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On the reclassification of Box Turtles (*Terrapene*): A response to Martin *et al.* (2014)

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Species delimitation is a central issue in all fields of biology, and it is closely tied to the species concept employed. Thousands of pages have been filled with debates about competing species concepts; for instance, Coyne & Orr (2004) counted no less than 25 distinct species concepts. Among these are several phylogenetic species concepts. The paper by Martin *et al.* (2013) on box turtles is one of many recent publications that raise formerly recognized subspecies to the species level, based on molecular genetic evidence and favouring implicitly or explicitly a phylogenetic species concept as the theoretical foundation. We wish to underline that there is growing concern with respect to this approach (see Zachos *et al.* 2013 for mammals), and this concern guided our recent taxonomic update for turtles (Fritz & Havaš 2013). Without intending to enter the debate on species concepts, we will briefly explain why we are not convinced by the conclusions of Martin *et al.* (2013).

Using sequence data of two mitochondrial genes (cyt *b*, COI) and one nuclear locus (GAPD), Martin *et al.* (2013) studied the relationships of box turtles (*Terrapene*) and recognized, like previous authors, the species *Terrapene coahuila*, *T. nelsoni* and *T. ornata*. However, with respect to the fourth generally accepted species, *T. carolina*, they proposed that this taxon should be split into two distinct polytypic species, *T. carolina* (containing the subspecies *T. c. carolina*, *T. c. bauri* and *T. c. major*) and *T. mexicana* (containing the subspecies *T. m. mexicana*, *T. m. triunguis* and *T. m. yucatana*). The three taxa referred to *T. mexicana* are fully allopatric, with the two subspecies from Mexico (*mexicana* and *yucatana*) occurring in completely isolated distribution ranges (Smith & Smith 1980; Ernst & Lovich 2009). However, in the southern USA, *triunguis* intergrades widely with other subspecies of *T. carolina* (Carr 1952; Ernst & Lovich 2009), a fact recently corroborated by microsatellite data together with evidence from morphology and mtDNA sequences (Butler *et al.* 2011). Based on microsatellite loci, Butler *et al.* (2011) found no population structuring in the contact zone, suggestive of a panmictic population with complete genetic admixture of the involved taxa. Furthermore, using extensive additional analyses of morphology and the rapidly evolving mitochondrial D-loop, Butler *et al.* (2011) concluded that "*T. c. major* is not a distinct evolutionary lineage but, instead, a mixture of extant taxa plus the extinct [subspecies] *T. c. putnami*." In contrast, Martin *et al.* (2013) treated *T. c. major* as a valid subspecies, without discussing these obviously contradictory results.

Martin *et al.* (2013) used their cyt *b* and GAPD data for phylogenetic inference. The COI sequences were used for a barcoding approach, which was inconclusive. It showed that several subspecies of *T. carolina*, including subspecies which are accepted as conspecific by Martin *et al.* (2013), differ by divergence values which exceed the universal barcoding threshold for distinct species (2%). This is in line with a recent review article on DNA barcoding using turtles as a case study, which pointed out that "no particular level of divergence can serve to identify species" (Shen *et al.* 2013), or in other words, whenever possible additional evidence is needed, and barcoding thresholds to be used as critical values for species delimitation need to be adjusted individually for smaller taxonomic groups (e.g., Fritz *et al.* 2012; Vargas-Ramírez *et al.* 2012).

Based on cyt *b* and GAPD sequences, Martin *et al.* (2013) discovered within *T. carolina* sensu lato two major clades. In the mitochondrial tree (Fig. 1: top left), one well-supported major clade was comprised of sequences of *T. c. mexicana*, *T. c. triunguis* and *T. c. yucatana*; and the other major clade contained the sequences of *T. c. carolina, T. c. bauri* and of what Martin *et al.* (2013) called *T. c. major*. However, mitochondrial sequences of "*T. c. major*" were paraphyletic with respect to the allopatrically distributed *T. coahuila*, a taxon which is accepted as a species distinct from *T. carolina* since its description in 1944 (Schmidt & Owens 1944; Smith & Smith 1980; Ernst *et al.* 2000; Dodd 2001; Butler *et al.* 2011; Martin *et al.* 2013), and support values for monophyly of the second major clade were low. Support for the two major nuclear clades was also weak (Fig. 1: top right). Moreover, these two nuclear clades conflicted with the species

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delimitation proposed by Martin *et al.* (2013) in that sequences of *T. c. carolina*, *T. c. bauri*, *T. c. major* and *T. c. triunguis* occurred in both clades. Consequently, we see no convincing phylogenetic evidence for splitting *T. carolina* sensu lato into two distinct species, regardless of the species concept applied.

