

RE-EVOLUTION OF LOST MANDIBULAR TEETH IN FROGS AFTER MORE THAN 200 MILLION YEARS, AND RE-EVALUATING DOLLO'S LAW

John J. Wiens

Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY 11794-5245

E-mail: wiensj@life.bio.sunysb.edu

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Dollo's law states that structures that are evolutionarily lost will not be regained. Recent phylogenetic studies have revealed several potential examples in which Dollo's law seems to be violated, where lost structures appear to have been regained over evolutionary time. However, these examples have recently been questioned and suggested to be methodological artifacts. In this article, I document a striking and incontrovertible phylogenetic example of the re-evolution of a lost, complex structure: mandibular teeth in the frog genus *Gastrotheca*. I use a time-calibrated phylogeny for 170 amphibian species to show that mandibular teeth were lost in the ancestor of modern frogs at least 230 million years ago (Mya) and have been regained in the last ~5–17 My. I review recent studies on trait re-evolution and show that this long period of trait absence prior to re-acquisition is largely unprecedented. I also argue that there are several methodological issues that may cause trait re-evolution to be hardest to detect under those conditions when it is most likely to occur, leading to erroneous failures to reject Dollo's law. Finally, I discuss a mechanism that may facilitate trait re-evolution, and the evolution of mandibular teeth in frogs as an example of developmental constraint.

KEY WORDS: Amphibians, developmental constraint, Dollo's law, morphology, phylogeny.

The loss of complex structures is a common feature of evolution. For example, among the vertebrates, humans and frogs have lost a functional tail, most snakes have lost their limbs, and birds and turtles have lost their teeth. Dollo's law (Gould 1970) states that once such complex features are lost, they will not be re-acquired with the same form (review in Collin and Miglietta 2008).

Yet, recent phylogenetic studies have shown many apparent exceptions to Dollo's law. For example, Whiting et al. (2003) concluded that wings were lost and re-evolved in stick insects (Phasmida). Collin and Cipriani (2003) showed evidence for re-evolution of coiling in the shells of limpets (snails). Chippindale et al. (2004) and Wiens et al. (2007) found apparent re-evolution of the larval stage (from direct development) in salamanders and frogs, respectively. Kohlsdorf and Wagner (2006) and Brandley et al. (2008) showed evidence for the re-evolution of lost digits in lizards (see also Galis et al. 2010; Kohlsdorf et al. 2010). Lynch

and Wagner (2010) found that eggshells (oviparity) seemingly re-evolved within boid snakes.

Recently, Goldberg and Igic (2008) suggested that violation of Dollo's law is a "spectacular claim" (p. 2730) and that many of these examples may simply be the result of "devastating flaws" (p. 2727) in the methods used. Specifically, they suggested that previous studies were compromised by assumptions about state frequencies at the root of these phylogenies and by failure to account for the possible impact of the character on patterns of diversification (speciation and extinction). However, two subsequent studies that accounted for both of these issues still found strong support for re-evolution of lost structures (Lynch and Wagner 2010; Kohlsdorf et al. 2010), and Goldberg and Igic (2008) did not actually test the ability of methods to detect when Dollo's law was violated (i.e., they only simulated cases in which Dollo's law was true). Galis et al. (2010) have also questioned the evidence for re-evolution of lost digits (but see Kohlsdorf

et al. 2010). Overall, despite an increasing number of studies that suggest Dollo's law can be violated, the question of whether lost complex traits can re-evolve is still in doubt, and indisputable examples are needed (if they can be found).

The goals of the present study are fivefold. First, I document a striking and incontrovertible phylogenetic example of the re-evolution of a complex morphological trait: the re-evolution of lost mandibular teeth in the frog genus *Gastrotheca*. In some ways, this example is well known in the older, pre-phylogenetic herpetological literature (e.g., Boulenger 1882; Noble 1931; Trueb 1973), but it has not been explicitly tested, nor brought into recent discussions of Dollo's law (e.g., Collin and Miglietta 2008; Goldberg and Igic 2008). Second, I use a time-calibrated phylogeny to show that the time-scale over which this reversal occurs is remarkable. Although most previous studies on Dollo's law have not considered the temporal scale over which trait re-acquisition occurs, I review previous studies and show that the time frame of trait re-evolution is generally similar across these examples, with *Gastrotheca* mandibular teeth providing a striking exception. Third, I discuss a general mechanism that may help explain how re-evolution after this lengthy period of absence is possible. Fourth, I discuss several issues that may make violations of Dollo's law challenging to detect, such that these violations may actually be most difficult to infer under conditions when they are most likely to occur. Finally, I discuss the re-evolution of mandibular teeth in frogs (and lack thereof) as a possible example of developmental constraint.

Materials and Methods

BACKGROUND

The taxon currently known as *Gastrotheca guentheri* has long been recognized as the only living anuran with true teeth on the lower jaw (and not simply tooth-like extensions of the dentary bone; Noble 1931; Trueb 1973; Duellman and Trueb 1994; Fabrezi and Emerson 2003). Because of this unique trait, the species was formerly placed in its own genus and family (Amphignathodontidae, *Amphignathodon*; Boulenger 1882). Later, it was placed in the family Hylidae as a separate genus within the subfamily Hemiphractinae, and then as a species in the hemiphractine genus *Gastrotheca* (Duellman et al. 1988). Recent molecular studies have shown that hemiphractines are not closely related to other subfamilies of hylid frogs (Darst and Cannatella 2004; Faivovich et al. 2005; Wiens et al. 2005b), and should be recognized as a distinct family (Hemiphractidae; Wiens et al. 2005b). Recent molecular studies have also confirmed the placement of *G. guentheri* within *Gastrotheca* (Wiens et al. 2007). For the present study, the questions to answer are: (1) do statistical phylogenetic analyses confirm that the presence of mandibular teeth in *G. guentheri* is a case of re-evolution of a lost trait? (2) how long ago did anurans lose their mandibular teeth? and (3) when did *G.*

guentheri (apparently) get them back? Answering these questions requires a time-calibrated phylogeny that includes many amphibian species, particularly those in the phylogenetic neighborhood of *G. guentheri*.

MOLECULAR DATA

The nuclear gene RAG-1 (recombination activating gene 1) has been used extensively in studies of higher level phylogeny and divergence times of amphibians (e.g., Hoegg et al. 2004; van der Meijden et al. 2004, 2005; San Mauro et al. 2005; Wiens et al. 2005a; Bossuyt et al. 2006; Wiens 2007) and vertebrates in general (e.g., Townsend et al. 2004; Hugall et al. 2007; Alfaro et al. 2009). It was also used to reconstruct the phylogeny of *Gastrotheca* and other hemiphractids, along with other genes (Wiens et al. 2007). Therefore, I estimated phylogeny and divergence times using RAG-1. Although other genes have been used in amphibian phylogenetics, they have not been as widely sampled as RAG-1 (e.g., mitochondrial 12S and 16S have been sequenced in many anurans, but sporadically in other amphibian groups). Further, because of their faster rates of change, mitochondrial genes may be problematic for phylogeny reconstruction and divergence-time estimation in ancient groups such as amphibians (>250 million years old; see below). The results of this study show that RAG-1 alone gives an estimated phylogeny and divergence times within amphibians that are generally similar to those based on multiple nuclear and mitochondrial genes, using comparable methods (e.g., Roelants et al. 2007).

