Short Communication

Multilocus phylogeny of the New-World mud turtles (Kinosternidae) supports the traditional classification of the group

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ABSTRACT

A goal of modern taxonomy is to develop classifications that reflect current phylogenetic relationships and are as stable as possible given the inherent uncertainties in much of the tree of life. Here, we provide an in-depth phylogenetic analysis, based on 14 nuclear loci comprising 10,305 base pairs of aligned sequence data from all but two species of the turtle family Kinosternidae, to determine whether recent proposed changes to the group’s classification are justified and necessary. We conclude that those proposed changes were based on (1) mtDNA gene tree anomalies, (2) preliminary analyses that do not fully capture the breadth of geographic variation necessary to motivate taxonomic changes, and (3) changes in rank that are not motivated by non-monophyletic groups. Our recommendation, for this and other similar cases, is that taxonomic changes be made only when phylogenetic results that are statistically well-supported and corroborated by multiple independent lines of genetic evidence indicate that non-monophyletic groups are currently recognized and need to be corrected. We hope that other members of the phylogenetics community will join us in proposing taxonomic changes only when the strongest phylogenetic data demand such changes, and in so doing that we can move toward stable, phylogenetically informed classifications of lasting value.

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1. Introduction

Over the last few decades, a clear consensus among the systematics community has emerged regarding the primacy of monophyly in defining named groups above the species level (e.g. Funk and Omland, 2003). In keeping with the goal of eliminating non-monophyletic groups, the classifications of virtually all taxa have been re-evaluated and changed, often with the resultant recognition of new, relatively restricted clades at the genus and family levels. However, a growing concern has also been voiced that the rush to eliminate seemingly non-monophyletic taxa has led to premature changes in traditional classifications. Phylogenetic analyses based on one or a few genes often recover spurious clades based on idiosyncratic gene trees rather than organismal lineages, producing sub-optimal classifications. The resulting taxonomic confusion (some would say chaos) has been identified as a growing concern, leading to inconsistent usage of established names, multiple competing nomenclatural schemes, and reduced, rather than improved communication among systematists and biodiversity scientists (Pauly et al., 2009; TITSG, 2012; Vences et al., 2013).

The turtle superfamily Kinosternoidea (Joyce et al., 2004) is a case in point, and the focus of our study. The Kinosternoidea currently contains 26 species including the monotypic family Dermatemydidae (Central American river turtle) and the family Kinosternidae (the mud and musk turtles) containing four genera: Kinosternon (18 species), Sternotherus (4 sp.), Staurotypus (2 sp.), and the monotypic Claudio (TTWG, 2012). The Kinosternoidea are highly aquatic turtles distributed from southern Canada south through Brazil and northern Argentina (TTWG, 2012). Since the late 1970s, several systematic analyses have been completed for Kinosternidae using morphological, karyotypic, and allozyme data. In one of the earliest of these studies, Seidel et al. (1986) analyzed allozyme data for the 18 kinosternid species recognized at that time, and a few years later Iverson (1991) combined the Seidel et al. (1986) allozyme data set with 27 morphological characters and generated phylogenies based on morphology alone and combined morphology + allozyme data (Fig. 1). Most recently, Iverson et al.
Fig. 1. Previous phylogenetic hypotheses for the Kinosternoidea including analyses of (a) morphological characters, (b) allozyme data, (c) combined morphology plus allozyme data, (d) supertree analyses, (e) COI data, and (f) mtDNA plus nuDNA. Panel f also contains nodes with Bayesian posterior probabilities as indicated. Citations for each are indicated in the figure and text. (See above-mentioned references for further information).
utilized combined mtDNA + nuDNA sequence data for all members of the Kinosternoidea and recovered a tree topology that strongly differs from previous analyses generated from allozyme and morphological data (Fig. 1). Consistent across all of these analyses, however, is the sister-group relationship between *Claudius* and *Staurotypus* (often recognized as Staurotypinae), and (usually) the sister group relationship between Staurotypinae and the remaining kinosternids (Kinosterninae). However, relationships within Kinosterninae have been quite difficult to resolve, and no consensus among the three major analyses based on the different types of data has emerged (Fig. 1).

