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Gene and species trees of a Neotropical group of treefrogs: Genetic diversification in the Brazilian Atlantic Forest and the origin of a polyploid species

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ABSTRACT

The Neotropical *Phyllomedusa burmeisteri* treefrog group includes four diploid (*P. bahiana*, *P. burmeisteri*, *P. distincta* and *P. iheringii*) and one tetraploid (*P. tetraploidea*) forms. Here we use mitochondrial and nuclear sequence variation from across its range to verify if recognized morphospecies correspond to phylogenetic clades, examine the origin of the polyploid *P. tetraploidea*, and compare range wide patterns of diversification to those of other BAF organisms. We compared single gene trees with one Bayesian multi-gene tree, and one Bayesian species tree inferred under a coalescent framework. Our mtDNA phylogenetic analyses showed that *P. bahiana*, *P. burmeisteri* and *P. iheringii* correspond to monophyletic clades, while *P. distincta* and *P. tetraploidea* were paraphyletic. The nuclear gene trees were concordant in revealing two moderately supported groups including (i) *P. bahiana* and *P. burmeisteri* (northern species) and (ii) *P. distincta*, *P. tetraploidea* and *P. iheringii* (southern species). The multi-gene tree and the species tree retrieved similar topologies, giving high support to the northern and southern clades, and to the sister-taxa relationship between *P. tetraploidea* and *P. distincta*. Estimates of ^{t}MRCA suggest a major split within the *P. burmeisteri* group at ≈5 Myr (between northern and southern groups), while the main clades were originated between ≈0.4 and 2.5 Myr, spanning the late Pliocene and Pleistocene. Patterns of geographic and temporal diversification within the group were congruent with those uncovered for other co-distributed organisms. Independent paleoecological and geological data suggest that vicariance associated with climatic oscillations and neotectonic activity may have driven lineage divergence within the *P. burmeisteri* group. *P. tetraploidea* probably originated from polyploidization of *P. distincta* or from a common ancestor.

1. Introduction

The Brazilian Atlantic Forest (BAF) has lost around 90% of its original area of more than one million square kilometers (Morellato and Haddad, 2000; Ribeiro et al., 2009). BAF is however among the most biodiverse regions of the world, harbouring one of the largest percentages of endemic species (Myers et al., 2000). The evolutionary processes that led to this extraordinarily diverse biome have long intrigued evolutionary biologists, but there are only a few studies unveiling the evolutionary processes that determined biological diversification in this megadiversity hotspot. Recently, some molecular studies have confirmed theoretical predictions that species' biogeographical histories have been impacted by the recurrent isolation and persistence of organisms in forest refugia throughout Quaternary climatic fluctuations – Pleistocene refugia hypothesis (*sensu* Haffer, 1969) – (e.g. Cabanne et al., 2007; Carnaval et al., 2009). Other studies, however, suggested that not only Quaternary climatic fluctuations but also climatic and geologic factors such as major rivers, mountain chains and Tertiary tectonic events associated with the formation of geographical landmarks determined current patterns of genetic diversity and diversification of many BAF taxa (Lara and Patton, 2000; Grazziotin et al., 2006; Pellegrino et al., 2005, but see review in Rull, 2008). Forest refugia and geographic barriers could have also acted in combination to produce taxon-specific idiosyncratic patterns of diversification (Grazziotin et al., 2006; Cabanne et al., 2008). To understand their relative importance in producing biological diversification will require detailed multi-species phylogeographical studies across organisms with distinct life histories. This will be especially relevant given that complex topography shaping the diverse phytophisiognomies of the BAF (Oliveira–Filho and Fontes, 2000).

Recent studies analyzing both molecular data and palaeoclimatic models of the BAF (Carnaval and Moritz, 2008; Carnaval et al., 2009) suggested spatial variation in forest persistence throughout the Pleistocene, predicting a large area of historical forest stability

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in the central corridor (Bahia) and in other small areas (Pernambuco) along the Brazilian coast, roughly concordant with current centres of endemism and mtDNA diversity patterns of few taxa (Costa et al., 2000; Costa, 2003; Cabanne et al., 2008), including three BAF treefrog species (Carnaval et al., 2009). Because the species of the *Phyllomedusa burmeisteri* group are widespread mostly in the BAF, inhabiting submontane to montane areas of rainforest, mixed ombrophilus, semi-deciduous and deciduous forest, they are appropriate model organisms for understanding diversification mechanisms and biogeographical patterns across the BAF. Five species are currently recognized within the *P. burmeisteri* species group based on external morphology, vocalization, and cytogenetics (Pombal and Haddad, 1992; Silva-Filho and Juncá, 2006): the diploid *P. burmeisteri*, *P. bahiana*, *P. distincta*, *P. iheringii*, and the tetraploid species *P. tetraploidea*. Their parapatric or allopatric species ranges replace each other along the BAF northeastern to southern Brazil, with the southernmost *P. iheringii* reaching the Uruguayan Pampas (Fig. 1). The tetraploid species is the only with an exclusively inland distribution in southern BAF, including areas in Argentina, Brazil and Paraguay. These treefrogs occur mainly in forested habitats of the BAF, breeding in ponds near the forest edge. The southern *P. iheringii* is an exception as it may occur and breed in the more open areas of the Pampas biome. Although the morphospecies occur mostly in non-overlapping distributions, areas of phenotypic intergradation between *P. bahiana* and *P. burmeisteri*, and one area of natural hybridization between *P. distincta* and *P. tetraploidea* were previously recognized (Pombal and Haddad, 1992; Haddad et al., 1994).

Phylogenetic relationships across the subfamily *Phyllomedusinae* based on several mitochondrial and slowly-evolving nuclear genes (exons) supported the monophyly of described *P. burmeisteri* morphospecies, but showing only moderate support to the sister-taxa relationship of *P. tetraploidea* with *P. distincta* (Faivovich et al., 2009). Because those results relied on a parsimony analysis of a concatenated dataset, including few representatives per species, were here undertake a rangewide more inclusive multilocus statistical phylogenetic analyses using both mitochondrial and more variable nuclear gene introns under a coalescent framework, to examine species diversification within the *P. burmeisteri* group, particularly the origin of *P. tetrapoidea*.

Speciation through polyploidization, once considered relatively rare in animals, has been reported for several vertebrates including

