Live birth among Iguanian lizards predates Pliocene–Pleistocene glaciations

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Among tetrapods, viviparity is estimated to have evolved independently within Squamata (lizards and snakes) more than 100 times, most frequently in species occupying cold climate environments. Because of this relationship with cold climates, it is sometimes assumed that many origins of squamate viviparity occurred over the past 2.5–4 Myr during the Pliocene–Pleistocene glaciations; however, this hypothesis is untested. Divergence-dating analysis on a 733-species tree of Iguanian lizards recovers 20 independent lineages that have evolved viviparity, of which 13 multispecies groups derived live birth prior to glacial advances (8–66 Myr ago). These results place the transitions from egg-laying to live birth among squamates in a well-supported historical context to facilitate examination of the underlying phenotypic and genetic changes associated with this complex shift in reproduction.

Keywords: squamata; Iguania; viviparity; phylogeny; molecular clock

1. INTRODUCTION

The shift from laying eggs to live birth is a complex evolutionary transition associated with significant modifications of a species' genome, morphology and ecology (Rothchild 2003; Crespi & Semeniuk 2004). Despite this, viviparity (live birth) is widespread among animals and has evolved independently numerous times in groups as disparate as reptiles, amphibians, fishes, molluscs, velvet worms, scorpions, insects and mammals. Among vertebrates, viviparous reproduction is inferred to have evolved no less than 141 times (Blackburn 1999; Blackburn 2005) with 102–115 potential origins within squamate reptiles (lizards and snakes). However, most of our functional understanding of this reproductive mode is derived from extensive studies on mammals. Unfortunately, reconstructing the evolutionary transition from egg-laying in monotremes to live birth in marsupials and placental mammals is difficult because of the age of this transition, which occurred at least 125 Ma in the early Cretaceous (Luo *et al.* 2003). Better understanding of the evolutionary requirements and timing associated with a shift in reproductive mode requires knowledge of the historical environmental conditions to which a particular lineage is exposed, its age of

origin and changes in the morphology, physiology and genetic architecture of that lineage.

Most origins of viviparity in squamate reptiles are inferred to have occurred in colder environments at either high elevations or high latitudes (Shine 1985). The most widely accepted hypothesis for this pattern is that the probability of offspring survival in harsher environments is increased when females actively thermoregulate and maintain embryos at optimal developmental temperatures until parturition (Shine 2005). Therefore, one hypothesis is that most origins of squamate viviparity evolved relatively recently during the Pliocene–Pleistocene glaciations within the last 4 Myr (Blackburn 1995). Intraspecific phylogenetic studies on reproductively bimodal species *Zootoca vivipara*, *Lerista bougainvillii*, *Saiphos equalis* and *Sceloporus bicanthalis* support this hypothesis (summarized in Blackburn 2005). Alternatively, average global temperatures have steadily declined since the Late Eocene about 40 Ma (Lear *et al.* 2000), so we may expect to see a steady increase in origins of viviparous squamate clades over this time span. To date, these evolutionary hypotheses have not been explicitly tested using modern phylogenetic and divergence dating techniques.

Iguanian lizards are an ideal group to test alternative hypotheses for the origin of viviparity because they have evolved this trait multiple times. This group occurs worldwide, occupying diverse habitats from Southeast Asian and Brazilian rainforests to Tierra del Fuego and the Gobi and Atacama deserts. Several species live above 5000 m in the Andes and Tibetan Plateau (Pough *et al.* 2003; Vitt & Caldwell 2009). Currently three families are recognized, Agamidae, Chamaeleonidae and Iguanidae (Schulte *et al.* 2003*b*), with each clade containing several viviparous species. This study uses a phylogenetic tree of 737 species and fossil-calibrated divergence times to test alternative hypotheses for the origins of viviparous iguanian lineages.

2. MATERIAL AND METHODS

(a) *Taxon and data sampling*

This study used 737 (733 iguanians, four outgroups) species including representatives of all major lineages of three iguanian families with 64 chamaeleonids, 196 agamids and 473 iguanids. GenBank accession numbers for the 712 previously published and 21 newly reported ingroup iguanian species, as well as four previously published outgroup squamate species are presented in the electronic supplementary material, table S1. Reproductive states for all species were obtained from the literature (electronic supplementary material, table S1) with exceptions where species states were inferred because literature sources were not available (some *Anolis, Cophotis dumbara*). Specimen voucher, locality information and citation information are available from GenBank flatfiles.

