



A reassessment of *Cuora cyclornata* Blanck, McCord and Le, 2006 (Testudines, Geoemydidae) and a plea for taxonomic stability

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Abstract

We analyze the phylogenetic variation present in the *Cuora trifasciata* species complex using mitochondrial and nuclear DNA sequence data. We use this information to evaluate the recent description of *Cuora cyclornata* Blanck, McCord, and Le (2006), and reinterpret this proposed species in light of likely mitochondrial introgression. Our results indicate that the pattern of variation within the *Cuora trifasciata* species complex is better explained by mitochondrial introgression coupled with hybridization and/or clinal variation, than it is by the presence of a previously unrecognized species. We also use our phylogeny as a framework for discussion of additional proposed changes to generic level taxonomy in this critically imperiled clade. Our results highlight the importance of appropriate data sampling in taxonomic revisions and suggest that *Cuora cyclornata* be treated as a junior synonym of *Cuora trifasciata*.

Introduction

The Asian box turtles (genus *Cuora*) could be the “poster children” of chelonian conservation biology. Seventy-five percent of the contained species (9/12) are in peril; seven species are critically endangered including *C. yunnanensis* Boulenger (which was declared extinct, but has since been found in the wild [Zhou and Zhao, 2004]), one is endangered, and one is vulnerable (IUCN, 2008). A sound, defensible taxonomy for these turtles is absolutely essential, as species-level taxonomy is key to understanding and protecting biodiversity (Turtle Taxonomy Working Group, 2007a). However, delimiting species for *Cuora* is complex because we know very little about patterns of intraspecific variation in the wild, some named taxa are very similar genetically, and there is widespread interspecific and intergeneric hybridization involving numerous *Cuora* species and related geoemydid genera (Parham *et al.*, 2001; Spinks & Shaffer, 2007; Spinks *et al.*, 2004; Stuart & Parham, 2004; Stuart & Parham, 2007; Wink *et al.*, 2000).

The greatest threat facing these turtles is over-exploitation for the Asian turtle trade. *Cuora* spp. are intensively collected to satisfy the increasing demands of the pet trade and food markets of China (Parham *et al.*, 2001; Stuart & Parham, 2004; van Dijk *et al.*, 2000). As a consequence, many species are extremely rare in the wild, thereby making population genetic or phylogeographic analyses all but impossible. For example, *C. pani* Song, and *C. aurocapitata* Luo and Zong are two recently described, Critically Endangered species (IUCN, 2008) for which almost no field-verified specimens are available (Luo & Zong, 1988, cited in Parham & Li, 1999). Since we have virtually no understanding of intraspecific patterns of genetic or phenotypic variation in nature, the differences among *C. aurocapitata* and *C. pani* could be attributed to clinal variation within a single taxon (McCord & Iverson, 1991; Parham & Li, 1999). Combined with the widespread movement of, and breeding among, species of *Cuora* for the Asian turtle market and the heavy past reliance on market-purchased specimens for taxonomic work, sorting out species boundaries and interspecific

phylogenies remains a daunting challenge requiring extensive taxon sampling from known-locality specimens (Fong *et al.*, 2007; Parham *et al.*, 2001).

Widespread hybridization among *Cuora* species, and between *Cuora* and other related genera including *Mauremys* and *Sacalia* further clouds our understanding of variation within the group. Over the last two decades, several new taxa have been erected, often based on morphologically distinctive individuals that were later shown to be interspecific or intergeneric hybrids. For example, “*Cuora serrata*” (Iverson & McCord, 1992a) is an interspecific hybrid between *Cuora galbinifrons* Boulenger (or the closely-related species *C. bouretti* Obst & Reimann) and *C. mouhotii* Gray (Parham *et al.*, 2001; Stuart & Parham, 2004; Stuart & Parham, 2007) while “*Mauremys iversoni*” (Pritchard & McCord, 1991), “*Ocadia philippeni*” (McCord & Iverson, 1992) and “*Sacalia pseudocellata*” (Iverson & McCord, 1992b) are now viewed as intergeneric hybrids between *Mauremys* sp. or *Sacalia quadriocellata* and *C. trifasciata* (Parham *et al.*, 2001; Spinks *et al.*, 2004; Stuart & Parham, 2007; Wink *et al.*, 2000).

