

Patterns of reproductive-mode evolution in Old World tree frogs (Anura, Rhacophoridae)

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The Old World tree frogs (Anura: Rhacophoridae), with 387 species, display a remarkable diversity of reproductive modes – aquatic breeding, terrestrial gel nesting, terrestrial foam nesting and terrestrial direct development. The evolution of these modes has until now remained poorly studied in the context of recent phylogenies for the clade. Here, we use newly obtained DNA sequences from three nuclear and two mitochondrial gene fragments, together with previously published sequence data, to generate a well-resolved phylogeny from which we determine major patterns of reproductive-mode evolution. We show that basal rhacophorids have fully aquatic eggs and larvae. Bayesian ancestral-state reconstructions suggest that terrestrial gel-encapsulated eggs, with early stages of larval development completed within the egg outside of water, are an intermediate stage in the evolution of terrestrial direct development and foam nesting. The ancestral forms of almost all currently recognized genera (except the fully aquatic basal forms) have a high likelihood of being terrestrial gel nesters. Direct development and foam nesting each appear to have evolved at least twice within Rhacophoridae, suggesting that reproductive modes are labile and may arise multiple times independently. Evolution from a fully aquatic reproductive mode to more terrestrial modes (direct development and foam nesting) occurs through intermediate gel nesting ancestral forms. This suggests that gel nesting is not only a possible transitional state for the evolution of terrestriality, but also that it is a versatile reproductive mode that may give rise to other terrestrial reproductive modes. Evolution of foam nesting may have enabled rhacophorids to lay a larger number of eggs in more open and drier habitats, where protection from desiccation is important. Terrestrial direct development allows frogs to lay eggs independent of bodies of water, in a diversity of humid habitats, and may represent a key innovation that facilitated the evolution of nearly half of all known rhacophorid species. Corresponding author: *Madhava Meegaskumbura, Department of Molecular Biology & Biotechnology, Faculty of Science, University of Peradeniya, Peradeniya, KY 20400, Sri Lanka. E-mail: madhava88m@gmail.com*

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Introduction

Among the orders of living vertebrates, the Anura (frogs and toads) show the greatest diversity of reproductive modes (Gomez-Mestre *et al.* 2012). Based on site of egg deposition, the presence or absence of free-swimming larvae, and the environment in which the larvae develop, Duellman & Trueb (1986) listed 29 different reproductive modes in the group, to which Haddad & Prado (2005) added 10. Iskandar *et al.* (2014) recently added one more (giving birth to tadpoles instead of eggs), which highlights that there may be still more amphibian reproductive modes to be discovered.

The Old World tree frogs of the family Rhacophoridae (*sensu* Bossuyt *et al.* 2006; Frost *et al.* 2006; Wiens *et al.* 2009) display a remarkable variety of reproductive modes. Broadly, the genus-level variation is as follows. In *Buergeria* and *Liuixalus*, tadpoles emerge from clumps of small eggs laid in streams or phytotelms (small bodies of water trapped in plants), completing their development entirely in water as feeding tadpoles (Smith 1953; Fukuyama & Kusano 1992; Nishizawa *et al.* 2011; AmphibiaWeb 2014). In *Beddomixalus*, *Feihyla*, *Gracixalus*, *Kurixalus*, *Mercurana*, *Nyctixalus* and *Theloderma*, terrestrial eggs are deposited in a gelatinous matrix (of varying thickness) on rocks, soil or vegetation above or adjacent to small pools, in phytotelms, or in areas about to be flooded, and then hatch into partially developed tadpoles that fall or otherwise find their way into water, where they complete development as feeding tadpoles (Kuramoto & Wang 1987; Kam *et al.* 1996; Orlov & Rybaltovsky 1999; Rybaltovsky 2000; Grosjean *et al.* 2008; Abraham *et al.* 2013). The eggs of *Chiromantis*, *Ghatixalus*, *Polypedates*, *Rhacophorus* and *Taruga* are laid in foam nests above water, into which larvae fall to complete their development (Kuramoto & Wang 1987; Wilkinson *et al.* 2002; Biju *et al.* 2008; Grosjean *et al.* 2008; Meegaskumbura *et al.* 2010). In *Philautus*, *Pseudophilautus* and *Raorchestes*, fully developed froglets emerge directly from eggs deposited on terrestrial vegetation, in leaf axils, in crevices, in leaf litter or under soil, there being no free-swimming or feeding larval stage (Alcala & Brown 1982; Dring 1987; Meegaskumbura *et al.* 2002; Biju 2003; Bossuyt *et al.* 2004; Bahir *et al.* 2005; Biju & Bossuyt 2005; Delorme *et al.* 2005; Meegaskumbura 2007). A single species of *Philautus* (*P. macroscelis*), however, is reportedly nidicolous: eggs hatch at an advanced, non-feeding larval stage but larvae remain within the gel mass before falling into water (Hertwig *et al.* 2012).

Despite advances in the analysis of rhacophorid phylogeny (e.g. Wilkinson & Drewes 2000; Meegaskumbura *et al.* 2002; Wilkinson *et al.* 2002; Li *et al.* 2008, 2009, 2013; Yu *et al.* 2008, 2009; Abraham *et al.* 2013) patterns of repro-

ductive evolution within the family have received relatively little attention. The family currently contains 387 species in 17 genera (AmphibiaWeb 2014). As such, it comprises nearly 6% of the world's anurans. It has a predominantly Asian distribution, with only one valid genus, *Chiromantis*, that extends into Africa. Almost a third of rhacophorid species, representing all rhacophorid genera, have been included in published phylogenies, and the monophyly of many groups (genera) is now well established using both mitochondrial and nuclear DNA data (Li *et al.* 2009, 2013; Yu *et al.* 2009). Despite the availability of phylogenies and an understanding of the major life-history characteristics of the various genera, interesting questions regarding reproductive-mode and life-history evolution remain to be addressed: (i) Which character states are plesiomorphic and which are derived? (ii) Did the major reproductive modes evolve multiple times or only once? (iii) Have there been reversions of major reproductive modes? (iv) How do complex modes of development, such as direct development, evolve (and which reproductive modes are the most versatile in terms of giving rise to other modes)? (v) Does current taxonomy reflect underlying life histories? (vi) What are the possible implications of these major traits?

