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Species limits, phylogeographic and hybridization patterns in Neotropical leaf frogs (Phyllomedusinae)

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The taxonomy of many species is still based solely on phenotypic traits, which is often a pitfall for the understanding of evolutionary processes and historical biogeographic patterns, especially between closely related species due to either phenotypic conservatism or plasticity. Two widely distributed Neotropical leaf frogs from the *Phyllomedusa burmeisteri* species group (*P. burmeisteri* and *Phyllomedusa babiana*) constitute a paramount example of closely related species with relatively unstable taxonomic history due to a large phenotypic variation. Herein, we analysed ~260 individuals from 57 localities distributed across the range of the two species to contrast individual phenotypic with an integrative phylogenetic and phylogeographic multilocus approach. We aim to clarify species limits, investigate potential undocumented diversity and examine to what extent taxonomic uncertainties could lead to misleading hypotheses on phylogeographic and interspecific hybridization patterns. Our molecular analysis supports the recognition of the two currently defined species, providing evidences for one novel and highly divergent evolutionary unit within the range of *P. burmeisteri*, which encompasses its type locality (Rio de Janeiro city). Spatial patterns of genetic and the colour of the hidden areas of the thigh was not congruent, varying considerably both within and between populations of both species. Genetic data showed signs of admixture between both species but do not corroborate the previously inferred wide area of introgression based on the distribution of the intermediate phenotype. Our results suggest that phenotypic variation can result from local adaptations, geographic isolation and/or evolutionary processes and, thus, cannot be used to reliably diagnose *P. burmeisteri* and *P. babiana*. Globally, this study underscores the need of a geographical broad sampling of widespread species and the combination of molecular and phenotypic data to delineate species limits and phylogeographic patterns in species with complex taxonomy.

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Introduction

Understanding the processes underlying biological diversity in the Brazilian Atlantic Forest (BAF) has been the focus of many molecular studies in past few years. Among the numerous proposed drivers of diversification, the refuge hypothesis, which is associated with the climatic-induced cyclical expansion and contraction of forests, and the tectonically mediated river dynamics are the predominant ones (e.g. Grazziotin *et al.* 2006; Thomé *et al.* 2010; Amaral *et al.* 2013). The accumulated data have shown that the origin and distribution of biodiversity in this hotspot cannot be explained by any general model, being more consistent with idiosyncratic and organism-specific response patterns to potential driving factors of diversification, generating overwhelming complexity (see review in Silva *et al.* 2012).

Despite of these studies, inadequate biodiversity inventories, taxonomic uncertainties, limited knowledge on the spatial distribution of taxa, and the absence of phylogenetic information are still serious drawbacks to undertake phylogeographic reconstructions in many taxonomic groups. The taxonomy of most Neotropical taxa or groups/species complexes is still based on phenotypic traits, which is often the cause for discordance with molecular data due to either phenotypic plasticity or morphological conservatism, often associated with highly homoplastic traits (e.g. Thomé *et al.* 2010). Indeed, molecular data have indicated that many recognized taxonomic clades are not monophyletic; while in other cases revealed deep genetic divergences within populations of species, which may often correspond to species complexes (e.g. Gehara *et al.* 2013). Moreover, the lack of integrative approaches (combining natural history with genetic, morphological, and ecological data) to clearly delimit taxonomic units associated with inconsistent criteria for species delimitation are still producing problematic taxonomies and either underestimating or overestimating the number of species (Vieites *et al.* 2009; Vences *et al.* 2013).

The Neotropical leaf frogs of the *Phyllomedusa burmeisteri* species group, with a widespread distribution throughout the BAF, constitute an excellent example of how a comprehensive phylogenetic/phylogeographic approach linked to newly developed species delimitation methods may be used to investigate the current taxonomy of the group and to explore any undocumented species diversity. Species of the genus *Phyllomedusa* are characterized by presenting eco-physiological adaptations linked to desiccation resistance, and aposematic colour patterns of flanks and thighs associated with skin bioactive peptides (Funkhouser 1957; Shoemaker *et al.* 1972; Faivovich *et al.* 2010; Calderon *et al.* 2011). Currently, the *P. burmeisteri* species group includes four diploid species, *P. burmeisteri*, *Phyllomedusa iheringii*, *Phyllomedusa distincta*, *Phyllomedusa bahiana* and one tetraploid form, *Phyllomedusa tetraploidea*. However, this group experienced a relatively unstable taxonomic history due to the large phenotypic variation and the absence of alternative diagnosable phenotypic characters (see revision in Pombal & Haddad 1992). Taxonomic uncertainty has been particularly evident in the case of *P. bahiana* and *P. burmeisteri*, because the first species was synonymized to the second by B. Lutz (1950), corroborated latter by Pombal & Haddad (1992) in a extensively review, and subsequently resurrected by Silva-Filho & Juncá (2006). Indeed, Pombal & Haddad (1992) observed a northeast/southeast phenotypic clinal pattern between *P. bahiana* and *P. burmeisteri*, showing a tendency towards the fixation of morphotypes of these species at the extremes of each respective distribution range, with the occurrence of the two morphotypes and 'intermediate' individuals in several locations of Espírito Santo and Minas Gerais states (Fig. 1A,B). The species revalidation was based on differences in larval morphology and vocalizations recorded in only one population (Serra de São José, Municipality of Feira de Santana, Bahia state).

More recently, Brunes *et al.* (2010) explored the phylogeny of the five morphospecies from *P. burmeisteri* group in a multilocus study. Mitochondrial (mtDNA) analyses recovered the monophyly of *P. bahiana*, *P. burmeisteri* and *P. iheringii*, showing that *P. distincta* and *P. tetraploidea* were paraphyletic, but not resolved the phylogenetic relationships among taxa. By contrast, both single locus nuclear (nuDNA) analysis only gave support for higher-level relationships revealing the presence of two main groups: (i) one formed by *P. bahiana* and *P. burmeisteri* (northernmost species) and other (ii) by *P. distincta*, *P. tetraploidea*, and *P. iheringii* (southernmost species). This closest relationship between both the northernmost and the southernmost distributed species was confirmed by the multi-gene tree and the species tree analysis. Although the hypothesis of retention of ancestral polymorphisms was advanced to explain the lack of monophyly at the nuDNA level for the currently recognized five morphospecies, the broad-scale nature of the study (i.e. small sampling of populations and loci) together with previous evidence for an extensive area of phenotypic intergradation between *P. bahiana* and *P. burmeisteri* (Pombal & Haddad 1992) pre-

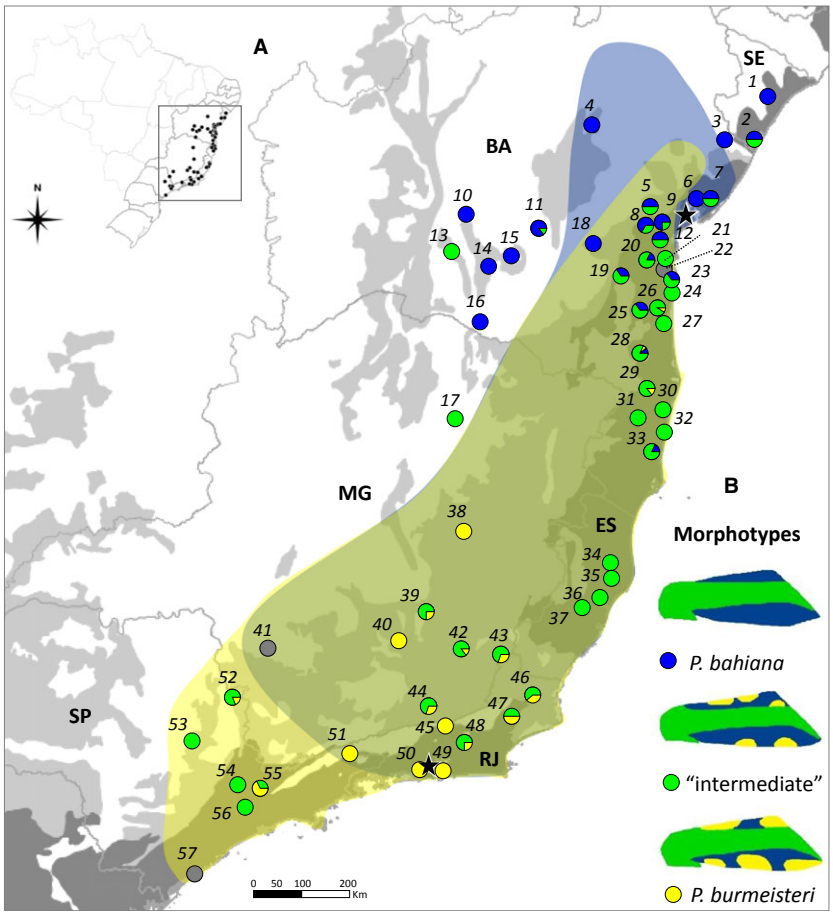

Fig. 1 Based on Pombal & Haddad (1992): (A) Geographic distribution of morphotypes: *Phyllomedusa bahiana* (blue), *Phyllomedusa burmeisteri* (yellow) and 'intermediate' individuals (overlapped area); and (B) scheme of morphotypes following the patterns of the hidden surfaces of the thigh. Sampling localities numbered of 1–57 (SL; Table 1). Circles on map represents the proportional occurrence of each individual morphotype analysed in this study (see Table 1 and Supporting Information Table S1). Grey circles indicates no morphotype information. Brazilian Atlantic Forest original cover: Ombrophylous (dark grey) and Semideciduous/Deciduous (light grey) forests are represented. Stars indicate type localities. Brazilian states: SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.

cluded a more detailed exam of alternative hypotheses to explain these results. Regarding the confusing patterns of phenotypic diversity and, in particular, the lack of comprehensive phylogeographic studies in face of the extensive distribution range of both forms (Brunes *et al.* 2010; Faivovich *et al.* 2010), we here contrasted the individual morphotype characterization with an integrative phylogenetic and phylogeographic multilocus approach (one mitochondrial and three nuclear genes) in combination with newly developed Bayesian species delimitation approaches to clarify species limits, investigate potential undocumented diversity across the ranges of *P. burmeisteri* and *P. bahiana*, and generally discuss the biogeographic patterns of diversification in BAF.