In the most recent comprehensive analysis, Iverson et al. (2013) utilized nucleotide sequence data from three mitochondrial genes (Cytb, 12S, 16S, 1958 bp of aligned data), and three nuclear loci (cmos, RAG1, RAG2, 2553 bp) for all living species of Kinosternidae. Among several novel phylogenetic results, Iverson et al. (2013) resolved *Sternotherus* and each of two subgroups of *Kinosternon* as reciprocally monophyletic. However, their analysis resolved *Kinosternon* as paraphyletic with respect to *Sternotherus*, with the latter identified as the sister group of a large subgroup of North, Central and South American *Kinosternon* (Fig. 1). Based on the non-monophyly of *Kinosternon*, Iverson et al. (2013) proposed the new genus *Cryptochelys*, comprising the six taxa previously classified as *K. acutum*, *K. angustipons*, *K. dunnii*, *K. creaseri*, *K. herreraei* and *K. leucostomum* to maintain monophyletic genera within Kinosternidae. Iverson et al. (2013) provided both character-based and phylogenetic definitions of *Cryptochelys*, noting that the group has never been identified in the previous literature; in fact, the new genus name refers to the cryptic hidden nature of this previously unrecognized clade. In addition, and following some earlier researchers, Iverson et al. (2013) elevated the subfamily Staurotypinae to the family Staurotypidae based on the estimated age of the clade and differences in morphology and sex-determination mechanisms. Finally, Iverson et al. (2013) noted that several polytypic species may contain additional species-level taxa, and elevated *K. subrubrum steindachneri* and *K. scorioides abaxillare* to full species, *K. steindachneri* and *K. abaxillare*, respectively. Although Iverson et al. (2013) had samples for only a single exemplar of these subspecies, they felt justified in elevating them to full species, based partly on levels of morphological and genetic divergence between these individuals and their putative conspecifics, and partly on geographical distributional data.

Given the long history of competing hypotheses arising from different datasets, and the recent taxonomic changes proposed for the group, we reevaluated the phylogeny of Kinosternoidea using an expanded, more informative nuclear dataset to further elucidate the evolutionary history of this group. The Iverson et al. (2013) analysis was based almost exclusively on mitochondrial DNA (60% of their informative characters were in the Cytb gene, and 84% in their concatenated mtDNA data set), while their nuclear genes were virtually uninformative (see Table 1 in Iverson et al., 2013). We collected data for 14 nuclear loci from a panel of 49 individuals that includes all species of Kinsterinoidea with the exception of *K. alamosae* and *K. angustipons*. Our results are broadly concordant with those arising from the combined morphology and allozyme analysis of Iverson (1991), recovering reciprocally monophyletic groups consisting of the traditional genera *Kinosternon* and *Sternotherus*, and rejecting the monophyly of *Cryptochelys* (sensu Iverson et al., 2013). In addition, our results shed additional light on the enigmatic relationships among members of the *Kinosternon scorioides* and *K. subrubrum* species groups. We compare our findings to previous analyses, discuss taxonomic implications for the group, and argue for maintaining stability in taxonomy of the Kinosternoidea.

### 2. Materials and methods

#### 2.1. Taxon and marker sampling

Our taxon sampling consisted of two individuals each of *Dermatemys mawii*, *Claudius angustatus*, all species of *Sternotherus*, and *Staurotypus* and most species of *Kinosternon*. The sole exceptions were *K. alamosae* and *K. angustipons* (no samples), and *K. acutum*, *K. chimalhuaca*, and *K. integrum* (one sample each). This sampling included eight of the same individuals included in Iverson et al. (2013), additional samples from field-collected turtles, and a small number of pet trade animals provided by colleagues (Appendix S1). Our data set consisted of nucleotide sequences for 49 individuals from up to fourteen nuclear loci (Appendix S1). DNA was extracted from blood or soft tissue samples using a salt extraction protocol (Sambrook and Russell, 2001). Partial sequences of all loci were generated using 20 µl volume PCR reactions, with an initial denaturation of 60 s at 95 °C, followed by 40 cycles of denaturation (94 °C for 30 s), annealing (45 s at 57–62 °C), and extension (72 °C for 60 s) with a final extension period (72 °C for 10 min) (see Table S1 for locus-specific annealing temperatures, extension times and primers). All PCR products were sequenced bidirectionally by Beckman Coulter Genomics (http://www.beckmangenomics.com/).

