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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.

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.

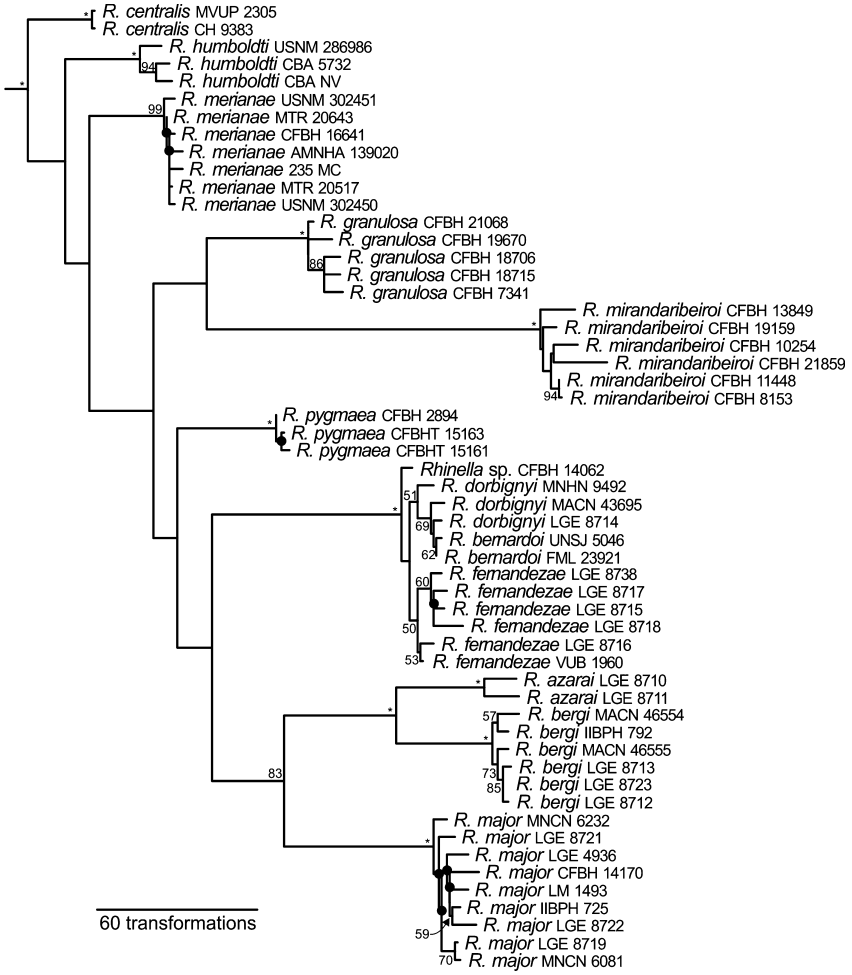

Fig. 1. One of the 6072 MPTs obtained from the analysis of mitochondrial genes under DO (length 6216 steps). Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analysed with parsimony 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.

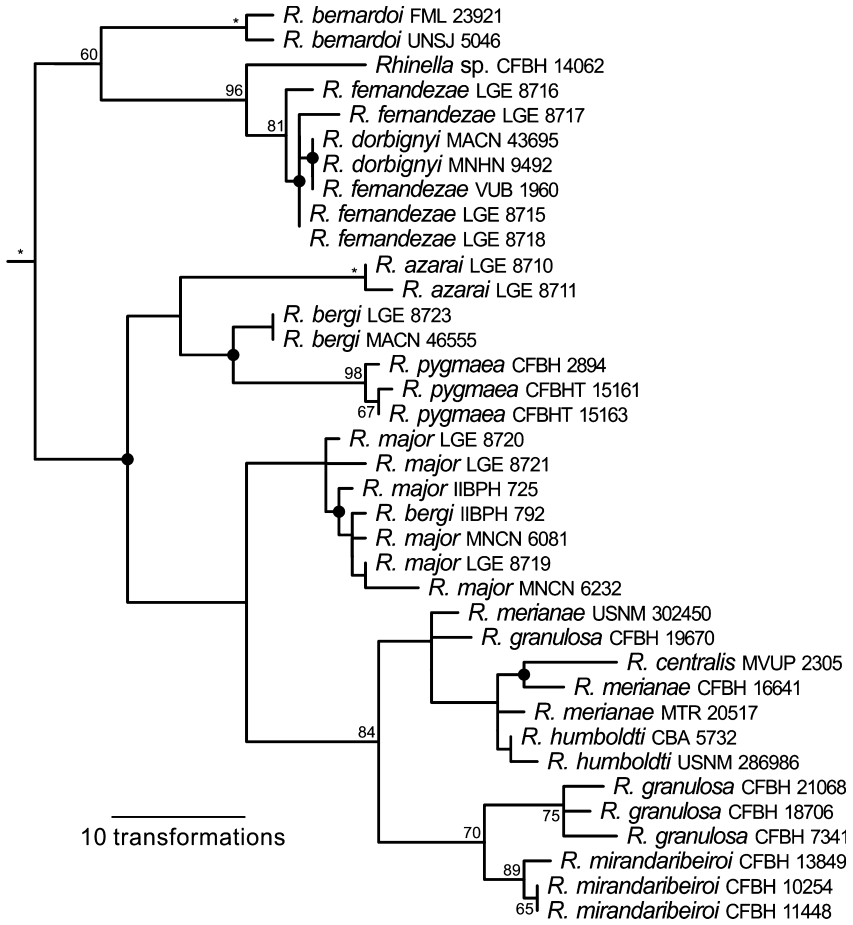

Fig. 2. One of the 270 MPTs obtained from the analysis of nuclear genes under DO (length 1192 steps). Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analysed with parsimony in TNT 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

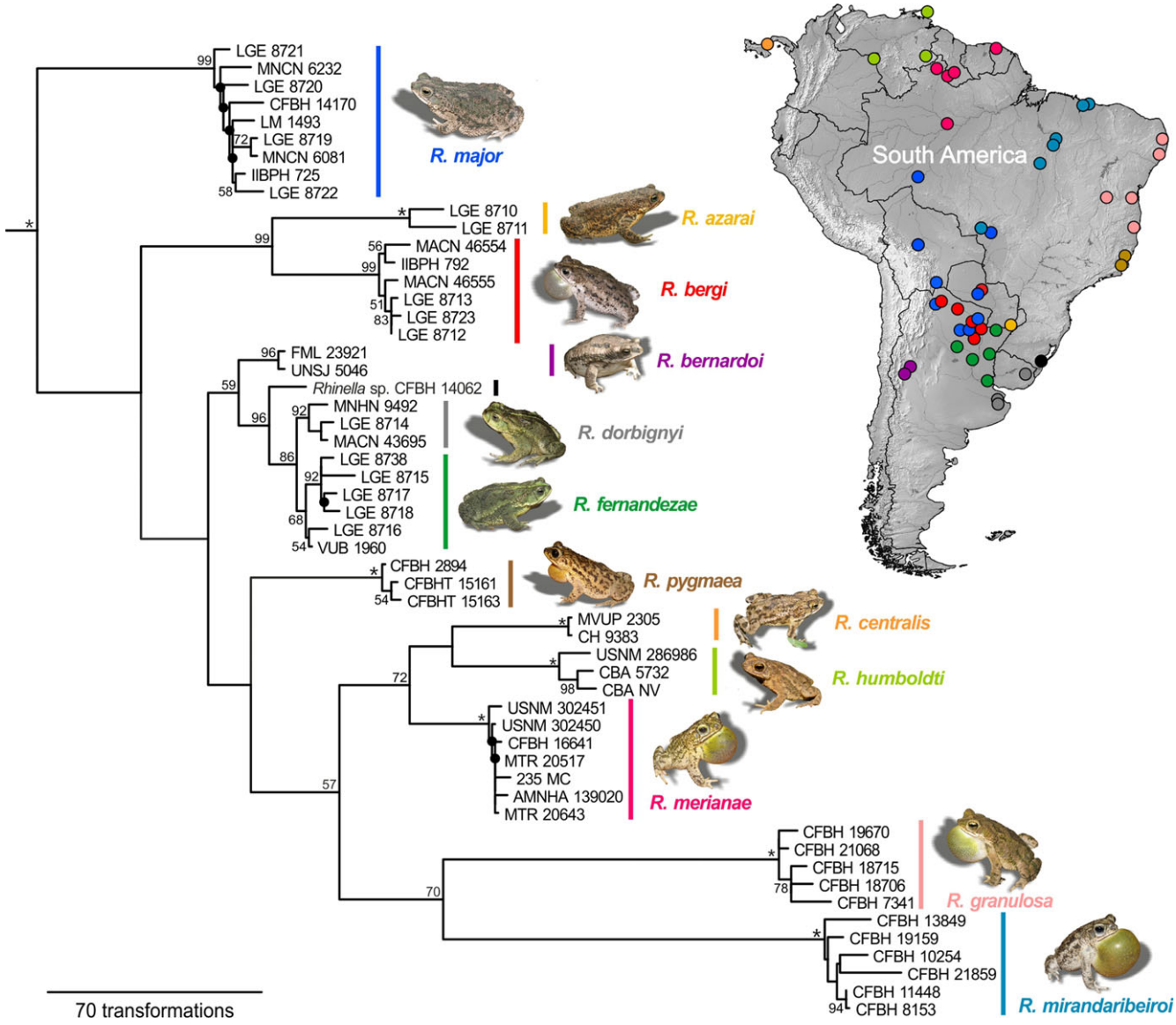

Fig. 3. One of the 264 MPTs obtained from the combined analysis of mitochondrial and nuclear genes under DO (length 7446 steps), after removal of the putatively introgressed sequences. Filled circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife absolute 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.

Appendix S1. Locality data for specimens sequenced in this study (GenBank numbers for these specimens are provided in **Appendix S3**). Collection abbreviations are as follow: CBA (field numbers of César Barrio-Amoros); CFBH (Collection Célio F.B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil); CHP (Círculo Herpetológico de Panamá, Panamá); FML (Fundación Miguel Lillo, Tucumán, Argentina); IIBP-H (Instituto de Investigación Biológica del Paraguay, Asunción, Paraguay); IMCN-UNSJ (Universidad Nacional de San Juan, San Juan, Argentina); LGE (Instituto de Biología Subtropical, CONICET-Universidad Nacional de Misiones, Posadas, Misiones, Argentina); MACN (Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina); MNCN (Museo Nacional de Ciencias Naturales, Madrid, Spain); MNHN (Museo Nacional de Historia Natural de Montevideo. Uruguay); MTR (Miguel T. Rodrigues field numbers, Universidade de São Paulo, São Paulo, Brazil); MVUP (Museo de Vertebrados de la Universidad de Panamá, Panamá).

Rhinella azarai: ARGENTINA: Misiones: Candelaria, Candelaria, intersection Ruta Nacional 12 and Ruta Provincial 3 (LGE 8710–1).

R. bergi: ARGENTINA: Chaco: General Güemes, 4 km W El Sauzalito (LGE 8712); San Fernando, Ruta Provincial 63, 3 km NE from its intersection with Ruta Nacional 16 (MACN 46555); Formosa: Pilcomayo, Ruta Provincial Nº 3, 1.5 km W Palma Sola (LGE 8723); Salta: Rivadavia, Rio Bermejo near Morillo (MACN 46554); Santa Fe: General Obligado, Ruta Provincial 32, 16 km S Villa Ana (LGE 8713). PARAGUAY: Presidente Hayes: Ruta Nacional Nº 5, 50 km E Concepción (IIBP-H 792).

R. bernardoi: ARGENTINA: San Juan: Valle Fértil, Parque Provincial Ischigualasto (IMCN-UNSJ 5046 - paratype); Caucete, Quebrada de Las Flores (FML 23921).

R. centralis: PANAMÁ: Coclé: Valle de Antón (MVUP 2305, CH 9383).

R. dorbignyi: ARGENTINA: Buenos Aires: Chascomús, Chascomús (LGE 8714); Dolores, Dolores (MACN 43695). URUGUAY: Treinta y tres: Bañado de los Oliveras (MNHN 9492).

R. fernandezae: ARGENTINA: Entre Rios: Chajarí, Chajarí (LGE 8716); Islas de Ibicuy, Ruta Nacional 12 between Brazo Largo and Arroyo Luciano (LGE 8738); Corrientes: General Paz, Ita Ibaté (LGE 8717); Santa Fe: San Justo, Ruta Provincial 62, 1.5 km SW El Laurel (LGE 8715); 9 de Julio, Tostado, Rio Salado and Ruta Provincial 2 (LGE 8718).