In a study that considered a majority of anuran families, Gomez-Mestre *et al.* (2012) analysed the evolution of reproductive modes, including direct development. They suggest that direct development arises as often from ancestors with exotrophic larvae ('larva that feeds on various materials not derived from a parent or trophic eggs provided by subsequent ovulations of the parent': McDiarmid & Altig 1999) as it does from those with terrestrial or aquatic eggs. They also suggest that anurans have skipped many seemingly intermediate stages in the evolution of direct development. However, the apparently rapid transition from primitive to derived reproductive modes may result from the absence of intermediate forms that either are now extinct or were excluded from their analysis. Because the impact of missing transitional reproductive modes on the analysis could be high, the evolution of reproductive modes deserves closer investigation through more complete taxon sampling within a reproductively diverse clade such as Rhacophoridae.

Here, we use DNA sequence data from three nuclear and two mitochondrial gene fragments from 53 rhacophorid species (66 haplotypes), including the type species of most genera, together with GenBank data for an additional 31 species (33 haplotypes), to infer genus-level phylogenetic relationships. We use the resulting phylogeny to analyse patterns of reproductive-mode evolution within the Old World tree frogs to better understand the evolution and diversification of anuran life histories.

Materials and methods

Mitochondrial and nuclear DNA sequences were obtained from 107 individuals representing 80 species (99 haplotypes) and 17 rhacophorid genera (AmphibiaWeb 2014), together with four species of Mantellidae (the sister group of Rhacophoridae; Vences & Glaw 2001) and four Ranidae/Dicroglossidae/Nyctibatrachidae outgroup species (Table S1). Tissues of 27 individuals were collected from the field in Sri Lanka and those of 39 others were obtained from natural history museums; the remaining sequences were retrieved from GenBank.

Tissue extraction, PCR amplification and DNA Sequencing

DNA was extracted from ethanol-preserved tissues using Qiagen tissue-extraction kits following manufacturer's protocols. A total of 3730 base pairs (bp) were sequenced, including two mitochondrial gene fragments with a total of ca. 1500 bp and three nuclear gene fragments with a total of ca. 2230 bp. Portions of the mitochondrial 12S and 16S ribosomal RNA genes were amplified by PCR and sequenced directly using dye-termination cycle sequencing. The following primer sets were used for both PCR and sequencing of mitochondrial genes: 12Sa and 12Sb (Palumbi 1996), which amplified a ca. 380-bp fragment of the 12S rRNA gene; and 16Sar and 16Sbr (Palumbi 1996), which amplified a ca. 600-bp fragment of the 16S rRNA gene. PCR conditions for amplification were as follows: denaturation at 95 °C for 40 s, annealing at 45 °C for 40 s and extension at 72 °C for 50 s, 35 cycles, with a final extension of 72 °C for 5 min.

Nuclear Rag-1 (recombination activating gene-1), BDNF (brain-derived neurotrophic factor) and Rhod (rhodopsin exon-1) partial gene fragments were amplified by PCR. Two sets of primers were used to amplify two fragments of the Rag-1 gene for a total sequence length of 1403 bp: Amp-Rag1F (5'-AGC TGC AGY CAR TAC CAY AAR ATG TA-3') and Amp-Rag1R1 (5'-AAC TCA GCT GCA TTK CCA ATR TCA CA-3'); and Amp-Rag1F1 (5'-ACA GGA TAT GAT GAR AAG CCT GT-3') and Amp-Rag1R (5'-TTR GAT GTG TAG AGC CAG TGG TGY TT-3'; Mauro *et al.* 2004). Primers BDNFF (5'-CAT CCT TTT CCT TAC TAT GGT T-3') and BDNFR (5'-TTC CAG TGC CTT TTG TCT ATG-3'; Murphy *et al.* 2001) were used to amplify 550 bp of the BDNF gene; Rhod.ma (5'-AAC GGA ACA GAA GGY CC-3') and Rhod.md (5'-GTA GCG AAG AAR CCT TC-3'; Hoegg *et al.* 2004) were used to amplify 281 bp of Rhodopsin. PCR conditions for amplification of Rag-1 were as follows: denaturation at 95 °C for 45 s, annealing at 52–58 °C for 45 s, and extension at 72 °C for 60 s, 35–38 cycles, with a final extension at 72 °C for 10 min. PCR parameters for amplification of BDNF and Rhodopsin gene fragments were the same as for Rag-1 except for the annealing

temperatures, which were 50 and 45 °C, respectively. All products were gel purified and sequenced on an ABI 3100 automated sequencer following manufacturer's protocols.

Alignment of sequences and phylogenetic analyses

Chromatograms were edited using 4 peaks (v. 1.7.1). The program MEGA v. 5.0 (Tamura *et al.* 2011) was used to align Rhodopsin, BDNF and Rag-1 using translated amino acid sequences. Following a ClustalW analysis as implemented in MEGA v. 5.0 (Tamura *et al.* 2011), the 12S and 16S rRNA gene sequences were aligned using secondary-structure models and edited visually. Regions of sequences that were difficult to align, and in which we had low confidence of positional homology, were excluded from the phylogenetic analyses.

All 107 species were included in a single data set (Tax107). A Bayesian analysis (Tax107_BA) and a maximum-likelihood analysis (Tax107_ML) were performed to infer tree topology and assess support. jModelTest v.2.1.4 (Guindon & Gascuel 2003; Darriba *et al.* 2012) was used to choose the model of nucleotide substitution that best fitted the combined data sets. The general time-reversible model with invariant sites and gamma distribution (GTR+I+G) yielded the best fit for all data sets (using the Akaike information criterion; Akaike 1974). Obtained best model and parameters were implemented in the Bayesian and maximum-likelihood (ML) analyses.

The tree was rooted using *Lankanectes corrugatus* (Nyctibatrachidae), *Hylarana temporalis*, *Rana kukunoris* (Ranidae) and *Nannophrys ceylonensis* (Dicroglossidae) as outgroups. These taxa represent the closest relatives of the ranoid clade containing the Rhacophoridae and Mantellidae and hence are useful to root the rhacophorid tree (Bossuyt *et al.* 2006; Frost *et al.* 2006).