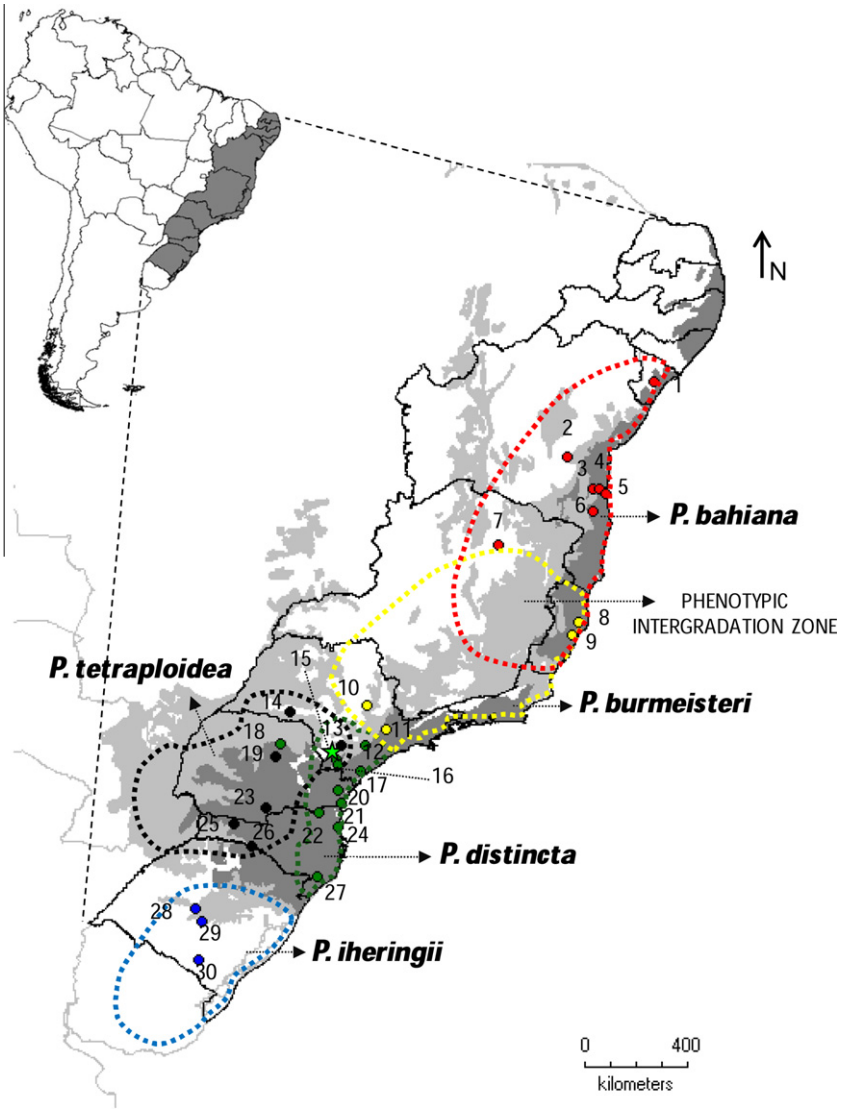

Fig. 1. Geographic distribution of *Phyllomedusa burmeisteri* species group with sampling localities numbered 1–30. Brazilian Atlantic Forest original cover: ombrophylous (dark gray) and semi-deciduous (light gray) forests are represented. The green star marks the reported hybridization between *Phyllomedusa distincta* and *Phyllomedusa tetraploidea*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anuran amphibians (see reviews in Otto and Whitton (2000), Mable (2004), Gregory and Mable (2005)). In most cases, vertebrate polyploid species were originated through autopolyploidization (e.g. Keller and Gerhardt, 2001), but with some rare examples of species that resulted from hybridization between distinct species followed by genome duplication, such as in *Xenopus* and *Bufo viridis* subgroup (Tymowska, 1991; Evans et al., 2004; Stöck et al., 2006, 2010). Haddad et al. (1994) suggested that *P. tetraploidea* resulted from recent autopolyploidy of *P. distincta*, taking into account their hybrid zone, the geographic distribution of the other species in the group, cytogenetic data and similar breeding vocalizations. This is noteworthy given that differences in ploidy are frequently accompanied by both distinct geographical distributions and advertisement calls (e.g. Stock, 1998; Martino and Sinsch, 2002).

Phylogenetic analyses are one of the most widely methods used to trace the evolutionary history of polyploid species. In most cases, mitochondrial DNA was the marker of choice (see several examples in Ptacek et al. (1994), Evans et al. (2004)), given its higher mutation rate and informativeness to examine diversification among closely related species (Pamilo and Nei, 1988; Hudson and Coyne, 2002). Recently, the additional use of nuclear loci increased (e.g. Evans et al., 2005; Stöck et al., 2010), cautioned by the fear that high levels of recombination could obscure phylogenetic signal (Osborn et al., 2003; Evans et al., 2005). Recombination should however not be problematic if it is low or absent, and the reconstruction of a coalescent-based species-tree combining nuclear and mitochondrial genes should overcome the putative source of inference errors of a single gene tree due to stochastic variation in the coalescent process of each gene (e.g. Bachtrog et al., 2006; Edwards et al., 2007).

Here we analyze mitochondrial and nuclear sequence variation from across the range of the *P. burmeisteri* species group to verify (i) the hypothesis that recognized morphospecies correspond to phylogenetic clades, (ii) the origin of the polyploid *P. tetraploidea*, and (iii) if range wide patterns of diversification in the group parallel those recently described for other BAF. To this purpose, we compared single gene trees with one multi-gene tree estimated using partitioned Bayesian analysis of concatenated datasets, and one species tree resulting from Bayesian inference under a coalescent framework.

2. Materials and methods

2.1. Population sampling and DNA extraction

In this study we obtained tissue samples from 72 field collected individuals of the *Phyllomedusa burmeisteri* group and one outgroup taxa (*Phyllomedusa boliviana*). We chose this species as outgroup based on published molecular data (Faivovich et al., 2009) (Appendix 1). We sampled all the five currently recognized species of the group in 30 georeferenced localities from all over the species range (Fig. 1, Table 1). We removed a portion of liver or muscle prior to specimen fixation in 10% formalin and storage in 100% ethanol. Vouchers are deposited in the Célio F.B. Haddad amphibian collection at Departamento de Zoologia, Universidade Estadual Paulista "Júlio de Mesquita Filho" (CFBH), Museu de Zoologia da Universidade de São Paulo (MZUSP), Miguel T. Rodrigues collection at Universidade de São Paulo (MTR), and Museu de Ciência e Tecnologia/Pontifícia Universidade Católica do Rio Grande do Sul (MCT/PUCRS).

2.2. DNA extraction, amplification, and sequencing

Tissue samples were digested in lysis buffer and Proteinase K. Purified whole genomic DNA was obtained using QIA Quick DNEasy columns (Qiagen, Inc.) according to the manufacturer's protocol. We sequenced the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and two nuclear genes, β-fibrinogen intron 7 (hereafter referred to as β-fibint7) and a segment of exon 2 and

Table 1
Locality information and sample sizes for 30 populations sampled across the range of the *Phyllomedusa burmeisteri* species group. State abbreviations: SE, Sergipe; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.

Code	Locality	State	Taxon	N	Longitude	Latitude
1	Areia Branca	SE	*P. bahiana*	1	−37.3153	−10.7578
2	Maracas	BA	*P. bahiana*	4	−40.4308	−13.4411
3	Aurelino Leal	BA	*P. bahiana*	4	−39.5395	−14.5734
4	Urucuca	BA	*P. bahiana*	5	−39.2844	−14.5931
5	Ilheus	BA	*P. bahiana*	1	−39.0636	−14.7822
6	Camacan	BA	*P. bahiana*	1	−39.5087	−15.4164
7	Grao Mogol	MG	*P. bahiana*	3	−42.9024	−16.5909
8	Linhares	ES	*P. burmeisteri*	1	−40.0722	−19.3911
9	Aracruz	ES	*P. burmeisteri*	1	−40.2733	−19.8203
10	Rio Claro	SP	*P. burmeisteri*	1	−47.6755	−22.3472
11	Jundiai	SP	*P. burmeisteri*	1	−46.9439	−23.2496
12	Pilar do Sul	SP	*P. distincta*	1	−47.7164	−23.8131
13	Buri	SP	*P. tetraploidea*	4	−48.5928	−23.7975
14	Assis	SP	*P. tetraploidea*	1	−50.3933	−22.5992
15	Ribeirao Branco	SP	*P. tetraploidea* (6) *P. distincta* (7)	13	−48.7430	−24.3586
16	Iporanga	SP	*P. distincta*	1	−48.7003	−24.5328
17	Pariquera-Acu	SP	*P. distincta*	1	−47.8811	−24.7150
18	Sao Jeronimo da Serra	PR	*P. distincta*	1	−50.7411	−23.7275
19	Ortigueira	PR	*P. tetraploidea*	1	−50.9494	−24.2083
20	Antonina	PR	*P. distincta*	1	−48.7119	−25.4286
21	Guaratuba	PR	*P. distincta*	3	−48.6324	−25.8694
22	Sao Bento do Sul	SC	*P. distincta*	1	−49.3786	−26.2503
23	Cruz Machado	PR	*P. tetraploidea*	4	−51.2583	−26.0784
24	Barra Velha	SC	*P. distincta*	3	−48.7657	−26.7222
25	Sao Domingos	SC	*P. tetraploidea*	1	−52.4550	−26.6314
26	Piratuba	SC	*P. tetraploidea*	2	−51.7719	−27.4197
27	Treviso	SC	*P. distincta*	5	−49.4575	−28.5156
28	Santa Maria	RS	*P. iheringii*	2	−53.8069	−29.6842
29	Sao Sepe	RS	*P. iheringii*	3	−53.5653	−30.1606
30	Candiota	RS	*P. iheringii*	1	−53.6725	−31.5581

intron 2 of the cellular myelocytomatosis (hereafter referred to as C-myc2). We obtained mtDNA sequences for 72 samples, and nuclear sequences for a representative subsample of those for phylogenetic inferences only (15 individuals for both β-fibint7 and C-myc2). 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). GenBank Accession Numbers:HQ262424–HQ262491.