Material and methods

Sampling

We collected individuals of *P. burmeisteri*, *P. bahiana*, and their morphological intermediates from all over the species ranges (Pombal & Haddad 1992). We obtained a total of 269 individual's tissue samples from 57 localities (see Sampling localities, SL) by field collecting trips from November of 2010 to February of 2012 and by several tissue donations from different Brazilian herpetological collections (see Acknowledgements and Supporting Information Table S1). Most of the occurrence points were georeferenced with GPS coordinates and the remaining was based on the coordinates from the closest town (Table 1 and Fig. 1A). Both species were collected in places ranging from ~5 to 1031 metres above sea level. Samples consisted of liver, toe clips or muscle preserved in 100% ethanol. All collected individuals were fixed in 10% formalin solution for a week, preserved in 70% ethyl alcohol and deposited in three Brazilian Herpetological collections: Célio F. B. Haddad amphibian collection (CFBH), Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP; Coleção Zoológica da Universidade Estadual do Sudoeste da Bahia (CZUESB), Jequié, BA; and, Coleção Herpetológica da Universidade Federal de Juiz de Fora (CHUFJF), Departamento de Zoologia, MG, Brazil (vouchers from Juiz de Fora locality).

Table 1 Columns indicate sampling locality information, sample sizes, mitochondrial haplotype number, mitochondrial clade and morphotype proportional values of *Phyllomedusa babiana* and *Phyllomedusa burmeisteri*. Coordinates are in decimal degrees on the WGS84 datum

							mtDNA			Morphotypes		
SL	Locality	ST	Longitude	Latitude	*N*	Haplotypes (H)	BAH	BUR	BUR RJ	U	I	S
1	Areia Branca	SE	−37.3153	−10.7578	1	1	1.0	–	–	1.0	–	–
2	Indiaroba	SE	−37.5131	−11.5194	2	2, 3	1.0	–	–	0.5	0.5	–
3	Acajutiba	BA	−38.0576	−11.5724	4	2, 4, 5	1.0	–	–	1.0	–	–
4	Itaitú	BA	−40.4004	−11.3274	9	2, 4, 6, 7	1.0	–	–	1.0	–	–
5	Castro Alves	BA	−39.3880	−12.7239	2	2	1.0	–	–	0.5	0.5	–
6	Cachoeira	BA	−38.6200	−12.6231	8	2, 4, 8	1.0	–	–	0.9	0.1	–
7	Dias d'Ávila	BA	−38.2990	−12.6028	2	9, 10	1.0	–	–	0.5	0.5	–
8	Amargosa	BA	−39.3852	−13.0058	3	2, 4, 11	1.0	–	–	0.7	0.3	–
9	Santo Antônio de Jesus	BA	−39.1548	−12.9970	4	2, 11	1.0	–	–	0.75	0.25	–
10	Contendas do Sincorá	BA	−42.6831	−12.9164	1	10	1.0	–	–	1.0	–	–
11	Mucugê	BA	−41.4056	−13.1213	10	2, 12, 13	1.0	–	–	0.9	0.1	–
12	Valença	BA	−39.2229	−13.3241	2	14	1.0	–	–	0.5	0.5	–
13	Riacho Santana	BA	−42.9352	−13.5948	1	15	1.0	–	–	–	1.0	–
14	Caetité*	BA	−42.2816	−13.8323	3	15, 16	1.0	–	–	1.0	–	–
15	Livramento de Nª Senhora	BA	−41.8454	−13.6498	1	15	1.0	–	–	1.0	–	–
16	Jacaraci	BA	−42.4404	−14.8475	1	15	1.0	–	–	1.0	–	–
17	Grão Mogol	MG	−42.9024	−16.5909	4	17, 18	1.0	–	–	–	1.0	–
18	Maracás	BA	−40.4308	−13.4411	12	2, 4, 14, 19–21	1.0	–	–	1.0	–	–
19	Jequié	BA	−39.9376	−13.9834	14	4, 7, 11, 22, 23	1.0	–	–	0.3	0.7	–
20	Gandu	BA	−39.4401	−13.7348	11	11, 14, 24–26	1.0	–	–	0.2	0.8	–
21	Ituberá	BA	−39.1542	−13.7318	1	7	1.0	–	–	–	1.0	–
22	Igrapiúna	BA	−39.1710	−13.8212	8	11, 27	1.0	–	–	–	–	–
23	Camamu	BA	−39.1082	−13.9704	3	11, 28	1.0	–	–	0.3	0.7	–
24	Itacaré	BA	−39.0279	−14.2918	2	29	1.0	–	–	–	1.0	–
25	Aurelino Leal	BA	−39.5395	−14.5734	6	11, 29, 30	1.0	–	–	0.3	0.7	–
26	Uruçuca	BA	−39.2844	−14.5931	8	11, 29, 31	1.0	–	–	–	0.9	0.1
27	Ilhéus*	BA	−39.1724	−14.7957	12	11, 32, 33	1.0	–	–	–	1.0	–
28	Camacan*	BA	−39.5087	−15.4164	14	11, 29, 33–39	1.0	–	–	0.2	0.7	0.1
29	Itapebi	BA	−39.5041	−16.0045	5	40, 41, 42	–	1.0	–	–	0.8	0.2
30	Porto Seguro	BA	−39.1805	−16.4081	2	40, 43	–	1.0	–	–	1.0	–
31	Itabela	BA	−39.6781	−16.5653	2	44, 45	–	1.0	–	–	1.0	–
32	Caraíva	BA	−39.1478	−16.8081	1	29	1.0	–	–	–	1.0	–
33	Prado	BA	−39.3976	−17.1606	6	40, 41, 46, 47	–	1.0	–	0.2	0.8	–
34	Sooretama	ES	−40.0978	−19.1969	2	48	–	1.0	–	–	1.0	–
35	Linhares	ES	−40.0722	−19.3911	5	49–52	–	1.0	–	–	1.0	–
36	Aracruz	ES	−40.2733	−19.8203	8	11, 29, 52–54	0.5	0.5	–	–	1.0	–
37	Santa Teresa	ES	−40.6050	−19.9525	2	55, 56	–	1.0	–	–	1.0	–
38	São João Evangelista	MG	−42.7494	−18.5461	1	57	–	1.0	–	–	–	1.0
39	Catas Altas	MG	−43.4378	−20.0562	4	58–60	–	1.0	–	–	0.75	0.25
40	Congonhas	MG	−43.8759	−20.4883	1	61	–	1.0	–	–	–	1.0
41	Furnas	MG	−46.2459	−20.6564	1	62	–	1.0	–	–	–	–
42	Viçosa	MG	−42.7500	−20.7500	7	62–64	–	1.0	–	–	0.8	0.2
43	Carangola	MG	−42.0836	−20.7986	11	56, 63, 65	–	1.0	–	–	0.7	0.3
44	Juiz de Fora	MG	−43.3696	−21.7325	13	61, 63, 66, 67	–	1.0	–	–	0.7	0.3
45	Três Rios	RJ	−43.2159	−22.1124	1	66	–	1.0	–	–	–	1.0
46	Campos dos Goytacazes	RJ	−41.4906	−21.5276	5	68–70	–	–	1.0	–	0.6	0.4
47	Santa Maria Madalena	RJ	−41.9384	−21.8811	4	68, 70, 71	–	–	1.0	–	0.5	0.5
48	Cachoeiras de Macacu*	RJ	−42.7491	−22.4257	7	72, 73	–	–	1.0	–	0.7	0.3
49	Niterói	RJ	−43.1045	−22.8798	1	73	–	–	1.0	–	–	1.0
50	Rio de Janeiro	RJ	−43.5315	−22.8278	1	74	–	–	1.0	–	–	1.0
51	Queluz	SP	−44.7739	−22.5369	1	75	–	1.0	–	–	–	1.0
52	São José do Rio Pardo	SP	−46.8727	−21.5493	5	62, 76	–	1.0	–	–	0.8	0.2
53	Rio Claro	SP	−47.6755	−22.3472	1	62	–	1.0	–	–	1.0	–
54	Jundiaí*	SP	−46.8172	−23.1647	11	77, 78	–	1.0	–	–	1.0	–

Table 1 Continued

SL	Locality	ST	Longitude	Latitude	*N*	Haplotypes (H)	mtDNA			Morphotypes		
							BAH	BUR	BUR RJ	U	I	S
55	Nazaré Paulista	SP	−46.4191	−23.2373	3	77, 79	–	1.0	–	0.3	0.7	–
56	"Grande" São Paulo*	SP	−46.6361	−23.5475	4	77, 80	–	1.0	–	–	1.0	–
57	Iguape	SP	−47.5414	−24.6981	3	77, 81	–	1.0	–	–	–	–

State abbreviations (ST): SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo. Morphotype classification: U = *Phyllomedusa bahiana*, I = 'intermediate', and S = *Phyllomedusa burmeisteri*.

*Localities with different sample sizes in genetic and phenotypic analysis (see Supporting Information Table S1).

