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Phylogeny and biogeography of the *Rhinella marina* species complex (Amphibia, Bufonidae) revisited: implications for Neotropical diversification hypotheses

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A number of distinct hypotheses have been proposed to account for the origin of the considerable biological diversity found in the Neotropics, which is still a matter of intense debate. Here, we conducted a phylogenetic analysis of the *Rhinella marina* complex, a group of species widely distributed in Central and South America, combining published data with new sequences of three mtDNA genes (12S, 16S and cyt b) in order to clarify the evolutionary relationships and biogeographical history of the group. We included eight of the ten currently recognized *R. marina* group species and several outgroups. Maximum parsimony, maximum likelihood, and Bayesian inference analyses produced similar topologies, with two well-supported main clades, each characterized by a deep subdivision. One of these major clades includes the samples of *R. marina* from Central America and Ecuador (west of the Andes), whereas the other comprises the remaining species of the group and samples of *R. marina* from the Amazon basin and other areas east of the Andes. A Bayesian coalescent-based method (BEAST) dated the divergence between the two major clades, and between the Central American and Ecuadorian clades to the Miocene, matching the timing of other Central-South American faunal divergences. Taken together, the results highlight the importance of Tertiary events such as the Pebas/marine incursions into the Amazon basin and Andean uplift for the diversification and historical biogeography of *R. marina*, making such taxa paraphyletic, and provide new perspectives on the debate on its species status.

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Introduction

The Neotropics encompass a number of important biodiversity hotspots, characterized by large numbers of endemic species and high α-diversity (e.g. Myers *et al.* 2000; Pitman *et al.* 2001). Several hypotheses have been proposed to account for such high diversity. Traditional models have concentrated on vicariant events (see Haffer 1997), such as those caused by the climatic instability of the Pliocene-Pleistocene (e.g. Refugia theory; disturbance-vicariance hypothesis) or resulting from the formation of the Amazon River and its tributaries (e.g. riverine hypothesis). However, more recent models (e.g. Moritz *et al.*

2000; Nores 2004; Wesselingh & Salo 2006; Rull 2008) have emphasized the role of major paleogeographical events such as the Andean uplift, closure of the Panama Isthmus, and changes in sea levels and the formation of river systems on the geographical patterns of Neotropical organisms. Recent phylogenetic and phylogeographic studies have emphasized the influence of such events on patterns of diversification, reporting profound genetic divergence between populations in the eastern and western Amazon basin, and from Central and South America in many taxonomic groups, including birds, mammals and amphibians (Hoffmann & Baker 2003; Brumfield & Edwards 2007; Nyári 2007; DaCosta & Klicka 2008; Gamble *et al.* 2008; Santos *et al.* 2009).

While molecular studies are still incipient, there is growing evidence that the factors determining biogeographical patterns are largely dependent on the biology of each taxon (e.g. Eberhard & Bermingham 2005; Velazco & Patterson 2008). Given this, a comprehensive understanding of diversification in the Neotropics will depend on the phylogeographic and phylogenetic reconstruction of a wide range of organisms with distinct ranges, ecological requirements and dispersal capabilities. In this context, one especially appropriate group of species for the investigation of the different hypotheses of diversification is the *Rhinella marina* complex (formerly *Bufo marinus* complex), a group of Neotropical toads. The group is diagnosed morphologically by a combination of traits, such as the presence of a 'scalloped' suture at the point of articulation between the medial ramus of the pterygoid and parasphenoidalae, the anterior edge of the sacral diapophyses angled posterolaterally to the longitudinal axis of the vertebrae, and the presence of distinctively large parotoid glands (Pramuk 2006). Traditionally, the group has encompassed seven species (Frost 2007) – *R. marina*, *R. arenarum*, *R. ictericus*, *R. jimi*, *R. poeppigii*, *R. rubescens* and *Rhinella schneideri* – although three new species have been described in recent years: *R. veredas* (Brandão *et al.* 2007), *R. achavalli* (Maneyro *et al.* 2004) and *R. cerradensis* (Maciel *et al.* 2007). With the exception of *R. marina*, which ranges as far north as the southern tip of Texas, the group is endemic to South America, where it is found as far south as Argentina (Fig. 1A and B) (Blair 1972; Cei 1972; Pauly *et al.* 2004; Frost 2007).

Previous phylogeographic/phylogenetic studies have focused on the widespread *R. marina*. Slade & Moritz (1998) analysed the genetic divergence of populations from Central and South America, and found *R. marina* to be paraphyletic, with *R. schneideri* grouping with the *R. marina* populations from South America. The results of this study indicated that the South and Central American populations of *R. marina* diverged during the Tertiary orogeny of the Andes. More recently, Mulcahy *et al.* (2006) showed that specimens of *R. marina* from Central America were very different from a sample from Ecuador, with an estimated time of divergence in the Miocene (≈10 Ma). The most complete recent phylogenetic study of the *R. marina* group was that of Pramuk (2006), which combined sequences of mitochondrial (12S, tRNAVal, and 16S) and nuclear (POMC; Rag-1) genes with the analysis of 83 morphological characters of six species. The results of this study indicated that *R. marina* forms a monophyletic clade, which is sister group to the *R. schneideri–R. poeppigii* clade, while *R. rubescens* is the most basal species. However, the evolutionary relationships inferred from this analysis should be treated with caution, given that samples of *R. marina* from only two regions (Central America and Ecuador) were included. In addition, this study focused on the entire South America *Rhinella* (former *Bufo*) genus and not only the *R. marina* species group.

In this study, we combine published data with new mitochondrial sequences of representatives of the *R. marina* species complex, including populations of *R. marina* from the eastern Amazon Basin, and the first samples of the recently described *R. achavalli* and *R. jimi* (Fig. 1C). We analysed two sets of sequences (12S rDNA + 16S rDNA and 16S rDNA + Cyt b) separately in order to provide a more ample perspective on the evolutionary history and interspecific relationships within the *marina* group. Using phylogenetic and recently developed Bayesian coalescent-based methods, we aimed to estimate the extent of divergence among clades and interpret temporal variables in the context of our current knowledge of general biogeographic patterns in the region.