#### 2.2. Phylogenetic analyses

Nuclear exons were translated using Geneious v5.1 (Drummond et al., 2010) to check for the presence of unexpected stop codons, frame shifts, and other indicators that pseudogenes may have been unintentionally sequenced. Alignments were carried out using the MAFFT software (Katoh et al., 2002) implemented in Geneious (Drummond et al., 2011). We used PartitionFinder (Lanfear et al., 2012) to choose both a partitioning strategy and models of molecular evolution. We performed Bayesian phylogenetic analyses using MrBayes v3.2 (Husonbeck and Ronquist, 2001; Ronquist and Huelsenbeck 2003; Ronquist et al., 2012) both for a concatenated data set and for each locus individually. Bayesian analyses consisted of two independent runs each comprising four incrementally heated chains that ran for 10,000,000 generations. We sampled the posterior distribution every 1000 generations, and checked for stationarity by ensuring that the potential scale reduction factor equaled 1, and the average standard deviation of split frequencies between independent runs approached 0. We examined the MCMC samples in Tracer and AWTY (Rambaut and Drummond, 2007; Nylander et al., 2008; Wilgenbusch et al. 2004) to ensure that all chains were sampling from the same target distribution. We discarded the first 25% of samples as burnin, provided the chains had reached stationarity prior to that point. Iverson et al. (2013) analyzed their mitochondrial data only as a single concatenated data set. To more thoroughly assess their mitochondrial results, we reanalyzed the mitochondrial data from Iverson et al. (2013) and performed Bayesian phylogenetic analyses on each mitochondrial gene individually using MrBayes with the settings listed above and the models of molecular evolution specified in Iverson et al. (2013). We again assessed convergence in Tracer and AWTY. Most MrBayes analyses were carried out through the CIPRES Web portal (www.phylo.org).

### 3. Results

#### 3.1. Concatenated nuDNA and single-gene phylogenies

Our nuDNA data set was composed of up to 10,305 base pairs (bp) for 49 individuals. The matrix was nearly complete with
~8.3% missing data. All generated sequences were submitted to GenBank (Appendix S1), and our nuDNA matrix was submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2: S15454). We found no indication of pseudogenetic sequences, and the optimal partitioning strategy selected via PartitionFinder was a single partition with the HKY + I + G model of sequence evolution.

The majority-rule consensus of the posterior distribution of trees from the Bayesian analysis of the concatenated, unpartitioned data set was generally well resolved, with 29/48 nodes supported with a posterior probability of 1.0. Support was particularly strong at the deeper phylogenetic levels. Staurotypinae (Staurotypus plus Claudius) was strongly supported, as was its sister-group relationship to Kinosterninae. *Sternotherus* was well supported both as a clade and as the sister group to a monophyletic *Kinosternon* (Fig. 2). Within *Sternotherus*, *S. carinatus* was recovered as the sister clade to *S. odoratus* with strong support, but our two *S. minor* individuals were paraphyletic (but without strong statistical support) with respect to a well-differentiated, monophyletic *S. depressus* (Fig. 2). Within *Kinosternon*, we recovered two major clades including a well-supported Clade “A” that was composed of *K. acutum*, *K. chimalhuaca*, *K. creaseri*, *K. dunni*, *K. herrerai*, *K. hirtipes*, *K. integrum*, *K. leucostomum*, *K. oaxacae*, *K. scorioides*, and *K. sonoriense*, and an additional Clade “B”, which was not well supported and included *K. arizonense*, *K. baurii*, *K. durangoense*, *K. flavescens*, and *K. subrubrum* (Fig. 2). Although we lacked a sample for *K. angustipons*, we recovered strong support (Bayesian posterior probabilities [PP] = 1.0) for the reciprocal monophyly of *Kinosternon*, including the taxa assigned to *Cryptochelys* in Iverson et al. (2013); i.e., *K. acutum*, *creaseri*, *dunni*, *herrerai* and *leucostomum* and *Sternotherus*. In our analysis, the clade containing taxa assigned to *Cryptochelys* also included *K. chimalhuaca*, *K. hirtipes*, *K. integrum*, *K. oaxacae*, *K. scorioides*, and *K. sonoriense* rendering *Cryptochelys* as defined by Iverson et al. (2013) paraphyletic with respect to these six taxa (Fig. 2). We performed additional phylogenetic analyses of the concatenated data set under the multispecies coalescent model using the BEAST software (Heled and Drummond, 2010). The resultant phylogeny differed from the concatenated tree with respect to some interspecific relationships, but was identical at the deeper nodes. Critically, these analyses recovered a monophyletic *Sternotherus* as the sister group to a monophyletic *Kinosternon* (including all those taxa assigned to *Cryptochelys*), and clades “A” and “B” (see Fig. 2). Because results from both models were similar, we report the concatenated tree as our preferred phylogenetic hypothesis, and do not show the tree generated under the multispecies coalescent model. However, results from the BEAST analyses are available from the authors.

Our phylogenetic reanalyses of the mitochondrial data from Iverson et al. (2013) (Cytb, 12S and 16S) revealed that analyses of the Cytb data alone recovered *Kinosternon* paraphyletic with respect to *Sternotherus*, and it did so with only weak support (Fig. S1). The gene tree generated from 12S was less informative, and recovered a monophyletic Kinosterninae, but neither *Kinosternon* nor *Sternotherus* were monophyletic (Fig. S2). The phylogeny generated from 16S recovered *Kinosternon* paraphyletic with respect to *Sternotherus*, *Claudius* and *Staurotypus* (Fig. S3), and a poorly-supported *Sternotherus*.