Qualitative phenotypic variation

Qualitative phenotypic characterization was focused on the pattern of the hidden surface of the thigh, as proposed by Pombal & Haddad (1992): *P. burmeisteri* presenting yellowish rounded spots on a bluish background (S); *P. bahiana* with a coloured blue uniform without the presence of rounded blotches (U); and the 'intermediate' individuals with smaller and fewer yellowish rounded blotches (I) (Fig. 1B). Assuming that the pattern observed for 'intermediate' individuals could be under- or overestimated when compared with that of *P. burmeisteri*, we adopted a conservative approach and considered only individuals with three yellowish rounded blotches in both thighs as *P. burmeisteri*, while individuals with less than three were considerate intermediate.

Laboratory procedures

Genetic analyses included a total of 269 individuals of *P. bahiana*, *P. burmeisteri* and their intermediates. With exception of *P. tetraploidea*, we used three *P. distincta* and two *P. iheringii* individuals as representatives of the remaining species of the group, and *P. boliviana* as outgroup. The data include some sequence information from previous studies (Brunes *et al.* 2010; Faivovich *et al.* 2010) available in GenBank (see Supporting Information Table S1). Whole-genomic DNA was obtained through tissue samples digested in lysis buffer and Proteinase *K* and using QIA Quick DNEasy columns (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. We sequenced a fragment of the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) and three nuclear genes: (i) a segment of exon 2 and intron 2 of the cellular myelocytomatosis (C-myc2), (ii) a segment of exon 2 of chemokine receptor 4 (CXCR4) and (iii) a segment of β-fibrinogen intron 7 (β-fibint7).

PCR amplification and sequencing protocols for ND2 and β-fibint7 were performed following Brunes *et al.* (2010). For C-myc2 and CXCR4 PCR amplification, we specifically designed two pairs of internal primers: CMYC-PHYF (5′-TCCAAAGTTGGCTCTCAATGC-3′) and CMYC-PHYR (5′-GAGTCTCTGCCCTAAACT ATTC-3′); CXCR4-PHYF (5′-GTCCAGGACCATGACT GACAAG-3′) and CXCR4-PHYR2 (5′-TTCGGTGA TGGCGATCCACTTG-3′), respectively. PCRs were performed in 20 μL reaction volume containing 10 μL Qiagen PCR Master Mix, ~50 ng of genomic DNA and 0.2 μM each primer. Cmyc2 amplification consisted of a predenaturing step of 3 min at 92 °C, followed by 40 cycles of a denaturing step of 30 s at 92 °C, annealing at 58 °C for 30 s and extension at 72 °C for 90 s. The final extension was accomplished at 72 °C for 5 min. CXCR4 amplification conditions consisted of an initial denaturation at 95 °C for 15 min, followed by a touchdown program with 10 cycles of 95 °C for 30 s, 56 °C to 52 °C for 30 s, decreasing 0.5 °C in each cycle, and 72 °C for 45 s, followed by 32 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 45 s with a final extension at 60 °C for 30 min. Sequencing was performed from enzymatically purified PCR products in the ABI Prism BigDye Terminator Cycle sequencing protocol in an ABI PRISM 3130 XL Genetic Analyser and by the Macrogen Corporation sequencing facility (http://www.macrogen.com). All electropherograms were checked for errors and assembled contiguous sequences using the CodonCode Aligner (CodonCode Corporation, Dedham, MA). Alignments were edited in BIOEDIT v.7.0.5.2 (Hall 1999) and correct by eye.

Sequence variation

Heterozygous insertions or deletions (indels) of nuclear DNA sequences were identified with the mutation detection tool in CodonCode Aligner and through eye inspection of chromatograms. Next, we used the PHASE software v.2.1 (Stephens *et al.* 2001) with the assistance of SeqPHASE (Flot 2010) to resolve the haplotype phases. Multiple independent runs were performed for each gene with different seeds for the random-number generator and 1.0×10^6 iterations with the default values. Haplotype estimation was checked through consistency analysis across runs. We selected the haplotype reconstructions above $P = 0.90$ or the most likely phased. Additionally, we detected multiple-base indels in two nuclear loci and

reduced it to a single evolutionary step following Brunes *et al.* (2010). The presence of recombination events was evaluated through the Difference of Sums of Squares (DSS) test implemented in TOPALi v.2.5 (Milne *et al.* 2004) using all phased haplotypes, a window size of 70 bp, steps 10 bp-long and 100 bootstrap repetitions. Alignment Transformation Environment, ALTER (Glez-Peña *et al.* 2010) was used to transform datasets to the input formats of down-the-line software used for analyses. Polymorphism values including segregation sites (S), number of haplotypes (h), haplotype diversity (Hd), and population mutation parameter (Θ) were calculated for mitochondrial and nuclear data.

Phylogenetic analyses and haplotype networks

We reconstructed a phylogenetic tree under Bayesian Inference (BI) for mitochondrial data and maximum parsimony (MP) haplotype genealogies for nuclear sequences. To select the best-fit partitioning schemes we used the PARTITIONFINDER v.1.0.1 (Lanfear *et al.* 2012) and the models of molecular evolution were selected in jMODELTEST v.0.1.1 (Posada 2008) under the Akaike information criterion (AIC; Akaike 1974), following Posada and Buckley (2004). BI analyses were performed in MRBAYES version v.3.2 (Ronquist & Huelsenbeck 2003) through two replicate searches using the stop rule command fixed with a standard deviation of 0.01 (stop value) sampling every 1000 generations. Four Markov chain Monte Carlo (MCMC) were run simultaneously in each analysis. To analyse the nuclear data we used the haplotype genealogy method to avoid the presence of reticulation and provide a 'clear' scenario to sets of closely related species. They were produced starting from a MP tree though DNAPARS available in PHYLIP v.3.69 package (Felsenstein 2005) following results of Salzburger *et al.* (2011). The best parsimony tree was converted in a haplotype genealogy in a beta version of the Haploviewer (http://www.cibiv.at/~greg/haploviewer). Detailed information of geographical locality, voucher number and sequenced genes for individuals is shown in Supporting Information Table S1.

Pairwise distance and neutrality tests

For mtDNA, we calculated the uncorrected pairwise distances (*p*-uncorrected) between the major mtDNA clades, between subclades and within each subclades. For ncDNA, the same genetic distance was calculated between major groups as revealed by STRUCTURE analysis (see Results). Estimates of 95% confidence intervals were generated with 10 000 coalescent simulations. To evaluate whether mitochondrial subclades and nuclear groups present deviations from the neutral theory, we computed Tajima's D (Tajima 1989) and R^2 test (Ramos-Onsins & Rozas 2002) and significance of values were checked also through 10 000 coalescent simulations. All analyses were performed in DNASP v.5.10 (Librado & Rozas 2009).

Species delimitation, species tree and divergence times estimates

To investigate species delimitation, we performed several multilocus analyses with different methods: (i) a Bayesian clustering method (STRUCTURE, Pritchard *et al.* 2000) that not require *a priori* assignment of individuals to clusters; and three methods that assume *a priori* assignment of individuals to clusters: (ii) a genetic distance matrix network (POFAD, Joly & Bruneau 2006); and two additional methods based on the coalescent theory: (iii) *BEAST (Heled & Drummond 2010); and (iv) Bayesian species delimitation (BPP, Rannala & Yang 2003; Yang & Rannala 2010). The *BEAST was used to estimate a species tree and the time of the most recent common ancestor (^{t}MRCA). The resulting species tree was used to evaluate the limits of the putative species using the software BPP (e.g. Tsai & Carstens 2013). Regarding the no accommodation of recent admixture in both *BEAST and BPP, we used individuals only from allopatric areas. This 'allopatric data set' comprises eight *P. bahiana* individuals from Sergipe and north Bahia state (BAH clade), nine *P. burmeisteri* individuals from south São Paulo state (BUR clade) and 10 *P. burmeisteri* individuals from Rio de Janeiro state (BUR-RJ clade; see details in Supporting Information Table S1). For comparative purposes, we used the same allopatric data set for POFAD analysis.

Genetic distances between individuals were calculated for each nuDNA fragment in MEGA v.5.2.2 (Tamura *et al.* 2011) and combined into a multilocus distance matrix in POFAD v.1.03. Both standardized and non-standardized matrices were produced to evaluate the possible understatement of attribution of the same weight for all matrices. The multilocus distance network was visualized in Splits Tree v.4.12.3 (Huson & Bryant 2006) via NeighborNet method. To access the genetic assignment to population clusters within the northern distributed species from the *P. burmeisteri* group, we used the Bayesian model-based algorithm implemented in the program STRUCTURE v.2.3.4. To that purpose, the sequence information for at least two nuDNA fragments per individual were converted to allele frequency data through the program xmfa2struct (available at: http://www.xavierdidelot.xtreemhost.com/clonalframe.htm) for a total of 114 individuals from 41 localities (see details in Supporting Information Table S1). Analysis was performed under the admixture ancestry model, with five independent runs for each *K* ranging from 1 to 10. The first 1×10^5 MCMC iterations were discarding as burn-in and the next 25×10^4 repetitions were counted. The best

K value was found via the on-line program STRUCTURE HARVESTER v.0.6.93 (Earl & Von Holdt 2012) to monitor the estimated log posterior probability of the data (ln Pr (*X*/*K*); Pritchard *et al.* 2000) and estimate the second-order rate of change of the likelihood function (Δ*K*; Evanno *et al.* 2005). Finally, the results of the five independent runs were assembled in the program CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) and checked for biologically meaningful population clusters.