R. granulosa: BRAZIL: Alagoas: Passo de Camarajibe (CFBH 7341); Bahia: Caetité, Povoado Senote (CFBH 21068); Aurelino Leal, Fazenda Pedras Pretas (CFBH 18715); Espírito Santo: Linhares: Fazenda Luzitânia (CFBH 18706); Pernambuco: Itamaracá, Praia do Forno da Cal (CFBH 19670).

R. henseli: BRAZIL: Rio Grande do Sul: Mato Castelhano (MNRJ 33006).

R. humboldti: VENEZUELA: Bolivar: Chivaton, Gran Sabana (CBA 5732); Amazonas: Puerto Ayacucho (CBA unvouchered).

R. major: ARGENTINA: Chaco: 9 de Julio, Las Breñas (LGE 8719), General Güemes, Ruta Provincial, 1 Km W El Sauzal (LGE 8722); Formosa: Pirané, Ruta Provincial 81; 1.5 km NW from its intersection with Ruta Provincial 3 (LGE 8721); Salta: Rivadavia, Ruta Provincial 13, 10 km W El Ocultar (LGE 8720). BOLIVIA: Cochabamba: Chaparé, Paractito - Los Guácharos (MNCN 6232); Tarija: Villa Montes, Gran Chaco (MNCN 6081). BRAZIL: Mato Grosso: Poconé, Fazenda

Ipiranga (CFBH 14170). PARAGUAY: Presidente Hayes: Parador Búfalo Bill, 15 km N Pozo Colorado (IIBP-H 725).

R. merianae: BRAZIL: Amazonas: Manaus, Reserva Ducke (CFBH 16641); Roraima: Estação Ecológica de Maracá (MTR 20517), Fazenda Salvamento (MTR 20643).

R. mirandaribeiroi: BRAZIL: Tocantins: Aguiarnópolis (CFBH 8153), Araguacema (CFBH 10254), Babaçulândia (CFBH 11448); Maranhão: Santo Amaro do Maranhão, Parque Nacional dos Lençois Maranhenses (CFBH 13849), Alcântara (CFBH 19159); Mato Grosso: Estação Ecológica Serra das Araras (CFBH 21859).

R. ornata: ARGENTINA: Misiones: 9 de Julio (LGE 8729).

R. pygmaea: BRAZIL: Rio de Janeiro: São João da Barra (CFBH 2894), Mimoso do Sul (samples CFBHT 15161 and 15163 from two specimens of series CFBH 27102–5).

***Rhinella* sp.**: BRAZIL: Rio Grande do Sul: Rio Grande, Ilha dos Marinheiros (CFBH 14062).

R. sternosignata: VENEZUELA: Barinas: Caño Los Monos, Acequias (CBA unvouchered).

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**).

Rhinella fernandezae: URUGUAY (VUB 1960, as *Rhinella* cf. *granulosa*: van Bocxlaer et al., 2010).

R. humboldti: TRINIDAD: Arima (USNM 286986, as *Bufo humboldti*: Pramuk, 2006; see also Narvaes and Rodrigues, 2009).

R. major: BRAZIL: Rondia: Porto Velho (LM 1493, as *Bufo granulosus*: Pramuk et al., 2001).

R. merianae: FRENCH GUIANA: Mana (235 MC, as *Chaunus granulosus* A: Fouquet et al., 2007); GUYANA: Upper Takutu-Upper Essequibo (AMNHA 139020, as *Bufo granulosus*: Frost et al., 2006; see also Narvaes and Rodrigues 2009); BRAZIL Roraima: Caracaranã (USNM 302450: Pramuk 2006, as *Bufo granulosus* 1; see also Narvaes and Rodrigues, 2009. USNM 302451, as *Bufo granulosus*: Pauly et al., 2004; see also Narvaes and Rodrigues, 2009).

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

The sequences generated for this article are in bold. Abbreviations: *CXCR4*: *chemokine receptor 4* gene; *NCX1*: *sodium-calcium exchanger subunit 1* gene; *POMC*: *proopiomelanocortin A* gene; *RAG1a*: *recombination activating protein 1* gene (fragment A); *RAG1a*: *recombination activating protein 1* gene (fragment B); *RHO*: *rhodopsin* gene; *12S-16S*: ribosomal genes *12S* and *16S*; *ND1*: *NADH dehydrogenase subunit 1* gene; *CytB*: *cytochrome b* gene.

Especie	Voucher	*CXCR4*	*NCX1*	*POMC*	*RAG1a*	*RAG1b*	*RHO*	*CytB*	*12S*	*16S*	*ND1*
Amazophrynella minuta	QCAZ 17377	DQ306496	–	DQ158262	DQ158346	–	–	–	DQ158420		–
Amietophrynus brauni	unvouchered	DQ306514	–	DQ158279	DQ158361	–	DQ284021	–	FJ882822		
Anaxyrus boreas	MVZ 223292	DQ306499	–	DQ158278	HM563973	–	–	HM563929	DQ158436		–
Barbarophryne brongersmai	VUB 1786	FJ882718	FJ882663	–	–	–	–	–	FJ882817		
Bufo gargarizans	CAS 228184 - USNM 292081	FJ882708	FJ882654	DQ158270	DQ158353	–	–	–	FJ882808		
Duttaphrynus melanostictus	FMNH 255309	DQ306508	–	DQ158317	DQ158394	–	–	–	DQ158475		–
Incilius coccifer	KU 290030	DQ306526	–	DQ158284	DQ158366	–	–	AY927863	DQ158443		–
Ingerophrynus galeatus	FMNH 256443	DQ306506	–	DQ158293	DQ158374	–	–	–	DQ158452		–

Especie	Voucher	*CXCR4*	*NCX1*	*POMC*	*RAG1a*	*RAG1b*	*RHO*	*CytB*	*12S*	*16S*	*ND1*
Leptophryne borbonica	VUB 673	EF107450	EF107224	–	EF107287	–	–	–		FJ882799	
Peltophryne lemur	A. Goebel	DQ306513	–	DQ158306	DQ158386	–	–	AY028506		DQ158465	–
Phrynoidis juxtaspera	VUB 649 - FMNH 231245	FJ882710	FJ882656	DQ158304	DQ158385	–	–	–		FJ882805	
Rhinella arenarum	AR 305 - MACN 38639	DQ306529	–	DQ158271	DQ158354	AY844370	AY844547	AY843795		DQ158429	JX204061
R. arunco	KU 217369	DQ306552	–	DQ158283	DQ158365	–	–	–		DQ158442	–
R. cf. *margaritifera*	QCAZ 13896	DQ306554	–	DQ158313	DQ158390	–	–	–		DQ158471	–
R. henseli	MNRJ 33006	**KP684942**	–	**KP685077**	**KP685113**	**KP685143**	GU907407	–		**KP685183**	GU907246
R. marina	KU 217482	DQ306544	–	DQ158316	DQ158393	–	–	DQ415597		DQ158474	–
R. martyi	MW 1006	FJ882729	FJ882675	–	–	–	–	–		FJ882832	
R. nesiotes	UTA 53310	DQ306500	–	DQ158320	DQ158397	–	–	–		DQ158478	–
R. ornata	USNM 303015 - LGE 8729	–	**KP685015**	DQ158288	–	–	–	DQ415596		DQ158447	–
R. schneideri	KU 289057 – VUB 1965	DQ306528	FJ882674	DQ158322	DQ158399	–	–	DQ415598		FJ882831	
R. spinulosa	IDLR 3837	DQ306566	–	DQ158328	DQ158405	–	–	–		DQ158487	–
R. sternosignata	CBA unvouchered	**KP684943**	**KP685016**	**KP685078**	**KP685114**	**KP685144**	**KP685163**	–		**KP685184**	**KP685035**
R. azarai	LGE 8710	**KP684944**	–	**KP685079**	**KP685115**	**KP685145**	–	**KP684986**		**KP685185**	**KP685036**
	LGE 8711	**KP684945**	**KP685017**	**KP685080**	**KP685116**	–	**KP685164**	**KP684987**		**KP685186**	**KP685037**

Especie	Voucher	*CXCR4*	*NCX1*	*POMC*	*RAG1a*	*RAG1b*	*RHO*	*CytB*	*12S*	*16S*	*ND1*
R. bergi	LGE 8723	**KP684946**	**KP685018**	**KP685081**	**KP685117**	**KP685146**	**KP685165**	**KP684988**	**KP685187**		**KP685038**
	IIBP-H 792	**KP684947**	–	**KP685082**	**KP685118**	**KP685147**	–	**KP684989**	**KP685188**		**KP685039**
	LGE 8713	**KP684948**	–	–	–	–	–	–	**KP685189**		**KP685040**
	LGE 8712	**KP684949**	–	–	–	–	–	–	**KP685190**		**KP685041**
	MACN 46554	–	–	**KP685083**	–	–	–	–	**KP685191**		**KP685042**
	MACN 46555	**KP684950**	–	**KP685084**	**KP685119**	–	–	**KP684990**	**KP685192**		**KP685043**
R. bernardoi	UNSJ 5046	**KP684951**	**KP685019**	**KP685085**	**KP685120**	**KP685148**	**KP685166**	–	**KP685193**		**KP685044**
	FML 23921	**KP684952**	–	**KP685086**	**KP685121**	–	–	**KP684991**	**KP685194**		–
R. centralis	MVUP 2305	**KP684953**	**KP685020**	**KP685087**	**KP685122**	**KP685149**	**KP685167**	**KP684992**	**KP685195**		**KP685045**
	CH 9383	**KP684954**	–	–	–	–	–	–	**KP685196**		–
R. dorbignyi	LGE 8714	–	–	**KP685088**	–	–	–	–	**KP685197**		**KP685046**
	MACN 43695	**KP684955**	–	**KP685089**	–	–	–	**KP684993**	**KP685198**		**KP685047**
	MNHN 9492	**KP684956**	**KP685021**	**KP685090**	**KP685123**	–	**KP685168**	**KP684994**	**KP685199**		**KP685048**
R. fernandezae	VUB 1960	FJ882728	FJ882673	–	–	–	–	–	FJ882774	FJ882775	
	LGE 8738	–	–	–	–	–	–	**KP684995**	**KP685200**		**KP685049**
	LGE 8717	**KP684957**	–	**KP685091**	**KP685124**	**KP685150**	–	**KP684996**	**KP685201**		**KP685050**
	LGE 8718	**KP684958**	**KP685022**	**KP685092**	–	–	**KP685169**	**KP684997**	**KP685202**		**KP685051**
	LGE 8715	**KP684959**	–	**KP685093**	**KP685125**	–	–	**KP684998**	**KP685203**		**KP685052**
	LGE 8716	**KP684960**	–	**KP685094**	**KP685126**	–	–	**KP684999**	**KP685204**		**KP685053**
R. granulosa	CFBH 7341	**KP684961**	**KP685023**	**KP685095**	**KP685127**	**KP685151**	**KP685170**	**KP685000**	**KP685205**		**KP685054**
	CFBH 18706	**KP684962**	**KP685024**	**KP685096**	**KP685128**	**KP685152**	**KP685171**	**KP685001**	**KP685206**		**KP685055**
	CFBH 21068	–	–	**KP685097**	**KP685129**	–	**KP685172**	–	**KP685207**		–
	CFBH 18715	–	–	–	**KP685130**	–	–	–	**KP685208**		**KP685056**
	CFBH 19670	**KP684963**	–	**KP685098**	–	–	–	**KP685002**	**KP685209**		**KP685057**

