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The serpent and the egg: unidirectional evolution of reproductive mode in vipers?

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Abstract

Dollo's law, that complex characters are not regained in evolution, is a pattern applied to many systems. Recent work has evaluated unidirectional evolution in a number of contexts, and several violations of this law have been documented. These methods have also been criticized for potentially overestimating reversals. We test the hypothesis that the ancestral reproductive mode of oviparity can be regained in vipers, in opposition to Dollo's law. We use model comparison and ancestral character state reconstruction methods that address recent criticisms, and find evidence both supporting and refuting Dollo's predictions from different analyses. We discuss our results in the context of unidirectional evolution and review factors required for strong inference of violations of Dollo's law.

Key words: Ancestral character state reconstruction – BiSSE – evolutionary constraint – macroevolution – oviparity – viviparity – reverse-jump Markov Chain Monte Carlo Bayesian methods

Introduction

The original formulation of Dollo's law states that an organism cannot return, even partially, to an identical condition expressed by an ancestor (Dollo 1893, 1905; see also Simpson 1953; Collin and Miglietta 2008). This pattern of unidirectional evolution has been rejected by a number of recent phylogenetic studies (e.g. Collin and Cipriani 2003; Whiting et al. 2003; Chippindale et al. 2004; Kohlsdorf and Wagner 2006; Domes et al. 2007; Wiens et al. 2007; Brandley et al. 2008; Kohlsdorf et al. 2010), but several of these have been criticized for methodological flaws (Goldberg and Igić 2008; Galis et al. 2010). Unidirectional evolution remains the assumed pattern for a number of life history characteristics; therefore, we evaluate the evidence for this pattern for a key trait in vertebrate life history: reproductive mode.

In vertebrates, reproductive mode is commonly understood to mean laying eggs (oviparity) or producing free-living offspring (viviparity) and is a prominent yet perplexing variable in life history evolution. Oviparity is primitive and often exclusively characterizes entire animal lineages, whereas viviparity has arisen multiple times (Blackburn 1982). Within the two basal amniote clades, mammals and reptiles, we see a major difference in the number of reproductive mode changes. In mammals, monotremes retain oviparity and viviparity probably arose only once in the stem leading to marsupials and placental mammals. Among living and fossil reptiles, there are no known viviparous turtles, archosaurs (including birds) or rhynchocephalians, yet livebearing has arisen almost a hundred times among living squamates (Fitch 1970; Blackburn 1985). Here, we capitalize on reproductive diversity within one subclade of squamate reptiles, the vipers, to rigorously appraise the possibilities and conceptual implications of evolutionary reversals in reproductive mode.

In Viperidae, an estimated 80% of species bear live young (Table S1), and viviparity has arisen multiple times over tens of millions of years (Wüster et al. 2008; this study). This allows

preliminary evaluation of the timing of transitions. Interestingly, recent phylogenetic hypotheses (Lenk et al. 2001; Castoe and Parkinson 2006; Wüster et al. 2008; Pyron and Burbrink 2009) place oviparous taxa within groups containing viviparous taxa, suggesting potential reversals from viviparity to oviparity.

The transition from oviparity to viviparity involves multiple complex changes: endocrine modifications to postpone parturition, suppression of nesting behaviour, reduction or loss of organs and pathways needed in eggshell formation and gain of adaptations for foetal respiration and nutrition (Blackburn 1995; Lee and Doughty 1997; but see de Fraipont et al. 1999). Because of the modifications required for a transition to viviparity in animals, a reversal to oviparity is considered unlikely on theoretical grounds (Neill 1964; Fitch 1970; Tinkle and Gibbons 1977), although strong empirical evidence or detailed justification is lacking (Lee and Doughty 1997).

Evolutionary reversals from viviparity to oviparity in squamate reptiles have been addressed in the past, but there is little evidence to definitively support reproductive mode reversal. Benabib et al. (1997) suggested a possible reversal to oviparity with a lizard species, but the inference had little support. de Fraipont et al. (1996) inferred multiple apparent reversals from viviparity to oviparity throughout squamate evolution. Criticisms of de Fraipont et al. (1996) highlighted multiple uncertainties in the phylogenies, counting particular transitions more than once, and other errors (Blackburn 1999; Shine and Lee 1999; Surget-Groba et al. 2001). Reanalysis of the data set by de Fraipont et al. (1999) found equivocal evidence for reversibility of viviparity. Blackburn (1999) argued that reversals to oviparity cannot be ruled out theoretically, but no convincing empirical evidence has yet been found. Lynch and Wagner (2009) subsequently found strong evidence for reversal to oviparity in a sand boa, and Lynch (2009) concluded that among vipers, a model that included apparent reversals was best supported by likelihood methods, albeit at a much lower rate than transitions from oviparity to viviparity. Lynch thus provided the first strong cases against Dollo's law for reproductive mode in snakes, but as we will show later, additional model tests refine that

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conclusion for vipers. Furthermore, because transitions to ancestral states should be long separated from origins of derived states to qualify as reversals (Marshall et al. 1994), their timing should be evaluated with explicit phylogenetic methodology.

