

PHYLOGENETIC ANALYSES REVEAL UNEXPECTED PATTERNS IN THE EVOLUTION OF REPRODUCTIVE MODES IN FROGS

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Understanding phenotypic diversity requires not only identification of selective factors that favor origins of derived states, but also factors that favor retention of primitive states. Anurans (frogs and toads) exhibit a remarkable diversity of reproductive modes that is unique among terrestrial vertebrates. Here, we analyze the evolution of these modes, using comparative methods on a phylogeny and matched life-history database of 720 species, including most families and modes. As expected, modes with terrestrial eggs and aquatic larvae often precede direct development (terrestrial egg, no tadpole stage), but surprisingly, direct development evolves directly from aquatic breeding nearly as often. Modes with primitive exotrophic larvae (feeding outside the egg) frequently give rise to direct developers, whereas those with nonfeeding larvae (endotrophic) do not. Similarly, modes with eggs and larvae placed in locations protected from aquatic predators evolve frequently but rarely give rise to direct developers. Thus, frogs frequently bypass many seemingly intermediate stages in the evolution of direct development. We also find significant associations between terrestrial reproduction and reduced clutch size, larger egg size, reduced adult size, parental care, and occurrence in wetter and warmer regions. These associations may help explain the widespread retention of aquatic eggs and larvae, and the overall diversity of anuran reproductive modes.

KEY WORDS: Amphibians, clutch size, development, egg size, life history, parental care.

A major challenge in evolutionary biology is to explain the phenotypic diversity of traits and organisms, and why traits evolve multiple times. The observation that a given character state evolves multiple times is often taken as evidence that it is strongly favored by natural selection under some conditions (i.e., the basis for the comparative method; Harvey and Pagel 1991). However, if a derived state is strongly favored, it may simply evolve once and become widespread among species, leaving few opportunities for repeated origins (e.g., Wiens et al. 2006). Thus, explaining multiple origins of a trait may also require that the primitive state be maintained for long periods of time or re-evolve frequently (both

providing opportunities for the derived state to arise repeatedly). Therefore, an underappreciated challenge in understanding patterns of trait evolution (and phenotypic diversity) is to uncover the selective factors that favor long-term maintenance of ancestral traits, as well as multiple origins of the derived states (but see the related literature on evolutionary stasis, e.g., Hansen and Houle 2004; Uyeda et al. 2011).

Anuran amphibians (frogs and toads) exhibit a remarkable diversity of reproductive modes, which appears to be driven by the repeated evolution of terrestrial reproduction and direct development from ancestors that bred entirely in water (Duellman

and Trueb 1986; Wells 2007). Anuran reproductive modes range from those in which there are large numbers of small, unprotected, aquatic eggs to those in which a few, large, terrestrial eggs are zealously guarded by adults. There is also a bewildering diversity of seemingly intermediate modes (including placement of eggs in burrows, foam nests, bromeliads, skin pouches, stomachs, and vocal sacs; reviews in Duellman and Trueb 1986; Haddad and Prado 2005; Wells 2007).

Direct developing amphibians have terrestrial eggs and lack a free-living larval stage (Duellman and Trueb 1986; Wells 2007). Because direct development frees species from their dependence on water bodies for reproduction, direct development is considered the “most complete form of adaptation to terrestrial life” in amphibians (Wells 2007, p. 483). In addition to variation in the placement of eggs, there is also remarkable variation in the biology of larvae. For example, some species have free-feeding larvae that complete development in large water bodies (exotrophic), whereas others have free-living but nonfeeding tadpoles that rely on maternal provisioning in the egg (endotrophic), a seemingly intermediate stage between modes with exotrophic larvae and direct development, because all direct developers are endotrophic. This great diversity of reproductive modes (unparalleled among terrestrial vertebrates; Pough et al. 2009) raises the questions of how these modes arose and how they might be related to each other and to the evolution of direct development.

The evolution of terrestrial reproduction and direct development in amphibians also offers perhaps the best extant system for understanding the origin of amniote reproduction. The anamniote–amniote transition was a major step in vertebrate evolution, representing total independence from water for reproduction, and set the stage for the evolution of reptiles and the radiation of birds and mammals (Martin and Sumida 1997). The amniotic egg is hypothesized to have evolved in species that were similar to modern direct developing amphibians (Carroll 1982; Packard and Seymour 1997). Thus, studying the evolution of direct development in amphibians can offer insights into the anamniote–amniote transition.

Numerous hypotheses have been proposed to explain the evolution and diversity of reproductive modes in anurans, but there have been few phylogenetic tests of these hypotheses. The evolution of direct development is often thought to have proceeded in an ordered, gradual sequence through prior stages involving fully aquatic eggs and free-feeding larvae, to terrestrial eggs but aquatic larvae, then to terrestrial eggs and terrestrial larvae, before evolving direct development (Salthe and Duellman 1973; Duellman and Trueb 1986; Wells 2007). However, this hypothesis has yet to be tested with a broad-scale phylogenetic analysis across anurans. All direct developing species have endotrophic embryos (feeding exclusively from yolk), but it is unknown whether direct development originated through intermediate stages that included

free-living endotrophic larvae. Terrestrial eggs are also thought to have evolved in response to intense aquatic predation on aquatic eggs and larvae, especially in tropical regions (Duellman and Trueb 1986; Magnusson and Hero 1991). However, it remains unclear whether modes that involve placing eggs and larvae in locations removed from aquatic predators actually precede direct development. Furthermore, terrestrial breeders and direct developers seem to lay larger eggs than aquatic breeders, with fewer eggs per clutch (Wells 2007), but there have been no phylogenetic tests of this hypothesis. A relationship between increased egg size and reduced clutch size may render each terrestrial egg more valuable (in terms of fitness), potentially favoring parental care in terrestrial egg layers (Summers et al. 2006), but this hypothesis has also not been tested phylogenetically. Finally, terrestrial reproductive modes seem to occur primarily in humid tropical and subtropical areas (Haddad and Prado 2005), but the relationship between climate and reproductive modes has only been tested on a limited scale (e.g., Wiens et al. 2007). The increased egg size, reduced clutch size, and restricted climatic distribution of terrestrial breeders may be important constraints on the evolution of terrestrial reproductive modes, which may help explain the widespread retention of the primitive aquatic breeding modes. However, these long-standing hypotheses have yet to be tested across anuran families using modern phylogenetic comparative methods.

Here, we use a phylogenetic approach to investigate the evolution of reproductive modes in anurans. We first generate a time-calibrated phylogeny for 720 species and a matched database on reproductive modes, life-history traits, and climatic variables. We then test whether direct development is preceded by reproductive modes with terrestrial eggs versus aquatic eggs, those with exotrophic versus endotrophic larvae, and those with eggs or larvae that are seemingly protected from aquatic predators. We also test for associations between terrestrial reproduction and female adult size, egg size, clutch size, and parental care. Finally, we test whether terrestrial reproduction (including direct development) is associated with different climatic conditions than aquatic reproduction.

