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Short Communication

Fourteen nuclear genes provide phylogenetic resolution for difficult nodes in the turtle tree of life

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ABSTRACT

Advances in molecular biology have expanded our understanding of patterns of evolution and our ability to infer phylogenetic relationships. Despite many applications of molecular methods in attempts at resolving the evolutionary relationships among the major clades of turtles, some nodes in the tree have proved to be extremely problematic and have remained unresolved. In this study, we use 14 nuclear loci to provide an in depth look at several of these troublesome nodes and infer the systematic relationships among 11 of the 14 turtle families. We find strong support for two of the most problematic nodes in the deep phylogeny of turtles that have traditionally defied resolution. In particular, we recover strong support for a sister relationship between the Emydidae and the monotypic bigheaded-turtle, *Platysternon megacephalum*. We also find strong support for a clade consisting of sea turtles, mud and musk turtles, and snapping turtles. Within this clade, snapping turtles (Chelydridae) and mud/musk turtles (Kinosternidae) are sister taxa, again with strong support. Our results emphasize the utility of multi-locus datasets in phylogenetic analyses of difficult problems.

1. Introduction

Progress in the field of molecular biology has facilitated a rapid increase in the resolution of the tree of life. Our understanding of the evolutionary relationships among the major clades of turtles has greatly improved, primarily due to the recent increase in the extent of taxon and data sampling. Analyses of individual mitochondrial (mtDNA) or nuclear loci, mitochondrial genomes, concatenated data sets, and supermatrices have recovered well-resolved trees that agree in many aspects of their topology (Fujita et al., 2004; Krenz et al., 2005; Parham et al., 2006; Shaffer et al., 1997; Thomson et al., 2008; Thomson and Shaffer, in press) and much of the deep phylogeny of extant turtles is now well understood. However, relationships among several turtle families and higher taxa remain uncertain, including the placement of the bigheaded-turtle (*Platysternon megacephalum*, family Platysternidae), the sea turtles (Chelonioidea, families Cheloniidae and Dermochelyidae), the mud/musk and Central American river turtles (Kinosternoidea, families Kinosternidae and Dermatemydidae), and the snapping turtles (family Chelydridae). Two particularly problematic groups have been the placement of *Platysternon* and the snapping turtles.

Over the last decade, the phylogenetic position of *P. megacephalum* has been the focus of several analyses. *Platysternon megacephalum*, a freshwater turtle found in east Asia, is the sole member of the monotypic Platysternidae. Morphologically, both *Platysternon* and Chelydridae (consisting of the New World genera *Chelydra* and *Macrochelys*) have large heads, longs tails, and a similar overall body morphology. Based on these and other morphological features, Gaffney (1975) assigned *Platysternon* to the family Chelydridae. A subsequent analysis of 115 morphological characters combined with the mitochondrial cytochrome *b* (cyt*b*) gene + 12s rDNA data also supported this arrangement (Shaffer et al., 1997), with the bulk of that support derived from the morphological data (Fig. 1).

However, a more recent molecular analysis employing the cyt*b*/12s data from Shaffer et al. (1997) and some nuDNA data call the hypothesized *Platysternon* and Chelydridae sister group relationship into question. Based on an analysis of concatenated cyt*b*, 12s rDNA, and nuclear recombination activase gene (RAG-1) sequences, Krenz et al. (2005, see their Fig. 5b) and Near et al. (2005) recovered moderate support for a sister group relationship between *Platysternon* and the Testudinoidea, (collectively, the Emydidae, Geoemydidae [Old World pond turtles], and Testudinidae [tortoises]), while an analysis of U17 small nucleolar RNA