These previous studies were primarily based on parsimony analysis of character evolution. Maximum likelihood and Bayesian methods are now often used because they provide probabilistic estimates of character states at a node, and they can be used to statistically treat hypotheses about character evolution (Huelsenbeck and Bollback 2001; Collin and Miglietta 2008). Additionally, reverse-jump Markov Chain Monte Carlo Bayesian methods (RJ-MCMC) include models of evolution in the analysis and provide a means to determine which models are best supported by posterior probability (Pagel et al. 2004). RJ-MCMC has been used for character state reconstruction in only a few studies (see Ekman et al. 2008; Xiang and Thomas 2008; Montgomery et al. 2010; Rasmussen and Cameron 2010), and it has not yet been applied to tests of unidirectional evolution.

The goal of the present study is to re-examine the evolution of reproductive mode in vipers, incorporating multiple analyses and methods to best assess whether this character follows Dollo's law of unidirectional evolution. We hypothesize that, contrary to this law, reversals are possible. This possibility of reversal may be due to selective constraints on pleiotropic effects of underlying genes that result in the conservation of developmental pathways over long periods of time, making phenotypic change easily reversible. In accordance with this hypothesis, we predict that vipers have experienced at least one evolutionary reversal from viviparity to oviparity. We test our hypothesis using multiple model comparison and ancestral character state reconstruction approaches, summarize our results identifying reversals and discuss these in the context of Dollo's law.

Materials and Methods

Phylogenetic estimation

Independent estimation of phylogeny and character evolution is optimal for strong inferences; therefore, the data set for phylogeny reconstruction was independent of the character of interest (Lee and Doughty 1997). As several character reconstruction methods assume that the phylogeny includes all extant taxa, we included members of all of the approximately 270 species of Viperidae that had DNA sequences available (Table S1). This sampling resulted in data for over 65% of the approximately 70 species of true vipers (Viperinae) and almost 80% of the approximately 200 species of pitvipers (Crotalinae), as well as *Azemiops feae*, the single species of Azemiopinae. Recent work (FitzJohn et al. 2009) suggests that accuracy and precision of binary-state speciation and extinction model (BiSSE) inference are essentially unaffected for phylogenies 75–100% complete. Published sequences constituted the majority of the data set, and we added new information for 17 species. Four of these species had no published sequence data in GenBank prior to this study.

The mitochondrial loci used in this study – rRNA genes 12S and 16S, and protein-coding genes cytochrome *b* (*cyt-b*) and NADH dehydrogenase subunit 4 (ND4) – are commonly used to infer inter-specific and intergeneric relationships in snakes (e.g. Parkinson 1999; Austin 2000; Parkinson et al. 2002; Malhotra and Thorpe 2004; Castoe et al. 2007; Wüster et al. 2007; Pyron and Burbrink 2009). Sequences were aligned with the Muscle algorithm (Edgar 2004) in MEGA 5.05 (Tamura et al. 2011) using default parameters. Internal gaps in the alignment represented by <50% of taxa were deleted; all other gaps were treated as missing data in analysis. We chose *Acrochordus granulatus* as the far outgroup for comparison with the

most recent family-level phylogeny (Wüster et al. 2008), with 22 other colubroid species also included as outgroups (Table S1). We partitioned the data set into eight segments: one for each rRNA gene (two total) and one for each codon position in protein-coding genes (six total). We calculated model likelihoods for each partition in PAUP* and estimated best-fit models of nucleotide evolution with MrModelTest 2.2 (Nylander 2004) using the Akaike information criterion (AIC). We conducted partitioned-model phylogenetic inference with BEAST 1.6.1 (Drummond and Rambaut 2007) using a Yule speciation process and a relaxed uncorrelated lognormal clock. Based on fossil data (Holman 2000; Parmley and Holman 2007), we set the following lognormal prior constraints: tMRCA of *Agkistrodon piscivorus*, *Agkistrodon bilineatus* and *Agkistrodon taylori* set to 4.7 Ma \pm 0.4 SD, tMRCA of *Sistrurus* + *Crotalus* set to 9 Ma \pm 0.2 SD. Based on a well-documented geologic event, we set a normal prior on the tMRCA of *Crotalus atrox* and *Crotalus ruber* to be 3.5 Ma \pm 0.4 SD. We ran two independent Markov chains for 4×10^7 iterations, sampling every 1×10^5 iterations. We used Tracer 1.5 (Rambaut and Drummond 2007) to verify stationarity of the Markov chain and conservatively discarded the first 1×10^7 generations as burnin, resulting in a sample of 600 independent topologies with associated ultrametric branch length estimates. We also generated a phylogeny with oviparous and viviparous species constrained to separate clades and compared the likelihoods using Bayes factors in Tracer 1.5 (Rambaut and Drummond 2007).