It is always the case that taxonomic revision should be approached cautiously to ensure that conclusions are based on rigorous and thorough analyses (Parham *et al.*, 2006; Pauly *et al.*, in press; Turtle Taxonomy Working Group, 2007a). However, this caution is particularly important when applied to seriously imperiled taxa, simply because the stakes are high. Importantly, these high stakes apply both to over and under-splitting species. Named taxa that are not true evolutionary lineages may trigger legal protection and conservation efforts for entities that are not real in any evolutionary sense, while unrecognized diversity may rob real, endangered species of the protection they require for survival. The current taxonomic instability within *Cuora*, coupled with our lack of understanding of intraspecific variation, known interspecific hybridization, and high conservation priority, should inspire caution when working in this clade. Failure to do so has led to widespread taxonomic confusion, and has clouded our understanding of these critically imperiled turtles.

A case in point is the recent description of a new *Cuora* species, including two contained subspecies. Blanck, McCord & Le (2006) purportedly assessed phenotypic and mitochondrial DNA (mtDNA) variation in *C. trifasciata*, and described two new taxa, “*Cuora cyclornata cyclornata*” and “*C. c. meieri*”, from the variation they observed within nominate *C. trifasciata*. The conclusions of Blanck, McCord & Le (2006) are based on two primary lines of evidence; a phylogenetic analysis of 884 base pairs (bp) of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) gene, and descriptions of morphological variation largely relating to coloration and shell shape. Here, we re-examine the methods and conclusions of Blanck, McCord & Le (2006) and discuss what we view as weaknesses in the design and execution of their study, and interpretations of their results. In particular, 1) available mitochondrial sequence data were excluded from their molecular phylogenetic analysis, 2) including all relevant data would have suggested the need for a multi-gene approach, 3) the morphological component of the study employed no analytical or statistical methods, and 4) they failed to provide a clear definition of what they mean by a species, and a clear method by which species should be delineated. Based on results from a more thoroughly sampled phylogenetic analysis performed here (see below), and results from Spinks & Shaffer (2007) we address some of these methodological and analytical shortcomings and interpret relationships among *C. aurocapitata*, *C. pani*, and *C. trifasciata* (this trio of species is hereafter referred to as the *C. trifasciata* complex following Parham *et al.*, 2004) in light of probable hybridization and introgression (Spinks & Shaffer, 2007).

Results and discussion

Phylogenetic evidence

Analyses undertaken to describe and quantify variation within a group often necessitates the inclusion of related, but very similar taxa. To accomplish this goal, Blanck, McCord and Le (2006, pg 36) included “four other species of *Cuora*: *C. amboinensis* (GenBank Accession No. AY364609), *C. flavomarginata* (No. AY364610), *C. galbinifrons* (No. AY364617), and *Cuora mouhotii* (No. AY699016) to show the relative

phylogenetic positions of different forms of *Cuora trifasciata*.” However, virtually all authors working on this clade have suggested a close relationship among *C. aurocapitata*, *C. pani*, and *C. trifasciata* (Blanck, 2005; Honda *et al.*, 2002; McCord & Iverson, 1991; Parham *et al.*, 2004; Spinks & Shaffer, 2007; Spinks *et al.*, 2004; Stuart & Parham, 2004). Indeed, the close relationship among these three taxa is congruent with Blanck’s (2005, pg. 25) observations:

“...the morphological aspects clearly suggest a close cladistic relationship with the *Cuora pani-aurocapitata-trifasciata* group...”

Three of the four GenBank sequences utilized by Blanck, McCord & Le (2006) were from the analysis of Stuart & Parham (2004) where *C. aurocapitata* and *C. pani* sequences were also presented, and where these two taxa were recovered as closely related to *trifasciata*. Thus, sequence data for these *C. aurocapitata*, *C. pani* and additional *C. trifasciata* were available to Blanck, McCord & Le (2006) on GenBank. However, Blanck, McCord & Le (2006) chose to exclude from their analysis the available *C. aurocapitata* and *C. pani* sequences, as well as additional samples of *C. trifasciata* that were posted on GenBank. This omission was particularly unfortunate since these GenBank *C. trifasciata* sequences were known to be virtually indistinguishable from *C. aurocapitata* and *C. pani* (Stuart & Parham, 2004) and in light of the recognized close mitochondrial relationships among the “*Cuora pani-aurocapitata-trifasciata* group” (Blanck, 2005; McCord & Iverson, 1991).

