Animal Conservation. Print ISSN 1367-9430

A genetic survey of heavily exploited, endangered turtles: caveats on the conservation value of trade animals

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Keywords
Mauremys annamensis; *Mauremys mutica*; Asian turtle crisis; turtle trade; turtle farm; reintroduction; repatriation; translocation.

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Received 14 May 2007; accepted 20 June 2007

doi:10.1111/j.1469-1795.2007.00131.x

Abstract

Asian turtles face an extinction crisis, and so it is imperative that systematists accurately determine species diversity in order to guide conservation strategies effectively. We surveyed mitochondrial and nuclear DNA (mtDNA and nuDNA) variation of the heavily exploited *Mauremys mutica* complex, a clade of Asian turtles that contains the endangered *M. mutica* from Japan, Taiwan, China and Vietnam, and the critically endangered *Mauremys annamensis* from central Vietnam. We discovered extensive mtDNA and nuDNA variation among samples that did not correspond to the currently recognized taxonomy. Both nuDNA and mtDNA data suggest that *M. mutica* is paraphyletic with respect to *M. annamensis*. Surprisingly, *M. annamensis* exhibits a previously unknown mtDNA structure in the form of two clades that are paraphyletic to *M. mutica*. These data reveal that the currently recognized taxonomy of the *mutica* complex does not reflect the genetic diversity of our samples. Unfortunately, many conservation-oriented captive-breeding efforts for turtles are also based on trade samples such as the ones studied here. These efforts include plans to breed trade-rescued individuals and release their progeny into the wild. Because our genetic survey reveals that the taxonomic identity of these samples does not reflect genetic diversity, we raise serious questions about the efficacy of these programs. In order to address conservation issues and provide more accurate estimates of evolutionary lineages within *Mauremys*, we recommend continued surveys for wild populations of the *mutica* complex to provide new genetic material and additional distributional data, attempts to extract DNA from historic museum specimens and a shift in conservation focus to *in situ* preservation of wild populations and associated habitat.

Introduction

Asian turtles face an extinction crisis (van Dijk, Stuart & Rhodin, 2000; Turtle Conservation Fund, 2003), underscoring the importance of accurately determining species diversity to guide conservation strategies (Parham *et al*., 2001; Stuart & Parham, 2004). Unfortunately, determining species boundaries of Asian turtles has proven challenging because of the widespread hybridization of distinct lineages in the wild and in captivity (Parham *et al*., 2001; Spinks *et al*., 2004; Shi *et al*., 2005; Stuart & Parham, 2007; Spinks & Shaffer, in press), a phenomenon that is exacerbated by the pervasive transportation and captive propagation of turtles for profit (Parham *et al*., 2001; Shi & Parham, 2001). These challenges faced by systematists are directly relevant to the captive-breeding efforts that are increasingly being championed as one of the long-term solutions to the survival of Asian turtles (Hudson & Buhlmann, 2002; Turtle Conservation Fund, 2003).

One heavily exploited species complex in dire need of detailed systematic study is the *Mauremys mutica* (Cantor, 1842) complex ('the *mutica* complex' hereafter). This group of medium-sized (~20 cm), aquatic turtles is widely distributed (~2600 km west to east) from central Vietnam to the islands of southern Japan (Fig. 1a). Currently, two named species are recognized, *M. mutica* and *Mauremys annamensis* (Siebenrock, 1903), although previous studies using morphological and genetic data conflict in the inference of their relationship, and suggest that the current taxonomy may not accurately reflect lineage diversity (Honda, Yasukawa &