Character evolution estimates

Information on reproductive mode for each species was taken from the literature (Table S1). Two species (*Garthius chaseni* and *Trimeresurus malabaricus*) do not have reproductive modes reported; in analyses that do not allow unknown states, we treated these as having either mode, similar to species that show both reproductive modes (*Echis carinatus* and *Protobothrops jerdonii*). In addition, we treated *Atheris barbouri* as having unknown reproductive mode because of weak evidence for oviparity; Rasmussen and Howell (1998) mentioned *A. barbouri* was 'apparently oviparous like the species of *Atheris*', but all other species of *Atheris* are viviparous.

Parsimony

We compared character state changes across the sample of 600 trees under reversible, irreversible and Dollo models using MacClade 4.08 (Maddison and Maddison 2005). We estimated character history at all nodes across all trees using the Trace Character History module in Mesquite followed by the Step Through Trees command (Maddison et al. 2007). Character values for nodes were calculated as the number of nodes reconstructed with the character state over the total tree sample to incorporate node confidence into character estimates.

Likelihood

Models of character evolution were tested with likelihood methods using the program Multistate in the package BayesTraits (Pagel et al. 2004; available at <http://www.evolution.rdg.ac.uk>). Using our posterior sample of 600 topologies and the character states for extant taxa, we tested three competing models of character transitions: (1) a Dollo model in which the transition probability for the change from viviparity to oviparity was constrained to be 0, (2) an equal rates model that constrained changes in both directions to have equal probability and (3) a variable rates model that estimated transition probabilities for both directions independently. For all models, outgroups were eliminated to better conform to assumptions of complete taxon sampling. Additionally, the root node representing the ancestor of viperids was constrained to oviparity based on prior work asserting that this is the ancestral state for this group (e.g. Blackburn 1985) and that constraining the root is necessary for an appropriate test of Dollo's law (Nosil and Mooers 2005). This was carried out using the 'fossil' command. By constraining the root node instead of allowing the root state frequency to be determined by the

tip frequencies, we avoid overestimating the frequency of viviparity at the root node and provide a conservative test of unidirectional evolution.

Additionally, we used an evolutionary model that allows speciation and extinction rates to vary based on different states of a given character using the BiSSE module in Mesquite (Maddison et al. 2007). The BiSSE model has six parameters: speciation rates when lineages are in (1) state 0 and (2) state 1, extinction rates for lineages in each character state (3–4), and rates of character transitions (5) from state 0 to state 1 and (6) from state 1 to state 0. State-dependent speciation (λ) and extinction (μ) rates either were constrained to be equal or varied independently; state transition rates were constrained to be equal, varied independently or only allowed transitions from oviparity to viviparity (Dollo model). We constrained the root node representing the ancestor of viperids to oviparity using a revised BiSSE module designed by R. G. Fitzjohn and E. E. Goldberg (personal communication). We increased the number of optimizations for each tree from the default of 2 to 5 to increase the probability of convergence.

For all maximum likelihood analyses, harmonic mean likelihoods across all 600 trees were compared using AIC, calculated as $-2 * \ln(\text{likelihood}) + 2K$, and K being the number of parameters estimated from the data. Subtracting a model of interest from the model with the minimum AIC score produces a ΔAIC score, allowing comparisons among non-nested models. Models with ΔAIC of two or less have substantial support; models with ΔAIC of 10 or more are considered to have no support (Posada and Buckley 2004).

Bayesian

Our fourth model comparison used RJ-MCMC to simultaneously determine the model and parameters with the highest posterior probability given the reproductive mode data (Pagel and Meade 2006). We again used the program Multistate in the package BayesTraits (Pagel et al. 2004; available at <http://www.evolution.rdg.ac.uk>). As the distribution of character transition rates was not known *a priori*, we tested uniform, exponential and gamma distributions for the rate parameters. As recommended by the authors of BayesTraits (Pagel and Meade 2006), we did not specify the parameters of the chosen distribution but rather seeded them from a uniform (0–10) hyperprior distribution. We ran each Markov chain for 1.0×10^8 generations, sampling every 500 generations after a 1.0×10^7 generation burnin. We ran three chains each for the chosen distribution to ensure convergence on the same parameters and also used this analysis to reconstruct ancestral character states at generic-level nodes. Nodal character state estimates were determined by defining a clade with the `addNode` command, which estimates support over the subset of trees that contain that clade. This value was then multiplied by the posterior probability estimate for that node in the phylogeny.

Results

Phylogeny

The final alignment consisted of 2289 characters, of which 1233 were parsimony informative (12S 411, 216 informative; 16S 494, 189 informative; *cyt-b* 716, 416 informative; ND4 668, 412 informative). The consensus phylogeny was congruent with recent phylogenies (e.g. Wüster et al. 2008), and most nodes were resolved with strong support (Figures S1–S4). A notable area of low support in this phylogeny is intergeneric relationships within Viperinae, which were also resolved with low support in previous work (e.g. Lenk et al. 2001; Wüster et al. 2008). We used the phylogeny that did not constrain oviparous and viviparous species to separate clades because it fit the data significantly better, with harmonic mean log likelihood of -105100 ± 1.776 SE compared with -106000 ± 4.329 for the constrained phylogeny ($\Delta\text{BIC} -413.9$ for constrained model).