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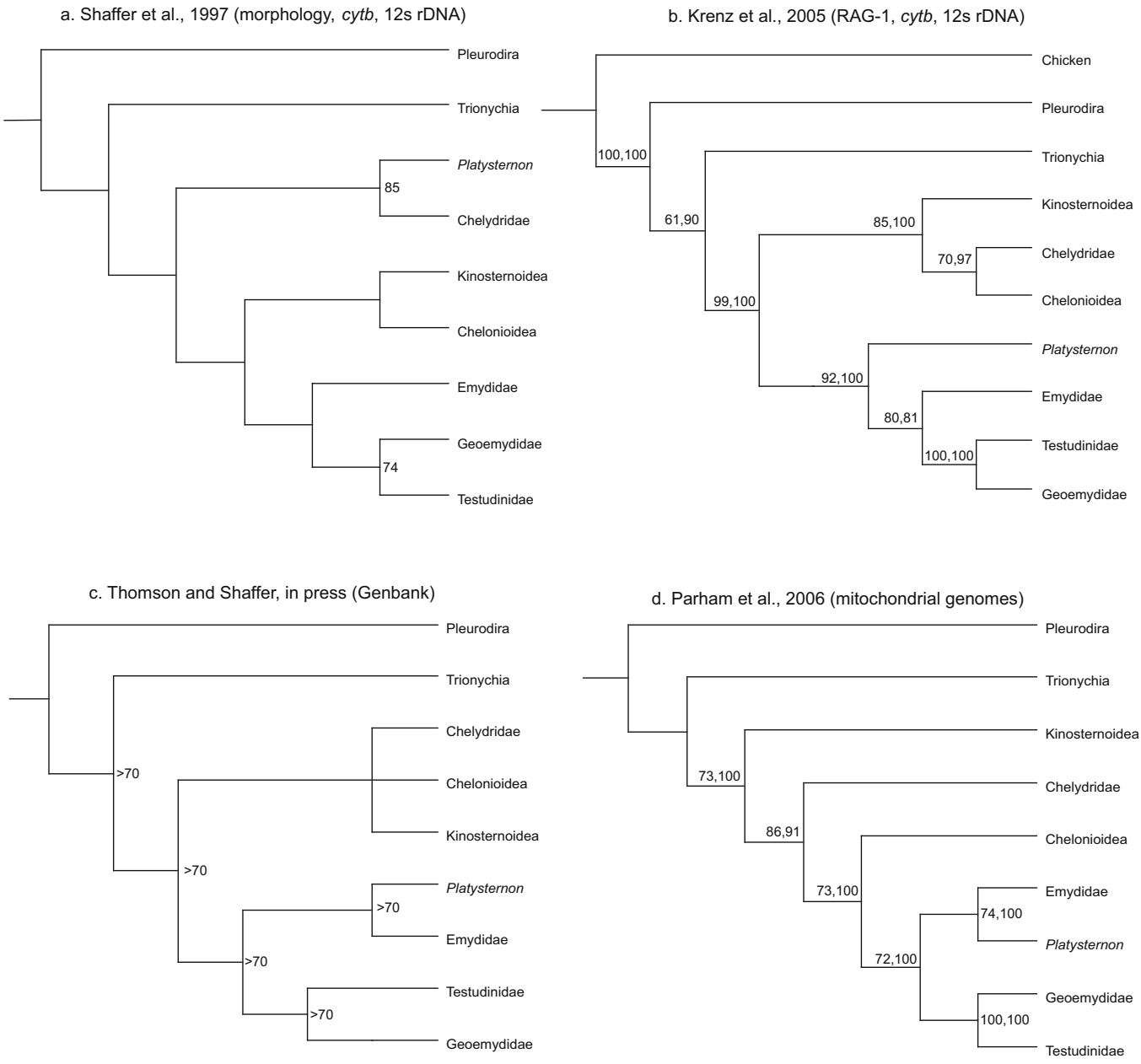

Fig. 1. Phylogenetic trees showing the higher level systematic relationships between the major turtle groups recovered by previous analyses. Source papers are provided for each panel. (a) Values represent MP bootstrap proportions, nodes without values represent nodes with less than 50% support. (b) ML/Bayesian tree from Krenz et al. (2005); values represent ML bootstrap proportions and BI posterior probabilities, respectively. (c) Values on nodes indicate MP bootstrap proportions (>70 but <95). (d) Values represent ML bootstrap proportions and BI posterior probabilities, respectively.

