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Programa de Pós-Graduação em Ecologia e Evolução

Evolução de atributos reprodutivos em anuros

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Dissertação apresentada à Universidade Federal de Goiás como parte das exigências do Programa de Pós-graduação em Ecologia e Evolução para a obtenção do título de mestre.

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Elisa Barreto Pereira

Evolução de atributos reprodutivos em anuros

Dissertação apresentada à Universidade Federal de Goiás como parte das exigências do Programa de Pós-graduação em Ecologia e Evolução para a obtenção do título de mestre.

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Goiânia, março de 2014

ñ*Das coisas*
que fiz a metro
todos saberão
quantos quilômetros são

Aquelas em
centímetros
sentimentos mínimos
ímpetos infinitos
não?ò

Paulo Leminski

Fico feliz em poder expressar meus sinceros agradecimentos a todos que me ajudaram e me apoiaram durante o mestrado.

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O estudo evolutivo de atributos de histórias de vida é um amplo ramo da ecologia evolutiva (Pianka 1982). Conhecer a origem e os padrões de diversificação destes atributos permite compreender mais sobre a evolução das espécies e a seleção natural (Schluter 2001). Com o advento da sistemática filogenética e a atual facilidade em gerar hipóteses filogenéticas, estudos evolutivos tem vem se tornando mais frequentes e vários métodos tem sido desenvolvidos (Pagel 1999; O'Meara 2012; Pyron e Burbrink 2013). Especialmente métodos para o estudo de atributos de história natural, que passou a ter uma perspectiva evolutiva (e.g. Huelsenbeck e Rannala 2003; Ree 2005; Magnuson-Ford e Otto 2012). O fato de que informações da história evolutiva dos grupos podem ser recuperadas a partir de hipóteses filogenéticas permite que esta seja usada para inferir como ocorreu o processo de evolução dos atributos de história natural. Métodos que unem estas duas fontes de informações permitem reconstruir estados ancestrais e descrever como estes atributos evoluíram, testar modelos de evolução pontual ou gradual, correlacionar a evolução destes atributos com outros, ou com variáveis ambientais ou com taxas de especiação e extinção (Pagel 1999; Maddison et al. 2007; Magnuson-Ford e Otto 2012; Pyron e Burbrink 2013).

A ampla diversidade de atributos de história de vida de anfíbios anuros os torna interessantes para estudos de evolução de caracteres. Sua marcada diversidade de modos reprodutivos, que variam com características de oviposição, desenvolvimento, desova, e cuidado parental (Salthe e Duellman 1973; Haddad e Prado 2005) é um dos atributos de história de vida mais marcantes neste grupo de organismos. A existência de modos aquáticos e terrestres, que variam na existência ou não de girinos, permitiu a ocorrência de diversas hipóteses sobre a evolução destas características. Entretanto, muitas delas ainda não foram

ecente trabalho em larga escala mostrou padrões inesperados na evolução dos modos reprodutivos de anuros (Gomez-mestre et al. 2012).

Uma hipótese que recorrentemente é citada na literatura sobre a evolução de modos reprodutivos em anuros é a de que nas rãs da subfamília Leptodactylinae há uma tendência à terrestrialidade ou graduação dependência de ambientes aquáticos para a reprodução (e.g., Duellman 1989; De La Riva 1995; Prado et al. 2002, 2005; Gibson e Buley 2004; Haddad e Prado 2005). De acordo com Heyer (1969), a evolução de modos reprodutivos nestas espécies segue uma sequência, onde o modo basal tem maior dependência da água e o mais derivado tem menor dependência. Neste trabalho, reconstruímos hipóteses filogenéticas para espécies desta linhagem de rãs com o objetivo de compreender como ocorreram os padrões e processos que culminaram em diferentes atributos relacionados à reprodução como, por exemplo, a tendência à terrestrialidade. Testamos também correlações entre esses modos e outros atributos.

Outro interessante atributo reprodutivo presente em diversas linhagens de anfíbios anuros é o ninho em forma de espuma. De acordo com a revisão mais recente, esta estrutura está presente em cinco de 39 modos reprodutivos (Haddad e Prado 2005) e em ao menos 326 espécies distribuídas em continentes distintos. A espuma é composta por albumina e é conhecida por ter diversas funções, tais como: evitar dessecação, diminuir a predação, auxiliar a difusão de oxigênio, e servir de alimento aos girinos (Dobkin e Gettinger 1985; Tanaka e Nishihira 1987; Seymour e Loveridge 1994; Fleming et al. 2009). Sua indiscutível importância se reflete no número de artigos que lidam com aspectos ecológicos desta estrutura. Entretanto, estudos que busquem padrões evolutivos relacionados a esta estrutura de ninho ainda são escassos (Faivovich et al. 2012; Fouquet et al. 2013). Neste sentido, testamos se a presença desta estrutura afeta a probabilidade da linhagem se diversificar ou se extinguir.

hipóteses filogenéticas e atributos reprodutivos de linhagens de anfíbios anuros, foi possível compreender como se deu a evolução de tais estruturas e se elas estão correlacionadas com algum outro fator, tais como número de ovos por desova, pigmentação do ovo e taxa de diversificação.

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CAPÍTULO I

RECONSTRUÇÃO DE ESTADOS ANCESTRAIS DE ATRIBUTOS REPRODUTIVOS INDICA AUSÊNCIA DE TENDÊNCIA À TERRESTRIALIDADE EM LEPTODACTYLIDEOS

ANCESTRAL RECONSTRUCTION OF REPRODUCTIVE TRAITS SHOWS NO TENDENCY TOWARD TERRESTRIALITY IN LEPTODACTYLID FROGS

Ancestral reconstruction of reproductive traits shows no tendency toward terrestriality in leptodactylid frogs

Running title: Life-history evolution and *Adenomera* phylogeny

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Leptodactylinae frogs are a remarkable example of anurans outstanding diversity of reproductive features. The major distinctions among the four reproductive modes presented by this group are the relationship with water and the predicted gradual tendency towards terrestriality. To study the evolution of Leptodactylinae reproductive traits and recognize their patterns we used ancestral reconstruction methods. We also tested correlations among reproductive modes and other life-history traits by using stochastic inferences. First we reconstructed a phylogenetic hypothesis of Leptodactylinae lineages including *Leptodactylus*, *Adenomera*, and *Lithodytes* genera based on four DNA fragments. This hypothesis comprises the most complete phylogeny of *Adenomera* to date and confirm its monophyletism with *Lithodytes* as sister taxon. Our ancestral reconstruction analysis indicated that even though shifts from aquatic to terrestrial breeding occurred in the history of *Leptodactylus* and *Adenomera*, shifts from terrestrial to aquatic happened in almost the same frequency, indicating that Leptodactylinae frogs do not always evolve towards terrestriality and that reproductive modes with semi-terrestrial tadpoles is not necessarily an intermediate form between aquatic and terrestrial breed. Correlations among reproductive modes and other life-history traits suggest that tadpole environment, clutch size, nuptial spines, and egg pigmentation are coevolving driven by water dependence.

Evolution of life-history traits in different lineages is one of the biggest questions in evolutionary biology (Avise 2006). Concerning the evolution of reproductive features in amphibians, many uncertainties are still unsolved due to the remarkable diversity of lifestyles, from purely aquatic to arboreal and fossorial (Haddad and Prado 2005). Anuran reproductive features are classified into reproductive modes based on oviposition, development, hatchling, and parental care (Salthe and Duellman 1973; Haddad and Prado 2005).

Shifts from aquatic to terrestrial breeding occurred repeatedly and independently in many vertebrates (Pough et al. 2001). Evolution of terrestrial reproduction in anurans from ancestors that bred in water has been traditionally claimed (Salthe and Duellman 1973; Duellman and Trueb 1994), specially because aquatic mode with exotrophic tadpoles is the most representative (Haddad and Prado 2005) and probably the ancestral state for anurans (Gomez-Mestre et al. 2012). Besides, the existence of intermediate stages such as species that lay eggs close to water (e.g., in burrows) instead of inside the water bodies supports the hypothesis of an ordered and gradual evolution in the direction of a more terrestrial reproduction (McDiarmid 1978; Duellman and Trueb 1994). Nonetheless this conventional view has recently been challenged by Gomez-Mestre et al. (2012), who showed the lack of intermediate stages in some groups and the evolution of direct development from both terrestrial and aquatic reproductive modes.

In addition, shifts between aquatic and terrestrial breeding may occur in consonance with modifications on morphological and ecological features (Heyer 1969; Zimkus et al. 2012), originating opportunities for coevolution between traits. Even though reproductive modes are frequently studied, the only few well-known associations that are commonly tested show: i) negative correlation between ovum and clutch size (number of eggs per spawning);

ze and hatchlings dimensions; and iii) positive correlation among clutch volume, egg size and female body size within a given reproductive mode (see Duellman and Trueb 1994). Under a cladistic perspective, a recent study shed light on some unexplored associations, such as the correlation of terrestrial reproduction with reduced clutch and adult size, and with parental care (Gomez-Mestre et al. 2012). However, little is known about other traits that may be correlated with reproductive modes, such as tadpoleôs features and adult morphological characters other than size.

Analyzes of character history, such as ancestral state reconstruction (revised by Pagel 1999) and stochastic mutational mapping (Nielsen 2002), are powerful methods to study the origin and maintenance of phenotypic diversity (Schluter 2001; Rundle and Nosil 2005). Moreover, the analysis of character associations may provide important clues about the correlation between life-history traits (Huelsenbeck and Rannala 2003). These analyzes have been used as important cladistic approaches to understand the origin and evolution of life-history traits in different living organisms (e.g., Hart et al. 1997; Pagel 1999; Chippindale et al. 2004; Ikeda et al. 2008), but these powerful methodologies are usually constrained by the availability of well-supported phylogenetic hypotheses.

Amphibianôs systematics underwent pronounced changes in the last decade (e.g., Frost et al. 2006; Pyron and Wiens 2011). *Leptodactylus* genus, the most diverse among Leptodactylidae, contains 75 species distributed from North America (southern Texas) throughout Central and South America. Formerly, the genus was assembled in five groups based on behavioral, morphological and ecological features (Heyer 1969): *Leptodactylus ocellatus* - now *L. latrans* (Lavilla et al. 2010), *L. melanonotus*, *L. pentadactylus*, *L. fuscus* and *L. marmoratus*. However, since Heyerôs (1969) suggestion that the group *Leptodactylus marmoratus* was not closely related to the other groups, the phylogenetic position of the

s placement in a different genus, *Adenomera* (Heyer 1974). Recent molecular data confirm *Adenomera* as a natural group with a single common ancestor (Pyron and Wiens 2011; Fouquet et al. 2013). Distributed throughout almost all South America, the genus may comprise cryptic species leading to underestimation of the number of species (Angulo and Icochea 2010).

Leptodactylus and *Adenomera* (Anura: Leptodactylidae) are good models to understand the patterns and processes of the evolutionary history of reproductive traits. Those foam-nesting species present at least four different reproductive modes varying in the place of oviposition and biology of larvae. The diversity of reproductive modes among *Leptodactylus* and *Adenomera* and its relationship with phenetic groups lead to the prediction of a gradual evolutionary tendency from a more aquatic to a more terrestrial breeding, with evidence of intermediary stages. Heyer (1969) hypothesized that the *Leptodactylus melanonotus* and *L. latrans* groups have the most primitive reproductive modes, with higher water reliance. The *Leptodactylus pentadactylus* group would represent the first step towards terrestriality, with eggs placed in the water accumulated in constructed basins, followed by the *L. fuscus* group, in which eggs are placed inside constructed subterranean chambers. Finally, *Adenomera* (formerly the *L. marmoratus* group) would represent the most derived reproductive mode with lower dependence on water for reproduction, since some species have endotrophic tadpole (develops entirely in the subterranean chambers). Since Heyerôs (1969) suggested that *Adenomera* is an independent lineage, he also postulated that the evolution of terrestrial reproduction had occurred twice, one in *Leptodactylus* and another in *Adenomera*. Although this hypothesis of gradual increase of terrestriality in some Leptodactylinae frogs has never being tested, it repeatedly appears in literature (e.g., Duellman 1989; Riva 1995; Prado et al. 2002, 2005; Gibson and Buley 2004; Haddad and Prado 2005). Recently some authors have

esis (Downie and Nokhbatolfoghahai 2006; Faivovich et al. 2012).