Materials and methods

Taxon sampling and laboratory methods

To examine the relationships among species of the *R. marina* group, all the currently recognized species were included in this study (Table 1), except for the recently proposed *R. cerradensis* and *R. veredas*, for which samples were unavailable. We collected samples of *R. achavalli*, *R. jimi*, *R. poeppigii*, *R. arenarum*, *R. marina* and *R. granulosa* (outgroup). Sequences of *R. marina* were obtained from four populations in the eastern Amazon Basin in Brazil (Fig. 1C; Table 1).

Total genomic DNA was extracted from samples (which were preserved in 70% ethanol) using the standard phenol-chloroform method, followed by sodium acetate precipitation (Sambrook *et al.* 1989). A 1946 bp-long fragment of the 12S and 16S ribosomal mtDNA genes and a 327 bp-long fragment of the mitochondrial cytochrome subunit b (cyt b) were amplified by polymerase chain reaction (PCR) using the 12SKH and 16H13 (Pramuk 2006),

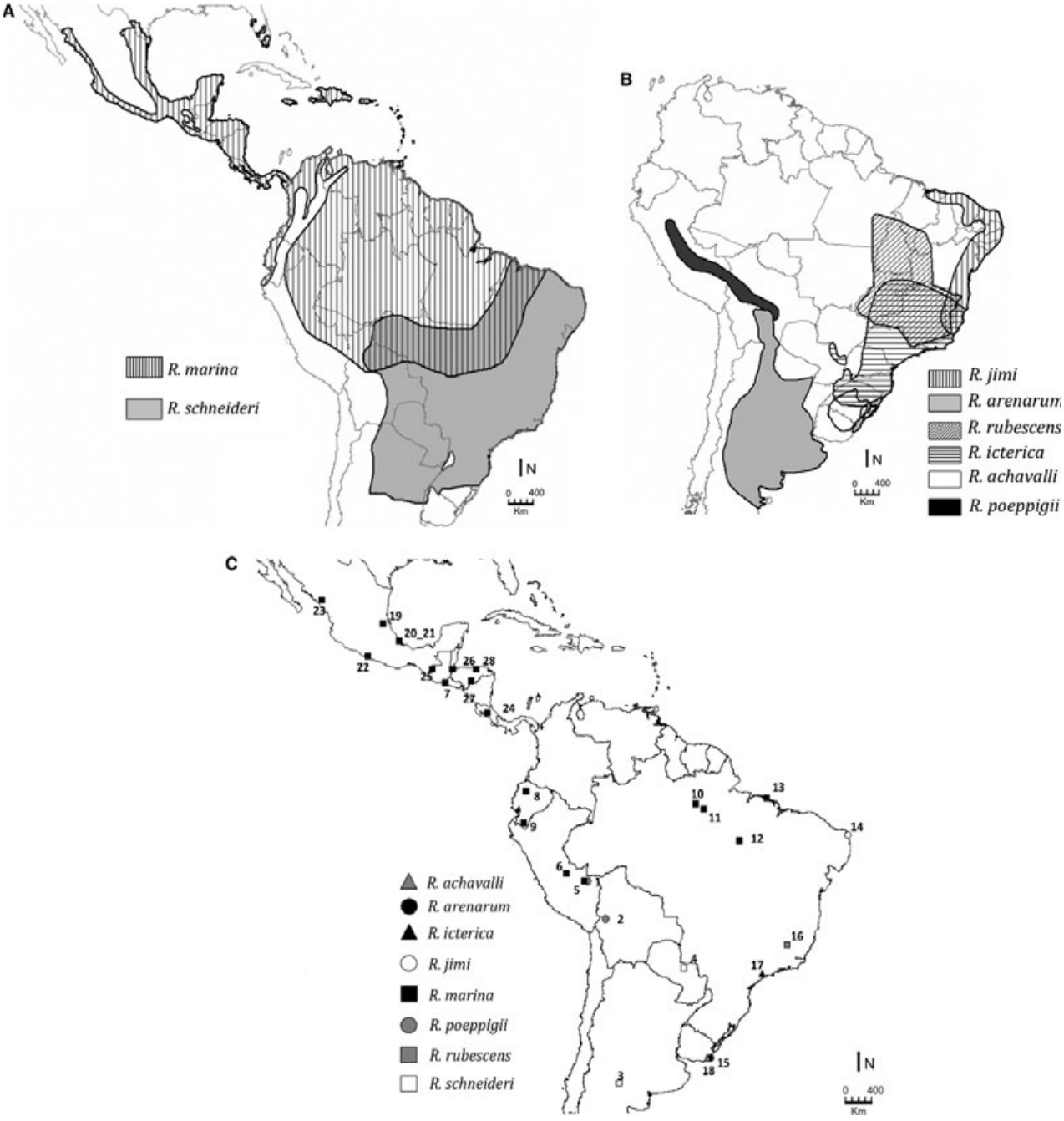

Fig. 1 Map showing geographic distributions of species of the *Rhinella marina* complex (A and B) and the collecting localities for those samples used in the present study (C). See Table 1 for specific information on each locality.

and cytbFor and cytbRev (Lamb *et al.* 2000) primers, respectively. Amplifications were performed using ~10 ng of genomic DNA, Tris–HCl pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 5 pMol of each primer and 1 U Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA). The PCRs were performed in a MJ Research thermocycler with a cycling procedure of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 and 54 °C (12S–16S and cyt b, respectively) for 1 min, 72 °C for 2 and 1 min (12–16S and cyt b, respectively), and a single final step at 72 °C for 5 min. For sequencing, we used the primers mentioned above, and in the case of

Table 1 Specimens and respective sample code and voucher number (numbers in parenthesis are used for geographical representation in Fig. 1), sampling localities, and GenBank accession numbers for sequences analysed in this study.