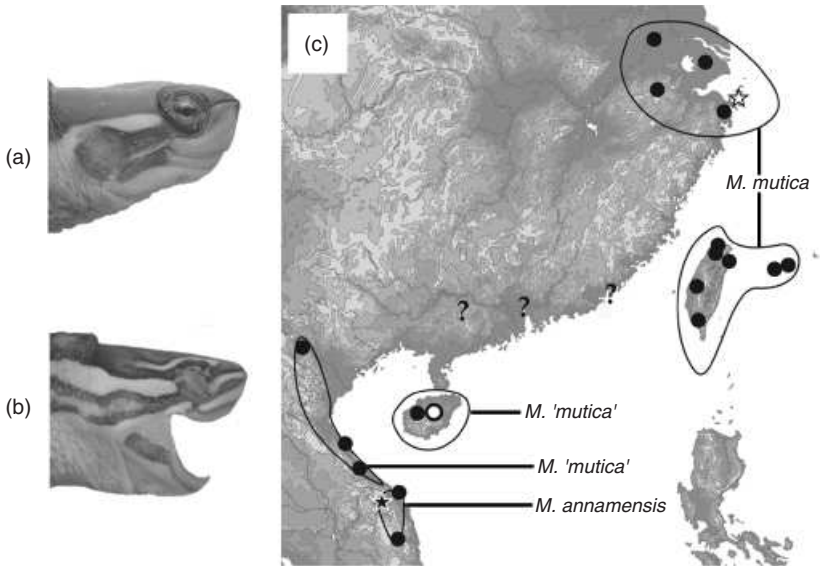

Figure 1 (a) Lateral view of the head of *Mauremys mutica*; (b) lateral view of the head of *Mauremys annamensis*; (c) map showing reported localities for the *mutica* complex in the Tonkin Gulf region and eastern Asia. Black lines encircle the hypothesized distribution of the *mutica* complex both in the Tonkin Gulf region (Hainan and Vietnam) as well as eastern China, Taiwan and southern islands of the Japan archipelago. All shapes (circles, stars, question marks) indicate historical records. The stars show the type localities of the taxa (*M. mutica* in eastern China and *M. annamensis* in central Vietnam), while the question marks represent questionable localities of the *mutica* complex in southern China due to the turtle trade. The white-colored shapes show the locality of genetic material used in this study. Records from other areas in Japan (excluding the Yaeyama Archipelago) are believed to be introduced and are not shown on this map (Goris & Maeda, 2004).

Ota, 2002; Barth *et al*., 2004; Feldman & Parham, 2004, hence '*mutica* complex'). Despite its once-widespread distribution, *M. mutica* is listed on the IUCN Red List as endangered (IUCN, 2006) because it is one of the most heavily exploited turtles in the food and medicine trade in China (Lau & Shi, 2000; van Dijk, 2000). *Mauremys annamensis* has a much more limited distribution and is considered to be one of the world's most endangered turtles (Turtle Conservation Fund, 2003); it has only been observed in the wild by scientists twice since 1939 (ATCN, 2006*a*; Parham, Stuart & Orlov, 2006). All reported localities are restricted to central and southern Vietnam, an area of intense wildlife harvesting (Le, Hoang & Le, 2004).

Establishing patterns of genetic variation among the *mutica* complex is important for guiding the usage of limited conservation resources and informing captive-breeding efforts (Parham *et al*., 2004; Stuart & Parham, 2004). The latter issue is especially relevant for *M. annamensis* because it is essentially unknown in the wild and trade specimens are being used in captive-breeding programs with the ultimate goal of reintroduction into the wild (Schaffer, 2004, 2006; ATCN, 2006*b*). Among the numerous problems associated with the translocation and release of confiscated wildlife (outlined by IUCN, 2002), the risk of genetic pollution through artificial introgression (e.g. Fitzsimmons *et al*., 2002) is especially high for heavily exploited Asian turtles (Buskirk, Parham & Feldman, 2005). In this study, we survey the genetic diversity within the *mutica* complex using mitochondrial and nuclear DNA (mtDNA and nuDNA) sequence data. Most of our specimens are from trade seizures, markets or turtle farms, although we also include, to our knowledge, every known-locality genetic sample obtained from the wild.

Methods

Specimens of *M. mutica* were morphologically diagnosed by having a weakly tricarnate to smooth carapace that is usually brown, a plastron that is marked with few or moderate black marks and a single, pale yellow temporal stripe that does not extend past the eye (Iverson & McCord, 1994; Yasukawa, Ota & Iverson, 1996). *Mauremys annamensis* is easily diagnosed from *M. mutica* in that it has a black, moderately tricarnate carapace, plastron marked heavily with black, and two to three yellow temporal stripes that meet at the tip of the nose (Iverson & McCord, 1994; Yasukawa *et al*., 1996). All specimens used in this study were categorized according to these criteria.

Our sampling included five individuals previously sequenced by Parham *et al*. (2001) and Feldman & Parham (2004) (Supplementary Material Appendix S1). These studies included a single field-collected, known-locality specimen of *M. mutica* (from the type locality at Zhoushan Island, Zhejiang Province, China). Our study added two additional known-locality specimens of *M. mutica* from Hainan Province, China (Wanling, Qiongzhong County), eight individuals of *M. annamensis* that were confiscated from an illegal shipment within Vietnam, 12 individuals of *M. mutica* and *M. annamensis* from a turtle farm in Hainan

(profiled by Shi & Parham, 2001 and Parham & Shi, 2001) and one individual of *M. annamensis* from the pet trade. Thirteen of our total samples (including four outgroups) were from preserved museum specimens and 24 were photo-vouchered using digital images (Supplementary Material Appendix S1). The 24 photo-vouchered specimens were a combination of farm, illegal trade seizures and wild-caught specimens that were (at the time of this paper) either living in the care of the turtle farmer, Cuc Phuong National Park Turtle Conservation Center in Ninh Binh Province, Vietnam, or with one of us (H. S.) at Hainan Normal University, respectively. Photo-vouchers are deposited in the Museum of Vertebrate Zoology at the University of California, Berkeley (Supplementary Material Appendix S1). Although we do not have precise locality data for the *M. annamensis* specimens from the trade seizure, we believe that these samples were from the wild because there are no known turtle farms in Vietnam breeding *M. annamensis*, trade with China is unidirectional and the specimens were confiscated off a truck in northern Vietnam (Ninh Binh Province). These individuals were probably wild caught in Vietnam and therefore most likely represent lineages that are not influenced by genetic admixture in captivity.

