Toward consilience in reptile phylogeny: miRNAs support an archosaur, not lepidosaur, affinity for turtles

Daniel J. Field, Jacques A. Gauthier, Benjamin L. King, Davide Pisani, Tyler R. Lyson, and Kevin J. Peterson

SUMMARY Understanding the phylogenetic position of crown turtles (Testudines) among amniotes has been a source of particular contention. Recent morphological analyses suggest that turtles are sister to all other reptiles, whereas the vast majority of gene sequence analyses support turtles as being inside Diapsida, and usually as sister to crown Archosauria (birds and crocodilians). Previously, a study using microRNAs (miRNAs) placed turtles inside diapsids, but as sister to lepidosaurs (lizards and Sphenodon) rather than archosaurs. Here, we test this hypothesis with an expanded miRNA presence/absence dataset, and employ more rigorous criteria for miRNA annotation. Significantly, we find no support for a turtle + lepidosaur sister-relationship; instead, we recover strong support for turtles sharing a more recent common ancestor with archosaurs. We further test this result by analyzing a super-alignment of precursor miRNA sequences for every miRNA inferred to have been present in the most recent common ancestor of tetrapods. This analysis yields a topology that is fully congruent with our presence/absence analysis; our results are therefore in accordance with most gene sequence studies, providing strong, consilient molecular evidence from diverse independent datasets regarding the phylogenetic position of turtles.

INTRODUCTION The phylogenetic position of turtles represents one of the most recalcitrant problems in vertebrate biology, with contrasting hypotheses arising from different datasets. In recent years, three mutually-exclusive hypotheses have been put forth for the phylogenetic placement of turtles: (i) turtles represent the sister group to all diapsid reptiles (mainly supported by morphological datasets and developmental data, e.g., Gauthier et al. 1988; Lee 1997; Werneburg and Sánchez-Villagra 2009; Lyson et al. 2010, 2013a); (ii) turtles are the sister group to Lepidosauria (Sphenodon and lizards), including snakes; supported mainly by expressed miRNAs (Lyson et al. 2012), as well as some morphological analyses, e.g., Rieppel and deBraga 1996; deBraga and Rieppel 1997; Rieppel and Reisz 1999; Li et al. 2008); and (iii) turtles are the sister taxon to, or are nested within, Archosauria (birds and crocodilians); supported mainly by gene-sequence datasets, e.g., Zardoya and Meyer 1998; Hedges and Poling 1999; Kumazawa and Nishida 1999; Iwabe et al. 2005; Shen et al. 2011; Tzika et al. 2011; Chiari et al. 2012; Crawford et al. 2012; Fong et al. 2012; Lu et al. 2013; Shaffer et al. 2013; Wang et al. 2013). In the absence of a well-resolved phylogenetic hypothesis for Amniota, outstanding macroevolutionary questions, including those regarding the acquisition of the unique turtle body plan, cannot be adequately addressed.

Although in contradiction to most molecular studies, the miRNA data supporting a turtle + lepidosaur clade (Lyson et al. 2012) was not entirely unexpected (Rieppel and deBraga 1996; Becker et al. 2010). miRNAs are approximately 22-nucleotide noncoding RNA molecules that have been heralded as especially useful phylogenetic characters due to their continuous addition to animal genomes through time, comparatively low rates of secondary loss, and the largely conservative nature of the mature gene product’s primary sequence. These characters have been used to reconstruct the phylogenetic interrelationships of numerous animal clades at all levels in metazoan phylogeny (Sperling and Peterson 2009; Tarver et al. 2013). Lyson et al. (2012) showed that
the lizard Anolis carolinensis and the turtle Chrysemys picta shared four putative miRNAs, and that these nucleotide sequences were not recovered in a small RNA library derived from a total RNA preparation of an alligator, nor present in any sequenced bird genome. On the basis of these apparent synapomorphic miRNAs, these authors concluded that turtles were likely the extant sister group of the lepidosaurs.