Character evolution

The reversible model of character evolution was most parsimonious, with an average of 17.16 and a range of 17–19 steps across all trees. Irreversible evolution resulted in an average of 24.45 and range of 20–27 steps; Dollo parsimony had an average of 24.94 and range of 23–27 steps. Parsimony character mapping showed similar patterns to character maps from other methods, but with higher node confidences (Fig. 1, Figures S1 and S2). One well-supported reversal from viviparous ancestors to oviparous descendants was recovered: *Lachesis* was oviparous in 100% of trees, with the common ancestor of New World pitvipers viviparous in 99% of trees (Fig. 1, Figure S2). Three other reversals were recovered with low support: oviparous *Parias* (100%) had three viviparous ancestors with 85% support, oviparous *Protobothrops* (95%) had two viviparous ancestors with 85% support and oviparous *Ovophis okinavensis* had a viviparous direct ancestor (94%).

In Multistate maximum likelihood comparison, the character evolution model that best fit the data was variable rates, with average $\ln(\text{likelihood})$ ($\ln L$) of -49.30 ± 0.830 SD (Table 1). Higher $\ln L$ scores represent more optimal models. Equal rates and Dollo models had lower likelihoods with ΔAIC values of 6.2 and 7.8 compared with the optimal model; these values suggest some support for the non-optimal models. The preferred model estimated the rate of transitions from oviparity to viviparity at 0.03405 ± 0.00374 SD, approximately 10 times higher than the rate of apparent reversals (0.003227 ± 0 SD).

BiSSE estimates found no significant effect of character state on speciation or extinction rates, with the optimal model constraining speciation and extinction rates to be equal for oviparous and viviparous lineages, and constraining reversals from viviparity to oviparity to minimum rates (Table 2). The only other model with ΔAIC support was the Dollo model allowing speciation and extinction rates to vary with character state. Models allowing reversals were significantly less likely, with ΔAIC values of 14.65–19.13.

Reverse-jump Markov Chain Monte Carlo Bayesian analysis with exponential and gamma-distributed hyperpriors had the highest harmonic mean likelihoods, and the exponential prior was used in further analysis to reflect the philosophical preference for explanations requiring fewer events (Occam's razor, FitzJohn et al. 2009). RJ-MCMC sampled the Dollo model most often, with that model used in 84.62% of the posterior probability sample. The next model, with support was the equal rates model, found in 14.82% of the posterior probability sample. Character state transition rates were estimated from all postburnin samples, with average $q_{\text{ovip to vivip}} = 0.0407 \pm 0.0002$ SD and $q_{\text{vivip to ovip}} = 0.0181 \pm 0.0002$ SD. Ancestral states reconstructed under RJ-MCMC generally had strong support for shallow, genus-level nodes, with low support for deeper nodes (Fig. 1, Figures S3 and S4). This finding led to a lack of support for apparent reversals in this analysis. Constraint of backbone nodes to oviparity or viviparity led to support for different models of character evolution: oviparity constraints supported Dollo models and viviparity constraints supported equal transition rate models. Oviparity constraints (-50.54 to -50.30) had greater log likelihoods than viviparity constraints (-53.38 to -53.24), but viviparity models had some support under AIC (Table S2). Nodal support for backbone nodes generally showed support for the character state of the additional node constraint.