Sample code	Species	Voucher	Locality	GenBank			Reference
				12S–16S	16S	Cyt b	
Rpo1 (1)	*R. poeppigii*	USNM 268824	Peru: Madre Dios	DQ158481			Pramuk 2006
Rpo2 (2)	*R. poeppigii*	MNCN/ADN6044	Bolivia: La Paz	GU178779	GU178790	GU178801	This study
Rsc1 (3)	*R. schneideri*	BB 1224	Argentina: Santiago del Estero	DQ283065			Frost *et al.* 2006
Rsc2 (4)	*R. schneideri*	KU 289057	Paraguay: Parque Nacional San Luis de la Sierra	DQ158480			Pramuk 2006
Rsch (4)	*R. schneideri*	KU 289057	Paraguay: San Luis de la Sierra		DQ415572	DQ415598	Mulcahy *et al.* 2006
Rma1 (5)	*Rhinella marina*	AY325994	Peru: Cuzco	AY325994			Mulcahy *et al.* 2006
Rma2 (6)	*R. marina*	MJH 3678	Peru: Puerto Inca	DQ283062			Frost *et al.* 2006
Rma3 (7)	*R. marina*	KU 289750	El Salvador: Ahuachapan	DQ158473			Pramuk 2006
Rma4 (8)	*R. marina*	KU 202274	Ecuador: Pichincha	AY680259			Pauly *et al.* 2004
Rma5 (9)	*R. marina*	KU 217482	Ecuador: Loja, Vilcabamba	DQ158474			Pramuk 2006
Rma5 (9)	*R. marina*	KU 217482	Ecuador: Loja, Vilcabamba		DQ415571	DQ415597	Mulcahy *et al.* 2006
Rma6 (10)	*R. marina*	Toe-clip only	Brazil: Porto Trombetas	GU178780	GU178791	GU178802	This study
Rma7 (11)	*R. marina*	Toe-clip only	Brazil: Santarém	GU178781	GU178792	GU178803	This study
Rma8 (12)	*R. marina*	Toe-clip only	Brazil: Canaã dos Carajás	GU178782	GU178793	GU178804	This study
Rma9 (13)	*R. marina*	Toe-clip only	Brazil: Viseu	GU178783	GU178794	GU178805	This study
Rji (14)	*R. jimi*	Toe-clip only	Brazil: Natal	GU178784	GU178795	GU178806	This study
Rar1	*R. arenarum*	AR 305	Argentina	DQ158429			Pramuk 2006
Rar2 (15)	*R. arenarum*	Toe-clip only	Uruguay: Rocha	GU178785	GU178796	GU178807	This study
Rru (16)	*R. rubescens*	AF 388	Brazil: Minas Gerais: Santa Barbara	DQ158486			Pramuk 2006
Ric1 (17)	*R. icterica*	AF 312	Brazil: São Paulo: Carapicuiba	DQ158462			Pramuk 2006
Ric2 (17)	*R. icterica*	Toe-clip only	Brazil: São Paulo	GU178786	GU178797	GU178808	This study
Rac (18)	*R. achavalli*	ZVCB 3801	Uruguay	GU178787	GU178798	GU178809	This study
Mx8a (19)	*R. marina*	UTA A-54875	Mexico: Veracruz: north of Palma Sola		DQ415547	DQ415573	Mulcahy *et al.* 2006
Mx8b (19)	*R. marina*	UNAM-JRM 4846	Mexico: Veracruz: north of Palma Sola		DQ415548	DQ415574	Mulcahy *et al.* 2006
Mx8c (19)	*R. marina*	UNAM-JRM 4848	Mexico: Veracruz: north of Palma Sola		DQ415549	DQ415575	Mulcahy *et al.* 2006
Mx8d (19)	*R. marina*	UNAM-JRM 4844	Mexico: Veracruz: north of Palma Sola		DQ415550	Q415576	Mulcahy *et al.* 2006
Mx9a (20)	*R. marina*	UTA A-54882	Mexico: Veracruz: near El Viejón		DQ415551	DQ415577	Mulcahy *et al.* 2006
Mx9b (20)	*R. marina*	UTA A-54873	Mexico: Veracruz: near El Viejón		DQ415552	DQ415578	Mulcahy *et al.* 2006
Mx9c (20)	*R. marina*	UNAM-JRM 4834	Mexico: Veracruz: near El Viejón		DQ415553	DQ415579	Mulcahy *et al.* 2006
Mx9d (20)	*R. marina*	UNAM-JRM 4835	Mexico: Veracruz: near El Viejón		DQ415554	DQ415580	Mulcahy *et al.* 2006
Mx9e (20)	*R. marina*	UTA A-54881	Mexico: Veracruz: near El Viejón		DQ415556	DQ415582	Mulcahy *et al.* 2006
Mx9f (20)	*R. marina*	UTA A-54879	Mexico: Veracruz: near El Viejón		DQ415555	DQ415581	Mulcahy *et al.* 2006
Mx9g (20)	*R. marina*	UTA A-54877	Mexico: Veracruz: near El Viejón		DQ415557	DQ415583	Mulcahy *et al.* 2006
Mx10a (20)	*R. marina*	UTA A-54878	Mexico: Veracruz: near El Viejón		DQ415558	DQ415584	Mulcahy *et al.* 2006
Mx10b (21)	*R. marina*	UTA A-54871	Mexico: Veracruz: south of Cardel		DQ415559	DQ415585	Mulcahy *et al.* 2006
Mx11a (22)	*R. marina*	UTA A-54869	Mexico: Guerrero: near Atoyac		DQ415560	DQ415586	Mulcahy *et al.* 2006
Mx11b (22)	*R. marina*	UTA A-54870	Mexico: Guerrero: near Atoyac		DQ415561	DQ415587	Mulcahy *et al.* 2006
Mx12 (23)	*R. marina*	UTA A-54868	Mexico: Sinaloa: near Cosala		DQ415562	DQ415588	Mulcahy *et al.* 2006
C1 (24)	*R. marina*	Toe-clip only	Costa Rica: Heredia: at Chilamate		DQ415563	DQ415589	Mulcahy *et al.* 2006
E1 (7)	*R. marina*	KU 289750	El Salvador: Ahuachapan: El Imposible		DQ415564	DQ415590	Mulcahy *et al.* 2006
E2 (7)	*R. marina*	KU 289772	El Salvador: Ahuachapan: El Imposible		DQ415565	DQ415591	Mulcahy *et al.* 2006
G1 (25)	*R. marina*	UTA A-50876	Guatemala: Huehuetenango: near Nenton		DQ415566	DQ415592	Mulcahy *et al.* 2006
G5 (26)	*R. marina*	UTA A-50870	Guatemala: Izabal: Montanas del Mico		DQ415567	DQ415593	Mulcahy *et al.* 2006
H3 (27)	*R. marina*	UTA A-50638	Honduras: El Paraiso: Las Manos		DQ415568	DQ415594	Mulcahy *et al.* 2006
H4 (28)	*R. marina*	USNM 534124	Honduras: Colon: Quebrada Machin		DQ415569	DQ415595	Mulcahy *et al.* 2006
Rcr	*R. crucifer*	USNM 303015	Brazil: São Paulo	DQ158447			Pramuk 2006
Rcr	*R. crucifer*	USNM 303015	Brazil: São Paulo		DQ415570	DQ415596	Mulcahy *et al.* 2006
Rgr1	*R. granulosa*	USNM 302450	Brazil: Roraima	DQ158457			Pramuk 2006
Rgr2	*R. granulosa*	Toe-clip only	Brazil: Porto Trombetas	GU178788	GU178799	GU178810	This study
Rgr3	*R. granulosa*	Toe-clip only	Brazil: Porto Trombetas	GU178789	GU178800	GU178811	This study
Aex	*A. exsul*	MVZ 137717	USA: California, Buckhorn Spring	DQ158450			Pramuk 2006
Afo	*A. fowleri*	USNM 314864	USA: Mississippi: Oktibbeha, Starkville	DQ158451			Pramuk 2006
Ami	*A. microscaphus*	USNM 320147	USA: New Mexico	DQ158476			Pramuk 2006
Cal	*C. alvarius*	USNM 320001	USA: Arizona	DQ158425			Pramuk 2006
Cva	*C. valliceps*	USNM 534129	Mexico: Vera Cruz	DQ158493			Pramuk 2006
Clu	*C. luetkenii*	KU 289850	El Salvador: Usulutan, Cerro del Tigre	DQ158467			Pramuk 2006
Bbu	*B. bufo*	MVZ 230209	Turkey: Bursa Province	DQ158438			Pramuk 2006