ND2. Amplification and sequencing of the ND2 gene was accomplished using primers L4437 (Macey et al., 1997) and int_Halbo2 (Prado et al., submitted for publication; 5′-GTCTAATTTATCC TAAGTTTC-3′). PCR was performed in 25 μl volume using PuReTaq Ready-To-Go PCR Beads (GE Healthcare 2006) with ~50 ng of genomic DNA and 0.2 μM each primer. Amplification conditions consisted of a pre-denaturing step of 3 min at 92 °C, followed by 40 cycles of a denaturing step of 30 s at 92 °C, annealing at 50 °C for 30 s and extension at 72 °C for 90 s. The final extension was accomplished at 72 °C for 5 min.

β-fibint7. At a preliminary stage, amplification of the β-fibint7 gene was accomplished using primers FIB-B17U and FIB-B17L (Prychitko and Moore, 1997) following the same protocol and amplification conditions described by Godinho et al. (2005), but lowering the annealing temperature to 50 °C. However, because sequencing success was low with these set of primers, sequencing was accomplished using the primers BFXF and BFXR described in Sequeira et al. (2006). Finally, due to the lack of success of both primer combinations for *P. iheringii,* the definitive amplification of this gene was accomplished using primer FIB-B17L and the new internal primer BFIint (5′-ATGCATGCCCAGATGTGCAGTAG-3′), while sequencing was performed with BFXR and BFIint. Amplifications were performed under the same PCR conditions described above for ND2, in 25 μL volumes, containing 2 μL 10× reaction buffer (Promega), 2 mM MgCl2, 0.4 mM each dNTP, 0.2 μM each primer, 1 U of Go taq DNA polymerase (Promega) and ~100 ng of genomic DNA.

C-myc2. Amplification of C-myc2 was accomplished using primers cmyc1U (Crawford, 2003a) and cmyc3cat (5′-GTTGYTGCTG ATCTGTTTGAG-3′). The amplified fragments were sequenced using primer cmyc1U and the new internal primer cmycF (5′-ATAGGAACCTGTAGGACCAG-3′). Amplifications were performed with similar PCR conditions described above for β-fibint7, with the exceptions of annealing temperature (54 °C).

2.3. Sequence variation and genetic analyses

For nuclear sequences we produced two different alignments. First, polymorphic positions in β-fibint7 and C-myc2 were coded using the IUPAC ambiguity codes (nuclear dataset 1). Second, we used three approaches to resolve the haplotype phase of nuclear DNA sequences (nuclear dataset 2): (i) the method of Flot et al. (2006) for sequences that were heterozygous for insertions or deletions; (ii) cloning the PCR product for heterozygous samples of *P. tetraploidea*. The purified PCR product was ligated to a plasmid and inserted in *Escherichia coli* using the pGEM-T Easy Vector Cloning kit. Bacteria were grown in solid LB medium, with Ampicilin, X-gal and IPTG. The amplification of each positive clone insert was performed with the universal primers M13(-20) e M13(-26). We sequenced the positive clones with an automatic sequencer (ABI PRISM 3130 XL Genetic Analyser); and (iii) the Bayesian algorithm implemented in the PHASE software v. 2.1 (Stephens et al., 2001), using known phases of haplotypes determined by the previous method. This analysis was run multiple times (3) with different seeds for the random-number generator and checked if haplotype estimation was consistent across runs. Each run was conducted for 1.0×10^6 iterations with the default values. In both nuclear genes we detected multiple-base insertions or deletions (indels). For analyses we pruned the data assuming that indels likely resulted from a single evolutionary step. We left only the first base of the indel (in the case of insertion) or reduced them to one single step (deletion). We choose this approach rather than completely removing indels because this would significantly reduce the number of polymorphic sites and disregard some of the information contained in the data sets. After these procedures, pruned sequences were aligned manually using BIOEDIT v. 7.0.5.2 (Hall, 1999).

For both mtDNA and nuclear fragments (dataset 2), we calculated summary statistics in DNAsp v. 4.50 (Rozas et al., 2003). Haplotype sequence divergence (*p*-uncorrected distance) was estimated in MEGA v. 4.0.2 (Tamura et al., 2007). Although we were not able to resolve the haplotype phase for some nuclear DNA sequences of β-fibint7 (three individuals), we used them for calculating the following summary statistics: π (Nei, 1987), θ (Watterson, 1975) and number of segregating sites (S). To that purpose, each haplotype of unknown phase was randomly separated in two putative haplotypes on the basis of the ambiguity codes i.e., assigning the two alleles at each polymorphic site to one of two sequences (see Borge et al., 2005). In the case of β-fibint7, haplotype diversity and number of haplotypes were estimated removing all unresolved ambiguity sites. To evaluate the possibility of recombination we computed Hudson and Kaplan, 1985) R_m statistic (minimum number of recombination events) using DNAsp. Because this statistic is likely to be highly affected by homoplasy, we also used the software PHIPACK (http://www.mcb.mcgill.ca/~trevor) to test for recombination using the pairwise homoplasy index (Φ_w statistic) of Bruen et al. (2006). We used the two available options to estimate such P-values of PHIPACK: an analytical approach and a permutation test (1000 permutations).

2.4. Phylogenetic analysis

2.4.1. Gene trees

Phylogenetic analyses of the mitochondrial and nuclear sequence datasets (both nuclear dataset 1 and 2) were performed under Maximum Likelihood (ML), and Bayesian Inference (BI). For ML and BI analyses we first used a more traditional analysis, applying the best fit model of sequence evolution for each of the three genes here analysed. Following recent studies (e.g. Brandley et al., 2005; Wiens et al., 2010) we also performed a partitioning scheme based on intron and exon regions (C-myc2) and codon positions in the protein-coding ND2 mitochondrial gene, using an independent model for each partition. For C-myc2, analysis of codon position in the protein-coding region (exon) was not performed due extremely low variation. Bayes Factors (BF) were used to discriminate the most appropriate strategy. 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).

ML analyses were conducted using RAxML GUI (Silvestro and Michalak, 2010), a graphical front-end for RAxML-VI-HPC (Randomized Axelerated Maximum Likelihood; Stamatakis, 2006). ML with the thorough bootstrap option was run ten times from starting random seeds to generate 1000 nonparametric bootstrap replicates. All ML analyses used the general time-reversible (GTR) with gamma model of rate heterogeneity. Bayesian analyses were performed in MrBayes version 3.0b4 (Ronquist and Heulsenbeck, 2003) using two replicate searches with 20×10^6 generations each, sampling every 1000 generations. Four MCMC (Markov chain Monte Carlo) were run simultaneously in each analysis. For the analysis of ND2 by codon position, the nucleotide frequencies were considered fixed for the first and the second positions, and we used

the Dirichlet process model for the third position (see Bofkin and Goldman (2007)). We employed three strategies for MCMC convergence diagnostics. First, we assessed the convergence of the chains by plotting the log-likelihood values *versus* generation number using the program Tracer v.1.5 (Rambaut and Drummond, 2007). Second, we used the on-line AWTY program (Nylander et al., 2008) to analyse the trace plot of the log-likelihood and the cumulative split frequencies across all post-burn-in generations within each analysis. Third, we checked the standard deviation of split frequencies as a convergence index (<0.001). After chain convergence analysis, we discarded all samples obtained during the first 4 million generations as burn-in. Post burn-in trees from all replicates were combined, and a 50% majority-rule consensus tree was estimated. The frequency of any particular clade in the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001).

2.4.2. Species trees

We used two different methods for species-tree reconstruction: a more traditional partitioned Bayesian analysis, combining mtDNA *ND2* with nuclear gene alignments into a single data matrix, consisting of 15 alleles of the 5 ingroup taxa; and the coalescent-based Bayesian species tree inference method implemented in the software *BEAST (Bayesian Inference of Species Trees from Multilocus Data; Heled and Drummond, 2010).