A data matrix for RAG-1 (~1400 bp) was assembled primarily by combining the matrices of Wiens (2007) for frogs (based mostly on data assembled from GenBank, including those from Hoegg et al. 2004; van der Meijden et al. 2004, 2005; San Mauro et al. 2005), Wiens et al. (2007) for hemiphractids (RAG-1) only, and Wiens et al. (2005a) for salamanders. Additional taxa, particularly caecilians and nonamphibian outgroups, were added from San Mauro et al. (2005), Hugall et al. (2007), and other sources. Finally, I searched GenBank for representatives of key taxa lacking in these previous studies. In general, taxa were only added if they had at least 700 bp of RAG-1 data that matched the other sampled taxa (50% completeness). However, exceptions were made for a few key taxa with ~550 bp that would otherwise be entirely unrepresented (e.g., centrolenids, dendrobatids). Although highly incomplete taxa can be accurately placed in phylogenetic analyses with sufficient character sampling (e.g., Wiens 2003; Philippe et al. 2004; Wiens and Moen 2008) the effect of missing data on divergence-time estimation is less clear, and so incomplete taxa were added sparingly. All taxa, GenBank numbers, and literature sources are listed in Appendix S1. Taxonomy follows AmphibiaWeb (2010; <http://amphibiaweb.org/>).

The major goals of the taxon sampling were to establish when frogs lost mandibular teeth and when they were (potentially)

regained in *Gastrotheca*. Taxon sampling therefore emphasized the base of frog phylogeny, Hemiphractidae, the larger groups to which hemiphractids belong (Neobatrachia, Hyloidea), and important taxa for establishing divergence dates (see below). Not all available RAG-1 sequences in amphibians were used, particularly within groups that are not closely related to hemiphractids (e.g., ranoid frogs, plethodontid salamanders). Taxon sampling included nearly all families of amphibians.

Several outgroup taxa were initially used outside of amphibians, including a dipnoan (*Lepidosiren*), actinistian (*Latimeria*), and several amniotes. However, preliminary analyses showed the sampled amniotes to be polyphyletic with respect to amphibians, clearly an artifact of incomplete taxon sampling (i.e., analyses of RAG-1 with more complete taxon sampling do not support this pattern; Hugall et al. 2007). Rather than adding more amniotes until the desired results were found, the amniotes were simply excluded and only the dipnoan and actinistian taxa were used as outgroups.

New sequences were added to the existing alignments manually, and then the final alignment was checked by translating to amino acid sequences using MacClade version 4.0 (Maddison and Maddison 2000). Indels appeared to be very rare among the sampled taxa, making alignment relatively unambiguous.

ESTIMATION OF PHYLOGENY AND DIVERGENCE TIMES

Two general approaches were used to estimate the phylogeny and divergence times of amphibian clades. First, I estimated the phylogeny using maximum likelihood (using RAxML version 7.2.0; Stamatakis 2006) and then used the reconstructed phylogeny to estimate divergence times with penalized likelihood (using r8s; Sanderson 2002, 2003). Second, the phylogeny and divergence times were estimated simultaneously using Bayesian analysis with an uncorrelated lognormal distribution of branch lengths (using BEAST; Drummond et al. 2006; Drummond and Rambaut 2007).

These two general approaches to estimating divergence dates were both used because recent simulations (Battistuzzi et al. 2010) suggest that the relative accuracy of different dating methods depends critically on whether the actual rates of molecular evolution for a given dataset are phylogenetically autocorrelated among branches (in which case methods such as penalized likelihood may be most accurate) or uncorrelated (favoring BEAST). Unfortunately, these same simulations suggest that currently available tests are powerless to distinguish these two models of evolution for a given dataset, and that the safest approach may be to consider the range of dates estimated when both types of methods are used (Battistuzzi et al. 2010).

RAxML analyses used a bootstrapping search with 500 replicates, combined with a heuristic search for the optimal tree (with 100 replicates). Previous analyses of RAG-1 data for amphibians

(e.g., Wiens et al. 2005a, 2007; Wiens 2007) showed that these data best fit a GTR + I + Γ model with separate partitions for each codon position. For RAxML, I used the recommended GTR + Γ model, which accounts for invariant sites (I) by utilizing a large number of rate categories for Γ (25, instead of the typical 4). The optimal likelihood tree was then used in analyses of divergence dates using penalized likelihood.

Penalized likelihood analyses used 25 fossil calibration points (see below), the largest number (so far) in any study of amphibian divergence dates. The most distant outgroup (*Latimeria*) was excluded to facilitate estimation of branch lengths at the root. Penalized likelihood analyses were implemented in r8s (version 1.71 for Unix; Sanderson 2003) using the TN (truncated Newton) algorithm. Cross-validated assessment was used to select the best-fitting smoothing parameter, initially using values from 10^0 to $10^{5.5}$ in exponential increments of 0.5. This analysis showed that values of $10^{2.5}$ and 10^3 had the lowest Chi-square error. To further refine this estimate, a range of values from $10^{2.5}$ to $10^{4.0}$ in exponential increments of 0.1 was then tested. This analysis showed that a value of $10^{2.8}$ gave the lowest Chi-square error. This value was then used to estimate the ages of clades, using 10 replicate optimizations. Confidence intervals for select clades were estimated by generating 200 likelihood trees from bootstrap resampling of characters with RAxML, and then summarizing the distribution of ages of these clades across these replicates using the “profile” command in r8s (using the smoothing factor selected from the analysis of the original data, two optimizations per replicate, and treating the standard deviation of ages $\times 2$ as the 95% confidence interval). However, these confidence intervals were narrow relative to the broad range of dates estimated by r8s and BEAST (at least for the age of frogs).

Bayesian analyses used BEAST version 1.5.4, with the uncorrelated lognormal relaxed clock model. Trees were estimated using random starting trees but constraining 24 nodes associated with fossil calibration points (see below) to be monophyletic. Analyses were conducted with the GTR + I + Γ model (with four rate categories for Γ), with separate partitions for different codon positions, estimated base frequencies, and with trees generated using a Yule speciation process. Fossil calibration points were treated as priors on clade ages, with a lognormal distribution, a standard deviation of 1 million years (arbitrary but standard), and offsets equal to the fossil age (such that the fossils were treated as minimum age estimates). For the root age, I used a normal distribution with no offset.