To estimate a species tree of the northern distributed species of the *P. burmeisteri* group, we used two data sets (mtDNA and nuDNA combined and only nuDNA), while diversification times (^{t}MRCA) were estimated only with the mtDNA and nuDNA combined dataset. For that, we used the Bayesian Markov chain Monte Carlo method for the multispecies coalescent method implemented in the software *BEAST v.1.8.0. We added the sequence information of *P. boliviana*, *P. distincta* and *P. iheringii* (see details in Supporting Information Table S1) to the 'allopatric data set' as explained above. Diversification times were based on a ND2 mutation rate (0.00957 mutations/site/million years; see Crawford 2003), primarily estimated for *Eleutherodactylus* species (Anura: Leptodactylidae). However, given the lack of fossil data and independent calibration points, this mutation rate has been previously applied in several anuran phylogeographic studies with appropriate prior adjustments (Carnaval & Bates 2007; Brunes *et al.* 2010; Thomé *et al.* 2010; Nuñez *et al.* 2011). We recognize that this type of non-specific approach is not free of drawbacks and could be a potential source of inference error (see Edwards & Beerli 2000). Thus, diversification times should be interpreted carefully. We performed preliminary analysis (data not shown) searching for the best model of molecular clock rate variation (Strict Clock or Relaxed Clock: Uncorrelated Lognormal) for each fragment and for the best tree model prior ('Yule' or 'Birth and Death') for the data. Performance and accuracy of analysis were checked in the software TRACER v.1.5 (https://web.archive.org/web/20130926165911/http://beast.bio.ed.ac.uk/Tracer). The final analysis was performed with a speciation 'Birth and Death' process as tree prior and relaxed clock with an uncorrelated lognormal distribution for ND2 and C-myc2, and a strict clock for β-fibint7 and CXCR4 in an independent run sampling every 10 000 generations for 100 million generations with 10% of burn-in. The final tree was obtained in the TREEANNOTATOR v.1.7.4 ((https://web.archive.org/web/20130806231432/http://beast.bio.ed.ac.uk/TreeAnnotator) and previewed in FIGTREE v.1.1.2 ((https://web.archive.org/web/20140221095451/http://beast.bio.ed.ac.uk/FigTree).

Species delimitation analysis was conducted using the Bayesian species delimitation method (BPP). This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. We also performed two analysis with different datasets as in *BEAST. To test the species limits of the northern distributed species of *P. burmeisteri* group, we incorporate *P. distincta*, *P. iheringii* and *P. boliviana* information and used the species tree estimate by *BEAST as rooted guide tree. Considering the low number of taxa and its close relationships (see Leaché & Fujita 2010), we adopted conservative values for the gamma prior (G) in population size (θ = 2, 2000) and for the divergence time parameter for the root in the species tree (τ0 = 2, 2000). These values means relatively small ancestral population sizes and shallow divergences among species, while the other divergence time parameters are assigned the Dirichlet prior (Yang & Rannala 2010: equation 2). Each rjMCMC analysis was run at least twice to confirm consistency between runs using 200 000 generations and 20 000 as burn-in.

Results

Qualitative phenotypic variation

Of the total of 269 individuals analysed, 233 were phenotypically characterized following the pattern of the hidden surfaces of the thigh proposed by Pombal & Haddad (1992). Figure 1A shows morphotype distributions, and Table 1 presents their proportional values in each locality. In general, the 'intermediate' morphotype was more frequent (60%) and showed a widespread distribution from north to south of BAF. The *P. burmeisteri* morphotype was the least frequent (11%), although it showed a moderate distribution along the BAF. The uniform blue morphotype (*P. bahiana*) was observed in 29% of the specimens and showed a more restricted distribution mainly in north of Bahia state, with the exception of three specimens located in southeast of Bahia state (SL 28 and 33). This last morphotype showed a particular distribution pattern mostly occupying some patches of BAF besides the continuous ombrophilous area. The sympatric occurrence of the three morphotypes was observed only in one locality (SL 28) with the prevalence of the intermediate one. We highlighted morphotype localities represented by only a single specimen and therefore the morphotype assigned to these localities should be interpreted with caution.

Genetic variation

We analysed one mitochondrial gene for the 267 individuals and three nuclear genes for a representative subsample of *P. bahiana* and *P. burmeisteri* individuals (see details in Additional Supporting Information Table S1). We obtained a mitochondrial fragment (ND2) of 993 base pairs (bp) which revealed a total of 81 haplotypes with 203 segregating sites.

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For the nuclear fragments, individuals of each species were grouped according to the STRUCTURE results (see section 3.3 and Fig. 5B). Overall, we analysed 250 gene copies of 609 bp for CXCR4, 122 gene copies of 600–604 bp for β-fibint7 and 144 gene copies of 345–348 bp for C-myc2. The number of segregating sites ranged from 29 in C-myc2 to 65 in β-fibint7, haplotypes numbers from 31 in CXCR4 to 71 in β-fibint7 and the population mutation parameter (Θ) ranged from 0.86% in CXCR4 to 2.1% in β-fibint7 (Table 2). The DSS test did not detect recombination in any of the fragments.

Phylogenetic analyses and haplotype genealogies

The best-fit partitioning schemes for the mtDNA fragment favoured the use of distinct evolutionary models for each codon position (AIC: 7134.03). Models of nucleotide substitution used were as follows: TrN+I+G (p-inv = 0.3330, gamma shape = 0.4770) for the 1st position, TrN+I (p-inv = 0.8000) for the 2nd position and GTR+G (gamma shape = 3.3920) for 3rd position. BI mtDNA analysis revealed the presence of three highly divergent well-supported clades (Fig. 2). The first one supported the monophyly of *P. bahiana* comprising haplotypes distributed from Sergipe state up to Espírito Santo state in which *P. burmeisteri* haplotypes also occur in the same locality (SL 36; Table 1; Figs 5A). The *P. bahiana* clade (BAH) also presented two subclades, Subclade A (Sc-A) and Sc-B, with high and moderate support, respectively. While Sc-A comprises haplotypes found in more deciduous and xeric (Caatinga) areas, Sc-B consists mostly of haplotypes from coastal and moister areas. Four localities in the transition between subclades areas shared haplotypes from Sc-A and Sc-B (SL 8, 9, 19 and 20; Table 1 and Fig. 2). The second main clade occupies the largest portion of the *P. burmeisteri* range (BUR), with the exception of the majority of the haplotypes from Rio de Janeiro state (SL 46–50) that form their own divergent clade (BUR-RJ; Figs 2 and 5A). The BUR clade also showed well-supported subclades: Sc-C including southern Bahia haplotypes; Subclade D (Sc-D) with haplotypes in the Espírito Santo, Minas Gerais and São Paulo states, and haplotypes from southern Bahia (SL 29, 31, and 33); and, subclade E (Sc-E) presenting one unique haplotype from the Rio de Janeiro state (SL 45), and exclusive haplotypes from the Minas Gerais and São Paulo states (SL 42, 43, 44, 56 and 57; Table 1 and Fig. 2).

Single locus nuclear genealogies did not recover any of the three clades presented in the mtDNA analysis (Fig. 3). However, two of the three fragments (CXCR4 and C-myc2) showed a strong tendency to separate two haplogroups: one formed by *P. bahiana* individuals (BAH) and another with *P. burmeisteri* individuals. All nuclear genealogies were congruent in presenting shared polymorphism, as six haplotypes in β-fibint7, four in both CXCR4 and C-myc2 are shared by individuals belonging to two or three different mtDNA-defined groups (BAH, BUR and BUR-RJ). In both C-myc2 and CXCR4, some haplotypes are clustered in the BAH group, but their corresponding

Table 2 Fragment information, summary statistics, genetic distances (*p*-uncorrected) and neutrality tests within the main mitochondrial clades and nuclear groups (see STRUCTURE results) of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*

			Polymorphism						Neutrality test's	
Fragment	Clade/Group	Length	*N*	S	h	% Hd	% θ	*P* [95% C.I.]	Tajima's D	R^2
ND2	All	993	267	203	81	95	3.3	–	–	–
	BAH		154	55	39	89	1	–	–	–
	Sc-A		71	27	19	88	0.5	0.27 [0.05–0.7]	−1.6291 ns	0.0491 ns
	Sc-B		83	22	20	71	0.4	0.16 [0.02–0.4]	−1.9207**	0.0335**
	BUR		95	71	35	95	1.4	–	–	–
	Sc-C		11	5	5	62	0.17	0.1 [0–0.28]	−1.7910**	0.1311*
	Sc-D		44	41	22	93	0.95	0.6 [0.2–1.5]	−1.2118 ns	0.0695 ns
	Sc-E		38	12	7	75	0.3	0.4 [0.1–1.1]	−1.9257 ns	0.1910 ns
	BUR-RJ		18	17	7	86	0.5	0.53 [0.1–1.3]	0.2478 ns	0.1549 ns
CXCR4	All	609	250	32	31	84	0.86	–	–	–
	P. bahiana		72	20	10	53	0.68	0.26 [0.03–0.72]	−1.8315*	0.0468*
	P. burmeisteri		178	22	24	78	0.63	0.41 [0.1–1.07]	−0.9326 ns	0.0567 ns
β-fibint7	All	600–604	122	65	71	98	2.1	–	–	–
	P. bahiana		44	34	28	96	1.3	1.4 [0.3–2]	0.0118 ns	0.1185 ns
	P. burmeisteri		78	42	47	97	1.4	0.5 [0.16–1.2]	−1.3593 ns	0.0592 ns
C-myc2	All	345–348	144	29	62	96	1.5	–	–	–
	P. bahiana		64	21	31	94	1.3	0.3 [0.05–0.7]	−1.3918 ns	0.0606 ns
	P. burmeisteri		88	14	34	93	0.8	0.15 [0.01–0.36]	−1.5226 ns	0.0454 ns

*$P < 0.05$ **$P < 0.01$ ns = no significance.

mtDNA haplotype belongs to BUR group, and vice versa (SL 31, 33 and 36). Throughout the visual inspection of the network topologies, only BAH group from CXCR4 exhibits a more pronounced pattern reflected by a star-like topology, which is indicative of population demographic expansion.