Including these data would have had a tremendous impact on the phylogenetic positions of Blanck, McCord & Le’s (2006) *C. trifasciata* samples, and, therefore, on the systematic interpretation of their reported variation within *C. trifasciata*. Blanck, McCord & Le (2006) recover *C. trifasciata* as two separate clades (A + B, and C, Fig. 1A). However, when all of the relevant data that were available at the time are included, the picture becomes more complex. We replicated the analysis of Blanck, McCord & Le (2006) using their sequence data (generously provided to PQS by M. Le), but also included GenBank *Cuora* mitochondrial DNA sequences that were also available to Blanck, McCord & Le (2006) at the time of their work. The resulting gene tree (Fig. 1B) reveals the confusion among the *C. trifasciata* complex for this mitochondrial gene. In particular, 1) the two main clades of *C. trifasciata* (clades A+B and C, Fig. 1B) are now widely disjunct rather than being sister clades, 2) *C. trifasciata* sensu Blanck, McCord & Le (2006) is rendered paraphyletic with respect to *C. aurocapitata* and *C. pani*, 3) the apparent paraphyly of Blanck, McCord & Le’s clade A+B+C (the old concept of *C. trifasciata*) with respect to *C. amboinensis* Daudin and *C. mouhotii* disappears into an unresolved polytomy, and 4) the *C. trifasciata* complex is now reconstructed as more closely related to *C. zhoui* Zhao, *C. flavomarginata* Gray, and *C. yunnanensis* (with strong support) than it is to “*C. cyclornata*”, even though “*C. cyclornata*” is phenotypically similar to the other members of the *C. trifasciata* complex.

Based on these data, a key inference can be made; if morphologically-similar *C. trifasciata* had split into two lineages over evolutionary time as proposed by Blanck, McCord & Le (2006), we would expect the two daughter lineages to be sister taxa, as the limited genetic sampling reported by Blanck, McCord & Le (2006) indicated. However, including all samples indicates that *C. trifasciata* and “*C. cyclornata*” are not each other’s closest relatives based on mitochondrial DNA, and (assuming that the mtDNA tree represents the true species tree) have not descended from an immediate common ancestor. One potential explanation for this pattern is that “*C. cyclornata*” is actually a distantly related, but morphologically-cryptic species. However, results from a combined mitochondrial and nuclear DNA analysis are in strong conflict with this interpretation (Spinks & Shaffer 2007, and below). Alternatively, recovering two widely disjunct mitochondrial clades from a single species is often indicative of mitochondrial introgression or nuclear-mitochondrial pseudogenes (numts) (Bates, 2004; Bernatchez *et al.*, 1995; Good *et al.*, 2003, 2008; Melo-Ferreira *et al.*, 2005; Peters *et al.*, 2007; Robertson *et al.*, 2006; Sorenson & Quinn, 1998; Wilson & Bernatchez, 1998), and we feel that the evidence supports these alternative interpretations in this case.

When introgression or numts are encountered, nuclear DNA (nuDNA) data can often illuminate the mitochondrial discrepancies. In their phylogenetic analysis of *Cuora*, Spinks & Shaffer (2007) included all GenBank mitochondrial sequences of the *C. trifasciata* complex plus additional new data from two *C. pani* and 21 *C. trifasciata* including five with mitochondrial haplotypes and phenotypes that are consistent with “*C.*

To further explore the mitochondrial landscape of *Cuora*, we performed an additional phylogenetic analysis incorporating all *Cuora* ND4 data available from GenBank including data from Parham *et al.* (2004), Stuart and Parham (2004), He *et al.* (2007), and Spinks & Shaffer (2007), plus the sequences from Blanck, McCord & Le (2006) (which have not been deposited in GenBank at the time of this writing). We performed maximum parsimony (MP) and maximum likelihood (ML) analyses in order to estimate both phylogenetic trees (MP and ML) and genetic distances among clades (ML). For MP, we employed the same methods as Blanck, McCord & Le (2006). For the ML analysis, we performed model selection using DT-ModSel (Minin *et al.*, 2003), estimated parameters of the model in PAUP*4.0b10 (Swofford, 1998), and performed a heuristic search with 10 random sequence addition replicates under the best fitting model (HKY+I+G), and 100 bootstrap replicates.