Tissue samples in the form of liver, blood or tail tips were frozen in liquid nitrogen, stored in 95% ethanol or stored in lysis buffer. Total genomic DNA was extracted via a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's recommendations. An 892 bp fragment of mtDNA that encodes part of the NADH dehydrogenase subunit 4 (*nad4*) gene, the complete tRNAs histidine (*trnH*) and serine (*trnS*) and part of the tRNA leucine (*trnL*) was amplified by PCR using the primers L-ND4 and H-Leu (Stuart & Parham, 2004) and standard conditions. Purified PCR products were sequenced in both directions using the same primers for amplification plus the internal primers L-ND4int and H-ND4int (Stuart & Parham, 2004). Sequences were aligned and the protein-coding region was translated into amino acids to ensure that there were no erroneous stop codons using Macvector v. 7.1.1 (Accelrys Inc., San Diego, CA, USA). For a subset of 25 samples, an ~1133 bp fragment of the RNA fingerprint protein 35 (R35) gene, intron 1, was amplified following Fujita *et al.* (2004).

Phylogenies were reconstructed using the maximum parsimony (MP) and maximum likelihood (ML) criteria as implemented in PAUP* v. 4.0b10 (Swofford, 1998) and Bayesian inference using MrBayes v.3.1.2 (Hulsenbeck & Ronquist, 2001). For mtDNA, *Cuora galbinifrons*, *Mauremys caspica*, *Mauremys nigricans* and *Mauremys reevesii* were used as outgroups following Parham *et al.* (2001) and Stuart & Parham (2004), while *Cuora amboinensis* was used for nuDNA. Our MP analysis was conducted using the branch-and-bound algorithm, with transitions and transversions weighted equally for 1000 random addition replicates and 100 pseudoreplicates. Characters were unweighted because the third codon position transitions accumulated in a linear fashion and showed no indication of saturation. Nodal support was assessed using 1000 nonparametric bootstrap pseudoreplicates (Felsenstein, 1985), with 100 random addition sequence replicates per pseudoreplicate.

For ML analyses, Modeltest 3.5 (Posada & Crandall, 1998) was used to infer the best-fit model of sequence evolution under the hLRT criterion. Analyses were initially performed with 1000 random addition replicates with stepwise addition of taxa and tree bisectional-reconnection (TBR) branch swapping. A single tree from the results was chosen, and analyses were iterated in a successive-approximations approach until the parameters stabilized (Sullivan *et al.*, 2005). Bootstrap proportions were calculated using RAxML 2.2.3 (Stamatakis, 2006) with 1000 pseudoreplicates under the GTRCAT model of sequence evolution.

For Bayesian analysis, models were selected for each partition using MrModelTest v.2 (Nylander, 2004) under the hLRT criterion. The mtDNA dataset was partitioned using first, second and third codon positions and tRNAs (Brandley, Schmitz & Reeder, 2005) while a single model was used for the entire nuDNA dataset. Searches were run using four chains, four million generations and sampling every 1000th tree. Burn-in was estimated in the program AWTY (Wilgenbusch, Warren & Swofford, 2004) by plotting the cumulative posterior probability of nodes against the generation time. The analysis was run twice with random starting trees and the results were compared.

Results

mtDNA

An 892 bp fragment of mtDNA including *nad4* and adjacent tRNAs was obtained for all samples. Products from sequencing produced single peaks and there were no erroneous stop codons within the protein-coding regions. The average nucleotide frequencies in the fragment were A = 0.349, C = 0.266, G = 0.134 and T = 0.252, showing the characteristic bias in the light strand of mtDNA against G (Kocher *et al.*, 1989). From our dataset, 179 nucleotides were variable and 117 were parsimony informative, while uncorrected pairwise distances within our ingroup ranged from 0 to 7.5%. The MP analysis resulted in a single most parsimonious tree (length = 255; CI = 0.749; RI = 0.902). For ML, The model HKY + Γ was used with a Ti/Tv ratio = 8.7318, γ distribution shape parameter = 0.1822 and base frequencies of A = 0.3539, C = 0.2606, G = 0.1337 and T = 0.2518. After the iterative likelihood approach, the result was a single tree ($-\ln L = 2531.126$; Ti/Tv = 8.7317; shape = 0.182171). Bayesian analyses were run under the following models: first position – HKY + I + Γ, second position – HKY, third position – GTR + Γ and tRNA – HKY + I + Γ. The first 500 generations were discarded as burn-in. Both independent Bayesian runs gave essentially identical likelihood estimates and tree topologies. The same tree topology resulted from all three analytical methods, although the support values differed (Fig. 2).

