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Late Miocene diversification and phylogenetic relationships of the huge toads in the *Rhinella marina* (Linnaeus, 1758) species group (Anura: Bufonidae)

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ABSTRACT

We investigated the phylogeny and biogeography of the *Rhinella marina* group, using molecular, morphological, and skin-secretion data, contributing to an understanding of Neotropical faunal diversification. The maximum-parsimony and Bayesian analyzes of the combined data recovered a monophyletic *R. marina* group. Molecular dating based on Bayesian inferences and fossil calibration placed the earliest phylogenetic split within the *R. marina* group at ~10.47 MYA, in the late Miocene. Two rapid major diversifications occurred from Central Brazil, first northward (~8.08 MYA) in late Miocene and later southward (~5.17 MYA) in early Pliocene. These results suggest that barriers and dispersal routes created by the uplift of Brazilian Central Shield and climatic changes explain the diversification and current species distributions of the *R. marina* group. Dispersal-vicariance analyzes (DIVA) indicated that the two major diversifications of the *R. marina* group were due to vicariance, although eleven dispersals subsequently occurred.

1. Introduction

One of the fundamental goals of modern systematic investigation is the use of inferred phylogenies to interpret biogeographical patterns (e.g. Pramuk et al., 2008; Santos et al., 2009). Several authors noted the importance of Tertiary–Quaternary geoclimatic events for the origin and diversification of Neotropical fauna and flora (e.g. Wesselingh and Salo, 2006; Rull, 2008). Events such as Andean uplift, the closure of Panama Isthmus, marine incursions, the formation of Neotropical river basins, and climatic changes drove diversification in Neotropical fauna, as previously described for amphibians (e.g. Santos et al., 2009) and squamates (e.g. Gamble et al., 2008). Nevertheless, the biogeography of the Neotropical herpetofauna is still poorly understood (e.g. Colli, 2005; Giugliano et al., 2007; Santos et al., 2009; Gamble et al., 2008; Vallinoto et al., 2010).

The species-rich true-toad family Bufonidae was considered until recently an old group originating before the splitting of Gondwanaland (Savage, 1973; Pramuk, 2006). However, Pramuk et al. (2008) found that the bufonids originated later than previously suggested (78–99 MYA), after the breakup of Gondwana, based on an analysis of divergence times with fossil calibrations under a relaxed modern molecular clock assumption. The phylogeny of Bufonidae was poorly understood in the past, mainly because of the high conservation of morphological and ecological traits across the family, and homoplastic characters used to reconstruct their relationships (e.g. Cei, 1972). In the last decade, molecular phylogenetic analyzes have elucidated the phylogenetic relationships and diversification of Bufonidae and of major amphibian groups (Faivovich et al., 2005; Frost et al., 2006; Grant et al., 2006; Hedges et al., 2008; Pramuk et al., 2008; Van Bocxlaer et al., 2009). A phylogenetic hypothesis focusing on all lineages of Bufonidae (Pramuk et al., 2008), based on 2521-bp of DNA data (mitochondrial and nuclear gene fragments) suggests that the genus *Rhinella* Fitzinger, 1828, should include almost all species of the former South American *Bufo* Laurenti, 1768.

The *Rhinella marina* group is currently composed of 10 species: *R. marina* (Linnaeus, 1758), *R. icterica* (Spix, 1824), *R. poeppigii* (Tschudi, 1845), *R. arenarum* (Hensel, 1867), *R. schneideri* (Werner, 1894), *R. rubescens* (A. Lutz, 1925), *R. jimi* (Stevaux, 2002),

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R. achavali (Maneyro, Arrieta and de Sá, 2004), *R. veredas* (Brandão, Maciel and Sebben, 2007), and *R. cerradensis* Maciel, Brandão, Campos and Sebben, 2007. These large-bodied species range from Texas (USA) to Uruguay, occurring throughout Central and South America (Frost, 2010) (Fig. 1a and b). They inhabit both open and forested areas and are characterized by extremely well ossified and exostosed crania, ornamented with deep striations, pits, and rugosities. A total-evidence analysis of Pramuk (2006) supports monophyly of the species belonging to the *R. marina* group by sharing the following unique and unreversed morphological synapomorphy: the point of articulation between the medial ramus of the pterygoid and parasphenoid alae formed by a jagged or "scalloped" suture

