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Phylogenetic relationships of toads of the *Rhinella granulosa* group (Anura: Bufonidae): a molecular perspective with comments on hybridization and introgression

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Abstract

The *Rhinella granulosa* group consists of 13 species of toads distributed throughout open areas of South America and Panama. In this paper we perform a phylogenetic analysis considering all but one species of the group, employing five nuclear and four mitochondrial genes, for up to 7910 bp per specimen. Separate phylogenetic analyses under direct optimization (DO) of nuclear and mitochondrial sequences recovered the *R. granulosa* group as monophyletic and revealed topological incongruence that can be explained mainly by multiple events of hybridization and introgression, both mitochondrial and nuclear. The DO combined analysis, after the exclusion of putatively introgressed or heterozygous genomes, resulted in a phylogenetic hypothesis for the *R. granulosa* group in which most of the species are recovered as monophyletic, but with interspecific relationships poorly supported. The optimization of morphological (adult and larval), chromosomal, and behavioural characters resulted in 12 putative phenotypic synapomorphies for this species group and some other synapomorphies for internal clades. Our results indicate the need for additional population genetic studies on *R. dorbignyi* and *R. fernandezae* to corroborate the taxonomic status of both taxa. Finally, we discuss biological and genetic characteristics of Bufonidae, as possible explanations for the common occurrence of hybridization and introgression observed in some lineages of this family.

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Introduction

Rhinella is one of the most diverse genera of true-toads of the nearly cosmopolitan family Bufonidae, comprising 87 species naturally distributed throughout different Neotropical ecoregions (Frost, 2014). This genus was resurrected by Frost et al. (2006) and redelimited by Chaparro et al. (2007) to include most of the South American species previously assigned to *Bufo*. Most of these species were included in species groups traditionally recognized (as part of *Bufo*) on

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the basis of osteological characters and external morphology (Tihen, 1962; Cei, 1972; Martin, 1972; Duellman and Schulte, 1992): the *R. crucifer*, *R. granulosa*, *R. margaritifera*, *R. marina*, *R. spinulosa*, and *R. veraguensis* groups. Pramuk (2006) studied the phylogenetic relationships of these toads based on a combined analysis of morphological and molecular data. She found no evidence of monophyly for the *R. spinulosa* and *R. veraguensis* groups, and recovered *Rhamphophryne* nested within *Rhinella*. Recently, Grant and Bolivar-G. (2014) defined the *Rhinella acrolopha* group to include the species of the former *Rhamphophryne*. Currently, the monophyly of some species groups (e.g. *R. marina*, *R. spinulosa*, and *R. veraguensis* groups) are still not corroborated, and several species are not assigned to any group (La Marca and Mijares-Urrutia, 1996; Pramuk, 2006; Chaparro et al., 2007; Padial et al., 2009; Vallinoto et al., 2010; Pyron and Wiens, 2011; Moravec et al., 2014).

The *Rhinella granulosa* group is one of the most morphologically distinct and widely distributed species groups of *Rhinella*, and comprises small to medium-sized toads having heavily ossified skulls, well-developed heavy keratinized cephalic crests, and body densely covered by granules and spicules (Gallardo, 1965; Duellman and Schulte, 1992; Narvaes and Rodrigues, 2009). This group currently comprises 13 species distributed throughout open areas from South America to Panama (Narvaes and Rodrigues, 2009; Frost, 2014). In her phylogenetic analysis, Pramuk (2006) recovered the three included species of the *R. granulosa* group (*R. humboldti* [as *Bufo humboldti*], *R. merianae* [as *B. granulosus* 1], and *R.* cf. *granulosa* [as *Bufo granulosus* 2]) as a well-supported monophyletic group, having two unique and unreversed morphological synapomorphies (other characters that optimize as synapomorphies but with some level of homoplasy were not listed): the presence of prenasal bones and the presence of an expanded dorsal crest of the ilium (Pramuk, 2006). van Bocxlaer et al. (2010) and Pyron and Wiens (2011) included in their molecular phylogenetic analyses sequences of *R. fernandezae* (as *R.* cf. *granulosa*) in addition to those generated by Pramuk (2006), and also recovered this group as monophyletic and highly supported.

The taxonomic history of the *Rhinella granulosa* group is somewhat intricate. Gallardo (1965) made the first comprehensive revision of this group and, on the basis of external morphology (head shape, parotoid gland shape, shape of cephalic crests, and dorsal skin texture), recognized 14 subspecies within *Rhinella granulosa* (then *Bufo granulosus*): *B. g. azarai*, *B. g. barbouri*, *B. g. beebei*, *B. g. dorbignyi*, *B. g. fernandezae*, *B. g. goeldii*, *B. g. granulosus*, *B. g. humboldti*, *B. g. lutzi*, *B. g. major*, *B. g. merianae*, *B. g. minor* (later substituted by *B. g. mini*, Gallardo, 1967), *B. g. mirandaribeiroi*, and *B. g. pygmaeus*. This author stated that several of these subspecies were highly associated with the main river basins in South America (Gallardo, 1965, 1969). Subsequently, several nominal subspecies were considered to be species (e.g. *B. beebei*, *B. dorbignyi*, *B. fernandezae*, *B. pygmaeus*; Cei, 1972; Frost, 1985; Rivero et al., 1986; Duellman and Schulte, 1992), and other forms were described as subspecies (*B. granulosa nattereri*, Bokermann, 1967) or species (*B. bergi*, Céspedez, 1999). Narvaes and Rodrigues (2009) reviewed the taxonomy of this group on the basis of external morphology and morphometry. Following their results, most of the subspecies were raised to specific status, others were considered junior synonyms (i.e. *Bufo granulosus barbouri*, *B. g. beebei*, *B. g. goeldii*, *B. g. lutzi*, and *B. g. mini*), and a new species was described from Panama (*R. centralis*). Narvaes and Rodrigues (2009) suggested that the distribution of taxa within the *R. granulosa* group is associated with open areas and congruent with the morphoclimatic domains defined by Ab'Saber (1977), instead of being linked to hydrographic basins as proposed earlier (Gallardo, 1965, 1969). Subsequently, Sanabria et al. (2010) described a new species from San Juan, western Argentina (*R. bernardoi*). Finally, Jansen et al. (2011) pointed to morphological and molecular differentiation in a population assigned to *R. mirandaribeiroi* of Bolivia, suggesting that more studies are necessary to confirm its taxonomic status.

Rhinella sternosignata is a species with controversial relationships (La Marca and Mijares-Urrutia, 1996) that some authors have considered related to the *R. margaritifera* (Cei, 1972; Hoogmoed, 1990; Duellman and Schulte, 1992) or *R. granulosa* groups (Gallardo, 1962). Based on osteological data Vélez-Rodriguez (2005) suggested that *R. sternosignata* could be allied to the *R. granulosa* group, and proposed some character states that support this relationship.

The reproductive behaviour of species of the *Rhinella granulosa* group, as in many other bufonids, is characterized by explosive breeding congregations with scramble competition, lasting for a few nights during or after rains (Wells, 1977, 2007; Narvaes and Rodrigues, 2009). These dense aggregations occur in temporary ponds or puddles, and also in permanent water reserves (as shallow ponds), where males call from the peripheral vegetation (Hoogmoed and Gorzula, 1979; Cei, 1980; Gallardo and Varela de Olmedo, 1993; Lescure and Marty, 2000; Lynch, 2006; Narvaes and Rodrigues, 2009; Guerra et al., 2011). During the day, these species can be found sheltered under fallen tree trunks or stones, cracks in the soil, and particularly in characteristic holes in the ground that they build by digging with their hindlimbs (Gallardo, 1957, 1969; Hoogmoed and Gorzula, 1979; Gallardo and Varela de Olmedo, 1993; Carvalho e Silva and Carvalho e Silva, 1994; Achaval and Olmos, 1997; Rosset and Alcalde, 2004; Narvaes and Rodrigues, 2009).