Bayesian inference as implemented in MrBayes (v. 3.1.2; Huelsenbeck & Ronquist 2001) was used to generate a phylogenetic hypothesis of relationships with the parameters of a general time-reversible model of sequence evolution with gamma-distributed rate variation among sites and a proportion of invariant sites (GTR+I+G) estimated as obtained from the jModelTest. Four Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were run for ten million generations, and the summed likelihood of the four chains converged on a stationary value by 350 000 generations (burn-in time). The frequency of clades in trees that were sampled every thousand generations from the last 250 000 generations (a total of 5000 trees) was used as an estimate of the posterior probabilities of those clades (Huelsenbeck *et al.* 2001). Uniform priors were specified, and branch lengths, topology and nucleotide substitution parameters were unconstrained. For the maximum-likelihood analysis implemented in GARLI 1.0

(Zwickl 2006), we used the default GTR+I+G model of nucleotide substitution. Trees that were obtained from Bayesian and ML analyses were visualized using PAUP* 4.0b10 (Swofford 2002). In addition to the Bayesian analysis (to assess posterior probabilities at nodes), bootstrap analyses were conducted in maximum parsimony (MP) and ML frameworks to evaluate clade stability. MP bootstrap analysis was executed with 1000 replicates and a random stepwise addition set as 100 replicates in PAUP* 4.0b10 (Swofford 2002). ML bootstrap analysis on the partitioned (by gene fragments) data set was run in RAxML 7.2.8 (Stamatakis 2006) using GTRGAMMA model with one thousand RAxML searches and 1000 iterations.

Testing alternative phylogenetic hypotheses

To determine whether a given developmental mode shared across genera was synapomorphic, we compared the likelihood of unconstrained trees with the likelihood of trees in which reproductive modes were constrained to be monophyletic. We used BEAST v1.8.0 (Drummond et al. 2012) to calculate the harmonic means of the $-\ln$ likelihood values for unconstrained and constrained trees. Likelihood values of the MCMC samples from our combined data set of five genes were obtained using 5 million generations, a sample frequency of 1000 and a burn-in of 1000, with 4000 trees sampled for subsequent analyses. For the constrained analyses, direct developers (*Philautus*, *Pseudophilautus*, *Raorchestes*), foam nesters (*Chiromantis*, *Ghatixalus*, *Polypedates*, *Rhacophorus*, *Taruga*) and gel nesters (*Feihyla*, *'Feihyla'*, *Gracixalus*, *Kurixalus*, *Nyctixalus*, *Theلودerma*) were constrained to be monophyletic in three separate analyses where one group was constrained in each instance. Sampling of trees and their likelihoods followed the same procedure as for the unconstrained analysis. The log Bayes Factors were then calculated, taking twice the difference between the logarithms of the harmonic means estimated from the unconstrained and relevant constrained topologies (Kass & Raftery 1995).

Evolution of reproductive mode

We reconstructed the evolution of reproductive modes using the 5000 trees (Tax107_BA.tre) obtained from the Bayesian analysis (Tax107_BA). We used BayesTraits v1.0 (www.evolution.rdg.ac.uk/BayesTraits.html; Pagel et al. 2004; Pagel & Meade 2006) to reconstruct ancestral states.

Four main characters that capture the essence of rhacophorid reproduction and development were analysed the following: (i) aquatic breeding (AB) – eggs and tadpoles fully aquatic, eggs laid directly in water (e.g. streams, rock pools, phytotelmata), tadpoles feeding in water: *Buergeria* (Fukuyama & Kusano 1992; Nishizawa et al. 2011) and *Liuixalus* (Li et al. 2008); (ii) terrestrial gel nesting (GN) –

partially aquatic development, with eggs deposited above or near water in a viscous gel matrix from which partially developed (late hatching) tadpoles hatch and fall into water, where they feed (exotrophic) and complete larval development as free-living forms: *Beddomixalus* (Abraham et al. 2013), *Feihyla* (Li et al. 2009), *'Feihyla'* (Grosjean et al. 2008; Fei et al. 2010), *Gracixalus* (Rowley et al. 2011), *Kurixalus* (Kuramoto & Wang 1987; Kam et al. 1996), *Mercurana* (Abraham et al. 2013), *Nyctixalus* (Liem 1970; Grosjean et al. 2008) and *Theلودerma* (Orlov & Rybaltovsky 1999; Rybaltovsky 2000); (iii) terrestrial foam nesting (FN) – partial aquatic development, with eggs laid on vegetation or rocks in a foam nest above water, with partially developed tadpoles (late hatching) emerging to feed (exotrophic) and complete development as free-living forms in water: *Chiromantis* (Fei et al. 2010), *Ghatixalus* (Biju et al. 2008), *Polypedates* (Wilkinson & Drewes 2000; Meegaskumbura et al. 2002; Manamendra-Arachchi & Pethiyagoda 2005), *Rhacophorus* (Wilkinson et al. 2002; Rowley et al. 2012) and *Taruga* (Meegaskumbura et al. 2010); and (iv) terrestrial direct development (DD) – eggs laid in moist, non-aquatic environments, such as leaves, leaf litter or crevices, or buried in soil with no free-swimming larval stage (embryonic development, endotrophic); fully developed terrestrial froglets emerge from the eggs: *Philautus* (Alcala & Brown 1982; Dring 1987), *Pseudophilautus* (Meegaskumbura et al. 2002; Bahir et al. 2005; Meegaskumbura 2007) and *Raorchestes* (Biju 2003; Grosjean et al. 2008; Biju & Bossuyt 2009). Each genus has been assumed to possess only a single mode based on the constituent species for which data on reproduction are available.

Ancestral states at specified nodes in the trees were reconstructed using BayesTraits to estimate the likelihood of each character state. States were free to vary in relation to each other, with no constraints. The model 'multistates' was selected for the MCMC analysis, with the reversible-jump hyperprior command selected to specify an exponential prior seeded from a uniform distribution in the interval 0–30 (rjhp exp 0 30), and with rate dev specified as eight to produce acceptance rates of 15–40%. (If the acceptance rate is too high, the model accepts nearly all changes, resulting in excessive autocorrelation among successive states of the chain. If too low, the model fails to explore the parameter space effectively.) A burn-in of 50 000 generations, with sampling of each chain every 1000 generations, and run for 3 000 000 generations, was applied.

Outgroups and mantellids were coded as being aquatic breeders (AB). Mantellids have a wide range of reproductive modes but basal genera are aquatic breeders, and while we assume that AB is regarded as the primitive state for the family a test of that hypothesis awaits resolution of phylogenetic relationships among the Boophinae, Laliostominae

and Mantellinae (Glaw *et al.* 2000; Heying 2001; Glaw & Vences 2003; Lehtinen 2003; Glaw & Vences 2006; Vejarano *et al.* 2006).