Here we studied the evolution of life-history traits among Leptodactylinae lineages reconstructing ancestral states, mapping mutations and testing the correlation among six characters. For this we first obtained a phylogenetic hypothesis for *Adenomera* and *Leptodactylus* based on Bayesian analysis. Afterwards, we reconstructed ancestral states under stochastic inference and quantified the association between characters based on Bayesian analysis implemented in SIMMAP. We herein tested: (1) *Adenomera*ôs monophyletism; (2) the hypothesis of tendency towards terrestriality, with shifts from aquatic to terrestrial breeding and the existence of intermediate stages; and (3) the association between reproductive modes and morphological and ecological features potentially related to water dependence.

Materials and Methods

TAXON SAMPLING

We sampled 35 Leptodactylinae species, 11 *Adenomera*, 23 *Leptodactylus* representing all phenetic groups, and the monotypic *Lithodytes lineatus* (Supporting Information Table S1). *Physalaemus cuvieri* and *Eupemphix nattereri* were used as outgroups based on their relationships with Leptodactylinae (Frost et al. 2006; Pyron and Wiens 2011). Most sequences were obtained in this work and some were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/, see Supporting Information Table S1).

GENETIC DATA

or liver tissue preserved in ethanol and tissue-storage buffer, using the DNeasy Tissue Kit (Qiagen®). We sequenced four DNA fragments. The nuclear Rhodopsin exon I (*Rhod*) fragment was sequenced using Rhod1A and Rhod1C primers (Bossuyt and Milinkovitch 2000). The mitochondrial regions 12S and 16S were sequenced using 12Sa, 12Sb, 16Sar and 16Sd (Reeder 1995). For cytochrome B (c*ytB*) we used MVZ15 (Moritz et al. 1992) and H15149 primers (Kocher et al. 1989) (PCR protocols on Supporting Information Table S2). The PCR products were purified using shrimp alkaline phosphatase (SAP) and exonuclease I (EXOI) enzymes (Biotech Pharmacon, ASA). Purified PCR products were sequenced in both directions on an ABI 3100 automated DNA sequencer (Applied Biosystems, CA) using the DYEnamic^{TM} ET terminator sequencing kit (GE HealthCare, Sweden), according to manufacturerôs instructions.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

Sequences were edited using the software SeqScape (v2.1) and aligned in MUSCLE 3.8 (Edgar 2004). Sequences not available were coded as missing data (Table S1). Coding sequences were tested for saturation plotting transitions and transversions against TN93 distance (Tamura and Nei 1993) using the software DAMBE (Xia and Xie 2001).

Phylogenetic hypotheses were obtained for the combined datasets based on Bayesian and maximum-parsimony methods. Evolutionary model selection was performed using Akaike Information Criterion (AIC) implemented in jModelTest 2 (Darriba et al. 2012). Then, Bayesian analyses were conducted in MrBayes v.3.1.2 (Ronquist et al. 2011) with randomly generated starting trees. Four Markov Chains and four million generations were sufficient to obtain a standard deviation of split frequencies below 0.01. Trees and parameter values were sampled every 500 generations. After discarding the first 250 trees (ócburn-inò) of the two

consensus and calculate the Bayesian credibility values (BC) for each branch. Clades with BC equal to or exceeding 95% were considered strongly supported (Leaché and Reeder 2002). The maximum parsimony (MP) analysis was carried out using PAUP* 4.0 (Swoford 2003). We used heuristic search with multiple tree bisection reconnection (TBR) branch swapping. Bootstrap re-sampling (Felsenstein 1985) was applied to assess the support for individual clades using 1,000 bootstrap replicates and full heuristic searches with 10 replicates of random stepwise addition and TBR branch swapping. Clades with bootstrap values higher than 75% were considered well-supported following Hillis and Bull (1993).

ANCESTRAL STATE RECONSTRUCTION, MUTATIONAL MAPPING, AND CORRELATION ANALYSIS

To study the evolution of life-history traits among Leptodactylinae lineages, we inferred ancestral states, mapped character state mutations and tested the correlation of six ecological and morphological traits using SIMMAP 1.5 (Bollback 2006). At least four reproductive modes are known for Leptodactylinae (Haddad and Prado 2005): i) mode ó11ô includes species that produce floating foam nests in ponds with exotrophic tadpoles; ii) mode ó13ô also presents exotrophic tadpoles, but with foam nests placed in water accumulated in constructed basins; iii) mode ó30ô groups species which foam nests are placed inside a subterranean chamber and after a period of development the tadpoles float to water bodies; iv) mode ó32ô is the most terrestrial one, with endotrophic tadpoles (develop entirely in subterranean chambers using only the yolk as source of energy). Other life-history traits studied here were considered directly (clutch size, tadpole environment, nuptial spines and egg pigmentation) or indirectly (habitat) related to reproductive modes in frogs. Character states were retrieved

, Table S3), based on personal observation or from specialists on reproductive traits of Neotropical anurans (information by authority) and coded following Table 1.

Table 1. Character codification used in the ancestral state reconstruction, mutational mapping and correlation analysis of life-history traits of Leptodactylinae. Polymorphic data were coded as missing data.

Character	State 0	State 1	State 2	State 3
Reproductive mode	Mode 11	Mode 13	Mode 30	Mode 32
Clutch size	Less than 50	Between 50 and 1,000	More than 1,000	-
Habitat	Open areas	Forest formations	-	-
Tadpole environment	Lotic water bodies	Lenthic water bodies	Terrestrial tadpole	-
Nuptial spine	Absence	Presence	-	-
Egg pigmentation	Absence	Presence	-	-

We reconstructed the ancestral states of the six characters using Bayesian stochastic character mapping (Huelsenbeck et al. 2003) on the 50% majority-rule consensus tree obtained from the Bayesian phylogenetic analysis. The analysis evaluates the consistency between character history and character states observed at the tips and then estimates the posterior probabilities of ancestral states. The results were visualized as pie charts using a function developed by Dr. Marion Chartier in R software (R Development Core Team 2011). We also mapped the mutations of character states along the phylogeny to estimate the number of transformations between states (Nielsen 2002). To perform this analysis, we randomly

ylogenetic analysis after the convergence.

Afterwards we calculated the overall character correlation (D statistic) between reproductive mode and five life-history traits (i.e., clutch size, habitat, tadpole environment, nuptial spine, egg pigmentation), and the correlation state-by-state (*dij*). For this analysis we randomly selected 300 trees generated after the convergence by the Bayesian phylogenetic analysis. The *dij* statistic represents the divergence between the observed and expected association of states *i* and *j*. The expected association is the product of the marginal probabilities of finding these states (*i* and *j*) in the same phylogenetic node (Huelsenbeck and Rannala 2003).

Results

PHYLOGENY ESTIMATION

The combined dataset alignment consisted on a fragment of 1,526 base pairs (Table 2). The third codon position of *cytB* was excluded from the final alignment due to the high saturation (Supporting information Figure S1). Ambiguous alignments from 12S and 16S sequences were also excluded from the analysis. For both 12S and 16S datasets the best evolutionary model was GTR+I+G, whereas for the *cytB* fragment was TIM2+I+G and for the *Rhod* fragment was TPM3uf+I+G (Table 2).

The Bayesian analysis recovered a monophyletic and highly supported *Adenomera* clade with *Lithodytes lineatus* as sister species (Figure 1). The basal clade is comprised by *A. heyeri* and *A. lutzi*. Species of *Leptodactylus* also formed a high supported monophyletic group, subdivided in two major clades (Figure 1).

evolutionary model for each DNA region used in phylogenetic analyses for 35 Leptodactylinae species.

	16S	12S	Cytochrome B	Rhodopsin 1
Original length (bp)	517	435	405	330
Final length (bp)	503	423	270	330
Base frequencies				
%A	0.314	0.308	0.211	0.234
%C	0.230	0.262	0.216	0.283
%G	0.210	0.203	0.230	0.195
%T	0.246	0.227	0.343	0.288
Parsimony informative characters (PIC)	116	107	28	44
PIC without outgroup	108	101	27	27
Best fit model	GTR+I+G	GTR+I+G	TIM2+I+G	TPM3uf+I+G
Model likelihood	2904.84	2865.12	868.58	1081.27

The maximum parsimony analyses produced 50 most parsimonious trees with 1,435 steps. The strict consensus tree had 1,472 steps (CI = 0.41, RI = 0.56) and also showed *Lithodytes lineatus* as sister species of the monophyletic genus *Adenomera*. Parsimony analyses generate a consensus tree similar to the Bayesian analysis, but with some polytomies (Figure 1).

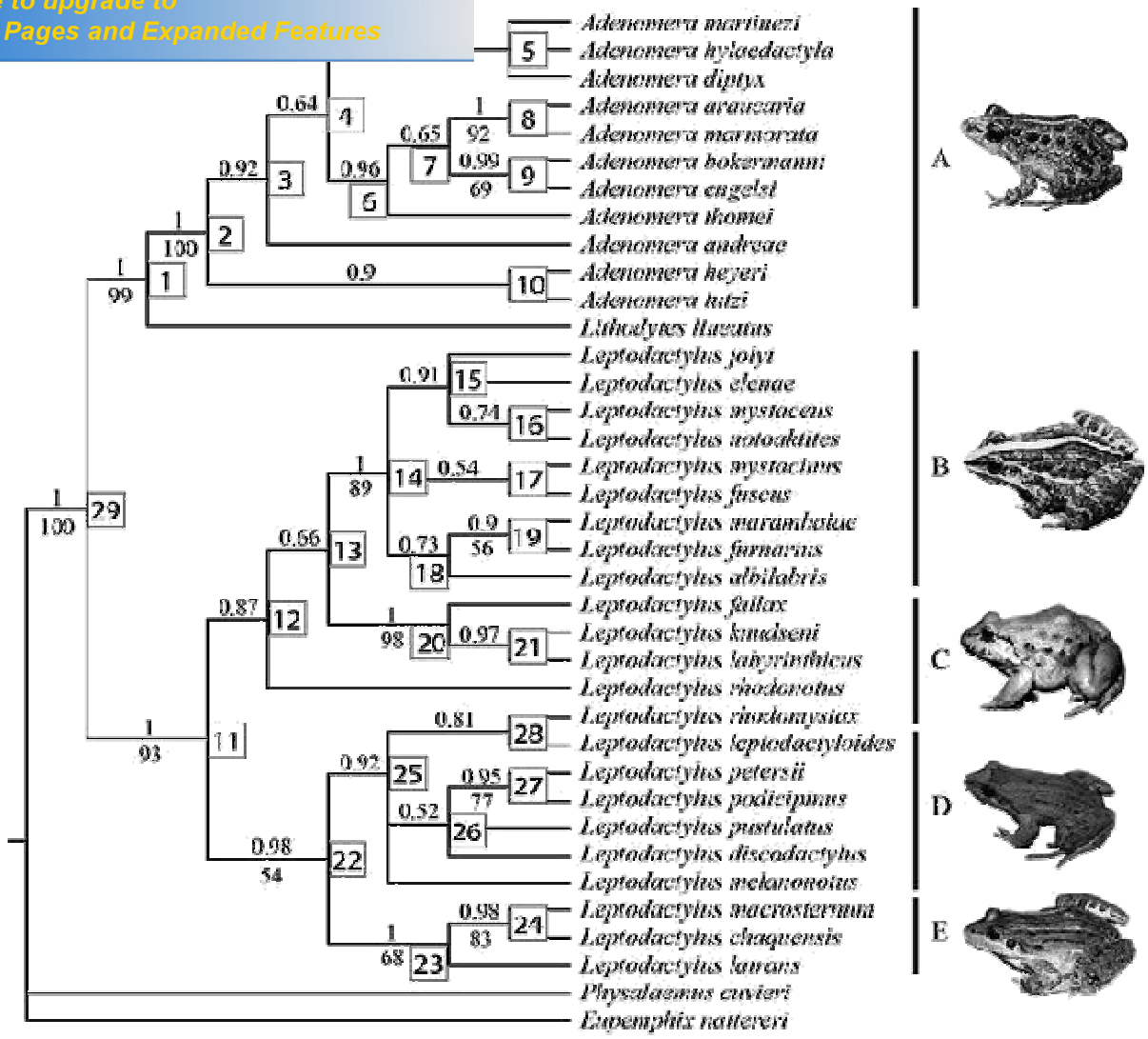
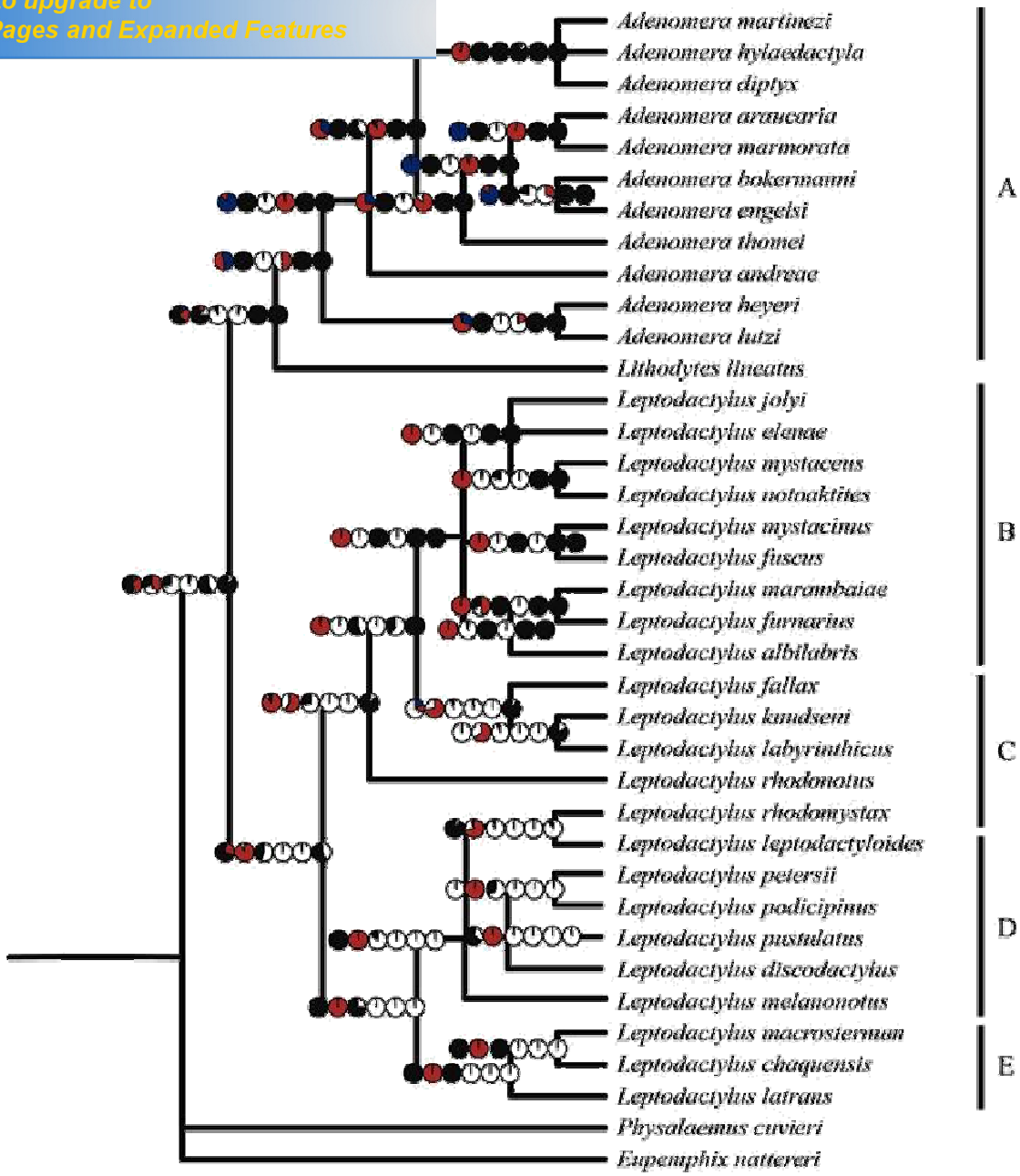