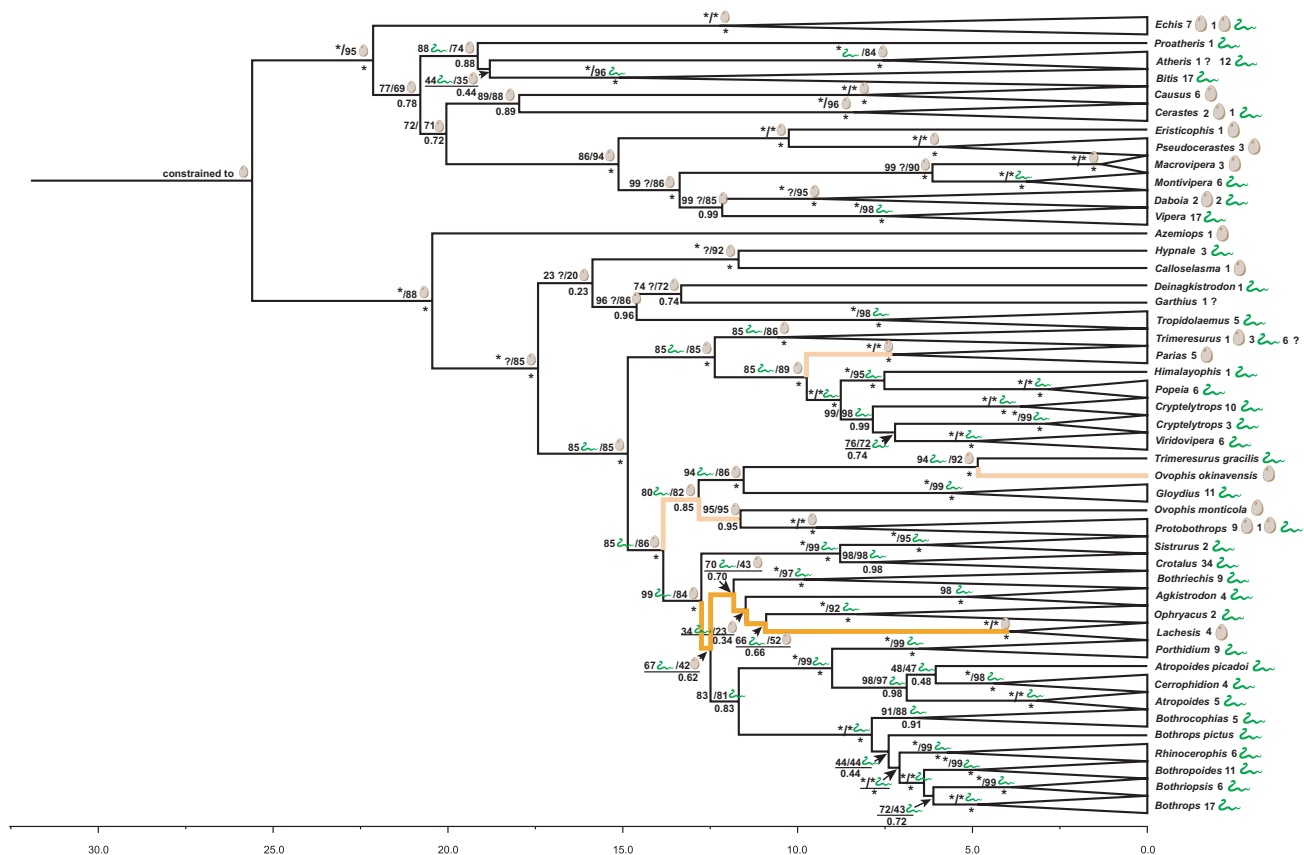


Fig. 1. Phylogram of viperid relationships showing the evolution of reproductive mode. Eggs denote oviparity, snakes denote viviparity, question marks denote species with unknown character states. Percentage of nodes recovered by parsimony/posterior probability for character reconstruction shown above node; posterior probability for phylogeny reconstruction shown below node. Branch lengths correspond to millions of years

Table 1. Maximum likelihood models tested. All models have some support under AIC, optimal model is bold. Parameter values are averages taken over the sample of 600 trees including standard deviations. Eggs symbolize rates under oviparity; snakes symbolize viviparity

Model	Parameters	LnL	Character state transition rate (q)		AIC	Δ AIC
			○→○	○→○		
Variable rates	2: $q_{\text{ovip to vivip}} \neq q_{\text{vivip to ovip}}$	-49.30 ± 0.83	0.0340 ± 0.0037	0.00323 ± 0.000	102.6	0
Equal rates	1: $q_{\text{ovip to vivip}} = q_{\text{vivip to ovip}}$	-53.42 ± 0.84	0.0136 ± 0.0014	0.0136 ± 0.0014	108.8	6.244
Dollo	1: $q_{\text{vivip to ovip}} = 0$	-54.21 ± 1.45	0.0487 ± 0.0057	0	110.4	7.824

AIC, Akaike information criterion.

Discussion

Evolution of reproductive mode in vipers







We postulate multiple gains of viviparity in vipers (Fig. 1, Figures S1–S4), but find equivocal support for reversals. Parsimony results showed apparent reversals in the ancestor of *Lachesis* with low support for reversals in the ancestors of *Parias*, *Ovophis okinavensis* and *Protobothrops* + *Ovophis monticola* (Fig. 1, Figures S1 and S2). Parsimony can take phylogenetic uncertainty into account but generally ignores uncertainty in character reconstruction; therefore, we expect the support for these reversals to be overestimates. The *Lachesis* parsimony result, yet, continues to provide an avenue for further study.

Maximum likelihood analyses found models allowing apparent reversals to be optimal (Table 1), but BiSSE

likelihood (Table 2) and RJ-MCMC analysis found the Dollo model optimal; the latter did not infer strongly supported reversals from oviparity to viviparity in the phylogeny (Fig. 1, Figures S3 and S4). BiSSE models found no significant effect of reproductive mode on speciation or extinction rates, supporting the validity of results from all model tests.