Our conclusion is supported by the alternate phylogeny reported by Spinks *et al.* (2009), a study not mentioned by Martin *et al.* (2013, 2014). Using sequences of the cyt *b* gene and seven nuclear loci, Spinks *et al.* (2009) found *T. c. bauri* clustering with *T. c. triunguis* (Fig. 1: bottom).

Like Martin *et al.* (2013), Spinks *et al.* (2009) found *T. carolina* to be paraphyletic with respect to *T. coahuila*. However, for the rapidly evolving D-loop Butler *et al.* (2011) found *T. carolina* not to be paraphyletic with respect to *T. coahuila*. Yet, they reported nearly identical haplotypes of a *T. carolina* from Escambia County (Florida) and *T. coahuila*, but these haplotypes were highly distinct from all other haplotypes of *T. carolina*. This confusing situation demands further research, and this issue is beyond the current discussion.

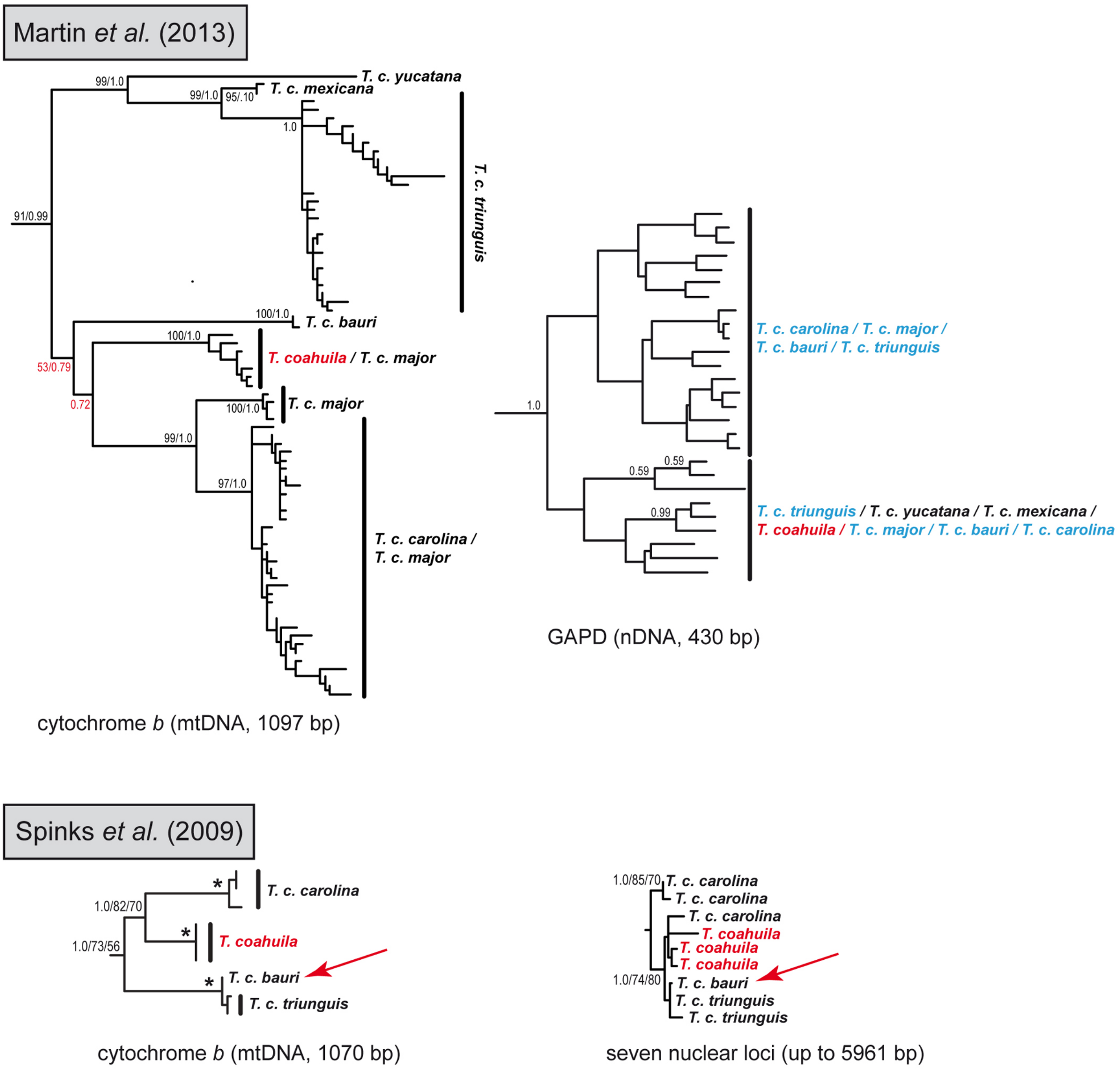

FIGURE 1. Phylogenetic trees for *Terrapene carolina* and *T. coahuila*. Top: Trees based on Maximum Likelihood and Bayesian analyses, redrawn from Martin *et al.* (2013). Shown are all support values provided in the original publication (bootstrap values and posterior probabilities). Note the weak support values (red) for the clades within *T. carolina*, the paraphyly of *T. carolina* with respect to *T. coahuila* (red) and the shared taxa in the nuclear tree (blue). The missing support values on the basal nodes of the nuclear tree suggest weak support. Bottom: Maximum Likelihood trees redrawn from Spinks *et al.* (2009). Support values are Bayesian posterior probabilities, ML and MP bootstrap values. Asterisks indicate support values ≥ 0.95/90/90. Note the placement of *T. c. bauri* (red arrow) conflicting with the results of Martin *et al.* (2013).

To justify their species delimitation, Martin *et al.* (2014) now argue that many species of animals may hybridize, and hence that intergradation among *T. carolina* subspecies does not invalidate their splitting of *T. carolina* in two distinct species. Martin *et al.* (2014) cite, among others, a paper reporting that up to 14% of box turtles from eastern Texas are hybrids between *T. carolina* and *T. ornata*. However, if 14% are hybrids, 86% of the concerned populations must be pure, and this is evidence for the maintenance of largely distinct gene pools in sympatry. By contrast, *T. carolina* forms panmictic populations whenever different subspecies meet (Butler *et al.* 2011), and this is fundamentally different from the situation in *T. carolina* and *T. ornata*. Consequently, the recognition of *T. mexicana* as a species distinct from *T. carolina*, as proposed by Martin *et al.* (2013, 2014), is unwarranted.