Evidence of different nature, such as morphology, bioacoustics, serological analyses, cytogenetics, and DNA sequences, has demonstrated that natural hybridization is common in some groups of Bufonidae (Blair, 1972; Feder, 1979; Masta et al., 2002; Azevedo et al., 2003; Green and Parent, 2003; Yamazaki et al., 2008; Fontenot et al., 2011), and mitochondrial and nuclear introgression (= gene flow) has been demonstrated in some well-studied clades (e.g. Green and Parent, 2003; Fontenot et al., 2011; Sequeira et al., 2011; cf. Garcia-Porta et al., 2012). Hybridization events are apparently common in the *Rhinella granulosa* group and there are reports of hybrid specimens of *R. bergi* × *R. major*, *R. dorbignyi* × *R. fernandezae*, *R. fernandezae* × *R. major*, *R. granulosa* × *R. mirandaribeiroi*, and *R. major* × *R. mirandaribeiroi* (Gallardo, 1969; Narvaes and Rodrigues, 2009; Guerra et al., 2011). However, virtually nothing is known about fertility of the hybrid progeny or the existence of gene flow between species of the group.

In this study we present a maximum-parsimony (MP) phylogenetic analysis under direct optimization (DO) of the *Rhinella granulosa* group on the basis of DNA sequences, including five nuclear and four mitochondrial genes from 55 individuals of all but one species (*R. nattereri*) included in the group. Individual and combined analyses (DO) of nuclear and mitochondrial sequences were performed to identify discordance between both genomes. Our goals were to (i) test for the monophyly of the *R. granulosa* group; (ii) explore the phylogenetic relationships and taxonomic status of its species; (iii) determine the occurrence of genetic discordance between mitochondrial and nuclear lineages and discuss the putative causes that explain the observed patterns (e.g. hybridization, introgression, incomplete lineage sorting); and (iv) discuss morphological and behavioural character states that represent putative synapomorphies for this species group or its internal clades.

Materials and methods

Taxon sampling

Our analyses included samples from most species of this group from several localities in Argentina, Bolivia, Brazil, Panama, Paraguay, Uruguay, and Venezuela (see Fig. 3 inset, and supplementary Appendix S1). We only included additional sequences from GenBank that were associated with a voucher specimen and locality information (Appendix S2). The only species from the group that we were unable to secure tissue samples is *Rhinella nattereri*. We include tissue samples of *R. sternosignata* to test its proposed relationships with the *R. granulosa* group (Vélez-Rodriguez, 2005).

Considering the phylogenetic hypotheses of Pramuk (2006), Frost et al. (2006), van Bocxlaer et al. (2010), and Pyron and Wiens (2011), we included 11 species of *Rhinella* as exemplars of the phylogenetic diversity of the genus to be used as outgroups. Additionally, we produced sequences of *R. henseli*, the most basal species of the *R. crucifer* group (Thomé et al., 2010), to provide a stringent test of the relationships of the *R. granulosa* group with other species groups of *Rhinella*. Because the phylogenetic position of *Rhinella* among other genera of Bufonidae remains controversial (Frost et al., 2006; Pramuk, 2006; Pramuk et al., 2008; Pyron and Wiens, 2011; van Bocxlaer et al., 2010), we also included exemplars of 11 other genera of this family. *Amazophrynella minuta*, a basal Bufonidae, was used to root the analyses. Appendix S3 provides a list of all included specimens, collection numbers, and GenBank accession numbers.

Laboratory protocols

We extracted total genomic DNA from ethanol-preserved tissues (liver, muscle, or fingertips) using the Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). We carried out PCR amplification in 25-μL reactions using 0.2 μL Taq (Fermentas, Vilnius, Lithuania). The PCR protocol consisted of an initial denaturation step of 3 min at 94 °C; 35 (for mitochondrial genes) or 45 (for nuclear genes) cycles of 30 s at 94 °C, 40 s at 48–62 °C, and 30–60 s at 72 °C; followed by a final extension step of 10–15 min at 72 °C. We cleaned PCR-amplified products using 10 U of Exonuclease plus 1 U of alkaline phosphatase per reaction. We sequenced the products with an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) in both directions to check for potential errors and nuclear polymorphisms. We processed the chromatograms using the software Sequencher 4.5 (Gene Codes) and edited the complete sequences with BioEdit (Hall, 1999). Sequences are deposited in GenBank under accession numbers KP684942-KP685232.

Character sampling

The mitochondrial loci sampled for the phylogenetic analyses include: (i) the ribosomal genes *12S* and *16S*, and the intervening $tRNA_{Val}$ (*12S–16S*; ~2450 bp); (ii) a fragment comprising the upstream section of the *16S* gene, the intervening $tRNA_{Leu}$, *NADH dehydrogenase subunit 1*, and $tRNA_{Ile}$ (*ND1*; ~1250 bp); and (iii) a fragment of the *cytochrome b* gene (*CytB*; 700 bp), comprising ~4400 bp of the mitochondrial genome. The nuclear loci include: (i) the *chemokine receptor 4* gene (*CXCR4*; 676 bp); (ii) the *sodium-calcium exchanger subunit 1* gene (*NCX1*; 715 bp); (iii) the *proopiomelanocortin A* gene (*POMC*; 559 bp); (iv) two non-overlapping fragments

of the *recombination activating protein 1* gene (here called *RAG1a* and *RAG1b*; 815 and 429 bp, respectively), and (v) the *rhodopsin* gene (*RHO*; 316 bp), making a total of 3510 bp sampled from the nuclear genome. Primers and their sources are detailed in Appendix S4. No phenotypic dataset is available for the *Rhinella granulosa* group. For this reason we make only general comments about a few morphological (adult and larval), chromosomal, and behavioural characters whose optimizations in the optimal combined tree (DO) suggest that they are putative synapomorphies of some of the major clades.

Phylogenetic analyses

Three molecular data sets were analysed: (i) all the mitochondrial sequences (M); (ii) all the nuclear sequences (N); and (iii) non-introgressed nuclear and mitochondrial sequences (M + N). The phylogenetic analyses of each data set were performed under direct optimization in POY 4.1.2.1 (Varón et al., 2010), using equal weights for all transformations (substitutions and insertion/deletion events). We considered parsimony as the optimality criterion because the cladogram that minimizes transformations to explain the observed variation is the simplest, maximizes evidential congruence, and has greatest explanatory power (Farris, 1983; Kluge and Grant, 2006; Wheeler et al., 2006). Sequences were first aligned using the online software MAFFT version 6.240 (Katoh and Toh, 2008) under the strategy E-INS-i (for the *12S–16S* fragment) and L-INS-i or G-INS-i (the remaining fragments) with default parameters for gap opening and extension. Final alignments for each gene are available from DRYAD (doi:10.5061/dryad.k4g78). The ribosomal genes (*12S–16S*) were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and equal-length sequences of coding genes (*ND1*, *Cytochrome b*, and nuclear genes) were considered as static alignments to accelerate the searches (Faivovich et al., 2010).

All the phylogenetic analyses under DO were performed using the command "Search," which implements a driven search building Wagner trees using random addition sequences (RAS), Tree Bisection and Reconnection (TBR) branch swapping followed by Ratchet (Nixon, 1999), and Tree Fusing (Goloboff, 1999). The shortest trees stored for each independent run were pooled as a source of topological diversity for a final round of tree fusing (Wheeler et al., 2006). Each independent "Search" run was followed by a round of TBR swapping holding up to 40 trees and final calculation of tree lengths using static approximation. The optimal trees from all searches were diagnosed using iterative pass optimization (Wheeler, 2003) and converted to static approximation for a final TBR swap of all unique topologies storing up to five trees each. Finally, a swapping of the implied alignment were done in TNT to check the occurrence of additional most parsimonious trees (MPTs).

The DO analyses of both M and N datasets were executed in an Intel Core i5-2500 3.3 GHz with 8 GB (4 × 2GB) RAM, performing 12 independent analyses composed of 6-h (M) or 3-h (N) runs. Meanwhile, DO phylogenetic analyses of the combined datasets (M + N) were executed in parallel using the Museu de Zoologia da Universidade de São Paulo's high-performance computing cluster Ace, which consists of 12 quad-socket AMD Opteron 6376 16-core 2.3-GHz CPUs, 16 MB cache, 6.4 GT/s compute nodes (= 768 cores total), eight with 128 GB RAM DDR3 1600 MHz (16 × 8 GB), two with 256 GB (16 × 16 GB), and two with 512 GB (32 × 16 GB), and QDR 4x InfiniBand (32 GB/s) networking. We performed nine independent analyses composed of two 6-h runs, one 3-h run, and six 2-h runs. Additional information about the phylogenetic analyses is given in Appendix S5.