An alternative approach for ancestral-state reconstruction using BEAST (v. 2.2.0 Bouckaert *et al.* 2014), where trees and ancestral-state reconstruction occur simultaneously in a Bayesian framework, was also tested (File S1, Data S1, Data S2).

Results

The complete data set initially comprised 3730 bp. A total of 3470 bp remained after removing regions of the mitochondrial 12S and 16S rRNA genes for which alignment was ambiguous and confidence of positional homology was low. All nuclear gene positions were retained. The Partition Homogeneity test showed that the data were congruent ($P > 0.01$) and that the analysis of combined data was justified. jModelTest results showed that, based on the AIC, GTR+I+G was the best fit of the 56 models considered for all gene partitions as well as the combined dataset.

The topology of the 50% majority-rule consensus tree from the Bayesian analysis is identical to that from the maximum-likelihood analysis (Figs 1 and 2) and yields several significant, highly supported phylogenetic conclusions regarding the evolution of reproductive modes. *Buergeria* and *Liuixalus* are sequential sister groups characterized by AB; they form the first diverging lineages sister to all other rhacophorids (Fig. 1). The next reproductive mode to arise is GN (Fig. 1, node 27), which evolved from an aquatic ancestral form; clades of GN *Tbeloderma* + GN *Nyctixalus* are sister to the remaining Rhacophoridae (Fig. 2). Both FN clades (Fig. 1, nodes 16 and 20) arise from GN ancestral forms (Fig. 1, nodes 21 and 18). GN ‘*Feihyla*’ + FN *Chiromantis* also constitutes a highly supported clade (Fig. 2), and GN *Feihyla* is basal to the clade [FN *Ghatixalus* + FN *Rhacophorus* + FN *Taruga* + FN *Polypedates*]. *Chiromantis* (*sensu* Li *et al.* 2013) contains species having both GN and FN; all African species and some Asian species that form a tight clade are FN, whereas ‘*Feihyla vittata*’ is a GN that forms a distinct clade separate from other African and Asian FN. GN *Gracixalus* forms a sequential sister clade to DD *Philautus*. The remaining DD group, [DD *Raorchestes* + DD *Pseudophilautus*], is well separated from DD *Philautus* by three GN clades: GN *Kurixalus*, GN *Beddomixalus* and possibly GN *Mercurana*. The position of *Mercurana* is not well supported (Fig. 1).

Hypothesis testing for direct development

The harmonic mean of the unconstrained search was –28119.79. Corresponding values for three constrained searches are (i) *Pseudophilautus*, *Raorchestes* and *Philautus*, –28180.77; (ii) all FNs, –28144.15; and (iii) all GNs,

–28245.50. Bayes Factors (BF) calculated using the statistic $2[\log(\text{harmonic mean (better model)})] - \log[\text{harmonic mean (worse model)}]$ for these harmonic means against that of the unconstrained tree were 121.96, 48.7 and 251.43, respectively.

Bayes Factor values >10 offer strong support for one hypothesis, whereas values greater than 100 offer decisive support (Kass & Raftery 1995). Hence, the tree in which all three DDs were constrained was much weaker in comparison with the unconstrained topology, which had *Philautus* separate from the other two DD clades. The tree with all FNs constrained was much weaker than the unconstrained topology, which had the FNs as two distinct clades separated by GN taxa. Finally, the tree with all GNs constrained as well was much weaker than the unconstrained topology, which had GNs at several points in the phylogeny.

Evolution of reproductive mode

There is strong support for aquatic breeding (AB), which is shared by the common ancestor of *Buergeria* and *Liuixalus* (Fig. 1, node 31), as the ancestral state of Rhacophoridae. Gel nesting (GN) was the first derived mode from AB. It likely evolved only once, early in rhacophorid evolution, in the common ancestor of all other non-AB rhacophorids (Fig. 1, node 27). Foam nesting likely evolved twice, once in the common ancestor of *Chiromantis* species (Fig. 1, node 20) and once in the common ancestor of *Ghatixalus*, *Polypedates*, *Rhacophorus* and *Taruga* (Fig. 1, node 16). In both instances, foam nesting probably evolved from a GN ancestor. Direct development evolved at least twice independently – once in *Philautus*, again from a GN ancestral form, and once or twice more, depending on whether *Pseudophilautus* and *Raorchestes* are or are not sister taxa (Table S3).

The ancestral-state reconstructions performed using BEAST give a very similar result to that of BayesTraits in terms of likelihoods at the major nodes supporting the genera (Fig. S1). One of the main differences is that the *Mercurana*, *Raorchestes* and *Pseudophilautus* polytomy is shown as a resolved clade in the BEAST reconstruction. For brevity, we discuss only the BayesTraits analysis below.

Discussion

Buergeria and *Liuixalus*, the extant rhacophorid genera that arose first, have fully aquatic eggs and larvae, whereas more derived forms show a marked shift towards terrestrial egg deposition and development (Fig. 1). Gel nesting appears to have evolved early in the rhacophorid phylogeny from ancestors that had aquatic eggs and larvae, which subsequently gave rise to foam nesting and direct-developing lineages. This trend is well supported:

