MicroRNAs support a turtle + lizard clade

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Despite much interest in amniote systematics, the origin of turtles remains elusive. Traditional morphological phylogenetic analyses place turtles outside Diapsida—amniotes whose ancestor had two fenestrae in the temporal region of the skull (among the living forms the tuatara, lizards, birds and crocodilians)—and allied with some unfenestrate-skulled (anapsid) taxa. Nonetheless, some morphological analyses place turtles within Diapsida, allied with Lepidosauria (tuatara and lizards). Most molecular studies agree that turtles are diapsids, but rather than alloying with lepidosaurs, instead place turtles near or within Archosauria (crocodilians and birds). Thus, three basic phylogenetic positions for turtles with respect to extant Diapsida are currently debated: (i) sister to Diapsida, (ii) sister to Lepidosauria, or (iii) sister to, or within Archosauria. Interestingly, although these three alternatives are consistent with a single unrooted four-taxon tree for extant reptiles, they differ with respect to the position of the root. Here, we apply a novel molecular dataset, the presence versus absence of specific microRNAs (miRNAs)—genes that encode approximately 22 nucleotide non-coding regulatory RNAs—to this problem. miRNA-based phylogenetics have been successfully applied to many metazoan clades, including vertebrates [18], as miRNAs show a number of characteristics that make them ideal phylogenetic characters, including the fact that new miRNAs are continually added to metazoan genomes through time and, once added, are rarely lost in most metazoan taxa [19–21]. In addition, miRNAs show extreme nucleotide conservation of the mature sequence, and structural considerations based on the requirement to fold into the canonical miRNA hairpin structure make convergence highly unlikely, resulting in little homoplasy [19,20]. Because the acquisition of a novel miRNA family represents the gain of a de novo trans-acting gene class, where the outgroup state (absence) can be established with certainty, miRNAs are ideal candidates for delineating the position of the root.

1. INTRODUCTION

The phylogenetic position of turtles remains labile, owing in part to the fact that, while many primitive cranial features suggest a basal position outside Diapsida, most molecular studies agree on the position of its root (figure 1). Thus, the problem is not one of deciding inter-relationships per se, but one of simply determining polarity. Correct rooting in morphological studies relying on highly divergent outgroups is often hampered by difficulty in establishing polarity for individual characters [16], while rooting in traditional molecular phylogenetic analyses is affected by rate and compositional heterogeneity [17]. Given that there is clear homoplasy in reptile morphology irrespective of the true phylogenetic position of turtles [8], and clear rate heterogeneity in molecular sequences in amniotes [12], an alternative data source is needed to correctly root the reptile tree.

Here, we apply a novel molecular dataset, the presence versus absence of specific microRNAs (miRNAs)—genes that encode approximately 22 nucleotide non-coding regulatory RNAs—to this problem. miRNA-based phylogenetics have been successfully applied to many metazoan clades, including vertebrates [18], as miRNAs show a number of characteristics that make them ideal phylogenetic characters, including the fact that new miRNAs are continually added to metazoan genomes through time and, once added, are rarely lost in most metazoan taxa [19–21]. In addition, miRNAs show extreme nucleotide conservation of the mature sequence, and structural considerations based on the requirement to fold into the canonical microRNA hairpin structure make convergence highly unlikely, resulting in little homoplasy [19,20]. Because the acquisition of a novel microRNA family represents the gain of a de novo trans-acting gene class, where the outgroup state (absence) can be established with certainty, miRNAs are ideal candidates for delineating the position of the root.

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Figure 1. The inter-relationships among the major groups of reptiles. Using an unrooted tree, it is possible to show that although each of the three previous hypotheses concerning turtle relationships—morphology 1 [4–7], morphology 2 [4–7] and the molecular results [10–12]—agree on the topology, they disagree on the position of the root (arrows).