An initial analysis of 20 million generations was run on the high-performance computer cluster available at the Cornell Computational Biology Service Unit (<http://cbsuapps.tc.cornell.edu/best.aspx>). This analysis showed that the likelihoods of the trees increased slowly over time. Therefore, five additional analyses were run with 20 million generations each (note

that longer runs timed out), and I used the results from the analyses with highest likelihood values. For this analysis, likelihood values appeared to stabilize after 12 million generations (using Tracer, version 1.5, A. Rambaut and A. J. Drummond, available from <http://beast.bio.ed.ac.uk/Tracer>), and the trees from the first 12 million generations were discarded as burnin. The 8 million post-burnin trees had a mean likelihood of -36110 . Using Tracer, the post-burnin trees yielded effective sample sizes (ESS) on the likelihood, posterior, and prior close to or over 200 (>175), and ESS on almost all of the clade ages >200 (among the 25 clades used for calibration), with two between 200 and 130. For analyses of character evolution, I used the maximum clade credibility tree from the post-burnin sample of trees, using mean clade heights for clade ages. I also performed some analyses on sets of 1000 trees sampled from the set of 8000 post-burnin trees (8 million trees sampled every 1000 generations). The results showed very little variation in tree topology (i.e., most clades had posterior probabilities of 1), and so these analyses primarily addressed the robustness to variation in branch lengths.

A total of 25 fossil calibration points were used (see Appendix S2). For r8s, 24 calibration points were used as minimum-age estimates, and one was used as a fixed root age. For BEAST, all 25 calibration points were treated as priors on the ages of clades. Most calibration points were taken from Wiens (2007) with some corrections and additions (e.g., Roelants et al. 2007). In general, these calibration points correspond to the oldest fossil taxon that can be confidently assigned to a given clade, and the minimum age of the fossil is used (based on the end of the time period that the fossil taxon is known from). In many cases, a fossil assigned to a given higher taxon (e.g., family) could not be confidently assigned to that taxon's crown group, and therefore it was used to estimate the age of the stem group instead (i.e., the age of the most recent common ancestor of that family and its sister group).

ESTIMATING PATTERNS OF TRAIT EVOLUTION

Mandibular teeth are known to be absent in all known frogs except for *G. guentheri*, and present in all salamanders and caecilians (reviews in Trueb 1973; Duellman and Trueb 1994). These general patterns are also supported by my own observations for many of these taxa (e.g., Wiens et al. 2005a,b; J. Wiens, unpubl. data). The 172 taxa sampled for molecular analyses were scored for the presence (state 0) or absence (state 1) of mandibular teeth assuming these broad-scale patterns. Note that although modern dipnoans lack dentary teeth, fossil taxa show that they were present ancestrally within this clade (Ahlberg et al. 2006), and dipnoans were therefore scored as having mandibular teeth. However, I found that coding dipnoans as having state 1 or deleting dipnoans entirely yielded very similar results (i.e., strongly supporting trait regain, not shown).

Patterns of evolution in mandibular teeth were estimated using maximum likelihood in Mesquite, version 2.72 (Maddison and Maddison 2010). All analyses were conducted on the time-calibrated trees from both penalized likelihood (r8s) and Bayesian analyses (BEAST).

First, I compared the relative fit of a model with different estimated rates for gains and losses of mandibular teeth (Mk-2; Lewis 2001) to one in which mandibular teeth are only lost and never regained. For the latter analysis, the forward rate (0 to 1, teeth present to absent) was estimated and the backward rate was set to zero. Following the recommendations of Goldberg and Igic (2008), the state at the root of the tree was set to an equal probability of either state (as opposed to the default use of stationary state frequencies). However, I found that use of equal versus stationary state frequencies gave very similar results (not shown). Fixing of the presumed ancestral state at the root may be desirable for model testing (Goldberg and Igic 2008; FitzJohn et al. 2009), but is not possible in current versions of Mesquite. However, I used BayesTraits (M. Pagel and A. Reade; www.evolution.rdg.ac.uk) to compare the likelihood of these models when the root state was set to state 0 (mandibular teeth present) to the apparent default option (root states at equal frequencies) and found that the impact on likelihood scores was negligible (for both r8s and BEAST trees; unconstrained Mk-2: with equal frequencies $-\ln L = 13.0861$; with fixed root $-\ln L = -13.0871$; Mk-2 no reversal model: with both equal frequencies and fixed root $-\ln L = -39.8088$). These results strongly suggest that use of equal frequencies versus a fixed root state has no impact on these analyses.

I also estimated the support for different states being present on each branch, to identify where (and when) state changes occurred, using the best-fitting reconstruction model. For a given branch, a difference in ln-likelihoods of 2.0 or greater units was considered significant support for the reconstructed state, and a branch was considered ambiguously reconstructed if the difference was less than 2.0.

I also used the binary state speciation and extinction (BiSSE) approach (Maddison et al. 2007; implemented in Mesquite) to test for potential effects of mandibular teeth on diversification rates and for possible character-associated effects of diversification rates on the reconstruction of the evolution of mandibular teeth (see also Goldberg and Igic 2008). I compared the relative fit of a model in which estimated rates of losses and gains of mandibular teeth were allowed to vary to a model in which no reversals to the primitive state were allowed, but accounting for the potential effects of the different states on diversification rates for both models. I also compared models in which rates of diversification associated with each character state were allowed to differ, and in which these rates were set to be equal, to evaluate whether there is any evidence that this trait influences diversification. All BiSSE analyses used 10 likelihood optimization

iterations (comparison to results with 500 replicates suggested that 10 were adequate). Initial analyses showed that BiSSE was unable to calculate likelihoods when the rate of reversals (re-evolution of teeth) was set to zero. Instead, I set the rate of regains to a very small value (1.0×10^{-14} ; the smallest value for which a likelihood was estimated).

To compare models, I followed Goldberg and Igic (2008) in using differences in the Akaike information criterion (AIC), with $AIC = 2k - 2 \ln \text{likelihood}$, where k is the number of parameters that are estimated. An AIC difference between models of 10 or greater shows no support for the alternate model (Burnham and Anderson 2002).

One issue to note that is that the sampling of amphibian species here is incomplete (170 of >6700 species), which could bias estimates of character-state associated diversification rates (FitzJohn et al. 2009) or other analyses. However, all major clades are represented, and in approximate proportion to their relative species richness (e.g., the sampled proportions of species are 18% salamanders, 6% caecilians, and 76% frogs, whereas the actual proportions are 9%, 3%, and 88% respectively; AmphibiaWeb: <http://amphibiaweb.org/>). Further, there is no evidence from the sampled species that mandibular teeth influence diversification rates (see Results), nor is there a clear reason why they should.

In theory, the issue of incomplete taxon sampling could be incorporated using the methods developed by FitzJohn et al. (2009) and implemented in the R package *diversitree*. However, both of the methods would be problematic for this dataset. The “skeletal tree” method requires that the included species be randomly sampled from the underlying phylogeny, which is not the case here (e.g., many hemiphractids were sampled). The other approach (terminally unresolved clades) cannot be used here because it can only accommodate clades that have fewer than 200 species, whereas many frog clades have many more (e.g., Bufonidae, Dendrobatidae, Hylidae, Microhylidae, Ranidae, Strabomantidae; AmphibiaWeb 2010).