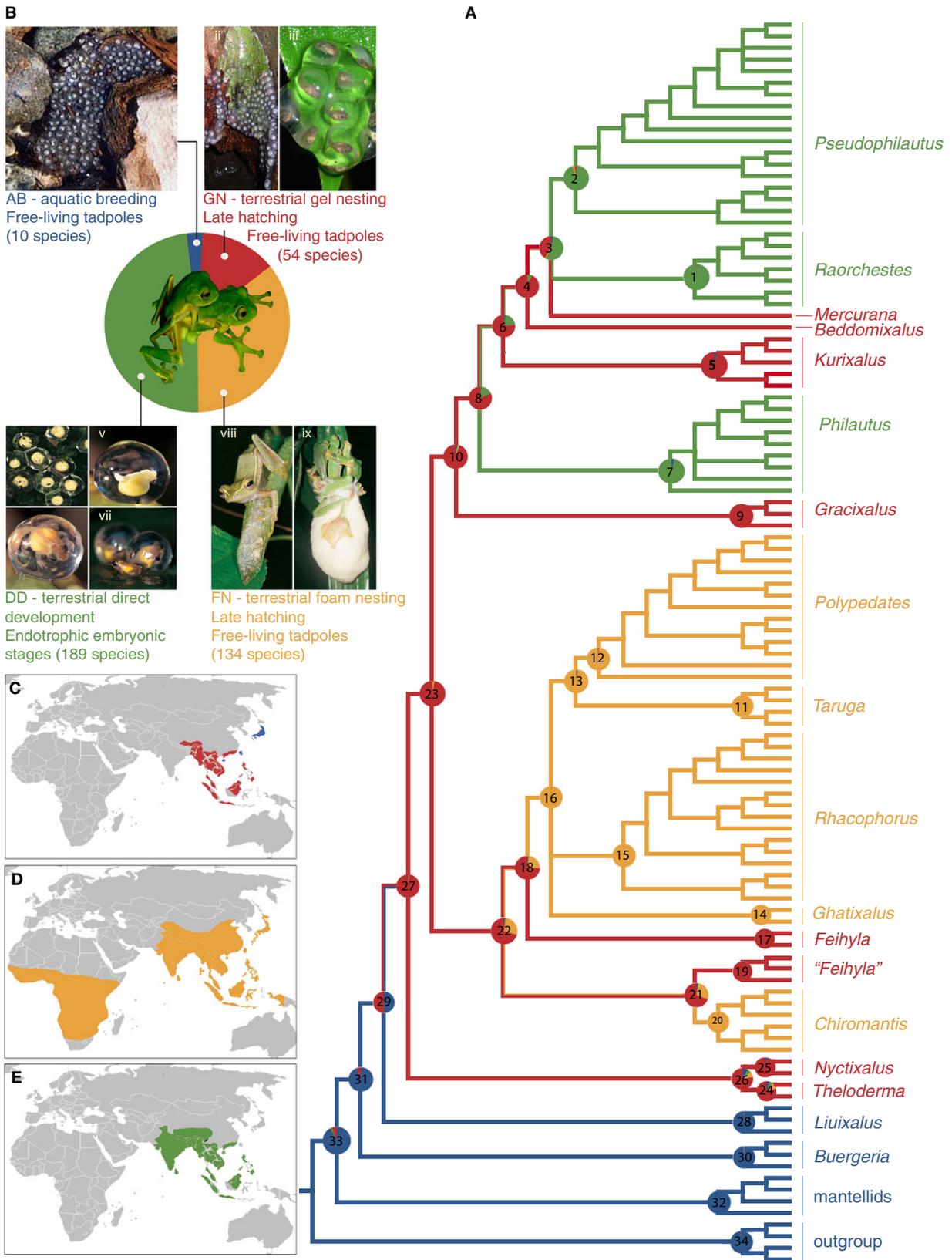


Fig. 1 —A. 50% majority-rule consensus tree from the Bayesian analysis. Major developmental modes of Rhacophoridae indicating Bayesian ancestral-trait reconstruction probabilities for the following life-history characteristics: blue – fully aquatic development (AB); red – terrestrial, gel-encapsulated eggs laid over water with initial phases of larval development in the egg and later stages in the water (GN); yellow – terrestrial eggs in foam nest over water with later stages of larval development in the water (FN); green – fully terrestrial development (DD). The basal life-history strategy for rhacophorids was AB; GN arose multiple times, subsequently giving rise to FN twice and DD two or three times. —B. The proportionate number of all known rhacophorid species characterized by AB (10 spp.), GN (54 spp.), FN (134 spp.) and DD (189 spp.); (i) *Buergeria* sp. eggs in water; (ii) *Gracixalus lumarius* eggs above a tree hole; (iii) *Gracixalus quangi* tadpoles on leaf overhanging water; (iv–vii) *Raorchestes resplendens* embryonic stages and fully developed metamorphs; (viii) *Rhacophorus lateralis* and (ix) *Rhacophorus malabaricus* foam nests (FN). —C–E. Worldwide distribution of AB, GN, FN and DD. FN is the most widespread, whereas AB is the least.

the backbone of the rhacophorid phylogeny is well resolved, with high posterior probabilities and high support for ML and/or MP bootstrap values (Fig. 2). Indeed, the same pattern of reproductive-mode evolution is seen in many other lineages of anurans, with basal lineages being aquatic and a marked shift towards increased terrestriality in derived groups (Duellman & Trueb 1986; Gomez-Mestre *et al.* 2012; Zimkus *et al.* 2012; Müller *et al.* 2013).

Ancestral-trait reconstruction suggests not only that fully aquatic breeding and development are basal in rhacophorid frogs, but also that this state is unlikely to occur in any of the nodes beyond [(*Buergeria* + *Liuixalus*) (*Theلودerma* + *Nyctixalus* + remaining rhacophorids)] (Fig. 1, nodes 29 and 31). Gel nesting, which now characterizes eight lineages (genera), probably arose just once. Foam nesting, on the other hand, probably evolved twice, once in Africa and again in the widespread Asian and Indo-Lankan group; monophyly of the foam nesting genera is strongly rejected. Direct development evolved independently two or three times; monophyly of the direct-developing genera is also strongly rejected.