Parsimony jackknife (Farris et al., 1996) absolute frequencies were estimated from the static alignment with TNT, Willi Hennig Society Edition (Goloboff et al., 2008), generating 50 RASs + TBRs per replicate for a total of 1000 replicates, and considering gaps as a fifth state. Editing of trees and character optimizations were performed with Winclada (Nixon, 2002).

We also performed a bayesian analysis for the combined dataset (including all the same putative non-introgressed sequences used in the combined DO analysis), employing the original multiple alignment used for the estimation of jackknife absolute frequencies. Models for each gene were chosen with jModelTest version 0.1.1 (Posada, 2008). First, second, and third codon positions were treated as separate partitions for each protein-coding gene. Additionally, *12S*, *16S*, and *tRNAs* (*Val*, *Leu*, and *Ile*) were also treated as separated partitions for model selection. The Akaike information criterion (AIC) was used to select the best fitting model for each partition (Pol, 2004; Posada and Buckley, 2004). The best-fit models for each partition are detailed in Appendix S6. Bayesian analyses were performed in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Each analysis consisted of four runs, each consisting of two replicate Monte-Carlo Markov chains. Each run used four chains and default settings of priors (Dirichlet for substitution rates and state frequencies, uniform for the gamma shape parameter and proportion of invariable sites, all topologies equally likely *a priori*, and branch lengths unconstrained:exponential). Four analyses using 30 million generations (with a burn-in fraction of 0.3) were first performed. The resulting parameters were evaluated using Tracer 1.6 (Rambaut et al., 2013) and showed that likelihood values appeared to stabilize before 4 million generations in some replicates. Consequently,

we performed an additional run of 50 million generations, sampling every 1000 generations, and trees from the first 15 million generations were discarded as burn-in in this analysis.

Taxonomic evaluation

Individuals were morphologically determined following the diagnoses proposed by Narvaes and Rodrigues (2009). We considered the following approximations to test the taxonomic status of each individual: (i) cladogram topology resulting from the DO analysis of mitochondrial sequences (see above) only (Fig. 1), and (ii) uncorrected pairwise distances (UPDs; Appendix S7), which were calculated in PAUP* (Swofford, 2002) for a dataset of the *16S* gene (comprising a fragment of 583 bp, aligned in MAFFT under the strategy G-INS-i) and containing only sequences of species of the *Rhinella granulosa* group.

Evaluation of genetic introgression between species

We use the term "heterozygous genotype" to refer to nuclear genotypes resulting from two non-conspecific genomic contributions (i.e. different parental species). The recognition of heterozygous genotypes could be controversial in some cases mainly as a result of ancestral introgression followed by genetic recombination and/or incomplete lineage sorting. We evaluated the occurrence of putative hybridization and/or gene introgression between species, following these approaches:

(1) The MPTs resulting from the independent DO analyses of mitochondrial (Fig. 1) and nuclear (Fig. 2) sequences were compared. Thus, it was possible to evaluate the mitochondrial–nuclear discordance using the topology of well-supported clades: incongruence of terminals in these optimal trees was considered to indicate putative conflict.

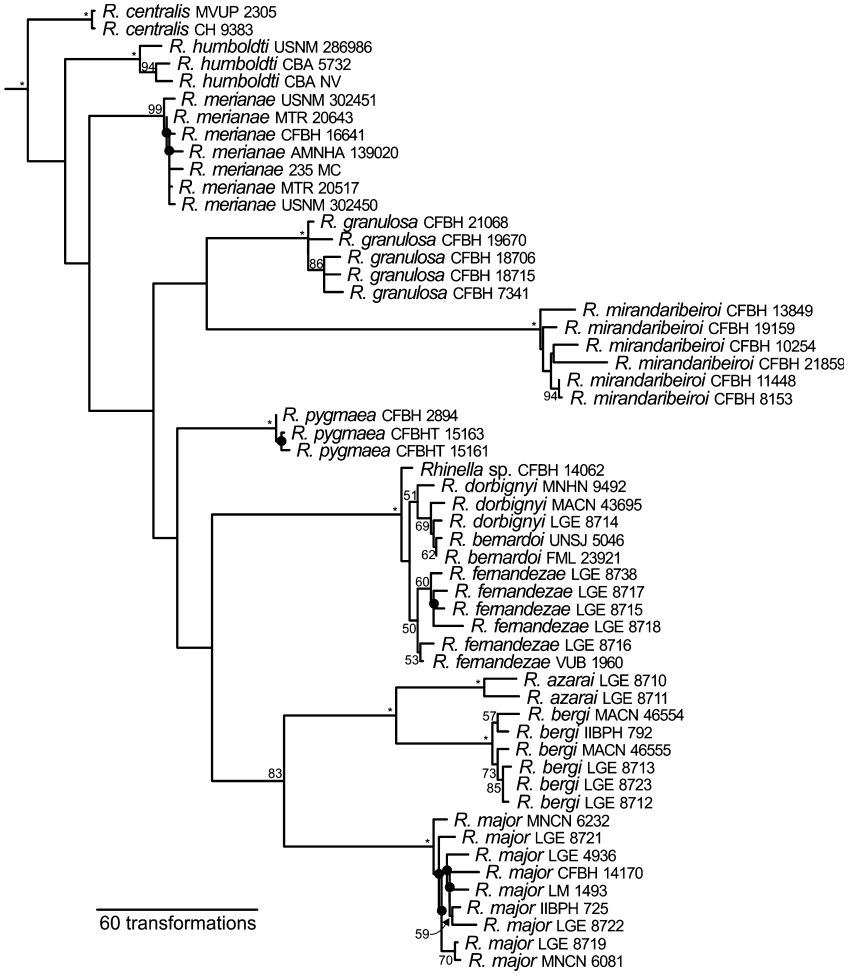

Fig. 1. One of the 6072 MPTs obtained from the analysis of mitochondrial genes under DO (length 6216 steps). Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analysed with parsimony in TNT with gaps as fifth state. Asterisks indicate groups with 100% support for both parsimony jackknife frequencies; only jackknife frequency values > 50% are shown. Relationships among outgroups are shown in Appendix S10.

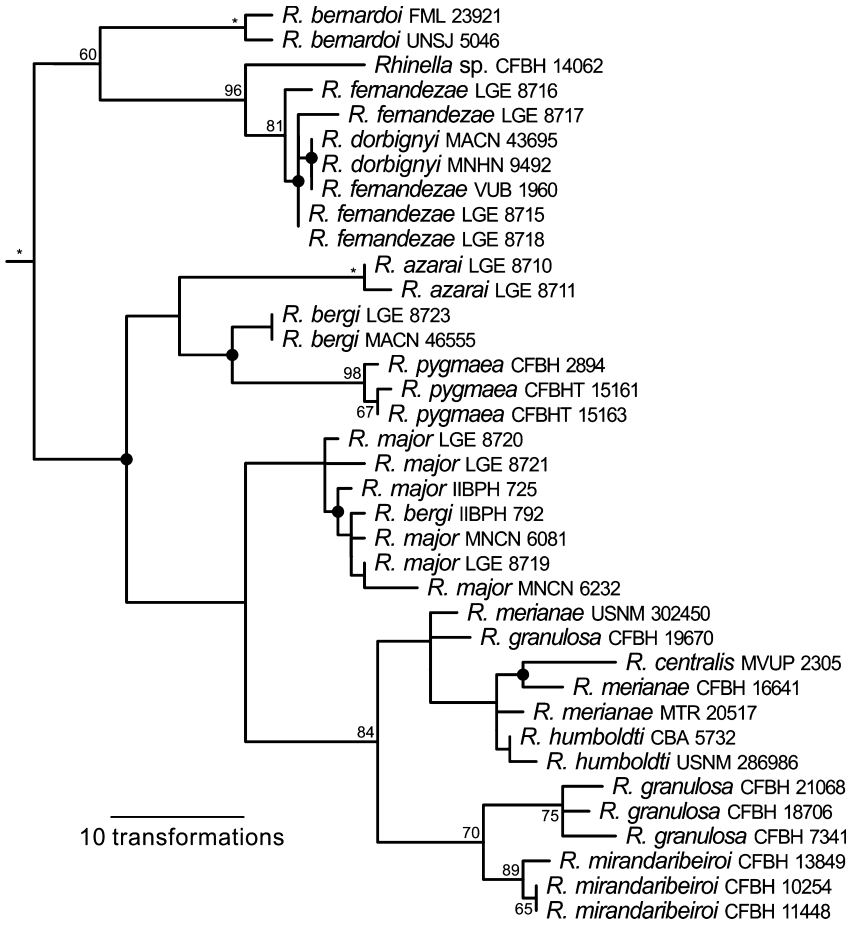

Fig. 2. One of the 270 MPTs obtained from the analysis of nuclear genes under DO (length 1192 steps). Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analysed with parsimony in TNT with gaps as fifth state. Asterisks indicate groups with 100% support for both parsimony jackknife frequencies; only jackknife frequency values > 50% are shown. Relationships among outgroups are shown in Appendix S11.