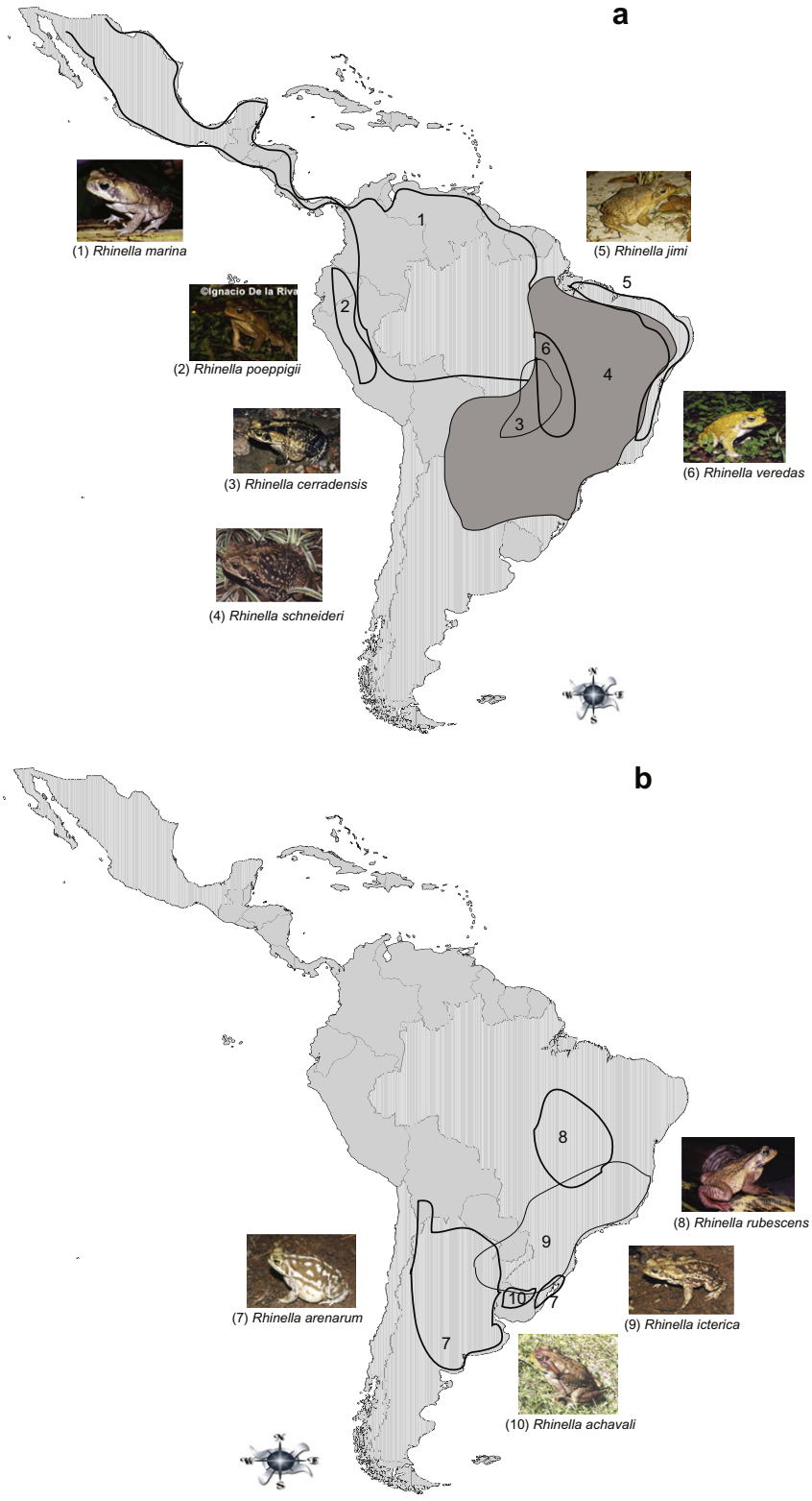

Fig. 1. Maps showing current geographic distributions of each species of the *Rhinella marina* group (a and b). The distributions are approximate. Photos by Natan Maciel except *R. poeppigii* (Ignacio De la Riva); *R. schneideri* and *R. rubescens* (Rubens H. Matsushita); *R. jimi* (José Roberto Leite); and *R. achavali* (Raúl Maneyro).

(see Pramuk, 2006; Maciel et al., 2007). A close relationship between *R. crucifer* and the *R. marina* group was proposed based on overall similarity (Cei, 1972), mtDNA data (Pauly et al., 2004) and total-evidence analyzes (Pramuk, 2006).

However, a recent study (Vallinoto et al., 2010) suggested that the *R. marina* group is paraphyletic with respect to *R. crucifer*. Vallinoto et al. (2010) included a 1946-bp-long fragment of the 12S and 16S ribosomal mtDNA genes along with a 327-bp-long fragment of the mitochondrial cytochrome subunit *b*. The phylogenetic analysis was based on a sampling of eight of the 10 currently recognized species of the group, excluding *R. veredas* and *R. cerradensis*. The *R. marina* species group is particularly interesting because of its wide geographic distribution.

Both molecular and morphological datasets have been used to infer the relationships of amphibians (Haas, 2003; Frost et al., 2006; Pramuk, 2006). In addition, alternative characters have also been employed, such as sperm ultrastructure (Braz et al., 2004; Garda et al., 2004) and skin-secretion chemical compounds (Maciel et al., 2006; Grant et al., 2006). The granular glands of amphibian skin produce a great variety of substances responsible for its noxious or poisonous character. They act as passive defense mechanisms against predators and microorganisms, and evolved independently in different groups of amphibians (Toledo and Jared, 1995). Indolealkylamines, chemical aromatic compounds derived from tryptophan, are noteworthy for their variety, abundance, and widespread distribution in *Rhinella* skin and parotoid-gland secretions (Erspamer, 1994). Several studies described differences in indolealkylamine composition in skin-secretion of bufonids (e.g. Cei et al., 1972), and used them in phylogenetic analyzes (Cei iotti et al., 1989; Maciel et al., 2003, 2006).

Herein, we present a total-evidence phylogenetic hypothesis of all known species in the *R. marina* group based on three different datasets (molecular, morphological, and parotoid-gland secretions). We also advance a biogeographic scenario based on molecular dating and dispersal-vicariance analysis (DIVA). The results provide new insights regarding the spatio-temporal distribution of the *R. marina* species group with general implications regarding the origins of Neotropical biodiversity.

2. Materials and methods

2.1. Taxon sampling

We assembled molecular, morphological, and parotoid-gland secretion datasets including all species in the *Rhinella marina* group. Four other bufonid taxa were used as outgroups: *Melanophryniscus* Gallardo, 1961; *R. margaritifera* (Laurenti, 1768); *R. granulosa* (Spix, 1824); and *R. crucifer* (Wied-Neuwied, 1821). Depending on the particular dataset, we used *Melanophryniscus stelzneri* (Weyenbergh, 1875), *M. klappenbachi* Prigioni and Langone, 2000, or *M. fulvoguttatus* (Mertens, 1937). Voucher information and GenBank accession numbers of the specimens used are presented in Tables 1 and 2. Outgroups were chosen based on their

Table 1
Species, locality, source, acronym and collection number, and GenBank accession numbers to each fragment gene partition.