For newly reported DNA sequences, genomic DNA extraction, polymerase chain reaction conditions, primers used and sequencing were performed following Schulte *et al.* (2003*a,b*). These sequences represented the mitochondrial-encoded region spanning *ND1* to *COI*. For this analysis, only the protein-coding regions, part of *ND1*, all of *ND2* and part of *COI* were used, as intervening tRNA regions were highly variable among sampled taxa, making unambiguous alignment of these regions unreliable. Base positions inferred to have ambiguous homology at the ends of *ND1* and *ND2* were excluded from phylogenetic analyses (198 out of 1281 aligned positions). Alignment is available in TREEBASE (study accession number S2475, matrix accession number M4714).

(b) *Phylogeny and divergence date estimation*

Maximum likelihood-based phylogenetic trees were estimated with RAxML (Stamatakis *et al.* 2008) in the CIPRES portal, with *a*

Table 1. Divergence times for viviparous iguanian lizard clades and immediate oviparous ancestor in millions of years. (Clades are listed by family and in order of mean age.)

	clade age (mean)	confidence interval (ABCq[a])	oviparous ancestor age (mean)	confidence interval (ABCq[a])
Agamidae				
Cophotis spp.	8.4	3.6–12.2	35.1	27.3–45.2
Chamaeleonidae				
B. pumilum clade	28.9	22.1–39.8	63.3	53.3–74.2
C. jacksonii clade	29.9	20.3–36.3	44	31.2–52.9
Iguanidae				
L. isabelae clade	3	0–4.6	24.3	14.4–33
L. ornatus clade	15.6	1.1–15.3	29	14.3–32.2
L. nigroviridis clade	17.8	9.1–24.8	32.2	18.8–39.2
L. pictus clade	25.7	14.6–31.8	29.5	18.6–38.4
S. poinsettii clade	32.1	13.8–33.5	58.2	37.4–62.7
L. capillitas clade	35.2	32.3–52.4	68.8	52.6–78.5
S. adleri clade	44.2	21.6–47	62.7	43.4–70.6
L. montanus series	46.1	23.1–49.9	70.3	46.9–75.9
Phrynosoma orbicularae clade	50.4	31.9–61.5	62	37.5–71.7
L. lineomaculatus series	52.6	34.3–60.7	79.3	58.5–86.2
Phymaturus spp.	66.4	48.7–82.2	125.3	99.1–125.8

[a]ABCq is approximate bootstrap confidence quadratic method of Burbrink & Pyron (2008).

priori partitioning by codon position for all three mtDNA genes combined. Analyses assumed a general time reversible sequence evolution model with gamma-distributed rate variation and proportion of invariant sites (GTR+Γ+I). Model parameter values were estimated from the data. This model was chosen because it is the most complex available in RAxML and almost all previously published studies using this mtDNA region have found that this model best explains the data. Eight independent runs were performed using all program defaults and the run with the highest likelihood was used as the tree for divergence analyses. All iguanian lizards were constrained to be monophyletic as long-branch attraction is hypothesized to explain a spurious sister–taxon relationship of snakes and acrodont lizards using these mtDNA sequences (Townsend *et al.* 2004). Phylogenies reconstructed using nuclear genes and morphology strongly support iguanian monophyly (Acrodonta and Iguanidae). Bootstrap resampling was applied using RAxML with 100 pseudoreplicates and parameter values estimated for each pseudoreplicate. Search conditions were identical with the initial search. The detailed bootstrap tree is available in the electronic supplementary material, figure S1. Bayesian (phylogenetic and divergence time estimation) analyses were not performed owing to computational and time limitations.

For ancestral state reconstructions of reproductive mode, Mk1 (one parameter) and AymmMk (two parameter) likelihood-based character evolution models were examined in MESQUITE (v. 2.71 build 486; Maddison & Maddison 2009). There was bias in favour of oviparity to viviparity transitions ($q01/q10 = 1.163$) but a likelihood ratio test for a difference between Mk1 and AsymmMk models was not significant ($p > 0.10$). Oviparity was assumed to be the ancestral condition for this tree and was corroborated by likelihood analysis. MESQUITE implements a likelihood threshold decision at $t = 2$ as a measure of statistical support for ancestral state reconstructions.