For the combined analysis (concatenated dataset), we used the nuclear dataset1 (unphased) together with a reduced mtDNA dataset (same individuals as analysed for nuclear data). The largest possible number of partitions (C-myc2 exon, C-myc2 intron, β-fibint7, and ND2 codon positions) was used as the partitioning strategy (see Brandley et al. (2005)) (data not shown). Because gene flow can bias the analysis accuracy, we used only representatives from outside of the described hybrid zone between *P. tetraploidea* and *P. distincta*. ML and BI analyses were conducted as described above for individual gene trees, with the exception of the option "per-partition brl" that was used in this case for ML analysis in the software RAxML.

The software *BEAST (an extension of BEAST v. 1.5.4; Drummond and Rambaut, 2007) also implements a Bayesian MCMC analysis, and is able to co-estimate species trees and gene trees simultaneously (Heled and Drummond, 2010). The input file was properly formatted with the BEAUti utility included in the software package, using the same partition scheme of the concatenated analysis. We unlinked substitution model parameters for each partition and specified a relaxed clock with an uncorrelated lognormal distribution (Drummond et al., 2006), and a speciation Yule process as tree prior were used. We also used this program to infer the time to the most recent common ancestor (^{t}MRCA) and their credibility intervals (95%) for nodes of interest. It is important to note that in the absence of accurate calibration points from external and independent data (e.g. dated fossil records, known biogeographic events, or paleoclimatic reconstructions), or due to the heterogeneity rate of evolution between the calibrated and uncalibrated taxa, temporal estimates by means of molecular data could be a potential source of inference error, and, therefore, they should be treated with caution (e.g. Heads, 2005). In this study, we used the ND2 mutation rate (0.00957 mutations/site/million years) based on a calibration for the Neotropical frog *Eleutherodactylus* (Anura: Leptodactylidae; Crawford (2003b). This calibration has already been applied in some studies of Neotropical amphibians producing congruent results (e.g. Carnaval and Bates, 2007; Thomé et al., 2010). It has also been shown that mutation rate estimates of amphibian protein-coding mtDNA vary only slightly depending on the marker and group of species (Macey et al., 1997; Crawford, 2003b). Despite the limitations mentioned above, we believe divergence time estimates are still valuable to provide a proxy for the temporal window of evolutionary diversification in our species group of interest. We performed two independent runs for 150 million generations, sampling every 15,000 generations, from which 10% were discarded as burn-in. To check for convergence we used the program Tracer v1.4 (Rambaut and Drummond, 2007). The results were obtained in the TreeAnnotator v1.4.8 (Drummond and Rambaut, 2007; http://beast.bio.edu.ac.uk) and visualized in FigTree1.1.2 (Rambaut, 2008).

3. Results

3.1. Genetic variation and phylogenetic analyses

3.1.1. Mitochondrial DNA

A fragment of 993 base pairs of the ND2 gene was obtained from 72 individuals of the *P. burmeisteri* group and from one exemplar of *P. boliviana* (outgroup). The ingroup alignment revealed 35 haplotypes defined by 208 polymorphic sites, of which 192 were parsimony informative (Table 2). The alignment required a gap with 3 bp- long present in one individual of *P. iheringii* in the position 931–933, which translates the Serine amino acid. Analysis with a distinct evolutionary model for each codon position was preferred (2lnBF = 245) following Kass and Raftery (1995). The Bayesian ND2 tree showed basically unresolved relationships among species of the *P. burmeisteri* group (Fig. 2). Five main well-supported clades correspond to the currently recognized morphospecies of the group: *P. bahiana*, *P. burmeisteri*, *P. distincta*, *P. tetraploidea*, and *P. iheringii*. Three of these species are monophyletic, while *P. distincta* and *P. tetraploidea* form a paraphyletic clade. Five haplotypes of *P. distincta* sampled in the hybrid zone between *P. tetraploidea* and *P. distincta* are grouped together with *P. tetraploidea* haplotypes, whereas one haplotype of *P. tetraploidea*

Table 2
Summary statistics, recombination tests and genetic distances (*p*-uncorrected) within the main mitochondrial clades.

Fragment	Clade	Length	Polymorphism						Distance	Recombination	
			N	*S*	*h*	% Hd	% *n*	% θ	% *p-uncorrected*	R_m	Φ_w
ND2	All	993	72	208	35	96	6.2	4.3		–	–
	P. bahiana		19	28	9	87	0.9	0.8	0.88(0.10–2.12)		
	P. burmeisteri		4	33	4	100	1.8	1.8	1.83(0.61–2.22)		
	P. distincta		20	17	7	75	0.8	0.5	0.78(0.10–1.51)		
	P. tetraploidea		23	17	11	92	0.5	0.5	0.50(0.10–1.21)		
	P. iheringii		6	19	4	80	0.7	0.6	0.69(0.10–1.30)		
P-fibint7	All	600–604	36	39	18	97	1.7	1.5		4	0.09 (0.07)
	Northern group		12	20	9	100	1.3	1.3			
	Southern group		24	15	9	92	0.8	0.7			
C-myc2	All	449–454	36	31	19	96	1.4	1.6		1	0.60 (0.32)
	Northern group		12	13	10	98	0.7	0.9			
	Southern group		24	12	9	93	0.6	0.7			

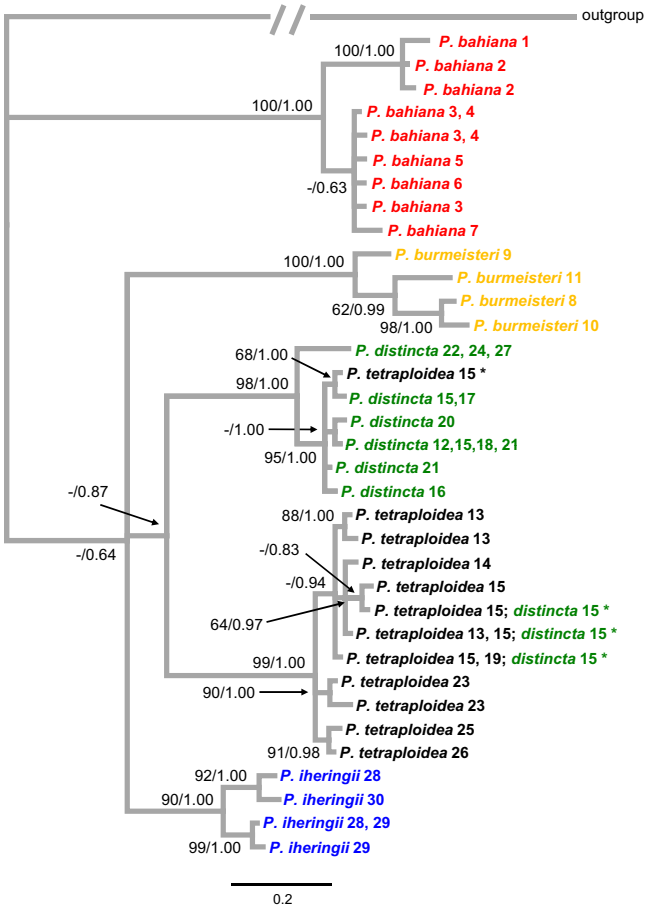

Fig. 2. Tree derived from Bayesian analysis of 993 bp of the mitochondrial ND2 sequences in the *P. burmeisteri* species group. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–". Haplotype from hybrid zone are represent by "*".

sampled in the same area is placed within *P. distincta* clade. All clades showed highly supported sub-clades but only in the case of *P. bahiana* and *P. distincta* a coherent geographic distribution is apparent (Figs. 1 and 2). ML method yielded an identical topology (not shown).

3.1.2. Nuclear DNA

We obtained 36 sequences from 15 samples of the *P. burmeisteri* group. For the nuclear C-myc2, the analysis with a distinct evolutionary model for each partition (exon and intron) was preferred (2lnBF = 7.4) following Kass and Raftery (1995). We found 22 out of 31 polymorphic sites in the ingroup alignment (449–454 bp long) that were parsimony informative. This alignment included four indels 1 bp long. C-myc2 sequences presented one recombinant event according to Hudson and Kaplan, 1985 statistics, but the evidence of recombination was not significant according to the PHI test, both using the analytical approach ($P = 0.60$) or the permutation test ($P = 0.32$) (Table 2). For Bayesian inference, we used the K80 substitution model for the partial exon 2 fragment (134 bp), and TPM2uf substitution model for partial intron 2 (314–320pb) fragment. The Bayesian trees resulting from analysis of datasets 2 and 1 are depicted in Fig. 3A and in Appendix 2A, respectively. ML analyses yielded similar topologies for both datasets (not shown).