(snoRNA) recovered a polytomy composed of *Platysternon* and all of the Testudinoidea (Cervelli et al., 2003). A recent analysis of nearly complete mtDNA genomes of 12 turtle taxa recovered support for a sister group relationship between *Platysternon* and the Emydidae (Parham et al., 2006), a relationship also recovered from a supermatrix analysis of mtDNA and nuDNA data (Thomson and Shaffer, in press) (Fig. 1). Thus, while a close relationship between *Platysternon* and some/all testudinoids appears to be emerging, the precise placement of *Platysternon* remains to be resolved with confidence. However, the weight of available evidence strongly indicates that *Platysternon* is not closely related to chelydrid turtles.

Relationships among Chelonioidea, Kinosternoidea, and Chelydridae (excluding *Platysternon*) are even more obscure. Chelonioidea consists of the family Cheloniidae (5 genera, 6 species of hard-shelled marine turtles) and the monotypic leatherback sea turtle, *Dermochelys coriacea* (Dermochelyidae). The Kinosternoidea consists of the family Kinosternidae (4 genera, 23 species of New World turtles) and the monotypic Dermatemydidae (containing the Central American River turtle *Dermatemys mawii*). Shaffer et al. (1997) recovered Chelydridae + *Platysternon* as sister to (Testudinoidea, (Chelonioidea + Kinosternoidea)). Krenz et al. (2005) recovered a clade sister to the Testudinoidea that consisted of Chelydridae, Chelonioidea, and Kinosternoidea, within which *Chelydra* and Chelonioidea were sister taxa (Fig. 1). Based on full mitochondrial genomes, Kinosternoidea was recovered as the sister group to the entire clade of Chelydridae + Chelonioidea + Testudinoidea, but only with moderate support (Parham et al., 2006). The goal of Parham et al. (2006) was to assess the phylogenetic position of *Platysternon*, and they resolved this issue with strong support. However, their representative kinosternid con-

tained a relatively high proportion of missing data (~50%), and the instability of Chelydridae + Chelonioidea + Testudinoidea might be an artifact of that missing data (Lemmon et al., 2009). The supermatix analysis of Thomson and Shaffer (in press) also contained a high proportion of missing data, and provided no additional clarification of these relationships. Finally, a recent analysis aimed specifically at resolving the phylogenetic position of Chelydridae had limited success. Chandler and Janzen (2009) assessed the interfamilial relationships of turtles using three nuclear loci, and found no support for the position of Chelydridae. Based on a power analysis of their data, they concluded that the uncertain higher-level phylogenetic relationships among these deep turtle lineages might represent a hard polytomy.

Based on available evidence, it appears that *Platysternon* may be the sister taxon to the Emydidae (or minimally, fall within or be sister to Testudinoidea), but relationships among chelydrids, kinosternids, and sea turtles are less well understood, and the prospects for resolving these relationships may appear to be poor (at least with molecular data). Mitochondrial sequence data have been exhausted, and the signal from available nuDNA sequences is low, suggesting that increased genetic sampling may do little to help the problem (Chandler and Janzen, 2009). However, the nuclear markers employed by Krenz et al. (2005) and Chandler and Janzen (2009) represent some of the least variable markers used in recent analyses of turtles, and thus it remains possible that the addition of more, faster evolving nuclear markers could help resolve this issue.

Our goal was to use several additional loci in an attempt to resolve these areas of phylogenetic controversy. To place our results into a comparative context, we evaluate our data and topology against the best-resolved current hypotheses of deep level turtle relationships. Finally, we reanalyzed the Parham et al. (2006) mitochondrial data set in an attempt to rectify any incongruence that we find between the mtDNA and nuDNA data.

2. Materials and methods

2.1. Taxon sampling and marker identification and sequencing

We selected 19 taxa for our study, including exemplars from 11 of the 14 recognized turtle families. The representative species we chose for each taxonomic group were: *Emys marmorata* and *Chrysemys picta* (Emydidae), *Mauremys reevesii, Rhinoclemmys annulata*, and *Batagur trivittata* (Geoemydidae), *Psammobates pardalis*, *Manouria emys*, and *Testudo graeca* (Testudinidae), *Dogania subplana*, *Pelodiscus sinensis*, and *Carettochelys insculpta* (Trionychia), *Chelonia mydas* and *Dermochelys coriacea* (Chelonioidea), *Chelydra serpentina* and *Macrochelys temminckii* (Chelydridae), *Kinosternon flavescens* and *Sternotherus odoratus* (Kinosternoidea), *Pelomedusa subrufa* (Pleurodira), and *Platysternon megacephalum* (Platysternidae). We used chicken and/or alligator sequences as outgroups when available. Our taxonomic sampling included all of the species from the Parham et al. (2006) analysis in order to facilitate a direct comparison between mitochondrial and nuclear datasets (Appendix).