Species	Locality	Source	Acronym and number	GenBank Accession Nos.		
				Cyt *b*	Rhod 1	12S–16S
Melanophryniscus fulvoguttatus	Brazil: Mato Grosso do Sul: Bonito	Present work	CHUNB 43636	HM159223	–	–
Melanophryniscus klappenbachi	Argentina: Chaco, proximidades de Resistencia	Frost et al. (2006)	MACN 38531	–	DQ283765	–
Melanophryniscus stelzneri	Paraguai: Parque Nacional San Rafael	Pramuk (2006)	KU 289071	–	–	DQ158421
Rhinella gr. *margaritifera*	Brazil: Mato Grosso: Rondolândia	Present work	CHUNB 39957	HM159224	HM159242	–
Rhinella gr. *margaritifera*	Ecuador: Francisco de Orellana: Yasuní	Pramuk (2006)	QCAZ 10601	–	–	DQ158312
Rhinella gr. *granulosa*	Brazil: Goiás: São João da Aliança	Present work	CHUNB 39959	HM159225	–	–
Rhinella gr. *granulosa*	Guyana: Southern Rupununi Savanah, Aishalton	Frost et al. (2006)	AMNH A139020	–	DQ283966	–
Rhinella gr. *granulosa*	Brazil: Porto Trombetas	Vallinoto et al. (2010)	Not provided	–	–	GU178788
Rhinella crucifer	Brazil: Bahia: Prado	Present work	CHUNB 49567	HM159226	HM159239	–
Rhinella gr. *crucifer*	Brasil: São Paulo	Pramuk (2006)	USNM 303015	–	–	DQ158447
Rhinella achavali	Uruguay: Rivera: Rivera	Present work	CHUNB 48024	HM159227	HM159237	–
Rhinella achavali	Uruguay	Vallinoto et al. (2010)	ZVCB 3801	–	–	GU178787
Rhinella arenarum	Brazil: Rio Grande do Sul: Rio Grande	Present work	CHUNB 39950	HM159228	–	–
Rhinella arenarum	Argentina: San Luis, ruta 20	Faivovich et al. (2005)	MACN 38639	–	AY844547	–
Rhinella arenarum	Argentina	Frost et al. (2006)	AR 305	–	–	DQ158429
Rhinella rubescens	Brazil: Distrito Federal: Brasília	Present work	CHUNB 39969	HM159229	HM159244	–
Rhinella rubescens	Brazil: Minas Gerais: Santa Bárbara	Pramuk (2006)	AR 388	–	–	DQ158486
Rhinella icterica	Brazil: São Paulo: Atibaia	Present work	CHUNB 39960	HM159230	HM159240	–
Rhinella icterica	Brazil: São Paulo: Carapicuíba	Pramuk (2006)	AR 312	–	–	DQ158462
Rhinella veredas	Brazil: Minas Gerais: Buritizeiro	Present work	CHUNB 44609	HM159231	HM159245	–
Rhinella cerradensis	Brazil: Bahia: Cocos	Present work	CHUNB 38670	HM159232	HM159238	–
Rhinella poeppigii	Bolivia: Santa Cruz: Espejillos: Ibanéz	Present work	MNCN/ ADN6174	HM159233	HM159243	–
Rhinella poeppigii	Bolivia: La Paz	Vallinoto et al. (2010)	MNCN/ ADN6044	–	–	GU178779
Rhinella jimi	Brazil: Bahia: Arembepe	Present work	CHUNB 49570	HM159234	HM159241	–
Rhinella jimi	Brazil: Rio Grande do Norte: Natal	Vallinoto et al. (2010)	Not provided	–	–	GU178784
Rhinella schneideri	Brazil: Minas Gerais: Buritizeiro	Present work	CHUNB 44601	HM159235	–	–
Rhinella schneideri	Argentina: Santiago del Estero, Dto. Guasayaan	Frost et al. (2006)	BB 1224	–	DQ283791	DQ283065
Rhinella marina	Brazil: Pará: Belém	Present work	CHUNB 39967	HM159236	–	–
Rhinella marina	Peru: Huanuco, Rio Pachitea: Puerto Inca	Frost et al. (2006)	MJH 3678	–	DQ283789	–
Rhinella marina	Brazil: Porto Trombetas	Vallinoto et al. (2010)	Not provided	–	–	DQ283065

Museum abbreviations: Coleção Herpetológica da Universidade de Brasília (CHUNB); Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Sección Herpetología (MACN); University of Kansas, Museum of Natural History, Division of Herpetology (KU); Museo de Zoología, Pontifica Universidad Católica del Ecuador (QCAZ); American Museum of Natural History, Division of Vertebrate Zoology (Herpetology) (AMNH); National Museum of Natural History, Division of Amphibians and Reptiles (USNM); Colección de Zoología Vertebrados, Facultad de Ciencias, Universidad de la República (ZVCB); Alexander Robertson private collection (AR); Museo Nacional de Ciencias Naturales (MNCN); Boris Blotto field series (BB); Martin J. Henzl field series (MJH).

Table 2
Voucher specimens, locality, source, acronym and collection number used to obtain morphological and parotoid-gland secretion characters.

Species	Locality	Source	Acronym and number	
			Morphology	Parotoid-gland secretion
Melanophryniscus fulvoguttatus	Brazil: Mato Grosso do Sul: Bonito	Present work	–	CHUNB 43636*
Melanophryniscus stelzneri	Argentina: San Luis	Pramuk (2006)	KU 167631	–
Rhinella gr. *margaritifera*	Brazil: Mato Grosso: Rondolândia	Present work	–	CHUNB 39957*
Rhinella gr. *margaritifera*	Brazil: Amapa	Pramuk (2006)	KU 93138	–
Rhinella gr. *granulosa*	Brazil: Goiás: São João da Aliança	Present work	–	CHUNB 39954*
Rhinella gr. *granulosa*	Colômbia: Meta	Pramuk (2006)	KU 110431*	–
Rhinella crucifer	Brazil: Bahia: Itacaré	Present work	–	CHUNB 39954
Rhinella crucifer	Brasil: Espírito Santo	Pramuk (2006)	KU 93112	–
Rhinella achavali	Uruguay: Rivera: Rivera	Present work	CHUNB 48025	CHUNB 48024*
Rhinella arenarum	Brazil: Rio Grande do Sul: Rio Grande	Present work	ASUnB 2585	CHUNB 39951*
Rhinella arenarum	Uruguay: Artiga	Pramuk (2006)	KU 71161	–
Rhinella rubescens	Brazil: Distrito Federal: Brasília	Present work	ASUnB 2070*	CHUNB 39970*
Rhinella icterica	Brazil: São Paulo: Atibaia	Present work	–	CHUNB 39969*
Rhinella icterica	Brazil: São Paulo: São Paulo	Present work	ASUnB 2586*	CHUNB 39969*
Rhinella veredas	Brazil: Minas Gerais: Butirizeiro	Present work	CHUNB 43336*	CHUNB 43336*
Rhinella cerradensis	Brazil: Bahia: Cocos	Present work	–	CHUNB 38670*
Rhinella cerradensis	Brazil: Distrito Federal: Brasília	Present work	ASUnB 2553	–
Rhinella poeppigii	Bolivia: La Paz	Pramuk (2006)	KU 183234	–
Rhinella jimi	Brazil: Bahia: Arembepe	Present work	ASUnB 2588	CHUNB 49570*
Rhinella schneideri	Brazil: Distrito Federal: Brasília	Present work	–	CHUNB 39971*
Rhinella schneideri	Argentina: Santiago del Estero	Pramuk (2006)	KU 160307	–
Rhinella marina	Brazil: Pará: Belém	Present work	–	CHUNB 44601*
Rhinella marina	Nicarágua: Rivas	Pramuk (2006)	KU 84935	–

Museum abbreviations: Coleção Herpetológica da Universidade de Brasília (CHUNB); University of Kansas, Museum of Natural History, Division of Herpetology (KU); Coleção Antonio Sebben – Universidade de Brasília (ASUnB).
* Represent species where more than one specimen were analyzed.

inferred relationships with the species of the *R. marina* group. *Melanophryniscus* is considered the sister taxon to a clade comprising all other Bufonidae (Haas, 2003; Frost et al., 2006; Pramuk, 2006). The remaining outgroup species (*R. margaritifera*, *R. granulosa*, and *R. crucifer*) are more closely related to the *R. marina* group (Pramuk, 2006).