(2) The variable and parsimony-informative sites for each nuclear gene of each individual of the *Rhinella granulosa* group (see Appendices S9.1–S9.6) were revised: a relatively high rate of polymorphism in the sequences of individuals, in combination with controversial positions both in the MPTs of the DO nuclear analysis (Fig. 2) and in individual analysis of each nuclear gene (data not shown), was interpreted as differences between nuclear genomes originating from different parental species (i.e. heterozygous genotype from hybrid/introgressed nature). When these occurred, both the high intraspecific similarity and high interspecific divergence are sufficiently contrasting as to determine precisely the heterozygous sequences and identify the parental species by examination of its polymorphisms. Individuals were considered putatively heterozygous when the sequences of at least one marker displayed a high level of polymorphism even though other nuclear fragments were not evidently recombinant. This was the most conservative approach possible, and is based in the knowledge that some regions of the nuclear genome are more susceptible to introgression than others (Baack and Rieseberg, 2007; Petit and Excoffier, 2009; Sousa et al., 2013).

As an additional source of evidence we consider geographical distribution patterns of the polymorphisms: by comparing the sequences of different populations along the distribution of a given species, one can evaluate levels of intraspecific variation and possibly detect interspecific recombination (Baack and Rieseberg, 2007). Allopatric individuals/species are less expected to be introgressed and thus this condition was used as evidence to define the parental nuclear genotypes, and test the occurrence of incomplete lineage sorting when ancestral polymorphism is present in both allopatric and sympatric populations (Toews and Brelsford, 2012). Thus, although incongruence between nuclear and mitochondrial or among different nuclear topologies is not necessarily an indication of hybridization or introgression (Toews and Brelsford, 2012), we consider

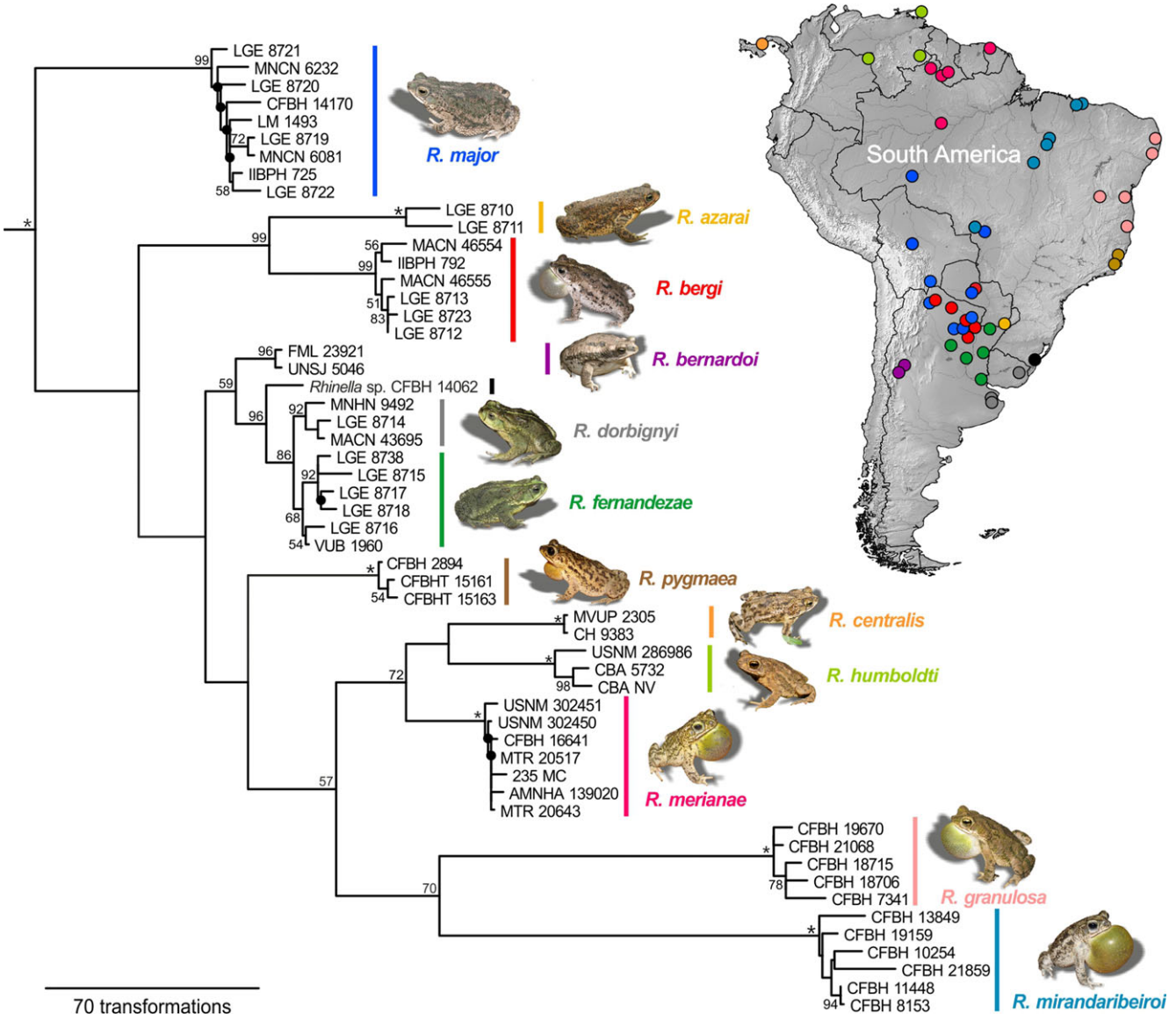

Fig. 3. One of the 264 MPTs obtained from the combined analysis of mitochondrial and nuclear genes under DO (length 7446 steps), after removal of the putatively introgressed sequences. Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analysed with parsimony in TNT with gaps as fifth state. Asterisks indicate groups with 100% support for both parsimony jackknife frequencies; only jackknife frequency values > 50% are shown. Relationships among outgroups are shown in Appendix S12. Inset, map showing collection sites for tissue samples used in this study. Exact localities are detailed in Appendices S1 and S2.

it to be a major cause of the genomic discordance when these previously described conditions were observed (Appendix S8).

Nuclear sequences of all specimens with potentially heterozygous genotypes were excluded, generating the non-introgressed nuclear and mitochondrial sequences dataset (M + N; Appendix S8) that we used in the definitive DO and bayesian phylogenetic analyses. We also excluded the mitochondrial sequences of both specimens of *Rhinella bernardoi* from this dataset because of complete mitochondrial introgression (see Discussion).

Results

DO parsimony analyses results in 6072 MPTs of 6216 steps (M), 270 MPTs of 1192 steps (N), and 264 MPTs of 7446 steps (M + N). The relationships in the *Rhinella granulosa* group resulting from these analyses are shown in Figs 1–3, whereas those of outgroups are displayed in Appendices S10–S12.

Rhinella was highly supported (jackknife = 87%) only in the DO combined analysis (M + N; Appendix S12), but poorly supported (< 50%) in the DO mito-

chondrial analysis (M; Appendix S10) or polyphyletic in the DO nuclear analysis (N; Appendix S11). All analyses recovered the *R. granulosa* group as monophyletic, with maximum support (100%). *Rhinella sternosignata* is recovered as distantly related to this group, and instead is grouped with the specimens of the *R. margaritifera* and *R. veraguensis* groups, with moderate support in the DO mitochondrial and combined analyses (> 66%), but poorly supported (< 50%) in the DO nuclear analysis. In the mitochondrial and combined analyses (DO), the *R. granulosa* group was recovered as the sister taxon of a clade composed of the exemplars of the *R. marina* group paraphyletic with respect to the *R. crucifer* group, and as the sister taxon of a largely paraphyletic *Rhinella* in the DO nuclear analysis.