For the β-fibint7 nuclear fragment, we obtained 36 sequences from 15 samples of the *P. burmeisteri* group. In the ingroup alignment (600–604 bp long) we found 39 polymorphic sites, of which 36 were parsimony informative (Table 2). The β-fibint7 fragment alignment also included two indels 1 bp long. β-fibint7 sequences presented four recombinant events according to the Hudson and Kaplan, 1985 statistics, but the evidence of recombination was not significant according to the PHI test, both using the analytical approach ($P = 0.09$) or the permutation test ($P = 0.07$). The Bayesian trees resulting from analysis of datasets 2 and 1 (Fig. 3B and Appendix 2B, respectively) were similar to ML trees for the same datasets (not shown).

All analyzes were congruent in showing one well-supported polyphyletic clade, that groups the more southern species *P. tetraploidea*, *P. distincta*, and *P. iheringii*, and another moderately supported clade grouping some sequences of the northern distributed *P. bahiana* and *P. burmeisteri*. Some sequences of *P. bahiana* and *P. burmeisteri* appeared more basally in the trees.

3.1.3. Species tree

The two multi-gene species tree methods (combined and *BEAST) recovered a similar topology, unveiling three well-supported monophyletic clades: one corresponding to the more northern distributed species *P. bahiana* and *P. burmeisteri* and the other grouping the southern distributed species (*P. tetraploidea, P. distincta* and *P. iheringii*). Within this group the species *P. tetraploidea* and *P. distincta* form a well-supported clade (Fig. 4).

3.2. Genetic distances and divergence time estimates

The mitochondrial average genetic distance (*p*-uncorrected) obtained within the main clades was 0.88% for *P. bahiana*; 1.83% for *P. burmeisteri*; 0.5% for *P. tetraploidea*; 0.78 for *P. distincta* and 0.69% for *P. iheringii* (Table 2). Between the main clades, *p*-uncorrected ranged from 5.3% to 10.2%. The highest divergence was found between *P. bahiana* and all other extant clades (≈10%), while the lowest values were obtained between *P. tetraploidea*, *P. distincta*, and *P. iheringii* (≈5%) (Table 3).

Nuclear sequence divergence ranged from ≈2% to 0.2% in C-myc2, while in β-fibint7 it ranged ≈1.4% to 0.3%. For both nuclear genes the highest divergence was between the northern species (*P. bahiana* and *P. burmeisteri*) and the southern species (≈2–1.5%) while the lowest values were obtained within the southern species group (*P. tetraploidea*, *P. distincta*, and *P. iheringii*; ≈0.2–0.6%) (Table 3).

Bayesian coalescent estimates for the time of the most recent common ancestor (^{t}MRCA) were: the main south–north split within *P. burmeisteri* started at around 5 Myr, *P. iheringii* diverged from the other southern species at around 2.5 Myr, *P. burmeisteri* from *P. bahiana at* 1.6 Myr, while the split between *P. distincta* and *P. tetraploidea* occurred around 0.4 Myr (see 95% confidence intervals in Table 4).

4. Discussion

We used mitochondrial and nuclear sequence variation to infer range wide processes of biological diversification within the *P. burmeisteri* species group. Mitochondrial and nuclear DNA phylogenies were not completely concordant. The first showed that three of the five recognized morphospecies (*P. burmeisteri*, *P. bahiana* and *P. iheringii*) corresponded to monophyletic clades, while *P. tetraploidea* and *P. distincta* were paraphyletic. Nuclear analysis sup-

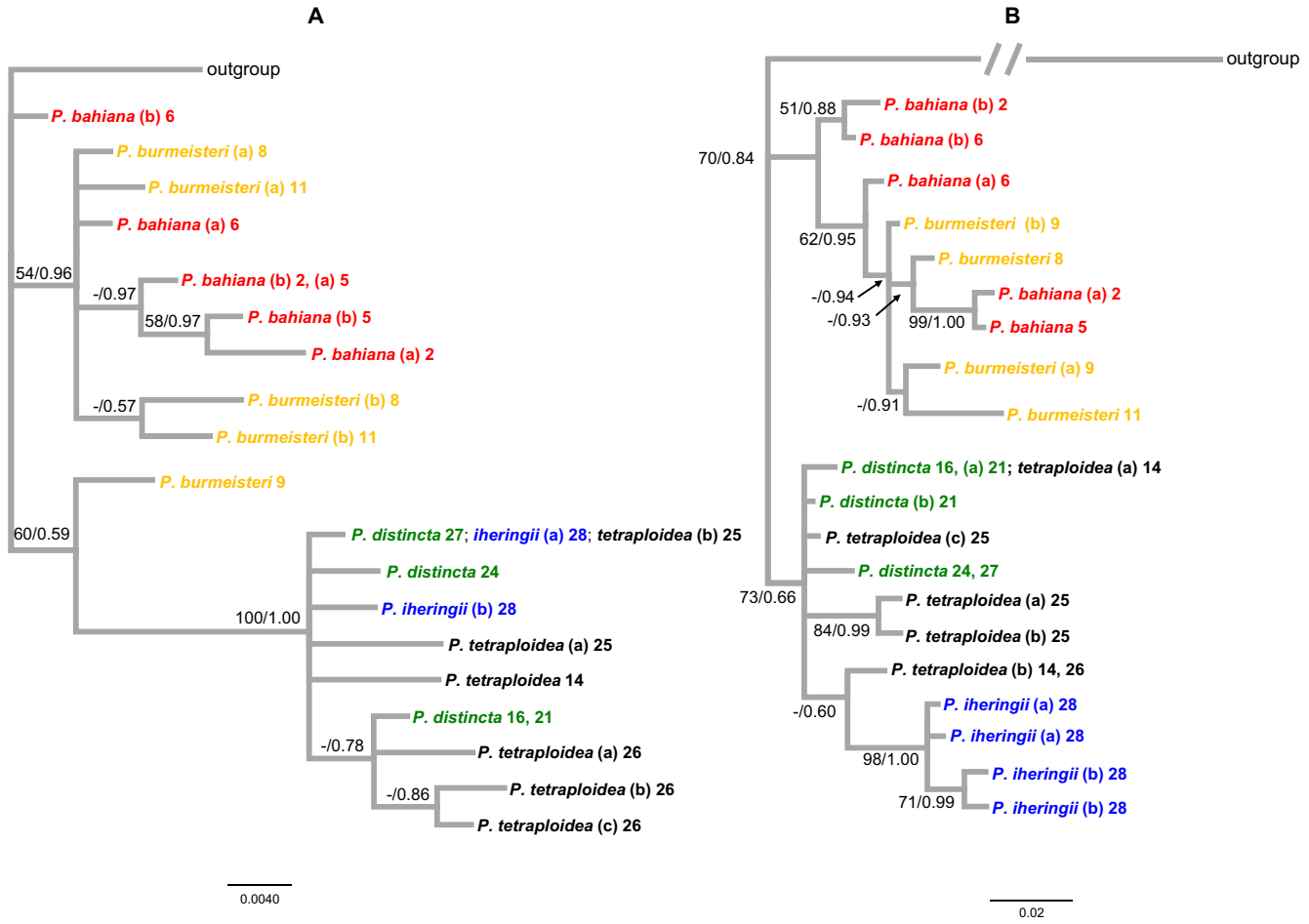

Fig. 3. Tree derived from Bayesian analysis of (data set 2) 454 bp of the C-myc2 (A) and 604 bp of the β-fibint7 (B) nuclear sequences in the *Phyllomedusa burmeisteri* species group. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–". Letters a, b and c represent different haplotypes found in heterozygote individuals.

ported a northern group composed of *P. bahiana* and *P. burmeisteri*, and a southern group with unresolved relationships between *P. distincta, P. iheringii* and *P. tetraploidea.* The two inferred species trees were basically concordant, giving high support for northern and southern groups, also supporting *P. distincta* and *P. tetraploidea* as sister-taxa. Our results support the hypothesis that *P. tetraploidea* is an autopoliploid resulting from the lineage of *P. distincta* (Haddad et al., 1994); temporal and geographical transitions between species are concordant with phylogeographic breaks uncovered for BAF organisms and with recognized herpetological biogeographic borders within the BAF.