Materials and Methods

LIFE HISTORY DATA

We collected data on reproductive modes, quantitative life-history traits, and parental care (Appendix S1) from literature sources (Appendix S2) and online databases (AmphibiaWeb 2011, IUCN 2011). We included only species with data on both reproductive mode and phylogenetic placement (see below), representing as many families, genera, and reproductive modes as possible. Our sampling spans 48 of 53 anuran families (sensu Pyron and Wiens 2011). The five families excluded (due to limited reproductive

or phylogenetic information) each include only two genera (Nyctibatrachidae) or one (Ceuthomantidae, Micrixalidae, Nasikabatrachidae, Telmatobiidae).

Reproductive modes can be categorized in many ways (Duellman and Trueb 1986; Haddad and Prado 2005; Wells 2007). Most species could be unequivocally assigned to a single mode (see also Wells 2007). However, both among-population variation (Utsunomiya and Utsunomiya 1983; Caldwell 1992; Haddad and Prado 2005) and within-population plasticity (Malone 2004; Touchon and Warkentin 2008) have been reported. In both cases, we coded species as having the most commonly reported mode.

We also obtained data on other relevant life-history traits, specifically, female size, egg size, and clutch size (Appendix S1). However, data for all three characters were available for a smaller set of species ($n = 470$), among those that were already included in the tree and in the life-history database. Following standard practice in anuran studies, adult female size is snout-vent length (SVL; mm), egg size is ovum diameter (mm), and clutch size is eggs per egg mass. These variables are usually reported as ranges of values and we used the range median of each trait (in some cases, combining ranges across populations and studies to obtain a single value for each species). Although it is theoretically possible to incorporate intraspecific variation in (some) phylogenetic comparative analyses, the available intraspecific data do not lend themselves to this sort of analysis (i.e., data are typically ranges and not variances). Further, our results show that there are still strong patterns in these trait data, even without incorporating intraspecific variation explicitly.

PHYLOGENY

We constructed a time-calibrated phylogeny for the 720 species included in our life-history database. Methods for character selection, sequence alignment, and phylogeny reconstruction generally follow Pyron and Wiens (2011), but we performed a new analysis of that matrix, including only the 720 species having life-history data. The new, reduced analysis facilitated estimating divergence dates and evaluating the robustness of comparative analyses to topological uncertainty (i.e., without encumbering these analyses with ~ 2100 species that would have to be excluded). In addition, our phylogenetic results are very similar to those including all 2871 species (see below). Sequence data were derived from literature sources (see Appendix S3 for GenBank accession numbers), and include most genes used in previous studies of higher level amphibian phylogeny (e.g., Frost et al. 2006; Roelants et al. 2007; Wiens 2007, 2011). The genes include nine nuclear loci (CXCR4, H3A, CX1, POMC, RAG1, RHOD, SIA, SLC8A3, and TYR) and three mitochondrial genes (*cyt-b*, 12S, and 16S).

The final matrix consisted of up to 12,433 bp for each of 720 frog species and three outgroups: a salamander (*Ambystoma mexicanum*), a caecilian (*Rhinatrema bivittatum*), and an am-

niote (*Homo sapiens*). The matrix contains sequence data for 682 species for 16S (94% coverage, up to 1855 bp per species), 666 for 12S (92%, 1230 bp), 359 for RAG-1 (50%, 2697 bp), 312 for *cyt-b* (43%, 1140 bp), 298 for TYR (41%, 600 bp), RHOD (36%, 315 bp), 242 for SIA (33%, 397 bp), 138 for POMC (19%, 651 bp), 119 for CXCR4 (16%, 753 bp), 203 for H3A (28%, 328 bp), 100 for NCX1 (14%, 1335 bp), and 93 for SLC8A3 (13%, 1132 bp). Although many taxa are missing data for some genes, our analyses of the full dataset (Pyron and Wiens 2011) and previous empirical and simulation studies (Wiens and Morrill 2011) suggest that the missing data need not adversely impact estimation of phylogeny and branch lengths.

We estimated the phylogeny using maximum likelihood and evaluated support for individual branches using nonparametric bootstrapping, implemented in RAxMLv7.0.4 (Stamatakis 2006). We used the rapid bootstrapping algorithm (100 nonparametric bootstrap replicates with estimated branch lengths) combined with the thorough likelihood search option (20 independent searches, starting from every fifth bootstrap replicate).

The estimated tree (Fig. S1) was generally similar to that from Pyron and Wiens (2011), and to other model-based, multi-locus estimates of higher level amphibian phylogeny (e.g., Roelants et al. 2007). Important points of congruence between our phylogeny and those of Roelants et al. (2007) and Pyron and Wiens (2011) include (1) Leiopelmatoidea as sister to all other frogs; (2) Discoglossoidea as sister to frogs excluding leiopelmatoids; (3) Pipoidea as sister to Pelobatoidea + Neobatrachia; (4) Heleophryinae as sister to other Neobatrachia; (5) monophyly of Hyloidea and Ranoidea; (6) Myobatrachidae + Calyptocephalellidae as sister to Hyloidea; (7) within hyloids, the clades bufonids + dendrobatids and leptodactylids + centrolenids (+ allophrynids); and (8) within ranoids, placement of Natatanura (Ranidae sensu lato) as sister to microhylids and (hemisotids + brevaceptids) + (arthroleptids + hyperoliids). There are also some differences, such as: (1) Sooglossidae as sister to Ranoidea, following Roelants et al. (2007) but not Pyron and Wiens (2011); (2) placement of Terrarana (eleutherodactylids, craugastorids, and related families) as sister to other Hyloidea (following Pyron and Wiens 2011 but not Roelants et al. 2007); (3) monophyly of Hylidae following Pyron and Wiens (2011) but not Roelants et al. (2007); and (4) a clade from Pyron and Wiens (2011) of alsodids, batrachylids, ceratophryids, cyclorhamphids, hylodids, rhinodermatids, odontophrynids, and telmatobiids is generally supported, but odontophrynids were not placed in this clade and telmatobiids were not included. Some differences between our results and previous studies may represent errors in our tree (e.g., *Sphaenorhynchus* as sister to other hyline hylids, ptychadenids placed with mantellids) but these potential errors all appear to be weakly supported. Importantly, despite some differences between this tree and other recent estimates, these conflicting clades are typically only weakly

supported in our tree and/or in earlier studies. We address the robustness of our comparative results to uncertainty in the phylogeny using the 100 trees from the bootstrapping analyses (see below). Thus, our conclusions about character evolution should be robust to weakly supported aspects of the phylogeny.