12S–16S, we used the internal primers 16H14(R) mod and 16L10 described by Pramuk (2006). Sequences were obtained on an ABI PRISM 3130 XL Genetic Analyser, following the ABI PRISM BigDye Terminator Cycle Sequencing protocol.

Phylogenetic analyses

In order to examine the phylogenetic relationships within the *R. marina* group, we sequenced a fragment of the 12S–16S fragment of ribosomal mtDNA of a number of different samples of *R. marina* from the eastern Amazon Basin, in addition to *R. jimi*, *R. poeppigii*, *R. achavalli*, *R. arenarum* and the outgroup *R. granulosa*. These sequences were combined with those available in the literature for other species of the *R. marina* complex, species of other *Rhinella* groups and of the genera *Anaxyrus*, *Cranopsis* and *Bufo*. The resulting matrix included a total of 31 individuals, belonging to eight *R. marina* complex species and eleven outgroup species (Table 1).

Because only three 12S–16S fragment sequences of *R. marina* are available in the literature (Pramuk 2006), representing Central America and Ecuador, we also analysed a combined data set of the 16S ribosomal (547 bp) and cyt b (327 bp) mtDNA genes in order to verify phylogenetic relationships. As sequences of these fragments are available for a large number of Central America populations (Mulcahy *et al.* 2006), the aim here was to trace historical biogeographic patterns. Similarly, we sequenced a fragment of both cyt b and 16S for all our samples, which were added to published sequences to produce a data set of 37 individuals, representing eight *R. marina* complex species, in addition to *R. crucifer* and two samples of *R. granulosa* as outgroups (Table 1).

The sequences were aligned in BioEdit 5.0.6 (Hall 1999). Maximum parsimony (MP) and maximum likelihood (ML) methods, and Bayesian inference (BI) were applied to the evaluation of phylogenetic relationships. The approach outlined by Huelsenbeck & Crandall (1997) was used to test alternative models of evolution, employing PAUP*4.0b10 (Swofford, 2001) and Modeltest 3.06 (Posada & Crandall 1998). Indels were coded as additional characters. The evolutionary model that best fit the data was determined by the Akaike Information Criterion (AIC) as implemented by Modeltest. Bayesian analyses were conducted using MrBayes v3.0b4 (Huelsenbeck & Ronquist 2001) with four default heated chains, each with five million generations, sampled every 100 generations, with the application of the stop rule command. Runs were checked for convergence using the AWTY graphical analysis (Nylander *et al.* 2007). The first 200 trees of each run were discarded as burn-in, and a consensus of the remaining trees was computed for the final outcome. Maximum parsimony and ML analyses were carried out in PAUP*4.0b10 (heuristic search, tree bisection-reconnection) and support for nodes was estimated by bootstrapping with 1000 pseudo-replicates (Felsenstein 1985), providing an estimate of the confidence limits for the resulting topologies.

Divergence time estimates

We used the combined 12S–16S rDNA data set to estimate divergence times because we had more sequences for this fragment and split time estimates based on this fragment are available for several other bufonid groups (Pramuk *et al.* 2008). These groups were used as calibration points for our Bayesian approach (see below). The Pramuk *et al.* (2008) estimate of the origin of the bufonids was used in a Bayesian coalescent approach, together with the relaxed (uncorrelated lognormal) molecular clock method implemented in BEAST version 1.4.8 (Drummond & Rambaut 2007), to estimate time to the most recent common ancestor (TMRCA) and the 95% credibility intervals (CI) for various clades. For calibration, we used the estimated time of divergence between the Old World *Bufo bufo* and the New world genera *Rhinella*, *Cranopsis* and *Anaxyrus* (43.3 Ma), and that between the South America genus *Rhinella* and the Central/North America genera *Cranopsis* and *Anaxyrus* (41 Ma) inferred by Pramuk *et al.* (2008) recovered based on different fossil calibrations and divergence times from the literature. We conducted two independent runs (chain length of 25 000 000; sampled every 1000 iterations; Yule speciation process; 10% burn-in). The results were checked for convergence, and the posterior age distributions were analysed using Tracer v1.4 (Rambaut & Drummond 2007). A GTR + I + G model of nucleotide substitution was used, based on ModelTest (see below). Tree topologies were assessed using TreeAnnotator v.1.4.6 (distributed as part of the BEAST package) and FigTree v.1.1.2 (Rambaut 2008).