Our analyses resulted in a strongly monophyletic *mutica* complex, with 100% support from all analyses (Fig. 2).

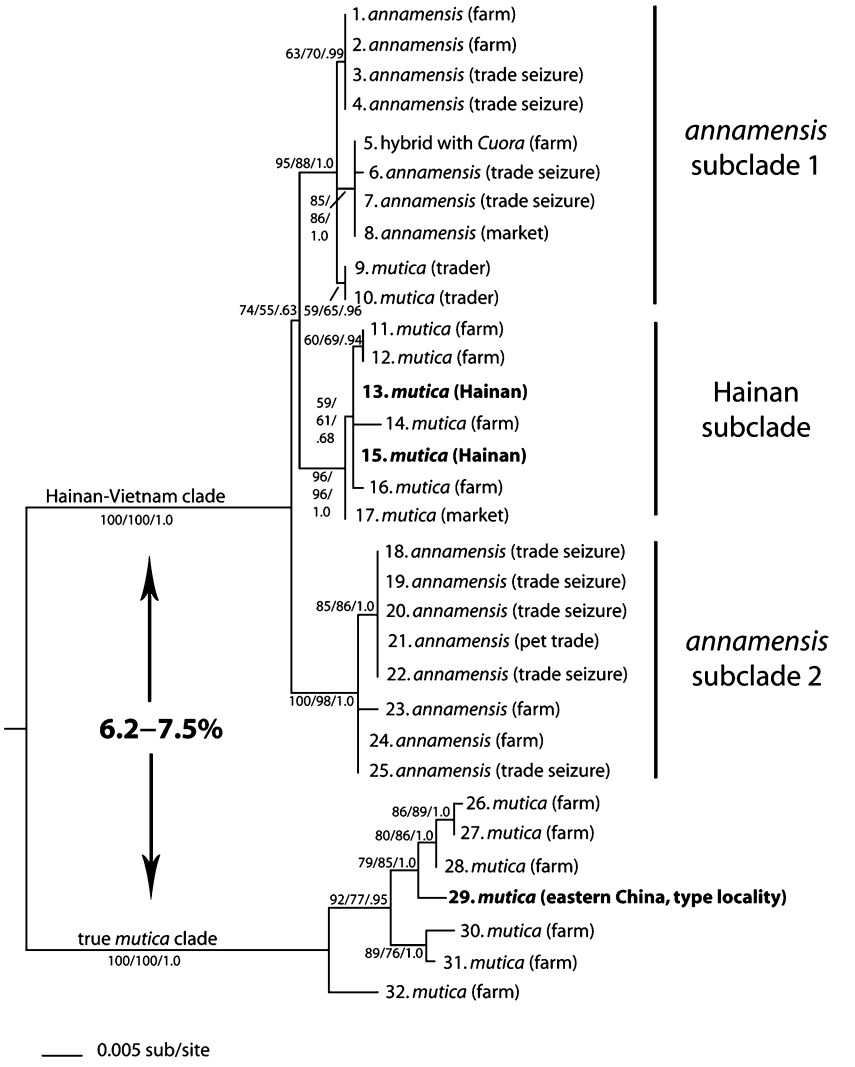

Figure 2 Phylogram resulting from maximum likelihood (ML) analysis of mitochondrial DNA from turtles of the *Mauremys mutica* complex. Trees from the maximum parsimony (MP) and Bayesian inference analyses had identical topologies. The *mutica* complex was strongly monophyletic with respect to the outgroups in all our analyses. The arrows indicate the presence of distinct mitochondrial clades that are 6.2–7.5% different with an *nad4*-based marker. Support values for each node are given in the following order: MP bootstrap, ML bootstrap and Bayesian posterior probabilities. The numbers before each species name correspond to the vouchers listed in (Supplementary Material Appendix S1) with the source of the specimen (farm, trade seizure, etc.). Specimens with reliable collection localities are shown in bold.

Contained in the *mutica* complex were two major, well-supported clades that differed by uncorrected pairwise sequence divergences of 6.2–7.5% (Fig. 2). One clade included the *M. mutica* specimen from the type locality in eastern China as well as many *M. mutica* specimens attributed to mainland China by the Hainan turtle farmer from whom the samples were obtained (see 'Discussion'). Because this clade included a specimen from the type locality, we call this the 'true *mutica*' clade (Fig. 2). The second clade included *mutica* from Hainan and *M. annamensis*, and is referred to as the 'Hainan–Vietnam' clade. The Hainan–Vietnam clade included three haplotype groups for which monophyly is strongly supported. We refer to these clades as *annamensis* 1, *annamensis* 2 and Hainan based on either known provenance samples or dominant morphotypes contained within.