The model testing and character mapping results seem to be due to low support for intergeneric phylogenetic relationships and for the character reconstructions at backbone nodes. This is additionally supported by the results from RJ-MCMC analyses constraining backbone nodes to oviparity or viviparity (Table S2). Phylogenetic and character information in the backbone of the phylogeny does not appear to be strong enough to overcome the influence of prior values on backbone nodes. An increase in phylogenetic resolution may help accept or reject unidirectional evolution for reproductive mode in

Table 2. BiSSE models tested. Model name includes number of parameters for that model. Model 5d has some support under AIC, optimal model is bold. Parameters not mentioned in models were allowed to vary independently of each other. Parameter values are harmonic means taken over the sample of 600 trees. Eggs symbolize rates under oviparity; snakes symbolize viviparity

Model	Parameters	LnL	Speciation rate (λ)		Extinction rate (μ)		Character state transition rate (q)		AIC	Δ AIC
										
6	All rates variable	646.7	0.113	0.196	2.33e-6	3.62e-7	0.0282	0.0031	-1283	19.13
5a	Speciation rates equal ($\lambda_{\text{ovip}} = \lambda_{\text{vivip}}$)	647.8	0.193	0.193	3.874e-6	6.350e-6	6.770e-7	0.0102	-1286	16.39
5b	Extinction rates equal ($\mu_{\text{ovip}} = \mu_{\text{vivip}}$)	647.8	0.093	0.193	4.925e-6	4.925e-6	1.355e-6	0.0104	-1286	16.45
5c	Character state transition rates equal ($q_{\text{ovip to vivip}} = q_{\text{vivip to ovip}}$)	646.7	0.113	0.197	2.635e-6	1.350e-6	0.0031	0.0031	-1283	18.65
5d	Dollo transition rates ($q_{\text{vivip to ovip}} = 0$)	654.0	0.122	0.196	1.039e-6	1.042e-6	0.0416	1.000e-7	-1298	4.00
4a	$\lambda_{\text{ovip}} = \lambda_{\text{vivip}}, \mu_{\text{ovip}} = \mu_{\text{vivip}}$	646.7	0.196	0.196	2.493e-6	2.493e-6	0.0282	0.0031	-1285	16.65
3a	$\lambda_{\text{ovip}} = \lambda_{\text{vivip}}, \mu_{\text{ovip}} = \mu_{\text{vivip}}, q_{\text{ovip to vivip}} = q_{\text{vivip to ovip}}$	646.7	0.197	0.197	1.159e-6	1.159e-6	0.003	0.003	-1287	14.65
3b	$\lambda_{\text{ovip}} = \lambda_{\text{vivip}}, \mu_{\text{ovip}} = \mu_{\text{vivip}}, q_{\text{vivip to ovip}} = 0$	654.0	0.196	0.196	1.328e-6	1.328e-6	0.042	1.000e-7	-1302	0

AIC, Akaike information criterion.

vipers, but current results emphasize the importance of looking for congruence in multiple analyses to confidently detect violations of a well-established pattern.

Our parsimony and maximum likelihood results are in agreement with the results of de Fraipont et al. (1996, 1999) in their detection of apparent reversals and show that a focus on species- or genus-level variation in character states can provide perspective on evolutionary patterns that are not apparent from analysis of higher taxonomic levels (Shine and Lee 1999). Our inferred patterns also contribute to the findings of Lynch and Wagner (2009), who used parsimony and likelihood methods to support an apparent reversal from viviparity to oviparity in the boid *Eryx jayakari*. Their work finding an apparent reversal in a terminal taxon is enlightening, but inference of apparent reversals at deeper nodes would better suggest violations of Dollo's law. Deeper inferred reversals are preferred because these nodes should be less affected if, through natural history research, an oviparous terminal is found to contain viviparous members. Our work points towards those possibilities, but better resolution is necessary.

Our results contrast with the results of Lynch (2009); we find that the model constraining speciation, extinction and character state transition rates to be equal is not significantly different from models allowing those parameters to vary. Lynch found higher speciation rates in viviparous lineages. However, our results agree with the results of Lynch that transitions to viviparity were at least ninefold higher than transitions to oviparity (Table 2). Our most optimal BiSSE model and the most optimal RJ-MCMC model inferred Dollo transition rates, which suggests an even more extreme difference in character state transition rates. The major difference between these studies appears to be taxon sampling, as this phylogeny contains more comprehensive sampling of pitvipers. Sampling differences can certainly contribute to differences in phylogeny estimation, and character reconstruction methods often assume complete taxon sampling. Because of the equivocal nature of the combined results from Lynch's (2009) and our study, we find no definitive support for a particular model of reproductive mode evolution in vipers.

Lee and Shine (1998) suggest that as neither viviparity nor oviparity is evolutionarily 'superior', there is no compelling reason to expect evolution to act unidirectionally. They

suggested the presence of five potential reversals in squamate reptiles, two of those occurring in viperid genera *Lachesis* and *Cerastes*. The apparent reversal in *Lachesis* is supported by parsimony, but apparent reversals in *Cerastes* were not found in any of our analyses, possibly because of low phylogenetic resolution among viperines. Lee and Shine's arguments are supported by our viper results and should certainly be evaluated in other squamate reptiles, as well as expanded to other groups containing oviparous and viviparous lineages.

Implications for studies of character evolution

Our results support the importance of addressing current criticisms of phylogenetic tests of Dollo's law and other patterns of character evolution (Goldberg and Igić 2008): taking phylogenetic uncertainty into account in character state reconstruction, fulfilling the assumptions of the analyses used, correctly assigning character state frequencies to the root node and accounting for character-state-specific rates of lineage diversification. In some cases, preliminary analyses that ignored one or more of these criticisms inferred different patterns of character evolution, which would have led to very different conclusions.