3. RESULTS

Two hundred and eighty two miRNA genes were annotated, belonging to 186 miRNA families [20] (electronic supplementary material, file S1), with 77 new miRNA families discovered that appear to be specific to Anolis (electronic supplementary material, file S1). As expected, given our understanding of how miRNAs evolve in most taxa [19–21], only two families appear to have been secondarily lost in the lizard: miR-457 and miR-2184. Similarly, in the turtle, reads were not detected for miR-147 and -208, and in the alligator, reads were not detected for miR-726 and -727 (the latter a potential shared loss with birds; electronic supplementary material, file S1). Thus, out of the 100 expected miRNA families in the last common ancestor of Tetrapoda (electronic supplementary material, file S3), each of these species has 98, making the occurrence of secondary loss in all these taxa no more than 2 per cent.

The phylogenetic analysis resulted in a single most parsimonious tree with a tree length of 36 (CI = 0.97; RI 0.99). Each of the reptile clades identified in this study is characterized by at least one miRNA acquisition—the alligator shares one miRNA with the birds (miR-1791), supporting the monophyly of Archosauria, and all reptiles analysed herein share miR-1677 (figure 2a). Importantly, we find that turtles and lizards share four of the 77 unique miRNA gene families identified in Anolis that are not found in any other organisms’ genome or small RNA library (figure 2a; electronic supplementary material, file S1). Thus, these sequences are indeed restricted to the reptiles, and a phylogenetic analysis of the presence/absence of miRNAs families in these taxa (electronic supplementary material, file S2) unambiguously supports a turtle + lizard sister group relationship (figure 2b), as no miRNAs were found in all diapsids but not in turtles, or in turtles and archosaurs but not in lizards. Other nodes including Amniota, Mammalia, Theria and Aves are each supported by one or more unique miRNA (figure 2b).

4. DISCUSSION

Turtles as sister group to lizards are recovered in some morphological analyses [4–7] and this is supported by some, mostly postcranial, characters including the fusion of the astragalus and calcaneum in postnatal ontogeny [25]. However, a diapsid affinity also requires several morphological reversals in turtles, including the closure of the temporal fenestrae [25]. In addition, it suggests that molecular analyses that recover an archosaur affinity might be the result of a systematic artefact caused by the attraction of the long-branched lizards towards the outgroup [8].

The consilience between at least some morphological apomorphies and the evolutionary acquisition of miRNAs suggests the validity of the sister group relationship between lepidosaurs and turtles. Hence, we propose ‘Ankylopoda’ (‘fused foot’ in reference to the fused ankle bones—astragalus and calcaneum—shared by lepidosaurs and turtles) as the name of the Lepidosauria + Testudines crown clade, which is defined as the last common ancestor of Chrysemys picta and A. carolinensis and all of its descendants living or extinct (electronic supplementary material, file S4). If Lepidosauria + Testudines is indeed a monophyletic group, then this implies significant convergence in molecular (nuclear and mitochondrial), developmental and morphological characters, including the loss of temporal fenestrae, in the early evolutionary history of turtles. Further, our study suggests that lizards are the appropriate outgroup comparison for understanding the origin of the turtle body plan.

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Figure 2. microRNAs support a turtle–lizard relationship. (a) Structures and alignments of the mature sequences for three of the 35 families analysed phylogenetically, the reptile-specific miR-1677, the archosaur-specific miR-1791 and miR-5390, a novel miRNA shared between Anolis carolinensis and C. pictus. Mature sequences within the context of the miRNA hairpin are shown in grey, changes in the mature sequence with respect to the reference sequence, either chicken or lizard, are shown in bold. (b) To arbitrate among these competing hypotheses (see figure 1), eight amniote taxa were scored for the presence/absence of 35 miRNA families with the frog Xenopus laevis as the outgroup. Using a combination of small RNA library sequencing coupled with genomic searches (electronic supplementary material, file S1), we find that the turtle C. picta shares four miRNA families with the lizard A. carolinensis that are not found elsewhere in the animal kingdom, supporting the rooting position of ‘morphology 2’ in figure 1 [4–7]. Bremer support indexes are indicated at each node. Character changes on branch: grey boxes, acquisition of miRNA family; filled triangle, loss of miRNA family.

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