Nu DNA

An ~1133 bp portion of the nuclear intron R35 (Fujita *et al.*, 2004) was collected for 25 samples. For the analyses, 31 nucleotides were variable and 14 parsimony-informative, with uncorrected pairwise distances within the ingroup ranging from 0 to 1.5%. The MP analysis resulted in a single most parsimonious tree (length = 51; CI = 0.902; RI = 0.945). For ML, the model HKY + Γ was used with a Ti/Tv ratio = 1.009, γ distribution shape parameter = 0.016 and base frequencies of A = 0.2792, C = 0.177, G = 0.212 and T = 0.3318. After the iterative likelihood approach, four equally likely trees resulted ($-\ln L = 1929.15$; Ti/Tv = 1.041; shape = 0.016), which only differed by the placement of terminal branches. Bayesian analyses were run under the HKY + Γ model. The first 500 generations were discarded as burn-in. Both independent Bayesian runs gave essentially identical likelihood estimates and tree topologies. Similar tree topologies resulted from all three analytical methods (Fig. 3).

Within the *mutica* complex, three clades were recovered corresponding to taxonomy and geography: *annamensis*, eastern China *mutica* and Hainan *mutica* (Fig. 3). Although not all the support values were strong, *M. annamensis*

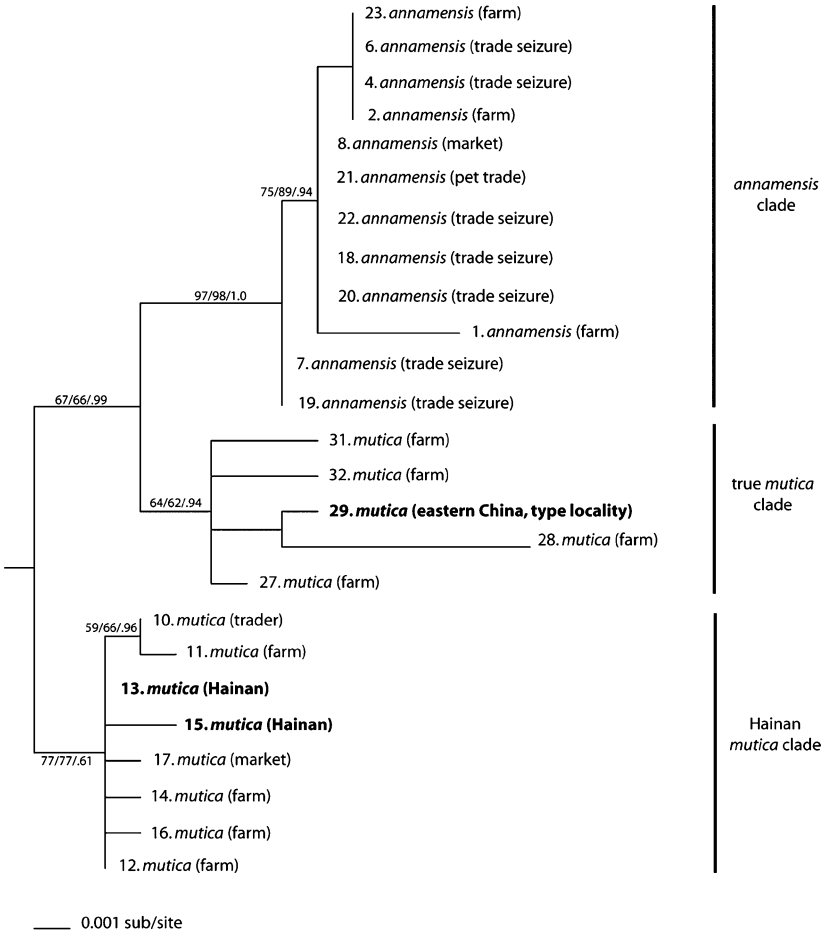

Figure 3 Phyogram resulting from maximum likelihood analysis of the R35 nuclear intron from turtles of the *Mauremys mutica* complex. Trees from the maximum parsimony (MP) and Bayesian inference analyses had almost identical topologies. Support values for each node are given in the following order: MP bootstrap, ML bootstrap and Bayesian posterior probabilities. The numbers before each species name correspond to the vouchers listed in (Supplementary Material Appendix S1) with the source of the specimen (farm, trade seizure, etc.). Specimens with reliable collection localities are shown in bold.

renders *M. mutica* paraphyletic, with eastern China *M. mutica* being more closely related to *M. annamensis* than Hainan *M. mutica*.