Results

Phylogenetic analysis

The addition of 12S–16S mtDNA sequences from GenBank to our data produced a combined character matrix of 1946 nucleotides (570 variables, 401 parsimony informatives). The most appropriate model of evolution for this data set was the GTR + I + G model with base frequencies: Lset Base = (0.3539 0.2057 0.1797) Nst = 6 Rmat = (3.0455 12.4172 7.4414 0.3123 39.8401) Rates = gamma Shape = 0.8256 Pinvar = 0.5542. Using this model, we found a ML tree of $-\ln L = 8404.85036$. Maximum parsimony analysis returned 68 equally parsimonious trees, 1259 steps in length (consistency index excluding uninformative sites = 0.614; retention index = 0.730). The Bayes-

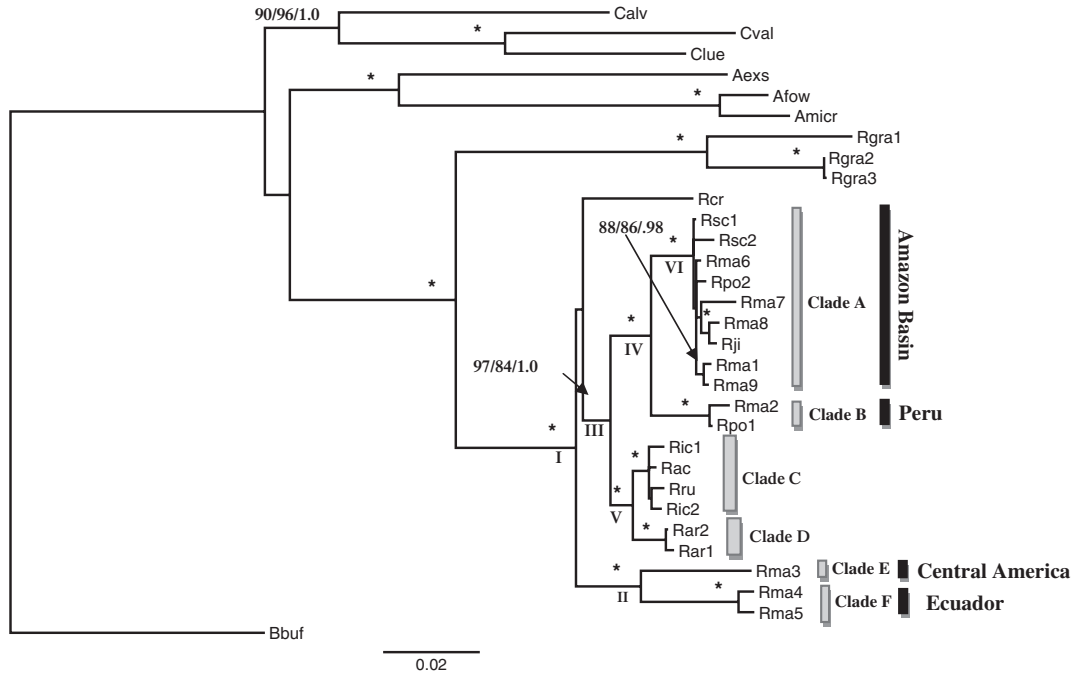

Fig. 2 Phylogenetic relationships among species of the *Rhinella marina* complex based on the ML analysis of the 12S–16S fragment. Numbers above the branches are maximum likelihood and maximum parsimony bootstrap values, and Bayesian posterior probability values, respectively. Maximum parsimony and ML bootstrap values higher than 90% and posterior probabilities higher than 0.9 are marked with an asterisk (*).

ian posterior probabilities tree is depicted in Fig. 2, with ML and MP bootstrap values overlaid.

The alignment of the concatenated 16S + Cyt b sequences contained 938 bp for phylogenetic analysis (184 variable and 144 parsimony-informative sites). The model selected for this data set was HKY + G (Lset Base = (0.2805 0.2559 0.1621) Nst = 2 TRatio = 7.7032 Rates = gamma Shape = 0.1604 Pinvar = 0). In Fig. 3 the Bayesian analysis is presented (180 trees and 310 steps; I.C. 0.716 and R.I. 0.897), together with posterior probability and bootstrap values from ML (−ln *L* = 2840.37609) and MP analyses.

The different trees derived from the 12S–16S data set presented almost identical topology (Fig. 2). The analyses were unanimous on the non-monophyletic status of the *R. marina* group, supporting instead a trichotomy involving: (i) *R. marina* from Central America and Ecuador; (ii) all other members of the *R. marina* group, including *R. marina* from the eastern Amazon Basin; (iii) *R. crucifer*, from eastern Brazil. Within the second group, high bootstrap values and Bayesian posterior probabilities support a clade comprising *R. achavalli*, *R. icterica*, and *R. rubescens*, which is the sister group of *R. arenarum*, a well as a polytomy involving *R. marina* from different Amazonian sites (in Peru and Brazil), *R. jimi*, *R. schneideri* and *R. poeppigii*.

Phylogenetic inferences derived from the analysis of the concatenated 16S + Cyt b data set were also nearly identical across the different methods, and are broadly congruent with the results of the previous analysis, despite lower bootstrap values and Bayesian probabilities. The same basic trichotomy was also identified, although, in this case, the phylogenetic positions of *R. arenarum*, *R. icterica*, *R. rubescens* and *R. achavalli* were not resolved.

Sequence divergence and estimation of divergence times

Sequence divergence (uncorrected *p*-distance) between *R. marina* group samples are presented in Table 2 (see also Fig. 4). Very high levels of nucleotide divergence (5.8–6.4%) were recorded between the two *R. marina* clades (eastern Amazon/Peru vs. Central America/Ecuador). Values between the Central America and Ecuadorian samples of *R. marina* were also relatively high (4.5%). Interestingly, these values are much higher than those found between the eastern Amazon/Peru clade and other *R. marina* complex species (2.5–3.2%), or between species of the latter group (Table 2).