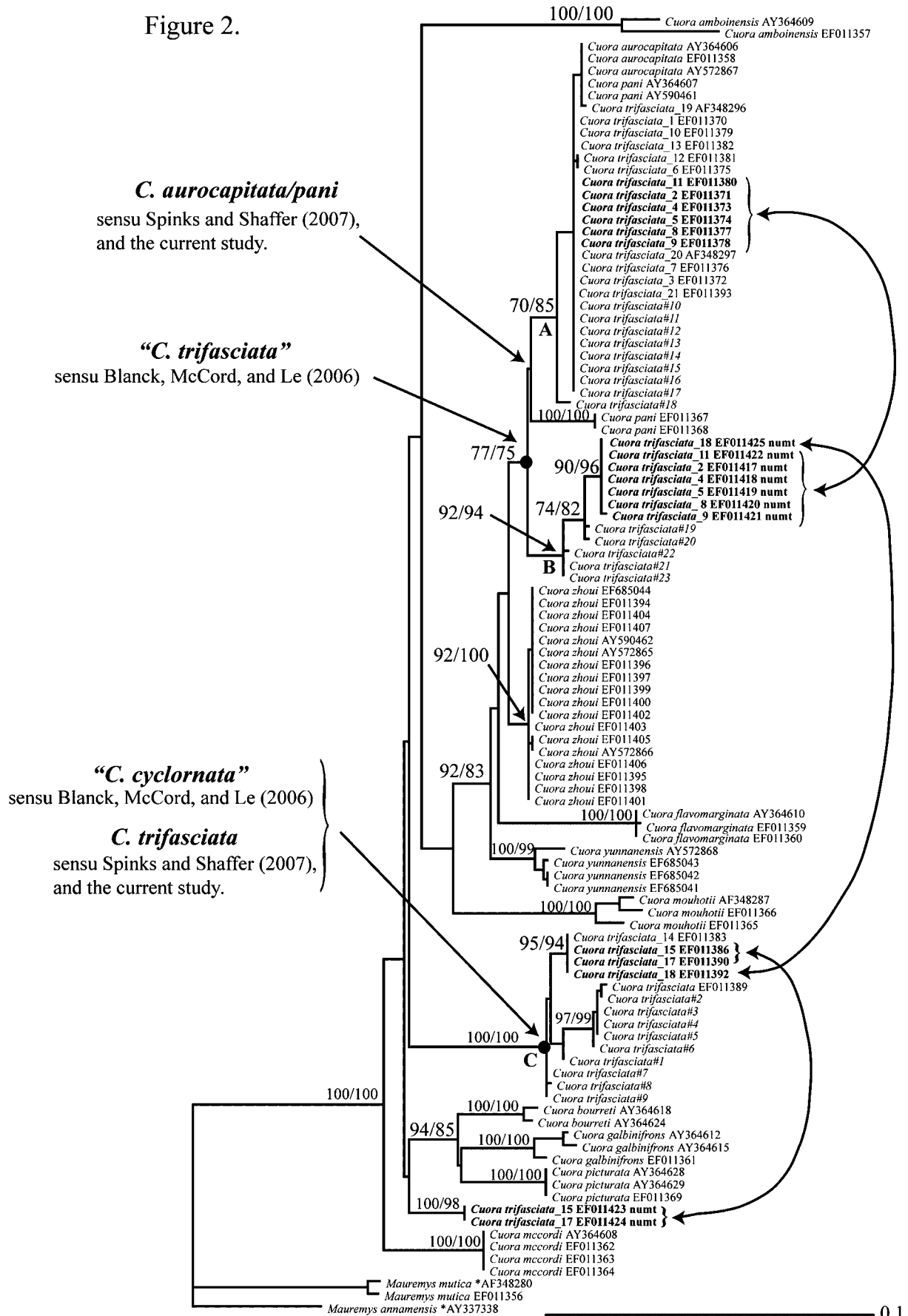
Figure 2 is one of two equally likely trees recovered from the ML analysis, and is congruent with the MP results discussed previously (i.e. Fig. 1B). In particular, 1) *C. trifasciata* sensu Blanck, McCord & Le (2006) is paraphyletic: 23 samples of *C. trifasciata* including eight from Blanck, McCord & Le (2006), and 14 from Spinks & Shaffer (2007) were virtually identical to three *C. aurocapitata* and two *C. pani* sequences, and 2) *C. flavomarginata* was not closely related to the *C. galbinifrons* species complex. In addition, our ML results present yet another conundrum in that clade B of Blanck, McCord & Le (2006) was recovered as sister (with strong support) to sequences identified by Spinks & Shaffer (2007) as putative numts. The arrows in Fig. 2 indicate the relative phylogenetic positions of the putative authentic and numt sequences recovered from each sample. For example, our ND4 primers amplified two different “ND4-like” sequences from specimen *Cuora trifasciata*_11. We interpret one of these as authentic ND4 sequence data (GenBank Accession #EF011380) and the other as a putative numt sequence (EF011422). These two sequences fell out in different parts of the tree, yet both were the result of single primer-pair amplifications from the same tissue sample.