We generated nucleotide sequence data for a total of 14 nuclear markers, eight of which have not previously been used for chelonian systematics. These markers included four exons: the aryl hydrocarbon receptor 1 gene (AHR, ~650 bp), the bone morphogenic protein 2 (BMP2, ~600 bp), the zinc finger homeobox protein gene (ZEB2, ~900 bp) (Townsend et al., 2008), and the nerve growth factor beta polypeptide gene (AIING, ~700 bp) (Kimball et al., 2009), and four introns: the high mobility group protein B2 (HMGB2, ~650 bp), the 26S protease regulatory subunit 4 gene (P26s4, ~1000 bp), the KIAA0398 gene (NB22519, ~700 bp) (Backstrom et al., 2008), and the paired box gene (PAX1P1, ~1000 bp) (Kimball et al., 2009). In some cases, we redesigned new, turtle-specific primers if the originals failed to work for a particular specimen. The redesigned primers and their annealing temperatures are listed in Appendix. We also included six loci that had been previously used in turtle systematic studies: the recombinase activating gene (RAG-1) (Krenz et al., 2005), the hepatocyte nuclear factor-1α (HNF-1α) (Spinks and Shaffer, 2007), the fingerprint protein 35 (R35) (Fujita et al., 2003), the brain-derived neurotrophic factor gene (BDNF) (Noonan and Chippindale, 2006), and two anonymous loci (TB01 and TB29) (Thomson et al., 2008).

Genomic DNA was extracted from various soft tissue types using a standard salt extraction protocol. AmpliTaq mediated 20 μL PCR amplifications were performed under the following conditions: an initial 60 s at 95 °C, followed by 38 cycles of denaturation at 94 °C for 30 s, 45 s at the primer specific annealing temperature and 45–90 s (depending on the size of the gene segment to be amplified) of extension time at 72 °C. This was followed by a final extension period at 72 °C for 10 min. All PCR products were sequenced in both directions unless one direction failed to sequence, in which case we resequenced the direction that worked. All sequencing was conducted either by Agencourt Bioscience Corporation, or the UC Davis College of Biological Science Sequencing facility.

2.2. Phylogenetic analysis

Sequences were edited and subsequently aligned using MUSCLE (Edgar, 2004) in Geneious Pro 4.6. The alignments were then visually examined and translated for coding regions where appropriate using MacClade 4.06 (Maddison and Maddison, 2003). For more problematic alignments, we did an initial alignment using DIALIGN (Morgenstern et al., 2006), and then adjusted the alignment using the refinement algorithm in MUSCLE and by eye. In some cases, the outgroup sequences were too divergent from chelonians to make confident homology statements. In these cases, we excluded chicken and/or alligator and used *Pelomedusa subrufa* as the outgroup. Gene trees without *P. subrufa* are unrooted. Alignments were deposited in TreeBase (accession # S2508).

We performed Maximum Likelihood (ML) and Bayesian inference phylogenetic analysis for each gene individually, and on the concatenated dataset. We selected models of molecular evolution for each partition using decision theory implemented by DT-ModSel (Minin et al., 2003). Maximum likelihood analyses were performed with PAUP* 4.0b10 (Swofford, 2002) using 10 random stepwise heuristic searches and TBR branch swapping. Support was assessed via 100 nonparametric bootstrap replicates. For the concatenated nuclear sequence dataset, we ran ML analysis using RaxML (Stamatakis et al., 2008) through the CIPRES web portal (http://www.phylo.org). For the RaxML analyses, the dataset was partitioned by gene and bootstrapped with 100 replicates.