Divergence time estimates for the main clades are shown in Table 3 (see also Fig. 2). Our estimates indicate that the Central America/Ecuador *R. marina* clade split from the remaining populations (including that of *R. cruci-*

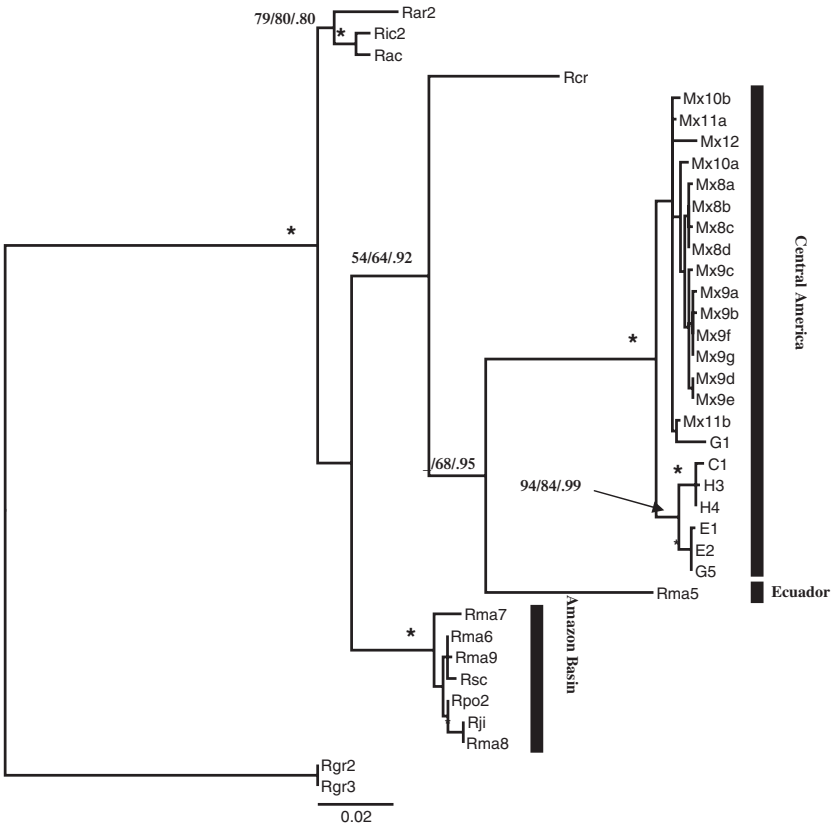

Fig. 3 Phylogenetic relationships among species of the *Rhinella marina* complex based on the ML analysis of Cyt *b* and 16S sequences. Numbers above the branches are maximum likelihood and maximum parsimony bootstrap values, and Bayesian posterior probability values, respectively. Maximum Parsimony and ML bootstrap values higher than 90% and posterior probabilities higher than 0.9 are marked with an asterisk (*).

Table 2 Pairwise uncorrected (*p*-distance) percentage of 12S–16S sequence divergence among *Rhinella marina* group. Sample codes are as in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *Rhinella marina* (Rma1)	–																		
2 *R. marina* (Rma2)	2.5	–																	
3 *R. marina* (Rma3)	5.8	6.4	–																
4 *R. marina* (Rma4)	5.7	6.5	4.5	–															
5 *R. marina* (Rma5)	5.9	6.8	4.4	0.6	–														
6 *R. marina* (Rma8)	0.8	2.8	6.1	5.8	6.0	–													
7 *R. marina* (Rma6)	0.4	2.5	5.8	5.7	5.9	0.6	–												
8 *R. marina* (Rma9)	0.3	2.4	5.9	5.8	6.0	0.8	0.4	–											
9 *R. marina* (Rma7)	1.1	3.2	6.3	6.2	6.2	1.1	0.9	1.0	–										
10 *R. schneideri* (Rsc1)	0.4	2.5	5.6	5.5	5.7	0.5	0.2	0.4	0.9	–									
11 *R. schneideri* (Rsc2)	0.8	3.0	6.0	6.0	6.2	1.0	0.6	0.8	1.3	0.5	–								
12 *R. jimi* (Rji)	0.8	2.8	6.0	5.7	5.8	0.4	0.5	0.7	1.0	0.5	0.9	–							
13 *R. poeppigii* (Rpo1)	2.3	0.5	5.9	6.0	6.2	2.6	2.3	2.2	2.9	2.2	2.7	2.5	–						
14 *R. poeppigii* (Rpo2)	0.5	2.6	5.9	5.8	6.0	0.7	0.3	0.5	1.0	0.3	0.7	0.6	2.4	–					
15 *R. icterica* (Ric1)	3.0	3.6	5.4	5.3	5.5	2.9	2.8	2.9	3.3	2.7	3.2	2.8	3.1	3.0	–				
16 *R. icterica* (Ric2)	3.0	3.5	5.3	5.4	5.6	3.0	2.9	3.0	3.5	2.8	3.3	2.9	3.0	3.0	0.6	–			
17 *R. arenarum* (Rar2)	3.2	3.7	5.6	5.2	5.4	3.1	3.0	3.1	3.4	2.9	3.4	3.0	3.2	3.2	1.4	1.2	–		
18 *R. arenarum* (Rar1)	3.4	4.0	5.9	5.6	5.8	3.4	3.3	3.4	3.7	3.2	3.7	3.3	3.4	3.4	1.5	1.3	0.2	–	
19 *R. achavalli* (Rac)	2.9	3.4	5.1	5.2	5.4	2.8	2.8	2.8	3.4	2.6	3.2	2.8	2.9	2.9	0.5	0.4	1.2	1.3	–

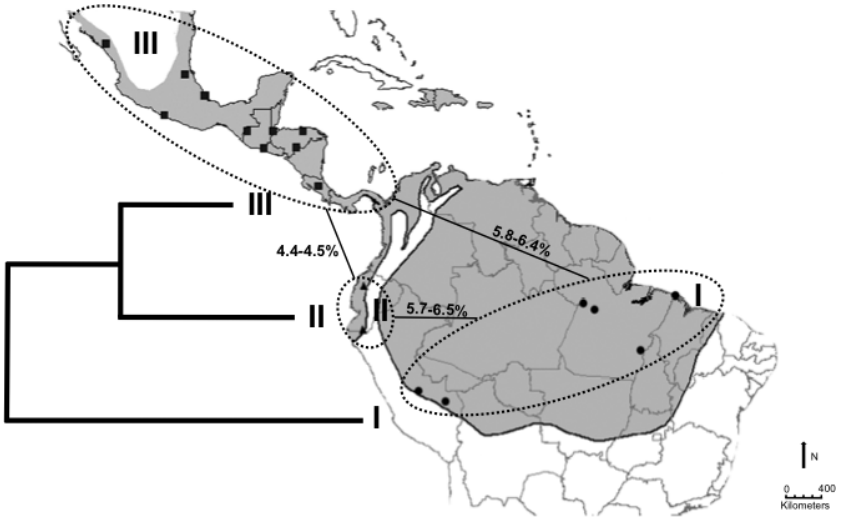

Fig. 4 Geographic locations and pairwise average uncorrected *p*-distances between the three main clades (delimited by the dotted line) of *Rhinella marina* defined by the phylogenetic analysis of the 12S–16S fragment. The shaded area represents the known present-day distribution of *R. marina* (Frost 2007).