TIME CALIBRATION

We converted the maximum-likelihood tree into a time-calibrated phylogeny using the penalized likelihood method implemented in r8s version 1.71 (Sanderson 2002, 2003). The appropriate smoothing parameter was evaluated by testing four values (1, 10, 100, and 1000). This range should include the optimal smoothing range for most datasets that depart from a molecular clock (Sanderson 2003). A log penalty function was selected to penalize differences between neighboring branches. We assessed the appropriateness of solutions for different rates with the checkGradient feature, and utilized the truncated Newton algorithm. Twelve fossil calibrations from the paleontological literature were initially used to constrain the potential ages of clades (Appendix S4). Although this is only a subset of the available fossil calibration points for anurans (e.g., Wiens 2011), only clades with strong (100%) bootstrap support were used (to avoid having some bootstrap replicates lacking certain calibration nodes). We then time-calibrated the 100 trees from the bootstrap analysis in r8s. Although other methods for divergence-time estimation are available and are widely used (e.g., BEAST; Drummond et al. 2006; Drummond and Rambaut 2007), such methods were not optimal for our study given the large number of taxa and incomplete sampling of characters within taxa.

The dates estimated were generally similar to those from other recent studies (e.g., Roelants et al. 2007; Wiens 2011). However, dates for some nodes differed by 40 million years (my) or more (e.g., root age of frogs, Neobatrachia). Therefore, we also performed an analysis in which we fixed the ages of major frog clades to those estimated by Wiens (2011; from r8s analyses of the RAG-1 gene), instead of using fossil calibrations. We fixed the following nodes: Leiopelmatoidea (202.04 my), Discoglossoidea (160.07 my), Pipoidea (190.42 my), Pelobatoidea (155.73 my), Ranoidea (111.9 my), and Hyloidea (73.53 my). The resulting dates were generally more similar to those estimated by Roelants et al. (2007) and Wiens (2011). We therefore used this latter chronogram (“fixed ages” hereafter) in all comparative analyses (see Figs. S2–S4), but we checked the robustness of most results to those from the fossil-constrained ages (“fossil ages” hereafter). However, the use of these different chronograms had almost no impact on the results.

CLIMATIC DATA

We obtained climatic data from the WorldClim dataset (Hijmans et al. 2005). We focused on four variables (annual mean temperature [Bio1], annual precipitation [Bio12], minimum temper-

ature of the coldest month [Bio6], and the precipitation of the driest quarter [Bio17]). We predicted that reproductive modes are influenced by overall levels of temperature and precipitation (annual values) and limited by extremes of cold and drought. The variables were projected globally at a spatial resolution of 2.5 min. Climatic data for each species were obtained using range maps and zonal statistics summaries. Range maps (polygons stored as ESRI shapefiles) were downloaded from the 2008 update of the IUCN Global Amphibian Assessment (<http://www.iucnredlist.org/initiatives/amphibians>), accessed in January 2009. When necessary, disjunct range segments were dissolved into single, multipart features to extract the centroids of each map. We then extracted mean values for climatic variables using zonal statistics summaries processed with Geospatial Modeling Environment v0.3.4b (Beyer 2010).

COMPARATIVE ANALYSES

To test hypotheses about the sequence of trait evolution, we summarized these modes with a limited number of states in three different ways. First, we tested whether terrestrial eggs (eggs placed outside water but which hatch into tadpoles) precede direct development, by coding species as having: (0) aquatic eggs, (1) terrestrial eggs without direct development, or (2) direct development. “Terrestrial eggs” can be placed in many different locations, including on the ground, in foam nests, on leaves over water, and on adults’ dorsum. Second, we tested whether endotrophy precedes direct development, coding species as having: (0) exotrophic larvae (feeding after hatching), (1) endotrophic larvae (nonfeeding larvae), or (2) direct development. Third, we tested whether direct development evolves from reproductive modes in which eggs and/or larvae are placed in sites that are separated from aquatic predators occurring in the main bodies of ponds, lakes, rivers, or streams (acknowledging that eggs and larvae are not intentionally placed in these locations for this purpose). We coded species as: (0) placing eggs and larvae in main bodies of water such as ponds or streams, (1) placing eggs in crevices, tree holes, excavated basins, and other hidden sites, but with larvae developing in main bodies of water, (2) placing both eggs and larvae away from main bodies of water (e.g., in bromeliads), or (3) having direct development.

We estimated changes among states using likelihood analyses on the time-calibrated tree (fixed ages). We then estimated ancestral states and changes between states using the “ace” and “fitDiscrete” commands in the R packages ape 2.7–2 (Paradis et al. 2004) and geiger 1.3–1 (Harmon et al. 2008), and with Mesquite 2.73 (Maddison and Maddison 2010). For all three hypotheses (characters), we first estimated the likelihood under the “equal rates” (ER) versus “all rates differ” (ARD) Markov models using “ace.” We then compared the fit of these models using the Akaike Information Criterion (AIC), where $AIC = -2\ln L + 2k$,

where k is the number of parameters in the model and LnL the log likelihood (Burnham and Anderson 2002). We then performed analyses using the best-fitting model (lowest AIC score). Differences in AIC between competing models were considered negligible if they were < 3 , very strong if > 10 , and moderately strong between 4 and 7 (Burnham and Anderson 2002).

To test our hypotheses about transitions between states, we estimated the number of changes between each pair of states for each character. For characters best fitting the ER model, we used the “summarize state changes” command in Mesquite. For those fitting the ARD model (which cannot be applied in Mesquite), we used the ancestral state reconstruction obtained with “ace,” and assumed the character state at any given node to be unambiguous as long as the proportional likelihood of the state at that particular node was 0.80 or higher. This value usually corresponds to a difference of 2 or more log-likelihood units between models (for which alternative character states have been fixed at that node), a standard threshold for assessing statistical support of likelihood reconstructions (Burnham and Anderson 2002). Then, we reconstructed all changes in states across the tree based on transitions between the states at each node. Ancestral-state reconstructions for all nodes for all three characters are shown in Figures S2–S4. To evaluate the robustness of our results to uncertainty in topology and branch lengths, these patterns of changes (or transition rates) were estimated across all 100 bootstrapped, time-calibrated trees (Appendix S5). An important issue in reconstructing ancestral states is the possibility that the states of interest will influence rates of speciation or extinction, and these effects on diversification will influence the reconstruction of ancestral states (e.g., Maddison et al. 2007). Therefore, we used our phylogeny and life-history data to test whether life-history states influence rates of net diversification (speciation – extinction), using the Binary State Speciation-Extinction method (BiSSE; Maddison et al. 2007), implemented in Mesquite. This analysis suggested that these life-history characters do not strongly influence diversification and so our reconstructions should be free of this potential artifact (Appendix S6; Table S1).

Prior to analyses of continuous traits (female size, egg diameter, clutch size, and the four climatic variables), we evaluated the best-fitting model and phylogenetic signal for each trait. We log-transformed continuous traits and estimated phylogenetic signal using Pagel’s lambda (Pagel 1999) with “fitContinuous” in geiger. We also used “fitContinuous” to compare the fit of Brownian motion (BM) and Ornstein–Uhlenbeck (OU) models. We assessed the robustness of our models of trait evolution for continuous traits by comparing the consistency of goodness of fit for BM and OU models across the fixed-age tree, the fossil-calibrated tree, and across 100 bootstrapped, fossil-calibrated trees. All continuous traits fit an OU model, and we therefore used phylogenetic generalized least squares (PGLS) with an OU variance–covariance

structure to test for associations among them using the “ppls.ou” function in ape (described in Duncan et al. [2007]; subsequently modified by M.E. Dillon). We also tested for association between continuous traits using SLOUCH (Hansen et al. 2008). We found that the results were generally robust to the choice of model of trait evolution and method (Appendix S5). Only the OU models are presented here. We tested the significance of the PGLS models by comparing the fit of models including an independent variable with the corresponding null model (e.g., egg size ~ clutch size + intercept vs. egg size ~ intercept) using likelihood ratio tests (LRT).