Pairwise distance and neutrality tests

Mitochondrial uncorrected pairwise distances values between clades and subclades are presented in Fig. 2. While a pairwise distance of about 10% was found between *P. burmeisteri* and *P. bahiana*, an unexpected high level (7%) was found between the two *P. burmeisteri* clades (BUR and BUR-RJ). All subclades presented similar distance values ranging from 1.5 to 1.9%. In general, intraclade distances were higher within *P. burmeisteri* than in *P. bahiana* (Table 2). Neutrality tests applied to the mtDNA data set were both in agreement showing that individuals from Sc-B (*P. bahiana*) and Sc-C (*P. burmeisteri*) might be undergoing a process of demographic expansion. Both tests performed with data from the nuclear CXCR4 fragment showed that *P. bahiana* could be undergoing expansion (Table 2).

Genetic distance network and population assignment analyses

The multilocus distance network was not congruent with the results from mtDNA. Instead of presenting three divergent groups, the POFAD analysis revealed two main groups: one clustering individuals from *P. bahiana* (BAH) and another with *P. burmeisteri* individuals from both BUR-RJ and BUR distribution (Fig. 4). This analysis also presented a strong tendency to separate BUR from BUR-RJ individuals with some transitional individuals between both lineages: five from BUR (SL 54 and 56) and two from BUR-RJ (SL 46 and 48). Individuals from the Jundiaí locality (SL 54) in São Paulo state, clustered with both the divergent BUR group and transitional individuals.

The Bayesian clustering analysis, according to the distribution of Ln Pr (*X*/*K*) and Δ*K*, recovered the nuclear DNA structure of the northern species of the *P. burmeisteri* group as $K = 2$, corresponding to *P. bahiana* individuals (BAH) from *P. burmeisteri* (BUR and BUR-RJ; Fig. 5B and Additional Supporting Information Fig. S1). The geographic break between these two clusters is located in southern of Bahia state, roughly coincident with the Jequitinhonha River with the exception of individuals of *P. bahiana* from SL 17 that penetrated into the interior part of northern of Minas Gerais state. STRUCTURE results also evidenced the presence of some putative hybrid individuals in Itabela and Prado localities (SL 31 and 33, respectively), showing in addition that all individuals from SL 36 (according to mtDNA four of which are clustered in *P. bahiana* group) belong to *P. burmeisteri* at nuclear level.

Species delimitation, species trees and divergence times estimates

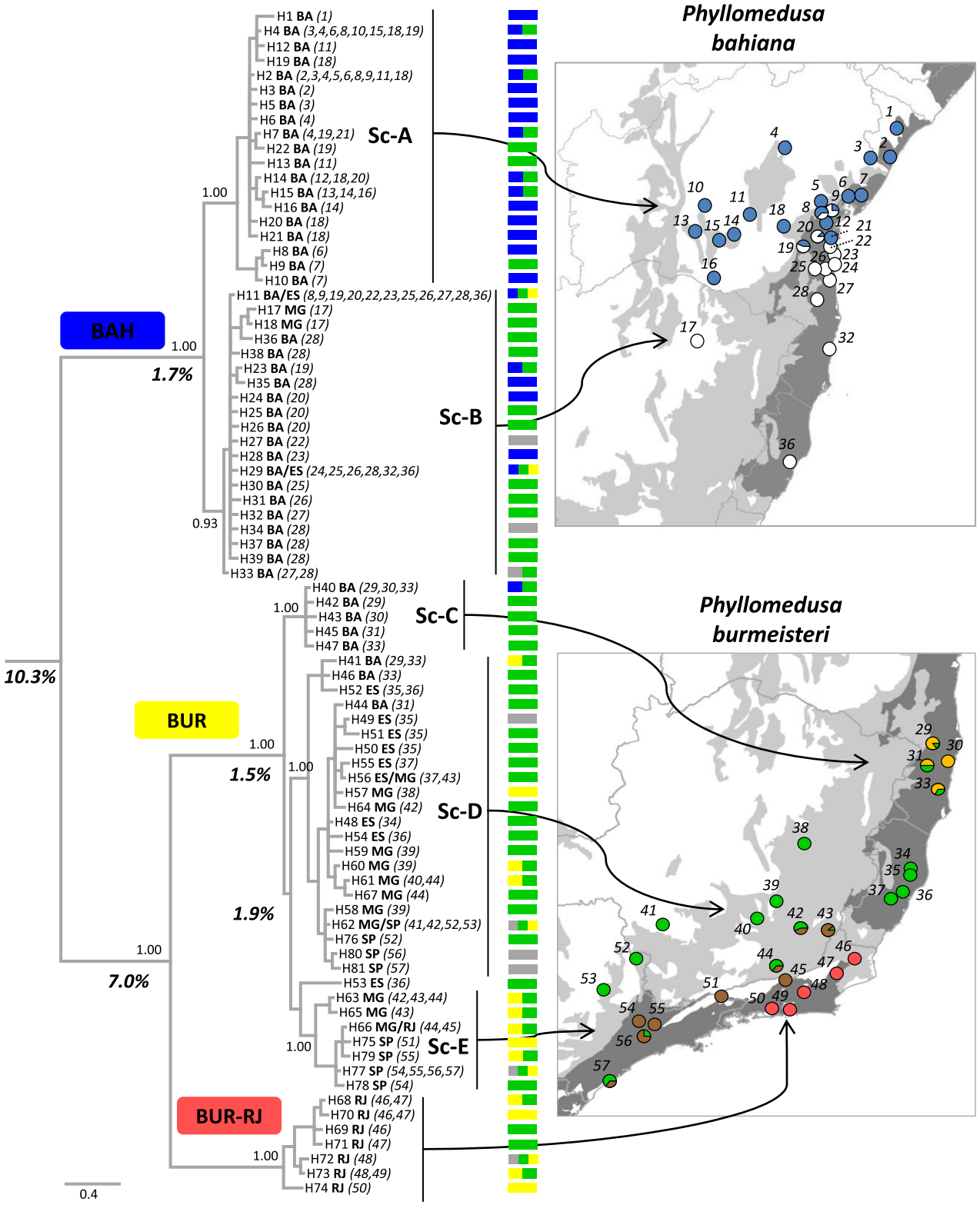
Both species trees showed a high support for two main clades; one grouping the southern species *P. distincta* (P. dis) and *P. iheringii* (P. ihe) and another with the northern species: *P. bahiana* (BAH), *P. burmeisteri* (individuals from BUR and BUR-RJ lineages). The species tree also supported the split between BUR and BUR-RJ lineages as sister-taxa (Fig. 6A and Additional Supporting Information Fig. S2A). The Bayesian coalescent estimate for ^{t}MRCA was ~2.9 Myr (95% CI; 1.4–4.4) for the split between *P. bahiana* and *P. burmeisteri* (BUR and BUR-RJ) and ~1.3 Myr (95% CI; 0.4–2.4) for the split between BUR and BUR-RJ lineages (Fig. 6A). The BPP species delimitation analyses, either combining mtDNA and nuDNA (Fig. 6B) or using only nuDNA data (Additional Supporting Information Fig. S2B), were strongly congruent and showed posterior probabilities equal or higher 0.99 in all nodes tested. Overall, the five units inferred in the BPP analysis (*P. distincta*, *P. iheringii*, BAH, BUR, and BUR-RJ) were highly supported as species or as candidate species in the case of BUR lineage.

Discussion

Phylogeny, genetic structure and cryptic diversity

The phylogenetic analysis of a mtDNA gene fragment (ND2) showed two deeply divergent (*p*-distance = 10%) and highly supported mtDNA clades, which exhibit a geographic cohesiveness broadly coincident with the previously inferred ranges of both *P. bahiana* and *P. burmeisteri* in BAF (Fig. 2). Our results showed also deep mitochondrial structure within each of the two main clades, corresponding broadly to previous results uncovered by Brunes *et al.* (2010). A new finding though was the observation of two highly divergent (*p*-uncorrected = 7%) and well-supported

Fig. 2 Mitochondrial gene tree (ND2) derived from Bayesian analysis of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri* and subclades (Sc-A-E) distribution on map. Bars indicate the morphotype classification: in blue, *Phyllomedusa bahiana*; in green, 'intermediate'; in yellow, *Phyllomedusa burmeisteri*, and in grey, no information. Terminal names indicate haplotype number, initial of states and locality code (see Fig. 1 and Table 1). Bayesian posterior probabilities (over 90%) are given near the branches. Percentages indicate *p*-uncorrected values between clades. Arrows indicate the respective subclade distribution: Sc-A, blue circles; Sc-B, white circles; Sc-C yellow circles; Sc-D green circles; Sc-E brown circles; and BUR-RJ, red circles. Brazilian Atlantic Forest original cover: Ombrophylous (dark grey) and Semi-deciduous/Deciduous (light grey) forests are represented.