Results

The phylogeny estimated by maximum likelihood (Fig. 1; likelihood = -46583.68) is generally concordant with those from previous analyses using additional nuclear and mitochondrial genes and model-based methods (e.g., Roelants et al. 2007). Frogs and salamanders are supported as sister taxa, and relationships among families and other higher clades within salamanders and frogs are similar to previous estimates. There are some differences, including some higher level relationships within caecilians (for which few taxa were available for full RAG-1) and frogs (i.e., the relative placement of pipoids and discoglossoids). However, these rearrangements involve taxa sharing the same state for mandibular

teeth. Most importantly, the phylogenies show that *G. guentheri* is nested deep within *Gastrotheca*, which is nested deep within Hemiphractidae, which is nested deep within frogs.

Penalized likelihood analysis using this phylogeny (Fig. 1) gives divergence-date estimates similar to those of Roelants et al. (2007). For example, my results show the most recent common ancestor of living frogs splitting at 230.5 Mya, whereas Roelants et al. (2007) estimated this node at ~ 240 Mya. My results show that *G. guentheri* split from its sister species (*G. weinlandii*) at 4.64 Mya.

All comparisons of models and rate estimates are shown in Table 1. I initially compared the Mk-2 model (with separate rates estimated for 0 to 1 and 1 to 0 changes) to one in which 1 to 0 changes (i.e., re-evolution of mandibular teeth) are not allowed. The former model has a $-\ln \text{likelihood} = 13.7349$ (AIC = 31.4698), whereas the latter has $-\ln \text{likelihood} = 64.0354$ (AIC = 130.0708). The difference in AIC values (98.6010) shows that there is no support for the model of irreversible evolution (Dollo's law).

Use of the unconstrained BiSSE model gives a $-\ln \text{likelihood}$ of 880.1524 (AIC = 1772.3048). Setting the rate of trait reversal to a very small value (1.0×10^{-14}) gives a significantly worse $-\ln \text{likelihood}$ of 902.0218 (AIC = 1814.0436). The difference in AIC values (42.4204) shows no support for Dollo's law. Furthermore, comparing the unconstrained BiSSE model to a model in which diversification rates for each state are set to be equal yields an almost identical likelihood ($-\ln \text{likelihood} = 879.8047$). Therefore, there is no evidence that the presence or absence of mandibular teeth influences diversification rates, so reconstruction of ancestral states should not be biased by differences in diversification rates in taxa having one state or another.

Mapping the evolution of mandibular teeth onto this phylogeny using the best-fitting, two-parameter model shows unambiguously that teeth were present in caecilians, salamanders, and the ancestor of frogs and salamanders, but were lost in the ancestor of living frogs (Fig. 1). This analysis shows mandibular teeth as being unambiguously absent throughout the phylogeny of frogs, including the ancestor of *G. guentheri* and *G. weinlandii*. Combining these reconstructions with the time-calibrated phylogeny suggests that mandibular teeth were absent in frogs for at least 225 million years before re-appearing in *G. guentheri*. However, in theory, mandibular teeth could have been lost anywhere on this long branch for stem anurans, which dates from 332.2 to 230.5 Mya. Thus, mandibular teeth may have been absent for ~ 225 –338 million years before being regained within *Gastrotheca*.

Bayesian analyses with BEAST yield a similar estimate of phylogeny, but older divergence dates (Fig. 2). Crown anurans are estimated at 279.32 Mya and the age for *G. guentheri* is also much older (17.48 vs. 4.64 Mya). Although I use this tree to assess the robustness of the analyses of trait evolution, some

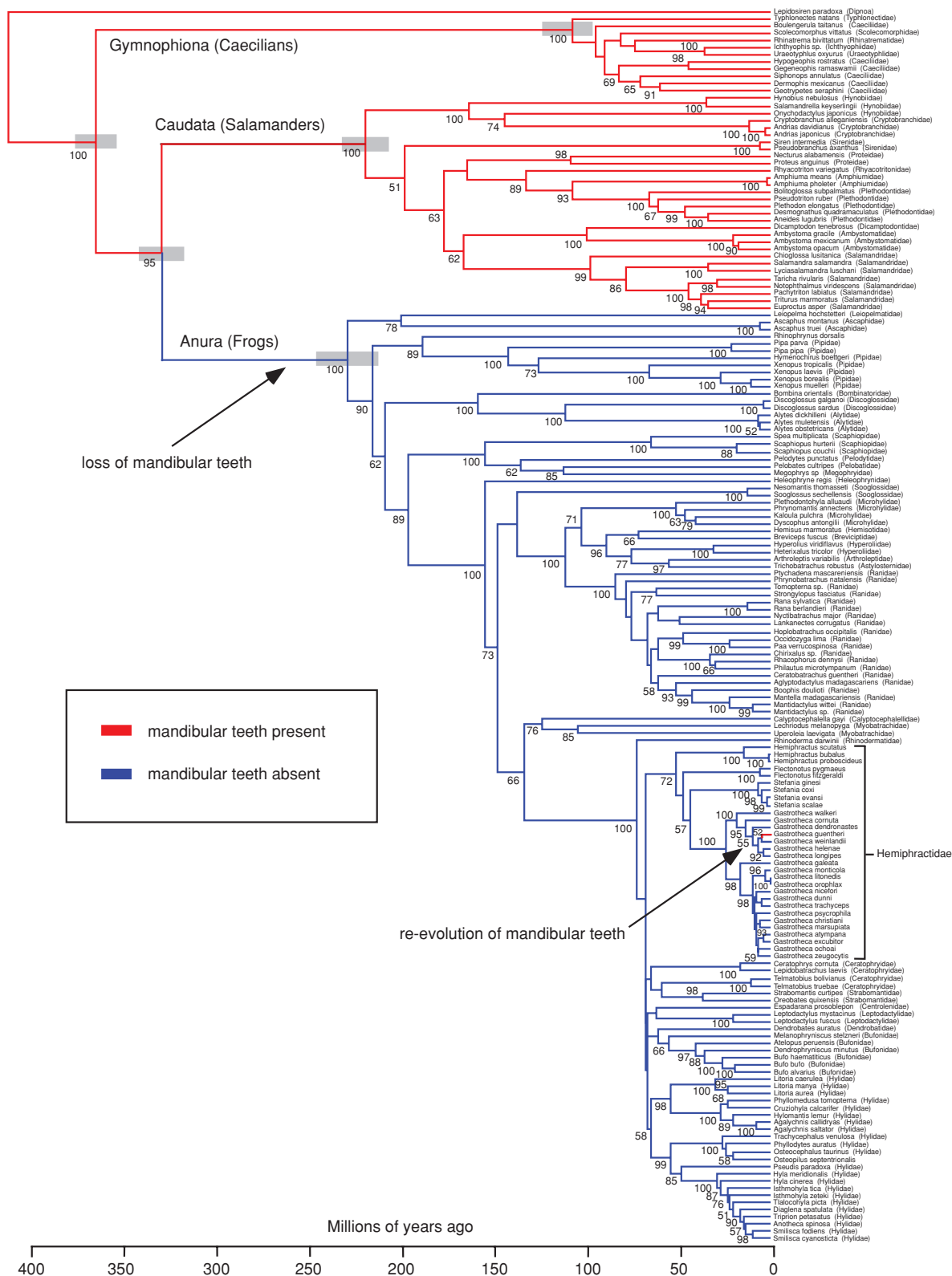


Figure 1. Re-evolution of lost mandibular teeth in frogs, based on maximum likelihood reconstruction (two-parameter model) on a phylogeny reconstructed using maximum likelihood, with branch lengths in units of time estimated using the penalized likelihood method. Numbers adjacent to nodes are bootstrap support values from the phylogenetic analysis. Bootstrap values <50% are not shown, and a few values within *Gastrotheca* are not shown due to space constraints. The gray bars associated with higher level clades represent the 95% confidence intervals for the estimated divergence times (due to the large number of taxa, these intervals are only shown for select clades). Ancestral character states for all branches are unambiguously reconstructed using maximum likelihood.