We used Penalized Likelihood implemented in r8s v. 1.71 (Sanderson 2003) for divergence time estimation. Cross-validation determined the optimal smoothing parameter with the additive-penalty function and truncated Newton method implemented. We assigned minimum age estimates to 12 internal nodes. Fossil placement on the inferred molecular phylogeny was determined primarily by whether a consensus exists among palaeontologists on the relative position of taxa in the squamate phylogeny. Minimum age estimates were assigned to stem groups, the most inclusive group of taxa that contains all extant and extinct clade members (Near *et al.* 2005). The root was assigned a maximum age of 218 Myr and minimum age of 144 Myr to span the age of Iguania inferred from several previous studies dating this node's age (see electronic supplementary material for dates, fossils and additional references, table S2). Divergence time confidence intervals were assessed using the bootstrap method outlined in Sanderson (2003) and calculated using the ABCq bias-correction method (Burbrink & Pyron 2008).

3. RESULTS

Phylogenetic relationships among the major clades of iguanian lizards are consistent with previous studies (Schulte *et al.* 2003*a,b*). In each family, viviparity has evolved multiple times and at least 20 events (Chamaeleonidae, two; Agamidae, two; and Iguanidae, 16) have been reconstructed (clades strongly supported by bootstrap analysis). In our 733-species tree we estimated divergence times for 14 out of 20 clades. Five viviparous lineages, represented by one species each, were excluded because divergence times could not be calculated using singleton species. The sixth inferred event was not included because samples for this lineage were unavailable (Tibetan Plateau viviparous *Phrynocephalus* species).

Our analyses revealed that 13 of the 14 iguanian lizard clades evolved viviparity prior to the initiation of the Pliocene–Pleistocene glaciations (table 1). The iguanid clade containing the Northern Chilean species *Liolaemus isabelae* and *L. paulinae* is estimated to have evolved viviparity at least by mid-Pliocene (3 Ma) while another South American iguanid group, *Phymaturus*, is estimated to have evolved this trait earliest by approximately 66.4 Ma in the Late Cretaceous. In the remaining nine iguanid clades viviparity evolves most often in the Eocene (35.2–52.6 Ma). Both chamaeleonid clades, *Chamaeleo jacksonii* clade and South African *Bradypodion pumilum* clade, evolved live birth at least by 29–30 Ma in the Oligocene. Within Agamidae, the Sri Lankan genus *Cophotis*, viviparity is estimated to have appeared about 8.4 Ma.

4. DISCUSSION

Our results are congruent with the hypothesis that most origins of viviparity in iguanian lizards occurred during the time of Earth's gradual cooling that began in the Eocene. The transition times between oviparity and viviparity for all but one of the 14 origins

examined here are estimated to have taken place over this time period. In only one instance (*Liolaemus isabelae* clade) is viviparity suggested to have evolved during the recent glacial periods 2.5–4 Ma. However, the oviparous ancestor of this clade is at least 14.4 Myr old (table 1), so it remains possible that the origin of viviparity in this group took place prior to the onset of glaciations.

A potential bias in this study is the lack of accurate age estimates for viviparous origins in the singleton species sampled here (e.g. *Sceloporus grammicus*, *Scel. jarrovii*) and reproductively bimodal species (*Zootoca vivipara*, *Lerista bougainvillii*, *Saiphos equalis*, *Scel. bicanthalis*) not sampled. It remains possible that viviparity in these species evolved within the last 4 Myr (Surget-Groba *et al.* (2001) for *Z. vivipara*). Modern divergence analyses applied to these taxa are required to further examine this hypothesis. Although our analysis of Iguania reconstructs 20 of the 102–115 origins within Squamata, future comprehensive sampling within other squamate clades, especially Serpentes, Anguidae and Scincidae, as well as detailed population-level analyses of reproductively bimodal species, will be an excellent complement to this study in determining whether most viviparous origins occurred pre-Pliocene.

Squamate reptiles are an ideal group in which to test alternative hypotheses for the evolution of viviparity owing to this trait's origination frequency. Identification of squamate reptile lineages that have evolved viviparity over a range of time scales will provide a temporal framework to better reconstruct genomic, ecological, physiological and morphological changes that take place during the transition from egg-laying to live birth (Blackburn 2005). Our results offer useful information in historical reconstruction of diverse squamate lineages, improve understanding of the evolution of a recurrent theme in the history of squamate reptiles and reveal much about evolutionary patterns in reproductive systems in general.

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