Phyllomedusa bahiana
Phyllomedusa burmeisteri
BAH
BUR
BUR-RJ
Sc-A
Sc-B
Sc-C
Sc-D
Sc-E
10.3%
1.7%
1.5%
1.9%
7.0%
1.00
0.93
0.4
H1 BA (1)
H4 BA (3,4,6,8,10,15,18,19)
H12 BA (11)
H19 BA (18)
H2 BA (2,3,4,5,6,8,9,11,18)
H3 BA (2)
H5 BA (3)
H6 BA (4)
H7 BA (4,19,21)
H22 BA (19)
H13 BA (11)
H14 BA (12,18,20)
H15 BA (13,14,16)
H16 BA (14)
H20 BA (18)
H21 BA (18)
H8 BA (6)
H9 BA (7)
H10 BA (7)
H11 BA/ES (8,9,19,20,22,23,25,26,27,28,36)
H17 MG (17)
H18 MG (17)
H36 BA (28)
H38 BA (28)
H23 BA (19)
H35 BA (28)
H24 BA (20)
H25 BA (20)
H26 BA (20)
H27 BA (22)
H28 BA (23)
H29 BA/ES (24,25,26,28,32,36)
H30 BA (25)
H31 BA (26)
H32 BA (27)
H34 BA (28)
H37 BA (28)
H39 BA (28)
H33 BA (27,28)
H40 BA (29,30,33)
H42 BA (29)
H43 BA (30)
H45 BA (31)
H47 BA (33)
H41 BA (29,33)
H46 BA (33)
H52 ES (35,36)
H44 BA (31)
H49 ES (35)
H51 ES (35)
H50 ES (35)
H55 ES (37)
H56 ES/MG (37,43)
H57 MG (38)
H64 MG (42)
H48 ES (34)
H54 ES (36)
H59 MG (39)
H60 MG (39)
H61 MG (40,44)
H67 MG (44)
H58 MG (39)
H62 MG/SP (41,42,52,53)
H76 SP (52)
H80 SP (56)
H81 SP (57)
H53 ES (36)
H63 MG (42,43,44)
H65 MG (43)
H66 MG/RJ (44,45)
H75 SP (51)
H79 SP (55)
H77 SP (54,55,56,57)
H78 SP (54)
H68 RJ (46,47)
H70 RJ (46,47)
H69 RJ (46)
H71 RJ (47)
H72 RJ (48)
H73 RJ (48,49)
H74 RJ (50)

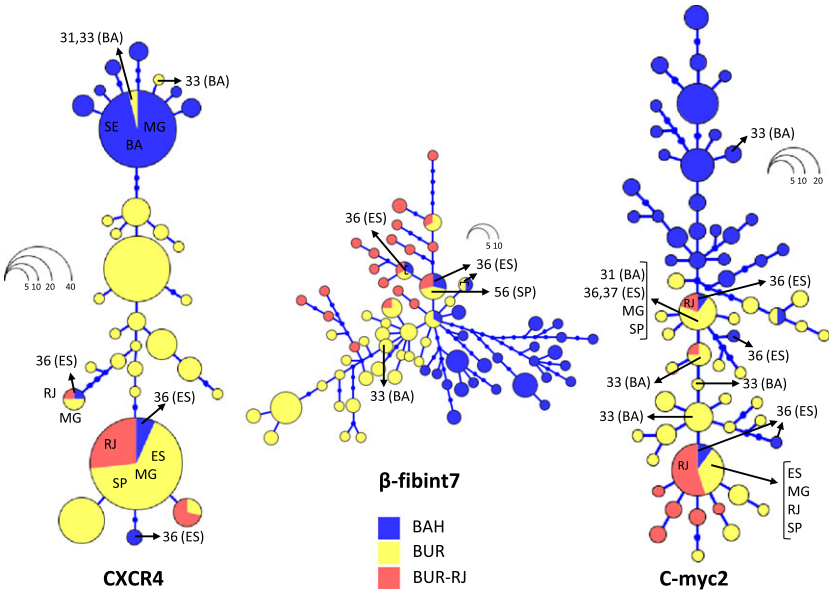


Fig. 3 Nuclear haplotype genealogies converted from parsimony trees in the software Haploviewer of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*. The circle area of each haplotype is proportional to its frequency. Mutations are edges. Colours represents the three major mitochondrial lineages (see Fig. 2). Arrows indicate sampling localities (SL) and Brazilian states, respectively (see Table 1 and Fig. 1).

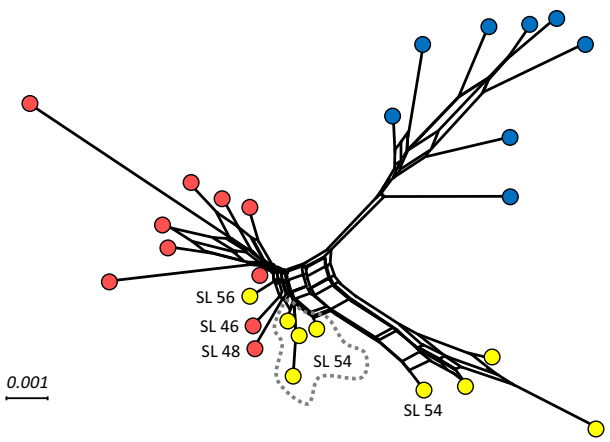


Fig. 4 Multilocus genetic distance network of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri* based on three nuclear fragments (CXCR4, β-fibint7, C-myc2) of the allopatric data set. Circles were coloured following the three major mitochondrial groups (see Figs 2 and 5). Sampling localities numbers (SL) are in Table 1 and Fig. 1.

monophyletic clades (BUR and BUR-RJ) within *P. burmeisteri*. This novel clade recovered by our current analysis shows geographical cohesiveness, corresponding to a previously unsampled geographic area of the Rio de Janeiro State (Fig. 2), including the city of Rio de Janeiro which is the type locality of *P. burmeisteri* (Fig. 5A, Boulenger 1882).

The single locus nuclear gene genealogies did not fully recover the same population structure observed in the mitochondrial tree (Fig. 3). Discordance between nuclear and mitochondrial markers is expected in analyses of closely related species (e.g. Degnan & Rosenberg 2009). However, both single and multilocus networks showed a general trend towards uncovering two groups concordant with the *P. bahiana* and *P. burmeisteri* (BAH and BUR/BUR-RJ) mtDNA clades. This result is corroborated by our Bayesian cluster assignment which consistently recovered two clusters that largely correspond to the two deeply diverged mtDNA clades, therefore suggesting the signature of a major evolutionary genetic break. Bayesian assignment of individuals showed signs of admixture at the nuclear level between both clusters in southern Bahia state (SL 31 and 33). Interestingly, no signs of nuclear admixture were found in SL 36 (Espírito Santo state), the single locality where mtDNA haplotypes of *P. burmeisteri* and *P. bahiana* co-occur (Fig. 5A,B). Although we cannot exclude that retention of ancestral polymorphism may explain the pattern of sharing haplotypes in our single locus nuclear genealogies (Fig. 3), the pattern of mtDNA distribution together with nuclear Bayesian assignment results may provide additional evidence for past or current gene flow between these two evolutionary genetic units (see detailed discussion below).

Within *P. burmeisteri*, only the multilocus distance network was roughly congruent with the occurrence of two clusters of haplotypes that correspond to two divergent mtDNA clades (BUR and BUR-RJ). Possible explanations for the lack of reciprocal monophyly in the nuclear

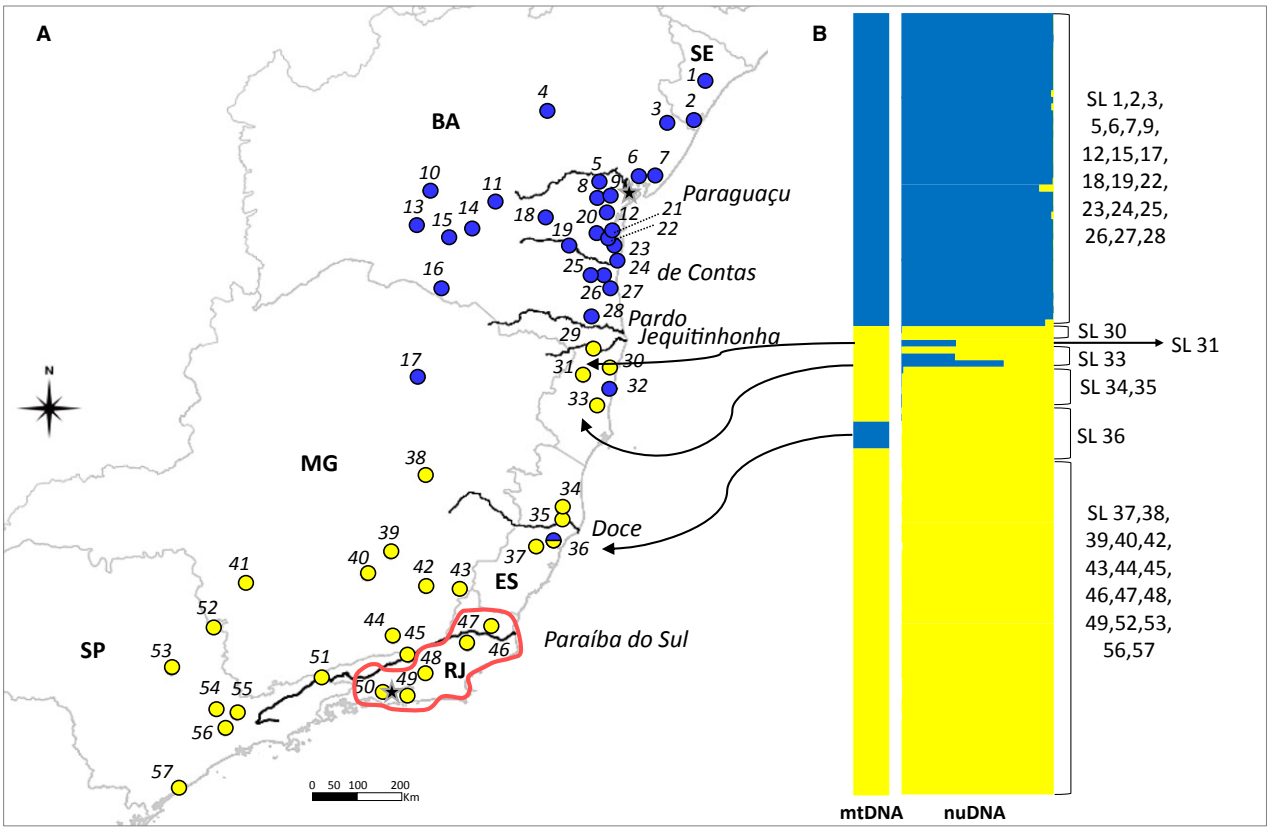


Fig. 5 Mitochondrial DNA distribution (A) and STRUCTURE results based in nuDNA allele frequencies for $K = 2$ (B) of *Phyllomedusa bahiana* (blue) and *Phyllomedusa burmeisteri* (yellow). Individuals are represented as bars, with colours representing the proportion of assignment. Bars on the left represent the respective mitochondrial main clades. Surrounded area in red represents the BUR-RJ clade (Fig. 2). Sampling localities (SL) follow Fig. 1 and Table 1. Major rivers of the Brazilian East Atlantic Basin are indicated. Stars indicate type localities.