2.2. DNA extraction, amplification, purification, and sequencing

Total DNA was extracted from muscle and/or liver tissue preserved in ethanol and tissue-storage buffer, using the DNeasy Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was used to amplify the cytochrome *b* sequences, using the primers described by Graybeal (1993), and rhodopsin 1 gene, with the primers used by Bossuyt and Milinkovitch (2000). All PCR products were amplified under standard conditions and with the following PCR profile: (1) initial heating for 2 min at 94 °C; (2) 35 cycles of: 94 °C for 60 s, 50–58 °C for 60 s, and 72 °C for 60 s; and (3) final extension for 8 min at 72 °C. PCR products were then purified using 1 unit/μl PCR of the enzymes "shrimp alkaline phosphatase" (SAP) and exonuclease I (EXO) (Biotech Pharmacon ASA). Reactions were incubated at 37 °C for 90 min and then at 80 °C for 20 min. Purified PCR products were sequenced in both directions on an ABI 377 automated DNA sequencer (Applied Biosystems, CA) using the DYEnamic™ ET terminator cycle sequencing kit (GE HealthCare, Sweden), according to manufacturer's instructions. When possible, two individuals of the same species were sequenced to control sequencing contamination.

2.3. Morphology (cleared-and-stained and macerated preparations)

We examined 83 morphological qualitative characters, mainly osteological as described by Lynch (1971), Martin (1972a,b), Pregill (1981), Cannatella (1985), Morrison (1994), Mendelson (1997) and Pramuk (2000, 2002). Some characters were scored from soft anatomy and integument and one was obtained from inguinal fat bodies (da Silva and Mendelson, 1999). From these characters, 13 were multistate. Morphological characters were obtained directly from the literature: *Melanophryniscus stelzneri*, *Rhinella* gr. *margaritifera*, *R.* gr. *granulosa*, *R. crucifer*, *R. arenarum*, *R. poeppigii*, *R. schneideri* and *R. marina* (Pramuk, 2006), or by the present work: *R. achavali*, *R. arenarum*, *R. rubescens*, *R. icterica*, *R. veredas*, *R. cerradensis*, and *R. jimi* (Table 1) following the protocols described below.

Cleared-and-stained specimens were prepared following the protocols of Hanken and Wassersug (1981) or Taylor and Van Dyke (1985). Macerated specimens were buried for a period of 3 months and then cleaned. Observations were done by bare eye or under stereoscopic microscope. Damage to structures and logical inconsistencies were coded as missing characters. The polarity of transformations was determined by examination of outgroups. All characters were considered unordered, and the osteological terminology follows Trueb (1993). When available, two or more individuals of the same species were examined to allow for polymorphisms. The complete data matrix containing all morphological character states is presented in Appendix (Supplementary data).

2.4. Parotoid-gland skin-secretion extraction (indolealkylamines) and thin layer chromatography (TLC)

Parotoid-gland skin secretions were obtained by gland compression, resuspended in deionized water, lyophilized, and then stored at −20 °C. All the species of the *Rhinella marina* species group were sampled with the exception of *R. poeppigii*. Dried secretion was prepared in methanol to the concentration of 20 mg/ml. To fractionate indolealkylamines, we used thin layer chromatography (TLC). Indolealkylamine standards were prepared following Maciel et al. (2003). The identification of bufothionin (DHB-S) followed Deulofeu and Duprat (1944). Chromatographies were performed in ascendant unidimensional, 20 × 20 cm silica-gel H plates (Sigma Chemical Company) using a mobile phase as described by Maciel et al. (2003). The plates were sprayed with *o*-phthalaldehyde reagent (OPT), according to Narasimhacheri and Plaut (1971), and the visualization of spots was done under UV

light (375 nm). The qualitative indolealkylamines analysis was done by the respective retention factor (Rf), determined by the distance (cm) moved by the amine divided by the distance (cm) moved by the solvent front. We considered as characters all spots, even traces. When available, two or more individuals of the same species were examined to search for polymorphisms. TLC produced sixteen indolealkylamine characters. Five characters were chemically identified and presented the following Rf's: 0.053 to BTD, 0.273 to DHB, 0.366 to DHB-S, 0.593 to 5-HT, and 0.780 to BTN. The remaining eleven, non-indolealkylamines presented the following Rf's: 0.820, 0.880, 0.440, 0.660, 0.093, 0.133, 0.313, 0.013, 0.860, 0.213, and 0.626. The complete data matrix containing all parotoid-gland secretion characters is presented in Appendix (Supplementary data).

2.5. Sequence alignment and phylogenetic analyzes

Sequences were analyzed and edited using the software SeqScape (v2.1®) and aligned using Clustal X (Thompson et al., 1997). Equal weights were assigned for transitions and transversions.

We also used rhodopsin 1 nuclear sequences from GenBank (*Melanophryniscus klappenbachi*, *Rhinella granulosa*, *R. arenarum*, *R. schneideri*, and *R. marina*) produced by Faivovich et al. (2005) and Frost et al. (2006), and 1877-bp long 12S–16S mtDNA sequences from GenBank produced by Frost et al. (2006), Pramuk (2006) and Vallinoto et al. (2010) (Table 1). For the non-coding region 12S–16S, indels were excluded from phylogenetic analyzes due to ambiguous alignments. As 12S and 16S sequences are not available for *R. veredas* and *R. cerradensis*, we coded characters as missing data following Wiens et al. (2005).

We performed phylogenetic analyzes of the combined datasets (total-evidence analyzes), using Bayesian and maximum-parsimony methods. Prior model selection for each gene fragment was determined using Akaike Information Criterion implemented in Modeltest 3.7 (Posada and Crandall, 1998). Gamma distribution rates of morphological and parotoid-gland secretion datasets were employed to estimate the variation among characters.

Bayesian analyzes were conducted using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). Analyzes (MCMC runs) started with randomly generated trees to reach 4×10^6 generations. Two Markov Chains with default heating values were used. Trees and parameter values were sampled every 100 generations producing 40,000 saved trees per analysis. We assessed stationarity by plotting the $-\ln L$ per generation and checking for no average improvement in likelihood scores. We discarded the first 200 trees ("burn-in") of the two runs. Hence, estimates of trees and parameter values were based on 39,800 trees sampled from each run. PAUP was used to generate the 50% majority-rule consensus and to 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). Maximum-parsimony (MP) analyzes were performed using PAUP 4.0b 10 (Swofford, 1998), considering equal weighting of characters and equal costs for state transformations. Optimal trees were estimated using branch-and-bound searches with 1000 replicates of random stepwise addition and tree bisection reconnection (TBR) branch swapping. Bootstrap re-sampling (Felsenstein, 1985) was applied to assess the support for individual clades using 1000 bootstrap replicates and full

Fig. 2. Eight major geographic areas used in the dispersal-vicariance analysis of the *Rhinella marina* group. Amazonian basin (A), Central Oriental Andes (B), Guyana Shield (C), Chocoan Rain Forest (D), Central and North America (E), Brazilian Central Shield (F), Meridional South America (G), and Atlantic Coast (H).