Especie	Voucher	*CXCR4*	*NCX1*	*POMC*	*RAG1a*	*RAG1b*	*RHO*	*CytB*	*12S*	*16S*	*ND1*
R. humboldti	CBA 5732	**KP684964**	**KP685025**	**KP685099**	**KP685131**	**KP685153**	**KP685173**	–	**KP685210**		**KP685058**
	CBA unvouchered	**KP684965**	–	–	–	–	**KP685174**	–	**KP685211**		–
	USNM 286986	–	–	DQ158276	DQ158358	–	–	–	DQ158434		–
R. major	LGE 8720	**KP684966**	**KP685026**	**KP685100**	**KP685132**	–	**KP685175**	**KP685003**	**KP685212**		**KP685059**
	IIBP-H 725	**KP684967**	–	**KP685101**	**KP685133**	**KP685154**	–	**KP685004**	**KP685213**		**KP685060**
	LGE 8722	**KP684968**	–	–	–	–	–	–	**KP685214**		**KP685061**
	CFBH 14170	**KP684969**	–	–	–	–	–	–	**KP685215**		**KP685062**
	LGE 8721	**KP684970**	–	**KP685102**	**KP685134**	–	–	**KP685005**	**KP685216**		**KP685063**
	LGE 8719	**KP684971**	**KP685027**	**KP685103**	**KP685135**	–	–	**KP685006**	**KP685217**		**KP685064**
	MNCN 6081	**KP684972**	**KP685028**	**KP685104**	**KP685136**	**KP685155**	–	**KP685007**	**KP685218**		–
	MNCN 6232	**KP684973**	**KP685029**	**KP685105**	–	**KP685156**	–	**KP685008**	**KP685219**		–
	LM 1493	–	–	–	–	–	–	AY028508	AY028483	AY028496	–
R. merianae	CFBH 16641	**KP684974**	**KP685030**	**KP685106**	**KP685137**	**KP685157**	**KP685176**	**KP685009**	**KP685220**		**KP685065**
	235 MC	–	–	–	–	–	–	–	EF364280	EF364306	–
	AMNH 139020	–	–	–	–	–	DQ283966	–	DQ283332		–
	USNM 302450	DQ306557	–	DQ158298	–	–	–	–	DQ158457		–
	USNM 302451	–	–	–	–	–	–	–	AY680261		–
	MTR 20517	**KP684975**	**KP685031**	**KP685107**	–	–	–	**KP685010**	**KP685221**		**KP685066**
	MTR 20643	–	–	–	–	–	–	–	**KP685222**		–
R. mirandaribeiroi	CFBH 10254	**KP684976**	**KP685032**	**KP685108**	**KP685138**	**KP685158**	**KP685177**	**KP685011**	**KP685223**		**KP685067**
	CFBH 13849	**KP684977**	–	**KP685109**	**KP685139**	**KP685159**	**KP685178**	**KP685012**	**KP685224**		**KP685068**
	CFBH 21859	**KP684978**	–	–	–	–	–	–	**KP685225**		**KP685069**
	CFBH 8153	**KP684979**	–	–	–	–	–	–	**KP685226**		**KP685070**

Especie	Voucher	*CXCR4*	*NCX1*	*POMC*	*RAG1a*	*RAG1b*	*RHO*	*CytB*	*12S*	*16S*	*ND1*
R. mirandaribeiroi	CFBH 11448	**KP684980**	–	–	**KP685140**	–	**KP685179**	–	**KP685227**		**KP685071**
(cont.)	CFBH 19159	**KP684981**	–	–	–	–	–	–	**KP685228**		**KP685072**
R. pygmaea	CFBH 2894	**KP684982**	**KP685033**	**KP685110**	**KP685141**	**KP685160**	**KP685180**	**KP685013**	**KP685229**		**KP685073**
	CFBHT 15163	**KP684983**	–	**KP685111**	–	–	**KP685181**	–	**KP685230**	–	**KP685074**
	CFBHT 15161	**KP684984**	–	–	–	**KP685161**	**KP685182**	–	**KP685231**	–	**KP685075**
Rhinella sp.	CFBH 14062	**KP684985**	**KP685034**	**KP685112**	**KP685142**	**KP685162**	–	**KP685014**	**KP685232**		**KP685076**

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

Gene	Primer	Direction	Primer sequence (5'→3')	Source
12S-tRNA$_{Val}$-16S	MVZ59	Forward	ATAGCACTGAAAAYGCTDAGATG	Graybeal, 1997
	Phe2-L	Forward	AAAGCATAACACTGAAGATGTTAAGATG	Wiley et al., 1998
	12S F-H	Reverse	CTTGGCTCGTAGTTCCCTGGCG	Goebel et al., 1999
	12S A-L	Forward	AAACTGGGATTAGATACCCCACTAT	Goebel et al., 1999
	tRNAval-H	Reverse	GGTGTAAGCGARAGGCTTTKGTTAAG	Goebel et al., 1999
	12Sm	Forward	GGCAAGTCGTAACATGGTAAG	Pauly et al., 2004
	L13	Forward	TTAGAAGAGGCAAGTCGTAACATGGTA	Feller and Hedges, 1998
	Titus I	Reverse	GGTGGCTGCTTTTAGGCC	Titus and Larson, 1996
	L2A	Forward	CCAAACGAGCCTAGTGATAGCTGGTT	Hedges, 1994
	H10	Reverse	TGATTACGCTACCTTTGCACGGT	Hedges, 1994
	AR	Forward	CGCCTGTTTATCAAAAACAT	Palumbi et al., 1991
	Wilkinson2	Reverse	GACCTGGATTACTCCGGTCTGA	Wilkinson et al., 1996
16S-tRNA$_{Leu}$-ND1-tRNA$_{Ile-Gln}$	16S-frog	Forward	TTACCCTRGGGATAACAGCGCAA	Wiens et al., 2005
	tMet-frog	Reverse	TTGGGGTATGGGCCCAAAAGCT	Wiens et al., 2005
	ND1 F1	Forward	AGCCATAATCATCTGAACC	Smith et al., 2005
	ND1 R1	Reverse	TCCTCCCTATCAAGGAGGTCC	Smith et al., 2005
CytB	CytbDen3-L	Forward	AAYATYTCCRYATGATGRAAYTTYGG	Santos and Cannatella, 2011
	CytbDen1-H	Reverse	GCRAANAGRAAGTATCATTCNGGYTTRAT	Santos and Cannatella, 2011
CXCR4	CXCR4-C	Forward	GTCATGGGCTAYCARAAGAA	Biju and Bossuyt, 2003
	CXCR4-G	Reverse	AGGCAACAGTGGAARAANGC	Biju and Bossuyt, 2003
POMC	POMC-F	Forward	GAATGTATYAAAGMMTGCAAGATGGWCCT	Wiens et al., 2005
	POMC2B	Reverse	GCATTYTTGAAAAGAGTCATTARTGGAGTCTG	Pramuk, 2006
RAG1a	MartFl1	Forward	AGCTGCAGYCARTAYCAYAARATGTA	Hoegg et al., 2004
	AmpR1	Reverse	AACTCAGCTGCATTKCCAATRTCA	Hoegg et al., 2004
RAG1b	R1-GFF	Forward	GAGAAGTCTACAAAAAVGGCAAAG	Faivovich et al., 2005
	R1-GFR	Reverse	GAAGCGCCTGAACAGTTTATTAC	Faivovich et al., 2005
RHO	Rhod1A	Forward	ACCATGAACGGAACAGAAGGYCC	Bossuyt and Milinkovitch, 2000
	Rhod1C	Reverse	CCAAGGGTAGCGAAGAARCCTTC	Bossuyt and Milinkovitch, 2000

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Appendix S5. Results for the different dataset analyzed under direct optimization in POY.

Dataset	**M (n = 77)**	**N (n = 59)**	**M + N (n = 77)**
Number of terminals	77	59	77
Number of RAS+TBR	151	72	63047
Number of rounds of fusion	1413	787	579071
Number of rounds of ratcheting	98	48	31513
Number of MPT	6072	270	264
Length of optimal trees	6216	1192	7446

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

Gene	Partition	Selected model
tRNAs	Fragment	GTR+I+G
12S	Fragment	GTR+G
16S	Fragment	HKY+G
CytB	By codon	SYM+G for 1st, F81 for 2nd, and GTR+G for 3rd
ND1	By codon	SYM+I+G for 1st, GTR+I+G for 2nd, and GTR+I+G for 3rd
CXCR4	By codon	GTR+G for 1st, F81+G for 2nd, and HKY+G for 3rd
NCX1	By codon	HKY+G for 1st, F81+G for 2nd, and GTR+G for 3rd
POMC	By codon	GTR+G for 1st, HKY+G for 2nd, and SYM+G for 3rd
RAG1a	By codon	HKY+G for 1st, HKY+G for 2nd, and HKY+I for 3rd
RAG1b	By codon	HKY for 1st, F81 for 2nd, and SYM+G for 3rd
RHO	By codon	K80+I for 1st, F81 for 2nd, and K80 for 3rd

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

	1												
1-*R. bernardoi* (2)	0.00	**2**											
2-*R. dorbignyi* (3)	0.00–0.35	0.17–0.52	**3**										
3-*Rhinella* sp. (1)	0.17	0.17–0.35	–	**4**									
4-*R. fernandezae* (6)	0.35–0.52	0.35–0.69	0.17–0.35	0.00–0.35	**5**								
5-*R. pygmaea* (1)	3.11	3.11–3.46	3.45–3.63	3.45–3.63	–	**6**							
6-*R. azarai* (2)	2.43–2.61	2.43–2.94	2.60–2.77	2.77–3.11	1.73–2.08	0.70	**7**						
7-*R. bergi* (6)	2.95–3.90	3.11–4.36	3.11–4.13	3.29–4.59	1.73–2.53	0.86–1.73	0.00–0.57	**8**					
8-*R. major* (9)	3.80–4.74	3.97–5.13	3.97–4.93	4.15–5.31	1.21–1.90	1.73–3.24	2.25–3.70	0.00–0.94	**9**				
8-*R. granulosa* (5)	4.33–6.14	4.31–6.11	4.49–6.34	4.66–6.81	2.93–4.31	2.94–5.27	3.28–5.42	2.77–4.79	0.00–1.14	**10**			
10-*R. mirandaribeiroi* (6)	4.49–6.15	4.50–6.38	4.32–5.92	4.32–6.37	4.32–5.65	3.29–4.56	3.98–5.89	4.32–6.34	3.81–5.95	0.00–0.86	**11**		
11-*R. centralis* (2)	3.98–4.15	3.97–4.48	4.14–4.31	4.31–4.66	3.28–3.46	2.93–3.47	3.80–4.94	3.11–4.35	3.11–4.51	3.80–5.21	0.17	**12**	
12-*R. humboldti* (3)	4.49–5.15	4.66–5.33	4.67–5.33	4.84–5.70	3.11–3.87	2.94–4.06	3.46–5.14	2.94–4.55	3.47–5.04	3.97–5.41	2.25–2.75	0.91–1.28	**13**
13-*R. merianae* (7)	4.85–7.40	4.67–7.90	5.02–7.65	5.20–7.67	3.81–5.41	3.47–5.95	4.34–7.13	3.64–6.15	3.64–5.94	3.98–6.97	2.77–4.42	2.57–3.93	0.00–0.96

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

Species	Acronym	Mitochondrial sequences			Nuclear sequences				Observations
		Analysis M	**Analysis M+N**	**Taxonomic and phylogenetic justification**	**Analysis N**	**Analysis M+N**	**Phylogeny-based justification**	**Sequences analysis justification**	
R. azarai	**LGE 8710**	Included	Included	Recovered in a well-supported monophyletic *R. azarai*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. azarai* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
	LGE 8711	Included	Included	Recovered in a well-supported monophyletic *R. azarai*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. azarai* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
R. bergi	**MACN 46554**	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	Nuclear polymorphisms in the *POMC* gene in agreement with a hybrid origin between *R. major* and *R. bergi* parentals	Sympatric with *R. major*
	IIBPH 792	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	Included	**Not included**	Nested in a poorly supported (<50%) *R. major* in N analysis	Nuclear polymorphisms in *POMC*, *RAG1a*, and *RAG1b* genes in agreement with a hybrid origin between *R. major* and *R. bergi* parentals	Sympatric with *R. major*
	LGE 8723	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Sympatric with *R. major*
	LGE 8712	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Sympatric with *R. fernandezae* and *R. major*
	LGE 8713	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	Nuclear sequence of the *CXCR4* gene highly similar to the sympatric species *R. fernandezae* (p-distance = 0–0.15%)	Sympatric with *R. fernandezae* and *R. major*