The DO analysis of all mitochondrial sequences (Fig. 1) recovered *Rhinella centralis* as the most basal species of the group with low support (< 50%). The interspecific relationships have low support values in general (< 50%) except for the following clades, *Rhinella* sp. CFBH 14062 (from Rio Grande do Sul, Brazil) + (*R. fernandezae* + *R. dorbignyi* (including *R. bernardoi*)) (100%), *R. major* + (*R. bergi* + *R. azarai*) (83%), and *R. bergi* + *R. azarai* (100%). All species were recovered as individually monophyletic with high jackknife support values (≥ 99%) except *R. dorbignyi* which was paraphyletic with respect to *R. bernardoi*.

The DO analysis of all nuclear sequences (Fig. 2) recovered three main clades whose relationships are unresolved: (i) a poorly supported clade (< 50%) composed of *Rhinella azarai*, *R. pygmaea*, and most of the specimens of *R. bergi*; (ii) a weakly supported clade (60%) composed of *R. bernardoi*, *Rhinella* sp. CFBH 14062, and *R. fernandezae* paraphyletic with respect to *R. dorbignyi*; and (iii) a poorly supported clade (< 50%) composed of a poorly supported and paraphyletic *R. major* (with respect to the specimen *R. bergi* IIBPH 792) and sister to a well-supported monophyletic group (84%) comprising the remaining species of the group. This latter group consists of a moderately supported clade (70%) comprising *R. mirandaribeiroi* and most of the specimens of *R. granulosa*, which is the sister taxon of a poorly supported clade (< 50%) consisting of a specimen of *R. granulosa* (CFBH 19670), a polyphyletic *R. merianae*, a monophyletic *R. humboldti*, and the only exemplar of *R. centralis*. In summary, six of the 12 included species of the *R. granulosa* group (as defined by Narvaes and Rodrigues, 2009 and Sanabria et al., 2010) were recovered as non-monophyletic in the DO nuclear analysis: *R. bergi*, *R. dorbignyi*, *R. fernandezae*, *R. granulosa*, *R. major*, and *R. merianae*.

The DO analysis of mitochondrial sequences (Fig. 1) and the comparison of uncorrected p-distances (Appendix S7) are congruent with the morphological–taxonomic determination of all specimens. Most species of the *Rhinella granulosa* group were recovered as monophyletic with high jackknife support values and relatively high genetic distance values in the *16S* gene, except *Rhinella* sp. CFBH 14062, *R. dorbignyi*, *R. bernardoi*, and *R. fernandezae*. *Rhinella fernandezae* was recovered as monophyletic in the DO mitochondrial analysis and as the sister taxon of *R. dorbignyi* (with *R. bernardoi* nested inside it), while *Rhinella* sp. CFBH 14062 is the sister taxon of this clade. The genetic distances between these taxa are low (≤ 0.69%).

Corroborating the results of the DO analysis of nuclear sequences (Fig. 2), examination of the parsimony-informative sites of nuclear sequences (Appendices S9.1–S9.6) allows us to identify several potentially heterozygous sequences that differ notably from putatively non-introgressed sequences (see Discussion). All the nuclear sequences for individuals with putative heterozygous sequences were excluded from the DO and bayesian combined analyses, as well as the mitochondrial sequences of *R. bernardoi* (see Discussion).

In the DO combined analysis (Fig. 3) *Rhinella major* was the sister taxon of all remaining species of the group, with low support. The following clades were recovered: (i) *R. azarai* + *R. bergi* (jackknife = 99%)—this clade is sister to all remaining species of the *R. granulosa* group, excluding *R. major*; (ii) *R. bernardoi* + (*Rhinella* sp. CFBH 14062 + (*R. fernandezae* + *R. dorbignyi*)) (59%); (iii) *R. pygmaea* (100%); (iv) *R. merianae* + (*R. centralis* + *R. humboldti*) (72%); and (v) *R. granulosa* + *R. mirandaribeiroi* (70%). The internal relationships among all these clades were poorly supported (< 57%).

The bayesian analysis of nuclear and mitochondrial sequences (Appendix S13) recovered the same major clades as the DO combined analysis under MP within the *Rhinella granulosa* group. However, the relationships among these clades differ with regards to the former analysis, recovering the clade *R. bernardoi* + (*Rhinella* sp. CFBH 14062 + (*R. fernandezae* + *R. dorbignyi*)) as the most basal. The relationships between the clades composed of *R. merianae* + (*R. centralis* + *R. humboldti*) and *R. granulosa* + *R. mirandaribeiroi* are identical to the DO combined analysis.

Discussion

Relationships among outgroups

The inclusion of multiple outgroups had as its only goal to provide a stringent test of the monophyly of the *R. granulosa* group and not to construct a critical test of previous analyses regarding relationships among other clades of Bufonidae (e.g. Frost et al.,

2006; Pramuk, 2006; Pramuk et al., 2008; van Bocxlaer et al., 2010; Mendelson et al., 2011; Pyron and Wiens, 2011).

In the optimal tree resulting from the DO combined analysis, *Rhinella* is monophyletic and the sister taxon of a clade comprising species of Bufonidae from Africa and Eurasia (Appendix S11). *Rhinella* was recovered as polyphyletic by Frost et al. (2006), with a clade consisting of species of the *R. crucifer*, *R. granulosa*, *R. marina*, *R. spinulosa*, and *R. veraguensis* groups (defined as *Chaunus*), which was related to *Incilius*, and another clade consisting of species of the *R. acrolopha* (formerly *Rhamphophryne*) and *R. margaritifera* (defined as *Rhinella*) groups related to *Pelophryne*. Subsequent studies recovered *Rhinella* as monophyletic and the sister taxon of *Anaxyrus* + *Incilius* (Pramuk, 2006; Pramuk et al., 2008; Pyron and Wiens, 2011), or a clade comprising African and Eurasian bufonids by van Bocxlaer et al. (2010). Our parsimony-based results are coincident with this latter phylogenetic hypothesis with regard to the sister clade of *Rhinella*. Conversely, in the bayesian analysis, *Rhinella* was recovered as sister to the clade *Anaxyrus* + *Incilius* (Appendix S12).

Nuclear–mitochondrial discordance

The evident para-/polyphyly of some species of the *Rhinella granulosa* group in the DO nuclear analysis (e.g. *R. bergi*, *R. granulosa*, *R. merianae*; Fig. 2) in relation to the taxonomic determination based on morphological evidence and mitochondrial information (Fig. 1, Appendix S7) provides evidence of putative hybridization and/or introgression of nuclear genomes between species in this group. The high level of polymorphism observed in the parsimony-informative sites of nuclear fragments in contrasting specimens supports this view. However, it is possible that there are some levels of incomplete lineage sorting in the nuclear genes that cause part of the polymorphism in the sequences. Otherwise, *Rhinella bernardoi* shows complete mitochondrial introgression from *R. dorbignyi* (see below).

Our results suggest a wide introgression of nuclear genes between *Rhinella bergi* and *R. major* throughout extensive geographical areas, which is supported by the large number of polymorphisms in the nuclear sequences of both species (Appendices S9.1–S9.6) from different (and sympatric) localities. Guerra et al. (2011) reported the occurrence of hybrids between both species calling actively in breeding sites, and some other adult specimens with intermediate morphological traits between these species were observed in collection material (M.O.P. and D.B., pers. observ.). Meanwhile, some additional cases of apparent genetic introgression occur in the specimens *R. bergi* LGE 8713, *R. granulosa* CFBH 19670, and *R. merianae* USNM 302450 (Appendix S8). These observations strongly suggest the occurrence of hybridization/introgression between *R. bergi* × *R. major* and *R. bergi* × *R. fernandezae*, and possibly nuclear genetic introgression in at least *R. bergi*, *R. granulosa*, and *R. merianae*. The fact that a relatively low number of specimens were analysed, together with the occurrence of extensive areas of sympatry (Narvaes and Rodrigues, 2009), allows us to infer the occurrence of intensive gene flow between these species.

Taxonomic remarks

The DO analysis of mitochondrial sequences and comparison of uncorrected p-distances are congruent with the morphological–taxonomic determination of all the specimens. All species of the *Rhinella granulosa* group are monophyletic in this analysis with high support (Fig. 1) and with genetic distances in the *16S* gene (Appendix S7), except for *Rhinella* sp. CFBH 14062, *R. bernardoi*, *R. dorbignyi*, and *R. fernandezae*.