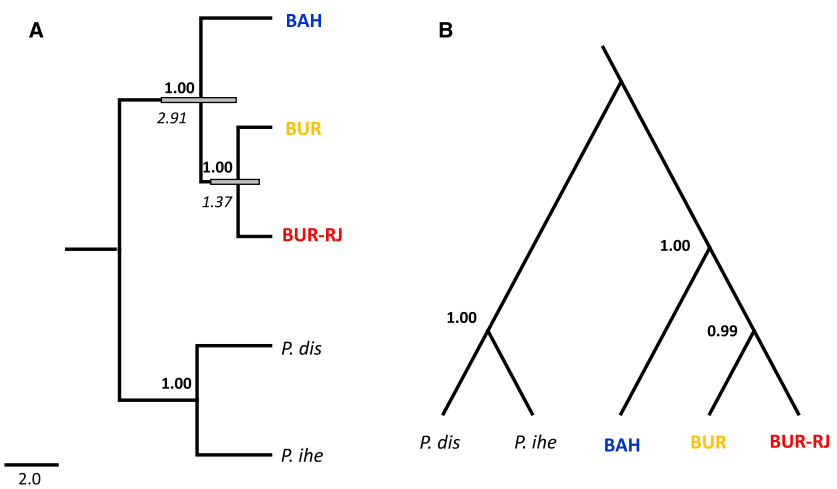


Fig. 6 Coalescent multilocus analysis combining mitochondrial and nuclear DNA information of partial *Phyllomedusa burmeisteri* group: (A) species tree in *BEAST; (B) species delimitation in BPP. Posterior probabilities are shown above of the branches and the mean times to the most recent common ancestor (tMRCA) are bellow of the branches. The node grey bars represent the 95% confidence intervals. P. dis, *Phyllomedusa distincta*; P. ihe, *Phyllomedusa iheringii*.

genealogies includes the possibility of gene flow, since BUR-RJ clade is geographically surrounded by the BUR clade, incomplete lineage sorting due to recent diversification as previously described for the *P. burmeisteri* species group (Brunes *et al.* 2010), and the longer times of neutral coalescence of nuclear DNA compared with the mitochondrial genome (e.g. Zink & Barrowclough 2008). Indeed, the Bayesian coalescent estimates of divergence time (^{t}MRCA) were twice as old for the split between *P. bahiana* and *P. burmeisteri* clades (~2.8 Myr) than for the split between BUR and BUR-RJ clades (~1.3 Myr; Fig. 4A).

The Bayesian clustering analyses did not recover any genetic substructure within the *P. burmeisteri* clade, which could additionally be explained by the reduced power of reliably inferring the optimal number of clusters due to low number of loci here analysed (e.g. Thomé *et al.* 2012). In recent years, many studies have used nuclear sequence data coupled with assignment methods for revealing cryptic genetic structure and investigate species delimitation using similar or slightly higher numbers of loci (3–6) in groups with complex taxonomy (Leaché & Fujita 2010; Thomé *et al.* 2012; Fusinatto *et al.* 2013; Welton *et al.* 2013). Although the performance of these methods has been varied with different levels of genetic divergence, in most cases, they have successfully detected relevant genetic structure. The power to detect genetic structure with a reduced number of loci in Bayesian model-based assignment methods must also depend on the markers used, adequate sampling of individuals, as well as the species evolutionary history (Yang *et al.* 2005; Hausdorf & Hennig 2010). Here, the use of relatively conserved nuclear markers did not allow the detection of further genetic structure within the major clades (i.e. finer-level structure). The use of fast-evolving markers such as microsatellites (see Brunes *et al.* 2013) may be instrumental in this respect to increase the power of detecting genetic clustering at shallower lineage divergences.

Patterns of genetic and phenotypic variation

An overall discordance between the genetic data and the variation in colour pattern on the hidden surfaces of the thigh (morphotype) was observed in this study. The morphotypes previously assigned to *P. bahiana* and *P. burmeisteri* (pure morphotypes) were reported to occur within the distribution range of each species albeit in a few cases of sympatry (Pombal & Haddad 1992). Our results showed that the intermediate morphotype is clearly the most frequently observed across the ranges of both species, co-occurring with pure morphotypes at several localities (this includes the two phenotypically indistinguishable evolutionary units of *P. burmeisteri*, BUR and BUR-RJ). Most of individuals assigned to each of the two identified nuclear clusters with a probability of approximately 100% (q = 1.0) therefore exhibit the intermediate morphotype, suggesting that the colour patterns of the hidden surfaces of the thigh is not a diagnostic character between *P. bahiana* and *P. burmeisteri*.

Discordance between phenotypic traits and patterns of genetic divergence is a widespread phenomenon among amphibians (e.g. Ohmer *et al.* 2009; Rodríguez *et al.* 2012; Brusa *et al.* 2013; Bruschi *et al.* 2013; but see *Agalychnis callidryas* in Robertson *et al.* 2009). Colour polymorphism can arise and be maintained by the action of isolated or combined evolutionary processes such as natural and sexual selection (Sandoval & Nosil 2005; Richards & Knowles 2007), genetic drift (Hoffman *et al.* 2006; Abbott *et al.* 2008), gene flow (Slatkin 1985) and antipredator strategy and conspecific communication (Toledo & Haddad 2009; Rudh & Qvarnström 2013). The occurrence of an intermediate morphotype has been previously interpreted as the result of extensive gene flow between *P. bahiana* and *P. burmeisteri* (Pombal & Haddad 1992). Based on this hypothesis, we expected that high levels of gene flow combined with limited localized selection have contributed to the widespread geographic distribution of the intermediate morphotype. However, the signature of extensive admixture between both species was not detected in this study, as either individuals with an assignment probability lower than 70% (q < 0.7; three) or with 100% (q = 1.0) but with introgressed mtDNA (four individuals classified as *P. burmeisteri* with nuclear DNA have mtDNA from *P. bahiana*), are relatively few and restricted to some localities in southern Bahia and Espírito Santo. Alternatively, the spatial pattern and frequency of the intermediate morphotype may result from natural selection acting on defensive strategies. In species of *Phyllomedusa*, the colour patterns of the hidden surfaces of flanks and/or thighs (bright uniform colours or mixed with yellow spots or irregular stripes) have been interpreted as aposematic, given that these patterns are displayed when individuals move on tree branches stimulated by the presence of potential predators (Toledo & Haddad 2009; Toledo *et al.* 2011; but see Rudh & Qvarnström 2013). The most important point of the theory explaining aposematism (Müller 1879) is that populations of distinct species should converge into one similar aposematic phenotype because in this way the predator's behaviour will be reinforced to avoid that specific colouration pattern (e.g. Endler 1982; Harmon *et al.* 2005). In this situation, selection is expected to favour the most conspicuous and common colour pattern (directional selection). So, it is possible that the highest frequency and widespread distribution of the intermediate morphotype reflects the most successfully pattern of colouration as a defensive mechanism. While our sample of a single phenotypic trait and the use of neu-

tral markers cannot conveniently address this hypothesis, intra-populational variability in colour patterns present in both species deserves further study, with particular emphasis on the role of sexual selection, ecological traits, and behavioural strategies.

Species tree and estimates of species delimitation

Estimates of coalescent species trees are efficient to accommodate inconsistencies between gene genealogies due to incomplete lineage sorting in recently diversified populations (Heled & Drummond 2010). In this study, we used the Bayesian coalescent species tree implemented in *BEAST to contrast the phylogenetic relationships inferred by mtDNA and nuclear data when separately analysed. To avoid the confounding effect of contact zones between adjacent groups, as we detected potential gene flow, we used only individuals from geographic regions distant from the contact zones ('allopatric' data set) to estimate a species tree and to determine species limits between *P. bahiana* and *P. burmeisteri*. The species trees (*BEAST) recovered a sister-taxa relationship between *P. bahiana* and *P. burmeisteri*, and two sister-clades within *P. burmeisteri* (BUR and BUR-RJ), with high posterior probability support for all nodes. The Bayesian species delimitation method (BPP) consistently supported the three distinct evolutionary units (BAH, BUR and BUR-RJ). In both cases, results were therefore concordant with the mtDNA tree topology, irrespectively of using only the nuclear DNA data set (Additional Supporting Information Fig. S2A,B) or combining mtDNA and nuclear data sets (Fig. 6A,B).