We used phylogenetic analyses of variance (ANOVAs) to evaluate differences in continuous traits (see above) between discrete reproductive modes (i.e., aquatic, terrestrial, direct development) using the “phylANOVA” function in phytools 0.0–8 (Revell 2011) with 1000 simulation replicates. We adjusted branch lengths to fit the OU model using “ouTree” in geiger.

To test the association between discrete traits (e.g., terrestrial breeding or direct development vs. parental care), we compared likelihood models in which traits evolve dependently or independently using BayesTraits (Pagel 1994; Pagel and Meade 2006, 2007), evaluating their relative fit using AIC scores.

CLIMATE AND REPRODUCTIVE MODES ACROSS LOCAL SITES

As an alternative to testing for the relationship between climate and reproductive modes among species across the phylogeny, we also compiled data on climate and reproductive modes from 51 globally distributed sites to determine if the frequency of modes among species at a site is related to climate. We compiled lists of anuran species for these sites based on various sources, including books, articles, and checklists (Appendix S7). The area of sites varied somewhat, but this should not influence the proportion of species with different modes, and we also used more specific localities within larger parks or reserves (Appendix S7). Our sampling of localities is clearly not exhaustive but we included similar numbers of localities across major regions, to avoid biasing the global results by oversampling from some regions. We also attempted to represent different habitat types within each major region (e.g., mesic vs. arid). Given the latitude and longitude for each site (Appendix S7), we obtained climatic data (Appendix S7) using the WorldClim database (Hijmans et al. 2005). We used the same four climatic variables used in the comparative analyses.

Given the list of species from each site, we compiled data on their reproductive modes, utilizing our database for 720 species (Appendix S1) and summaries provided in AmphibiaWeb (2011) and the Global Amphibian Assessment (2011). Specifically, we recorded whether species had aquatic eggs or terrestrial eggs and whether species had direct development. We then determined the proportion of species with aquatic eggs in each community and the

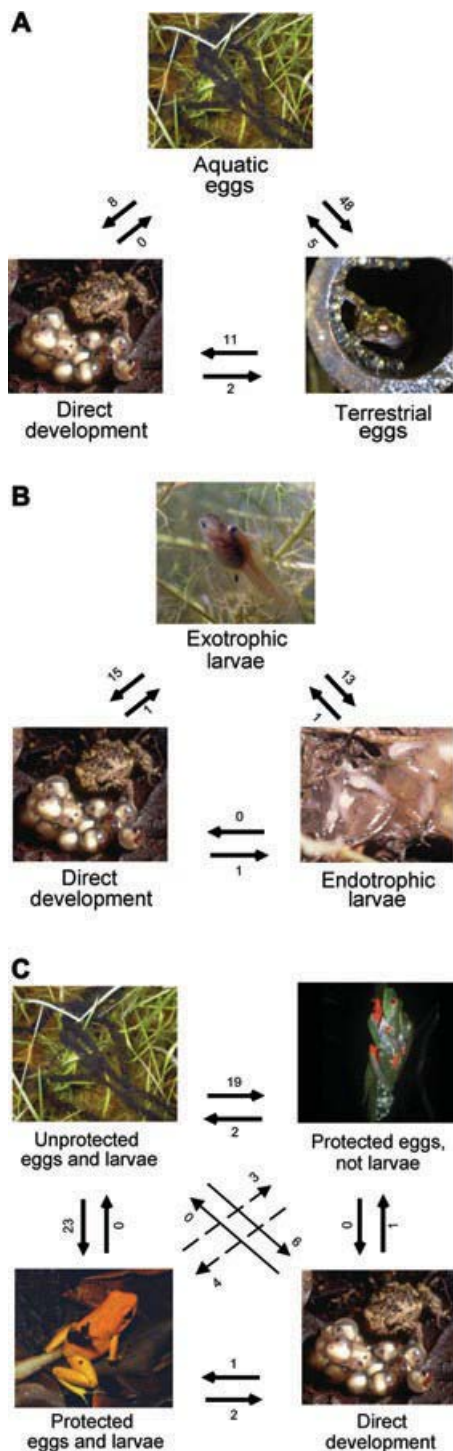


Figure 1. Estimated number of evolutionary changes between reproductive modes in anuran amphibians. (A) Number of evolutionary changes between aquatic eggs, terrestrial eggs, and direct development estimated from an ancestral-state reconstruction using the best-fitting all-rates-differ (ARD) likelihood model. Direct development has frequently evolved from aquatic eggs through a seemingly intermediate state with terrestrial eggs, but appears to have evolved directly from aquatic ancestors nearly as often. There have been five reversals from terrestrial to aquatic

proportion with direct development (Appendix S7). In some cases, data were unavailable for one or both traits (i.e., typically, the exact site of egg deposition was unclear). The average proportion of species with data for both characters ranged from 44% to 100% (mean = 87%). Finally, we tested for a correlation between the proportion of species with each reproductive mode and each climatic variable using Spearman's rank correlation (given that reproductive traits are proportions and not normally distributed).

Results

SEQUENCE OF TRAIT EVOLUTION

We first estimated changes among aquatic eggs, terrestrial eggs, and direct development. A model in which all rates differ (ARD) among states was preferred over an ER model (ER: LnL = -315.4, AIC = 640.4; ARD: LnL = -294.9, AIC = 601.7). Based on the ARD model, aquatic eggs represent the ancestral state for anurans (proportional likelihood = 99.94%; Fig. S2). Aquatic egg-laying is also the most frequent mode among these species (55%). Terrestrial egg-laying has evolved from aquatic egg-laying 48 times (Fig. 1A). Direct development has evolved frequently from terrestrial egg-laying (11 times), but surprisingly, seemingly evolved directly from the primitive state (aquatic eggs, larvae) nearly as often (eight times; Fig. 1A). In theory, these latter cases may simply indicate that a transitional form (with terrestrial eggs and aquatic larvae) was undetected among extant species. Although we cannot rule out this possibility, seemingly direct transitions from aquatic eggs to direct development occurred more rapidly (mean = 27.81 my \pm 7.76 SE, based on summed branch lengths) than changes from aquatic eggs to terrestrial eggs to direct