Figure 1. Phylogenetic relationships among Leptodactylinae species based on the 50% majority-rule consensus cladogram reconstructed using Bayesian analysis. Numbers above nodes are clade *posteriori* probability, below nodes are bootstrap supports for the maximum parsimony analysis, and inside box are node number. A: *Adenomera martinezi* (*Adenomera* genus); B: *Leptodactylus fuscus* (*L. fuscus* group); C: *L. labyrinthicus* (*L. pentadactylus* group); D: *L. podicipinus* (*L. melanonotus* group); and E: *L. latrans* (*L. latrans* group). Photos: A, Pedro Peloso, B and D, Ariovaldo Giaretta, and C and E, Antonio Sebben.

ANCESTRAL STATE RECONSTRUCTION, MUTATIONAL MAPPING, AND CORRELATION

Bayesian character state reconstruction indicated that reproductive mode 11, in which eggs

y on the top of water, is the most probable ancestral state of the most recent common ancestor (MRCA) of Leptodactylinae (node 29, Figure 2). This reproductive mode was also the ancestral state of *Lithodytes* + *Adenomera* (node 1) and of *Leptodactylus* (node 11). While reproductive mode 11 had one origin, modes 13 and 32 originated twice and mode 30 originated at least three times (Figure 2). In addition, the analysis showed that transitions from aquatic to terrestrial (or at least to a less aquatic) reproductive mode happened in Leptodactylinae at least four times: 1) a shift from mode 11 to modes 30 or 32 in the ancestral *Adenomera* (nodes 1 and 2, Figure 2); 2) a shift from mode 30 to 32 in some *Adenomera* species (nodes 6 and 7); 3) a shift from mode 11 to 30 in some *Leptodactylus* ancestral (nodes 11 and 12); and a shift from 11 to 13 in two species of the *L. melanonotus* group (nodes 26 and 27). Moreover, transitions from terrestrial to aquatic (or at least a less terrestrial) reproductive mode were also found: 1) a shift from mode 32 to 30 on some *Adenomera* species (nodes 3 and 4); and 2) a shift from mode 30 to 13 on species of the *Leptodactylus pentadactylus* group (nodes 13 and 20). Besides those, transformation direction in node 2 was undetermined because it had equal probability for reproductive modes 30 and 32 (node 2, see Figure 2). Thus, reproductive mode may have changed from 30 to 32 in node 3 or from 32 to 30 in node 10 (Figure 2).



Character state	Reproductive mode	Clutch size	Habitat	Tadpole environment	Nuptial spines	Egg pigmentation
0 ●	Mode 11	Less than 50	Open areas	Lotic water bodies	Absent	Absent
1 ○	Mode 13	Between 50 and 1,000	Forest formations	Lenthic water bodies	Present	Present
2 ●	Mode 30	More than 1,000	-	Terrestrial	-	-
3 ●	Mode 32	-	-	-	-	-

Figure 2. Ancestral states of the six life-history traits reconstructed for 35 Leptodactylinae species using stochastic inference. Pie charts represent the probability of each character state

g table order. A: *Adenomera* genus; B: *Leptodactylus fuscus* group; C: *L. pentadactylus* group; D: *L. melanonotus* group; and E: *L. latrans* group. For probability of each character state in each clade see Table S3.

While clutch size, tadpole environment, nuptial spines and egg pigmentations presented clear evolutionary pattern with few independent origins of states (Figure 2), multiple reversals between ópen areasô and óforest formationsô were recovered by the habitat reconstruction. The MRCA of *Adenomera* and *Lithodytes* (node 1) placed less than 50 eggs per clutch and lacked nuptial spines and melanin on eggs, while *Leptodactylus* MRCA (node 11) had big clutch sizes, tadpoles on lenthic water bodies, presence of nuptial spines and absence of egg pigmentation.

The mutational mapping analysis retrieved the estimated number of changes in the ancestral nodes together with the probable transformations along branches (Table 3). Habitat had the highest expected number of changes (approximately 32), being almost the same amount from one state to another. Reproductive mode had 20 changes, most of them between modes 32 to 30. Though, nuptial spine presented the lowest number of transformations (8 changes).

ransformation, amount of time and rate of transformation for each character of 35
chastic Bayesian mutational mapping in 600 trees (See characters codes in Table 1).

		Expected	Expected number of character state transformation											Amount of time				
		number of												State	State	State	State	
Character	**Replications**	**transformations**	**0-1**	**0-2**	**0-3**	**1-0**	**1-2**	**1-3**	**2-0**	**2-1**	**2-3**	**3-0**	**3-1**	**3-2**	**0**	**1**	**2**	**3**	**Rate**
Reproductive																			
mode	60000	19.87	2.53	2.6	1.1	1.04	1.3	0.5	1.9	1.4	1.8	1	0.6	4.1	0.30	0.16	0.32	0.22	8.16
Clutch size	60000	14.30	1.78	1.9	-	1.72	2.6	-	2.3	4	-	-	-	-	0.33	0.31	0.36	-	5.62
Habitat	60000	31.60	16.4	-	-	15.2	-	-	-	-	-	-	-	-	0.46	0.54	-	-	11.8
Tadpole																			
environment	60000	16.39	1.83	1	-	2.66	4.8	-	1.5	4.6	-	-	-	-	0.08	0.69	0.23	-	6.33
Nuptial spines	60000	7.94	2.91	-	-	5.03	-	-	-	-	-	-	-	-	0.58	0.42	-	-	2.64
Egg																			
pigmentation	60000	13.10	6.25	-	-	6.85	-	-	-	-	-	-	-	-	0.69	0.31	-	-	4.37

icant correlations only between reproductive mode and clutch size or nuptial spines (Table 4). However, correlations between specific reproductive mode and other character states were pointed by the *dij* statistics (Table 4). For example, although reproductive mode and egg pigmentation presented no significant correlation (D = 0.47, p > 0.05), egg pigmentation had positive association with reproductive modes 11 and 13 (respectively *dij* = 0.09 and 0.02, p Ò0.05), and negative association with mode 32 (*dij* = -0.05, p Ò0.05).

Table 4. Positive and negative correlations values obtained by the D and *dij* statistic for 35 Leptodactylinae species. Bolded values indicate p Ò0.05.

	Reproductive mode *D*		**Reproductive mode *dij***			
			Mode 11	**Mode 13**	**Mode 30**	**Mode 32**
Clutch size	**0.72**	Less than 50	**-0.07**	**-0.04**	-0.01	**0.12**
		Between 50 and 1,000	-0.03	-0.01	**0.08**	**-0.05**
		More than 1,000	**0.09**	0.04	**-0.07**	**-0.07**
Habitat	0.30	Open areas	-0.01	-0.02	0.06	-0.03
		Forest formations	0.01	0.02	-0.06	0.03
Tadpole environment	0.49	Lotic water bodies	**-0.02**	**-0.01**	0.02	-
		Lentic water bodies	0.07	**0.03**	0.01	-
		Terrestrial	-	-	-	-
Nuptial spines	**0.65**	Absent	**-0.10**	-0.06	0.08	**0.08**
		Present	**0.10**	0.06	-0.08	**-0.08**
Egg pigmentation	0.47	Absent	**-0.09**	**-0.02**	0.06	**0.05**
		Present	**0.09**	**0.02**	-0.06	**-0.05**

Our results showed that the evolution of reproductive modes in Leptodactylinae did not happened linearly as predicted by Heyer (1969) and not necessarily with intermediate stages, as suggested by McDiarmid (1978). Besides more than two shifts were retrieved from an aquatic to a more terrestrial reproduction in Leptodactylinae lineages, independent origins of less aquatic modes occurred in *Leptodactylus* and *Adenomera*, as suggested by Heyer (1969). To achieve this result, we derived a phylogenetic hypothesis with the highest number of nominal species of *Adenomera*, and confirmed that *Adenomera* and *Leptodactylus* are monophyletic as obtained elsewhere (Heyer 1974; De Sá et al. 2005; Frost et al. 2006; Ponssa 2008; Pyron and Wiens 2011; Fouquet et al. 2013). Distinct phylogenetic results in which

Adenomera and *Leptodactylus* are paraphyletic have also been found (Heyer 1998; Kokubum and Giaretta 2005; Giaretta et al. 2011). One author even suggests the *Spumoranuncula* clade to refer to some species of *Leptodactylus* and *Adenomera* (Giaretta et al. 2011), but this clade is paraphyletic according to our results. The greatest number of *Adenomera* species used in this study resulted in a different phylogenetic hypothesis (see Fouquet et al. 2013). While in our hypothesis *Adenomera lutzi* and *A. heyeri* comprehends the basal clade of the genus, in Fouquetôs results these species are considered derived.