4.1. Phylogenetic analysis

4.1.1. Gene trees

Phylogenetic analyses of mtDNA *ND2* showed five main clades with high support but with their relationships unresolved. The currently recognized morphospecies *P. bahiana*, *P. burmeisteri*, and *P. iheringii* represent exclusive monophyletic lineages, while *P. tetraploidea* and *P. distincta* are paraphyletic. Haplotypes of five *P. distincta* were placed in a clade that includes all haplotypes of *P. tetraploidea* but one that was placed in the clade with the remaining of *P. distinta* (Fig. 2). Paraphyly of closely related species can result from incomplete lineage sorting associated to recent divergence or hybridization (e.g. Funk and Omland, 2003; Machado and Hey, 2003). In the case of *P. tetraploidea* and *P. distincta*, previous work using morphological and cytogenetic data reported a limited zone of hybridization between the two species (Haddad et al., 1994). All individuals of *P. distincta* with haplotypes of the *P. tetraploidea* clade and vice versa were sampled in the hybrid sites described by Haddad et al. (1994) and are triploid hybrids according to cytogenetic analysis (S. Kasahara and C.F.B. Haddad, unpublished data). Samples of *P. distincta* and *P. tetraploidea* outside of this hybrid zone show exclusive haplotypes from two well-supported diverged clades.

Nuclear C-myc2 and β-fibint7 gene tree topologies were generally similar and contrasted with that of mtDNA (Figs. 2 and 3). The tree for C-myc2 showed high support for a clade grouping *P. tetraploidea*, *P. distincta*, and *P. iheringii* and a clade including most of the *P. burmeisteri* and *P. bahiana* haplotypes, but the position of four haplotypes of these two species was uncertain (Fig. 3a). The *β-fibint7* tree showed two exclusive clades with moderate support, one including *P. burmeisteri* and *P. bahiana*, the other including *P. tetraploidea*, *P. distincta*, and *P. iheringii* (Fig. 3b). Nuclear genes therefore showed a subdivision between a northern (*P. bahiana* and *P. burmeisteri*) and southern (*P. tetraploidea*, *P. distincta*, and *P. iheringii*) lineages. The two nuclear genes revealed less power than mtDNA for resolving species-level phylogenetic relationships in the *P. burmeisteri* group. This type of cytonuclear nonconcordance was described in many other cases of closely related species

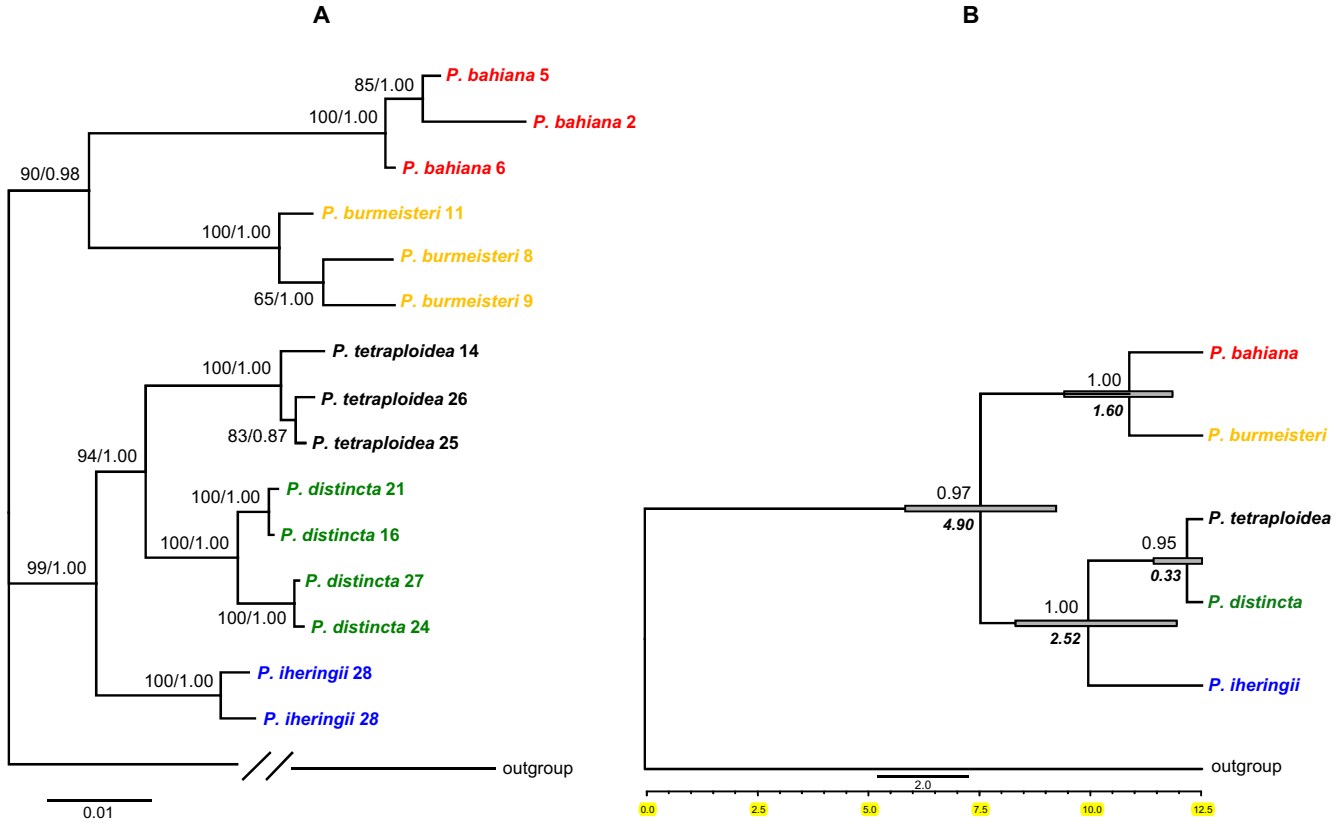

Fig. 4. Species tree from the combined mitochondrial and nuclear DNA data: (A) partitioned Bayesian analysis. Bootstrap values for ML and Bayesian posterior probabilities are given near the branches, respectively. (B) Bayesian Inference of Species Trees (*BEAST). Clade posterior probabilities are shown above branches and the mean times to the most recent common ancestor (†MRCA) below branches. The node gray bars represent the 95% confidence intervals (see also Table 4).

Table 3
Average sequence divergence (*p*-uncorrected %) between species of the *Phyllomedusa burmeisteri* group for both mitochondrial and nuclear genes.

Clades	*P. bahiana*	*P. burmeisteri*	*P. distincta*	*P. tetraploidea*	*P. iheringii*
P. bahiana	–	0.39 (0.35)	1.95 (0.84)	2 (0.75)	1.7 (1.1)
P. burmeisteri	10.1	–	1.87 (1.2)	1.93 (1.1)	1.7 (1.45)
P. distincta	10.2	7.5	–	0.33 (0.26)	0.17 (0.61)
P. tetraploidea	9.8	7.8	5.2	–	0.23 (0.35)
P. iheringii	9.4	7.5	5.3	5.4	–

Table 4
Mean time to the most common ancestors (†MRCA; 95% confidence intervals in parentheses) corresponding to the nodes of the multilocus coalescent species tree.