Discussion

Our study further elucidates the genetic diversity and major genetic groupings within the *mutica* complex reported by Parham *et al.* (2001) and Feldman & Parham (2004). We also recover a deep mitochondrial divergence (6.2–7.5%) between the eastern Chinese *M. mutica* (the true *mutica* clade) and those from the Tonkin Gulf region (The Hainan–Vietnam clade). Both mitochondrial and nuclear datasets agree that the true *mutica* clade is genetically distinct and that *M. mutica* is paraphyletic with respect to *M. annamensis*. Developing a more accurate picture of the geographic distribution of the two *mutica* clades (true *mutica* vs. Hainan–Vietnam *mutica*), and determining where they come into contact (if at all) will prove challenging because the distributional gap between known-locality specimens of both clades is in southern mainland China, the most active region in the consumption and import of turtle goods. This highlights a recurring problem associated with studying the phylogeography of Asian turtles: there are so few unambiguously field-collected specimens that establishing accurate geographical distributions is difficult (Parham & Li, 1999; Parham & Wang, 2000; Fong, Parham & Fu, 2002). However, if this distributional gap is real and ancient, it could explain the high sequence divergence between the *mutica* clades.

The high level of divergence of mtDNA used in this study between the true *mutica* and Hainan–Vietnam clade (6.2–7.5%) exceeds that between many pairs of widely accepted turtle species sequenced for the same marker (<5%; Feldman & Parham, 2002; Parham *et al.*, 2004; Stuart & Parham, 2004). Therefore, within *M. mutica*, current taxonomy is potentially underestimating lineage diversity due to the presence of *M. mutica* in three mtDNA clades (true *mutica*, *annamensis* 1 and Hainan; Fig. 2) and two nuDNA clades (Hainan, eastern China; Fig. 3). Previous systematic studies have noted considerable regional morphological variation within *M. mutica* (Iverson & McCord, 1989, 1994), with individuals in the north-east of its range tending to be brown in coloration and black in the south-west of its range. We compared these findings with additional literature and specimens (from museums, turtle

farms, trade seizures and markets; Supplementary Material Appendix S1 and S2). There seems to be some concordance from these results, for example, the turtle farmer's categorizations of individuals (*mutica* samples 11–12, 14, 16, 26–28, 30–32) into 'mainland' and 'Hainan' were all correct based on similar criteria as Iverson & McCord (1989, 1994). However, with our limited known-locality specimens, we are unable to definitively tie morphotypes of *M. mutica* to geography. If consistent diagnostic characters can be adequately demonstrated, it may be justified to resurrect junior synonyms of *M. mutica* [e.g. *Mauremys schmackeri* (Boettger, 1894) for Hainan *mutica* and *Mauremys grochovskiae* (Dao, 1957) for Vietnam *mutica*]. But an adequately informed taxonomy of the *mutica* complex cannot be generated without detailed genetic and morphologic studies of specimens with reliable locality data. For now, we urge taxonomic restraint and feel that it is most judicious to maintain the current taxonomy, while treating the genetically distinct *M. 'mutica'* populations from Hainan and Vietnam as distinct units for conservation (with quotes to denote their uncertain taxonomic status) separate from true *M. mutica* from eastern China and *M. annamensis*.

Although our mtDNA and nuDNA trees have different topologies, a similar pattern arises – *M. annamensis* is nested within *M. mutica*. In the case of the mtDNA phylogeny, *M. annamensis* is more closely related to *M. mutica* from Hainan, while the nuDNA infers *M. annamensis* to be more closely related to *M. mutica* from eastern China. Differences in the topologies may be due to biological factors (e.g. introgression or retention of ancestral polymorphisms) and/or anthropomorphic factors (e.g. translocation or artificial hybridization), but we are reluctant to favor one explanation over the other based on the available sampling. One unexpected result from our study was that *M. annamensis* samples seized within Vietnam had haplotypes corresponding to two distinct mitochondrial subclades (*annamensis* 1 and *annamensis* 2) that were paraphyletic to those of *mutica* from Hainan and Vietnam. Based on this high level of genetic diversity within *M. annamensis*, we predict that the range of *M. annamensis* may be larger than currently realized (Iverson, 1992; Parham *et al.*, 2006). If so, this genetic break may correspond to phylogeographic patterns seen in several other Vietnamese vertebrates (Garza & Woodruff, 1992; Roos & Nadler, 2001; Stuart & Parham, 2004).

The incongruent morphological and molecular variation among our trade samples also reveals their limitation as a conservation resource. Because current captive-breeding efforts for endangered Asian turtles rely entirely on taxonomic assignments based on morphology, the potential for these programs to foster the admixture of formerly discrete genetic lineages is extremely high. In the case of *M. annamensis*, established programs include plans to breed trade-rescued turtles and release their progeny into the wild in Vietnam (Schaffer, 2004, 2006; ATCN, 2006*b*). Without a better understanding of the geographic patterns of genetic diversity in the *mutica* complex, these efforts may inadvertently lead to the disruption of natural population substructure through the release of animals into the incorrect geographic area, outbreeding depression and/or the release of hybrid individuals.