The absence of a gradual increase of terrestriality with no mandatory intermediate stages was confirmed by transitions from mode 11 to modes 30 or 32, as shown for other terrestrial modes of reproduction (Gomez-Mestre et al. 2012). This shift may have happened two times: 1) from the *Leptodactylus* ancestral to the *L. fuscus* and *L. pentadactylus* groups; and 2) from the *Lithodytes* and *Adenomera* ancestral to the MRCA of this last genus. In this last case, even with the robustness of the phylogenetic position of *Lithodytes lineatus* (which was also shown by De Sá et al. 2005; Frost et al. 2006; Ponssa 2008; Pyron and Wiens 2011;

rtainty on its ancestral state due to its reproductive mode. The breeding site of *Lithodytes lineatus* is uncommon among Leptodactylinae species, and its association with the water inside ant nests doesnôt fit any of the reproductive modes assigned to the subfamily. Additionally, the absence of evolutionary linearity in reproductive modes is confirmed by reversions from terrestrial to aquatic breeding (e.g., from mode 30 to 13 or from 32 to 30, Figure 2) that are not uncommon among Anura (Gomez-Mestre et al. 2012). Also, the mutational mapping analysis showed that these transitions occurred not only in ancestral nodes, but also along the branches. The transitions from a more terrestrial breeding to a less one may also be noticed by summing up the expected number of transformations in this direction and comparing to transformations from aquatic to terrestrial breeding (see Table 3). Both presented nearly 10 transformations, showing that terrestrial egg-laying is not necessarily an evolutionary tendency, but actually an alternative strategy with no implied directionality.

The analysis of ancestral state reconstruction also showed evidence that clutch size is phylogenetically structured. The ancestor of *Leptodactylus* species presented more than 1,000 eggs per clutch, while *Adenomera* had less than 50 eggs as the most probable ancestral state (see Figure 2). Even though a reversion was recovered from mode 32 to 30 in *Adenomera*, the hypothetical ancestor holds the oviposition of few eggs. It is unlikely that the exotrophic *Adenomera*ôs tadpoles simply float to water bodies by chance (e.g., due to topography or great rain incidence) because they have functional mouthparts and spiracle (Heyer 1973; Almeida and Angulo 2006), while the endotrophic tadpoles donôt have these morphological traits.

A clear association pattern between clutch size and reproductive modes was found as predicted by Heyer (1969) and demonstrated for many anuran genera (Gomez-Mestre et al. 2012). The higher dependence of water, which is an unpredictable environment, together with

ich may allocate energy to increase the number of eggs per clutch. Opposing, species with low dependence of water, in this case egg-burrowing species, may allocate energy to parental care (Pianka 1970; Price 1974). The correlation analyses showed that *Leptodactylus* and *Adenomera* species presents both kinds of reproductive strategies, the first favors productivity, while the other favors parental care. The construction of the subterranean chambers by males of the *Leptodactylus fuscus* group and *Adenomera* species is considered a type of parental care, providing more suitable microhabitat for offspring development (McDiarmid 1978; Clutton-Brock 1991). When compared to water environments, subterranean chambers increase the chance of offspring survivorship by reducing predation, desiccation and interspecific competition (Heyer et al. 1975; McDiarmid 1978; Magnusson and Hero 1991). Egg-burrowing species have limitation on the number of eggs due to both, the amount of energy spent in the burrow construction, and the limited space inside the chamber (Crump 1996). Thus, space may be an important restrain, since terrestriality in anurans demands increased amounts of yolk to feed the endotrophic tadpole, which consequently increase egg dimensions (Heyer 1969; Salthe and Mecham 1974; Crump 1996). The amount of yolk needed in reproductive mode 32 is higher than in mode 30, because the tadpole completes the development using exclusively yolk as source of energy. Therefore, species with mode 30 only depends on the yolk for a brief period of tadpole development, which may lead to contrasting correlations of modes 32 and 30 and clutch sizes. An opposite relationship is noticed in more aquatic reproductive mode with tadpoles using external sources of energy since the beginning of the development, leading to a smaller egg dimension and larger clutch size and consequently to negative correlation with the oviposition of less than 50 eggs per clutch.

of phylogenetic constrain in Leptodactylinae habitat usage (open and forest formations) since this trait presented the highest number of expected transformations and no significant correlations with reproductive modes. The lack of correlations diverges from the hypothesis that the evolution of terrestrial breeding is linked to forest habitats due to the high humidity (Silva et al. 2012; Müller et al. 2013). However, air humidity may not limit the development of Leptodactylinae because the foam nest may protect eggs from desiccation.

Historical factors seem to have been more decisive for the evolution of tadpole environment in *Leptodactylus* than local factors, since all species shared the same state, lenthic environment. This tadpole environment appears in Leptodactylinae during almost 70% of the lineage history (see Table 3). Species with aquatic reproduction that place eggs in lenthic environments has an adaptive advantage when compared to those who uses lotic water bodies because lenthic waters facilitates the amplexus and provides a sheltered environment for eggs and tadpole first stage development. Consequently, reproductive modes 11 and 13, which are more related to aquatic breeding, are negatively correlated with lotic water bodies.

Our results showed similar evolutionary histories for nuptial spines and egg pigmentation. The presence or absence of these structures occurred together in almost the same species and ancestral nodes, with two major exceptions: the ancestral of *Leptodactylus* and *L. pentadactylus* group. Both lacked egg pigmentation but had nuptial spines, which help the male to anchor the female. The presence of nuptial spines in *Leptodactylus* ancestral was maintained in the ancestors of the groups *Leptodactylus latrans* and *L. melanonotus*, including *L. discodactylus*, but now in association with the presence of melanin on eggs. Although *Leptodactylus discodactylus* has not been assigned to any phenetic group yet, our results suggest that it belongs to the *L. melanonotus* group (see De Sá et al. 2005; Pyron and Wiens

al state of *Leptodactylus pentadactylus* group, which had nuptial spines but lacked melanin on eggs, corroborates with Heyerôs (1969) hypothesis that the presence of spines in this group have evolved must likely due to the large adult body size than to the water dependence. Whereas this species do not place eggs directly on the water body, the spines are used to facilitate the amplexus between large specimens, and not to assist aquatic amplexus in water bodies. The lack of egg pigmentation in the ancestor of this group, which is only needed in eggs exposed to ultraviolet lights, is another support to Heyerôs hypothesis. Nevertheless, we found positive correlation between mode 13, which is common among the *Leptodactylus pentadactylus* group and some species of the *Leptodactylus melanonotus* group, and egg pigmentation most likely due to the plasticity in constructed basins places (Prado et al. 2002), which in some cases can be exposed to ultraviolet light. The correlation analysis also confirmed the association between the presence of spines and egg pigmentation with a more aquatic reproductive mode (mode 11) and a negative correlation with terrestrial breeding (mode 32).

In conclusion, our results showed no evolutionary tendency toward terrestriality in Leptodactylinae. Indeed, we found reversals from terrestrial to aquatic tadpole development and no evidences of mandatories intermediate stages. Besides, we also found correlations between morphological and ecological trait driven by water dependence. Aquatic reproductive modes are associated with higher clutch sizes, lenthic waters, and presence of nuptial spines and egg pigmentation. No correlation was found between reproductive modes and habitat usage, which may not be constrained by the phylogenetic relationships. The robustness of the phylogenetic hypothesis, which confirmed *Adenomera* and *Leptodactylus* monophyletism with *Lithodytes* as sister taxon of *Adenomera*, enabled the study of reproductive traits

ly reinforces the usefulness of Bayesian stochastic character mapping to better understand the evolution of life history traits.

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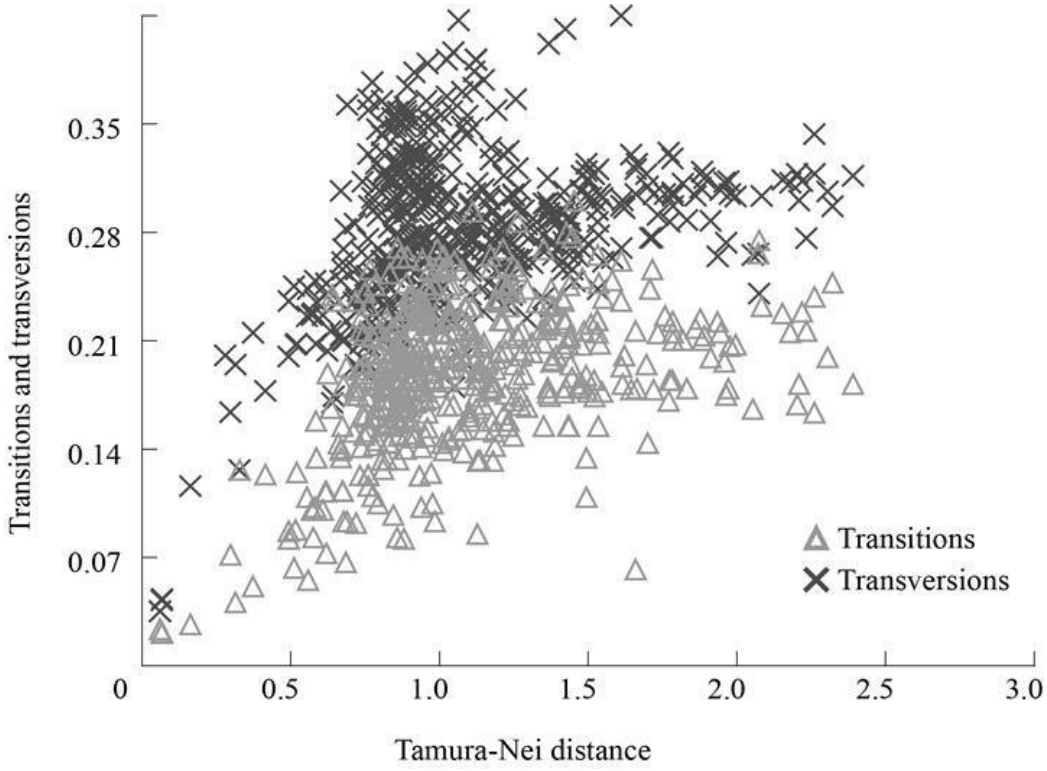

Figure S1. Saturation plot of the third codon positions of Cytochrome B fragment for 35 Leptodactylinae species. Transitions (indicated by triangles) and transversions (indicated by X) are plotted against the Tamura-Nei (1993) distance.

and GenBank accession numbers.

Species	Locality		GenBank accession numbers			
	(Country: State: Municipality)	Acronym and number	16S	12S	Cytochrome B	Rhodopsin 1
Adenomera andreae	Brazil: Amazonas: Manaus	AAG-UFU 4283	KC477261	KC470112	KF548069	KF613599
Adenomera araucaria	Brazil: Santa Catarina: Lebón Régis	MCP 9673	KC477241	KC470091	KF548070	KF613578
Adenomera bokermanni	-	CHUNB 46021	KC477243	KC470107	KF548072	KF613580
Adenomera diptyx	Brazil: Mato Grosso do Sul: Corumbá	LHUFCG 0173	KC477249	KC470099	KF548078	KF613587
Adenomera engelsi	-	GenBank	**KC603940**	**KC603940**	**KC603970**	**KC604100**
Adenomera heyeri	-	GenBank	**KC603948**	**KC603947**	**KC603972**	**KC604096**
Adenomera hylaedactyla	Brazil: Amazonas: Manaus	AAG-UFU 4736	KC477260	KC470111	KF548068	KF613598
Adenomera lutzi	Guyana	IRSNB 13951	KC477251	KC470100	KF548079	KF613588
Adenomera marmorata	Brazil: São Paulo: Caraguatatuba	LHUFCG 0165	KC477242	KC470092	KF548071	KF613579
Adenomera martinezi	Brazil: Distrito Federal: Brasília	AAG-UFU 4238	KC477244	KC470090	KF548065	KF613577
Adenomera thomei	-	GenBank	**KC603946**	**KC603945**	**KC603971**	**KC604101**
Eupemphix nattereri	-	GenBank	**AY326020**	**AY326020**	-	-

	Locality (Country: State: Municipality)	Acronym and number	GenBank accession numbers			
			16S	12S	Cytochrome B	Rhodopsin 1
Leptodactylus albilabris	-	GenBank	**EF091413**	**EF091410**	**EF091393**	-
Leptodactylus chaquensis	-	GenBank	**EF632055**	**AY943221**	-	-
Leptodactylus discodactylus	-	GenBank	**DQ283433**	**AY943226**	-	**DQ284033**
Leptodactylus elenae	Brazil: Mato Grosso do Sul: Corumbá	AAG-UFU 4211	KC477248	KC470098	KF548077	KF613586
Leptodactylus fallax	-	GenBank	**EF091415**	**EF091412**	**EF091407**	-
Leptodactylus furnarius	Brazil: Minas Gerais: Paracatu	CHUNB 25860	-	KC470108	KF548085	KF613595
Leptodactylus fuscus	Brazil: Minas Gerais: Buritizeiro	LHUFCG 0491	KC477246	KC470095	KF548074	KF613583
Leptodactylus jolyi	Brazil: Distrito Federal: Brasília	AAG-UFU 3124	KC477250	KC470093	KF548066	KF613581
Leptodactylus knudseni	-	GenBank	**EF632056**	**EF613180**	**EF091409**	-
Leptodactylus latrans	-	GenBank	-	**AY143353**	**AY843934**	**AY844681**
Leptodactylus labyrinthicus	-	GenBank	**AY947860**	**AY947874**	-	-
Leptodactylus leptodactyloides	Brazil: Acre: Rio Branco	AAG-UFU 4199	KC477247	KC470096	KF548075	KF613584