*BEAST	MRCA (Myr)
All	5 (3.28–6.63)
North group	1.6 (0.65–3.05)
South group	2.5 (0.55–4.12)
Pdi + Pte	0.4 (0–1.06)

(e.g. Broughton and Harrison, 2003; Machado and Hey, 2003; Dolman and Moritz, 2006; Gonçalves et al., 2007; Pinho et al., 2008; Kotaki et al., 2008) and is not unexpected given that the higher effective population sizes and lower mutation rates of nuclear loci (Slade et al., 1994; Moriyama and Powell, 1997; Johnson and Clayton, 2000) result in coalescent processes acting slower to produce complete lineage sorting (Edwards and Beerli, 2000). While monophyly of morphospecies is clearly supported by mtDNA (when 3n hybrids between *P. distincta* and *P. tetraploidea* are excluded), the retention of ancestral polymorphisms could explain the lack of monophyly at nuclear gene trees for the five species of the *P. burmeisteri* group.

Gene flow and introgression between closely related lineages/species may also cause incongruence between cytoplasmic and nuclear gene genealogies (e.g. Maddison, 1997; Nichols, 2001; Seehausen, 2004; Mallet, 2008). A hybrid zone between *P. tetraploidea* and *P. distincta* was documented previously using morphological and cytogenetic data (Haddad et al., 1994) and was now confirmed by an mtDNA dataset (present study). It is however difficult to distinguish ancestral polymorphism from gene flow in causing allele sharing in closely related species (revised in Arbogast et al. (2002); see also Charlesworth et al. (2005), Bull et al. (2006), Pinho et al., 2008). Concerning nuclear allele sharing between *P. tetraploidea*, *P. distincta*, and *P. iheringii*, the hypothesis of retained polymorphism is favoured by (i) the fact that hybrids between *P. tetraploidea* are *P. distincta* may be sterile or barely fertile (ii) *P. iheringii* is allopatric relative to the other two species (Haddad et al., 1994). The morphospecies *P. bahiana* and *P. burmeisteri* are separated by a hybrid zone with morphologically intermediate individuals (Pombal and Haddad, 1992). Introgression was not apparent in the mtDNA genealogy

which could favour the hypothesis of nuclear gene ancestral polymorphism to explain incomplete lineage sorting. The broad-scale nature of the present study (i.e., small sampling of populations and loci) precludes a more detailed exam of alternative hypotheses to explain the dissimilarities between nuclear and cytoplasmic markers.

4.1.2. Species-trees inference combining mitochondrial and nuclear markers

Gene trees may hide the phylogenetic signal of distinct evolutionary units because of retained ancestral polymorphism between species (Maddison, 1997; Belfiore et al., 2008), which may apply to the nuclear gene data in treefrogs of the *P. burmeisteri* group. Assuming that gene flow did not largely confound true species relationships (see Section 2), we reconstructed species trees under a Bayesian coalescent framework and also using the more traditional method of data concatenation. Although concatenation has been used for inference of multilocus datasets (e.g. Brandley et al., 2005; Bossu and Near, 2009), recent studies have shown that it may be inconsistent (e.g. Liu and Edwards, 2009) and less accurate in recovering species tree topology when compared to Bayesian coalescent-based species tree methods (Heled and Drummond, 2010). In the present study, phylogenies resulting from both approaches produced basically similar topologies and posterior probability estimates for species relationships. When compared with gene trees, the species trees give higher support for the northern (*P. bahiana* and *P. burmeisteri*) and southern (*P. tetraploidea*, *P. distincta*, and *P. iheringii*) groups as exclusive evolutionary units. Species trees additionally provided high support for the sister-taxa relationship between *P. tetraploidea* and *P. distincta*, which had low support in the mtDNA gene tree and was not at all apparent in any of the nuclear gene trees topologies.

Overall, phylogenetic relationships for the *P. burmeisteri* species group now recovered are roughly similar to those found by Faivovich et al. (2009). Specifically, the results from our much expanded dataset support the morphospecies *P. burmeisteri, P. bahiana* and *P. iheringii* as monophyletic clades, and support a close relationship between the southernmost species, in particular between the sister-taxa *P. distincta* and *P. tetraploidea*. The major distinction between northern and southern groups of *P. burmeisteri* species was not recovered by previous phylogenetic work but is in agreement with breeding call similarities within each group (single and regular *versus* paired and regular pulse rates, in northern and southern groups, respectively; see Pombal and Haddad (1992)). The present study also offers stronger support for the *distincta-tetraploidea* sister relationship, which was only moderately supported (parsimony bootstrap value = 88) in the phylogenetic analysis of Faivovich et al. (2009). This lends additional support for the hypothesis suggesting an origin of *P. tetraploidea* from *P. distincta* (Haddad et al., 1994).

4.2. The origin of P. tetraploidea

The species *P. tetraploidea* was previously suggested to result from recent autopolyploidization of *P. distincta* on the basis of undergoing hybridization between species, similar mating calls and chromosomic homologies of metaphase I trivalents in triploid hybrids (Haddad et al., 1994). Multivalents could however reflect not the complete homology of genomes, but close relatedness between genomes that are still able to pair (King, 1990). Cytologic analysis could also fail in revealing an autopolyploid origin when multisomic inheritance is lost as expected after a certain time with the re-establishment of dissomic inheritance (Dewet, 1980). Following the hypothesis of an autopolyploid origin of *P. tetraploidea* from *P. distincta*, it would be expected that both nuclear and mtDNA alleles of the tetraploid species cluster with *P. distincta*. However, sequence divergence (Table 3) between any pair of southern (*P. iheringii*, *P. tetraploidea* and *P. distincta*) species is actually very similar for both mtDNA (≈5%) and nuclear data (≈0.2–0.6), and with sharing of nuclear alleles. We cannot therefore exclude the hypothesis that *P. tetraploidea* resulted from hybridyzation between *P. iheringii* and *P. distincta* followed by genome duplication (allopolyploidy). Given the high bootstrap and posterior probabilities in both concatenated and coalescent-based Bayesian species-trees supporting *P. distincta* as sister taxa of *P. tetraploidea*, it is however likely that *P. tetraploidea* resulted from genome duplication of *P. distincta*.

Considering the estimates for time of divergence between *P. tetraploidea* and *P. distincta* at about ≈0.3 million years ago, and between those and their close relative *P. iheringii* at 2.5 million years ago (Fig. 4), it is likely that sharing of nuclear alleles between *P. tetraploidea*, *P. distincta*, and *P. iheringii* was due to incomplete lineage sorting (see above). On the contrary, the high level of mtDNA sequence divergence among those three species may result from the lower effective population size and higher mutation rate of ND2 relative to nuclear loci, leading to much faster sorting of ancestral polymorphisms. This would also explain the lack of resolution to infer phylogenetic relationships among these three species. Because gene trees will not necessarily converge onto a similar species-tree due to stochastic variation in the coalescent process (e.g. Maddison, 1997; Bachtrog et al., 2006), the present study exemplifies the need to use multiple loci for phylogenetic analysis of closely related species.

Given our multilocus inference and the evidence provided by Haddad et al. (1994), the most likely hypothesis to explain the origin of tetraploid species should be (i) autopolyploidization from *P. distincta* or, (ii) autopolyploidization from an unknown independent lineage that shares a common ancestor with *P. distincta* (e.g. Mable and Roberts, 1997; Stöck et al., 2006). In the latter case, replacement of the original 2n lineage by *P. tetraploidea* would not be surprising given that tetraploids may be more tolerant to different ecological conditions (for reviews, see Otto and Whitton (2000), Otto (2007)). Indeed, the geographic distribution range of *P. tetraploidea* includes regions with more pronounced seasonality compared to typical BAF rainforest areas occupied by the other species in the group, with exception of *P. iheringii* (Pombal and Haddad, 1992; Haddad et al., 1994). In the future, a genetic approach based on quantitative analyses of marker alleles transmitted in the progeny of laboratory controlled crosses could be particularly useful to better elucidate the origin of *P. tetraploidea* (see for example Chenuil et al. (1999)).