As with any dataset, however, the quality of the characters used directly dictates the robustness of the analysis. With miRNAs, care must be taken in distinguishing them from other types of RNA molecules including other small RNAs (e.g., piRNAs, tRNAs), and fragments of larger RNA molecules (in particular, fragments of rRNAs and mRNAs). Recent clarifications of the criteria for miRNA annotation have challenged the diagnosis of many sequences previously identified as miRNAs (Tarver et al. 2012). According to these more rigorous specifications, four miRNA sequences identified as turtle + lepidosaur synapomorphies by Lyson et al. (2012) do not meet the minimal criteria established for miRNA annotation (Kozomara and Griffiths-Jones 2011; Tarver et al. 2012), especially as none of the four putative miRNAs exhibited expression of both arms of the hairpin (see below). Thus, the discordance between the miRNA dataset of Lyson et al. (2012) and most sequence-based datasets to this point, including the recent phylogenomic analysis of Chiari et al. (2012), could be due to mistaken miRNA homologies in Lyson et al. (2012). To address this issue, we characterized the near-complete miRNA repertoire of the turtle Chrysemys picta using both small RNA library reads and genomic sequences, and compared this repertoire to the near-complete repertoires of the snake Python bivittatus, the crocodilian Alligator mississippiensis, and the avian Columba livia, in addition to previously published lizard (Lyson et al. 2012) and bird data (miRBase v.19; Kozomara and Griffiths-Jones 2011). We also sequenced small RNA libraries from three additional species representing major lineages from across the lizard tree—the gecko Coleonyx variegatus, the xantusiid Xantusia wigginsi, and the snake Chionactis occipitalis—and queried the genomes of one additional crocodilian (Alligator sinensis), and three other turtles (the chelonid Chelonia mydas, and the trionychids Pelodiscus sinensis and Apalone spinifera). Our analyses fully support an archosaur affinity for turtles: the original ‘miRNAs’ identified by Lyson et al. (2012) appear to be spurious, whereas we demonstrate that turtles share several bona fide miRNAs with archosaurs not found or expressed in lepidosaurs, mammals, or any other metazoans. Additionally, this conclusion is strongly supported by a Bayesian phylogenetic analysis of 238 precursor miRNA sequences; therefore, according to these analyses, turtles are inferred to be diapsid reptiles sharing a more recent common ancestor with archosaurs than with lepidosaurs. These results resolve a major discordance between miRNA and gene sequence datasets regarding the phylogenetic position of Testudines within Amniota.

METHODS

Total RNA (Wheeler et al. 2009) was extracted from homogenized individuals; preparations were made from single late-stage embryos of the pigeon Columba livia, an adult gecko Coleonyx variegatus, an adult xantusiid Xantusia wigginsi, and a juvenile snake Chionactis occipitalis, following standard animal care protocols (IACUC number 2009-11302). Small RNA libraries were prepared at the Yale W. M. Keck Facility according to manufacturer’s instructions, and sequenced on an Illumina Genome Analyzer II platform. The number of reads sequenced per library is detailed in Table 1.

An updated version of miRMiner (Wheeler et al. 2009) was used to identify both orthologues of previously identified miRNAs (miRBase v. 19; Kozomara and Griffiths-Jones 2011) and novel miRNA families from these four taxa, in addition to reanalyzing the raw data from Lyson et al. (2012) for the turtle Chrysemys picta, the alligator Alligator mississippiensis, and the lizard Anolis carolinensis. Because published genomes are now available for Chrysemys picta (Shaffer et al. 2013) and Alligator mississippiensis (St John et al. 2012), the near-complete complements of miRNAs from these two taxa were assembled (see Electronic Supplementary Material: ESM Files 1–2, respectively). In addition, the reads from Columba livia were used to query the recently released pigeon genome (Shapiro et al. 2013), and its near-complete miRNA repertoire was assembled (ESM File 3). Finally, the near-complete ancestral miRNA complement of macrostomate snakes (Lee et al. 2007) was assembled (ESM File 4) using the reads from the snake Chionactis occipitalis and the genome of the python Python bivittatus (Castoe et al. 2011).

Next, the miRNAs constituting each of these complements (ESM Files 1–4) were used as queries to search the genomes of three other turtles (the chelonid Chelonia mydas, and the two trionychids Pelodiscus sinensis and Apalone spinifera), the Chinese alligator Alligator sinensis, and the coelancanth

<table>
<thead>
<tr>
<th>Table 1. Read count and genome assembly information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>Alligator mississippiensis</td>
</tr>
<tr>
<td>Columba livia</td>
</tr>
<tr>
<td>Chrysemys picta</td>
</tr>
<tr>
<td>Chionactis occipitalis</td>
</tr>
<tr>
<td>Coleonyx variegatus</td>
</tr>
<tr>
<td>Python bivittatus</td>
</tr>
<tr>
<td>Xantusia wigginsi</td>
</tr>
</tbody>
</table>

