substitution. Journal of Molecular Evolution 39: 105-111.
- Yang, Z. 1994b. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. Journal of Molecular Evolution 39: 306-314.
- Yang, Z. 1996. Maximum likelihood models for combined analyses of multiple sequence data. Journal of Molecular Evolution 42: 587-596.

Appendix 1.

Specimens used and GenBank Accession numbers for DNA sequence data. Acronyms are: MVZ = Museum of Vertebrate Zoology, Berkeley, California; ROM = Royal Ontario Museum, Toronto, Ontario; AF or AY = GenBank (http://www.ncbi.nlm.gov).

Mauremys annamensis - Purchased in turtle farm in Hainan Province, China, no real locality data; MVZ 238937; AY337338, AY337346. Mauremys caspica -Field collected on Bahrain Island, Bahrain; MVZ 230971; AY337339, AY337347. Mauremys caspica -Field collected in West Azarbaijan Province, Iran; MVZ 234281; AY337340, AY337348. "Mauremys iversoni" -Purchased in turtle farm in Hainan Province, China, no real locality data; MVZ 230475; AF348275, AF34281. Mauremys japonica - Pet trade specimen, no locality data; MVZ 234647; AY337341, AY337349. Mauremys leprosa - Field collected in Tetouan Province, Morocco; MVZ 178059; AY337342, AY337350. Mauremys leprosa - Field collected in Cadiz Province, Spain; MVZ 231989; AY337343, AY337351. Mauremys mutica -Field collected in Zoushan Island, Zhejiang Province, China; MVZ 230476; AF348262, AF348278. Mauremys mutica - Purchased from a turtle trader in Yen Bai ROM 25613; AF348260, Province, Vietnam; AF348279. Mauremys mutica - Purchased from a turtle trader in Yen Bai Province, Vietnam; ROM 25614; AF348261, AF348280. Mauremys rivulata - Field-collected in Bursa Province, Turkey; MVZ 230212; AY337344, AY337352. Mauremys (=Ocadia) sinensis -Field-collected in Hainan Province, China; MVZ 230479; AY337345, AY337353. Mauremys nigricans -Pet trade specimen, no locality data, MVZ 130463; AF348264, AF348289. Mauremys reevesii - Pet trade specimen, no locality data, MVZ 230533; AF348263, AF348288. Cuora picturata - Purchased from a turtle trader in Dong Nai Province, Vietnam, ROM 37067; AF348265, AF348292. Cuora trifasciata - Pet trade specimen, no locality, MVZ 230636; AF348270, AF348297. Cuora mouhotii - Purchased from a turtle trader in Bac Thai Province, ROM 35003; AF348273, AF348286.