Table 1. Results from likelihood analyses comparing models with reversals allowed to those in which reversals are not allowed or are constrained to have a very low probability, using trees from penalized likelihood (Fig. 1) and Bayesian divergence time estimation (Fig. 2). Differences in the AIC scores (Δ AIC) show no support for models without reversal (regain of mandibular teeth). For simple two-parameter models (Mk2), parameter estimates include the rate of changes from 0 to 1 (r01; rate of mandibular tooth loss) and from 1 to 0 (r10; rate of regain of mandibular teeth). For the BiSSE model, parameter estimates also include r01 and r10, as well as a0 (speciation/extinction rate, with state 0) and a1 (speciation/extinction rate, with state 1), d1 (net diversification rate with state 1), and d0 (net diversification rate with state 0).

Penalized likelihood tree (from r8s)	
Mk2	Mk2 (no reversal)
–ln likelihood=13.7349	–ln likelihood=64.0354
AIC=31.4698	AIC=130.0708
r01=2.3396 $\times 10^{-4}$	r01=0.0041
r10=1.4804 $\times 10^{-4}$	r10=0
Δ AIC=98.6010	
BiSSE (unconstrained)	BiSSE (no reversal)
–ln likelihood=880.1524	–ln likelihood=902.0218
AIC=1772.3048	AIC=1814.0436
r01=2.5504 $\times 10^{-4}$	r01=1.9754 $\times 10^{-4}$
r10=2.7273 $\times 10^{-4}$	r10=1.0 $\times 10^{-14}$
a0=1.4573 $\times 10^{-6}$	a0=4.5753 $\times 10^{-5}$
a1=0.2264	a1=0.5346
d0=0.0098	d0=0.0113
d1=0.0154	d1=0.0106
Δ AIC=42.4204	
Bayesian tree (from BEAST)	
Mk2	Mk2 (no reversal)
–ln likelihood=13.7793	–ln likelihood=51.9826
AIC=31.5586	AIC=105.9652
r01=2.2494 $\times 10^{-4}$	r01=0.0040
r10=1.0979 $\times 10^{-4}$	r10=0
Δ AIC=74.4066	
BiSSE (unconstrained)	BiSSE (no reversal)
–ln likelihood=929.1332	–ln likelihood=951.7118
AIC=1870.2664	AIC=1913.4236
r01=1.2449 $\times 10^{-4}$	r01=1.2789 $\times 10^{-4}$
r10=1.3383 $\times 10^{-4}$	r10=1.0 $\times 10^{-14}$
a0=1.5806 $\times 10^{-5}$	a0=0.0211
a1=0.0808	a1=0.1440
d0=0.0118	d0=0.0116
d1=0.0116	d1=0.0112
Δ AIC=43.1572	

dates differ markedly from other recent estimates and so should be viewed with considerable caution.

Likelihood analyses of character evolution on the tree from BEAST give very similar results to the tree from r8s (Table 1). The Mk2 model has $-\ln$ likelihood = 13.7793 (AIC = 31.5586), whereas the no-reversal model has $-\ln$ likelihood = 51.9826 (AIC = 105.9652). Again, the difference in AIC values (74.4066) shows no support for the model of irreversible evolution. Furthermore, performing these comparisons on 1000 post-burnin trees from the BEAST analysis gives very similar results, with a mean $-\ln$ likelihood for the Mk2 model of 14.5105 (range = 12–20; AIC = 33.0210) and $-\ln$ likelihood of 54.0690 (range = 44–67; AIC = 110.1380) for the irreversible model, showing no support for the Dollo model (AIC difference = 77.1170).

The unconstrained BiSSE model (Table 1) gives a $-\ln$ likelihood of 929.1332 (AIC = 1870.2664). The BiSSE model with the rate of trait regain constrained to approach zero has significantly worse fit than the unconstrained model, with $-\ln$ likelihood of 951.7118 (AIC = 1913.4236). The AIC difference (43.1572) shows no support for the Dollo model, and there is again no evidence that mandibular teeth influence diversification rates (model with diversification rates for the two states set to be equal has $-\ln$ likelihood = 928.4163; AIC = 1866.8326; AIC difference with unconstrained model = 3.4338). Mapping mandibular teeth on the Bayesian phylogeny (Fig. 2) shows the same pattern found on the tree from penalized likelihood. The Bayesian phylogeny suggests that mandibular teeth were absent for at least ~262–298 million years before being regained within *Gastrotheca*.

Discussion

STRONG EVIDENCE FOR VIOLATION OF DOLLO'S LAW

Recent years have seen numerous potential examples of the re-evolution of lost features (e.g., Whiting et al. 2003; Collin and Cipriani 2003; Chippindale et al. 2004; Kohlsdorf and Wagner 2006), seemingly in violation of Dollo's law of irreversibility. Goldberg and Igic (2008) recently questioned the results of many of these studies on methodological grounds. The results presented here offer an incontrovertible phylogenetic example of trait re-evolution, showing that mandibular teeth were lost in the ancestor of all living frogs and then re-evolved in the hemiphractid species *G. guentheri*. The alternate hypothesis, that mandibular teeth were lost independently in each of the dozens of lineages leading up to *G. guentheri*, is statistically unsupported and seems incredibly unlikely. Although the hypothesis that *G. guentheri* re-evolved mandibular teeth may be unsurprising to experts in amphibian anatomy, this compelling example has been ignored in the recent literature on Dollo's law. Further, this example is made remarkable by the application of a time scale for this event: mandibular teeth