¹This number represents the number of non-redundant sequences, 20–25 nucleotides in length, that were expressed two or more times in the respective small RNA library and annotated to miRBase (v. 19) using miRMiner (Wheeler et al. 2009).
Latimeria chalumnae, using the default blastn parameters. A data matrix of 57 miRNAs (ESM File 5), including 32 new miRNA families specific to either the snake, alligator, or turtle lineages, was assembled that included all known miRNAs to have evolved in the reptile lineage since their split with the mammalian stem-group approximately 310 Ma ago (ESM File 6), excluding autapomorphies. Each putative miRNA was aligned with its known orthologues using MacVector v. 10.02 (MacVector, Inc., Cary, NC; alignments available upon request), and a dataset of presences/absences (ESM File 5) was assembled using MacClade v. 4.08 (Maddison and Maddison 2005). This character matrix was analyzed using both Dollo parsimony (PAUP* 4.0b10; Swofford 2002, with all characters given equal weight and using the branch and bound search algorithm), and Bayesian analysis (BEAST 1.8; Drummond et al. 2012, using the stochastic Dollo model and the standard binary data model, which assigns equal probability to all changes irrespective of directionality). For both BEAST analyses a Birth-Death prior on tree topologies was used. Because BEAST requires the use of a molecular clock in conjunction with standard phylogenetic analyses, we incorporated an uncorrelated exponential clock using eight calibration points (all of which were modeled as uniform distributions between a minimum and a maximum). Similarly, the root node was modeled using a uniform distribution (416–421 Ma with the starting root age set to 418; Benton et al. 2009). For both Bayesian analyses, three runs of 10,000,000 generations were performed. Convergence was ascertained by inspecting the log files of the three chains in Tracer (http://tree.bio.ed.ac.uk/software/tracer/). A burnin of 1,000,000 generations was deemed sufficient, and a majority rule consensus tree was built for each analysis by merging the trees sampled from the three chains after convergence. These majority rule consensus trees were built using PAUP* 4.0b10 (Swofford 2002). Clade support was estimated using Bremer support values (Bremer 1994) for the parsimony analysis, and posterior probabilities for the Bayesian analysis.

Finally, a concatenated dataset of 238 pre-miRNA sequences was assembled for 17 tetrapod taxa: the frog Xenopus tropicalis; Homo sapiens; the mouse Mus musculus; the marsupials Monodelphis domestica and Macropus eugenii; the platypus Ornithorhynchus anatinus; the lepidosaurs Anolis carolinensis and Python bivittatus; the birds Gallus gallus, Taenopygia guttata and Columba livia; the alligators Alligator mississippiensis and Alligator sinensis; and the turtles Chrysemys picta, Chelonia mydas, Pelodiscus sinensis, and Apalone spinifera; as well as two outgroup species, the coelacanth Latimeria chalumnae and the zebrafish Danio rerio. This dataset (ESM File 7) was assembled in two stages. First, orthology of each miRNA reconstructed as present in the last common ancestor (LCA) of Tetrapoda was determined for six taxa (H. sapiens, M. musculus, G. gallus, Anolis carolinensis, Xenopus tropicalis, and D. rerio) using both phylogenetic (MacVector v. 10.02) and syntenic (Ensembl release 72) analysis (ESM File 8). Because the current miRNA annotation system (Kozomara and Griffiths-Jones 2011) is not amenable to orthology analysis, a new nomenclature system was erected to make orthology recognition readily apparent among multi-gene families (ESM File 8). Once orthology was determined for all multi-gene miRNA family members for these six taxa, all members of each of these families from the remaining taxa were aligned and subjected to distance analysis using Neighbor Joining with uncorrected distances (MacVector v. 10.02). Subsequently, each miRNA gene was assigned to a particular paralogy group, giving a total of 238 miRNA genes reconstructed as having been present in the tetrapod LCA (ESM File 9). These 238 genes were then concatenated for each taxon, and finally subjected to Bayesian phylogenetic analysis. Phylogenetic analyses were performed under the GTR + G, the CAT–GTR + G, and the QMM + G models using Phylobayes (Lartillot et al. 2009). The difference between these models is that while a single GTR matrix is applied to an unpartitioned superalignment under GTR + G, under CAT–GTR + G, and QMM + G the data are automatically partitioned (during tree search) in an optimal number of compositionally defined partitions. In addition, in the CAT–GTR substitution rates are modeled using one GTR matrix (common to all the partitions), while in the QMM model each partition is assigned its own partition-specific GTR matrix. For each considered model, two independent analyses were run until convergence (for the two analyses the number of burnin generations were different, but the chains were always subsampled every 100 generations).