Rhinella fernandezae and *R. dorbignyi* (with *R. bernardoi* nested within it, see below) are two reciprocally monophyletic groups in the mitochondrial analysis (DO), and all specimens of *R. fernandezae* and *R. dorbignyi* (but not *R. bernardoi*) collapse in a polytomy in the nuclear analysis (DO). Otherwise, *Rhinella* sp. CFBH 14062 was the sister taxon of this clade, and displayed low uncorrected p-distances (0.17–0.35%) with respect to these species. This voucher is morphologically most similar to specimens of *R. fernandezae*, but has some differences in the cephalic crests, and cannot be reliably assigned to this species. Moreover, in the nuclear analysis this specimen has a relatively long branch length, similar to those of other distinctive species of the group (see Fig. 2). Currently there are no diagnostic morphological characters to differentiate between *R. fernandezae* and *R. dorbignyi* nor the specific distinctiveness of *Rhinella* sp. CFBH 14062, as these differ only in the development and shape of some cephalic crests (Gallardo, 1957; Narvaes and Rodrigues, 2009), and some authors have reported the absence of fixed differences between these taxa (Klappenbach and Langone, 1992; Prigioni and Achaval, 1992; Maneyro and Kwet, 2008). Furthermore, they also cannot be distinguished based on genetic distance (Appendix S7), tadpole morphology (Borteiro et al., 2006), advertisement and release call parameters (Guerra et al., 2011), or cytogenetic data (M.O.P. and D.B., pers. observ.), and thus our first hypothesis was to consider these taxa as conspecifics. However, at this time we cannot test for the occurrence of interpopulational events such as recent speciation, followed by gene flow, or ongoing speciation under the presence of

gene flow; either could generate a similar pattern to what we observed with our dataset. Therefore, we prefer to be cautious and wait for additional population genetic studies to understand more clearly the evolutionary history of these taxa.

Rhinella bernardoi is recovered nested in *R. dorbignyi* in the DO mitochondrial analysis (Fig. 1), but not in the DO nuclear analysis (Fig. 2) where it is recovered as the sister taxon of *R. dorbignyi* + *R. fernandezae*. The close mitochondrial similarity between *R. bernardoi* and some individuals of *R. dorbignyi* (Appendix S7) with a relatively high nuclear divergence and morphological distinctiveness with respect to *R. dorbignyi* suggests the occurrence of past events of hybridization between these species followed by a fixation of mtDNA haplotypes of *R. dorbignyi* in *R. bernardoi*. This phenomenon has been demonstrated in the closely related *R. marina* and *R. schneideri*; populations of *R. marina* south to the Amazon River have a massive introgression of a mitochondrial genome from *R. schneideri* (Sequeira et al., 2011). Although they share nearly identical mitochondrial haplotypes, we do not consider *R. bernardoi* to be conspecific with *R. dorbignyi* due its distinctive nuclear genotypic (Fig. 2) and morphological distinctiveness (see Sanabria et al., 2010). The habitats of *R. bernardoi* and *R. dorbignyi* are very different and separated by at least 1000 km (straight line). *Rhinella dorbignyi* inhabits grasslands and savannas of the Uruguayan savanna, Pampa, and Espinal ecoregions (between 700 and 1300 mm rainfall per year), whereas *R. bernardoi* inhabits the Monte ecoregion, a warm shrub desert (between 80 and 250 mm rainfall per year) restricted to the pre-Andean region of western Argentina (Olson et al., 2001).

Rejecting an "Unconfirmed Genealogical Lineage"

The informal category Unconfirmed Genealogical Lineages (UGL; Vieites et al., 2009) was used by Jansen et al. (2011) for specimens preliminarily assigned to a species, but that showed high genetic distances with respect to individuals reliably assigned to that species. Although having a relatively high genetic divergence, morphological or bioacoustic characters between these individuals are not clearly divergent.

The *16S* sequence of a specimen preliminarily assigned to *Rhinella mirandaribeiroi* (MNKA 9783) by Jansen et al. (2011) from San Sebastián (Santa Cruz, Bolivia) showed an uncorrected p-distance of 2.9% and some morphological differences (e.g. canthus rostralis less distinct; loreal region more tuberculate and less concave; tympanum smaller; Jansen et al., 2011) with respect to individuals of *R. mirandaribeiroi* from Pará, Brasil. It was then considered a UGL by Jansen et al. (2011); *R. mirandaribeiroi* A UGL. The revision of this sequence (JF790182) indicates that three of six bases in the 5′ extreme are polymorphic and can be coarsely aligned with other sequences. Due to the uniparental inheritance of the mitochondria, these polymorphisms are more probably due to ambiguities in the sequencing chromatograms than to actual heterozygosity. Thus, we determined the genetic distances between the six-base reduced sequence of the *R. mirandaribeiroi* A UGL with the available *16S* sequences of *R. mirandaribeiroi* and *R. major* used in the present study (Appendix S14). The individual MNKA 9783 and the other specimen identified by Jansen et al. (2011) as *R. mirandaribeiroi* (SMF 88236) displayed low uncorrected p-distances with respect to all included specimens of *R. major* (0.38–1.18%), but high genetic distances when compared with *R. mirandaribeiroi* (4.45–5.51%). The morphological difference noted by Jansen et al. (2011) in MNKA 9783 with respect to *R. mirandaribeiroi* can be attributed to the misidentification of the species and it cannot be considered a UGL, but simply a specimen of *R. major*, a species known to occur in the area (Narvaes and Rodrigues, 2009).

Relationships of the Rhinella granulosa *group*

The phylogenetic hypothesis resulting from our DO combined analysis (Fig. 3; Appendix S12) excluding putatively introgressed sequences is considered as the most stringent tests of the phylogenetic relationships among taxa of the *Rhinella granulosa* group, as it includes the greatest amount of evidence (i.e. sequences of nuclear and mitochondrial genomes) used so far to study this group.

In the DO combined analysis, the *Rhinella granulosa* group is well supported and is the sister taxon of a paraphyletic *R. marina* group, with the *R. crucifer* group nested within it (Appendix S12), as was recovered in the hypotheses of Vallinoto et al. (2010) and Pyron and Wiens (2011). Within the group (Fig. 3), *R. major* is recovered as the sister taxon of the remaining species of the *R. granulosa* group, and the clade *R. azarai* + *R. bergi* is the only highly supported clade within the group. The clade *R. granulosa* + *R. mirandaribeiroi* is also recovered in the DO analyses of mitochondrial and nuclear genes (Figs 1 and 2, respectively), always poorly supported. Alternatively, in the bayesian analysis (Appendix S13) the clade *R. bernardoi* + (*Rhinella* sp. CFBH 14062 + (*R. fernandezae* + *R. dorbignyi*)) was the most basal clade and all the interspecific relationships have high posterior probabilities (> 99%), except for the clade *R. centralis* + *R. humboldti* (61%).

The relatively long branch lengths in the most basal clades in the combined MPT (DO) are inconsistent with an early adaptive radiation as an explanation of

the low support in these nodes (Glor, 2010). Alternatively, the occurrence of incomplete lineage sorting can explain the short length of some internal branches. As hybridization and introgression seem to be common phenomena among species of the *R. granulosa* group, one possibility is that the low support for the clades is due to ancient introgression of genes followed by recombination.