	MACN 46555	Included	Included	Recovered in a well-supported monophyletic *R. bergi*. Agreement with the taxonomic determination	Included	**Not included**	-	Relative high levels of polymorphisms regard to the remaining specimens of *R. bergi*	Sympatric with *R. fernandezae* and *R. major*.
R. bernardoi	**FML 23921**	Included	**Not included**	Deeply nested within *R. dorbignyi* in M analysis but not in N analysis	Included	Included	Recovered in a well-supported monophyletic *R. bernardoi* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
	UNSJ 5046	Included	**Not included**	Deeply nested within *R. dorbignyi* in M analysis but not in N analysis	Included	Included	Recovered in a well-supported monophyletic *R. bernardoi* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
R. centralis	**CH 9383**	Included	Included	Recovered in a well-supported monophyletic *R. centralis*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric species
	MVUP 2305	Included	Included	Recovered in a well-supported monophyletic *R. centralis*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Allopatric species
***Rhinella* sp.**	**CFBH 14062**	Included	Included	Concordant relationships with N analysis	Included	Included	Concordant relationships with N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
R. dorbignyi	**LGE 8714**	Included	Included	Recovered in a monophyletic *R. dorbignyi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Putative allopatric population
	MACN 43695	Included	Included	Recovered in a monophyletic *R. dorbignyi*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	MNHN 9492	Included	Included	Recovered in a monophyletic *R. dorbignyi*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with N analysis	No nuclear polymorphisms associated with introgression	Allopatric population

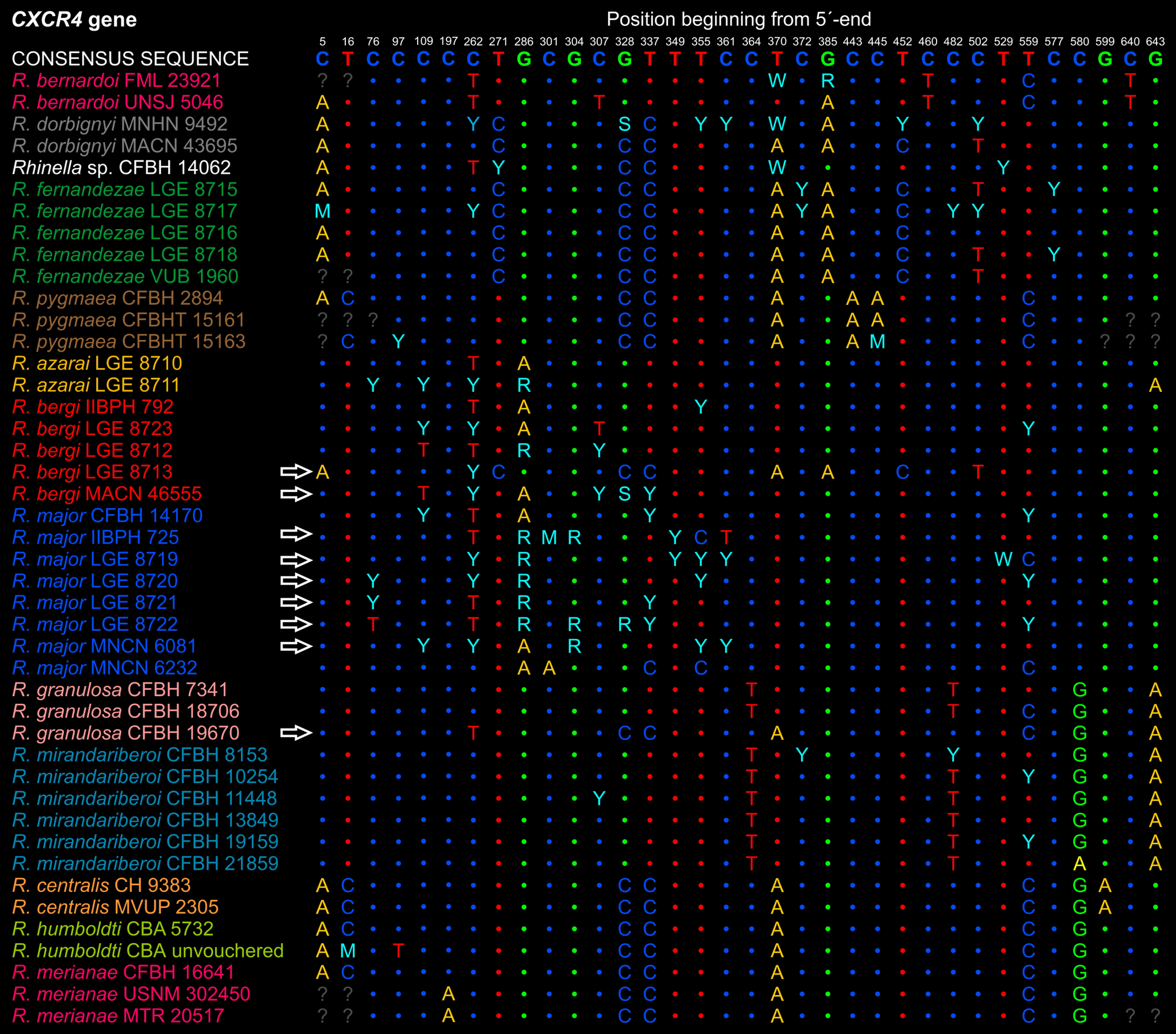
R. fernandezae	**LGE 8738**	Included	Included	Recovered in a monophyletic *R. fernandezae*. Agreement with the taxonomic determination	**No sequences available**	**No sequences available**	-	-	-
	LGE 8715	Included	Included	Recovered in a monophyletic *R. fernandezae*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Sympatric with *R. major* and *R. bergi*
	LGE 8717	Included	Included	Recovered in a monophyletic *R. fernandezae*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Allopatric population
	LGE 8716	Included	Included	Recovered in a monophyletic *R. fernandezae*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Allopatric population
	LGE 8718	Included	Included	Recovered in a monophyletic *R. fernandezae*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Sympatric with *R. major* and *R. bergi*
	VUB 1960	Included	Included	Recovered in a monophyletic *R. fernandezae*	Included	Included	-	No nuclear polymorphisms associated with introgression	-
R. granulosa	**CFBH 18706**	Included	Included	Recovered in a well-supported monophyletic *R. granulosa*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Allopatric population
	CFBH 18715	Included	Included	Recovered in a well-supported monophyletic *R. granulosa*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population
	CFBH 19670	Included	Included	Recovered in a well-supported monophyletic *R. granulosa*. Agreement with the taxonomic determination	Included	**Not included**	Collapsed in a polytomy with a specimen of *R. merianae* and a clade composed of *R. marianae*, *R. centralis*, and *R. humboldti*	Nuclear polymorphisms in *CXCR4* and *POMC* genes in agreement with a hybrid origin between *R. granulosa* and *R. merianae*	Putative allopatric population

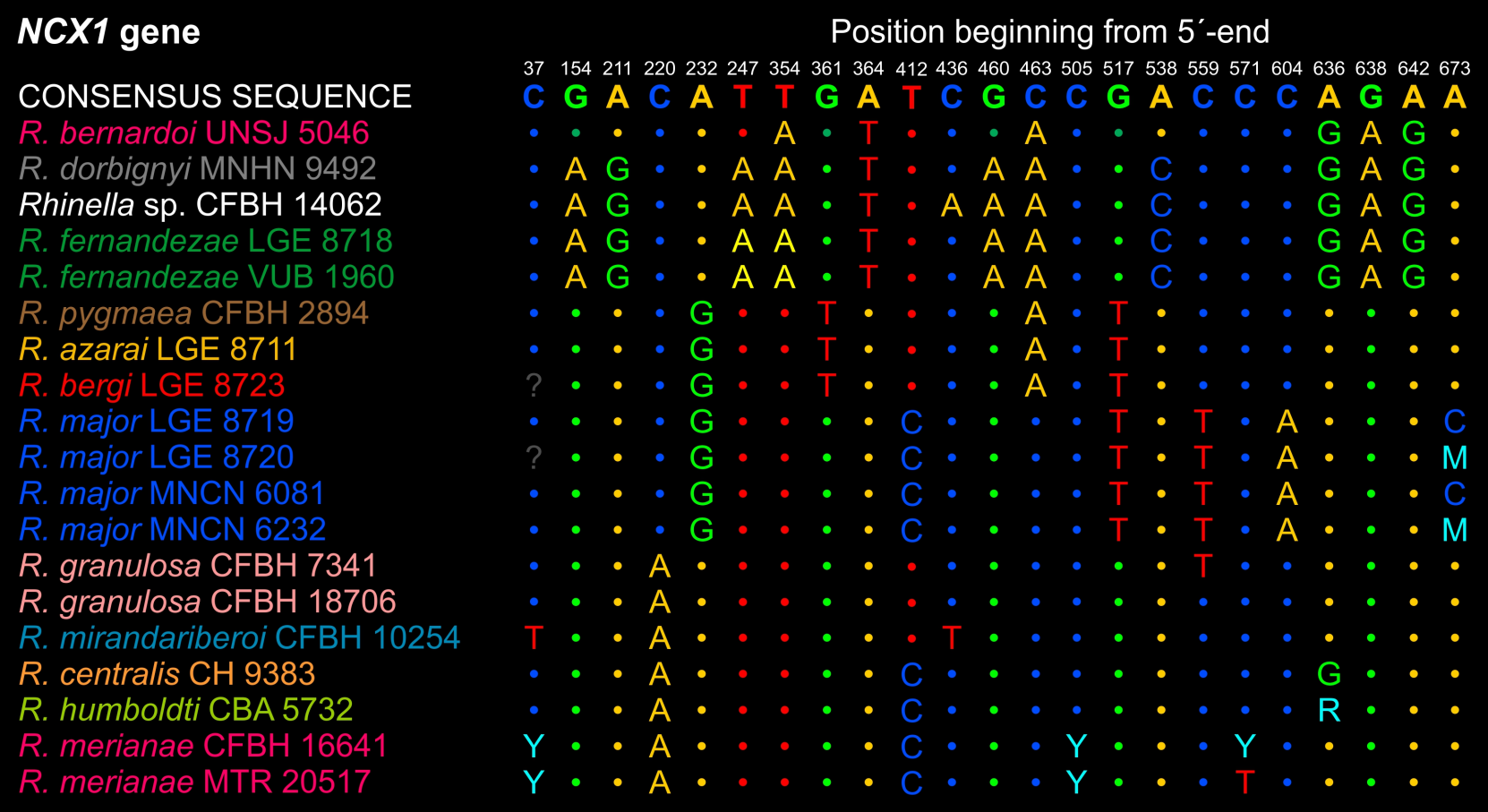
	CFBH 21068	Included	Included	Recovered in a well-supported monophyletic *R. granulosa*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	CFBH 7341	Included	Included	Recovered in a well-supported monophyletic *R. granulosa*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Allopatric population
R. humboldti	**CBA 5732**	Included	Included	Recovered in a well-supported monophyletic *R. humdbolti*. Agreement with the taxonomic determination	Included	Included	Recovered in a monophyletic *R. humboldti* in N analysis	No nuclear polymorphisms associated with introgression	Sympatric with *R. merianae*
	CBA NV	Included	Included	Recovered in a well-supported monophyletic *R. humdbolti*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population
	USNM 286986	Included	Included	Recovered in a well-supported monophyletic *R. humdbolti*	Included	Included	Recovered in a monophyletic *R. humboldti* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
R. major	**CFBH 14170**	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Putative sympatry with *R. mirandaribeiroi*
	IIBPH 725	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	**Not included**	-	Nuclear polymorphisms in *CXCR4*, *RAG1a*, and *RAG1b* genes in agreement with genetic introgression from *R. bergi*	Sympatric with *R. bergi*
	LM 1493	Included	Included	Recovered in a well-supported monophyletic *R. major*	**No sequences available**	**No sequences available**	Untested relationships	-	Putative allopatric population
	LGE 8719	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	**Not included**	-	Nuclear polymorphisms in *CXCR4* gene in agreement with genetic introgression from *R. bergi*	Sympatric with *R. bergi*