Spinks & Shaffer (2007) suggested that the “*C. cyclornata*” haplotypes represented non-introgressed *C. trifasciata* mitotypes, and the analysis presented here, which included data from Spinks & Shaffer (2007) and data from Blanck, McCord & Le (2006) is consistent with this interpretation of the data. Although the gene trees, and the history that they reveal, are complex, the total genetic picture points to mitochondrial introgression in some (but not all) *C. trifasciata*, rather than cryptic speciation, as the most likely explanation for all of the currently available data.

This interpretation of mitochondrial introgression raises the possibility that the holotype of *C. trifasciata* itself contains an introgressed mitochondrion from another species. Fortunately, the ICZN rules on this matter preserve name availability for taxa with hybrid history provided that the species is still considered valid. Two Articles are relevant to this point. Article 17 of the ICZN states that “Names found to denote ... taxa of hybrid origin” remain valid as long as the taxon to which they refer is considered valid, whereas Article 23.8 simply states that the name assigned to an invalid hybrid species cannot be reassigned to either parental species, even if it has priority (ICZN, 2008).

If the holotype of *Cuora trifasciata* possesses an introgressed mitotype, but is still considered to be a member of a valid species, then under Article 17 it remains the legitimate name-bearer of the species. If the holotype is a hybrid individual, and not a member of a good species, then the name *trifasciata* would be invalid under Article 23.8 of the code. While the code does not explicitly define what is and is not considered a hybrid, it seems clear that the intent of the code is that an invalid hybrid name refers to a recent generation hybrid. *Cuora serrata* is an example of such a name. However, the evidence so far only suggests that an introgression event occurred at some time in the past (a common phenomenon in *Cuora*, and in turtles in general), but no evidence currently exists that the holotype is a recent-generation hybrid. Morphologically, the holotype is a typical *Cuora trifasciata* and nuclear data are not available for the specimen. Because of this, our assessment is that the name *Cuora trifasciata* is valid, and should be retained to maintain taxonomic stability. If new data show definitively that the holotype is a recent-generation hybrid, then a name change would be appropriate under the Code.

Figure 2.