There is a growing body of research on exploring methods for testing species delimitation, as inferring boundaries between evolutionary lineages has become paramount to estimate and describe species diversity, particularly in cases of complex taxonomy (Leaché & Fujita 2010; Fusinatto *et al.* 2013; Satler *et al.* 2013; Sousa-Neves *et al.* 2013). However, these methods are not free of drawbacks (e.g. testing *a priori* delimited units based on a single guide tree), and the outcomes have greatly varied among studies. While several authors have presented taxonomic recommendations (Niemiller *et al.* 2012; Fusinatto *et al.* 2013) or even have described new taxonomic entities (Leaché & Fujita 2010; Satler *et al.* 2013), others have recognized only possible taxonomic implications without presenting formal recommendation (Setiadi *et al.* 2011; Camargo *et al.* 2012; Welton *et al.* 2013).

In the case of *P. burmeisteri*, there are several challenges for the taxonomic significance of the novel evolutionary unit unveiled in this study (BUR), as its delimitation is not fully congruent across different types of markers and methods. By contrast, our molecular analyses are congruent in supporting an independent evolutionary trajectory of the two current recognized species, *P. bahiana* and *P. burmeisteri*. Moreover, it is notable that the putative existence of a second taxa within *P. burmeisteri* (BUR-RJ clade) would imply a drastic reduction of the current known range of this species, as type locality of *P. burmeisteri* is from Rio de Janeiro city (Fig. 5A). Indeed, individuals from BUR clade occupy the largest portion of the known range assigned currently to *P. burmeisteri*, whereas individuals from the BUR-RJ clade are restricted to the Rio de Janeiro state. Notwithstanding, the taxonomic status of these two species and particularly of this novel evolutionary unit would benefit from an integrative approach including other sources of information, such as reproductive aspects (e.g. advertisement calls) related with prezygotic isolation.

Biogeography and spatiotemporal patterns of diversification

Patterns of diversification in the *P. burmeisteri* species group have been recently addressed by Brunes *et al.* (2010). Following these authors, species from this group were originated between ~0.3 and 2.5 Myr, spanning the late Pliocene and Pleistocene. Here, using geographically widespread sampling, the split time between *P. bahiana* and *P. burmeisteri* was estimated to occur at ~2.9 Myr (95% CI; 1.4–4.4). Although this time interval is within the confidence interval estimated by Brunes *et al.* (2010), the more ancient split time inferred here probably reflects the addition of one more nuclear DNA locus (CXCR4) and more individuals, especially those from the novel and highly divergent evolutionary unit uncovered within the *P. burmeisteri* clade (BUR-RJ). Following theoretical expectations, population subdivision can increase coalescence time between genes and inflate estimates of population divergence time (e.g. Edwards & Beerli 2000). In addition, it has been demonstrated that both increasing number of individuals and loci is an important factor affecting the accuracy of estimates, especially in systems with shallow histories (McCormack *et al.* 2009). The second major split was estimated to occur between the two sister-evolutionary units of *P. burmeisteri* (BUR and BUR-RJ) at around 1.3 Myr (95% CI; 0.4–2.4). These results corroborate previous findings on temporal patterns of diversification in many BAF organisms, suggesting that their populations were affected by historical events that occurred during the Pliocene and Pleistocene periods (e.g. Pellegrino *et al.* 2005; Grazziotin *et al.* 2006; Thomé *et al.* 2010; Amaro *et al.* 2012).

Our results from mitochondrial and nuclear analysis were concordant in defining the geographic boundary between *P. bahiana* and *P. burmeisteri* in the vicinity of the River Jequitinhonha, which disagree with the previously southernmost range limit (Doce River) inferred by Brunes *et al.* (2010). However, results presented herein showed the

co-occurrence of *P. bahiana* and *P. burmeisteri* mtDNA haplotypes in Espírito Santo state (SL 36) close to the Doce River (Fig. 5A). The individuals bearing the two *P. bahiana* haplotypes (H11 and H29; four individuals) found in Espírito Santo state were assigned to *P. burmeisteri* on the basis of nuclear markers, suggesting that the presence of both haplotypes in this region may be a remnant of a wider distribution of *P. bahiana* in the past. The presence of foreign mtDNA as a wake of past hybridization is a widespread phenomenon among animals, including several amphibians (Babik *et al.* 2003; Sequeira *et al.* 2005; but see Toews & Brelsford 2012). Indeed, mtDNA usually tends to be more sensitive to stochastic processes than nuclear loci due to lower effective population size, maternal inheritance and lack of recombination. Accordingly, these results may reflect a combination of distinct episodes of secondary contact between *P. bahiana* and *P. burmeisteri*: an older hybridization event in the Espírito Santo region, and a more recent secondary contact in southern Bahia.

The association of phylogeographic breaks to river barriers has already been mentioned for various BAF organisms including amphibians, lizards, and birds (Pellegrino *et al.* 2005; Thomé *et al.* 2010; Maldonado-Coelho 2012). It is well-documented that Plio-Pleistocene climatic oscillations induced sea-level fluctuations along the Brazilian coast that have contributed to change the coastal plains of rivers across time and were likely responsible for recurrent episodes of isolation and secondary contact between populations (Dominguez 2009; and references therein). There is a growing body of literature documenting that Pleistocene climatic oscillations have promoted cyclic changes on environmental conditions and concomitant alterations of habitats in BAF that profoundly impacted the evolutionary history of several species (e.g. Cabanne *et al.* 2007; Carnaval & Bates 2007; Valdez & D'Elía 2013). Regarding potential range shifts of *P. bahiana* and *P. burmeisteri*, the combined effects of complex river dynamics and Pleistocene induced extinction/recolonization events could explain both general phylogeographic patterns and more specifically the geographic location of the two putative secondary contact zones.

Regarding the ample debate about Pleistocene refuges in BAF (e.g. Carnaval *et al.* 2009), one of the most striking findings of our study was the detection of two evolutionary units (BUR and BUR-RJ) within the range of *P. burmeisteri*, whose split spanned the Pleistocene (~1.3 Myr). While BUR exhibits a wide distribution range, BUR-RJ is restricted to a small area coincident with the Serra do Mar in the Rio de Janeiro state, being roughly delimited by the Paraíba do Sul River (Fig. 5A). This area has been referred as a small putative Pleistocene refugium, exhibiting high levels of endemic species (Rocha *et al.* 2004) and evolutionary units (Pellegrino *et al.* 2005; Amaral *et al.* 2013; Gehara *et al.* 2013), which may corroborate by several assumptions for the existence of a large refugium in the southeast of Brazil based on various methodologies (see Porto *et al.* 2013, and references therein).

Additional evidence supporting a southeastern refugium hypothesis are provided by the high levels of genetic substructuring within *P. burmeisteri* (BUR clade), as suggested by the detection of three well-supported and geographically delimited mtDNA subclades (Sc-C, Sc-D, and Sc-E; *p*-uncorrected = 1.5–1.9%; Fig. 2). This hypothesis is however difficult to corroborate given the little evidence of population size changes. Indeed, Tajima's D and R^2 only detected consistently signatures of population expansion for the northernmost subclade Sc-C found in southern Bahia. Our results may favour instead the hypothesis that there have been at least several local refugia allowing the species to persist through multiple climatic cycles. Some authors suggested that some BAF regions such as the foothills of mountain ranges and valleys of large rivers could have provided suitable habitats for the persistence of organisms during adverse climatic periods of the Pleistocene (see Maldonado-Coelho 2012, and references therein).

The species *P. bahiana* also revealed high levels of genetic substructure, exhibiting two well-supported and geographically delimited mtDNA subclades (Sc-A and Sc-B; *p*-uncorrected = 1.7%; Fig. 2). Both Tajima's D and R^2 consistently detected signatures of population expansion only for Sc-B. This subclade is mostly distributed along the coast following the Ombrophylous forest-type and meets with *P. burmeisteri* in southern Bahia. Our results suggest that this area of sympatry between *P. bahiana* and *P. burmeisteri* in southern of Bahia could have resulted from the southern expansion of Sc-B and the northern expansion of Sc-C, establishing a relatively recent secondary contact zone. Regarding Sc-A, it occurs in a large area containing small patches of BAF surrounded by open Caatinga vegetation, with no signs of further substructure from the rest of the subclade range.

Taken together, our results suggest that a mosaic of habitats in heterogeneous landscapes could be of major importance for species of this group to persist though changing environmental conditions. While this hypothesis deserves further detailed molecular studies, including extended fine-scale sample sizes and the use of faster-evolving markers to examine routes and levels of gene flow among populations, the eco-physiological features (uricotelism, lipids glands and wiping behaviour) present in phyllomedusine frogs (see Faivovich *et al.* 2010, and references therein) could favoured the persistence of populations during extreme environmental changes, both those that putatively occurred during the Pleistocene and those that may be occurring in the present.

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Supporting Information

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

Fig. S1. *K* values curve for $K = 1–10$ (Ln Pr (*X*/*K*)), and value of ΔK for $K = 2$.

Fig. S2. Multilocus nuclear analysis: (A) species tree in *BEAST and (B) species delimitation in BPP. Posterior probabilities are shown near the branches. P. dis, *Phyllomedusa distincta*; P. ihe, *Phyllomedusa iheringii*.

Table S1. Individual geographic, phenotypic and genetic sample information of *Phyllomedusa bahiana* and *Phyllomedusa burmeisteri*. Morphotype classification: U = *Phyllomedusa bahiana*, I = 'intermediate', and S = *Phyllomedusa burmeisteri*.