Bayesian phylogenetic analyses were run using MrBayes V3.1.2 utilizing two replicates with four chains each for 10^7 generations, sampling every 10^3 generations (Huelsenbeck and Ronquist, 2001). Individual genes were analyzed using a single partition while the concatenated data set was partitioned by gene, utilizing a separate best-fitting model for each locus. For protein coding genes, we also performed ML and Bayesian analyses of individual genes with the dataset partitioned by codon. However, changes in topology or support values were not observed, and so we partitioned the dataset by gene in the concatenated analyses. To assess convergence between the chains, we checked that the average standard deviation of split frequencies approached zero. We also verified that the potential scale reduction factor approached 1 and that the log likelihood scores had reached a stationary value. We were unable to sequence every specimen for every nuclear marker (despite numerous attempts), thus we had small amounts

of missing data for six genes (Appendix). Missing data has been shown to affect topology in some situations (Lemmon et al., 2009), so we performed additional ML and Bayesian analyses on a reduced eight-gene data matrix with no missing data as a check for these effects.

The mitochondrial genome data set of Parham et al. (2006) contained 37 partitions overall, as well as ~50% missing data for their representative kinosternid. In order to determine if the unstable position of the Kinosternidae (or Kinosternoidea) might be due to an effect of model and partition choice or the effects of missing data, we performed four Bayesian analyses on the mitochondrial genome data set (1) as a single partition, (2) as partitioned in the original study, but with all partitions assigned the GTR + Γ model, (3) as a single partition, but with no missing data, and (4) on the original mitochondrial genome alignment. We also performed ML and BI phylogenetic analysis on our concatenated 14 gene dataset using only the 12 taxa from the Parham analysis to confirm that any differences we identified between our analysis were not due to taxon sampling differences.

Finally, we performed phylogenetic analysis on our full dataset of 14 genes using the program BEST (Liu and Pearl, 2007). BEST uses the joint posterior distribution of gene trees in order to estimate a species tree in a multiple locus analysis. The BEST software allows only one outgroup to be specified and we used chicken in our analysis. Convergence was assessed using the log likelihood values and by confirming that the average standard deviation of split frequencies was less than 0.10. Our initial MCMC runs had inefficient chain swapping, and decreasing the temperature parameter did not alleviate the problem. So, to assure that our runs were converging on a single global optimum, we performed four preliminary MCMC runs with four cold chains each for 1.5×10^7 generations and then used Tracer v1.4 (Rambaut and Drummond, 2007) to check that the chains were converging. We ran two final MCMC analyses with two runs and four chains each for 6×10^7 generations at a temperature of 0.01. This allowed for a small amount of chain swapping, and we again verified the convergence of all runs on a single optimum that matched the optimum reached by the four initial runs. We also did a final check of our analyses using Tracer, assuring that all the parameters and statistics of the final runs had reached stationarity and sufficient (>100) ESS values.

3. Results

Our nuDNA data set was composed of 10,648 base pairs (BP). This matrix was fairly complete with 7.3% missing data excluding the outgroups and 12.4% including the outgroups. The proportion of missing data was calculated using the total number of missing characters in the alignment. All single gene analyses resulted in trees with similar topologies, but usually with weaker support than recovered from the concatenated dataset (see Supplementary materials). Trees generated from individual loci were mostly congruent, recovering *Platysternon* as sister to the Emydidae at seven loci, while five loci recovered a polytomy, and two weakly supported an alternative topology. In addition, eight individual loci recovered Testudinoidea as sister to (Chelonioidea (Kinosternoidea, Chelydridae)), two recovered a polytomy, and four weakly supported an alternative topology.

Maximum likelihood and Bayesian analyses of the concatenated sequences recovered a single well-supported tree that placed *Platysternon* as sister to Emydidae, and Testudinoidea as sister to (Chelonioidea (Kinosternoidea, Chelydridae)), both with strong support (Fig. 2a). Results of our ML and Bayesian analyses of the reduced concatenated data set (containing only the 12 taxa from Parham et al., 2006) as well as the data set pruned of genes with missing data were identical to those of the full dataset, and had similar support levels (not shown). The BEST analysis (Fig. 2b) resulted in a well-resolved tree and recovered all of the same nodes as the concatenated analysis, except that the soft-shelled turtles were sister to all other turtles including, including the representative Pleurodire *Pelomedusa*, with strong support (Bayesian Posterior Probability (BPP) = 100).