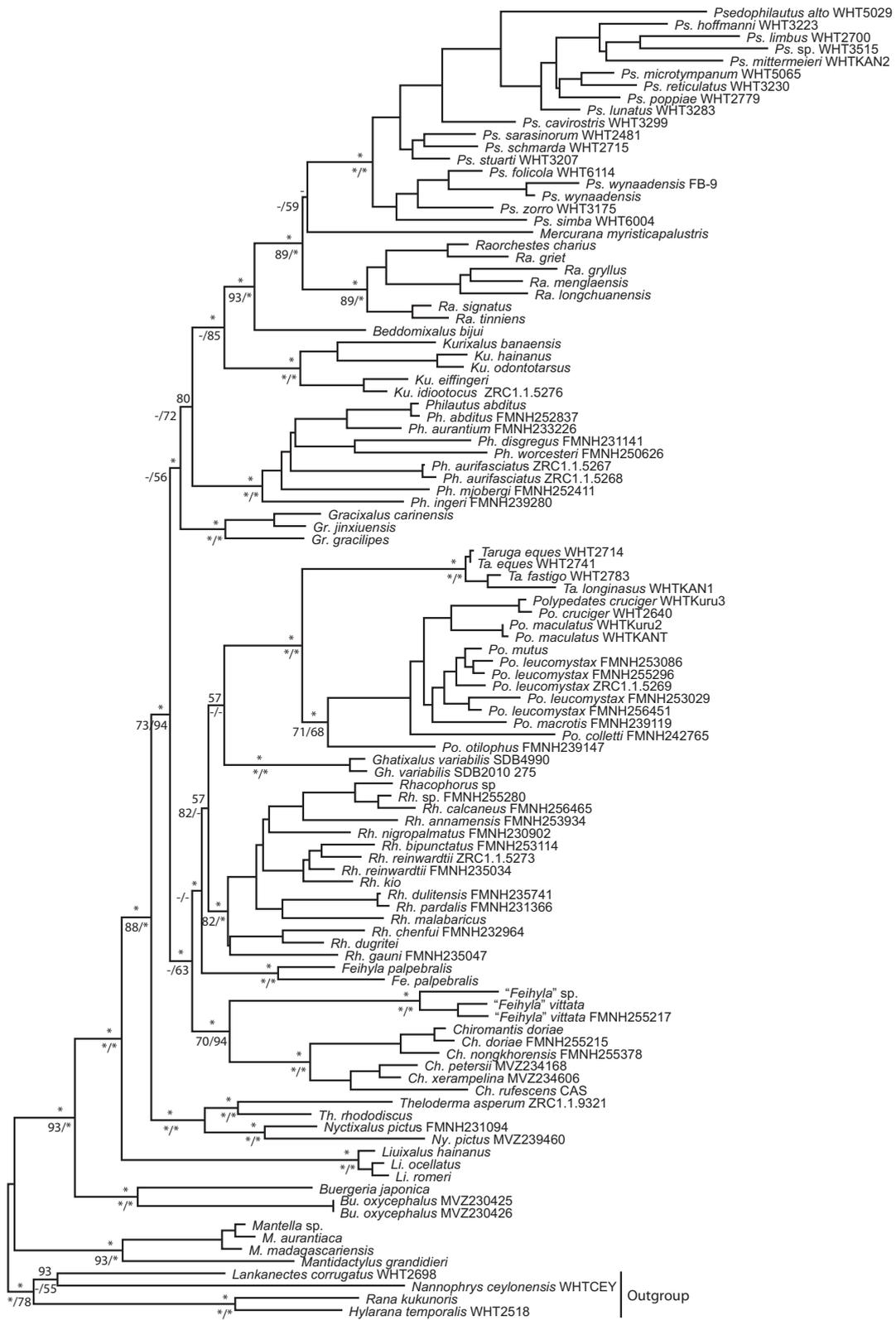
Foam nesting may evolve from gel nesting through increased production of ovarian and seminal fluids (perhaps facilitated by evolution of larger female body size) and a change in behaviour whereby one or both parents whip up the gel nesting material. Likewise, maximizing the allocation of nutrients to the eggs, which remain inside the gel-matrix for longer periods of time, would make possible the evolution of direct development from a gel nesting state. These are some of the tenable hypotheses that can be tested following delineation of the likely evolutionary progression of reproductive modes.

Evolutionary reversals are a distinct possibility in gel nesters, foam nesters and direct developers. Ancestral-state reconstructions show that *Philautus* (DD) probably arose from a gel nesting ancestor (Fig. 1, node 8). The probabilities of nodes 4 and 6 being gel nesters are also very high. However, the reproductive mode of the node-3 ancestor (where there is a polytomy), which gave rise to *Mercurana* (GN), *Pseudophilautus* (DD) and *Raorchestes* (DD), may have

been either direct development or gel nesting. The most parsimonious interpretation is that *Pseudophilautus* and *Raorchestes* are sister groups and that direct development evolved only once in their common ancestor. A test of this hypothesis, however, awaits resolution of the uncertain relationships among these two taxa and *Mercurana*. Finally, foam nesting seems to have evolved both times from a gel nesting ancestor (nodes 21, 22, 18).

Evolution of direct development and foam nesting from a gel nesting ancestor suggests that gel nesting is an important transitional state towards a more terrestrial life history, enhancing opportunities to occupy new niches. In a survey of reproductive modes across anurans, Gomez-Mestre *et al.* (2012) found that direct development may have arisen from both terrestrial and aquatic eggs. We do not find evidence for the latter in Rhacophoridae, and we think that direct development arising from aquatic eggs without a transitional gel nesting phase may be unlikely. While we do not discount the value of the Gomez-Mestre *et al.* (2012) analysis, we suggest reasons for their inference of an aquatic-breeding ancestry of direct-developing taxa. First, our study provides a finer phylogenetic and phenotypic resolution than that of Gomez-Mestre *et al.* (2012), who looked broadly across all anurans and who considered both foam nesting and gel nesting as ‘terrestrial eggs’. We sampled taxa at a finer phylogenetic resolution, thereby decreasing the likelihood of missing taxa whose absence might affect reconstruction of the ancestral state, and we differentiated gel nesting from foam nesting phenotypes (modes). Within Rhacophoridae, the phylogeny of Gomez-Mestre *et al.* recovers direct-developing *Philautus* as a basal taxon that is the sister group of aquatic-breeding *Liuixalus*, a phylogenetic position at odds with recent rhacophorid phylogenies (e.g. Li *et al.* 2008, 2013; Yu *et al.* 2008, 2009). The unusual phylogenetic placement of *Philautus* may have reinforced their claim that direct development arose from aquatic breeding. Finally, *Feihyla palpebralis*, a gel nesting form (‘terrestrial eggs’), was inadvertently coded as a direct developer in their analysis.

Fully terrestrial direct development appears to have evolved from foam nesting ancestors independently in *Phil-*



— 0.01 substitutions/site

Fig. 2 Maximum-likelihood phylogram for 107 taxa showing node support. Numbers above nodes indicate Bayesian posterior probabilities (percentages). Numbers below nodes left and right of slash show maximum parsimony and maximum-likelihood bootstrap values >50 respectively; all values >95 are indicated by *. Most of the nodes highlighted are well supported, except for the nodes involving *Pseudophilautus*, *Raorchestes* and *Mercurana*.

autus and in the shared ancestor of *Pseudophilautus* and *Raorchestes*. Evolution of terrestrial direct development involves major changes in development (Kerney *et al.* 2007), morphology and behaviour (Bahir *et al.* 2005; Vassilieva *et al.* 2013; Rowley *et al.* 2014). Deposition of eggs in a novel habitat can involve highly derived behaviours such as nest building on leaves or egg burying (Bahir *et al.* 2005) or deposition in bamboo stumps (Gururaja *et al.* 2007). Developmental changes are striking in that exotrophic larval stages and metamorphosis are eliminated, with development proceeding from zygote to fully formed froglet within the egg (Duellman & Trueb 1986; Bahir *et al.* 2005; Wells 2007). Evolution of terrestrial eggs with extended development requires changes in egg morphology as well as female reproductive morphology to produce and provision large eggs with sufficient resources to sustain development (Wells 2007). Can such changes evolve multiple times, and is reversion to a more primitive reproductive mode from a direct-developing ancestor likely?