	Locality		GenBank accession numbers			
	(Country: State: Municipality)	Acronym and number	16S	12S	Cytochrome B	Rhodopsin 1
Leptodactylus macrosternum	Brazil: Tocantins: Vale do Paranã	AAG-UFU 2679	KC477255	KC470106	KF548084	KF613594
Leptodactylus marambaiae	Brazil: Rio de Janeiro: Ilha de Marambaia	AAG-UFU 4193	-	KC470097	KF548076	KF613585
Leptodactylus melanonotus	-	GenBank	**DQ347060**	**AY943224**	-	**AY364405**
Leptodactylus mystaceus	Brazil: Acre: Rio Branco	AAG-UFU 4197	KC477252	KC470101	KF548080	KF613589
Leptodactylus mystacinus	Brazil: Distrito Federal: Brasília	-	KC477256	KC470105	KF548067	KF613593
Leptodactylus notoaktites	Brazil: Santa Catarina: Itapema	AAG-UFU 3129	KC477254	KC470104	KF548083	KF613592
Leptodactylus petersii	Brazil: Tocantins: Caseara	CHUNB45794	-	KC470109	KF548086	KF613596
Leptodactylus podicipinus	Brazil: Mato Grosso do Sul: Corumbá	LHUFCG 0244	KC477245	KC470094	KF548073	KF613582
Leptodactylus pustulatus	Brazil: Tocantins: Palmas	CHUNB11258	-	KC470110	KF548087	KF613597
Leptodactylus rhodomystax	Brazil: Acre: Rio Branco	AAG-UFU 4196	**AY947855**	KC470103	KF548082	KF613591
Leptodactylus rhodonotus	-	GenBank	**EU368908**	**AM039727**	**EU368908**	-

	Locality		GenBank accession numbers			
	(Country: State: Municipality)	Acronym and number	16S	12S	Cytochrome B	Rhodopsin 1
Lithodytes lineatus	Brazil: Acre: Rio Branco	AAG-UFU 4198	KC477253	KC470102	KF548081	KF613590
Physalaemus cuvieri	-	GenBank	JQ627212	AY819347	AY843975	AY844717

Museum abbreviations: Coleção Herpetológica da Universidade de Brasília (CHUNB), Coleção Ariovaldo A. Giaretta da Universidade Federal de Uberlândia (AAG-UFU), Institut Royal des Sciences Naturelles de Belgique (IRSNB), Laboratório de Herpetologia da Universidade Federal de Campina Grande (LHUFCG), and Museu de Ciências e Tecnologia da Pontifícia Universidade Católica de Porto Alegre (MCP).

agments.

r each of the amplified fragments

		ytB	*Rhod*
Deionized water	7.7 µL	8.5 µL	7.5 µL
DNA	2.0 µL	2.0 µL	3.0 µL
Forward primer (2 mM)	3.0 µL	3.0 µL	3.0 µL
Reverse primer (2mM)	3.0 µL	3.0 µL	3.0 µL
Buffer 1X*	2.0 µL	2.0 µL	2.0 µL
DNTPs (2,5 mM)	2.0 µL	1.2 µL	1.2 µL
Taq polymerase (5u/µL)	0.3 µL	0.3 µL	0.3 µL
Total volume	20 µL	20 µL	20 µL

*Buffer 1X (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2)

B. PCR thermal program for each of the amplified fragments

Fragment	Initial heating	Denaturation	Annealing	Extension	Final extension
			34 cycles		
16S	2 min at 94ºC	60s at 94ºC	60s at 54ºC - 58ºC	90s at 72ºC	10 min at 72ºC
			35 cycles		
12S, *cytB* and *Rhod*	2 min at 94ºC	60s at 94ºC	60s at 54ºC - 58ºC	60s at 72ºC	6 min at 72ºC

story traits for 35 Leptodactylinae species. Characters coded as in Table 1.

Species	Reproductive mode		Clutch size		Habitat		Tadpole environment		Nuptial pads or spines		Egg pigmentation	
Adenomera andreae	3	Rodríguez and Duellman 1994	0	Rodríguez and Duellman 1994	1	Rodríguez and Duellman 1994	2	Hero 1990	0	Heyer 1974	0	Rodríguez and Duellman 1994
Adenomera araucaria	3	Kwet and Angulo 2002	?	-	1	Kwet and Angulo 2002	2	Kwet and Angulo 2002	0	Kwet and Angulo 2002	0	Personal observation
Adenomera bokermanni	2	Haddad and Prado 2005	0	Heyer 1973	0	Heyer 1973	?	-	0	Heyer 1974	0	Heyer 1974
Adenomera diptyx	2	De La Riva 1995	0/1	De La Riva 1995	0	De La Riva et al. 2000	0	De La Riva et al. 2000	?	-	0	Personal observation
Adenomera engelsi	3	Kwet et al. 2009	?	-	1	Kwet et al. 2009	?	-	0	Kwet et al. 2009	?	-
Adenomera heyeri	?	-	?	-	1	Boistel et al. 2006	?	-	0	Boistel et al. 2006	0	Personal observation
Adenomera hylaedactyla	3	Rodríguez and Duellman 1994	0	Rodríguez and Duellman 1994	0	Menin et al. 2009	2	Heyer and Silverstone 1969	0	Heyer 1974	0	Rodríguez and Duellman 1994
Adenomera lutzi	?	-	0	Kok et al. 2007	1	Kok et al. 2007	?	-	0	Kok et al. 2007	0	Personal observation
Adenomera marmorata	3	Heyer 1974	0	Heyer et al. 1990	0/1	Heyer et al. 1990	2	Heyer et al. 1990	0	Heyer 1998	0	Heyer 1998

			lutch size		Habitat		Tadpole environment		Nuptial pads or spines		Egg pigmentation
Adenomera martinezi	2	Personal observation	0	Heyer 1974	0	Personal observation	0	Personal observation	0	Heyer 1974	0	Heyer 1974
Adenomera thomei	2	Almeida and Angulo 2006	0	Almeida and Angulo 2006	1	Almeida and Angulo 2006	?	-	0	Almeida and Angulo 2006	0	Almeida and Angulo 2006
Leptodactylus albilabris	2	Prado et al. 2002	?	-	?	-	?	-	0	Maxson and Heyer 1988	0	Dent 1956
Leptodactylus chaquensis	0	Haddad and Prado 2005	2	Heyer 1974	0	De La Riva et al. 2000	1	Heyer 1998	1	Heyer 1998	1	Heyer 1998
Leptodactylus discodactylus	?	-	1/2	Heyer and Bellin 1973	1	Rodríguez and Duellman 1994	1	Heyer 1998	0	Heyer 1970	0	Heyer and Bellin 1973
Leptodactylus elenae	2	Prado and D'Heursel 2006	?	-	0	De La Riva et al. 2000	1	Prado and d'Heursel 2006	0	Maxson and Heyer 1988	?	-
Leptodactylus fallax	?	-	2	Daltry 2002	1	Daltry 2002	2	Gibson and Buley 2004	1	Maxson and Heyer 1988	?	-
Leptodactylus furnarius	2	Sazima and Bokermann 1978	0/1	Giaretta and Kokubum 2004	0	Giaretta and Kokubum 2004	1	McDiarmid and Altig 1999	0	Heyer and Heyer 2004	0	Giaretta and Kokubum 2004
Leptodactylus fuscus	2	Haddad and Prado 2005	1	Lucas et al. 2008	0	de-Carvalho et al. 2008	1	de-Carvalho et al. 2008	0	Heyer 1998	0	Heyer 1998
Leptodactylus jolyi	2	Sazima and Bokermann 1978	?	-	?	-	0/1	Sazima and Bokermann 1978	0	Maxson and Heyer 1988	?	-

	lutch size		Habitat		Tadpole environment		Nuptial pads or spines		Egg pigmentation	
Leptodactylus knudseni	1	Rodríguez and Duellman 1994	1	Lima et al. 2006	1	Rodríguez and Duellman 1994	1	De Sá et al. 2007a	1	Rodríguez and Duellman 1994	0	Lima, AP
Leptodactylus latrans	0	Haddad and Prado 2005	2	Kwet and di-Bernardo 1999	0	De La Riva et al. 2000	1	Prado et al. 2002	1	Heyer et al. 1990	1	Kwet and di-Bernardo 1999
Leptodactylus labyrinthicus	1	Agostinho 1994	2	Zina and Haddad 2005	0	Giaretta et al. 2008	1	Eterovick and Sazima 2000	1	Eterovick and Sazima 2000	1	Zina and Haddad 2005
Leptodactylus leptodactyloides	0	Prado et al. 2002	1/2	Duellman 2005	0/1	De La Riva et al. 2000	1	Heyer 1998	1	Heyer 1974	1	Heyer 1998
Leptodactylus macrosternum	0	Haddad and Prado 2005	2	Uetanabaro et al. 2008	0	De La Riva et al. 2000	1	Prado et al. 2002	1	Maxson and Heyer 1988	1	Lima, AP
Leptodactylus marambaiae	?	-	?	-	?	-	?	-	0	Maxson and Heyer 1988	?	-
Leptodactylus melanonotus	0	Heyer 1974	2	Heyer 1974	?	-	1	Heyer 1998	1	Heyer 1998	1	Heyer 1998
Leptodactylus mystaceus	2	Heyer 1974	1	Rodríguez and Duellman 1994	0/1	Giaretta et al. 2008	1	Menin, M	0	Rodríguez and Duellman 1994	0	Heyer 1974
Leptodactylus mystacinus	2	Prado et al. 2002	1	Filho and Giaretta 2008	0	de-Carvalho et al. 2008	1	de-Carvalho et al. 2008	0	Maxson and Heyer 1988	0	Filho and Giaretta 2008
Leptodactylus notoaktites	2	Haddad and Prado 2005	?	-	?	-	0	De Sá et al. 2007a	0	Maxson and Heyer 1988	?	-

	lutch size			Habitat		Tadpole environment		Nuptial pads or spines		Egg pigmentation	
Leptodactylus petersii	1	Lima et al. 2006	2	Rodrigues, DJ	1	De La Riva et al. 2000	1	Prado et al. 2002	1	Maxson and Heyer 1988	?	-
Leptodactylus podicipinus	1	Prado et al. 2002	2	Prado et al. 2002	0	De La Riva et al. 2000	1	Prado et al. 2002	1	Maxson and Heyer 1988	1	Prado et al. 2002
Leptodactylus pustulatus	0	Prado et al. 2002	?	-	1	De Sá et al. 2007b	1	De Sá et al. 2007b	1	Maxson and Heyer 1988	1	Fenolio et al. 2006
Leptodactylus rhodomystax	1	Rodríguez and Duellman 1994	1	Rodríguez and Duellman 1994	1	De La Riva et al. 2000	1	Eterovick and Sazima 2000	1	Rodríguez and Duellman 1994	0	Lima, AP
Leptodactylus rhodonotus	?	-	?	-	1	De La Riva et al. 2000	1	Eterovick and Sazima 2000	1	Rodríguez and Duellman 1994	?	-
Lithodytes lineatus	?	-	1	Bernarde and Kokubum 2009	1	De La Riva et al. 2000	1	Heyer 1998	0	Heyer 1974	0	Rodríguez and Duellman 1994

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robabilities of each clade of the Bayesian

species. Clade numbers are indicated in Figure 1. Bolded values specify the highest probabilities.