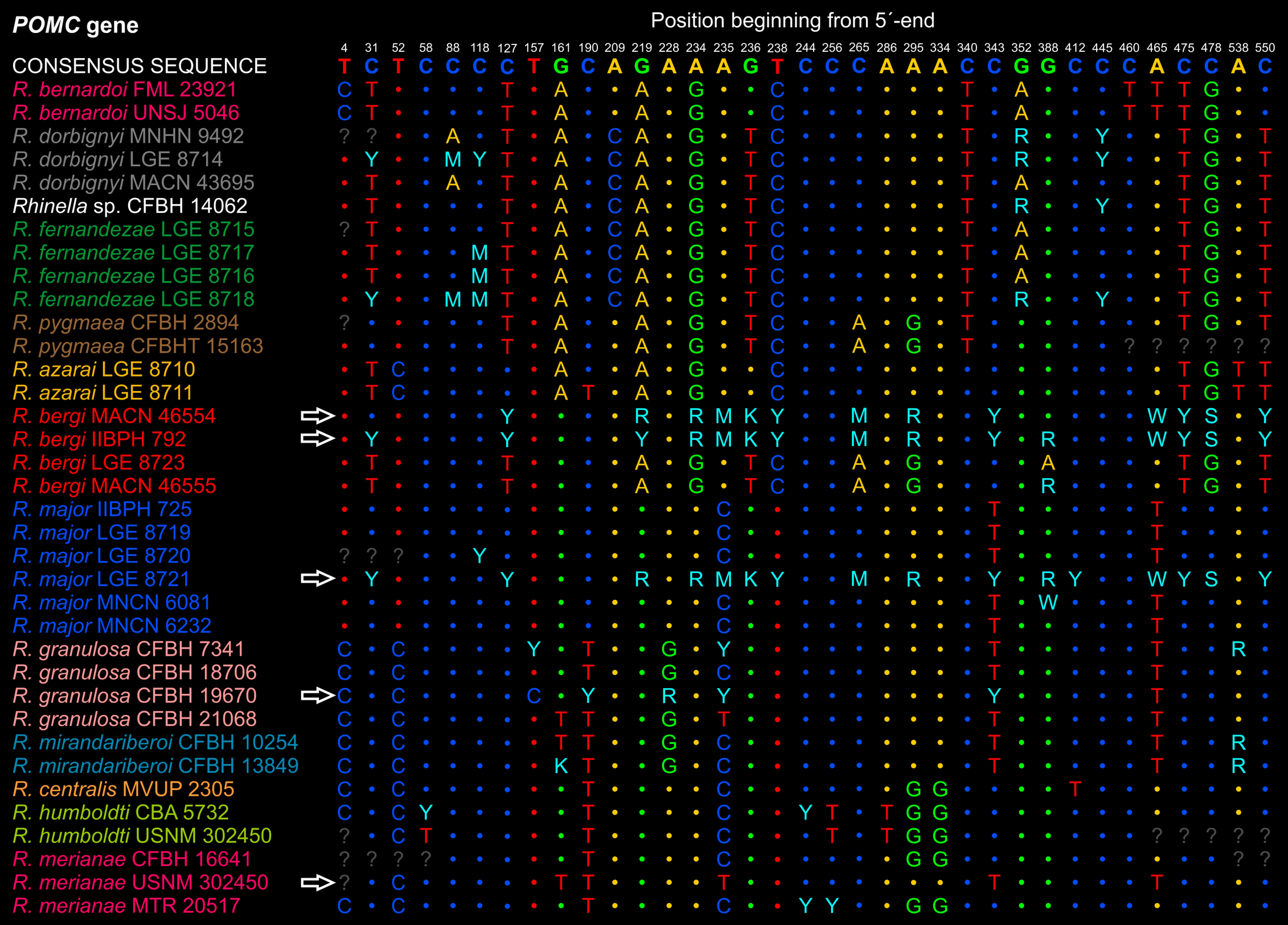
	LGE 8720	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	**Not included**	-	Nuclear polymorphisms in *CXCR4* gene in agreement with genetic introgression from *R. bergi*	Putative sympatry with *R. bergi*
	LGE 8721	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	**Not included**	-	Nuclear polymorphisms in *CXCR4* and *POMC* genes in agreement with genetic introgression from *R. bergi.*	Sympatric with *R. major*
	LGE 8722	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	Nuclear polymorphisms in *CXCR4* gene in agreement with genetic introgression from *R. bergi*	Sympatric with *R. bergi*
	MNCN 6081	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	**Not included**	-	Nuclear polymorphisms in *CXCR4* and *RAG1b* genes in agreement with genetic introgression from *R. bergi*	Putative allopatric population
	MNCN 6232	Included	Included	Recovered in a well-supported monophyletic *R. major*. Agreement with the taxonomic determination	Included	Included	-	No nuclear polymorphisms associated with introgression	Allopatric population
R. merianae	**235 MC**	Included	Included	Recovered in a well-supported monophyletic *R. merianae*. Agreement with the taxonomic determination	**No sequences available**	**No sequences available**	-	-	Putative allopatric population
	AMNHA 139020	Included	Included	Recovered in a well-supported monophyletic *R. merianae*	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population
	CFBH 16641	Included	Included	Recovered in a well-supported monophyletic *R. merianae*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	MTR 20517	Included	Included	Recovered in a well-supported monophyletic *R. merianae*. Agreement with the taxonomic determination	Included	Included	Concordant relationships with M analysis	No nuclear polymorphisms associated with introgression	Putative allopatric population

	MTR 20643	Included	Included	Recovered in a well-supported monophyletic *R. merianae*. Agreement with the taxonomic determination	**No sequences available**	**No sequences available**	-	-	Putative allopatric population
	USNM 302450	Included	Included	Recovered in a well-supported monophyletic *R. merianae*	Included	**Not included**	Collapsed in a polytomy with a specimen of *R. granulosa* and a clade composed of *R. marianae*, *R. centralis*, and *R. humboldti*	Nuclear polymorphisms in the *POMC* gene in agreement with genetic introgression (*R. mirandaribeiroi*? *R. granulosa*?)	Putative allopatric population
	USNM 302451	Included	Included	Recovered in a well-supported monophyletic *R. merianae*	**No sequences available**	**No sequences available**	-	-	Putative allopatric population
R. miran-daribeiroi	**CFBH 10254**	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	CFBH 11448	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	CFBH 13849	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric population
	CFBH 19159	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population
	CFBH 21859	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population
	CFBH 8153	Included	Included	Recovered in a well-supported monophyletic *R. mirandaribeiroi*. Agreement with the taxonomic determination	**Not included (<1200bp)**	**Not included (<1200bp)**	Untested relationships	-	Allopatric population

R. pygmaea	**CFBH 2894**	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
	CFBHT 15161	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species
	CFBHT 15163	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea*. Agreement with the taxonomic determination	Included	Included	Recovered in a well-supported monophyletic *R. pygmaea* in N analysis	No nuclear polymorphisms associated with introgression	Allopatric species


CXCR4 gene
Position beginning from 5´-end
5 16 76 97 109 197 262 271 286 301 304 307 328 337 349 355 361 364 370 372 385 443 445 452 460 482 502 529 559 577 580 599 640 643
CONSENSUS SEQUENCE C T C C C C C T G C G C G T T T C C T C G C C T C C C T T C C G C G
R. bernardoi FML 23921
R. bernardoi UNSJ 5046
R. dorbignyi MNHN 9492
R. dorbignyi MACN 43695
Rhinella sp. CFBH 14062
R. fernandezae LGE 8715
R. fernandezae LGE 8717
R. fernandezae LGE 8716
R. fernandezae LGE 8718
R. fernandezae VUB 1960
R. pygmaea CFBH 2894
R. pygmaea CFBHT 15161
R. pygmaea CFBHT 15163
R. azarai LGE 8710
R. azarai LGE 8711
R. bergi IIBPH 792
R. bergi LGE 8723
R. bergi LGE 8712
R. bergi LGE 8713
R. bergi MACN 46555
R. major CFBH 14170
R. major IIBPH 725
R. major LGE 8719
R. major LGE 8720
R. major LGE 8721
R. major LGE 8722
R. major MNCN 6081
R. major MNCN 6232
R. granulosa CFBH 7341
R. granulosa CFBH 18706
R. granulosa CFBH 19670
R. mirandariberoi CFBH 8153
R. mirandariberoi CFBH 10254
R. mirandariberoi CFBH 11448
R. mirandariberoi CFBH 13849
R. mirandariberoi CFBH 19159
R. mirandariberoi CFBH 21859
R. centralis CH 9383
R. centralis MVUP 2305
R. humboldti CBA 5732
R. humboldti CBA unvouchered
R. merianae CFBH 16641
R. merianae USNM 302450
R. merianae MTR 20517


NCX1 gene
Position beginning from 5´-end
37 154 211 220 232 247 354 361 364 412 436 460 463 505 517 538 559 571 604 636 638 642 673
CONSENSUS SEQUENCE C G A C A T T G A T C G C C G A C C C A G A A
R. bernardoi UNSJ 5046 . . . . . A . T . . . A . . . . . G A G .
R. dorbignyi MNHN 9492 . A G . . A A . T . . A A . . C . . . G A G .
Rhinella sp. CFBH 14062 . A G . . A A . T . A A A . . C . . . G A G .
R. fernandezae LGE 8718 . A G . . A A . T . . A A . . C . . . G A G .
R. fernandezae VUB 1960 . A G . . A A . T . . A A . . C . . . G A G .
R. pygmaea CFBH 2894 . . . . G . . T . . . . A . T . . . .
R. azarai LGE 8711 . . . . G . . T . . . . A . T . . . .
R. bergi LGE 8723 ? . . . G . . T . . . . A . T . . . .
R. major LGE 8719 . . . . G . . . . C . . . . T . T . A . . . C
R. major LGE 8720 ? . . . G . . . . C . . . . T . T . A . . . M
R. major MNCN 6081 . . . . G . . . . C . . . . T . T . A . . . C
R. major MNCN 6232 . . . . G . . . . C . . . . T . T . A . . . M
R. granulosa CFBH 7341 . . . A . . . . T . . . . . .
R. granulosa CFBH 18706 . . . A . . . .
R. mirandariberoi CFBH 10254 T . . A . . . . . T . . . .
R. centralis CH 9383 . . . A . . . . . C . . . . G . . .
R. humboldti CBA 5732 . . . A . . . . . C . . . . R . . .
R. merianae CFBH 16641 Y . . A . . . . . C . . . Y . . . Y . . . . .
R. merianae MTR 20517 Y . . A . . . . . C . . . Y . . . T . . . . .