0.1

Morphological evidence

Blanck, McCord & Le (2006) provide a table of 25 “diagnostic morphological characters” for five “populations” of *C. trifasciata* (their Appendix II), as well as plots of carapace width/carapace length (CW/CL) (their Appendix III). These two appendices, coupled with the actual species descriptions constitute the morphological evidence presented for “*Cuora cyclornata*” and its two named subspecies. While average ratios of some values are presented, no ranges, error bars or confidence intervals are presented, and no statistical tests are provided. The lack of any formal statistical analysis makes it virtually impossible to place this variation into a meaningful quantitative context. Moreover, the ‘diagnostic’ characters listed in Appendix II are at least equally consistent with two alternative interpretations; clinal variation and hybridization. *Cuora trifasciata* is widely distributed from Vietnam to southeastern China (Ernst & Barbour, 1989) and the morphological and phenotypic variation could be interpreted as clinal variation running roughly from north to south within the range of *C. trifasciata*. In addition, the morphology/phenotype of hybrid offspring are most often intermediate between that of the parental types. For example, “*Cuora serrata*” has physical and genetic characteristics intermediate between those of *C. galbinifrons* and *C. mouhotii*, its hypothesized parental species (Stuart & Parham, 2007). Thus, we would expect offspring of hybridization between *C. trifasciata*, and *C. aurocapitata/pani* to be somewhat intermediate between the parental taxa. Blanck, McCord & Le (2006) did not address the possibility of clinal variation, nor did they entertain the hypothesis of hybridization or present morphometric data on other members of *Cuora*. A more rigorous treatment of morphology in a species-delimitation context would have subjected the author’s measurement (plus measurements of the other, morphologically similar taxa) to a formal statistical morphometric analysis (see Lovich & McCoy, [1992] for a good example). Finally, the sample sizes examined by Blanck, McCord & Le (2006) were small enough that “diagnosability” should be viewed with caution. The descriptions of “*C. cyclornata*” on page 58 indicate that only five museum specimens were examined. We understand well the difficulty in gathering population-level information on *Cuora* species, and so the small sample sizes in Blanck, McCord & Le (2006) are both understandable and not a fault of the study. However, the small sample size combined with the lack of formal morphometric analysis suggests that the morphological assessment of Blanck, McCord & Le (2006) should be interpreted cautiously, particularly with respect to diagnosing new taxa.



FIGURE 2. One of two equally likely trees recovered from ML analysis of the 105-taxon mitochondrial ND4 data set (820 bp). This tree included ND4 sequences from Stuart & Parham (2004), Parham *et al.* (2004), Blanck, McCord & Le (2006) as well as sequences from He *et al.* (2007), and Spinks & Shaffer (2007) (including putative numts). Estimated ML model parameters conform to the HKY+I+G model of sequence evolution. $-\ln L = 3659.46827$. Base frequencies: A = 0.36, C = 0.27, G = 0.12, and T = 0.25. Ti/Tv ratio = 11.4782, proportion of invariant sites (I) = 0.4392, and γ -shape parameter = 0.9940. Numbers above branches are ML/MP bootstrap support values. Terminals are species followed by GenBank Accession number except for the *C. trifasciata* samples from Blanck, McCord & Le (2006) which were not deposited in GenBank and are indicated with the # sign. For example, *Cuora trifasciata*#10 is from Blanck, McCord & Le (2006). Samples containing putative numts are in **Bold**, and the arrows indicate the disjunct placement of the recovered sequences. For example, Spinks & Shaffer (2007) recovered two sequences from the sample *Cuora trifasciata*_15 including EF011386 and EF011423, and determined that sequence EF011386 represented authentic mtDNA, while EF011423 was a putative numt (see Spinks & Shaffer 2007 for detailed methods).