The number of nodes in which character states are not strongly supported (Fig. 1, Figures S1–S4) suggests the importance of using models of character evolution that take all sources of uncertainty into account in character state reconstruction. In some cases, a character state was inferred with >95% confidence, but low support for the existence of the node lowered the confidence in that reconstruction.

Additionally, we ran MCMC analyses that tested the effect of stem length on character state reconstruction, and found it had minimal impact. Replacing the stem estimated by outgroup rooting with one of minimal length resulted in estimates that were well within one standard deviation of the estimate using the outgroup root (e.g. $q_{\text{ovip to vivip}} = 1.03 \pm 0.32$ with outgroup rooting, 1.09 ± 0.33 without). Character state assignment was similarly unaffected with node estimates changing no more than 4% of posterior probability. In no case did the length of the stem affect conclusions. This suggests that the differences between our outgroup sampling and that of Lynch (2009) should have no impact on results.

Incomplete sampling violates the assumptions of most character reconstruction methods (e.g. Maddison et al. 2007), although most phylogenies at this scale do not include all species. Our sampling included >75% of viperids, and work by FitzJohn et al. (2009) suggests BiSSE inference should be little affected by this amount of missing data. Work by Lynch (2009) in vipers found little effect on model estimates for phylogenies over 70% complete, and our ingroup sampling is more complete. Although character mapping may be affected by incomplete sampling, missing potential reversals, it appears that reproductive mode is generally conserved at the generic level. We sampled >95% of genera, making future work unlikely to change our conclusions.

Including outgroups in character analysis strongly violates the assumption of complete taxon sampling, and preliminary analysis including outgroups found all model tests strongly rejecting the Dollo model in favour of models including apparent reversals. In the light of our results, finding only marginal evidence of reversals, it seems that the inclusion of outgroups can have a strong influence and lead researchers to potentially incorrect conclusions.

One of the most strongly criticized aspects of phylogenetic tests of character evolution is incorrect assignment of character state frequencies to the root node of the phylogeny. Preliminary analyses that did not constrain the ancestor of viperids to oviparity resulted in reconstructions with higher likelihoods, but tended to reconstruct that root node as viviparous, which is incorrect based on prior work and the character states of extant taxa (Blackburn 1985). This error is predicted because the high frequency of viviparity in vipers can lead to incorrect estimation of character state frequencies at the root node (Goldberg and Igić 2008). Therefore, we consider our constrained analyses (Fig. 1, Figures S1–S4) to be the most biologically realistic reconstructions.

Although character-dependent variation in speciation and extinction rates may lead to false inferences of apparent reversal, in vipers we found no significant effect of character state on either speciation or extinction rates. Lynch (2009) found speciation rates to be significantly different for oviparous and viviparous vipers, which would suggest BiSSE to be the most appropriate analysis in this group. Our BiSSE results are somewhat different than those of Lynch as they support Dollo models, while the prior work allows a low rate of reversals. Overall, we find no definitive evidence supporting or rejecting Dollo's law.

In contrast to methodological criticisms of studies finding character reversals, Wiens (2011) suggested in certain cases, methodological biases may favour Dollo's law. He cites a few situations where the law may be incorrectly supported or give ambiguous results, including if species with reversals have higher diversification rates or if they go extinct and are undetected among extant taxa. The most relevant situation to this study is if a trait is regained multiple times within a clade, a clear pattern of loss and regain may be replaced by a mosaic of trait presence and absence. As multiple oviparous and viviparous groups are spread throughout the tree of vipers causing a mixture of states to be recovered in ancestral nodes, this could certainly lead to the ambiguity recovered by our analyses. We agree with Wiens that a detailed simulation study should provide insight into the difficulties in rejecting Dollo's law when it is false as well as the difficulties in supporting it when it is correct.

Future work on reproductive mode evolution

Our study found equivocal support for unidirectional evolution of viviparity from oviparity. Some methods suggested reversals are possible, particularly in *Lachesis*. Later, we discuss additional considerations for inferring reversals: timing of changes and identification of developmental pathways.

The assumption underlying unidirectional evolution is that genes in the pathway leading to the ancestral character accumulate mutations once the derived character is fixed in the population. This means that transitions from derived to ancestral states occurring shortly after the origin of the derived state may be permitted by Dollo's law. The reversals that are most interesting are those separated from origins of a derived state by >10 million years (Marshall et al. 1994). A review of recent Dollo's law studies (Wiens 2011) finds several examples of apparent reversals occurring 15–60 million years after a complex character was lost. Timing of potential character state change in *Lachesis* supports continued research on this group. The estimated origin of viviparity was in New World pitvipers, occurring 13.8 mya (95% CI 11.0–16.5; 20.1–29.1 per Wüster et al. 2008), with the estimated reversal in *Lachesis* occurring 3.9 mya (95% CI 2.9–5.2; 3.5–9.8 per Wüster, also see Fig. 1, Figures S2). This suggests the potential reversal occurred 10 million years or more after the origin of viviparity in the group. Although Sanders et al. (2010) suggest Wüster's dates may be older than predicted by certain fossils, our relative results are generally congruent with the results of Wüster et al.