The relationships of Rhinella sternosignata

Rhinella sternosignata is recovered as distantly related to the *R. granulosa* group, being the sister taxon of the exemplars of the *R. margaritifera* and *R. veraguensis* groups (Appendix S12). Based on the results of an unpublished PhD dissertation on the phylogeny of the *R. margaritifera* group, Vélez-Rodriguez (2005) suggested a close relationship of *R. sternosignata* and *R. humboldti* (of the *R. granulosa* group). She noted that *R. humboldti* and *R. sternosignata* share some character states: (i) a close articulation between the nasals and the dorsal margin of the pars facialis of the maxilla. In *Rhinella*, this condition was only observed in other species of the *R. granulosa* group and *R.* cf. *margaritifera* (Pramuk, 2006). Based on the results obtained by Pramuk (2006), van Bocxlaer et al. (2010), and our phylogenetic hypothesis, this character state is a synapomorphy of the *R. granulosa* group (see below) and is homoplastic in *R.* cf. *margaritifera* and *R. sternosignata*. (ii) An anteroventral expansion of the zygomatic ramus of the squamosal reaching the medial level of the ventral ramus, but without articulating with the maxilla as in the *R. granulosa* group. The contact between both rami of the squamosal also occurs in all species of the *R. granulosa* group studied by Pramuk (2006) but not in other species of *Rhinella*. Thus, this state is a putative synapomorphy of the *R. granulosa* group with an instance of homoplasy in *R. sternosignata*. (iii) The presence of the m. adductor longus. This muscle is also present in species of the *R. marina* and *R. margaritifera* (except *R. cristinae*) groups, but absent in the *R. veraguensis* and *R. acrolopha* groups (Limeses, 1964; Trueb, 1971; McCranie et al., 1989; Vélez-R. and Ruiz-C., 2002; Frost et al., 2006; Chaparro et al., 2007; Grant and Bolivar-G., 2014). The condition is unknown in the *R. crucifer* and *R. spinulosa* groups. (iv) Inguinal fat bodies, which are also present in the *R. granulosa*, *R. marina*, *R. spinulosa*, and *R. veraguensis* groups, but absent in the *R. acrolopha*, *R. crucifer*, and *R. margaritifera* groups (Silva and Mendelson, 1999). According to the recovered phylogenetic relationships of *Rhinella*, the presence of these character states in *R. sternosignata* and in the *R. marina* (including the *R. crucifer* group) + the *R. granulosa* groups is homoplastic.

Putative phenotypic synapomorphies

Several morphological synapomorphies support the monophyly of the *Rhinella granulosa* group. Pramuk (2006) suggested two unique and unreversed synapomorphies: the presence of prenasal bones (ch42.1), and the presence of an expanded, "flag-shaped" dorsal crest of the ilium in lateral view (ch59.1). However, the optimization of the morphological characters analysed by Pramuk in our phylogenetic hypothesis (DO combined analysis) allowed us to identify five additional putative synapomorphies for this species group: (i) nasal bone articulates with the dorsal margin of the pars facialis of the maxilla from the preorbital process to the posterior margin of the narial opening (Pramuk, 2006; ch7.1; see above); (ii) articulation of zygomatic ramus of the squamosal with the maxilla, thereby completing the bony margin of the orbit (the "closed orbit condition" of Cei, 1972) (ch14.1, see above); (iii) jaw articulation lies anterior to the fenestra ovalis, in lateral view (ch25.2); (iv) alary process of the premaxillae angled to the anterior margin of the premaxillae (ch26.2); and (v) occipital condyles widely separated (ch33.0).

The optimization of morphological, chromosomal, and behavioural characters (see character states and references in Appendix S15) in our optimal tree provides some additional putative synapomorphies for the *Rhinella granulosa* group or internal clades that are described below.

Ability to build and inhabit holes in the ground. Species of the *Rhinella granulosa* group are commonly found sheltering in holes in the ground during the day. The holes are built in wet soil after rains using lateral and alternate movements of the hindlimbs (Gallardo, 1969; Gallardo and Varela de Olmedo, 1993; Narvaes and Rodrigues, 2009), a behaviour that should not be confused with sheltering in natural cracks or cavities not constructed by them. This character state has been reported for the following species of the *R. granulosa* group: *R. azarai*, *R. bergi*, *R. dorbignyi*, *R. fernandezae*, *R. granulosa*, *R. humboldti*, *R. major*, *R. merianae*, and *R. pygmaea* (Appendix S15), whereas it is unknown in *R. bernardoi*, *R. centralis*, *R. mirandaribeiroi*, and *R. nattereri*. As this behaviour has not been reported in any of the outgroups nor in any other known species of *Rhinella*, the ability to build and inhabit holes in the ground optimizes as a synapomorphy of the *R. granulosa* group.

Note composition of the advertisement call. The advertisement calls of the *Rhinella granulosa* group consist of long trills composed of pulsed notes (Guerra et al., 2011). There is a notable variation in the number of pulses per note among species: two in *R. bergi*; three in *R. azarai*, *R. dorbignyi*, *R. fernandezae*, and

R. pygmaea; four in *R. centralis*, *R. granulosa*, *R. humboldti*, *R. merianae*, and *R. mirandaribeiroi*; and six to eight in *R. major* (Appendix S15). There are no available data about advertisement call parameters of *R. bernardoi* and *R. nattereri*. The optimization of the states in the MPTs (either as additive or as non-additive) indicates that the notes with three pulses are plesiomorphic for the *R. granulosa* group, with three subsequent transformations from this ancestral condition, as autapomorphies in *R. major* (six to eight pulses) and in *R. bergi* (two pulses), and four pulses as a synapomorphy of the clade composed of ((*R. merianae* + (*R. centralis* + *R. humboldti*)) + (*R. granulosa* + *R. mirandaribeiroi*). Interestingly, the species that have autapomorphic conditions are sympatric and syntopic between them and with *R. fernandezae*.

Dorsal pigmentation pattern of tail musculature of tadpoles. Tadpoles of the genus *Rhinella* are in general similar in morphology and pigmentation pattern, and resemble the morphological pattern seen in many other bufonids. In most species of the *R. granulosa* and in some of the *R. margaritifera* groups, the dorsal region of the caudal musculature has irregular transverse whitish stripes due to the absence of melanocytes in these areas, which have been interpreted as a synapomorphy of the *R. granulosa* group or an internal clade (Blotto et al., 2014). Species of this group that display this pattern are: *R. azarai*, *R. dorbignyi*, *R. fernandezae*, *R. granulosa*, and *R. pygmaea*. Besides, *R. major* and *R. merianae* have a dorsal coloration of the tail musculature that is uniformly black, both patterns apparently occurs in *R. humboldti* (Appendix S15), and the character state is unknown for *R. bergi* (see discussion regarding the taxonomic identity of the tadpole described by Yanosky et al. (1993) in Blotto et al. (2014)), *R. bernardoi*, *R. centralis*, *R. mirandaribeiroi*, and *R. nattereri* (for which the tadpoles remain undescribed). The examination of a series of tadpoles (LGE 7977, Gosner stage 28) that hatched from an amplexus of a pair of *R. major* (LGE 8331 × 8332) indicates a transverse whitish striped pattern in the caudal musculature, rather than the one reported by Lavilla et al. (2000). These authors did not clearly state how their tadpoles were identified, so we consider only our observations for the optimizations of tadpole morphology in *R. major*. On the basis of our phylogenetic hypothesis, the striped dorsal pattern of the tail is a putative synapomorphy of the *R. granulosa* group. The ancestral state in the clade *R. merianae* + (*R. centralis* + *R. humboldti*) optimizes ambiguously, as it is unknown in *R. centralis*.

Posterior labial tooth rows of the larval oral disc. The tadpoles of some species of the *Rhinella granulosa* group are unique in the genus in having a reduction in the posterior labial tooth rows from three to two (see revision in Blotto et al., 2014; Appendix S15): *R. azarai*, *R. dorbignyi*, and *R. pygmaea*. Furthermore, we were able to determine this condition in the tadpoles of *R. major* (*contra* the presence of three rows reported by Lavilla et al., 2000). Optimization of this character indicates that the presence of two posterior rows is a putative synapomorphy in the *R. granulosa* group, whereas the reversion to three labial tooth rows represents a synapomorphy of the clade (*R. merianae* + (*R. centralis* + *R. humboldti*)) + (*R. granulosa* + *R. mirandaribeiroi*).

Rhinella humboldti and *R. granulosa* have a distinct medial flap bearing P3 (lower labial tooth row), which is absent in other species of the genus (unknown state in *R. merianae*). Our results suggest that *R. merianae* and the undescribed tadpoles of *R. centralis* and *R. mirandaribeiroi* also have this medial flap, and that this condition is a putative synapomorphy of the clade (*R. merianae* + (*R. centralis* + *R. humboldti*)) + (*R. granulosa* + *R. mirandaribeiroi*).

Submarginal papillae. Submarginal papillae absent in the oral disc of tadpoles is a typical state of the *Rhinella granulosa* group, but are apparently present only in some individuals of *R. fernandezae* and *R. major*. We note the absence of submarginal papillae in a series of *R. major*, and we used this state for the character optimization. These papillae are also absent in most species of the *R. margaritifera* group (present only in *R. margaritifera*), but are present in the *R. crucifer*, *R. marina* (polymorphic in *R. marina*), *R. spinulosa*, and *R. veraguensis* groups (see revision in Blotto et al., 2014; Appendix S15). The absence of submarginal papillae optimizes as a putative synapomorphy of the *R. granulosa* group in our phylogenetic hypothesis.