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).

2.6. Molecular dating

We estimated divergence times among lineages of the *Rhinella marina* species based on a Bayesian relaxed molecular clock with uncorrelated rates, using the software BEAST 1.4.8 (Drummond and Rambaut, 2007). This approach allows the incorporation of many constraints taking into account both fossils and divergence times as "points" of calibration to estimate the variance in divergence times. We used two fossil records and one divergence time to provide a minimum time constraint at three points of our phylogenetic hypothesis: the oldest fossil of Bufonidae (57 MYA, late Paleocene) (Baéz and Gasparini, 1979), the oldest fossil of *R. marina* (about 11 MYA, late Miocene) (Estes and Wassersug, 1963; Sanchíz, 1998), and the origin of *Rhinella* between 31 and 44 MYA (Pramuk et al., 2008). We conducted analyzes using the following parameters: chain length of 20,000,000; sample every 1000 interactions; Yule speciation process; 10% burn-in. Results were checked for convergence and posterior age distributions using Tracer v.1.4 (Rambaut and Drummond, 2007). The most appropriate model of evolution for this analysis (GTR + I + G) was assessed using combined data from all fragments using Modeltest v3.7 (Posada and Crandall, 1998). The morphological and parotoid-gland secretion characters were excluded from this analysis.

2.7. Ancestral areas

Ancestral areas were recovered by DIVA 1.2 (Ronquist, 1997). DIVA uses the maximum-parsimony method to estimate the most recent ancestral area of each clade, minimizing dispersal and extinction events (Ronquist, 1997). We used eight major Neotropical areas to explain the origin and diversification of the species of the *Rhinella marina* group (e.g. Cei, 1972; Ab'Sáber, 1977; Colli, 2005; Tuomisto, 2007; Pramuk et al., 2008; Santos et al., 2009): Amazonian Basin (A), Central Oriental Andes (B), Guyana Shield (C), Chocoan Rain Forest (D), Central and North America (E), Brazilian Central Shield (F), Meridional South America (G), and Atlantic Coast (H) (Fig. 2).

3. Results

3.1. Phylogeny estimation

A 710 pb region including cytochrome *b* (451-bp) and rhodopsin 1 (259-bp) was obtained. DNA sequences are published in GenBank–NCBI (Table 1). The sequences of cyt *b* and rhodopsin 1 were compared with the partial sequences of the genes (327-bp) of *Rhinella marina* (Vallinoto et al., 2010; GenBank Accession No. GU178803) and (316-bp) of *R. schneideri* (Frost et al., 2006; GenBank Accession No. DQ283791). Both fragments correspond to the 5′ region of the genes. Together with the 12S–16S 1877-bp a total of 2587-bp were used to construct the molecular dataset.

The Akaike Information Criterion indicated that the most appropriate model of evolution for both the cytochrome *b* and rhodopsin 1 datasets was HKY + G, whereas for the 12S–16S regions the best model was GTR + I + G (Table 3). The Bayesian analysis produced a monophyletic, well resolved, and highly supported *R. marina* group (Fig. 3). The analysis also indicated that *R. crucifer* is the sister species of the *R. marina* group and that this group is formed by two major clades (Fig. 3): one including *R. veredas*, *R. cerradensis*, *R. jimi*, *R. marina*, *R. schneideri*, and *R. poeppigii* (north-central clade) and another including *R. arenarum*, *R. rubescens*, *R. achavali*, and *R. ictterica* (south-central clade).

Table 3
Optimal models and model parameters selected by Modeltest for each gene alignment.

		Data sets		
		Cytochrome *b*	Rhodopsin 1	12S–16S
Model	Model	HKY + G	HKY + G	GTR + I + G
	AIC	2192.8860	2192.8860	6622.4189
	−ln*L*			
Base frequencies	%A	0.2487	0.2487	0.3552
	%C	0.2688	0.2688	0.2049
	%G	0.1747	0.1747	0.1726
	%T	0.3078	0.3078	0.2673
Rates nucleotide change	A–C	2.7511	2.7511	1.4280
	A–G	6.3807	6.3807	5.8640
	A–T	1.3625	1.3625	4.1196
	C–G	1.9792	1.9792	0.0000
	C–T	6.3807	6.3807	23.8246
	G–T	1.0000	1.0000	1.0000
Invariable sites (*I*)		0	0	0.2695
Gamma shape distribution (*G*)		0.3625	0.3625	0.2758
Ti/Tv ratio		1.8577	1.8577	

The maximum-parsimony analyzes produced four equally most parsimonious trees. From a total of 2686 characters, 296 were parsimony-informative. The consensus tree had 1696 steps (CI = 0.86, RI = 0.59) and also showed that the *R. marina* group is monophyletic with two main clades (Fig. 3). The topology of the strict consensus tree is nearly identical to the tree generated by the Bayesian analyzes (Fig. 3, see differences indicated by asterisks). The relationship among *R. cerradensis*, *R. jimi*, and *R. marina* was unresolved in the MP analysis. In the Bayesian analyzes *R. cerradensis* is the sister taxon of the clade formed by *R. poeppigii* and *R. schneideri* (Fig. 3). Topological differences occur mainly among species outgroup. Maximum-parsimony recovered *R. crucifer* as the sister taxon of a clade comprising all bufonids sampled in this analysis. In the MP topology, *R.* gr. *granulosa* is the sister taxon of a clade formed by *Melanophryniscus* and *R.* gr. *margaritifera*.

Our results also indicated that the following morphological characters are synapomorphies (character number follow Pramuk, 2006) for the *R. marina* group: 16 (in posterior view, the ventral ramus of the squamosal is ventrolateral), 31 (articulation between the medial ramus of pterigoid and parasphenoid alae is formed by a suture – unique and unreversible synapomorphy of *R. marina* group (*sensu* Pramuk, 2006)), 32 (the anterior extension of the cultriform process extends beyond the orbitonasal foramina), 34 (the sphenethmoid is lightly ossified, creating a large, triangular exposure of the planum antorbitale cartilage), 52 (the anterior edge of the sacral diapophyses can be angled approximately posteriorly) and 70 (the medial ramus of the pterygoid is relatively narrow). Although only the indolealkylamine with Rf = 0.093 is a synapomorphy of *R. marina* group among skin-secretion characters analyzed, only three were not informative to the analysis.