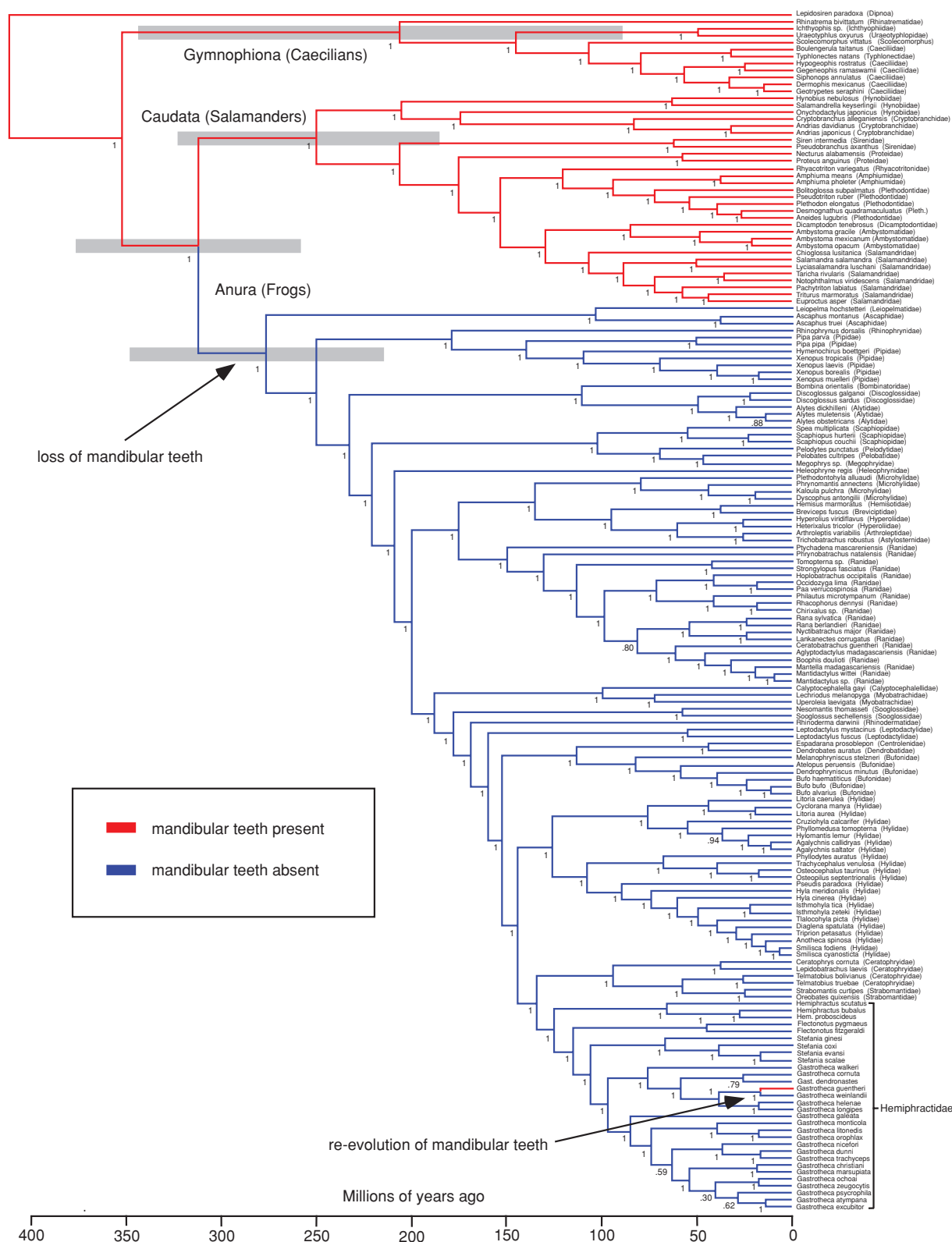


Figure 2. Re-evolution of lost mandibular teeth in frogs, based on maximum likelihood reconstruction (two-parameter model) on a phylogeny reconstructed (along with estimated divergence times) using Bayesian analysis with the uncorrelated lognormal model (in BEAST). Numbers adjacent to nodes are posterior probabilities for clades (but note that clades used for fossil calibration were constrained to be monophyletic). The gray bars associated with higher level clades represent the 95% highest posterior density interval for the estimated divergence times (due to the large number of taxa, these intervals are only shown for select clades). Ancestral character states for all branches are unambiguously reconstructed using maximum likelihood.

Table 2. The time frame over which lost traits may be regained. Ranges of values that include 0 are cases in which the trait has re-evolved in an extant species, and it is unclear at what point in the species' history the trait re-evolved. For *Bachia*, only the approximate age of the genus is known (~60 Mya; from Brandley et al. 2008), and the exact placements of digit loss and gain are also uncertain. The ultrametric tree of Kohlsdorf et al. (2010) suggests that digits were most likely lost by the latter half of the history of the genus (i.e., the clade including *B. bresslaui*) and regained in the final quarter (e.g., *B. panoplia*). However, estimated dates for *Bachia* can only confidently show that loss and re-evolution of digits occurred within the past ~60 million years.

Organism	Trait regained	When lost (Mya)	When regained (Mya)	Time lost (My)	Source for reconstruction	Source for time
Plethodontid salamanders	Larval stage	45–50	18–20	~25–32	Chippindale et al. (2004)	Kozak et al. (2009)
Hemiphractid frogs	Larval stage	~50	~10	~40	Wiens et al. (2007)	this study
Amphisbaenian lizards (<i>Bipes</i>)	Forelimb digits	~120	0–25	~95–120	Brandley et al. (2008)	Brandley et al. (2008)
Gymnophthalmid lizards (<i>Tretioscincus</i>)	Forelimb digits	~60	0–40	~20–60	Brandley et al. (2008)	Brandley et al. (2008)
Gymnophthalmid lizards (<i>Bachia</i>)	Digits	~30	~15	~15	Kohlsdorf et al. (2010)	Brandley et al. (2008); Kohlsdorf et al. (2010)
Scincid lizards (<i>Scelotes mirus</i>)	Digits	~35	0–15	~20–35	Brandley et al. (2008)	Brandley et al. (2008)
Boid snakes (<i>Eryx jayakeri</i>)	Eggshell	~80	0–50	~30–80	Lynch and Wagner (2010)	Noonan and Chippindale (2006)
Snail	Shell coiling	99–65	20–24	~41–79	Collin and Cipriani (2003)	Collin and Cipriani (2003)

were absent for at least 225 million years (and likely much longer) before being regained. In the sections that follow, I review the time frame of trait re-evolution in other studies, discuss a general mechanism that may have allowed mandibular teeth to re-evolve despite this long absence, discuss some potential methodological biases in testing for violations of Dollo's law, and finally discuss mandibular teeth in frogs as a possible example of developmental constraint.

THE TIME FRAME OF DOLLO'S LAW: IS THERE A STATUTE OF LIMITATIONS?

The molecular phylogenetic results presented here suggest that mandibular teeth were absent in anurans for ~225–338 million years before their re-evolution in *G. guentheri*. This range of dates is broad primarily because it is unclear where on the anuran stem branch mandibular teeth were lost. However, the fossil record also has direct bearing on estimates of the amount of time that mandibular teeth have been absent in frogs. Rocek (2000) described the late Jurassic (La Matilde formation; Callovian-Oxfordian; 164.7–155.7 Mya) anuran *Notobatrachus deguistoi* as clearly lacking teeth on the dentary, and this taxon seems to be closely related to modern anurans (e.g., only nine

presacral vertebrae). More importantly, the Triassic stem-group anuran *Triadobatrachus massinoti* (245–251 Mya, see above) also seems to lack teeth on the lower jaw (Rage and Rocek 1989). The absence of mandibular teeth in this taxon supports the idea that loss of mandibular teeth evolved before the origin of crown-group anurans, and that mandibular teeth were absent for at least 220 million years before re-evolving in *G. guentheri*.