If such major developmental reorganizations are associated with the evolution of direct development, then it could be difficult for gel nesting *Kurixalus* (which separates *Philautus* from the Indian–Sri Lankan direct developers) to revert to partially aquatic development from a direct-developing ancestor (Wake & Hanken 1996). Nevertheless, a comparable evolutionary transformation has been claimed in plethodontid salamanders (Chippindale *et al.* 2004). In addition, direct development may be the plesiomorphic state in the frog genus *Gastrotheca*, which includes both direct-developing species and ones with non-feeding, partially aquatic larvae, implying a reversal to partially aquatic larvae from direct-developing ancestors (Duellman *et al.* 1988; Mendelson *et al.* 2000). Thus, circumstantial evidence suggests that reversals of direct development are possible. However, the extinction of basal aquatic-breeding forms (or their absence from comparative analyses) may yield a pattern in which a highly derived, yet comparatively old direct-developing lineage appears to be basal, thus significantly confounding interpretations of life-history evolution.

In Sri Lankan *Pseudophilautus*, embryos retain several larval characters typically associated with aquatic tadpoles, such as the cement gland and a coiled gut, which are absent in the neotropical direct-developing genus *Eleutherodactylus* (Bahir *et al.* 2005; Kerney *et al.* 2007). Furthermore, the morphology of cartilaginous elements in embryonic stages of Sri Lankan *Pseudophilautus* shows a

greater resemblance to those of free-swimming larvae of metamorphosing anurans than to other direct developers (Kerney *et al.* 2007). For example, larval-specific upper-jaw cartilages – absent in many non-rhacophorid direct-developing species – develop in *Pseudophilautus*. Further, lower-jaw cartilages, which initially assume a larval morphology in *Pseudophilautus*, are not seen in other direct-developing groups. It is possible, therefore, that direct development in *Pseudophilautus* has not involved as many modifications to developmental pathways as those seen in *Eleutherodactylus* (Kerney *et al.* 2007). Reversal of direct development in *Pseudophilautus* thus may be more likely than in *Eleutherodactylus* (and perhaps other direct-developing lineages).

Differences in embryonic development between *Philautus* and (*Pseudophilautus* + *Raorchestes*) also suggest that direct development may have evolved independently in the two groups. *Philautus* embryos display a distinct operculum (Alcala & Brown 1982), which is absent in Indo-Lankan *Pseudophilautus* (Kanamadi *et al.* 1996; Bahir *et al.* 2005). Furthermore, the South-east Asian *Philautus petersi* lacks a larval beak, labial teeth, expanded lips and an oral sucker (Dring 1987), all of which are present in *Pseudophilautus*. Additionally, all *Philautus* have a female-specific snout tip tubercle, which is absent in *Pseudophilautus* (Bahir *et al.* 2005).

Several characteristics of reproductive mode distinguish *Pseudophilautus* from *Philautus*. Female *Philautus* lay eggs in constricted spaces, such as crevices in the ground and in the leaf axils of ferns, *Pandanus* trees and wild-banana plants, and in *Nepenthes* pitchers (Alcala 1962; Alcala & Brown 1982; Dring 1987; Brown & Alcala 1994). Most species of *Pseudophilautus*, however, lay eggs in excavations made in soil (Kanamadi *et al.* 1996; Bahir *et al.* 2005). *Raorchestes* adhere their eggs onto the upper surfaces of leaves (Biju 2003; Biju & Bossuyt 2005), whereas a small clade of Sri Lankan species do so on the undersides of leaves (Bahir *et al.* 2005). Soil-nesting species show an unusual behaviour in which the female, while in amplexus, makes a shallow excavation in the ground into which she deposits eggs. This is followed by a remarkable series of movements in which she mixes the eggs with soil and, presumably, sperm, and then covers the eggs with soil and litter (Bahir *et al.* 2005).

The distinction between *Pseudophilautus* (and presumably *Raorchestes*) and *Philautus* is thus supported by phylogenetic analysis of DNA sequences as well as by characteristics of reproductive mode and larval morphology. This, in turn,

supports the idea that direct development evolved independently in these two groups. Additional developmental studies in *Philautus* and *Raorchestes* are needed, however, to further test this hypothesis.

Hertwig *et al.* (2012) report *Philautus macroscelis* as being nidicolous, a rare state unknown in any other rhacophorid: eggs hatch at an advanced stage but continue to develop within a gel-matrix, later proceeding through free living but non-feeding (endotrophic) final stages prior to metamorphosis. In the absence of a tissue sample and corresponding DNA sequence data, this species is not included in our analysis. Moreover, the *Philautus* clade of Hertwig *et al.* (2012) shows a basal polytomy, which precludes determination of the position of this nidicolous form among other direct developers. Nevertheless, it is possible that the nidicolous condition, if placed at the base of the *Philautus* clade, could represent an intermediate stage in the transition from gel nesting to direct development. Future studies with wider taxon and gene sampling, together with more focused natural history observations, are needed to determine the patterns of reproduction in this biogeographically and developmentally intriguing *Philautus*.

One of the two major clades of *Pseudophilautus sensu prior* was recognized as *Raorchestes* by Biju *et al.* (2010). There are subtle life-history differences between the predominantly soil-nesting *Pseudophilautus* (Bahir *et al.* 2005) and leaf nesting *Raorchestes* (Biju *et al.* 2010), both of which have direct-developing eggs. There is, however, a clade of three leaf nesting species within *Pseudophilautus* (Meegaskumbura & Manamendra-Arachchi 2005; Meegaskumbura *et al.* 2008). Further research is needed to determine the full variation of life-history variation in these two groups.