Character	Clade	Pr(0)	Pr(1)	Pr(2)	Pr(3)
Reproductive mode	1	**0.582**	0.011	0.297	0.110
Reproductive mode	2	0.026	0.006	0.478	**0.490**
Reproductive mode	3	0.000	0.000	0.123	**0.876**
Reproductive mode	4	0.000	0.000	**0.650**	0.349
Reproductive mode	5	0.000	0.000	**0.993**	0.007
Reproductive mode	6	0.001	0.000	**0.718**	0.281
Reproductive mode	7	0.000	0.000	0.018	**0.982**
Reproductive mode	8	0.000	0.000	0.000	**1.000**
Reproductive mode	9	0.001	0.000	0.142	**0.856**
Reproductive mode	10	0.041	0.015	**0.649**	0.296
Reproductive mode	11	**0.675**	0.022	0.289	0.015
Reproductive mode	12	0.068	0.055	**0.859**	0.018
Reproductive mode	13	0.001	0.019	**0.975**	0.006
Reproductive mode	14	0.000	0.000	**1.000**	0.000
Reproductive mode	15	0.000	0.000	**1.000**	0.000
Reproductive mode	16	0.000	0.000	**1.000**	0.000
Reproductive mode	17	0.000	0.000	**1.000**	0.000
Reproductive mode	18	0.000	0.000	**1.000**	0.000
Reproductive mode	19	0.000	0.000	**1.000**	0.000
Reproductive mode	20	0.005	**0.672**	0.142	0.181
Reproductive mode	21	0.000	**0.998**	0.001	0.001
Reproductive mode	22	**0.998**	0.001	0.001	0.000
Reproductive mode	23	**1.000**	0.000	0.000	0.000
Reproductive mode	24	**1.000**	0.000	0.000	0.000
Reproductive mode	25	**0.998**	0.002	0.001	0.000
Reproductive mode	26	**0.661**	0.302	0.036	0.002
Reproductive mode	27	0.000	**0.999**	0.000	0.000
Reproductive mode	28	**0.900**	0.078	0.021	0.002
Reproductive mode	29	**0.508**	0.051	0.347	0.094

ade	Pr(0)	Pr(1)	Pr(2)	Pr(3)
	0.742	0.170	0.088	-
2	**0.999**	0.001	0.000	-
3	**1.000**	0.000	0.000	-
4	**1.000**	0.000	0.000	-
5	**1.000**	0.000	0.000	-
6	**1.000**	0.000	0.000	-
7	**1.000**	0.000	0.000	-
8	**0.994**	0.002	0.004	-
9	**0.994**	0.002	0.004	-
10	**0.997**	0.001	0.002	-
11	0.007	0.063	**0.931**	-
12	0.010	0.389	**0.601**	-
13	0.002	**0.960**	0.038	-
14	0.001	**0.997**	0.002	-
15	0.002	**0.994**	0.003	-
16	0.012	**0.970**	0.018	-
17	0.000	**1.000**	0.000	-
18	0.017	**0.957**	0.026	-
19	0.320	0.202	**0.478**	-
20	0.007	0.271	**0.722**	-
21	0.006	0.372	**0.623**	-
22	0.000	0.001	**0.999**	-
23	0.000	0.000	**1.000**	-
24	0.000	0.000	**1.000**	-
25	0.000	0.001	**0.999**	-
26	0.002	0.002	**0.997**	-
27	0.000	0.000	**1.000**	-
28	0.040	0.241	**0.719**	-
29	0.306	0.280	**0.414**	-
1	0.091	**0.909**	-	-
2	0.021	**0.979**	-	-
3	0.058	**0.942**	-	-

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	ade	Pr(0)	Pr(1)	Pr(2)	Pr(3)
	4	**0.633**	0.367	-	-
Habitat	5	**0.998**	0.002	-	-
Habitat	6	0.047	**0.953**	-	-
Habitat	7	0.014	**0.986**	-	-
Habitat	8	0.024	**0.976**	-	-
Habitat	9	0.221	**0.779**	-	-
Habitat	10	0.007	**0.993**	-	-
Habitat	11	0.435	**0.565**	-	-
Habitat	12	0.287	**0.713**	-	-
Habitat	13	**0.569**	0.431	-	-
Habitat	14	**0.997**	0.003	-	-
Habitat	15	**0.970**	0.030	-	-
Habitat	16	0.211	**0.789**	-	-
Habitat	17	**0.998**	0.002	-	-
Habitat	18	**0.997**	0.003	-	-
Habitat	19	**0.996**	0.004	-	-
Habitat	20	0.067	**0.933**	-	-
Habitat	21	0.056	**0.944**	-	-
Habitat	22	**0.738**	0.262	-	-
Habitat	23	**0.993**	0.007	-	-
Habitat	24	**1.000**	0.000	-	-
Habitat	25	0.141	**0.859**	-	-
Habitat	26	0.024	**0.976**	-	-
Habitat	27	0.363	**0.637**	-	-
Habitat	28	0.073	**0.927**	-	-
Habitat	29	0.290	**0.710**	-	-
Tadpole environment	1	0.002	**0.962**	0.036	-
Tadpole environment	2	0.019	0.489	**0.492**	-
Tadpole environment	3	0.015	0.025	**0.960**	-
Tadpole environment	4	0.076	0.027	**0.897**	-
Tadpole environment	5	**0.853**	0.091	0.056	-
Tadpole environment	6	0.011	0.146	**0.843**	-

	ade	Pr(0)	Pr(1)	Pr(2)	Pr(3)
	7	0.000	0.044	**0.956**	-
Tadpole environment	8	0.000	0.005	**0.995**	-
Tadpole environment	9	0.003	**0.681**	0.316	-
Tadpole environment	10	0.015	**0.782**	0.204	-
Tadpole environment	11	0.000	**1.000**	0.000	-
Tadpole environment	12	0.000	**1.000**	0.000	-
Tadpole environment	13	0.000	**0.998**	0.002	-
Tadpole environment	14	0.000	**1.000**	0.000	-
Tadpole environment	15	0.000	**1.000**	0.000	-
Tadpole environment	16	0.017	**0.982**	0.001	-
Tadpole environment	17	0.000	**1.000**	0.000	-
Tadpole environment	18	0.000	**1.000**	0.000	-
Tadpole environment	19	0.000	**1.000**	0.000	-
Tadpole environment	20	0.000	**0.977**	0.022	-
Tadpole environment	21	0.000	**0.999**	0.001	-
Tadpole environment	22	0.000	**1.000**	0.000	-
Tadpole environment	23	0.000	**1.000**	0.000	-
Tadpole environment	24	0.000	**1.000**	0.000	-
Tadpole environment	25	0.000	**1.000**	0.000	-
Tadpole environment	26	0.000	**1.000**	0.000	-
Tadpole environment	27	0.000	**1.000**	0.000	-
Tadpole environment	28	0.000	**1.000**	0.000	-
Tadpole environment	29	0.007	**0.956**	0.037	-
Nuptial spines	1	**0.964**	0.036	-	-
Nuptial spines	2	**1.000**	0.000	-	-
Nuptial spines	3	**1.000**	0.000	-	-
Nuptial spines	4	**1.000**	0.000	-	-
Nuptial spines	5	**0.999**	0.001	-	-
Nuptial spines	6	**1.000**	0.000	-	-
Nuptial spines	7	**1.000**	0.000	-	-
Nuptial spines	8	**0.999**	0.001	-	-
Nuptial spines	9	**0.999**	0.001	-	-

ade	Pr(0)	Pr(1)	Pr(2)	Pr(3)
0	**0.999**	0.001	-	-
Nuptial spines 11	0.012	**0.988**	-	-
Nuptial spines 12	0.015	**0.985**	-	-
Nuptial spines 13	0.395	**0.605**	-	-
Nuptial spines 14	**1.000**	0.000	-	-
Nuptial spines 15	**1.000**	0.000	-	-
Nuptial spines 16	**0.999**	0.001	-	-
Nuptial spines 17	**0.999**	0.001	-	-
Nuptial spines 18	**1.000**	0.000	-	-
Nuptial spines 19	**1.000**	0.000	-	-
Nuptial spines 20	0.011	**0.989**	-	-
Nuptial spines 21	0.001	**0.999**	-	-
Nuptial spines 22	0.000	**1.000**	-	-
Nuptial spines 23	0.001	**0.999**	-	-
Nuptial spines 24	0.000	**1.000**	-	-
Nuptial spines 25	0.000	**1.000**	-	-
Nuptial spines 26	0.005	**0.995**	-	-
Nuptial spines 27	0.001	**0.999**	-	-
Nuptial spines 28	0.004	**0.996**	-	-
Nuptial spines 29	**0.557**	0.443	-	-
Egg pigmentation 1	**0.986**	0.014	-	-
Egg pigmentation 2	**1.000**	0.000	-	-
Egg pigmentation 3	**1.000**	0.000	-	-
Egg pigmentation 4	**1.000**	0.000	-	-
Egg pigmentation 5	**1.000**	0.000	-	-
Egg pigmentation 6	**1.000**	0.000	-	-
Egg pigmentation 7	**1.000**	0.000	-	-
Egg pigmentation 8	**0.999**	0.001	-	-
Egg pigmentation 9	**0.992**	0.008	-	-
Egg pigmentation 10	**0.999**	0.001	-	-
Egg pigmentation 11	**0.598**	0.402	-	-
Egg pigmentation 12	**0.865**	0.135	-	-

	ıde	Pr(0)	Pr(1)	Pr(2)	Pr(3)
	3	**0.995**	0.005	-	-
Egg pigmentation	14	**1.000**	0.000	-	-
Egg pigmentation	15	**0.996**	0.004	-	-
Egg pigmentation	16	**0.967**	0.033	-	-
Egg pigmentation	17	**0.999**	0.001	-	-
Egg pigmentation	18	**1.000**	0.000	-	-
Egg pigmentation	19	**0.998**	0.002	-	-
Egg pigmentation	20	**0.917**	0.083	-	-
Egg pigmentation	21	**0.840**	0.160	-	-
Egg pigmentation	22	0.006	**0.994**	-	-
Egg pigmentation	23	0.001	**0.999**	-	-
Egg pigmentation	24	0.000	**1.000**	-	-
Egg pigmentation	25	0.002	**0.998**	-	-
Egg pigmentation	26	0.016	**0.984**	-	-
Egg pigmentation	27	0.014	**0.986**	-	-
Egg pigmentation	28	0.088	**0.912**	-	-
Egg pigmentation	29	**0.881**	0.119	-	-

CAPÍTULO II

NINHOS DE ESPUMA INFLUENCIARAM AS TAXAS DE DIVERSIFICAÇÃO EM LINHAGENS DE ANUROS? UM ESTUDO COMPARATIVO EM QUATRO CLADOS INDEPENDENTES

DOES ANURAN FOAM NEST INFLUENCED LINEAGE DIVERSIFICATION RATES? A COMPARATIVE STUDY IN FOUR INDEPENDENT CLADES

ity influenced lineage diversification rates? A comparative study in two independent clades

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Among all terrestrial vertebrates, anurans are the ones with greater variation in reproductive features. This conspicuous attribute includes fossorial, terrestrial, aquatic and arboreal modes, which may have eggs and tadpoles placed in foam nests. The foam is known to have many functions, such as prevent desiccation, protect from predators and microbes, and control oxygen supply. Knowing that a specific clade trait might influence rate and mode of lineage diversification, we hypothesized that foam-nesting lineages have higher diversification rates when compared to non-foaming. We tested this trait effect in speciation and extinction rates of four independent anuran clades using BiSSE and comparing models by AIC. To account for missing taxa in the phylogeny we used both existing methods: skeleton and terminally unsolved topology. The results of the second method were by far better than the first for all our datasets. To every lineage, the model in which speciation and extinction rates do not vary among foaming and non-foaming was among the best ones. For groups which we were not able to select only one model, the majority of other models pointed to higher diversification of foaming lineages caused by higher rates of speciation and/or lower rates of extinction.

1. Introduction

Evolutionary and ecological aspects of organismal life history traits have long interested biologists. The idea that some traits constitutes key innovations that allowed the invasion of a new ñadaptative zonesò [1] have persisted for decades. Currently, it was expanded to the idea that these traits also tends to promote diversification [2ï 4]. Even though the concept of key innovation have being applied in distinct contexts (reviewed

at some organismal attributes have played a major

Even though changes in diversification rate may result from other factors (e.g. ecological adaptation, sexual selection, and non-adaptative factors such as genetic drift), many studies have identified possible key innovations by demonstrating the correlation between speciation and/or extinction rate and life history traits (e.g. [6ï 8]). How a test for key innovation is conducted depends on trait evolutionary history. While homologous traits can be evaluated in one particular clade, homoplastic traits resulting from convergence or parallel evolution in distantly related clades need to be assessed in every lineage it evolved, which makes it possible to detect a general pattern (e.g. [6,9ï 11]).

Until now only a few key innovations have been hypothesized and evaluated in anurans [12ï 14]. Even though one of anurans most striking attribute is reproduction, presenting at least 39 reproductive modes that summarizes these attributes (reviewed by [15]), the only study to test if some of them influenced lineage diversification rates found no strong correlation [16]. Among these reproductive modes, ten are known to comprise foam nests, either in aquatic, terrestrial or arboreal habitats. Ecological aspects of anurans foam nests have received much attention in the past decades, but most of the studies were conducted in few species. Yet, it yielded a central concept that foam have many functions: (i) avoids aquatic predators [17], (ii) prevents desiccation [18], (iii) controls oxygen supply [19], (iv) provides adequate temperatures for egg and tadpole development [18], (v) works as food source for larvae [20], and (vi) defends eggs from microbial colonization [21].