3.2. Molecular dating

BEAST 1.4.8 (Drummond and Rambaut, 2007) also recovered two major clades based on the molecular dataset (Fig. 4). However, there were differences in the relationships among *R. veredas*, *R. jimi*, *R. marina*, *R. schneideri*, *R. cerradensis*, and *R. poeppigii*. In the Bayesian phylogeny, *R. poeppigii* is closest to *R. schneideri* (Fig. 3, north-central clade), whereas in the molecular dating *R. poeppigii* is closest to *R. cerradensis* (Fig. 4). Our estimates of divergence times indicate that the ancestor of the *R. marina* group originated

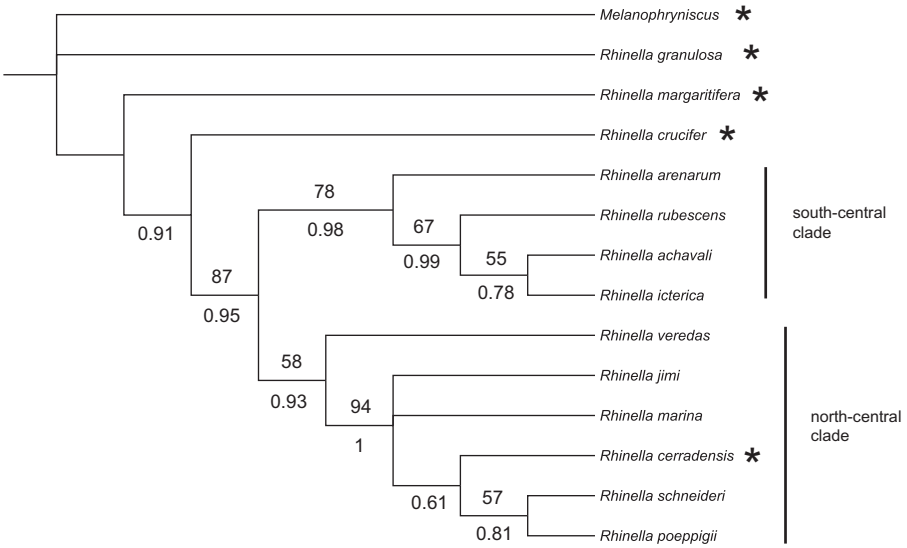

Fig. 3. Phylogenetic relationships among species of the *Rhinella marina* group based on Bayesian and maximum-parsimony (MP) inferences and produced by 2686 characters (a 2587-bp-long fragment including cytochrome subunit *b*, 12S and 16S ribosomal, rhodopsin 1 combined to 83 morphological characters and 16 from parotoid-gland skin secretions). Nonparametric bootstrap and posterior probability values are shown above and below nodes, respectively. The two major clades of the *R. marina* group are shown. Asterisks denote species whose topological positions differ in MP trees (see Section 3).

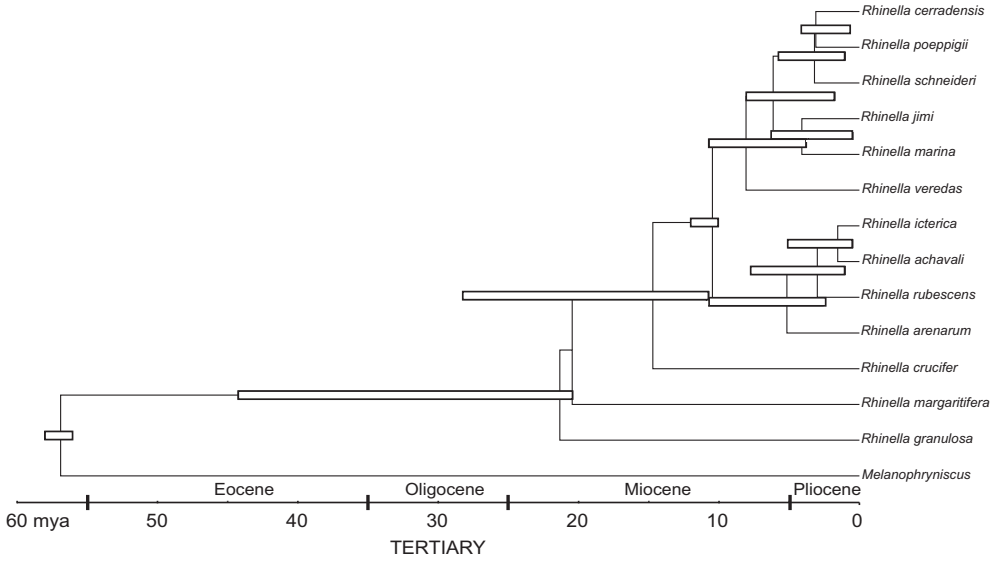

Fig. 4. Chronogram of the *Rhinella marina* species group and outgroups. Dating times were based on Bayesian relaxed molecular clock, combining molecular data (cytochrome *b*, rhodopsin 1, and 12S–16S) and calibrations (fossils and divergence times). Divergence times estimated were indicated by mean divergence time ± one standard deviation in boxes.

~10.47 MYA (10.01–11.96 MYA credibility interval) in the late Miocene (Fig. 4). The ancestor of the clade formed by *R. poeppigii*, *R. cerradensis*, *R. schneideri*, *R. marina*, *R. jimi*, and *R. veredas* originated about 8.08 MYA (3.75–10.68 MYA credibility interval) also in late Miocene, and the ancestor of the clade formed by *R. arenarum*, *R. rubescens*, *R. icterica* and *R. achavali* appeared ~5.17 MYA (2.58–10.89 MYA credibility interval) in early Pliocene. The last diversification in the *R. marina* group occurred in the Pleistocene, ~1.55 MYA (0.46–5.05 MYA, credibility interval).