At the shallowest phylogenetic levels, our multilocus nuclear data set failed to recover *Staurotypus salvinii*, *Sternotherus minor* and four *Kinosternon* species as monophyletic (Fig. 2). *Staurotypus salvinii* was paraphyletic with respect to *Staurotypus triporcatus*, and *Sternotherus minor* was paraphyletic with respect to *Sternotherus depressus* (Fig. 2). Within *Kinosternon*, paraphyletic species based on this dataset were fairly widespread. *Kinosternon baurii* and *K. subrubrum* were reciprocally paraphyletic, and non-monophyly among the two members of the *K. scorioides* complex (*K. scorioides*, *K. s. cruentatum*) in our sample was extensive. *Kinosternon oaxacae* was also broadly paraphyletic with respect to other *Kinosternon* species (Fig. 2). These examples of non-monophyly at the species level were generally based on two individuals, suggesting that additional within-species sampling will be required to accurately delimit many kinosternid species. In addition, it is always possible that occasional tissues were misidentified; additional, field-collected and specimen or photo-vouchered samples would help to identify such potential problems.

4. Discussion

Many in the systematics community, ourselves included, feel that taxonomic revision should be approached cautiously and, generally, only when a large, multilocus data set firmly suggests that the current taxonomy does not accurately convey phylogenetic history. Results from our analyses shed light on the systematics of the Kinosternidae and in particular on several of the recently proposed taxonomic changes for this group. They also provide an additional cautionary note on proposing taxonomic changes based on only one or a few linked genes.

Although there is some limited evidence to the contrary, the weight of evidence strongly supports *Kinosternon*, including those taxa recently assigned to *Cryptochelys*, as monophyletic with respect to *Sternotherus*, and we strongly support following the traditional classification of the family. We base this recommendation on two lines of evidence. First, the evidence for the paraphyly of *Kinosternon* with respect to *Sternotherus* presented in Iverson et al. (2013) stems largely from a single mitochondrial gene with weak statistical support, while other genes are either equivocal or provide strong evidence against its monophyly. Individual loci from our analysis generally provided limited resolution for these taxa. However, 4/14 loci (*BDNF*, *NR2B2519*, *PAX*, *TB29*) recovered *Kinosternon* (including those taxa assigned to *Cryptochelys*) as monophyletic and three of these did so with reasonably strong support (i.e., PP > 0.90), while 2/14 loci (*AIING*, *HNFL*) recovered *Kinosternon* as paraphyletic with respect to *Sternotherus*. Among 14 nuclear loci, only *AIING* did so with strong support (and *AIING* failed to recover a monophyletic *Sternotherus*, Supplementary Figs. S4–S10). In addition, we found compelling evidence that *Cryptochelys* as defined by Iverson et al. (2013) is not monophyletic for the concatenated or single gene analyses. For the single gene analyses, 6/14 loci recovered a paraphyletic *Cryptochelys* with strong support (*AIING*, *HNFL*, *HMGB2*, *P2654*, *PAX*, and *R35*), and the remaining loci were largely unresolved and therefore uninformative with respect to the monophyly of *Cryptochelys* (Supplementary Figs. S4–S10). Thus, only the mitochondrial loci *Cytb* and *12S* provide support for *Cryptochelys*.

Concatenated and single locus nuclear gene trees neither support the monophyly of *Cryptochelys* nor the non-monophyly of *Kinosternon* with respect to *Sternotherus*, and therefore the splitting of *Kinosternon* is neither necessary nor justified. It appears that the Cytb gene tree driving the analysis of Iverson et al. (2013) is likely artifactual with respect to the species tree for the family. Because *Cryptochelys* is only monophyletic at the mitochondrial locus, and half of the nuclear loci reject the monophyly of *Cryptochelys* with modest-to-strong support, we suggest that the name *Cryptochelys* should be rejected. Retaining *Kinosternon* (sensu lato) maintains nomenclatural stability and more accurately represents the current state of phylogenetic knowledge for the Kinosternoidea. This result also suggests that the morphological characters used by Iverson et al. (2013) to define *Cryptochelys*, and the important biogeographic and dating analyses derived from that study, should be reevaluated in light of the phylogeny presented here (Fig. 2).