Our reanalyses of the mitochondrial genome dataset recovered trees that were mostly congruent with those reported in Parham et al. (2006). Our reanalysis when all partitions were assigned a GTR + Γ model (Supplementary Fig. 4d) recovered a topology that was identical to Parham et al. (2006). However, our reanalysis of the original mitochondrial genome dataset (Supplementary Fig. 4a), the alignment without missing data (Supplementary Fig. 4b), and when the dataset was analyzed as a single partition (Supplementary Fig. 4c), all produced different trees from that in the Parham et al. (2006) analysis, and two of these new analyses recovered a sister relationship between chelydrids and kinoster-

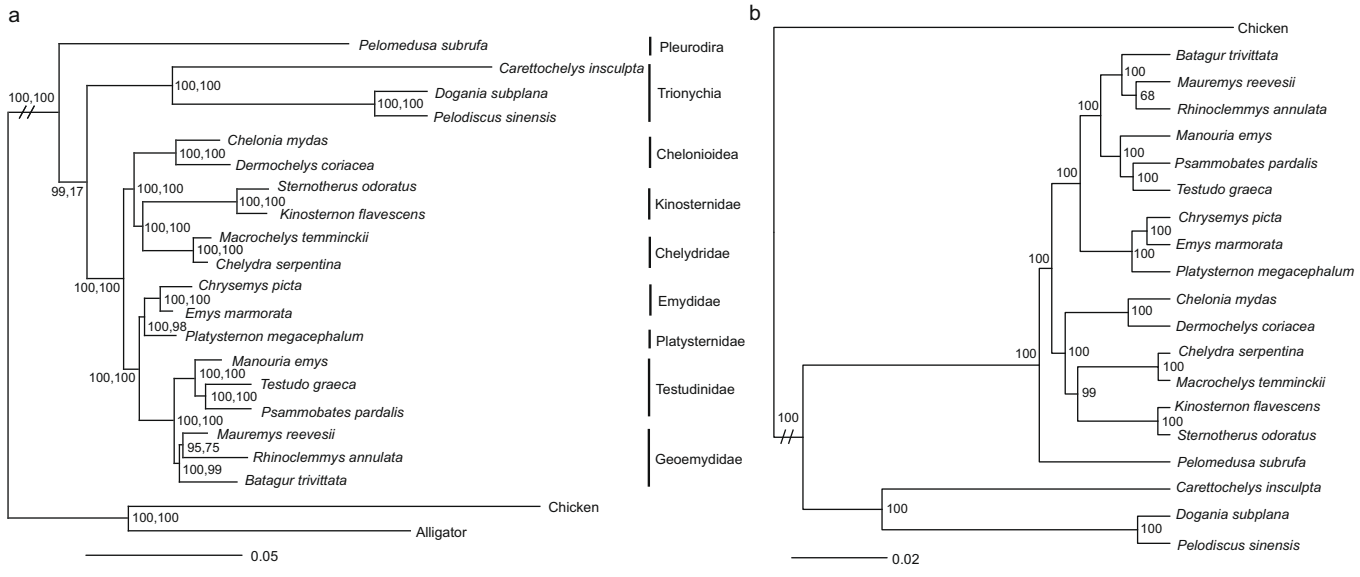

Fig. 2. (a) Phylogram generated from concatenated phylogenetic analysis of 14 nuclear genes. Numbers are BI posterior probabilities, followed by ML bootstrap proportions. (b) Species tree generated from BEST phylogenetic analysis of 14 nuclear genes. Numbers are BI posterior probabilities.

nids. However, this relationship received low support in our reanalyses (BPP 50 and 60).