Figure 1. Continued. reproduction, and two reversals from direct development to terrestrial eggs (but see Results). (B) Number of evolutionary changes among exotrophic larvae, endotrophic larvae, and direct development based on ancestral-state reconstruction with the best-fitting equal rates (ER) likelihood model. Direct development has apparently not evolved from an intermediate state with endotrophic larvae, but has frequently evolved directly from exotrophic larvae instead. (C) Number of evolutionary changes among character states related to the protection of eggs and larvae from aquatic predators by placing them outside main bodies of water (e.g., ponds and streams), based on an ER likelihood model. There have been many transitions from the ancestral state of unprotected eggs and unprotected larvae to states with protected eggs and/or larvae, but these protected states have rarely given rise to direct development. Photo credits are A. Portheault, D. Bickford, M. Largen, and A. Amézquita for the images corresponding to "aquatic eggs," "direct development," "endotrophic larvae," and "protected eggs and larvae," respectively.

development (mean = 53.20 my \pm 10.02 SE; ANOVA $F_{1,13} = 4.13$, $P = 0.06$). We also find that terrestrial egg-laying reverted to aquatic egg-laying five times, and a model with such reversals prohibited (i.e., rate set to zero) has a substantially poorer fit (unconstrained: LnL = -294.9, AIC = 601.7; reversals prohibited: LnL = -300.6, AIC = 611.3). In contrast, direct development never reverted directly to aquatic reproduction, and only rarely reverted to terrestrial nondirect development (Fig. 1A). In fact, the model with reversals from direct development prohibited showed better fit than the unconstrained model (unconstrained: LnL = -294.9, AIC = 601.7, reversals prohibited: LnL = -293.3, AIC = 596.6).

For the hypothesis of transitions from exotrophic to endotrophic larvae to direct development, the ER model has the best fit (ER: LnL = -167.4, AIC = 336.8; ARD: LnL = -166.9, AIC = 345.8). Based on this model, exotrophic larvae represent the ancestral state for anurans (proportional likelihood = 99.92%; Fig. S3). Remarkably, the results (Fig. 1B) show that direct development did not evolve from ancestors with endotrophic larvae (a seemingly intermediate state), but instead evolved directly from exotrophic larvae (Fig. 1B).

We also tested whether direct development evolved from reproductive modes with eggs and/or larvae that are seemingly protected from aquatic predators (e.g., deposited outside the main bodies of ponds or streams). The ARD model could not be optimized on the fixed-age tree. Optimizing this model on the bootstrapped trees failed in 75% of 100 replicates, but had an average likelihood across the remaining 25 trees of -516.9 (mean AIC = 1045.8). In contrast, the ER model had a much better fit on the fixed-age tree (LnL = -457.1; AIC = 916.2) and across the bootstrapped trees (average LnL = -459.4; average AIC = 920.7). According to the ER model, reproduction with aquatic, unprotected eggs and larvae is ancestral for anurans (proportional likelihood = 99.91%; Fig. S3). Again, our results do not support the expected sequence of changes (Fig. 1C). Although modes with unprotected eggs and larvae often give rise to modes with protected eggs and unprotected larvae, this latter mode only rarely gives rise to the mode with protected eggs and protected larvae. Further, modes with unprotected eggs and larvae give rise to direct development more often than modes with protected eggs and/or larvae. For all three characters, these general results are robust to use of the fossil-age chronogram, and across the 100 bootstrapped trees (Appendix S5).

LIFE HISTORY CHANGES ASSOCIATED WITH TERRESTRIALITY

We tested for potential constraints on the evolution of terrestrial breeding and direct development that are associated with variation in other life-history traits (body size, egg size, clutch size), using 470 species with complete data for all three traits and that are

included in our phylogeny. Phylogenetic ANOVAs indicate that aquatic egg-laying species have significantly larger adult female sizes than terrestrial egg layers, including here direct developers (hereafter means \pm SE; female SVL: aquatic 59.20 \pm 1.78 mm, terrestrial 41.93 \pm 2.01 mm; $F_{2,468} = 29.04$, $P = 0.001$). Among terrestrial breeders, direct developers did not significantly differ in size from nondirect developers (direct developers 39.72 \pm 3.03, nondirect developers 42.96 \pm 2.08; $P = 0.89$). Aquatic egg-laying species have significantly smaller eggs but larger clutches than terrestrial egg layers (average egg diameter: aquatic 1.77 \pm 0.08 mm, terrestrial 3.05 \pm 0.09 mm, $F_{2,468} = 66.80$, $P < 0.001$; average clutch size: aquatic 2057.51 \pm 197.10, terrestrial 320.63 \pm 222.16, $F_{2,468} = 124.39$, $P < 0.001$). Among species with terrestrial eggs, phylogenetic ANOVAs indicated no significant differences in egg diameter or clutch size between direct developers and nondirect developers (mean egg size of direct developers: 4.22 \pm 0.19 mm, terrestrial breeders with larvae: 2.48 \pm 0.13, $P = 0.25$; mean clutch size of direct developers: 23.78 \pm 1.96 eggs/clutch, other terrestrial species: 459.58 \pm 113.70; $P = 0.69$).

We tested for a general trade-off between egg size and clutch size, taking female size (as SVL) into consideration, using PGLS. Egg diameter was significantly and negatively associated with clutch size ($R^2 = 0.18$; LRT = 87.72, $P < 0.0001$; Fig. 2A). In turn, clutch size was significantly and positively associated with female size ($R^2 = 0.30$; LRT = 143.7, $P < 0.0001$; Fig. 2B), whereas egg diameter showed a much weaker positive association with female size ($R^2 = 0.007$; LRT = 4.12, $P = 0.042$; Fig. 2C). The results of PGLS and phylogenetic ANOVA analyses are robust to use of the fossil-age chronogram (Appendix S5).

Finally, we tested for an association between terrestrial egg-laying and parental care. Parental care and terrestrial egg-laying (including direct development) evolve dependently under the best-fitting model (independent: LnL = -471.3, AIC = 950.5; dependent: LnL = -428.6, AIC = 873.2). Similarly, parental care was also significantly associated with direct development (independent: LnL = -321.9, AIC = 651.8; dependent: LnL = -309.9, AIC = 635.9). Robustness of these results was confirmed using the fossil-ages tree and 100 bootstrapped trees (Appendix S5).