RESULTS

A total of 267 miRNA loci were found in the genome of the turtle Chrysemys picta (Table 2), with 251 supported with reads from at least one arm of the miRNA hairpin (ESM File 1). Of these 267 miRNAs, 20 are novel miRNA families, acquired hierarchically in the turtle lineage as expected (Fig. 1; Sperling and Peterson 2009; Tarver et al. 2013). These include six miRNA families specific to Chrysemys picta, not illustrated in Fig. 1. Three additional loci—miR-15-P4 (=Hsa-miR-497), miR-138-P1 (=Hsa-miR-138-1), and miR-150—are likely present in the Chrysemys picta genome, as reads were detected for these miRNAs in the Chrysemys picta small RNA library, and these loci are present in other turtle genomes. However, corresponding loci were not present in the deposited Chrysemys picta trace archives at Genbank. The number of loci annotated in Chrysemys picta is similar to those of the three unambiguous diapsids described herein (Table 2). The completeness of the Chrysemys picta genome appears to be slightly higher than that of the crocodilian Alligator mississippiensis and the avian Columba livia (at least by assessing the number of missing miRNA loci), but seems comparable to that of the python Python bivittatus. Alligator mississippiensis appears to be missing 10–11
are also present in the genomic sequence of a near relative. Cluster in all other amniotes (the miR loci, although six of these missing loci are linked in a single cluster in all other amniotes (the miR-103-P2 cluster), and were sequenced in the close relative Alligator sinensis, whereas Columba livia is missing 15–18 loci based on the appearance of reads in its small RNA library.

Of particular interest to us was confirmation of the presence of the four synapomorphic miRNAs used by Lyson et al. (2012) to demonstrate a phylogenetic affinity between the turtle Chrysemys picta and the lizard Anolis carolinensis: miR-5390, -91, -92, and miR-5393. Lyson et al. (2012) showed that these reads were present in both the Chrysemys picta and Anolis carolinensis small RNA libraries, and absent in that of Alligator mississippiensis; additionally, a locus for each of these reads was present in the genomic sequence of Anolis carolinensis. However, star reads were not recorded for any of these four putative miRNAs, which is problematic given that the relative position of the enzymatic cuts between the two arms of the putative hairpin is essential for recognizing bona fide miRNAs (Tarver et al. 2012). Using these four putative miRNA sequences as queries against all diapsid genomes curated in Genbank reveals that there are no corresponding loci in any other genome including that of Chrysemys picta, the supposed source of the shared reads with Anolis carolinensis (Lyson et al. 2012), for three of the four loci—only miR-5391 has a corresponding locus in other reptiles (including Alligator mississippiensis, contra Lyson et al. 2012). However, closer examination of this sequence reveals that the supposed mature miRNA read is actually the terminal portion of an exon, and a consensus splice site sits immediately 3’ of the putative mature read. Further, reads for none of these four miRNAs were found in any of our new libraries, including the lizards Coleonyx variegatus, Xantusia wigginsi, and the snake Chionactis occipitalis.

Instead, three miRNAs—miR-1720, miR-1791, and miR-2984—are present in archosaur genomes and in all four turtle genomes (ESM Files 10–12), but are absent in the lepidosaurs Anolis carolinensis and Python bivittatus. These miRNAs have not been reported in any other animal genome, although the squamate sister clade, Sphenodon punctatus, has yet to be assayed for them. Curiously, none of these miRNAs were detected in our single-ontogenetic-stage library of Chrysemys picta (which is why they were missed by Lyson et al. 2012, as publically available genomes for turtles were not available to the authors at that time), suggesting that these miRNAs are expressed either at very low levels in turtles, or (and more likely) at different ontogenetic stages. Indeed, reads for most of these miRNAs were found in the late-stage pigeon embryo, whereas neonatal turtle and alligator individuals were used for the Chrysemys picta and Alligator mississippiensis libraries in Lyson et al. (2012). Therefore, it is possible that profiling miRNAs from late-stage embryos would reveal transcripts of these relatively under-expressed miRNAs in turtles.

Both our maximum parsimony (BSI = 3) and Bayesian analysis (PP = 1.0 using the stochastic Dollo model, and PP = 0.79 using the standard binary model) strongly support an archosaur affinity for turtles, with no support for a lepidosaur affinity based on shared miRNA sequences (Fig. 1). Nonetheless, despite both archosaurs and turtles evolving a suite of novel miRNAs (ESM Files 1–3), no synapomorphic miRNAs appear to exist that enable resolution of the interrelationships among turtles and archosaurs (whether turtles are the extant sister group of archosaurs, or alternatively nested within Archosauria as the living sister to either birds or crocodilians).