Table 3 Divergence time estimates (with 95% credibility interval) in million of years (Ma) for the main nodes recovered in phylogenetic analysis of the *Rhinella marina* group (see Fig. 2).

Nodes	TMRCA (Ma)	95% Credibility interval
I	13.915	10.654–17.175
II	9.139	6.253–12.141
III	8.659	6.274–11.153
IV	5.828	4.025–7.72
V	4.039	2.379–5.837
VI	2.881	1.577–3.315

Cladogenetic events were dated based on Bayesian coalescent phylogenetic estimation of time to most recent common ancestor (TMRCA) assuming a relaxed molecular clock (as implemented in BEAST) for 12S–16S data set.

fer) at approximately 13.0 Ma (node I), while *R. marina* from eastern Amazonia separated around 9.1 Ma (node II). The split of the remaining species took place around 8.7 Ma (node III), and within this grouping, clades A and B split at approximately 6 Ma (node IV), while clades C and D split around 4 Ma (node V), and the most recent event (node VI) was dated at 2.8 Ma.

Discussion

Phylogenetic relationships

Our results provide the most comprehensive analysis to date of phylogenetic relationships within the *R. marina* species complex. We have expanded previous analyses by creating a data set that includes most of the currently recognized species, as well as including new specimens of *R. marina* from the eastern Amazon basin.

Our inferences from both 12S–16S and 16S + cyt b sequences do not support the monophyly of the *R. marina* complex, not least because the outgroup species *R. crucifer* is included in one of the major clades. The two primary clades indicate the phylogeographic role of the Andes, which separate the *R. marina* populations from El Salvador/Ecuador (west of the Andes) from the remaining species, including *R. crucifer* (Fig. 2). A close phylogenetic relationship between *R. crucifer* and the *R. marina* species group was found by both Cei (1972), based on their morphological similarities, and in the mtDNA data of Pauly *et al.* (2004). More recently, Pramuk (2006) placed *R. crucifer* at the base of a weakly supported monophyletic grouping with the *R. marina* species complex, based on a combined morphological and molecular dataset.

Within the *R. marina* group, the results of this study showed that the *R. marina* populations from the Amazon basin (east of the Andes) form a well-supported clade with the remaining species, which is highly divergent from the *R. marina* clade from west of the Andes (Fig. 2). These results are congruent with the paraphyly of *R. marina* reported by Slade & Moritz (1998) and Mulcahy *et al.* (2006). However, the inclusion of new sequences from the Amazon basin revealed, for the first time, a closer relationship between samples of *R. marina* from western Ecuador and Central America, when compared with populations east of the Andes. Phylogenetic relationships among other species of the *R. marina* group were, in general, poorly resolved, if at all. With the exception of *R. arenarum*, no other species form a monophyletic group. *Rhinella schneideri*, *R. jimi*, and *R. poeppigii* together with the Amazonian *R. marina* form a clade that is sister to that of *R. achavalli*, *R. icterica*, *R. rubescens* and *R. arenarum*. These two clades are basically allopatric, with exception of *R. schneideri* that extends its distribution range into C/D clade, likely reflecting a

secondary range expansion. These results suggest that the current taxonomy of this group may not accurately reflect its phylogeny. In the Neotropics, the determination of species, including those of the *R. marina* group, is still based primarily on traditional studies of anatomical and morphological characters (Frost *et al.* 2006; Kwet *et al.* 2006). It is thus not surprising that the recognition of non-monophyletic species has been a frequent outcome of molecular phylogenetic analyses (e.g. Funk & Omland 2003; Fouquet *et al.* 2007a,b; Maurício *et al.* 2008). However, in the case of the *R. marina* group, this conclusion should be treated with caution, given that our analysis is based on linked mtDNA genes and a limited geographic sample for most taxa. Indeed, the difficulties that arise from the study of closely related species – which include low levels of genetic divergence and lineage sorting of ancestral polymorphisms – have been well documented. Given this, we cannot eliminate entirely the possibility that variation at other loci may be more congruent with the current species taxonomy. The inclusion of multiple independent loci should provide a much greater probability of identifying the correct species tree, in comparison with one based on a single-gene (e.g. Nichols 2001; Machado & Hey 2003; Jennings & Edwards 2005).

Another potentially misleading factor here is introgressive hybridization (e.g. Funk & Omland 2003; Fouquet *et al.* 2007b). While natural hybridization has only been demonstrated in the *R. marina* group between *R. icterica* and *R. schneideri* (Azevedo *et al.* 2003), interspecific hybridization is common among bufonids (e.g. Masta *et al.* 2002). Considering that some species of *R. marina* group – such as *R. marina* and *R. poeppigii*, and *R. icterica* and *R. achavalli* (Kwet *et al.* 2006; Frost 2007) – occur in sympatry, it is possible that phylogenetic relationships within the group are strongly influenced by past or current hybridization events. The relative positions of different *R. poeppigii* specimens in our analysis appear to support this hypothesis. One *R. poeppigii* specimen (Rpo1) from Peru groups with the Peruvian *R. marina*, whereas the sample from Bolivia (Rpo2) appears in a highly divergent clade together with eastern Amazonian *R. marina*, and *R. schneideri* and *R. jimi*. The karyotypes of *R. poeppigii* and *R. marina* are also closely related (Blair 1972; Bogart 1972).

Further studies including a larger number of individuals and a much wider sampling area, more representative of the geographic range of each species, together with the inclusion of nuclear genes, will be essential to clarify the phylogenetic relationships within this group. This will also be necessary to validate the taxonomic status of some species, especially those that have been described recently, based only on morphological characters.