Location of nucleolar organizer regions (NORs). Most species of Bufonidae so far studied have $2n = 2x = 22$ chromosomes (except some species of *Amietophrynus*, see revisions of King, 1990; Kuramoto, 1990; Green and Sessions, 1991). Despite this karyotypic uniformity, there is evident variation in the location of NORs on different clades of Bufonidae. Baldissera et al. (1999) report NORs on chromosome pair 5 in *R. granulosa* and *R. pygmaea*. Among the bufonids, this condition was only reported for species of the *Melanophryniscus tumifrons* group (see revision in Baldo et al., 2012; Appendix S15). Thus, this character state represents an additional synapomorphy of the *R. granulosa* group or an internal clade.

Mating system and hybridization in Bufonidae

Species of the *Rhinella granulosa* group, as with some other species of true-toads of the genera *Amietophrynus*,

Anaxyrus, *Bufo*, *Bufotes*, *Incilius*, and *Rhinella* (see revisions of Wells, 1977; Han and Fu, 2013), show explosive reproductive aggregations and males exhibit classic scramble competition for females. During or after rains, males congregate for a few days/nights in small temporary water bodies forming large choruses to attract females to the reproduction site (Wells, 1977, 2007). Males of these species actively search for females around the breeding site and exhibit a remarkable promiscuity. They often attempt to amplect the first individual that approaches, occasionally form so-called "mating balls" or even amplect inert objects (Wells, 1977, 2007; Haddad et al., 1990; Haddad and Sazima, 1992; Goldberg et al., 2006; Fig. 4). This mating system implies a relatively low species-specificity during reproduction, as it decreases the effectiveness of prezygotic isolating barriers (e.g. advertisement calls) and probably explains the occurrence of interspecific amplexus between sympatric species (e.g. Eaton et al., 1999; Baldo and Basso, 2004; Mollov et al., 2010; Bezerra and Cascon, 2011; Machado and Bernarde, 2011; Fig. 4). In a few groups of toads, there are well-documented cases of natural hybridization (e.g. Hillis et al., 1984; Haddad et al., 1990; Malmos et al., 2001; Masta et al., 2002; Minter et al., 2004; Fontenot et al., 2011; Guerra et al., 2011), but the viability and fertility of these hybrids are mostly unknown (but see Haddad et al., 1990). However, under experimental conditions, it is known that some species of Bufonidae have high rates of interspecific hybridization and survival of hybrids (Blair, 1972; Malone and Fontenot, 2008). Overall, these biological and reproductive characteristics could provide recurrent opportunities for genetic exchange between different species, as is

Fig. 4. Scramble competition and hybridization in Bufonidae (a–i). "Mating balls" in *Rhinella arenarum* (a) and *Melanophryniscus cambaraensis* (b). Interspecific amplexus: *R. bergi* ♂ × *R. arenarum* ♀ (c), *R. ornata* ♂ × *R. icterica* ♀ (d), and *M. krauczuki* ♂ × *M. atroluteus* ♀ (e). Hybrid specimen (*R. major* × *R. bergi* MLP DB 2736; f). Non-specific amplexus: *M.* aff. *devincenzii* (♂) clasping a finger (g), *R. arenarum* (♂) clasping a boot (h), and *R. arenarum* (♂) clasping a piece of cow dung (i).

noticeable in the *R. granulosa* (this work), the *R. crucifer* (Thomé et al., 2012), and the *R. marina* (Sequeira et al., 2011) groups, *Bufotes* (Stöck et al., 2009; Colliard et al., 2010), the *Anaxyrus americanus* group (Fontenot et al., 2011), and *Bufo* (Yamazaki et al., 2008; Garcia-Porta et al., 2012; but see Arntzen et al., 2013) where extensive nuclear and/or mitochondrial introgression was observed. Moreover, Stöck et al. (2009) have proposed that phenomena of hybridization between diploid and tetraploid species can be implied in the origin of hybrid triploid and tetraploid taxa in *Bufotes*. Nevertheless, more detailed studies in other clades of toads are necessary to understand how widespread are the phenomena of hybridization and introgression in Bufonidae.

Currently, introgressive hybridization is considered a phenomenon that can contribute to adaptation and speciation in many species of animals (Baack and Rieseberg, 2007). Thus, it can play a considerable role in the evolution of populations/species by the acquisition of new adaptive phenotypic traits in one species from another, eventually leading to the origin of new species by hybrid speciation (Baack and Rieseberg, 2007; Schwenk et al., 2008; Twyford and Ennos, 2012). While introgression and hybridization in general could have an evident impact in phylogenetic analyses (Hennig, 1966; Posada and Crandall, 2002), this can be at least partially mitigated if detected, so special effort should be made when studying groups where these phenomena are known to occur widely. In this sense, a thorough revision of phylogenetic studies of several bufonids based mostly on mitochondrial sequences, and where cases of hybridization are reported, such as *Anaxyrus* (Pauly et al., 2004), *Amietophrynus* (Cunningham and Cherry, 2004), and the *Rhinella marina* group (Vallinoto et al., 2010), would be desirable.

In this study we detected discordant patterns of nuclear and mitochondrial variation across species of the *Rhinella granulosa* group due to both nuclear and mitochondrial introgression. The results highlight the need to identify the specimens carefully using phenotypic diagnosis and nuclear and mitochondrial sequences, avoiding the problem of species identification inherent to simple "taxonomic" solutions such as DNA barcoding (Hebert et al., 2003). Moreover, the use of multiple independent nuclear markers in addition to mitochondrial sequences is essential to understand more accurately the evolutionary history of toads because it mitigates the potential problem of genetic introgression (e.g. Chen et al., 2009).

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Supporting Information

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

Appendix S1. Locality data for specimens sequenced in this study (GenBank numbers for these specimens are provided in Appendix S3).

Appendix S2. Locality data and bibliographic reference for vouchers of the *Rhinella granulosa* group with sequences available in GenBank (accession numbers for these specimens are provided in Appendix S3).

Appendix S3. List of all voucher specimens and GenBank accession numbers of the sequences employed in this study.

Appendix S4. Primers used to amplify and sequence DNA in this study.

Appendix S5. Results for the different datasets analysed under DO in POY.

Appendix S6. Models of nucleotide substitution for the partitions used in the bayesian phylogenetic analysis.

Appendix S7. Uncorrected p-distances between *16S* sequences of species of the *Rhinella granulosa* group. Sample size in parentheses.

Appendix S8. List of mitochondrial and nuclear sequences for each terminal used in the nuclear (N) and combined (M + N) analyses (DO) and sources of evidence supporting their inclusion or exclusion in these analyses.

Appendix S9.1. Parsimony-informative sites of the *CXCR4* gene.

Appendix S9.2. Parsimony-informative sites of the *NCX1* gene. See Appendix S9.1 for details.

Appendix S9.3. Parsimony-informative sites of the *POMC* gene. See Appendix S9.1 for details.

Appendix S9.4. Parsimony-informative sites of a fragment of the *RAG1* gene (*RAG1a*). See Appendix S9.1 for details.

Appendix S9.5. Parsimony-informative sites of a fragment of the *RAG1* gene (*RAG1b*). See Appendix S9.1 for details.

Appendix S9.6. Parsimony-informative sites of the *RHO* gene. See Appendix S9.1 for details.

Appendix S10. Outgroup relationships of the MPT displayed in the Fig. 1 (DO mitochondrial analysis). See Fig. 1 for details.

Appendix S11. Outgroup relationships of the MPT displayed in the Fig. 2 (DO nuclear analysis). See Fig. 1 for details.

Appendix S12. Outgroup relationships of the MPT displayed in the Fig. 3 (DO combined mitochondrial + nuclear analysis). See Fig. 1 for details.

Appendix S13. Results of the bayesian analysis using the static alignment of the same dataset used in the combined M + N analysis under DO. Values around nodes are Posterior Probabilities. Asterisks indicate groups with values of 100% and nodes with values <50% are collapsed.

Appendix S14. Uncorrected p-distances between *16S* sequences of *Rhinella major*, *R. mirandaribeiroi*, and the specimens preliminarily assigned to *R. mirandaribeiroi* by Jansen et al. (2011). Sample size of *R. major* and *R. mirandaribeiroi* in parentheses.

Appendix S15. Literature sources for the taxonomic distribution of phenotypic characters.