To test this result, and to see if a more precise position of turtles relative to archosaurs could be inferred, we analyzed the primary nucleotide sequences of the precursor miRNAs for every miRNA sequence reconstructed as present in the last common ancestor of Tetrapoda (=Amphibia + Amniota; ESM File 7), including many new miRNA sequences not currently deposited in miRBase v. 20 (ESM Files 13–15), using standard Bayesian phylogenetics. When the super-alignment of the considered miRNAs was analyzed, we found that turtles resolve as sister to archosaurs under all considered models (PP = 1 for every node under all models investigated: GTR + G, CAT–GTR + G, and QMM + G; Fig. 2).

**DISCUSSION**

Contrary to the results reported by Lyson et al. (2012), both the pattern of acquisition of post-tetrapod miRNAs (Fig. 1), and a phylogenetic analysis of the primary sequences of pre-tetrapod pre-miRNAs (Fig. 2), robustly support an archosaur, rather than a
miRNAs support a turtle-archosaur relationship. Sixteen tetrapod taxa were scored for the presence/absence of 57 miRNA families using the coelacanth *L. chalumnae* and the zebrafish *D. rerio* as outgroups. A single shortest tree (tree length = 62) was found using Dollo parsimony (PAUP* 4.0b10, Swofford 2002) with all characters given equal weight and using the branch and bound search algorithm. Bremer support indexes (BSI) were calculated using PAUP*, and the values are indicated at the nodes. Posterior probabilities (PP) were calculated using Bayesian analysis (BEAST 1.8, Drummond et al. 2006) under the stochastic Dollo model and the standard binary data model. These data support an archosaur affinity for turtles (PP = 0.98, BSI = 3), as turtles share three miRNAs with archosaurs not found or expressed in any other tetrapod taxon. miRNAs that are not secondarily lost are shown as boxes, and those that are secondarily lost are shown as triangles, with the loss denoted by an upside-down triangle. Note that the paucity of available lepidosaur genomes does not allow for the recognition of potentially phylogenetically informative miRNAs within that clade, and hence this part of the tree is largely unresolved.

The reasons for the different results obtained in our analysis and by Lyson et al. (2012) are not due to problems with miRNAs per se (as suggested, e.g., Chiari et al. 2012), as turtles show both slow rates of miRNA evolution (Thorne et al. 1998) (ESM File 16) and minimal secondary miRNA gene loss (ESM File 17). Instead, the reason for the apparent incongruence is simply due to misrecognition of primary homologies by Lyson et al. (2012). The four miRNAs purported to be shared between the lizard Anolis carolinensis and the turtle Chrysemys picta did not express both arms of the hairpin in Anolis carolinensis (the ‘mature’ and the ‘star’; Ambros et al. 2003). Normally, this is not a problem; deep phylogenetic conservation can substitute for the absence of star reads when annotating miRNAs (Ambros et al. 2003), as star sequences are often expressed at much lower levels than their corresponding mature reads. However, in this case, deep phylogenetic conservation was the issue at hand, and thus Lyson et al. (2012) essentially made a circular argument: they used phylogenetic conservation to justify the robustness of the new miRNAs discovered in Anolis carolinensis, and then used these miRNAs to propose a close affinity between turtles and lepidosaurs. More recent work on miRNA annotation strongly indicates that obtaining reads from both arms of the hairpin is essential for the recognition of new miRNAs (e.g., Tarver et al. 2012) and, indeed, each of the three miRNAs shared between turtles and archosaurs presented here express both arms of the hairpin in at least one species (ESM Files 10–12).

One final contrast between our study and that of Lyson et al. (2012) is that none of the miRNAs supporting a turtle + archosaur grouping were expressed in our single-ontogenetic-stage turtle library, and thus, as suggested (Crawford et al. 2012), sampling biases—in this case the absence of sequenced genomes in key areas of the tree—resulted not only in the misrecognition of putative miRNAs, but also in the non-recognition of bona fide miRNAs. Nonetheless, given the concordance between our two independent analyses using miRNAs (one a presence/absence analysis, and the other a primary sequence analysis), and virtually every other study of gene sequences focused on amniote phylogeny, we conclude that molecular data in general strongly support an exclusive turtle + archosaur clade (but see Lu et al. 2013 for a discussion of gene heterogeneity in amniotes).