At a deeper phylogenetic level, Iverson et al. (2013) proposed elevating *Staurotypinae* to family status, even though the current
taxonomy with two subfamilies accurately reflects the known phylogeny of Kinosternidae. Iverson et al. (2013) justified elevating Staurotypinae largely on what those authors felt is a sufficient time of divergence (based on a time calibration for their tree), morphological distinctiveness, and because staurotypines have genetic sex determination while kinosternines have temperature dependent sex determination (Ewert et al., 2004). However, in the interest of maintaining taxonomic stability (Kaiser et al. 2013; TTWG, 2007), we suggest that the community maintain the historical treatment of Staurotypinae as a subfamily as has been done for decades. It is true that there are characters, like sex determination mechanism, that define these clades, but they can equally be used to define subfamilies or families. We recognize that the alternative, family-level arrangement has been suggested previously (e.g. Bickham and Carr, 1983), but also that the community has settled on the subfamilial taxonomic resolution.

Fig. 2. Majority-rule consensus of the posterior distribution of trees from the Bayesian analysis of the concatenated nuDNA data set (49 taxon, 10,305 bp). Bayesian posterior probabilities (PP) as indicated. Bold indicates those samples also utilized by Iverson et al. (2013).
Finally, our results are consistent with Iverson et al. (2013) in indicating that there is much uncertainty regarding species delimitation in the *K. scorioides* and *K. subrubrum* groups (and perhaps other species in Kinosternidae). Additionally, and as emphasized by Iverson et al. (2013), full resolution of species boundaries in these groups requires extensive additional geographic and genetic sampling. Given this uncertainty, we feel that taxonomic revisions within these groups are premature, and we recommend that *K. subrubrum steinadachneri* and *K. scorioides abaxillare* be retained as subspecies until analyses incorporating rangewide taxon sampling and many more markers can be brought to bear on these problematic groups. By taking this approach, we hope to avoid repeated taxonomic revision, and the instability that this generates at both the species and higher taxonomic levels.

4.1. Future research

Our results clearly reveal the need for additional work to clarify interspecific relationships within Kinosternidae, and especially species delimitations within several subclades of the group. For example, of 21 species with two (one with three) individuals, we recovered 14 as monophyletic, 13 of which had strong (PP = 1) support. However, *Sternotherus minor*, five *Kinosternon* and one *Staurotypus* were recovered as non-monophyletic with varying levels of support (Fig. 2). Iverson et al. (2013) reported similar levels of non-monophyly, with three of six species for which they had multiple (2–4) samples monophyletic and three non-monophyletic. Within the Kinosternidae, some species descriptions are based on very subtle morphological differences, and assigning individuals to species can be challenging. Thus, some instances of non-monophyly in both analyses could be due to incorrect species identification rather than true non-monophyly. Alternatively, some groups may contain unrecognized variation, or be so recent that lineage sorting is incomplete. In addition, recent work on other difficult turtle lineages in the genus *Pseudemys* (Spinks et al., 2013) has shown that single exemplar sampling can lead to well-supported, but spurious phylogenetic conclusions, suggesting that future work should include much greater taxon sampling coupled with extensive data sampling to better delimit species boundaries, particularly among shallowly diverged lineages.

*Kinosternon* serves as an excellent example of the current state of systematics for many clades where phylogenetic studies have proceeded from initial analyses of morphology, karyotype and allozymes to analyses of one or a few linked mitochondrial or nuclear DNA markers, to current analyses of multilocus nuclear DNA sequence data. As systematic analyses generally have proceeded through time, apparently paraphyletic groupings have often been suggested, only to be followed by more extensive analyses that confirm earlier hypotheses of monophyly. If an analysis provides multiple independent lines of evidence and strong support for non-monophyly, then taxonomic revisions may be warranted. Alternatively, if support for paraphyly is weak or based on only a one or a few markers or individuals, then taxonomic revision might not be an appropriate course of action until additional data are brought to bear and novel hypotheses can be subjected to more rigorous tests.

Phylogenetic incongruence among morphological characters, mtDNA data, and multiple nuclear loci is ubiquitous across the tree of life, and we agree with many systematists that caution is warranted before using any one phylogeny, especially one based largely or exclusively on a single locus, as the basis for taxonomic revisions. For understudied groups, we obviously do not know how well an existing phylogeny represents the evolutionary history of that group. However, given that phylogenetic incongruence is ubiquitous, and that the systematics community increasingly has access to the necessary tools for performing more thorough phylogenetic analyses, we hope that our community becomes more cautious in accepting what are de facto single-gene trees as the basis for taxonomic revisions. Instead, we should pursue analyses based on multiple individuals/species and multi-locus data. Only when results from these analyses show strong support for paraphyly, should taxonomic revisions be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.03.025.

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