Anurans foam is an interesting attribute not only by its many functions, but also for being an exceptional example of convergence. It originated independently in distantly related clades at least six times [22]. Within each lineage entire clades, genera

is ability: Hylidae (*Scinax rizibilis*), Hyperoliidae (*Opisthothylax immaculatus*), Leptodactylidae, Limnodynastidae, Microhylidae, and Rhacophoridae. Besides foam, structures that resembles it (e.g. aquatic bubble and jelly) are known to exist in few species of these lineages (*Chiasmocleis leucosticta* [23], *Feihyla vittata* and *Feihyla hansenae* [24], *Philoria loveridgei* [25], and some *Pleurodema* [22]).

Until now, evolutionary studies of foaming ability are mainly restricted to identify ancestral states with the purpose to detect multiple origins and/or reversals [22,26,27]. Previously, Heyer [28] and Martin [29] stressed the possibility that egg deposition in foam may have played a major role in allowing some Leptodactylidae and Myobatrachidae species to penetrate open areas of South America and Australia. It may have not only allowed these lineages to explore new ñadaptative zonesò and previously unavailable ecological resources, but also exposed them to distinct selection pressures, which may have influenced their diversification rates [30].

Here we tested evolutionary aspects of anuran foam nesting ability by testing if it constitutes a key innovation. Specifically, we target the following two questions: (i) does any of the studied groups experienced shifts in diversification rates? and (ii) does foam and non-foaming lineages have distinct rates of speciation and/or extinction? In order to compare results and search for general patterns, analyses were performed in all four groups of frogs from which more than one species generates foam.

2. Materials and methods

(a) ***Datasets***

We assembled from literature a list of anurans that produce foam nests (Supporting Information Table S1). Among these species we identified four independent groups in which the foam nest ability is present in more than one species: Leptodactylidae,

nylidae), and Rhacophoridae. All phylogenies used here were pruned from amphibian most comprehensive dated phylogeny ([31]). Taxonomic data that enabled us to know how many species were missing from the phylogeny (Supporting Information Table S2) were obtained in Amphibian Species of the World version 6.0 database [32].

Considering that foam nest ability may have originated only once in all these lineages (or twice in Leptodactylidae [30]) and that only few reversals are known, we included sister clades in the analysis to account for species without foaming ability to avoid high tip rate bias due to its influence in decreasing analysis power [33].

Leptodactylidae

We conducted the analysis with two sets of species. The first, consisted only of leptodactylid species, while the second also included the Leptodactylidae sister clade: Centrolenidae and Allophrynidae. To increase Leptodactylidae sampling we included position information of *Crossodactyloides*, *Rupirana* and *Hydrolaetare* which were missing from the original phylogeny [30].

In general, Leptodactylidae species are easy to classify as presenting or not foaming ability, with the exception of *Pleurodema*. This genus, besides encompassing foaming and non-foaming species, also include many species with structures that resembles foam [22]. In this case, these species were considered as lacking foam nest in the analysis of trait influence on diversification.

Limnodynastidae

As in Leptodactylidae, we used two species sets: one with only Limnodynastidae, and the other with Limnodynastidae e Myobatrachidae. *Philoria loveridgei*, a species that places eggs in jelly masses was considered as lacking foam ability [25].

Stumpffia (Cophylinae: Microhylidae)

ae only one genus reproduces using foam nest, the phylogenetic hypothesis used here covered the sub-family Cophylinae and its sister clade Hoplopryninae.

Rhacophorinae

Among Rhacophoridae, four of the 14 genus of the sub-family Rhacophorinae exhibit foaming ability. We considered that use a phylogenetic hypothesis for the sub-family would be enough to detect the influence of foam nest in this lineage evolution. Rhacophorinae present variation within the foam nesting clade, with *Feihyla vittata* and *Feihyla hansenae* (former *Chiromantis*) laying their eggs in a jelly with bubbles [24,34]. We considered these species as lacking foam ability, since this jelly is a specialized method distinct from traditional foam nesting [26].

(b) ***Evolutionary analyses***

We evaluated if any lineage in the present study has undergone a shift in diversification rates independently of the presence or absence of foam nest using MEDUSA (Modelling Evolutionary Diversification Under Stepwise AIC, [35]) in R package (http://www.r-project.org/) Geiger version 1.99-3.1. Taking into account phylogenetic incompleteness, MEDUSA estimates changes in net diversification rates by calculating the likelihood of alternative birth-death models after considering branch lengths and number of species. Each alternative model presents distinct breakpoints in the diversification rate (parameters), and by using AIC we identified the model with greater balance between likelihood and breakpoints in diversification rates.

To determine whether foaming ability affected diversification rates we used the Binary State Speciation Extinction (BiSSE [36]) in the R package Diversitree version 0.9-6 [37]. By using likelihood methods it estimates rates of speciation (ə), extinction (μ), and transition (q) associated with the absence (0) or presence (1) of the foam. To

nest influenced speciation or extinction rates we compared the maximum likelihood value obtained in unconstrained model - all six parameters were allowed to vary - against constrained models. The constrained models used were: equal speciation rates (ə0 = ə1), equal extinction rates (µ0 = µ1), and equal speciation and extinction rates (ə0 = ə1 and µ0 = µ1).

To account for unsampled species in the phylogenies used both available approaches, skeletal tree and terminally unsolved clades [38]. In the first, we specified the proportion of foaming and non-foaming species that were not included in the phylogeny (Supporting Information Table S3). In the alternative method, terminally unsolved trees, we used the highest amount of positioning information we had about missing species by indicating which clade it belongs according to many phylogenetic hypotheses proposals. However, since we lacked information at the tips we had to constrain terminal clades. Therefore, most of them were constrained to entire genus and we indicated the number of species with and without foaming ability in each of these terminally unsolved clades (Table S2).

Akaike criterion was used to identify the best fit model of BiSSE analysis. Models were compared by measuring the difference between each model and the one with lower AIC value (ɑAIC). This was performed for each model from the same phylogeny, including both ways to account for missing species. Models with ɑAIC<2 were considered equally plausible to explain the observed pattern. We also calculated a weighted Akaike (wAIC), representing the relative contribution of a model to explain the data given all other models. All AIC analysis were performed in the R package ñqpcRò [39].

Shifts in diversification rates were detected by MEDUSA in some lineages of the following anuran families sampled: Leptodactylidae, Allophrynidae, Centrolenidae, and Myobatrachidae (Figure S1a, b, and d). Among Leptodactylidae and its sister clade, a decrease was identified in both groups, foaming and non-foaming (Figure S1a and b): *Allophryne* (from 0.084 to 0.012 lineages per million years), *Ikakogy* + *Lithodytes* + *Scythrophrys* + *Edalorhina* (from 0.084 to 1.388e^{-10}). Conversely, in Limnodynastidae and its sister clade an increase of 0.562 in diversification rate was noticed in the non-foaming nest lineage: *Assa*, *Crinia*, *Geocrinia*, *Metacrinia*, *Myobatrachus*, *Paracrinia*, *Pseudophryne*, *Spicospina*, *Taudactylus*, and *Uperoleia* (Figure S1d).

BiSSE analysis showed that account for missing species on the phylogeny using terminally unsolved methodology increased maximum likelihood values in all datasets (Table 1). Although some species groups tested for differential lineage speciation and extinction rates regarding foam ability had low ΔAIC, implicating in equally fitted models, all of them had equal rates as one of the best model. Actually, only for Leptodactylidae this was not the model with lower AIC value. The equal rates model (herein Equal.λμ) offers evidence that foaming ability does not influence speciation and extinction rates, leading to similar rates of diversification between foaming and non-foaming lineages. Nevertheless, models which all rates were free to vary according to character state (herein called full) was always the worst fit, with AIC ranging from 1.95 in Leptodactylidae to 4.05 in Limnodynastidae + Sister Clade (Table 1).

Table 1 - Comparable values of all BiSSE models used to evaluate if foaming nest lineages have higher diversification rates than non-foaming. Grey indicates phylogenies which missing species were informed using terminally unsolved method, while in white they were informed using skeleton method.

			AIC	qpAIC	wAIC	wAIC
		97	161.94	1.95	0.13	0.13
	Equal.λ	-74.99	159.99	0	0.35	0.35
	Equal.μ	-75.20	160.40	0.41	0.29	0.29
	Equal.λμ	-76.46	160.91	0.92	0.22	0.22
Leptodactylidae	Full	-263.12	538.24	378.25	0.16	
	Equal.λ	-263.25	536.51	376.52	0.39	
	Equal.μ	-263.5	536.99	377	0.31	
	Equal.λμ	-265.28	538.56	378.57	0.14	
	Full	-176.87	365.74	2.44	0.14	0.14
	Equal.λ	-177.65	365.30	2	0.18	0.18
	Equal.μ	-177.59	365.19	1.89	0.19	0.19
	Equal.λμ	-177.65	363.30	0	0.49	0.49
Leptodactylidae + Sister	Full	-546.04	1104.10	740.80	6.7E-162	
	Equal.λ	-546.98	1104.00	740.70	7.0E-162	
	Equal.μ	-536.54	1103.10	739.80	1.1E-161	
	Equal.λμ	-546.90	1101.80	738.50	2.1E-161	
	Full	-89.059	190.12	3.12	0.11	0.11
	Equal.λ	-89.389	188.78	1.78	0.22	0.22
	Equal.μ	-89.059	190.12	3.12	0.11	0.11
	Equal.λμ	-89.499	187.00	0	0.54	0.55
Limnodynastidae	Full	-94.76	201.51	14.51	0.00	
	Equal.λ	-94.54	199.08	12.08	0.00	
	Equal.μ	-94.76	199.51	12.51	0.00	
	Equal.λμ	-94.758	197.52	10.52	0.00	
	Full	-177.65	367.30	4.05	0.07	0.07
	Equal.λ	-177.71	365.41	2.16	0.19	0.19
	Equal.μ	-177.85	365.70	2.45	0.17	0.17
	Equal.λμ	-177.63	363.25	0	0.57	0.57
Limnodynastidae + Sist	Full	-205.95	423.91	60.66	3.81E-14	
	Equal.λ	-205.96	421.93	58.68	1.03E-13	
	Equal.μ	-207.31	424.61	61.36	2.69E-14	
	Equal.λμ	-207.31	422.61	59.36	7.30E-14	
	Full	-83.46	178.91	2.17	0.13	0.13
	Equal.λ	-84.37	178.74	2	0.14	0.14
	Equal.μ	-83.46	176.92	0.18	0.35	0.35
	Equal.λμ	-84.37	176.74	0	0.38	0.38
Stumpffia	Full	-175.63	363.25	186.51	1.21E-41	
	Equal.λ	-175.7	361.4	184.66	3.04E-41	
	Equal.μ	-175.63	361.26	184.52	3.26E-41	

			AIC	qpAIC	wAIC	wAIC
		.7	359.4	182.66	8.27E-41	
Rhacophorinae	Full	-121.09	254.18	4	0.08	0.08
	Equal.λ	-121.09	254.18	4	0.08	0.08
	Equal.μ	-121.09	252.18	2	0.22	0.22
	Equal.λμ	-121.09	250.18	0	0.61	0.61
	Full	-452.35	916.71	666.53	1.12E-145	
	Equal.λ	-452.38	914.77	664.59	2.96E-145	
	Equal.μ	-452.35	914.71	664.53	3.05E-145	
	Equal.λμ	-452.38	912.77	662.59	8.05E-145	

Focusing exclusively on the results generated in terminally unsolved phylogenies, Leptodactylidae and Limnodynastidae, the two groups from which more than one dataset were used, presented distinct results when together with its sister clade. In Leptodactylidae, even having Equal.ƛμ as one of the best fit model, the other equally fitted models points to increased diversification rate in foaming lineages by increased speciation and/or decreased extinction rates (Table 2). However, when including its sister clade into the analysis (Leptodactylidae + Sister), besides Equal.ƛμ, a model in which extinction rates are equal can be considered equally good to explain the data. In this model (Equal.μ), diversification rates of lineages without foam ability was higher due to increased speciation rate (Table 2). As in Leptodactylidae, when analyzing only Limnodynastidae, two models can explain the data, Equal.ƛμ and Equal.ƛ (same speciation rates). In Limnodynastidae, a lower extinction rate of foaming lineages explains its higher diversification rates when compared to non-foaming. Otherwise, when including its sister clade (Limnodynastidae + Sister) the only best fit model was Equal.ƛμ, in which there is no difference in diversification (Table 2). This same result was found in Rhacophorinae, while equal rates or higher diversification rate caused by increased speciation rates in foaming lineages was pointed out in *Stumpffia*.

eciation (ə), extinction (μ), transition between states (q) and diversification (r) of non-foaming (0) and foaming (1) lineages using both methods to account for missing species, terminally unsolved and skeleton phylogenies. In the full model all these parameters were allowed to vary, while in the Equal models some parameters were constrained and not allowed to vary.