Many of the studies that have postulated re-evolution of lost structures did not address the time frame over which traits were absent before their putative re-evolution (but see Collin and Cipriani 2003). Nevertheless, many of these studies can be reinterpreted in the light of time-calibrated phylogenies, and the resulting estimates are summarized in Table 2. Some caveats should be made, however. First, I assume that the original authors' trait reconstructions are correct (see below for some evidence supporting this assumption). Second, I assume that the estimated divergence times are not grossly incorrect. Third, even if the reconstructions and divergence times are correct, it is not known where on a branch a state change occurs. For internal nodes, the minimum amount of time that a state has been absent can be inferred from the age of the node above the branch where it was seemingly lost. However, for long terminal branches associated

with extant species, it is still unclear where (or when) on that branch the trait re-evolved. Thus, there are broad ranges of times associated with several cases. Despite these uncertainties, in these eight cases in which the temporal scale of trait re-evolution can be most readily evaluated, traits generally re-evolved ~15–60 million years after being lost. One important exception is the putative re-evolution of lost digits in the amphisbaenian lizard genus *Bipes* after ~100 million years (Brandley et al. 2008). Thus, the re-evolution of lost teeth in *Gastrotheca* after ~240 million years is exceptional.

A LOOPHOLE IN DOLLO'S LAW?

Marshall et al. (1994) provided a mechanistic, theoretical explanation for why Dollo's law might only operate within a certain time frame. They suggested that genes and developmental pathways that are not maintained by selection will decay due to mutational changes, making re-evolution of lost structures very unlikely after >10 million years. Remarkably, my survey of eight studies with temporal information suggest that lost structures have generally re-evolved after >20 million years (Table 2). One way that such reversals might be facilitated is if the structure in question has a homologue elsewhere in the organism, such that most of the relevant genes and developmental pathways for making the structure are maintained (e.g., West Eberhard 2003; Collin and Miglietta 2008; Yedid et al. 2008). Several other putative examples of trait re-evolution also involve structures in a given location that have obvious homologues nearby (first vs. second molar teeth in *Lynx*; Kurten 1963; median vs. compound eyes in arthropods; Oakley and Cunningham 2002; lost digits in squamates; Brandley et al. 2008). This hypothesis may explain how mandibular teeth in frogs could re-evolve in *Gastrotheca* after their long absence, given that teeth on the upper jaw are maintained in most major anuran clades (Trueb 1973; Duellman and Trueb 1994). However, alternate explanations are also possible. For example, recent studies also suggest that most (but not all) of the developmental pathways for tooth development are maintained in birds (or at least the chicken), despite the absence of teeth in (adult) birds for >60 million years (review in Collin and Miglietta 2008). In addition, there are other examples of long-term trait re-evolution that do not involve structures with homologues elsewhere in the organism (e.g., Collin and Cipriani 2003).

METHODOLOGICAL BIASES FAVORING DOLLO'S LAW

Goldberg and Igic (2008) provide a detailed study suggesting that there were serious flaws in how Dollo's law was tested in many previous studies. They suggest that these flaws may have caused Dollo's law to be incorrectly rejected in previous studies. Their study offers several valuable methodological recommendations for testing Dollo's law, which have been adopted in this and other recent studies (e.g., Lynch and Wagner 2010; Kohlsdorf et al.

2010; but note that these studies still strongly support the idea that Dollo's law is violated and that complex features re-evolve, as does re-analysis of plethodontid life histories (Chippindale et al. 2004) using the tree of Kozak et al. (2009); J. Wiens, unpubl. data).

However, it is important to note that Goldberg and Igic (2008) base their paper on the assumption that Dollo's law is universally correct and that the only relevant methodological biases are therefore those that would cause an analysis to fail to support it. In fact, their study completely failed to address cases in which Dollo's law was violated. Thus, the methods that they recommend for testing Dollo's law may themselves have "devastating flaws" when applied to cases in which Dollo's law is actually violated. Clearly, additional simulations are needed to test the ability of methods to accurately detect when Dollo's law is violated, and not simply cases when it is true.

In fact, several issues could cause Dollo's law to be incorrectly supported, or at least give ambiguous results when it should actually be rejected. For example, Goldberg and Igic (2008) implicitly assume that a character state's impacts on diversification rates will only cause Dollo's law to be incorrectly rejected. But a character state's influence on diversification rates might also cause Dollo's law to be incorrectly supported instead. For example, if species that have re-evolved the complex trait have higher diversification rates (e.g., after regain of larval development in *Desmognathus*, Kozak et al. 2005) and become predominant within a clade, it may be difficult to infer that the few species with the simple trait gave rise to the many species with the complex trait. Or, if the taxa that have re-evolved the complex trait have a decreased diversification rate, they may go extinct and be undetected among extant taxa. For example, recent fossil finds (reviewed in Coates and Ruta 2000) and new phylogenies integrating molecular and fossil data (Wiens et al. 2010) suggest that some relatively derived but extinct fossil snakes (e.g., *Haasiophis*) have well-developed limbs with multiple digits. This pattern suggests that some snakes may have re-evolved limbs with multiple digits and then became extinct (although there presently is no basis for saying that they went extinct because of their digits).

Other biases may also make violations of Dollo's law very difficult to detect. For example, our ability to detect trait re-evolution may depend on where in the phylogeny the trait is regained, with re-acquisitions that occur soon after trait loss being much harder to detect than those that occur after a long period of trait absence (Fig. 3). In this study, the re-acquisition of mandibular teeth in frogs is particularly clear because mandibular teeth were lost in the ancestor of living frogs and because *G. guentheri* is deeply nested within frog phylogeny among the advanced frogs (neobatrachians). An illustrative counterexample is offered by the frog genus (*Ascaphus*), which is near the root of frog phylogeny, and in which males have a fleshy tail that is lacking in other living frogs (Duellman and Trueb 1994). Because

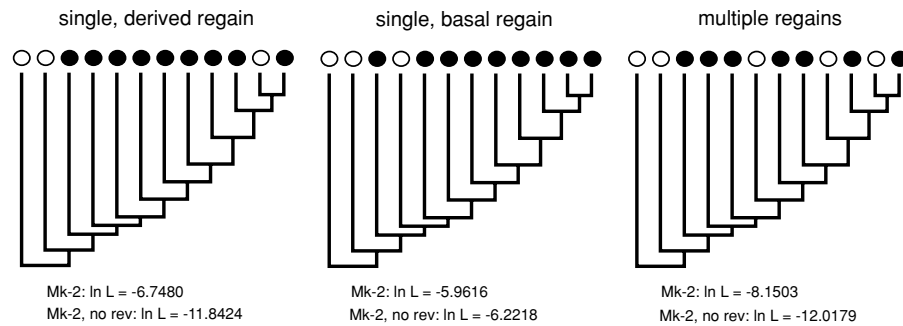


Figure 3. Hypothetical example showing that re-evolution of lost traits (reversals) may be easiest to detect when they occur later in the history of the group rather than earlier, and when they occur only once within a group rather than multiple times. Phylogeny was generated in Mesquite under a Yule model, and subsequently modified. Open circles indicate the structure is present, whereas filled circles indicate the structure is absent. Likelihoods are compared for a two-parameter model with forward and backward rates estimated ("Mk-2") and for a two-parameter model in which the backward rate is set to zero ("Mk-2, no rev").