The next steps in resolving the genetic underpinnings of the *mutica* complex are to increase efforts to locate and obtain genetic material from wild-caught specimens. This study includes three known-locality genetic samples collected by one of us (H. S.), or our colleagues, but without a concerted effort to obtain more known locality samples from China and Vietnam, we do not expect this small sample size to grow in the near future. However, another potential resource for genetic material is historical museum specimens. Natural history collections worldwide house voucher specimens that were collected before the Asian turtle survival crisis. Many of these specimens have detailed locality information and could ostensibly provide genetic data for extremely rare or extinct populations (e.g. Parham *et al.*, 2004). Unfortunately, most of these specimens are fixed with formalin, which makes DNA extraction a challenging and uncertain endeavor. Despite these difficulties, some workers have successfully extracted high quality DNA from formalin-fixed specimens (e.g. Shedlock *et al.*, 1997; Schander & Halanych, 2003), and so with some effort and the advent of new techniques, it may be possible to unlock a trove of genetic data from museum specimens.

Conclusions

Our study illustrates that the genetic diversity seen in the *mutica* complex is greater than believed previously. However, the genetic divisions within this group do not match the current taxonomy. There may be multiple natural and anthropogenic forces acting simultaneously to create the patterns of variation that we observed, but our ability to untangle these forces is hindered by the fact that most available genetic material originates from the turtle trade. Unfortunately, this trade also happens to be the same source of animals for captive-breeding efforts (Hudson & Buhlmann, 2002; Schaffer, 2004, 2006; ATCN, 2006*b*). Based on our findings, we seriously question the probability of success and efficacy of breeding and reintroduction efforts based on trade animals (Dodd & Seigel, 1991; IUCN, 2002; Lau, 2002). This is not to say that repatriation and augmentation (Reinert, 1991) are useless tools for conservation, but rather we currently know too little about the complex systematics of most Asian turtles (e.g. Spinks & Shaffer, 2007) to proceed properly. While genetic samples obtained from trade specimens can help elucidate minimum estimates of genetic diversity, their lack of provenance and the risk of genetic admixture in captivity severely limit their utility for understanding geographic patterns of genetic diversity or captive-breeding programs that recommend releasing animals into the wild. Breeding programs are useful in preserving individuals, educating the public and gathering data on reproductive biology (Schaffer, 2006), but it is prudent to shift more attention to *in situ* conservation of populations and habitats and understanding the geographic and genetic

diversity through fieldwork before reintroducing turtles into the wild.

The next steps towards unraveling the complicated patterns of incongruent molecular and morphological variation uncovered here will include: (1) intensive field surveys for new genetic material with known provenance, especially focusing on *M. annamensis* and Vietnam '*mutica*', and (2) increased effort to extract DNA from historic and formalin-fixed museum specimens.

Acknowledgments

This work represents partial fulfillment of the degree of Masters of Science for J.J.F. Funding for this project came from the Department of Natural Sciences, Loma Linda University (R.L.C.) as well as the Chelonian Conservation and Biology Linnaeus Fund (J.J.F.), and the Museum of Vertebrate Zoology (J.J.F.). This study was only possible with the help of many people: Mr Chen provided his knowledge of turtles as well as access to turtles on his farm, Ted Papenfuss for general advice and helping with the logistical issues working in China, Meiling Hong and Jichao Wang for helping with work in the field as well as translating of Chinese literature, Bui Dang Phong of the Cuc Phuong National Park Turtle Conservation Center for obtaining a CITES export permit from Vietnam for transport to the United States, Doug Hendrie for his help in obtaining *M. annamensis* samples from Vietnam, Adam Leaché and Dan Mulcahy for helping with the phylogenetic analyses, Chris Feldman for providing laboratory protocols, Roland Sosa and Sam Yamamoto for providing training, knowledge and support in the laboratory, J. Robert Macey for providing support with laboratory work, Lee Grismer for providing support and advising and the McGuire lab for reviewing this paper. Some of the sequencing was conducted in The Field Museum's Pritzker Laboratory for Molecular Systematics and Evolution operated with support from the Pritzker Foundation. This work is LBNL-59263 and was performed under the auspices of the US Department of Energy, Office of Biological and Environmental Research, by the University of California, Lawrence Berkeley National Laboratory, under contract No. DE-AC03-76SF00098. This is UCMP contribution # 1910.

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Supplementary material

The following material is available for this article online:

Appendix S1.