Higher-level taxonomic issues

Although our main goal is to address a problematic species description, a brief discussion of the higher-level taxonomic implications of this work is also warranted. The assessment of Blanck, McCord & Le (2006), the phylogenetic results of Spinks & Shaffer (2007) as well as the mitochondrial analyses presented here are all pertinent to an ongoing debate over the number of species as well as the generic allocation of the taxa currently classified as *Cuora*. For example, among four recent lists of the world's turtle species, two recognize 10 species and place all of them into the single genus *Cuora* (Fritz & Havas, 2007; Turtle Taxonomy Working Group, 2007b), one recognizes 10 species (including *C. "serrata"* – a known hybrid) and places them into three genera (Rodrigues *et al.*, 2008), and one recognizes 12 species, but places them into four genera (Iverson, 2008). This latter arrangement is also favored by Blank, McCord & Le (2006, p 14), and includes 1) retaining *Cuora mouhotii* in the previously-recognized, monotypic genus *Pyxidea*, 2) recognizing *Cuora bouretti* and *Cuora picturata* as full species, and relegating these taxa as well as *Cuora galbinifrons*, *C. flavomarginata*, and *C. mccordii* to the “high-domed” genus *Cistoclemmys*, 3) relegating *C. aurocapitata*, *C. pani*, *C. trifasciata* (including “*C. cyclornata*”), *C. yunnanensis* and *C. zhoui* to the genus “*Pyxiclemmys*”, and 4) restricting the new genus *Cuora* to contain a single species, *C. amboinensis*. Based on available evidence, including that presented here and our ongoing work on these turtles, we agree that *C. bouretti* and *C. picturata* are good species. However, we disagree with splitting *Cuora* into multiple genera. Such a change would shift the names of CITES-listed taxa, potentially invoking necessary legislative action by some countries to extend protection to the reclassified taxa (Turtle Taxonomy Working Group, 2007a). It would also require a complex nomenclatural shift for a small (~12 species), morphologically compact, demonstrably monophyletic clade of animals. Implementing these changes for *Cuora* would run contrary to what we view as best scientific practices. For example, the Turtle Taxonomy Working Group (2007a) enumerated six guidelines for consideration when contemplating taxonomic changes, and these proposed revisions are in conflict with four of the six recommendations. Since all previous analyses recover *Cuora* as a monophyletic clade, a taxonomic revision for the 12 contained species is *not* necessary. In addition, erecting three additional genera for this relatively small group would unnecessarily destabilize taxonomy. A further consideration is that the conflict between nuclear and mitochondrial phylogenies, combined with many low bootstrap support levels, suggests that we still do not understand the among-species phylogeny for *Cuora*, and any taxonomic changes based on phylogenetic considerations must be viewed as premature. Finally, erecting monotypic genera for *mouhotii* and *amboinensis* would be redundant (that is, it would provide no new information about evolutionary history beyond the recognition that these are distinct species), and would erase the phylogenetic information that higher-level names can, and in our minds should, provide (Parham & Feldman, 2002; Spinks *et al.*, 2004). On the whole, the single genus *Cuora* adequately describes the diversity present in the group and we view further revision, based on current data, to be unnecessary, unwarranted, and ultimately detrimental.

Conclusion

Naming new taxa is an important end product of systematic research that should be strongly data driven, and should be completed only when multiple lines of informative data have been collected and analyzed. Anything less can lead to confusing taxonomies that muddle rather than clarify the biological insights and conservation priorities that can be derived from phylogenetic research. While variation undoubtedly exists within *C. trifasciata* (as it does in many wide ranging species), Blanck, McCord & Le (2006) failed to assess crucial existing mitochondrial DNA sequences, did not consider alternative, biologically plausible explanations of the existing data, and did not operate under an explicit species delineation method. *Cuora trifasciata* may contain distinct lineages, and they may ultimately be shown to represent undescribed species. However, the evidence currently available, and available in 2006, does not support the view that “*C. cyclornata*” is a valid taxon. Thus, the most responsible approach to the systematics of this confused group of

endangered turtles is to treat “*Cuora cyclornata*” as a junior synonym of *Cuora trifasciata*.

Species delimitation is a difficult science and—in part, because of this—is of great interest in systematics. A voluminous literature exists on species criteria (reviewed by de Queiroz, [1998]), appropriate methods for operationalizing criteria to delimit species (reviewed by Sites & Marshall, [2003, 2004]), and pitfalls that should be avoided (Sites & Crandall, 1997), and a recent issue of *Systematic Biology* (vol. 56, #6) was devoted to species delimitation problems and solutions. In the case of very closely related species, well-known problems with gene coalescence times and single-gene introgression necessitate information from multiple genetic sources, non-traditional methodological approaches, and a healthy dose of caution (Cummings *et al.*, 2008; Knowles & Carstens, 2007; Parham *et al.*, 2006; Shaffer & Thomson, 2007). Given the complexities of species delimitations, not all species descriptions will end up being valid and all of us will undoubtedly make mistakes, regardless of the amount of care going into one’s work. Because of this, critical evaluation and careful science must be the basic tenet of our field as we move forward discovering, describing, studying and conserving the world’s diversity.

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