3.3. Ancestral areas

The analysis of ancestral areas revealed 11 equally most parsimonious reconstructions that required 10 dispersal and four vicariant events. The most likely reconstruction of ancestral areas for each clade is shown in Fig. 5. DIVA also recovered the ancestral area of the clade *R. arenarum*, *R. rubescens*, *R. achavali*, and *R. icterica* (south-central clade in Fig. 3) in area G (Meridional South America) and the ancestral area of the clade formed by the remaining species of *R. marina* group (north-central clade in Fig. 3) in areas A, B, C, D, E, F or H (see Fig. 2). However, South America was separated from Central and North America from the late Cretaceous to the Pliocene. Hence, the reconstruction of the ancestral area of the clade formed by *R. marina* + *R. jimi* + *R. cerradensis* + *R. schneideri* + *R. poeppigii* and north-central clade (Fig. 3) in Central and North America (area E) is misleading, because these clades diversified at about 6.14 and 8.08 MYA (late Miocene), before the formation of the Panamanian land bridge. The same happened to the ancestor of the *R. marina* species group that diversified earlier ~10.47 MYA also in late Miocene. Since it is not possible to constrain the analysis on

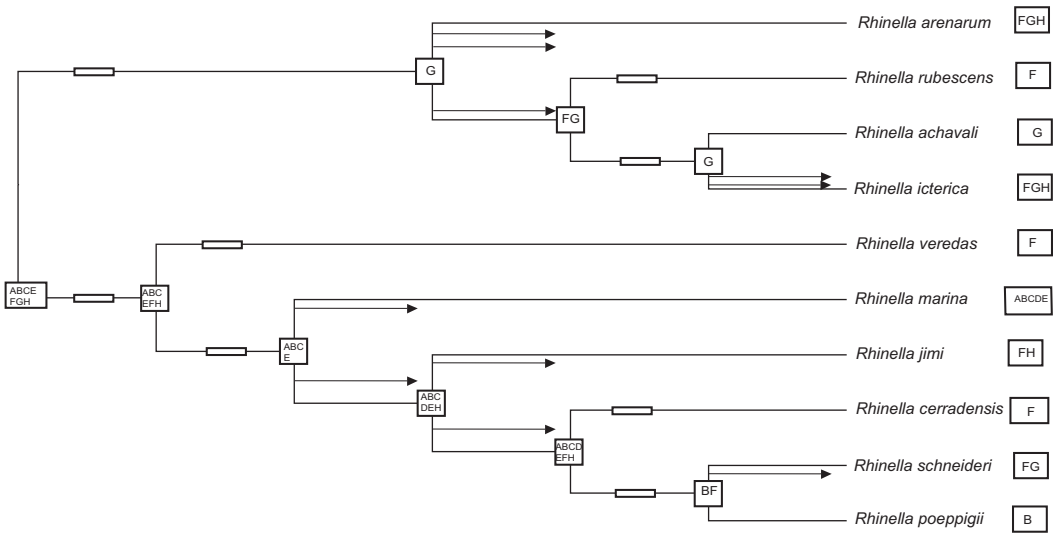

Fig. 5. Eight major geographic areas (see Fig. 2) used in the dispersal-vicariance analysis and reconstructed ancestral distributions for each node in one of the 11 most parsimonious solutions obtained (the most probable), based on the total-evidence (molecules + morphology + parotoid-gland skin-secretion) analysis of the *R. marina* species group. Arrows indicate dispersals and horizontal bars indicate vicariance events.

DIVA, we removed the area E as a candidate reconstructed ancestral area of these clades (Fig. 3).

The formation of the two main clades (north-central and south-central clades in Fig. 3) could be explained by a vicariant event. Among the minor clades (north-central and south-central clades) both vicariant and dispersal events have occurred. All reconstructions suggest less diversification within the south-central clade than within the north-central clade.

4. Discussion

4.1. Phylogeny estimation

We present here the most comprehensive phylogenetic hypothesis to date for the *Rhinella marina* species group. We expand previous analyzes by creating a large combined data set (molecular, morphological, and biochemical) that includes all currently recognized species and four outgroups. Our analyzes support monophyly of the *R. marina* group by both maximum-parsimony and Bayesian inferences. Bayesian analyzes showed that *R. crucifer* is the sister species of the *R. marina* group (see also Cei, 1972; Pauly et al., 2004; Pramuk, 2006). We also corroborated that the point of articulation between the medial ramus of the pterygoid and parasphenoid alae is formed by a jagged or "scalloped" suture as a synapomorphy of the *R. marina* group (see Pramuk, 2006; Maciel et al., 2007). A recent study suggests that the *R. marina* group is paraphyletic (Vallinoto et al., 2010), but it was based on 12S and 16S sequences with a high number of ambiguous alignment sites, which were not excluded from the analyzes. Together with mtDNA evidence, the present work added a nuclear gene and morphological and parotoid-gland skin-secretion characters. Our results divide the the *R. marina* group into two major clades. One clade is formed by *R. arenarum*, *R. rubescens*, *R. achavali* and *R. icterica* (Fig. 3, south-central clade) and the other by *R. veredas*, *R. cerradensis*, *R. jimi*, *R. marina*, *R. schneideri*, and *R. poeppigii* (Fig. 3, north-central clade). The history of the arrangement of the *R. marina* group, morphologically defined, is vast and was summarized by Brandão et al. (2007). Cei (1980) split the *R. marina* species into two groups based on skull development and tongue shape. One group contained *R. arenarum* and *R. rubescens* (called *R. arenarum* group) and the other group contained *R. icterica*, *R. marina*, *R. schneideri*, and *R. poeppigii* (called *R. marina* group). Maciel et al. (2007) conducted a principal-component analyzes (PCA) that summarized morphometric differences among all the currently recognized species of the *R. marina* group, recovering a similar arrangement found by Cei (1980). However, arrangement was done phenetically (i.e., grouping by overall similarity), and it is not supported by our phylogenetic findings. Besides, as already noted by Pramuk (2006), the osteological characters of the skull, mainly those related to the frontoparietal and used to infer morphological arrangements in the former genus *Bufo*, could be highly homoplastic and must be used with caution. Introgressive hybridization is another factor that could mislead the reconstruction of evolution in diverse taxa such as bufonids (Masta et al., 2002; Azevedo et al., 2003). Because many species of the *R. marina* group occur in sympatry (Frost, 2010), it is possible that the phylogeny could be strongly affected by introgressive hybridizations.

The use of different sets of data is very important to construct cladistic inferences (Hillis and Wiens, 2000). Even relatively small subsets (<5%) of morphological characters, in combination with large amounts of sequence data, can significantly influence the tree resulting from a combined analysis (Nylander et al., 2004). In addition, inclusion of morphology provides morphological characters for the diagnosis of phylogenetically based taxonomic groups (*sensu* de Queiroz and Gauthier, 1992). Although 37 of 99 characters of our morphological and skin-secretion data were not informative (constant or autopomorphies), it provided good resolution contributing to the overall topology. The cladogram of maximum-parsimony analyzes presented only two polytomies, recovering *R. marina* group as monophyletic (80% bootstrap value).