4. Discussion

Analyses of single genes and the concatenated data set recovered mostly well-resolved, well-supported trees. The fact that most gene trees had similar topologies also provides us with strong confidence in our resolution of several difficult deep nodes in turtle phylogeny. Our results confirmed, with strong support, the placement of *Platysternon* as sister to the Emydidae, a result that Parham et al. (2006) recovered from analysis of mostly complete mitochondrial genomes and Thomson and Shaffer (in press) recovered from an analysis of all available nuclear and mitochondrial data. Previous morphological, karyological and serum electrophoresis analyses also suggest this placement (Haiduk and Bickham, 1982; Frair, 1972; Whetstone, 1978), as did the earlier morphological hypothesis of Williams (1950). Thus, the morphological similarities between *Platysternon* and chelydrid turtles are most likely the result of convergence (Danilov, 1998; Krenz et al., 2005) and we feel that the weight of all evidence firmly places *Platysternon* as sister to the Emydidae.

Compared to previous analyses, our results differed on relationships among kinosternoid, chelonioid, and chelydrid turtles. Based on mtDNA only, Parham et al. (2006) found that Chelonioidea, Chelydridae, and Kinosternoidea were sequential sister groups to Testudinoidea (including *Platysternon*) (Fig. 1d). In contrast, we found that Kinosternoidea, Chelydridae, and Chelonioidea form a monophyletic group that is sister to Testudinoidea, a relationship that is consistent with the supermatrix analysis of Thomson and Shaffer (in press), the analysis of RAG-1 alone and in combination with cyt*b* and 12s (Krenz et al., 2005), and RAG-1 in combination with the cyt*b* and nuclear R35 genes (Near et al., 2005). Within this clade, Kinosternoidea (more precisely, Kinosternidae, since we lack data for *Dermatemys mawii*) was sister to Chelydridae with strong support – a relationship that has been suggested before with weak support in an analysis of RAG-1 data by itself under ML/BI but not MP (Krenz et al., 2005) and in a combined mtDNA and nuDNA dataset analysis (Near et al., 2005). Although the mtDNA genome and nuDNA results are incongruent (the data set from Parham et al., 2006 never recovered the (Kinosternoidea, Chelydridae, and Chelonioidea) clade, even with weak support), only one of the two nodes that differ was strongly supported in the mtDNA genome analysis. Some of our reanalyses of the mtDNA genome data set were fairly similar to our nuDNA results in that a *Chelydra/Kinosternon* sister relationship was found (as was the case under BI in Parham et al., 2006), although in all cases this support was extremely weak and dependent on the details of analysis.

Although the resulting trees from our concatenated nuDNA and BEST species tree analyses were nearly identical, there was one interesting difference: the placement of the soft-shelled turtles (the Trionychia) and side-necked turtles (the Pleurodira) with respect to the remaining turtle families. Results of our concatenated analysis placed *Pelomedusa* (our representative pleurodire) in its traditional position as sister to the Cryptodira (all other turtles in our analysis, including the Trionychia) with strong support from the Bayesian analysis, but with extremely weak ML support values (Fig. 2a). However, our BEST analysis resulted in a species tree with soft-shelled turtles sister to all other turtles including side-necked turtles. Several of our alignments did not include outgroup sequences, but out of the seven gene trees that contained chicken and/or alligator outgroups, two placed soft-shelled turtles sister to all other turtles, three placed *Pelomedusa* as the sister taxa to all other turtles, and two resulted in polytomies. Thus, we are unable to confidently resolve the root node of the living turtles with this dataset, although the lingering possibility of a paraphyletic Pleurodira nested within Cryptodira remains (see also Krenz et al., 2005).

An important methodological issue highlighted here is the critical role of marker selection. The rapid divergence of the Kinosternoidea, Chelydridae, and Chelonioidea led Chandler and Janzen (2009) to conclude that this node is best represented by a "hard polytomy" that cannot be resolved using molecular data. However, our analysis of 14 nuclear markers was able to resolve this short branch with strong support, a result that is very much in line with the multilocus simulation results for turtle phylogenetics presented in Spinks et al. (2009). Thus, selecting both enough markers, and those that are sufficiently variable to resolve the question of interest is an important consideration. With the growing availability of molecular markers and the ease of quickly developing them, assembling multi-locus datasets should no longer be a limiting factor in these types of analyses, illustrating the point that large, multi-locus datasets can be used to resolve complex phylogenetic problems that single or few-locus studies have been unable to answer confidently.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.11.005.

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