of the phylogenetic placement of *Ascaphus*, it is unclear whether its tail represents retention of the ancestral state combined with two instances of tail loss in other frogs, or one instance of tail loss in the ancestor of living frogs followed by tail re-evolution in *Ascaphus*. Reconstructing this trait with likelihood on the BEAST tree (assuming tail presence outside anurans), the Mk-2 model has a ln-likelihood of -9.6697 ($AIC = 23.3394$) and the reconstructions for key nodes are ambiguous, whereas an irreversible model has a ln-likelihood of -9.9407 ($AIC = 21.8814$), with no significant difference in the likelihoods. Thus, if a tail did re-evolve in *Ascaphus*, the placement of *Ascaphus* in frog phylogeny makes this pattern ambiguous and effectively undetectable.

Given this perspective, the observation that most well-documented cases of trait re-evolution occur after a period of trait loss of >15 million years (Table 2) may also reflect methodological bias. A complex structure that re-evolves may need to be absent for tens of millions of years before its re-acquisition can be confidently distinguished from multiple losses (e.g., given typical diversification rates, this period of time may be needed for a group to diversify enough to have species nested deep in the phylogeny). Yet, if the gain of lost traits is possible, a consideration of the underlying genetics suggests that it should be much more likely soon after the trait is lost (Marshall et al. 1994). Thus, trait re-evolution may actually be hardest to detect under the conditions when it is most likely to occur, raising the question of whether trait re-evolution might be more widespread but frequently undetected due to methodological biases.

Similarly, if a complex trait is regained multiple times within a clade instead of just once, trait re-evolution may actually become more difficult to detect, rather than easier (Fig. 3). If a trait re-evolves only once, there may be a clear pattern in which the regained state is nested deeply among species that lack it (e.g., mandibular teeth in frogs), making multiple losses relatively easy to reject. If the state re-evolves multiple times, this

clear pattern could be replaced by a mosaic of trait presence and absence, where multiple regains and multiple losses may be more difficult to distinguish from each other. Such a pattern could make trait re-evolution harder to detect under conditions when it is more prevalent. As a semi-hypothetical example, if mandibular teeth were regained in six arbitrarily selected major clades in addition to *G. guentheri* (e.g., bufonids, ceratophryines, hylids, microhylids, ranids, strabomantids), the AIC difference between reversible and irreversible models would actually shrink from 89.2168 to 55.4370 (i.e., offering less support for trait re-evolution). In summary, these issues seem worthy of a detailed simulation study that addresses the difficulties in rejecting Dollo's law when it is false, and not just the difficulty of supporting it when it is true.

MANDIBULAR TEETH AND DEVELOPMENTAL CONSTRAINT

The loss and re-evolution of mandibular teeth in frogs may also offer an intriguing example of developmental constraint. This example requires that we consider why mandibular teeth were lost in frogs in the first place.

Although teeth may play a vital role in prey capture in most carnivorous vertebrates, many frogs capture their prey lingually, flipping the anteriorly attached tongue downwards and outwards over the lower jaw (Nishikawa and Cannatella 1991; Duellman and Trueb 1994). Thus, the mandibular teeth may not be used in prey capture, and may therefore be unnecessary. Lingual prey capture is also used in some salamanders, which have retained mandibular teeth (Larsen et al. 1989; Duellman and Trueb 1994). In anurans, however, the lower jaws are further modified such that the ancestrally tooth-bearing dentaries are highly flexible, and are depressed anteriorly during prey capture to facilitate flipping the tongue out of the mouth (Duellman and Trueb 1994).

Lingual prehension of prey may not work well for all prey items. Many frogs that eat larger prey (e.g., other frogs) have evolved relatively inflexible mandibles and fang-like teeth on the upper jaw (e.g., the ranid *Ceratobatrachus* and the hemiphractid *Hemiphractus*), and these teeth are seemingly used in prey capture instead of the tongue (Duellman and Trueb 1994). It seems that selection would favor the evolution of mandibular teeth in these taxa, if they were to arise. Intriguingly, several species of frogs that have evolved fang-like teeth on the upper jaw have also evolved tooth-like structures (serrations, odontoids) on the lower jaw, but not actual teeth (e.g., *Ceratobatrachus*, *Hemiphractus*; review in Fabrezi and Emerson 2003). Thus, it appears that selection favors the evolution of tooth-like structures, but teeth have not evolved due to a developmental constraint on their re-evolution. Many questions remain, however, and it is particularly unclear why *G. guentheri* (and not other taxa) was able to break this apparent constraint and re-evolve mandibular teeth. Research on the development and genetics of mandibular teeth in *G. guentheri* and other amphibians could be particularly interesting. It should also be noted that the specific diet of *G. guentheri* in nature remains unreported, although its very large body size (J. Wiens, pers. obs.) strongly suggests that it may also eat small vertebrates and other large prey, given the general relationship between prey size and body size in treefrogs (e.g., Duellman 2005; Moen and Wiens 2009).

Conclusions

In this study, I use a time-calibrated phylogeny to show strong support for the re-evolution of lost mandibular teeth in frogs. This example of trait re-evolution is particularly intriguing because these complex structures have re-appeared after at least 200 million years of absence. A review of previous studies on Dollo's law suggests that this time frame is considerably longer than in any other example. The re-evolution of mandibular teeth after this very long period may be facilitated by the maintenance of teeth on the upper jaw, which may help preserve the genes and developmental pathways needed for tooth development on the lower jaw. Similar mechanisms may underlie other putative examples of trait re-evolution. Despite recent controversy (e.g., Goldberg and Igic 2008; Galis et al. 2010), this study confirms that striking phylogenetic examples of trait re-evolution are possible, and are not merely artifacts of statistical methods or questionable phylogenies. In fact, there are methodological biases that may make violations of Dollo's law hardest to detect under those conditions where it may be most common, and the ability of phylogenetic methods to detect cases in which Dollo's law is violated remains completely unstudied. Finally, I note that other obvious violations of Dollo's law may be present but dismissed as "common knowledge" in various organismal disciplines, and should also be

explicitly tested using modern methods with time-calibrated phylogenies (e.g., possible re-evolution of functional claws on the wings of the bird, the hoatzin, *Ophisthocomus hoazin*; Hughes and Baker 1999).

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Supporting Information

The following supporting information is available for this article:

Appendix S1. GenBank numbers for RAG-1 sequences used in phylogenetic analyses of amphibians.

Appendix S2. Twenty-five fossil calibration points used for divergence-time estimation.

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

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