		Terminally unsolved phylogeny								Skeleton phylogeny							
		ə0	ə1	μ0	μ1	q01	q10	r0	r1		ə0	ə1	μ0	μ1	q01	q10	r0	r1
Leptodactylidae	**Full**	0.084	0.073	0.051	0	0.004	0	0.033	0.073	**Full**	0.106	0.073	0.067	0	0.005	0	0.039	0.073
	Equal.λ	0.074	0.074	0.037	0	0.005	0	0.037	0.074	**Equal.λ**	0.073	0.073	0.027	0	0.006	0	0.046	0.073
	Equal.μ	0.049	0.073	0	0	0.006	0	0.049	0.073	**Equal.μ**	0.057	0.073	0	0	0.008	0	0.057	0.073
	Equal.λμ	0.066	0.066	0	0	0.007	0	0.066	0.066	**Equal.λμ**	0.07	0.07	0	0	0	0.003	0.07	0.07
		ə0	**ə1**	**μ0**	**μ1**	**q01**	**q10**	**r0**	**r1**		**ə0**	**ə1**	**μ0**	**μ1**	**q01**	**q10**	**r0**	**r1**
*Limnodynastidae*Sister	**Full**	0.082	0.244	0	0.208	0	0.001	0.082	0.036	**Full**	0.086	0.069	0	0	0	0.002	0.086	0.069
	Equal.λ	0.076	0.076	0	0	0.002	0	0.076	0.076	**Equal.λ**	0.081	0.081	0	0.013	0	0.002	0.081	0.068
	Equal.μ	0.077	0.073	0	0	0.002	0	0.077	0.073	**Equal.μ**	0.081	0.072	0	0	0.002	0	0.081	0.072
	Equal.λμ	0.076	0.076	0	0	0.002	0	0.076	0.076	**Equal.λμ**	0.077	0.077	0	0	0.002	0	0.077	0.077
Leptodactylidae +		**ə0**	**ə1**	**μ0**	**μ1**	**q01**	**q10**	**r0**	**r1**		**ə0**	**ə1**	**μ0**	**μ1**	**q01**	**q10**	**r0**	**r1**
	Full	0.098	0.04	0.092	0	0.002	0	0.006	0.04	**Full**	0.042	0.041	0	0	0	0.003	0.042	0.041
	Equal.λ	0.041	0.041	0.012	0	0.003	0	0.029	0.041	**Equal.λ**	0.044	0.044	0.021	0	0.004	0	0.023	0.044
	Equal.μ	0.037	0.04	0	0	0.003	0	0.037	0.04	**Equal.μ**	0.042	0.041	0	0	0	0.003	0.042	0.041
	Equal.λμ	0.039	0.039	0	0	0.003	0	0.039	0.039	**Equal.λμ**	0.041	0.041	0	0	0.007	0	0.041	0.041

		ylogeny								Skeleton phylogeny							
Limnodynastidae+Sister		ə0	ə1	μ0	μ1	q01	q10	r0	r1		ə0	ə1	μ0	μ1	q01	q10	r0	r1
	Full	0.046	0.053	0	0.023	0	0.004	0.046	0.03	**Full**	0.052	0.053	0.008	0.02	0	0.009	0.044	0.033
	Equal.λ	0.047	0.047	0.001	0.014	0	0.005	0.046	0.033	**Equal.λ**	0.052	0.052	0.009	0.019	0	0.009	0.043	0.033
	Equal.μ	0.049	0.043	0.004	0.004	0	0.005	0.045	0.039	**Equal.μ**	0.053	0.053	0.001	0.014	0.001	0.004	0.0516	0.039
	Equal.λμ	0.044	0.044	0.001	0.001	0	0.002	0.043	0.043	**Equal.λμ**	0.053	0.053	0.001	0.014	0.001	0.011	0.0516	0.039
		ə0	ə1	μ0	μ1	q01	q10	r0	r1		ə0	ə1	μ0	μ1	q01	q10	r0	r1
Stumpffia	**Full**	0.041	0.076	0	0	0.001	0	0.041	0.076	**Full**	0.041	0.047	0	0	0.002	0	0.041	0.047
	Equal.λ	0.045	0.045	0	0	0.001	0	0.045	0.045	**Equal.λ**	0.042	0.042	0	0	0.002	0	0.042	0.042
	Equal.μ	0.041	0.076	0	0	0.001	0	0.041	0.076	**Equal.μ**	0.041	0.047	0	0	0.002	0	0.041	0.047
	Equal.λμ	0.045	0.045	0	0	0.001	0	0.045	0.045	**Equal.λμ**	0.042	0.042	0	0	0.002	0	0.042	0.042
		ə0	ə1	μ0	μ1	q01	q10	r0	r1		ə0	ə1	μ0	μ1	q01	q10	r0	r1
Rhacophorinae	**Full**	0.058	0.058	0	0	0	0.001	0.058	0.058	**Full**	0.055	0.057	0	0	0.001	0.002	0.055	0.057
	Equal.λ	0.058	0.058	0.001	0	0	0.001	0.057	0.058	**Equal.λ**	0.055	0.055	0	0	0.001	0.002	0.055	0.055
	Equal.μ	0.058	0.058	0	0	0	0.001	0.058	0.058	**Equal.μ**	0.055	0.057	0	0	0.001	0.002	0.055	0.057
	Equal.λμ	0.058	0.058	0	0	0	0.001	0.058	0.058	**Equal.λμ**	0.055	0.055	0	0	0.001	0.002	0.055	0.055

…s some models had extinction rates estimated as close or equal to zero, there were also cases of high values such as 0.208 for foaming lineages of Leptodactylidae and its sister clade according to the full model. In some cases extinction rates, even being low, impacted diversification rate by shifting which state had higher rate (Table 2). Conversely, transition rate always presented low values between states, being virtually zero in many species group and models. Its highest value among terminally unsolved topology was 0.007 in the equal rates model for Leptodactylidae (Table 2).

4. Discussion

Anurans foam ability, despite of being a convergent behavior and having several relevant functions may not constitute a key innovation. In all groups tested, the model in which speciation and extinction rates were not influenced by foam presence or absence was always one of the best-fitted models. However, in four of the six phylogenies tested we were not able to select only one model. Equally fitted models pointed to increased diversification rates in foaming species among Leptodactylidae, Limnodynastidae, and *Stumpffia* and decrease in one Leptodactylidae and its sister clade.

Even though all groups may have equal rates of diversification independently of producing or not foam, the reliability of this result is questionable in some cases due to the lower AIC value. In four of the six phylogenies, we were not able to determine a single model to explain our data. The lack of accuracy in choosing a model is assigned to the relative low number of species in the phylogenies and also to the lack of detail in some terminals, which is known to affect BiSSE analysis [33,38]. Nevertheless, we were able to retrieve low transition rates between character states, which is already known for foaming species [22,25,27,30,34]. We choose then to discuss all scenarios pointed out by equally fitted models.

ıy was by far a better method to account missing species in our datasets. It was expected since it is known to perform better in cases of low phylogenetic resolution [38]. Besides, in our case, there are really few transitions between states, so use terminally unsolved method aggregate more information because it allows to inform how many species with each state are in the terminally clades, even though we are not certain about its resolution at terminal branches.

Considering that speciation and extinction rates based on a trait may be biased by shifts in lineages diversification independently of the trait presence [37], we first identified shifts without accounting for foaming ability. Although some foaming and non-foaming lineages had shifts in diversification rates pointed out by MEDUSA (Figure S1) it happened in lineages with low species richness, which should not greatly influence BiSSE results. MEDUSA identified shifts in two groups, Leptodactylidae and Limnodynastidae, both including or not the sister clade. In the first group we noticed a decrease in diversification rate in both foaming and non-foaming lineages, which apparently did not influenced BiSSE results. Even though some species of Leptodactylidae sister clade have decreased diversification rates, as detected by MEDUSA, when included in BiSSE analysis we identify a possibility that these non-foaming species may have increased speciation when compared to foaming. It is demonstrates that shifts not linked to foam ability did not influenced BiSSE results, since in this case we would expect a distinct outcome, in which non-foaming ability have decreased speciation. A distinct result was observed in Limnodynastidae and Myobatrachidae phylogeny, from which MEDUSA detected increased diversification rate in several lineages without foam ability. BiSSE results were apparently influenced by this increased diversification rate, since when considering only Limnodynastidae there is a possibility that foaming species have higher diversification rates, but when

ch MEDUSA detected the shift, the diversification ng species is considered equal.

Distinct environmental conditions are known to influence rates of speciation and extinction [4,40], but that may not be the case of anuran foaming ability. Even though this trait has independently originated in distinct continents and consequently evolved in distinct environmental conditions, all lineages presented a similar result, the rates of diversification may not be linked to foam ability. Nevertheless, we also had equally fitted models that points to foaming Leptodactylidae, Limnodynastidae and *Stumpffia* (Cophylinae) having increased diversification rates, while Leptodactylidae when together with its sister clade may also have decreased rates. Future studies relating environmental conditions to diversification rates, such as in [31,41,42], would help to understand why there may have distinct patterns of speciation and extinction in relation to foam ability. The foaming American lineage (Leptodactylidae) may have these rates influenced by foam ability, while in the Australia and New Guinea lineage (Limnodynastidae) only extinction rate was influenced, and conversely in the Madagascar lineage (*Stumpffia*) speciation rates differ.

In addition to environment, interactions with another taxa and other organismal attributes are hypothesized to influence how a trait affects diversification [40]. So far, few are known about anuran foam nest influences organismal interactions (e.g. helps avoiding aquatic predators [17] and defending eggs from microbial colonization [21]). The range from which it avoids predation is still unknown, since there are reports of foam predation by birds, snakes, ants, frogs, monkeys, and beetles and dipterans larvae [43ï 46]. In case of visually oriented predators, the foam may call attention, and consequently, increase predation rates.

Regarding other organismal attributes, foam may influence diversification when coupled with other specific reproductive modes attribute, such as oviposition site and

, considering foam importance in preventing desiccation, increased diversification rates may be related to both, foaming ability and water independence for reproduction. Although it can be tested using MuSSE [37], the lack of reproductive information for some species and the need to fit this information in binary categories makes it difficult to evaluate.

Besides these three factors (environment, species interaction and other traits), foam nest possible lack of influence in diversification rates may also result from foam and nest building characteristics, which varies on where it is built (e.g. floating on pond or water accumulated in constructed basins, axils of terrestrial bromeliads, subterranean chamber, humid forest floor), and by whom (adult male and tadpole) [17,28,47ï 49]. These variations in nest building are subjected to distinct selective pressures, which may have leaded to distinct patterns of diversification among lineages.

Fouquet et al. [30] pointed out that foam nest may be a key innovation in Leptodactylidae, leading to the high number of Leptodactylinae and Leiuperinae species when compared to the non-foaming subfamily Paratelmatobiinae. Even though we were not able to select a model of speciation and extinction for Leptodactylidae, we noticed that with the exception of the equal rates model (Equal.ə̧µ), diversification rate is higher among foaming lineages. The lack of power in detecting which model better explains the data may be due to the high number of foaming species in comparison to non-foaming, which is known to bias BiSSE analysis [33]. To avoid it, we included Leptodactylidae sister clade in a second analysis, on which the equal rates (Equal. ə̧µ) had the lower AIC and the model with equal extinction rates was equally considered to explain the data. Considering only this second model, we have evidence that lineages lacking foam ability may have higher speciation rates, and consequently, higher diversification. It supports Fouquet et. al. [31] final hypothesis, that the difference in

dae sub-families cannot be fully explained by foam

In conclusion, despite of being a convergent behavior and having several relevant functions, anurans foam nest may not constitute a key innovation. The model in which speciation and extinction rates were the same for foaming and non-foaming lineages was the only one considered adequate to explain the data of all independent origins of foaming lineages studied here. Nevertheless, in some cases we were not able to select only one model. In these cases, the other equally fitted models pointed to increased diversification of foaming lineages in three phylogenies, either by higher speciation and/or lower extinction. In only one case the results suggested a decreased diversification rate in foaming lineages caused by higher speciation in non-foaming.

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