POMC gene
Position beginning from 5´-end
4 31 52 58 88 118 127 157 161 190 209 219 228 234 235 236 238 244 256 265 286 295 334 340 343 352 388 412 445 460 465 475 478 538 550
CONSENSUS SEQUENCE
R. bernardoi FML 23921
R. bernardoi UNSJ 5046
R. dorbignyi MNHN 9492
R. dorbignyi LGE 8714
R. dorbignyi MACN 43695
Rhinella sp. CFBH 14062
R. fernandezae LGE 8715
R. fernandezae LGE 8717
R. fernandezae LGE 8716
R. fernandezae LGE 8718
R. pygmaea CFBH 2894
R. pygmaea CFBHT 15163
R. azarai LGE 8710
R. azarai LGE 8711
R. bergi MACN 46554
R. bergi IIBPH 792
R. bergi LGE 8723
R. bergi MACN 46555
R. major IIBPH 725
R. major LGE 8719
R. major LGE 8720
R. major LGE 8721
R. major MNCN 6081
R. major MNCN 6232
R. granulosa CFBH 7341
R. granulosa CFBH 18706
R. granulosa CFBH 19670
R. granulosa CFBH 21068
R. mirandariberoi CFBH 10254
R. mirandariberoi CFBH 13849
R. centralis MVUP 2305
R. humboldti CBA 5732
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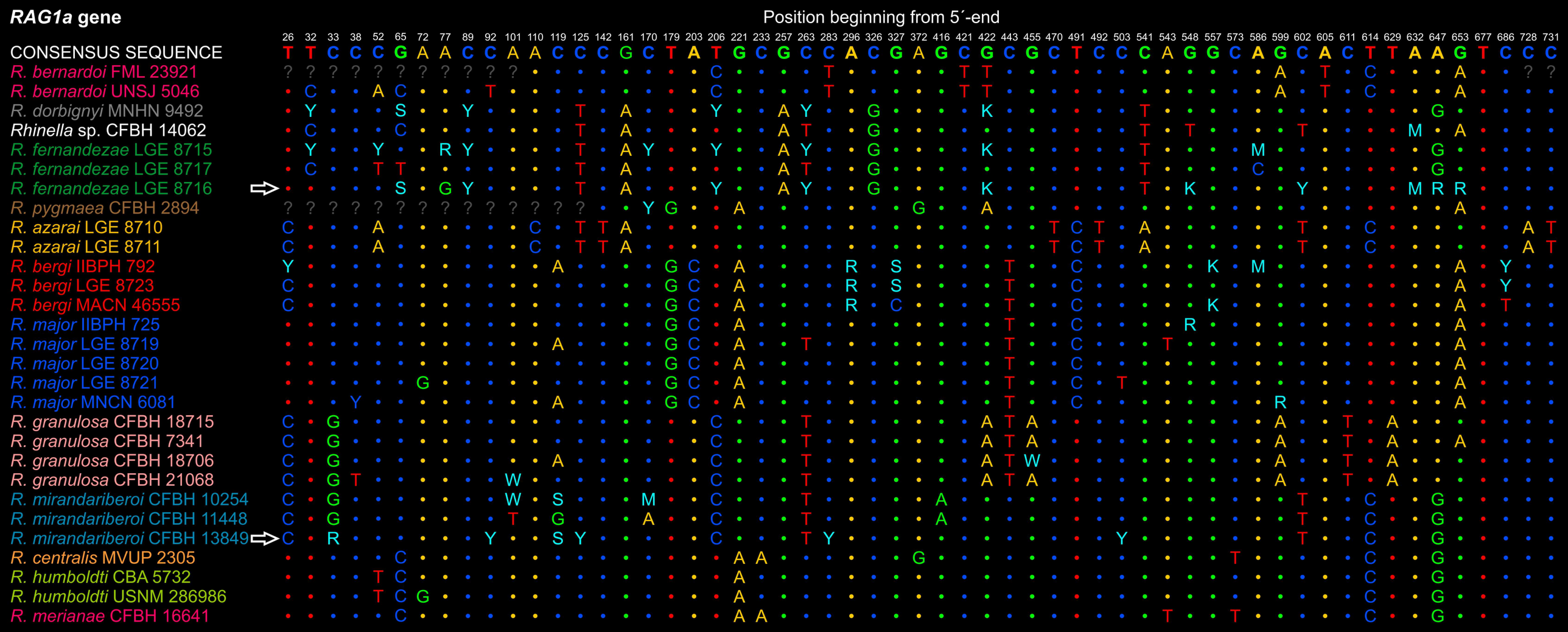
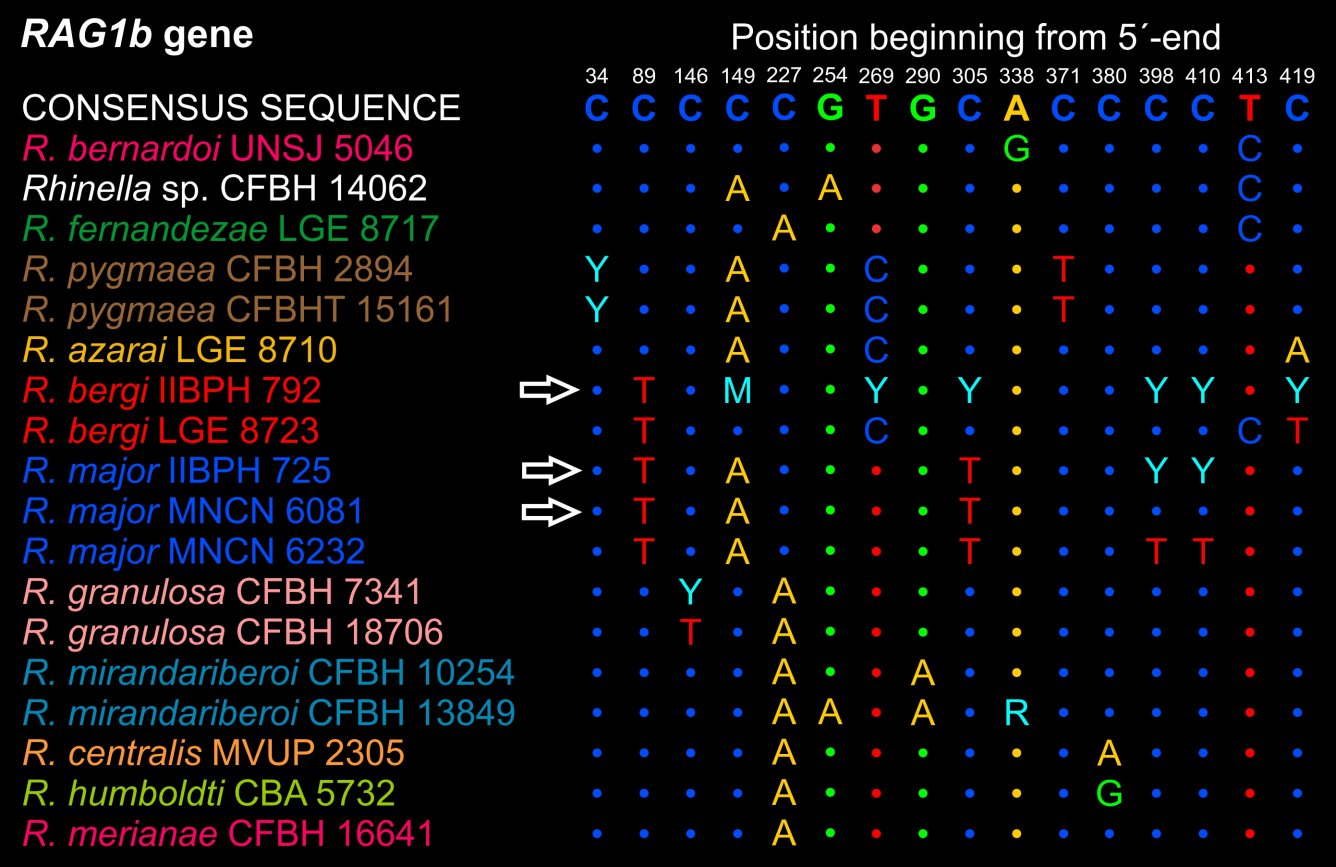
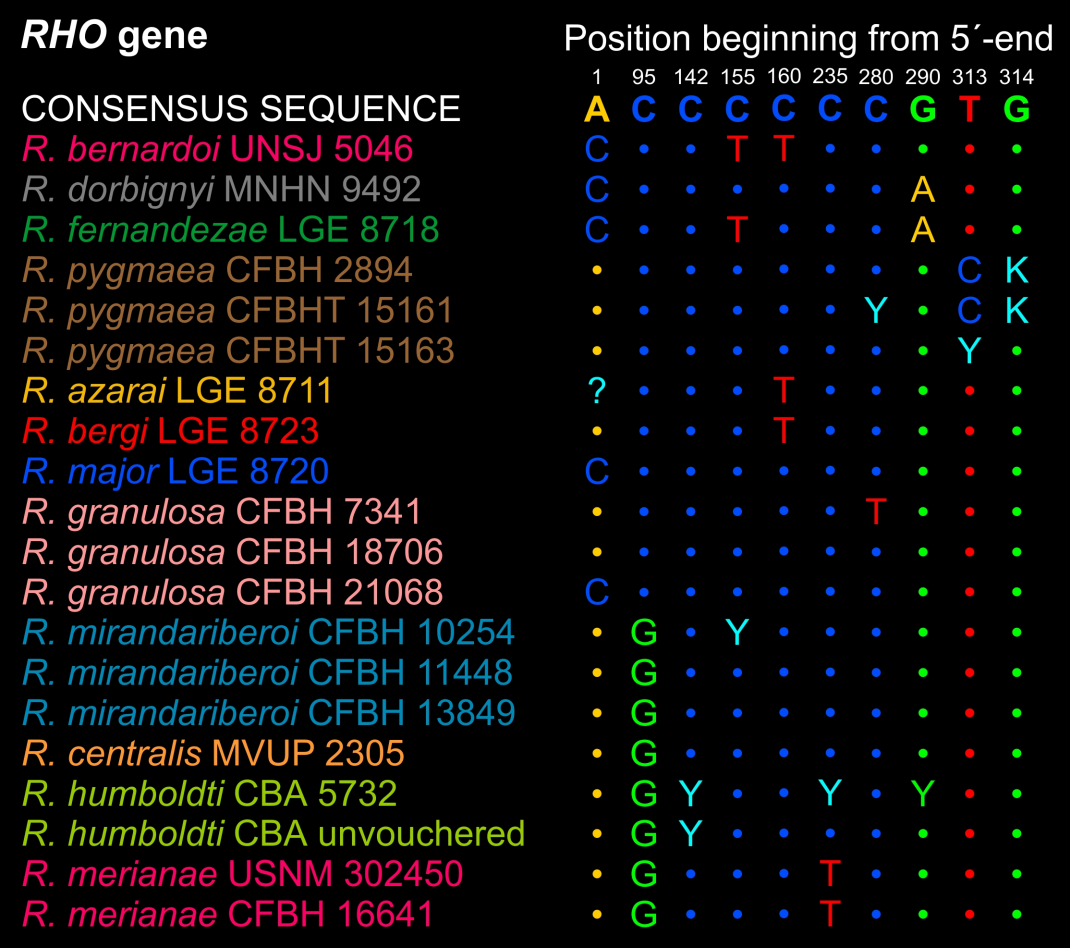
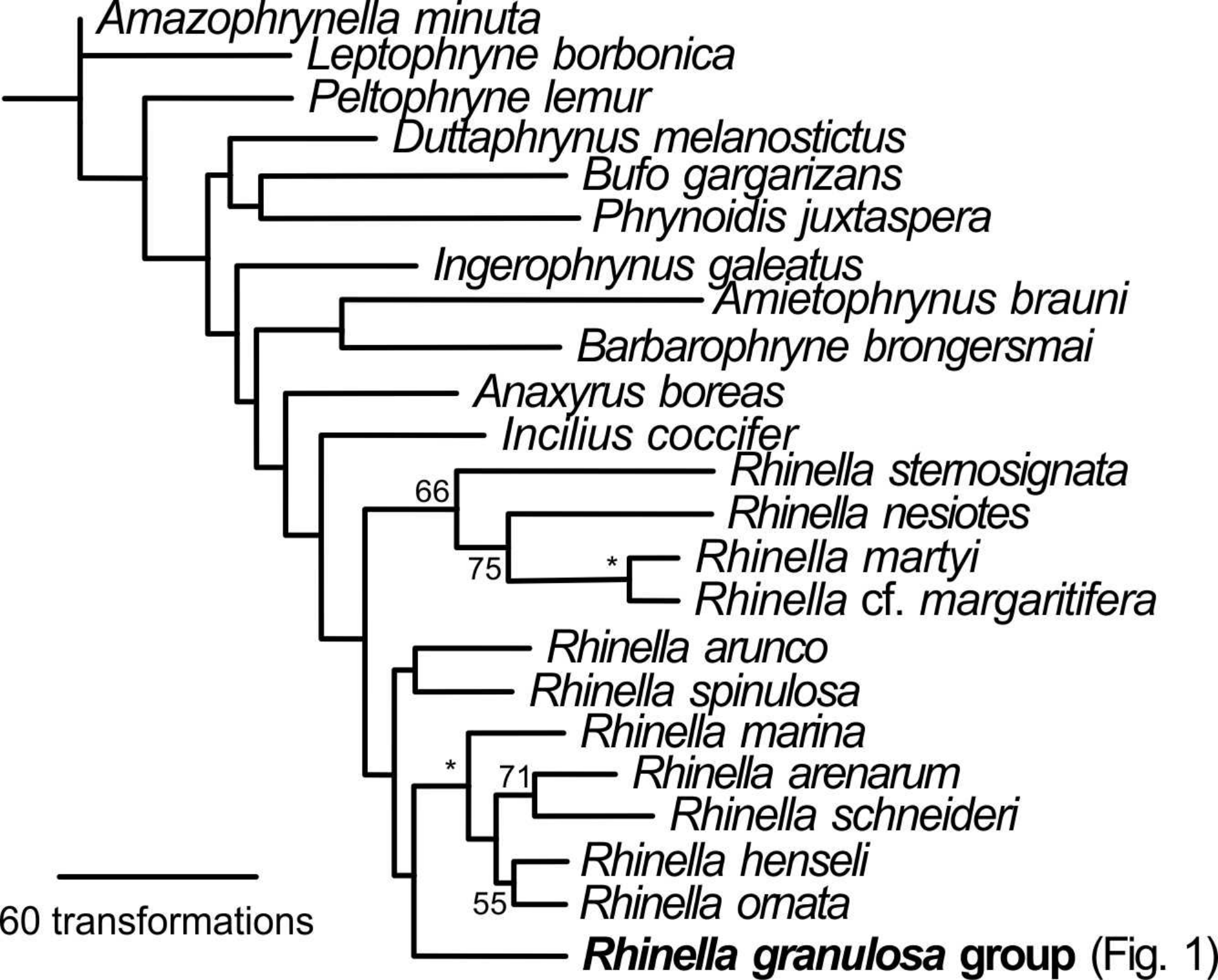
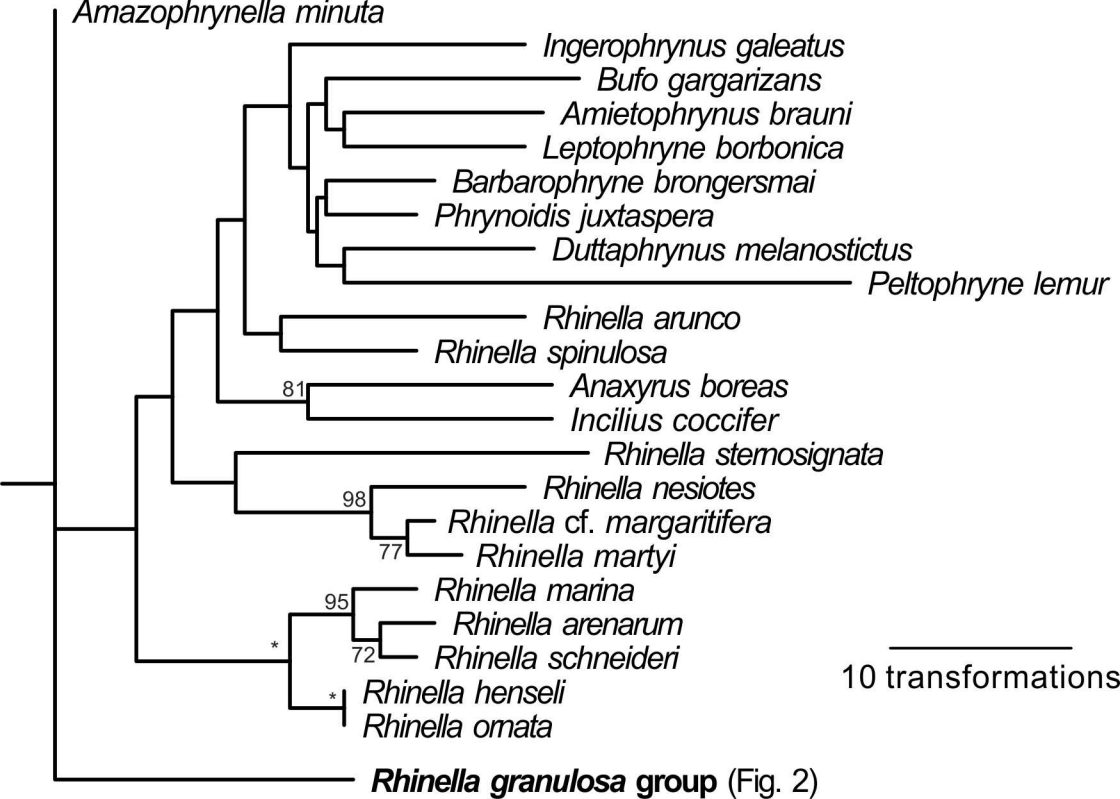
R. merianae CFBH 16641
R. merianae USNM 302450
R. merianae MTR 20517