Grant et al. (2006) used lipophilic alkaloids to infer relationships among dart-poison frogs (Athesphatanura) and their relatives. Although this skin-secretion chemical group is known to be of dietary origin, there is a hereditary aspect to the alkaloid-uptake system (Daly, 1998). Hypotheses of homology can therefore be proposed, albeit cautiously, for alkaloid profiles. Aromatic amines (including indolealkylamines) are known to originate from the metabolism of amphibians and can provide phylogenetic signal (Maciel et al., 2006). Also, an analysis of variation (individual, seasonal, and sexual variation) among parotoid-gland skin-secretion characters of *R. schneideri* (our unpublished data) indicates a lack

of variation among individuals within species, supporting usage of these characters for inferring interspecific relationships.

4.2. Biogeographic scenario (molecular dating and ancestral areas)

The results reinforce the importance of Tertiary events in the diversification of the Neotropical fauna. Our phylogenetic analyzes and molecular dating indicate that the ancestor of the *R. marina* group was present in late Miocene ~10.47 MYA (10.01–11.96 MYA credibility interval), somewhat later than previously proposed at ~13.91 MYA (10.65–17.17 MYA credibility interval) (Vallinoto et al., 2010). However, the results of Vallinoto et al. (2010) were presented considering the *R. marina* group paraphyletic with respect to *R. crucifer*. During late Miocene the uplift of the Brazilian Central Shield drastically changed the landscape, creating barriers and dispersal routes (Brasil and Alvarenga, 1989; Del'Arco and Bezerra, 1989). We hypothesize that this event caused the initial diversification of the *R. marina* species group. Although the ancestral area analysis performed with DIVA could not determine the exact ancestral area of the most recent common ancestor of all species in the *R. marina* group (Fig. 5), we suggest that the Brazilian Central Shield (area G) is the most likely ancestral area of the two major clades of *R. marina* (Fig. 3). *Rhinella rubescens* in the south-central clade and *R. veredas*, *R. schneideri*, and *R. cerradensis* in the north-central clade (Fig. 3) currently occur in the Brazilian central shield (Fig. 1a), and the remaining species of both clades occur only in the northern or southern parts of the continent (Fig. 1a). Besides, the ancestral area of the south-central clade was suggested to be meridional South America (area G), and the divergence of the two major clades was explained by vicariance (Fig. 5).

Our results also suggest that the ancestor of the north-central clade (Fig. 3) diversified at ~8.08 MYA (3.75–10.68 MYA credibility interval), later in the Miocene, northward, by the routes and dispersal barriers created by the uplift of the Brazilian Central Shield. All these species occur in Central Brazil (*R. veredas*, *R. cerradensis*, and *R. schneideri*) or northern South America (*R. jimi*, *R. marina*, and *R. poeppigii*) (see Fig. 1a). With the exception of *R. veredas* and *R. cerradensis*, which may have diversified by *in situ* vicariance and, as already stated, are distributed in Central Brazil, all other processes could be explained by dispersal in DIVA (Fig. 5). All speciation in this group appears to have occurred by contiguous geographic expansion of populations in times of widespread favorable habitat, followed by vicariant fragmentation as geographic barriers later intervened.

The south-central clade (Fig. 3) appeared at approximately 5.17 MYA (2.58–10.89 MYA credibility interval) in the early Pliocene. The new configuration of landscape and the continued uplift of Brazilian Central Shield, combined with the climatic changes (e.g. Moritz et al., 2000) could have driven the diversification of a later lineage of the *R. marina* group (south-central clade) in the south. All the species in this lineage, with the exception of *R. rubescens*, are currently distributed in the meridional part of South America (Fig. 1b). These species are small when compared with the other species of the *R. marina* group and also have a smoother skin texture. The northern *R. marina* and *R. poeppigii* (belonging to the first clade to radiate; north-central clade) also show a silky skin but are currently found in forested areas (Fig. 1a). The geoclimatic changes were obviously important in the interaction with the ecological properties of each group of organisms. The rough and keratinous skin of the species such as *R. veredas*, *R. cerradensis*, *R. jimi*, and *R. schneideri* are clearly adaptations to arid environments that could reflect the importance of past climatic changes in the evolutionary history of this group of toads. The marine incursion was an obvious dispersal barrier in the Miocene; however, its ecological impact did not cease when the waters receded, because it permanently altered soil properties and river water characteristics (Tuomisto, 2007). These events and the ecological traits (e.g. bimodal lifestyle of the group) could explain why the northern species of the *R. marina* group diversified earlier than did the southern ones.

In the Pliocene epoch, rainfall probably varied regionally in a mosaic of habitats controlled by river migration, sea level fluctuations, local dryness, and local uplift (Burnham and Graham, 1999) and could also drive local diversifications. The emergence of a continuous land bridge at 3 MYA between Central and South America is well documented and could explain the invasion of *R. marina* populations beyond the Panama Isthmus as well as the arrival of North American immigrants (Burnham and Graham, 1999). In fact, Mulcahy et al. (2006) hypothesize a Pliocene dispersal of populations of *R. marina* in the same area. The continuing uplift of the Andes, the formation of the Panamanian land bridge between the Chocó and Central America, which formed progressively until the Pliocene (Coates and Obando, 1996), and formation of the Amazon River could be important biogeographic events at the Miocene–Pliocene boundary, explaining the diversification of the forest northern species of the *R. marina* group. *Rhinella poeppigii* occurs at elevations of 600–1870 m of Central Oriental Andes (area B of DIVA in the present work) (Toft and Duellman, 1979) diversifying very recently at ~3.1 MYA (0.60–4.09 MYA credibility interval) in the Pliocene (Fig. 4). In fact, the events occurring in the Late Tertiary and Quaternary in the Andes and Amazon Basin created tremendous diversity such as that of the poison dart frogs (Santos et al., 2009). The formation of the other South American hydrological basins (e.g. Araguaia-Tocantins, Paraíba, São Francisco, and Paraná-Paraguai) could also be important in shaping the current distributions of the remaining species of the *R. marina* group. The formation of riverine basins comprises the modification of terrestrial environments through which water moves toward an outlet, and this alteration could also drive the diversification of a group of organisms. If information on the present-day environmental conditions may be difficult to obtain, it is certainly much more difficult to reconstruct for the past (Tuomisto, 2007). The *R. marina* species group is composed of species specialized for open (*R. veredas*, *R. schneideri*, *R. cerradensis*, *R. jimi*, *R. achavali*, and *R. rubescens*) and forested areas (*R. marina*, *R. poeppigii*, *R. arenarum*, and *R. icterica*) (Fig. 1a and b). If the species are specialized for a specific type of river, forest, or savannah, the possible dispersal barriers in the past might have been more extensive, and the dispersal routes more restricted, than is immediately apparent. These could also suggest a role for local and ecological components in explaining the presence of open and forested habitat species among *R. marina* species throughout the distribution of the *R. marina* group.

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The euthanasia methodology was authorized by the Ethical committee from the Universidade de Brasília (CEUA).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.08.025.

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