Divergence time and historical biogeographical models

The recent geological history of the Neotropical region has been characterized by complex geological and climatic changes that have been implicated in the determination of patterns of diversification, and the biogeographical history of many organisms (e.g. Haffer 1997 and references therein; Nores 2004; Wesselingh & Salo 2006; Tuomisto 2007). This idea has flourished in particularly in recent decades, with the emergence of a growing body of literature that has linked spatial and temporal patterns of genetic diversity with geoclimatic events for the reconstruction of the evolutionary history of a number of different organisms (see Rull 2008 and references therein). Although the reliability estimates of divergence times based on molecular data has improved in recent years, they are still a source of criticism, especially when precise independent data for time calibrations (e.g. a fossil record) are absent or they are based on single-gene clocks (e.g. Wakeley 2000; Arbogast *et al.* 2002; Heads 2005). In spite of these drawbacks, they should be considered important tools for the interpretation of phylogenetic histories. Considering this, we discuss below some of the historical hypotheses proposed to account for biogeographic patterns in the *R. marina* species complex, in the light of its phylogenetic relationships and spatio-temporal patterns of genetic diversity.

Our phylogenetic analyses combined with divergence time estimates, suggested that major diversification events within the *R. marina* group occurred in the Miocene, resulting in three highly divergent clades, that correspond to distinct geographical regions: (i) the eastern Amazon Basin (east of the Andes); (ii) Ecuador (west of the Andes); and, (iii) Central America. A pronounced phylogenetic break between populations settled in the eastern and western regions of the Andes. has been reported for many taxonomic groups, including mammals (da Silva & Patton 1993; Hoffmann & Baker 2003), birds (Brumfield & Capparella 1996; Eberhard & Bermingham 2005), reptiles (Gamble *et al.* 2008), frogs (Symula *et al.* 2003; Noonan & Wray 2006; Santos *et al.* 2009), and insects (Solomon *et al.* 2008).

Two geological episodes are central to explain such high divergence in a great variety of taxa – the uplift of the Andes and the formation of extensive floodplain system in the Amazon basin, the Pebas lake/wetland system (see revision in Duellman 1999; Noonan & Wray 2006; Wesselingh & Salo 2006; Santos *et al.* 2009). Based on our estimates of divergence times (10.7–17.2 Ma), the split between the eastern and western populations arose prior to the establishment of the Andes as a major geographic barrier, during the Miocene-Pliocene transition (Lundberg *et al.* 1998; Gregory-Wodzicki 2000). However, our esti-

mates do coincide with the proposed formation of the South American flood basin system in the mid-late Miocene (Hoorn 1993; Räsänen *et al.* 1995; Wesselingh & Salo 2006 and references therein), which has also been implicated in the diversification of other vertebrate groups, including amphibians (Symula *et al.* 2003; Noonan & Wray 2006; Santos *et al.* 2009) and reptiles (Gamble *et al.* 2008). This system, which resulted from periodic marine incursions into the Amazon Basin, promoted extensive flooding of lowlands and established an extensive mosaic of fluvial and lacustrine habitats in the western Amazon (e.g. Webb 1985; Nores 2004; Wesselingh & Salo 2006). Wesselingh & Salo (2006) proposed that this scenario enhanced east-west isolation in taxa with poor dispersal potential (Fig. 4).

Assuming a South American origin of *R. marina* (Pauly *et al.* 2004; Pramuk 2006; Pramuk *et al.* 2008), our results also support the conclusion that the species colonized Central America prior to the formation of the Isthmus of Panama (~3 Ma; Coates *et al.* 2004). This conclusion supports the split time (9.5–11.2 Ma) proposed by Mulcahy *et al.* (2006) for the Central American and Ecuadorian populations of *R. marina*, and contradict the hypothesis of Slade & Moritz (1998), i.e. that the species reached Central America during the Pliocene over the land bridge. However, it is important to note that these authors used a mean rate of DNA evolution of 1% per lineage per Ma, as estimated by Wilson *et al.* (1985) for a selection of vertebrate species, but which is probably much higher than the rates estimated for toad mtDNA (Macey *et al.* 1998; Mulcahy & Mendelson 2000; Mulcahy *et al.* 2006).

The role of the Isthmus of Panama in the exchange of Central and South American faunas has been controversial (e.g. Stehli & Webb 1985). While the emergence of the Panamanian land bridge paved the way for the major American Interchange, which involved mammals in particular (Webb 1985), Savage (1982) and Vanzolini & Heyer (1985) have suggested that exchange of amphibians took place prior to its formation. The development of DNA-based molecular phylogenies, and their more reliable estimates of divergence times, has led to increasing evidence that contact between South and Central American organisms – including vertebrates (Zamudio & Greene 1997; Bermingham & Martin 1998; Weigt *et al.* 2005; Santos *et al.* 2009), and invertebrates (Zeh *et al.* 2003) – predates the formation of the Panamanian land bridge. The divergence time between South and Central American *R. marina* estimated here (6.3–17.2 Ma) overlaps partially with those reported for other amphibians, such as the túngara frog (6-10 Ma; Weigt *et al.* 2005), and some poison frogs of the family Dendrobatidae (8.3–12.1 Ma; Santos *et al.* 2009).

Taxonomic implications

Our results provide important new insights into the phylogenetic relationships of the *R. marina* species group, and in particular, a new perspective on the classification of *R. marina* as a single evolutionary unit, as already indicated by Slade & Moritz (1998). Indeed, we recovered at least three highly divergent lineages of *R. marina* from distinct geographic regions that have been associated with vicariant events of the Tertiary period, suggesting independent biogeographical histories. Despite criticisms of the use of mtDNA data only for the differentiation of new species (Moritz & Cicero 2004; Meyer & Paulay 2005; Fouquet *et al.* 2007a), many authors still rely on a threshold of genetic distance for the definition of species status in both vertebrates and invertebrates (e.g. Bradley & Baker 2001; Vences *et al.* 2005; Hendrixson & Bond 2005; Fouquet *et al.* 2007b). The degree of sequence divergence found in the present study between the three main *R. marina* lineages (Fig. 4) is both greater than the genetic distances found between the remaining *marina* group species and the threshold traditionally used for the definition of frog species (Vences *et al.* 2005; Fouquet *et al.* 2007b; Alam *et al.* 2008). Although some amphibians present intraspecific divergence of the 16S and 12S rRNA genes of up to 6% (Vences *et al.* 2005), values of 4–5% are generally considered to be evidence of species-level differentiation (Fouquet *et al.* 2007b). However, while our mtDNA evidence indicates the existence of two or even three cryptic species within *R. marina*, we agree with Slade & Moritz (1999) on the need for further detailed morphological studies and the use of nuclear markers before deciding on the taxonomic status of the distinct *R. marina* lineages.

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