CLIMATE AND REPRODUCTIVE MODES

To test if the evolution of reproductive modes is constrained by climate, we used phylogenetic ANOVA to assess the association between reproductive modes and four climatic variables, using all 720 species. Direct developers did not differ significantly from terrestrial breeders with larvae (Appendix S5), so the results here contrast aquatic and terrestrial breeders (including direct developers). We find that species with terrestrial egg-laying occur in areas with significantly higher annual precipitation than species with aquatic eggs ($F_{1,718} = 38.84$, $P = 0.003$; terrestrial:

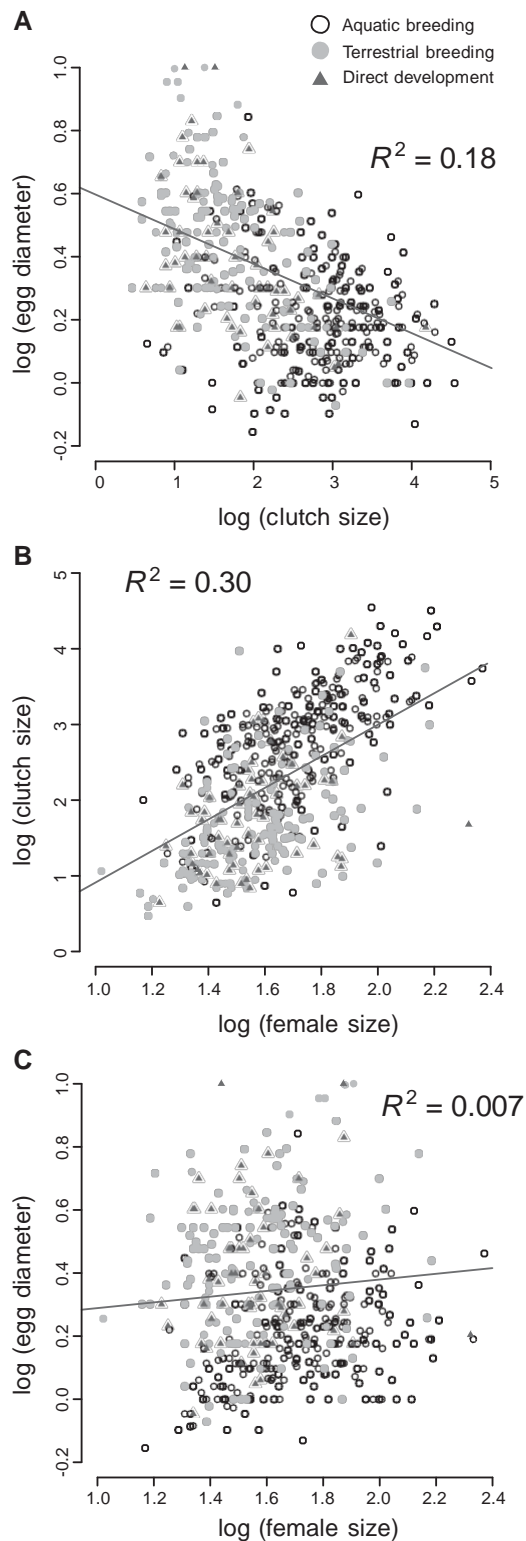


Figure 2. Relationships between egg diameter, clutch size, and female size across 470 anuran species based on phylogenetic generalized least-squares analysis under an Ornstein–Uhlenbeck model of evolution. There is a strong negative association (trade-off) between egg diameter and clutch size (A). Clutch size is strongly related to female body size (B) whereas egg diameter is much less so (C).

mean = 2053.30 ± 50.75 mm, aquatic: 1491.75 ± 38.98 mm), higher driest quarter precipitation ($F_{1,718} = 24.29$, $P = 0.004$; terrestrial: 234.95 ± 10.42 mm, aquatic: 153.01 ± 7.06 mm), higher mean annual temperature ($F_{1,718} = 20.12$, $P = 0.02$; terrestrial: 21.91 ± 0.23 °C, aquatic: 19.18 ± 0.34 °C), and milder coldest winter temperatures ($F_{1,718} = 46.37$, $P = 0.001$; terrestrial: 14.51 ± 0.31 °C, aquatic: 8.18 ± 7.06 °C).

Across the 51 communities surveyed globally (Appendix S7), the proportion of aquatic breeding species is negatively correlated with annual mean temperature (Spearman's rank correlation, $\rho = -0.55$, $P < 0.0001$), annual precipitation ($\rho = -0.54$, $P = 0.0001$) and coldest yearly temperature ($\rho = -0.68$, $P < 0.0001$) but not driest quarter precipitation ($\rho = -0.192$, $P = 0.174$). Likewise, the proportion of direct developing species was positively correlated with annual mean temperature ($\rho = 0.42$, $P = 0.0028$), annual precipitation ($\rho = 0.63$, $P < 0.0001$), minimum temperature ($\rho = 0.63$, $P < 0.0001$), and driest quarter precipitation ($\rho = 0.46$, $P = 0.001$).

Discussion

Anurans exhibit a remarkable diversity of reproductive modes and life-history strategies that is unparalleled among terrestrial vertebrate clades (Pough et al. 2009). In this article, we analyze the evolution of this diversity using a large-scale phylogeny and life-history database for 720 species. As expected, we find multiple origins of terrestrial reproduction (~48) and direct development (~19; Fig. 1A). However, we find many unexpected patterns in the sequence of changes among reproductive modes. First, although we find that modes with terrestrial eggs frequently give rise to direct development, we find that aquatic eggs give rise to direct development nearly as often (Fig. 1A). Second, we find that modes with endotrophic larvae do not give rise to direct development and that modes with exotrophic larvae often do so instead (Fig. 1B). Third, modes with protected eggs and larvae arise frequently from those with unprotected eggs and larvae (Fig. 1C), but rarely give rise to direct development. We also find many correlates with the evolution of terrestrial reproductive modes (involving body size, egg size, clutch size, parental care, and climatic distribution), which may act as constraints and help explain the long-term persistence and high frequency of aquatic modes (and ultimately, the repeated origins of terrestrial modes).

SEQUENCE OF CHANGES IN REPRODUCTIVE MODES

Our results challenge the conventional view that the diversity of anuran reproductive modes represents steps in a long sequence of changes leading to direct development. Instead, we find evidence suggesting that many seemingly intermediate modes are sometimes skipped (e.g., terrestrial eggs, endotrophic larvae, protected eggs and larvae). These seemingly rapid transitions

between aquatic eggs and direct development are particularly unexpected, given the profound developmental changes thought to be required for direct development to evolve (Fang and Elinson 1996; Callery et al. 2001; Kerney et al. 2010). Furthermore, in many cases, these diverse “intermediate” stages seem to represent terminal stages that are retained for tens of millions of years without proceeding to direct development (e.g., phyllomedusine hylids and centrolenids have maintained terrestrial egg-laying for approximately 54 and 62 my, respectively, see Fig. S2). Also contrary to a model of consistent stepwise changes toward direct development, we find some reversals from derived modes to more primitive conditions, including five reversals from terrestrial biphasic to aquatic modes (Fig. 1A). There are also cases where direct development may have reversed to modes with larval stages (although a model without these reversals has somewhat higher support). These cases include *Gastrotheca* (Wiens et al. 2007) and *Breviceps mossambicus* (given that all other *Breviceps* and closely related genera are direct developers; Wells 2007).

We make several caveats regarding our results on the inferred sequence of changes. First, some patterns may represent failure to observe intermediate states. For example, even though aquatic breeders apparently give rise to direct developers without passing through a terrestrial, biphasic mode (in some cases), the terrestrial biphasic mode may have evolved to direct development too rapidly to be observed (or was not retained among extant species). This possibility is difficult to fully rule out, but our analyses of the absolute times between transitions suggest that the time for a seemingly direct transition from aquatic eggs to direct development is (on average) roughly half as long as that for a change from aquatic eggs to direct development through an intermediate state with terrestrial, biphasic eggs.