Appendix S2.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-1795.2007.00131.x

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Appendix A

A total of 37 specimens from several different sources were used in this study. Numbered specimens correspond to those in Figure 2. Ten of the DNA sequences used in this analysis were obtained from Genbank. Following is a list of all specimens used in this study with their voucher number, Genbank accession number for *nad4*, and Genbank accession number for R35 (if applicable). Outgroups: *Cuora galbinifrons* (FMNH 255695; AY364617; n/a), *Mauremys caspica* (MVZ 234281; AY337340; n/a), *M. reevesii* **(**MVZ 236729; EF034110; n/a**),** *M. nigricans* **(**MVZ 234641; EF034111; n/a), *C. amboinensis* (FMNH 255262; n/a; DQ386654). Ingroup: 1) *M. annamensis* (MVZ 251449; EF034098; EF587934); 2) *M. annamensis* (MVZ 238937; AY337338; EF87933) 3) *M. annamensis* (MVZ 251454; EF034103; n/a); 4) *M. annamensis* (MVZ 251456; EF034105; EF587922) 5) *Cuora* X *Mauremys* hybrid (MVZ 230475; AF348241; n/a); 6) *M. annamensis* (MVZ 251453; EF034102; EF587929); 7) *M. annamensis* (MVZ 251460; EF034109; EF587926); 8) *M. annamensis* (MVZ 230462; EF034113; DQ386655) 9) Vietnam *M.* '*mutica*' (ROM 25613; AF348279; n/a); 10) Vietnam *M.* '*mutica*' (ROM 25614; AF348280; DQ386668); 11) Hainan '*mutica*' (MVZ 251447; EF034096; EF87929); 12) Hainan '*mutica*' (MVZ230477; EF034105; n/a); 13) Hainan '*mutica*' (MVZ237108; EF034104; EF587915); 14) Hainan '*mutica*' (MVZ 251448; EF034097; EF587925); 15) Hainan '*mutica*' (MVZ 251452; EF034101; EF587917); 16) Hainan '*mutica*' (MVZ 251446; EF034095; EF587930); 17) Vietnam '*mutica*' (MVZ230476; AF348278; DQ386664); 18) *M. annamensis* (MVZ 251455; EF034104; EF587923); 19) *M. annamensis* (MVZ 251457; EF034106; EF587928); 20) *M. annamensis* (MVZ 251458; EF034107; EF587924) 21) *M. annamensis* (FMNH 262238; EF034112; DQ386656) 22) *M. annamensis* (MVZ 252396; EF587914; EF587921); 23) *M.*

annamensis (MVZ 251450; EF034099; EF587919); 24) *M. annamensis* (MVZ 251451; EF034100; n/a); 25) *M. annamensis* (MVZ 251459; EF034108; n/a); 26) *M. mutica* (MVZ 251443; EF034092; n/a); 27) *M. mutica* (MVZ 251444; EF034093; EF587931); 28) *M. mutica* (MVZ 251440; EF034089; EF587932); 29) *M. mutica* (MVZ 230487; AF348278; DQ386666); 30) *M. mutica* (MVZ 251441; EF034090; n/a); 31) *M. mutica* (MVZ 251442; EF034092; EF587916); 32) *M. mutica* (MVZ 251445; EF034094; EF587927).

Institutional abbreviations: FMNH—Field Museum of Natural History, Chicago, IL, USA; MVZ—Museum of Vertebrate Zoology, University of California, Berkeley, CA, USA; ROM—Royal Ontario Museum, Toronto, Ontario, Canada.

Appendix B

For this project, additional material to Appendix A was examined but not sequenced: 1) CAS-SUR 9142, *Mauremys annamensis* (Holotype of *Annamemys merkleni*; Savage, 1953), Fai-Fo, Annam, Vietnam; 2) FU R177, *Mauremys mutica*, Shaoguan, Guangdong Province, China; 3) MNHN 1884-437, *Mauremys* sp., Saigon, Vietnam; 4) MNHN 6502, *Mauremys* sp., Saigon, Vietnam; 5) MNHN 1948-39, *Mauremys annamensis*, Fai-Fo, Annam, Vietnam; 6) MVZ 23937, *Mauremys mutica*, Kakchiek, Swatow, Guangdong Prov., China; 7) MVZ 23938, *Mauremys mutica*; locality: Kakchiek, Swatow, Guangdong Prov., China; 8) MVZ 230462, *Mauremys annamensis*; locality: Dongmen Market, Haikou, Hainan Province, China; 9) FMNH 6588, *Mauremys mutica*; locality: Hainan Prov., China; 10) FMNH 6589, *Mauremys* '*mutica*'; locality: Hainan Prov., China; 11) FMNH 6590, *Mauremys* '*mutica*'; locality: Hainan Prov., China; 12) FMNH 6592, *Mauremys* '*mutica*'; locality: Hainan Prov., China; 13) FMNH 168907, *Mauremys mutica*; locality: T'ai-chung Hsien, Taiwan; 14) FMNH 168920, *Mauremys mutica*; locality: T'ai-chung Hsien, Taiwan;

The following references provide images that aided our comparisons: Siebenrock (1903), Bourret (1941), Dao (1957), Petzold (1963), Iverson & McCord (1994), Yasukawa, Iverson, & Ota (1996).

Institutional abbreviations: CAS— California Academy of Sciences, San Francisco, CA, USA; FMNH—Field Museum of Natural History, Chicago, IL, USA; FU—Fudan University, Shanghai, China; MVZ—Museum of Vertebrate Zoology, University of California, Berkeley, CA, USA.