The second requirement to discover true bidirectional evolution is to investigate developmental mechanisms that give rise to a complex character, to distinguish between convergence and true reversal (Collin and Miglietta 2008). If a character state arises through different pathways in ancestral lineages compared to lineages with phylogenetic patterns of reversal, the apparent reversals are actually convergent and unidirectional evolution may still stand. Mechanistic examination suggests that oviparity in sand boas may in fact be an independent evolution of that character state and not a true reversal (Lynch and Wagner 2009). A separate consideration is that selection on pleiotropic effects of the genes underlying a character state may conserve the possibility for that state to re-evolve through one or few mutational changes. Conservation of genes with pleiotropic effects is likely the mechanism underlying the re-evolution of metamorphic development in salamanders after 20–42 million years (Chippindale et al. 2004) and the re-evolution of shell coiling in slipper limpets after more than 10 million years (Collin and Cipriani 2003). We consider selection on pleiotropic effects to be a mechanism driving true reversals to ancestral states. Comparison of reproductive mechanisms in the viperid groups as mentioned earlier is beyond the scope of our study, but our results suggest that detailed comparisons of these genera with their closest viviparous relatives should prove enlightening.

Conclusions

When challenging an accepted explanation of biological patterns, one must find strong inferences of a competing pattern and be confident in the accuracy of those inferences. For example, the growing number of reported exceptions to the pattern of Dollo's law (reviewed in Collin and Miglietta 2008) are accompanied by a growing number of criticisms of the methods used, citing overconfidence in the results (Lee and

Shine 1998; Blackburn 1999; Goldberg and Igić 2008). Our methods provide a conservative test of Dollo's law and find equivocal support for violations of that law, illustrating the validity of current criticisms. These methods are easily replicated and should provide a strong test for any examination of patterns of character evolution.

In the case of transitions between oviparity and viviparity, the difficulty of these changes has simply been asserted and not empirically demonstrated (Lee and Doughty 1997). Costs of oviparity such as lowered ability to keep eggs at the proper temperature have been discussed often (Shine 1985, 2004; Shine and Lee 1999), but the benefits of oviparity and the costs of viviparity are rarely considered (but see Lynch and Wagner 2009). Pregnant females are burdened and must thermoregulate, making them more vulnerable to predation and less able to capture prey. Viviparous females may require appropriate energy sources throughout gestation, while oviparous females are freed from reproductive constraints on energy intake after laying. These and other reasons suggest selection may favour bidirectional evolution. We suggest further study on the patterns and processes of reproductive mode changes, but place the burden of proof on adherents of the view that oviparity has not reversed within squamates.

Reproductive mode variation is a dramatic macroevolutionary pattern in amniotes, and as such reversals from viviparity to oviparity are interesting from a variety of developmental and evolutionarily ecological perspectives. Our analysis provides potentially rewarding avenues of research in this area. Detailed comparative studies of embryo-maternal relationships across potential transitions in viperid reproductive modes, as well as investigation into potential selective factors driving the retention of or reversal to oviparity, are clearly called for. Furthermore, within vipers the putative pattern of origins and reversals in reproductive mode warrant further analysis in the context of an equally complex pattern for the presence and absence of parental care in these snakes (Greene et al. 2002).

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Resumen

La serpiente y el huevo: evolución unidireccional del modo reproductivo en víboras?

La ley de Dollo, donde caracteres complejos no son ganados de nuevo en el transcurso evolutivo, es un patrón aplicado a muchos sistemas. Trabajo reciente ha evaluado la evolución unidireccional en un amplio contexto, y se han documentado varias violaciones de dicha ley. Sin embargo, los métodos analíticos han sido criticados por potencialmente sobre-estimar las reversiones. Nosotros probamos la hipótesis que el modo reproductivo ancestral de la oviparidad pudo haber sido re-adquirido en víboras, así oponiéndose a la Ley de Dollo. Usamos comparación de modelos y métodos de reconstrucción ancestral para abordar las recientes críticas y encontramos evidencia que soporta y refuta las predicciones de la Ley dependiendo de los diferentes análisis. Discutimos nuestros resultados en el contexto de la evolución

unidireccional y revisamos los factores requeridos para la inferencia robusta de la violación de la Ley de Dollo.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Ultrametric phylogram of viper relationships with nodes showing the evolution of reproductive mode inferred via parsimony.

Figure S2. Ultrametric phylogram of pitviper relationships with nodes showing the evolution of reproductive mode inferred via parsimony.

Figure S3. Ultrametric phylogram of viper relationships with nodes showing the evolution of reproductive mode inferred via Bayesian RJ-MCMC.

Figure S4. Ultrametric phylogram of pitviper relationships with nodes showing the evolution of reproductive mode inferred via Bayesian RJ-MCMC.

Table S1. Taxa and data used in analysis, with reproductive mode for each species.

Table S2. RJ-MCMC analyses with various nodes constrained. Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.