Furthermore, despite documenting many changes in reproductive modes overall, we find that some transitions are only rarely observed, or not at all. For example, we find no cases where nondirect developing endotrophs give rise to direct developers (despite repeated origins of both endotrophy and direct development; Fig. 1B), suggesting that this transition is unlikely and not merely unobserved. Similarly, we find many origins of protected eggs and/or larvae, but no transitions from modes with protected eggs (but unprotected larvae) to direct development. These biases suggest that the patterns we observe are not merely failures to observe transitional states.

The problem of unobserved transitional states may be exacerbated by incomplete sampling of extant species, given that we sampled only 12% of known frog species (Amphibia Web 2011). Nevertheless, our taxon sampling emphasized those groups with a diversity of reproductive modes (and sampling hundreds of additional species from the same clades with identical reproductive modes should have relatively little impact on our results). Therefore, we speculate that further taxon sampling should refine our

estimates of these relative transition patterns rather than completely overturn them. In addition, our phylogeny of frogs is still weakly supported in many parts (Fig. S1). However, the estimated transitions among states are relatively robust to weakly supported aspects of the tree (Appendix S5).

Reproductive modes are often thought to be invariant within species, and this seems to be the case for most anuran species (Wells 2007). However, there seems to be substantial variation in reproductive modes among populations within species, and even within populations. Amphibian development is highly modular (Chipman 2002) and highly responsive to the environment (Newman 1992; Denver et al. 2002) and therefore there has been great potential for evolution of reproductive modes in response to local conditions. Behavioral plasticity in oviposition site choice seems to have been key in anuran life-history evolution (Resetarits 1996) resulting in single species exhibiting several reproductive modes according to the local conditions experienced (Wells 2007). Moreover, there is recent experimental evidence for behavioral plasticity determining shifts in reproductive mode within species (Touchon and Warkentin 2008). Most discussions on the evolution of reproductive modes frequently describe how reproductive modes “merge into” one another (Duellman and Trueb 1986; Wells 2007) and how “flexible” reproductive modes are within species (Duellman and Trueb 1986). For instance, zealotness in egg attendance varies with humidity in *Hyalinobatrachium fleischmanni* (Hayes 1991), nest construction can be facultative in *Hypsiboas rosenbergi* (Höbel 1999), *Kalophrynus pleurostigma* in Borneo can lay eggs in either water-filled holes in logs, pools on the ground, or burrows in stream banks (Inger 1985), and *Smilisca sordida* can either attach eggs to the substrate in streams, bury them, or deposit them in a floating film inside a constructed basin on sandbars (Malone 2004). We expect further analyses of the genetic and developmental basis of within-species variation to make major contributions to our understanding of the evolution of reproductive modes.

LIFE-HISTORY CORRELATES AND THE RETENTION OF AQUATIC EGGS

We argue that a key to explaining the repeated origins of terrestrial reproduction and the overall diversity of anuran reproductive modes is the retention of the primitive mode over hundreds of millions of years and among thousands of species. Specifically, we show that aquatic reproduction has been maintained continuously for approximately 220 my across many lineages of anurans (Fig. S2), and is still the predominant mode among extant species (e.g., 55% of 720 species sampled; Appendix S1). The retention of this trait allows many opportunities for a diversity of derived states to evolve, as opposed to one or a few origins of terrestrial reproduction that are fixed in all or most subsequent species.

What then explains the long-term retention of the primitive, aquatic reproductive mode in anurans? We show several important correlates of terrestrial reproduction and direct development that may act as constraints on their evolution. These include reduced clutch size, increased egg size, reduced female size, parental care, and occurrence in relatively warm and wet regions. Thus, there may be important advantages to retaining aquatic, exotrophic, unprotected eggs, and these advantages may help explain the long-term maintenance of the primitive mode (e.g., ability to have large clutch sizes, large body sizes, and to colonize cooler and drier regions). We also find that neither aquatic nor terrestrial reproduction is associated with a dramatically higher diversification rate than the other (Table S1), which also supports the idea that neither mode has become predominant by either reducing extinction or accelerating speciation (although this should be tested further in analyses that include additional species).

Many of these correlations with life-history traits have been proposed in previous studies, but we provide the first large-scale phylogenetic tests of these hypotheses here. We support the long-standing hypotheses that terrestrial reproduction is associated with increased egg diameter, reduced clutch size, and parental care (Duellman and Trueb 1986; Summers et al. 2006; Wells 2007). Moreover, we show that terrestrial breeding anurans have reduced adult body size relative to aquatic breeding ones, a pattern only previously reported within some clades (Blackburn 2008).

The evolution of egg size and clutch size in terrestrial eggs may be strongly influenced by a trade-off between water balance and oxygen uptake. The tight packaging of eggs in terrestrial clutches and the physical barriers preventing water loss in these eggs (thick jelly layers, extra membranes) impede oxygen diffusion (Seymour and Bradford 1995; Warkentin et al. 2005). Bigger terrestrial eggs confer a better volume-to-surface ratio than smaller eggs, decreasing water loss but compromising oxygen diffusion, and they may have evolved reduced protective layers to improve oxygen diffusion (Seymour 1999; Elinson and Beckham 2002; Altig and McDiarmid 2007). Thus, large, jelly-free terrestrial eggs (such as those of direct developers) that have become effectively independent from large bodies of water may require high humidity (which may explain their climatic distribution, see below). Increased egg size may also be associated with greater maternal provisioning (Elinson and Beckham 2002). Larger egg sizes lead to smaller clutch sizes (a classic life-history trade-off; Salthe and Duellman 1973; Stearns 1992), especially given the small body sizes of terrestrial breeders, as we demonstrate here. Laying few large eggs may then favor the evolution of parental care (Salthe and Duellman 1973; Summers et al. 2006), especially if eggs are secluded in small confined spaces away from predators (Wells 2007).

The dependence of terrestrial eggs on high humidity may help explain the strong associations we observe between climatic variables and reproductive modes. Our results show that terrestrial breeders (including direct developers) generally occur in areas with higher annual precipitation and annual mean temperatures than aquatic breeders, areas that should have relatively high humidity. There are some exceptions, however. Some of the few direct developers that do venture into dry areas are fossorial and breed in burrows where humidity is higher, including some *Breviiceps* (South Africa) and *Myobatrachus gouldii* (Australia). Intriguingly, the same adaptive features that allowed direct developing embryos independence from large water bodies may have largely constrained them to moist tropical and subtropical areas with high humidity. Aquatic reproduction is less constrained by climate and may therefore have a greater potential for geographical expansion (Fig. 3), but requires ponds, streams, and other water bodies. Thus, each type of breeding is dependent upon water, but in different forms.