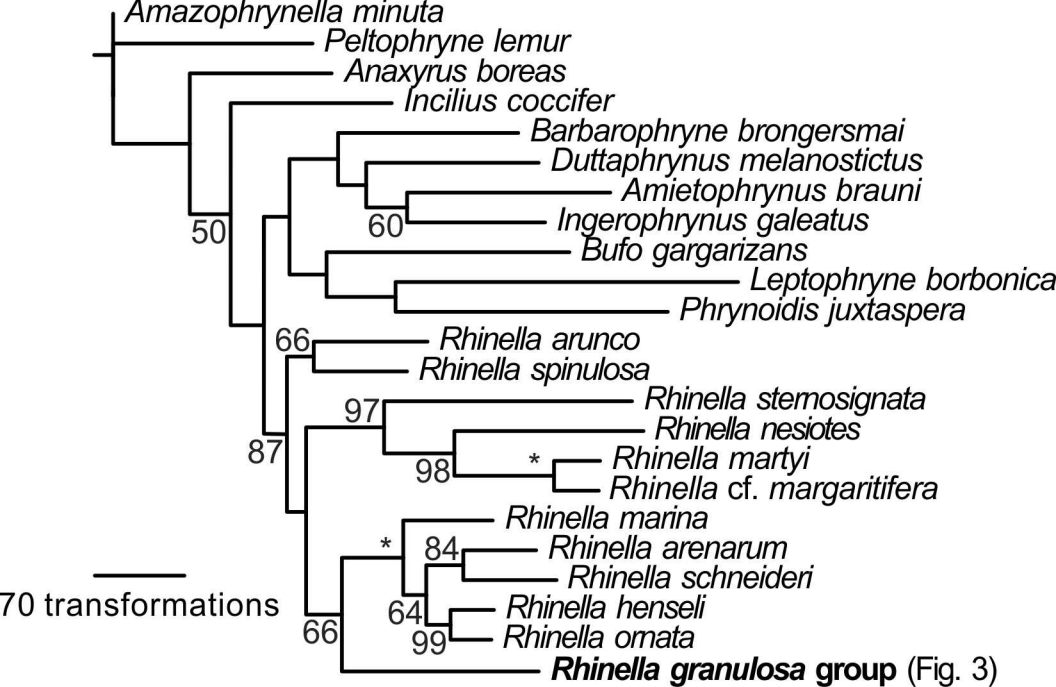

RAG1a gene
Position beginning from 5´-end
26 32 33 38 52 65 72 77 89 92 101 110 119 125 142 161 170 179 203 206 221 233 257 263 283 296 326 327 372 416 421 422 443 455 470 491 492 503 541 543 548 557 573 586 599 602 605 611 614 629 632 647 653 677 686 728 731
CONSENSUS SEQUENCE T T C C C G A A C C A A C C C G C T A T G C G C C A C G A G C G C G C T C C C A G G C A G C A C T T A A G T C C C
R. bernardoi FML 23921
R. bernardoi UNSJ 5046
R. dorbignyi MNHN 9492
Rhinella sp. CFBH 14062
R. fernandezae LGE 8715
R. fernandezae LGE 8717
R. fernandezae LGE 8716
R. pygmaea CFBH 2894
R. azarai LGE 8710
R. azarai LGE 8711
R. bergi IIBPH 792
R. bergi LGE 8723
R. bergi MACN 46555
R. major IIBPH 725
R. major LGE 8719
R. major LGE 8720
R. major LGE 8721
R. major MNCN 6081
R. granulosa CFBH 18715
R. granulosa CFBH 7341
R. granulosa CFBH 18706
R. granulosa CFBH 21068
R. mirandariberoi CFBH 10254
R. mirandariberoi CFBH 11448
R. mirandariberoi CFBH 13849
R. centralis MVUP 2305
R. humboldti CBA 5732
R. humboldti USNM 286986
R. merianae CFBH 16641

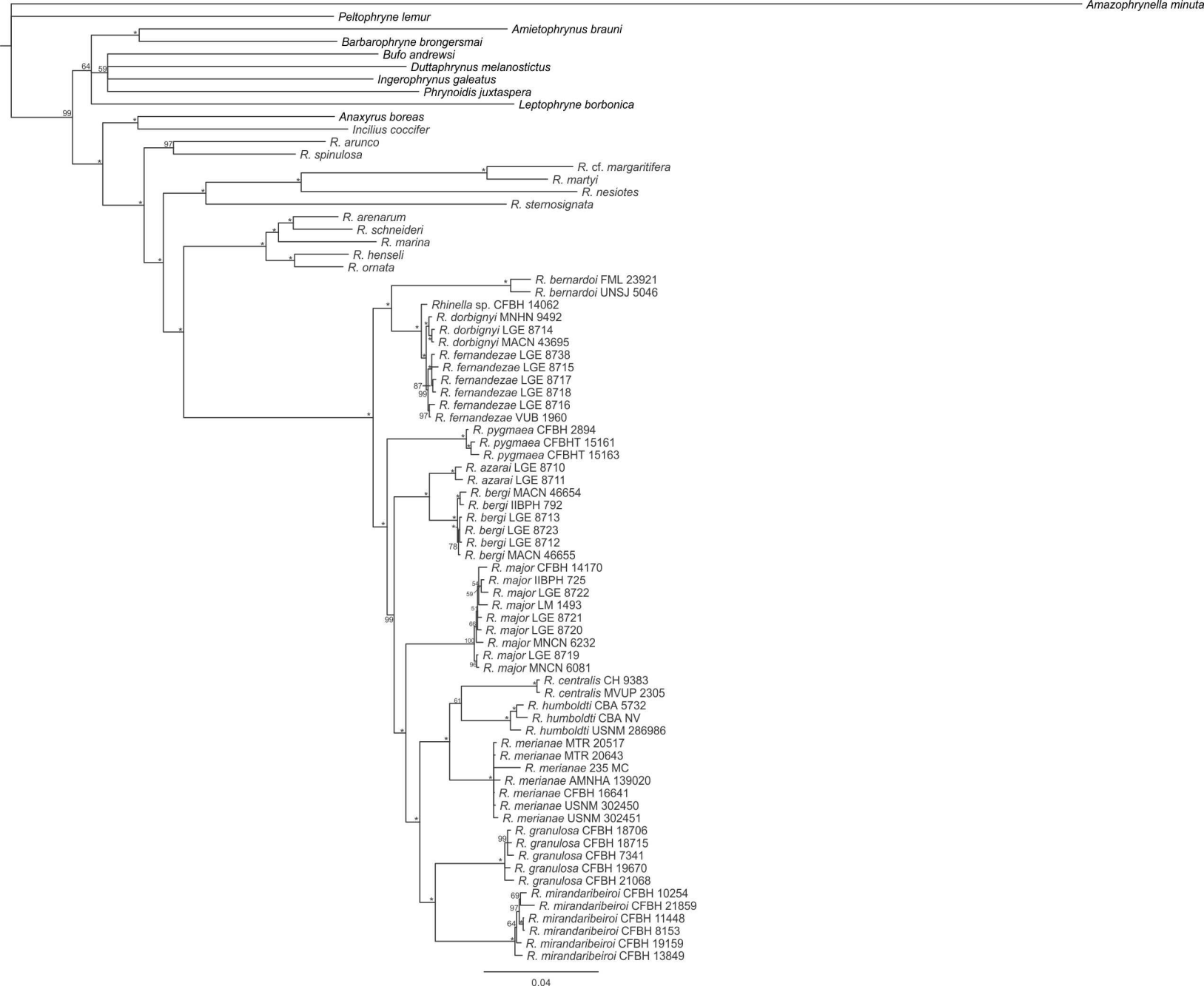

RAG1b gene
Position beginning from 5´-end
34 89 146 149 227 254 269 290 305 338 371 380 398 410 413 419
CONSENSUS SEQUENCE C C C C C G T G C A C C C C T C
R. bernardoi UNSJ 5046 . . . . G . . . . C .
Rhinella sp. CFBH 14062 . . . A . A . . . . C .
R. fernandezae LGE 8717 . . . . A . . . . C .
R. pygmaea CFBH 2894 Y . . A . . C . . . T . . . . .
R. pygmaea CFBHT 15161 Y . . A . . C . . . T . . . . .
R. azarai LGE 8710 . . . A . . C . . . . A
R. bergi IIBPH 792 . T . M . . Y . Y . . . Y Y . Y
R. bergi LGE 8723 . T . . . . C . . . . . . C T
R. major IIBPH 725 . T . A . . . . T . . . Y Y . .
R. major MNCN 6081 . T . A . . . . T . . . .
R. major MNCN 6232 . T . A . . . . T . . . T T . .
R. granulosa CFBH 7341 . . Y . A . . . .
R. granulosa CFBH 18706 . . T . A . . . .
R. mirandariberoi CFBH 10254 . . . . A . . A . . . .
R. mirandariberoi CFBH 13849 . . . . A A . A . R . . . . .
R. centralis MVUP 2305 . . . . A . . . . . A . . . .
R. humboldti CBA 5732 . . . . A . . . . . G . . . .
R. merianae CFBH 16641 . . . . A . . . .