Despite concordance among studies using molecules to address turtle affinities, the turtle + archosaur sister-group hypothesis has yet to find much support in morphological and fossil datasets, with rare apparent similarities emerging as convergent within a phylogenetic context (Bhullar and Bever 2009). Whereas the hypothesized composition of most major
amniote clades diverging in the Paleozoic—the total clades of mammals, reptiles, diapsids, archosaurs, and lepidosaurs—has remained remarkably stable across phylogenetic analyses regardless of data source, the phylogenetic position of crown turtles (as well as their most turtle-like proximal stem-sisters, e.g., Odontochelys and Proganochelys) has been contentious. Most morphological analyses argue for turtles as sister to total group pan-diapsids (Gauthier et al. 1986; Lee 1997; Wernig and Sánchez-Villagra 2009; Lyson et al. 2010, 2013a), or as being closely related to marine lepidosauromorph sauropterygians (Rieppel and deBraga 1996, deBraga and Rieppel 1997, Rieppel and Reisz 1999; although this latter hypothesis has not enjoyed much recent support). Although the putative ‘parareptilian’ affinities of turtles inferred from recent morphological datasets appear to stand in stark contrast to molecular results, recent work applying molecular scaffolds to turtle morphological datasets may suggest some potential for reconciliation of this incongruence. Although the morphological hypothesis that turtles are sister to all diapsids remained the most strongly supported topology across most analyses, Lee (2013) demonstrated that the inferred interrelationships of turtles, parareptiles and diapsids exhibit some variance according to different optimality criteria, ingroup compositions and character sets.

If the morphological hypothesis that turtles represent the extant sister group of living reptiles accurately reflects turtle origins, it would indicate that virtually the entire genome (Matsuda et al. 2005; Shedlock et al. 2007), including mitochondrial genes (Zardoya and Meyer 1998; Kumazawa and Nishida 1999), ribosomal RNA genes (Hedges and Poling 1999), protein coding genes (Hedges and Poling 1999; Iwabe et al. 2005; Shen et al. 2011; Tzika et al. 2011; Chiari et al. 2012; Feng et al. 2012; Lu et al. 2013; Shaffer et al. 2013; Wang et al. 2013), ultraconserved elements (Crawford et al. 2012), and miRNAs (this study), exhibit astonishing levels of homoplasy in a surprisingly congruent pattern. However, applying a ‘genes as characters’ approach, Lu et al. (2013) argued that the turtle + archosaur hypothesis could be an artifact of large, concatenated alignments overburdened by gene heterogeneity—likely the single largest source of systematic error in phylogenomic analyses (Jeffroy et al. 2006; Salichos and Rokas 2013). Moreover, in support of the idea that anomalous gene trees may be responsible for confounding phylogenomic analyses regarding the phylogenetic placement of turtles within amniotes, Lu et al. (2013) suggested that many of the gene trees supporting a turtle-archosaur sister group relationship may be under positive selection, or play important functional roles.

Although the results of Lu et al. (2013) shed light on the potentially confounding influence of gene heterogeneity in molecular systematics, consilience among independent datasets remains the most reliable way to adjudicate phylogenetic hypotheses. Given the persistent conflict between morphological and molecular hypotheses for the interrelationships of the major amniote clades, it may be premature to firmly conclude that turtles represent the extant sister taxon of archosaurs; however, the strongly-supported miRNA results presented herein add to the considerable (and ever-growing) body of evidence in support of this conclusion. This work is necessary to provide an accurate evolutionary framework from which patterns of trait evolution, such as the origin of the unique turtle body plan, and their fascinating physiology (Gilbert and Corfe 2013), can be inferred. The accurate interpretation of relevant fossils may await the development of a comprehensive morphological phylogenetic matrix incorporating all relevant taxa, and such work may be necessary for basal stem turtles with diapsid features to come to light. Although this pursuit will be a major undertaking, it is only fitting that a ‘slow and steady’ approach to reptile systematics will be necessary to confidently reconstruct the evolutionary history of this fascinating clade.

Acknowledgments

The authors thank K. Kuester for providing an egg of Columba livia, J. Carrell and G. Watkins-Colwell for assistance processing the pigeon, A. Heimberg and A. Hsiang for comments, J. Tarver and J. Vinther for laboratory assistance, and G. Wagner for providing lab space. Funding for this project was provided by a NSERC CGS and Lougheed Award of Distinction to D. J. F., and Yale Peabody Museum of Natural History to J. A. G. NASA-Ames supported K. J. P and B. L. K.

REFERENCES