IMPLICATIONS FOR AMNIOTE ORIGINS

The shift from anamniote to amniote reproduction was a major transition in vertebrate evolution, allowing total independence from water during reproduction (Martin and Sumida 1997). Although the ecological changes associated with this shift may be difficult to ascertain from the fossil record, our results on the evolution of terrestrial reproduction in anurans suggest several generalities that may be relevant. We find that shifts from aquatic to terrestrial reproduction can occur relatively rapidly (although still over tens of millions of years, on average), and that many seemingly intermediate steps may either be unnecessary or relatively fleeting. Furthermore, our results suggest that this shift to terrestrial reproduction may have been associated with reduced body and clutch size, increased egg size, parental care, and occurrence in relatively warm, wet climates. Of course, there may have been many differences between the immediate ancestors of amniotes and frogs. Further insights into the generality of these patterns (and their relevance for amniote origins) could also be gained from studying the evolution of reproductive modes in the other major amphibian clades (caecilians, salamanders), in which there has also been repeated origins of terrestrial reproduction and direct development (e.g., Wells 2007).

AREAS FOR FUTURE RESEARCH

Our results suggest several other important areas for future research on the evolution of reproductive modes in anurans, and our phylogenetic reconstructions (Figs. S2–S4) provide a framework for future developmental, evolutionary, and ecological studies. Most importantly, more fine-scale ecological work is needed on the selective pressures that drive the origins of terrestrial reproduction and direct development. Intense aquatic predation risk

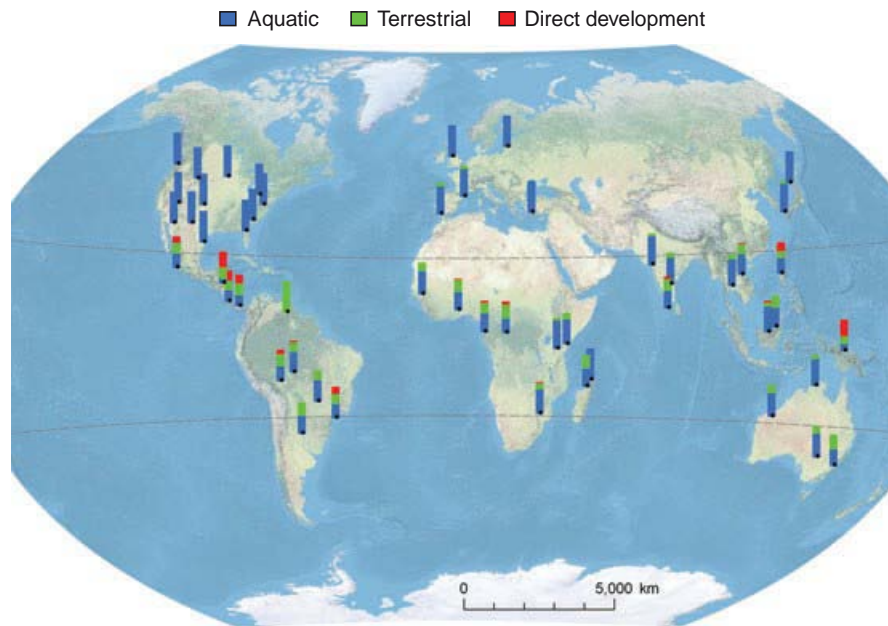


Figure 3. Frequency of different reproductive modes among anuran species at 51 sites (Appendix S7), indicating the proportion of species with aquatic reproduction (blue fraction of bars), terrestrial reproduction (green), or direct development (red). Direct developing species are largely confined to tropical and subtropical regions, and many temperate sites are dominated by aquatic breeders.

is thought to have been the most important factor driving the evolution of terrestrial reproduction (Duellman and Trueb 1986; Haddad and Prado 2005). However, larval competition, risk of pond drying, and low oxygen may have also contributed (Heyer 1969; Wells 2007), possibly depending on regions and clades. In support of the importance of aquatic predation, the proportion of terrestrial breeding species in tropical communities has been shown to be associated with predation risk for early aquatic life stages (Magnusson and Hero 1991). Nonetheless, predation on terrestrial clutches may be high as well (Gomez-Mestre and Warkentin 2007). Thus, whether aquatic predation rates are generally higher than terrestrial predation rates remains to be confirmed. Interestingly, our results (Fig. 1C) show that although there have been many transitions from unprotected aquatic eggs to eggs and/or larvae that are protected in some way, we find few subsequent transitions from these protected modes to direct development.

The role of phenotypic plasticity in the evolution of terrestrial egg-laying and direct development also needs further study. Some evidence suggests that these transitions may have been initiated by plastic changes in the reproductive behavior of adults. Behavioral plasticity in oviposition site is common (Resetarits and Wilbur 1991; Resetarits 1996) and single species that exhibit different reproductive modes depending on local conditions have been reported (Wells 2007; Touchon and Warkentin 2008). Adult behavioral plasticity (Touchon and Warkentin 2008) and developmental plasticity in response to environmental changes

(Thibaudeau and Altig 1999) may be very important in the evolution of reproductive modes. Thus, studies of plasticity and variation within and among closely related species in a phylogenetic context are particularly needed (e.g., Gomez-Mestre et al. 2008).

Finally, the importance of long-term maintenance and high frequency (among species) of a primitive state in explaining repeated origins of derived states should be modeled quantitatively. It seems intuitively obvious that if the primitive state becomes very rare or absent (at a given point in time), this pattern may reduce the probability that the derived state can evolve repeatedly from it. In addition, if selection does strongly favor the initial origin or spread of the derived state, this trend may be reduced as the derived state becomes widespread (e.g., no open niche to fill; Wiens et al. 2006). Therefore, explaining the long-term maintenance and/or high frequency of the primitive state may be critical for explaining multiple origins of a derived state within a clade. However, this hypothesis requires explicit quantitative testing with simulations or related approaches, to understand the specific conditions under which it may (or may not) be true.

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Supporting Information

The following supporting information is available for this article:

Appendix S1. Data used in the comparative analyses.

Appendix S2. Literature sources from which life-history data were extracted.

Appendix S3. GenBank accession numbers.

Appendix S4. Calibration points for estimates of divergence times.

Appendix S5. Robustness analysis of models of trait evolution for reproductive modes, life-history traits, and climatic data.

Appendix S6. Life history trait evolution and net diversification rates.

Appendix S7. Analysis of climate's effect on the distribution of reproductive modes around the world.

Table S1. Comparison between models in which character states influence diversification rates (unconstrained model) and those in which diversification rates are the same for both states (constrained model).

Figure S1. Time-calibrated phylogeny estimated using maximum-likelihood.

Figure S2. Ancestral state reconstruction on the fixed-age phylogeny of reproductive modes categorized as aquatic breeding, terrestrial breeding or direct developing.

Figure S3. Ancestral state reconstruction on the fixed-age phylogeny of reproductive modes categorized as having exotrophic larvae, endotrophic larvae or direct development.

Figure S4. Ancestral state reconstruction on the fixed-age phylogeny of reproductive modes categorized as having unprotected eggs and larvae, protected eggs but not larvae, protected eggs and larvae, or direct development.

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

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