RHO gene
Position beginning from 5´-end
1 95 142 155 160 235 280 290 313 314
CONSENSUS SEQUENCE A C C C C C C G T G
R. bernardoi UNSJ 5046 C • • T T • • • • •
R. dorbignyi MNHN 9492 C • • • • • • A • •
R. fernandezae LGE 8718 C • • T • • • A • •
R. pygmaea CFBH 2894 • • • • • • • • C K
R. pygmaea CFBHT 15161 • • • • • • Y • C K
R. pygmaea CFBHT 15163 • • • • • • • • Y •
R. azarai LGE 8711 ? • • • T • • • • •
R. bergi LGE 8723 • • • • T • • • • •
R. major LGE 8720 C • • • • • • • • •
R. granulosa CFBH 7341 • • • • • • T • • •
R. granulosa CFBH 18706 • • • • • • • • • •
R. granulosa CFBH 21068 C • • • • • • • • •
R. mirandariberoi CFBH 10254 • G • Y • • • • • •
R. mirandariberoi CFBH 11448 • G • • • • • • • •
R. mirandariberoi CFBH 13849 • G • • • • • • • •
R. centralis MVUP 2305 • G • • • • • • • •
R. humboldti CBA 5732 • G Y • • Y • Y • •
R. humboldti CBA unvouchered • G Y • • • • • • •
R. merianae USNM 302450 • G • • • T • • • •
R. merianae CFBH 16641 • G • • • T • • • •


Amazophrynella minuta
Leptophryne borbonica
Peltophryne lemur
Duttaphrynus melanostictus
Bufo gargarizans
Phrynoidis juxtaspera
Ingerophrynus galeatus
Amietophrynus brauni
Barbarophryne brongersmai
Anaxyrus boreas
Incilius coccifer
Rhinella stemosignata
66
Rhinella nesiotes
75
*
Rhinella martyi
Rhinella cf. margaritifera
Rhinella arunco
Rhinella spinulosa
Rhinella marina
*
71
Rhinella arenarum
Rhinella schneideri
Rhinella henseli
55
Rhinella ornata
60 transformations
Rhinella granulosa group (Fig. 1)


Amazophrynella minuta
Ingerophrynus galeatus
Bufo gargarizans
Amietophrynus brauni
Leptophryne borbonica
Barbarophryne brongersmai
Phrynoidis juxtaspera
Duttaphrynus melanostictus
Peltophryne lemur
Rhinella arunco
Rhinella spinulosa
81
Anaxyrus boreas
Incilius coccifer
Rhinella stemosignata
98
Rhinella nesiotes
Rhinella cf. margaritifera
77
Rhinella martyi
95
Rhinella marina
Rhinella arenarum
*
72
Rhinella schneideri
*
Rhinella henseli
Rhinella omata
10 transformations
Rhinella granulosa group (Fig. 2)


Amazophrynella minuta
Peltophryne lemur
Anaxyrus boreas
Incilius coccifer
Barbarophryne brongersmai
Duttaphrynus melanostictus
Amietophrynus brauni
Ingerophrynus galeatus
Bufo gargarizans
Leptophryne borbonica
Phrynoidis juxtaspera
Rhinella arunco
Rhinella spinulosa
Rhinella stemosignata
Rhinella nesiotes
Rhinella martyi
Rhinella cf. margaritifera
Rhinella marina
Rhinella arenarum
Rhinella schneideri
Rhinella henseli
Rhinella ornata
Rhinella granulosa group (Fig. 3)
50
60
66
97
87
98
*
*
84
64
99
66
70 transformations


Amazophrynella minuta
Peltophryne lemur
Amietophrynus brauni
Barbarophryne brongersmai
Bufo andrewsi
Duttaphrynus melanostictus
Ingerophrynus galeatus
Phrynoidis juxtaspera
Leptophryne borbonica
Anaxyrus boreas
Incilius coccifer
R. arunco
R. spinulosa
R. cf. margaritifera
R. martyi
R. nesiotes
R. sternosignata
R. arenarum
R. schneideri
R. marina
R. henseli
R. ornata
R. bernardoi FML 23921
R. bernardoi UNSJ 5046
Rhinella sp. CFBH 14062
R. dorbignyi MNHN 9492
R. dorbignyi LGE 8714
R. dorbignyi MACN 43695
R. fernandezae LGE 8738
R. fernandezae LGE 8715
R. fernandezae LGE 8717
R. fernandezae LGE 8718
R. fernandezae LGE 8716
R. fernandezae VUB 1960
R. pygmaea CFBH 2894
R. pygmaea CFBHT 15161
R. pygmaea CFBHT 15163
R. azarai LGE 8710
R. azarai LGE 8711
R. bergi MACN 46654
R. bergi IIBPH 792
R. bergi LGE 8713
R. bergi LGE 8723
R. bergi LGE 8712
R. bergi MACN 46655
R. major CFBH 14170
R. major IIBPH 725
R. major LGE 8722
R. major LM 1493
R. major LGE 8721
R. major LGE 8720
R. major MNCN 6232
R. major LGE 8719
R. major MNCN 6081
R. centralis CH 9383
R. centralis MVUP 2305
R. humboldti CBA 5732
R. humboldti CBA NV
R. humboldti USNM 286986
R. merianae MTR 20517
R. merianae MTR 20643
R. merianae 235 MC
R. merianae AMNHA 139020
R. merianae CFBH 16641
R. merianae USNM 302450
R. merianae USNM 302451
R. granulosa CFBH 18706
R. granulosa CFBH 18715
R. granulosa CFBH 7341
R. granulosa CFBH 19670
R. granulosa CFBH 21068
R. mirandaribeiroi CFBH 10254
R. mirandaribeiroi CFBH 21859
R. mirandaribeiroi CFBH 11448
R. mirandaribeiroi CFBH 8153
R. mirandaribeiroi CFBH 19159
R. mirandaribeiroi CFBH 13849
0.04

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.

	1			
1-*R. major* (11)	0.00–0.76	**2**		
2-"*R. mirandaribeiroi*" SMF 88236	0.18–0.77	-	**3**	
3-"*R. mirandaribeiroi* A" MNKA 9783	0.57–1.18	0.38	-	**4**
4-*R. mirandaribeiroi* (6)	4.32–6.25	4.52–5.56	4.45–5.51	0.00–0.86

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

Ability to build and inhabit holes in the ground.

Rhinella azarai: D.B. pers. observ.

R. bergi: Céspedez (1999).

R. dorbignyi: Milstead (1956), Gallardo (1969), and Braun (1978).

R. fernandezae: Gallardo (1969).

R. granulosa: R. Montesinos (pers. comm.) and photograph in Toledo et al. (2007)

R. humboldti: Renjifo and Lundberg (1999).

R. major: F. Brusquetti pers. comm.

R. merianae: Hoogmoed and Gorzula (1979).

R. mirandaribeiroi: C.F.B.H. pers. observ.

R. pygmaea: Carvalho e Silva and Carvalho e Silva (1994).

Note composition of the advertisement call.

***Rhinella granulosa* group**

Rhinella azarai, *R. bergi*, *R. centralis*, *R. dorbignyi*, *R. fernandezae*, *R. major*, and

R. merianae: Guerra et al. (2011).

R. granulosa: Sao Pedro et al. (2011).

R. humboldti: Tárano (2010), Torres-Suarez and Vargas-Salinas (2013).

R. mirandaribeiroi: Morais et al. (2012).

R. pygmaea: Carvalho et al. (2013).

Outgroups

Anaxyrus boreas: inapplicable, Blair (1972) and Marco et al. (1998); but see Long (2010).

Bufo gargarizans: inapplicable, Blair (1972) and Martin (1972).

Duttaphrynus melanostictus: 5–8, Ngo and Ngo (2013).

Rhinella arenarum: 3, Salas et al. (1998).

R. cf. *margaritifera*: 1–4, Köhler et al. (1997); 5–7, de la Riva et al. (1996); and 2 Fouquet et al. (2007).

R. marina: 3, Ibañez et al. (1999).

R. martyi: 2, Fouquet et al. (2007).

R. ornata: 4–7, Heyer et al. (1990).

R. schneideri: 3, Köhler et al. (1997).

R. spinulosa: inapplicable, Wells (1977) and di Tada et al. (2001).

Tadpole morphology (Dorsal pigmentation pattern of tail musculature of tadpoles, Posterior labial tooth rows of the larval oral disc. and Submarginal papillae).

References: B: Banded pattern of dorsal pigmentation of tail; UP: Uniformly pigmented; LTR: Number of posterior labial tooth rows; SPa: Submarginal papillae absent; SPp: present; SP?: Unknown character state.

***Rhinella granulosa* group**

Rhinella azarai: Blotto et al. (2014).

R. dorbignyi: Borteiro et al. (2006).

R. fernandezae: Fernández (1927), Lavilla et al. (2000), and Borteiro et al. (2006).

R. granulosa: Almeida Mercês et al. (2009).

R. humboldti: Kenny (1969) and Lynch (2006).

R. major: the tadpole of this species was described by Lavilla et al. (2000), but we use our own observations for the optimizations (see Putative phenotypic synapomorphies section).

R. merianae: Hero (1990).

R. pygmaea: Carvalho e Silva and Carvalho e Silva (1994).

Outgroups

Amazophrynella minuta: UP, LTR = 3, SPa, Duellman and Lynch (1969) and Duellman (1978).

Amietophrynus brauni: UP, LTR = 3, SPp, Sprague and Zimkus (2011).

Anaxyrus boreas: UP, LTR = 3, SPa, Orton (1952) and Altig (1970).

Barbarophryne brongersmai: UP, LTR = 3, SPp, Hoogmoed (1972) and Grillitsch et al. (1989).

Bufo gargarizans: UP, LTR = 3, SPp, Schmidt and Liu (1940) and Liu (1950).

Duttaphrynus melanostictus: UP, LTR = 3, SPp, van Kampen (1923) and Inthara et al. (2005).

Incilius coccifer: B, LTR = 3, SPa, McDiarmid and Foster (1981).

Ingerophrynus galeatus: B, LTR = 3, SPp, Hendrix et al. (2009).

Leptophryne borbonica: UP, LTR = 3, SPa, Berry (1972), Inger (1985), and Iskandar (1998).

Peltophryne lemur: UP, LTR = 3, SP?, Rivero et al. (1980).

Rhinella arenarum: UP, LTR = 3, SPp, Fernández (1927), Cei (1980), Fabrezi and Vera (1997), and Vera Candioti (2007).

R. arunco: UP, LTR = 3 (SP?), Müller and Hellmich (1932) and Cei (1962).

R. cf *margaritifera*: UP, LTR = 3, SPp, Duellman (1978, 2005) and Caldwell (1991).

R. marina: UP, LTR = 3 (SPa or SPp), Kenny (1969), Ford and Scott (1996), and Duellman (2005).

R. ornata: UP, LTR = 3, SPp, Heyer et al. (1990).

R. schneideri: UP, LTR = 3, SPp, Cei (1980), Fabrezi and Vera (1997), and Rossa-Feres and Nomura (2006).

R. spinulosa: UP, LTR = 3, SPp, Fernández (1927), Donoso Barros (1975), Aguilar et al. (2007), and Vera Candioti (2007).

Location of Nucleolar Organizer Regions (NORs). References: *q*: long chromosome arm; *p*: short chromosome arm; per: pericentromeric band; int: interstitial band; term: terminal or telomeric band; * :NORs location inferred from Secondary Constrictions.

***Rhinella granulosa* group**

R. granulosa and *R. pygmaea*: 5*q* term, Baldissera et al. (1999).

Outgroups

Amietophrynus brauni: 6*q* int* [2*n* = 20], Bogart (1968).

Anaxyrus boreas: l*p* term, Schmid (1978).

Barbarophryne brongersmai: 6*q* term, Herrero et al. (1993).

Bufo gargarizans: 6*q* term, Shang and Deng (1983).

Duttaphrynus melanostictus: 7*p* int, Saba et al. (2014).

Rhinella arenarum: 7*p* int, Schmid (1978) and Baldissera et al. (1999).

R. arunco: 7*p* int*, Formas (1978).

R. cf. *margaritifera*: 10*q* int, Baldissera et al. (1999).

R. marina: 7*p* int, Schmid (1978), Beck and Mahan (1979), and Baldissera et al (1999).

R. ornata: 7*p* int, Baldissera et al. (1999).

R. schneideri: 7*p* int, Baldissera et al. (1999) and Amaro-Ghilardi et al. (2007).

R. spinulosa: 10*p* int*, Formas (1978).

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