Foam nesting groups form two major clades, which are separated by *Feihyla* (GN) – the first clade contains *Polypedates*, *Taruga*, *Rhacophorus* and *Ghatixalus*; the second is represented by *Chiromantis* (Figs 1 and 2). At least four previous molecular-systematic studies recover each of the foam nesting genera as monophyletic (Richards & Moore 1998; Meegaskumbura *et al.* 2002; Wilkinson *et al.* 2002; Frost *et al.* 2006). Among previous morphological analyses, Wilkinson & Drewes (2000) place the foam nesters in a monophyletic group, whereas Liem (1970) and Channing (1989) do not. Li *et al.* (2013) also recover foam nesters split by a gel nesting group, a conclusion that is supported by our analysis.

The sister group of each foam nesting clade is a gel nesting clade. *Feihyla sensu stricto*, the clade including *F. palpebralis*, its type species) is a gel nesting clade whose sister group is the foam nesting clade comprising *Polypedates*, *Taruga*, *Rhacophorus* and *Ghatixalus*. Another gel nesting clade, which contains '*Feihyla vittata*', has the foam nesting clade

Chiromantis as its sister group (Figs 1 and 2). Our phylogeny thus recovers *Feihyla sensu* Frost (2014) as polyphyletic. Indeed, the (pairwise uncorrected) genetic distances for the combination of all genes between these two gel nesting clades (i.e. those containing *F. palpebralis* and '*Feihyla vittata*') are remarkably large, ranging from 4.9 to 7.6%, which is comparable to distances between other valid genera within the 'foam nesting' clade (Fig. S2). Pending further study, we suggest that '*Feihyla vittata*' may warrant placement in a distinct genus. While it is a close relative of *Chiromantis*, it differs from that foam nesting genus by being a gel nester, although it remains to be determined whether parental care, observed in *C. hansenae* (Sheridan & Ocock 2008), extends more widely through this clade. Li *et al.* (2013) recovered '*Feihyla vittata*' and *Feihyla palpebralis* as monophyletic but with low support. Our phylogeny recovers these two taxa as separate clades with higher support (Figs 1 and 2).

Gel nesting ancestors have contributed not only to life-history evolution but also to lineage diversification (here we regard genera as the major lineages); gel nesting ancestors have given rise to 8–9 lineages, whereas foam nesting and direct-developing ancestors have given rise to 5 and 0–1 lineages, respectively. Despite the lineage-diversification propensity of gel nesters, the other two derived reproductive modes have given rise to more species of foam nesters (134 species) and direct developers (189 species) (Table S2). Furthermore, foam nesting lineages are characterized by the largest geographic ranges across potentially xeric and open habitats.

The broad adaptive values of the three derived reproductive modes can be predicted based on their evolutionary patterns, numbers of species and distributions. Gel nesting rhacophorid species are relatively small and lay fewer eggs in humid 'closed-canopy' habitats. Presumably, a primary function of the gel-matrix of gel nests is to protect the developing embryos from desiccation. However, in the neotropical *Agalychnis callidryas* and a few other leaf nesting species, gel nests also protect embryos from snakes and wasps, sometimes even indirectly by facilitating the early hatching of embryos (Warkentin 1995, 2000; Gomez-Mestre *et al.* 2008). The evolution of foam nesting may have enabled rhacophorids to lay a larger number of eggs, often in more open and drier habitats. For instance, foam nesting *Chiromantis* show the widest geographic range of any rhacophorid genus, extending from South-East Asia to open and dry habitats in Africa. The thick foam nest of these frogs protects the eggs and developing larvae from sunlight (Gorzula 1977) and desiccation (Downie 1988). Direct developers comprise the largest number of species (Table S2), which suggests that this reproductive mode may represent a key innovation that enables diversification of anurans

into a variety of terrestrial habitats. However, a deeper analysis is required to evaluate these patterns comparatively.

The phylogenetic hypotheses (trees) for rhacophorids published to date (e.g. Meegaskumbura *et al.* 2002; Wilkinson *et al.* 2002; Li *et al.* 2008, 2009, 2013; Yu *et al.* 2008, 2009; Abraham *et al.* 2013) exhibit poor support at several nodes on the backbone or between major lineages, which has precluded the delineation of patterns of character evolution. The poor support has been due to a combination of insufficient representation of major lineages (genera) and dearth of genetic data. We have attempted to address these deficiencies by including all valid rhacophorid genera and more sequence data (five genes – three nuclear and two mitochondrial). As a result, genus-level systematic relationships are well supported except for *Pseudophilautus*, *Raorchestes* and *Mercurana*, which form a polytomy (Fig. 2). Surprisingly, the addition of *Mercurana* destabilizes the clade of *Pseudophilautus* and *Raorchestes*, which had high support even in the mtDNA phylogeny of Meegaskumbura *et al.* (2002) and in all subsequent rhacophorid phylogenies that included these latter two taxa but omitted *Mercurana*.

Incomplete taxon sampling increases the likelihood of drawing incorrect conclusions with regard to patterns of evolution of reproductive modes, especially in old lineages in which intermediate modes may have gone extinct. Focusing instead on relatively young lineages that represent both basal and derived reproductive modes may provide an improved understanding of the evolution of these modes. However, consideration of broad-scale phylogenies and life-history patterns (especially involving ancient lineages) will be useful in understanding the differential survival of lineages characterized by contrasting life histories.

Our study delineates the patterns of reproductive-mode evolution observed in Rhacophoridae by including all known major clades within the family. Results suggest that terrestrial gel nesting is a versatile mode of reproduction between fully aquatic breeding and more derived modes, viz, direct development and foam nesting. Further analyses of these patterns across disparate lineages will help better elucidate the evolution of reproductive modes in amphibians, the pioneering vertebrates of the terrestrial realm.

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Supporting Information

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

File S1. Material and Methods of the BEAST analysis.

Fig. S1. Ancestral-state reconstructions using *BEAST.

Fig. S2. Pairwise ‘uncorrected p’ genetic distances among foam nesting genera and two associated gel nesting genera, based on the three nuclear and two mitochondrial gene fragments used in the study.

Table S1. Taxonomic information, collection reference numbers*, geographic regions, gene fragments for which sequences are not available (X), GenBank reference numbers, and modes of development of the 107 species used in this study.

Table S2. Major life history characteristics and number of species within each rhacophorid genus that share a given life history.

Table S3. Reconstruction of ancestral states for four characters using Bayes Traits (A – DD; B – GN; C – FN; D – AB).

Data S1. Input file of the *BEAST analysis.

Data S2. Output summarized.tre file with posterior probabilities for each node.