We are not disputing that distinct species may hybridize, and there are many cases known, especially in turtles and tortoises, as well as in other taxa (e.g., Kraus *et al.* 2012). However, interspecific hybridization is different from intergradation among subspecies. The example of *T. carolina* and *T. ornata* shows that distinct species are capable of maintaining largely discrete gene pools, allowing them to occur together in widely overlapping distribution ranges. The region of sympatry for *T. carolina* and *T. ornata* corresponds to a vast area including the eastern parts of Texas, Oklahoma and Kansas, most of Missouri, western Arkansas, western Louisiana, and parts of Illinois and Indiana (Ernst & Lovich 2009). In contrast, the genetic and morphological distinctness of the subspecies of *T. carolina*, including *T. c. triunguis*, dissolves in their contact zone (Butler *et al.* 2011), which constitutes a true genetic melting pot. As a legacy of the distinct subspecies, only the deeply divergent mtDNA lineages remain, which are inherited through the maternal line (without recombination). A similar case of genetic admixture is known for subspecies of spur-thighed tortoises (*Testudo graeca*) in the Caucasus region (Mashkaryan *et al.* 2013).

We respect the conservation-oriented motives of Martin and coauthors for their proposal to revise the taxonomy of *Terrapene carolina*. Martin *et al.* (2014) argue "because the *Terrapene* are of conservation concern throughout their range […], and because many conservation efforts are species-based and tend to ignore subspecies, it is imperative that their classification be correctly resolved". However, we are also convinced that only well-founded taxonomic decisions serve conservation and science (see also Karl & Bowen 1999; Zachos *et al.* 2013), and will therefore continue to treat *T. c. mexicana*, *T. c. triunguis* and *T. c. yucatana* as subspecies of *Terrapene carolina*.

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