4.3. Patterns of diversification in burmeisteri species group

MtDNA *ND2* genetic diversity across the range was strikingly high with five well-supported clades showing pairwise divergences ranging from 5–10%. Further genetic substructuring in *P. burmeisteri* species group was suggested by well-supported subclades within major clades, but should be confirmed with increased sampling. The divergence between northern and southern clades started around 5 Myr. This time estimate (^{t}MRCA) roughly coincides with that of other BAF widespread organisms (*Bothrops jararaca*, Grazziotin et al., 2006; *Rhinella crucifer*, Thomé et al., 2010). *P. distincta*, *P. tetraploidea* and *P. iheringii* may have diverged at around 2.5 Myr, while species in the northern group may have diverged more recently (≈1.6 Myr; Table 4). These levels of divergence are compatible with those for lineages of *Hypsiboas bishoffi* (2.4 Myr; J; Alexandrino, unpublished data). Diversification in these organisms should therefore be explained in the context of historical population events that occurred since the Late Tertiary (Pliocene) and throughout the Pleistocene.

Recent analyses combining phylogeographic data and paleodistribution modeling supported that endemism and genetic diversity in BAF is associated with range stability since the Last Glacial Maximum (LGM; Carnaval and Moritz, 2008; Carnaval et al., 2009). Two major stability areas (refugia) were identified: the Pernambuco and the Bahia refugia. The region south of São Paulo may have been unstable since the LGM and would have been recently recolonized from adjacent northern refugia (Carnaval et al., 2009), explaining the comparatively lower genetic diversity of two anurans observed south of the refugia areas (*H. albomarginatus*, *H. semilineatus*). Genetic diversity patterns not compatible with recent colonization have however been also recently described for reptiles, birds and other amphibians (Grazziotin et al., 2006; Cabanne et al., 2008; Fitzpatrick et al., 2009; Thomé et al., 2010; J. Alexandrino, unpublished data). In the *P. burmeisteri* group, fragmentation in northern (between mtDNA haplotypes of *P. bahiana*) and central BAF (between haplotypes of *P. burmeisteri*) would be compatible with long-term regional persistence of populations. In southern BAF, we found high genetic diversity that corresponds to divergence between *P. distincta* and *P. tetraploidea*. Because their geographic distributions both include areas in southern São Paulo, it cannot be ruled out that there were parallel southward colonizations from refugia in that region (e.g. Carnaval et al., 2009). The additional structure in southern BAF apparent from the mtDNA gene tree (haplotypes in localities 22, 24, 27 and 23, 25 and 26, respectively, in *P. distincta* and *P. tetraploidea*) may suggest instead longer term population persistence, but this must be confirmed with more detailed sampling.

Independent BAF palinological data suggest that forest fluctuations occurred during the Pleistocene. While the studies made in the inland region showed good support for forest fragmentation (Ledru, 1993; Ledru et al., 1996; Behling and Lichte, 1997; Behling, 2002; Gouveia et al., 2002), data on the coastal region indicated always the presence of forest in the LGM (Saia et al., 2008; Ledru et al., 2009). This scenario of fragmentation could have impacted the diversification of the southern *P. distincta* and *P. iheringii*, as much as genetic diversity within *P. distincta* and *P. tetraploidea*, but this can only be properly examined with further studies including detailed sampling. *P. iheringii* is an ecological outlier within the treefrog group as it occurs in a southern drier area, well within the Pampas biome, and cannot be associated to the forest dynamics of the BAF. We speculate that diversification of *P. iheringii* may be associated to genetic divergence accompanied by ecological differentiation.

Phylogeographic concordance in BAF was revealed with breaks separating major *P. burmeisteri* group clades, northern (*P. burmeisteri* and *P. bahiana*) and southern (*P. tetraploidea, P. distincta*, and *P. iheringii*), coinciding with those separating major clades of the treefrogs *H. faber* (Carnaval et al., 2009), in the whole of BAF, *H. bishoffi* (J. Alexandrino, unpublished data), *Rhinella crucifer*

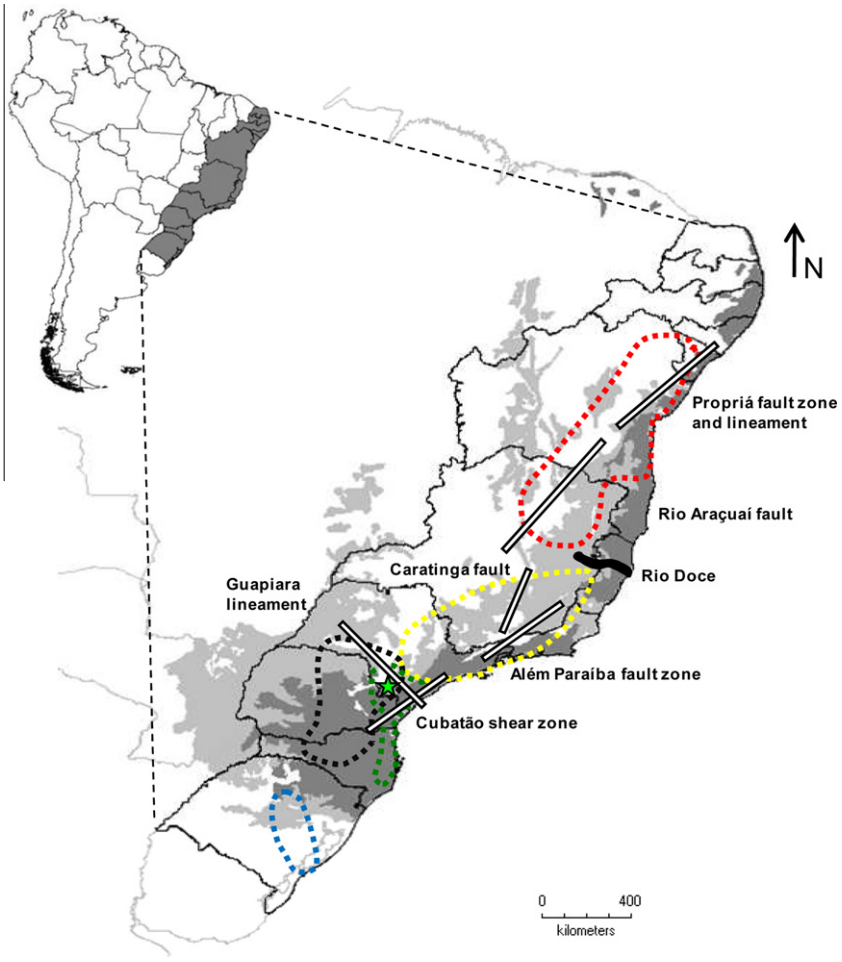

Fig. 5. Location of putative geographic barriers in BAF *versus* mtDNA clades of *Phyllomedusa burmeisteri* species group. Brazilian Atlantic Forest original cover, ombrophylous (dark gray) and semi-deciduous (light gray) are represented. Clade colours correspond to Fig 1 and 2. The green star marks the reported hybridization between *Phyllomedusa distincta* and *Phyllomedusa tetraploidea*.

(Thomé et al., 2010), and the snake *B. jararaca* (Grazziotin et al., 2006), in southern São Paulo. Similar phylogeographic breaks were also revealed for BAF birds (Cabanne et al., 2008; and the spatial pattern is also coincident with patterns of endemism for BAF amphibians and mammals (Lynch, 1979; Costa et al., 2000). Coincident spatial patterns of genetic and species diversity suggests common processes acting to promote biological diversification.

Geomorphological events since the Tertiary to the present day may have impacted the diversification process of BAF organisms, such as the remake of parts of the Brazilian east coast due to older tectonic and more recent neotectonic phenomena (e.g. Ribeiro, 2006; Saadi et al., 2002). The main break in the *P. burmeisteri* group (*P. burmeisteri*/*P. bahiana* and *P. tetraploidea*/*P. distincta*/*P. iheringii*) is coincident with spatial movements of the Guapiara lineament and the Cubatão shear zone, with this one being also associated to more recent additional structure in *P. distincta* (Fig. 5; Saadi et al., 2002). The Doce River system is coincident with break between *P. burmeisteri* and *P. bahiana*. This complex and dynamic geological formation may have been a barrier for other BAF organisms (Pellegrino et al., 2005; Saadi et al., 2005; Sigrist and Carvalho, 2008). The Propriá and Rio Araçuaí fault zone or Caratinga and Além Paraíba fault zone can acted together in promoting the diversification of *P. bahiana* and *P. burmeisteri* and also of a well-supported sub-clade of *P. bahiana* (Figs. 2 and 5). *P. burmeisteri* and *P. bahiana* have however an area of phenotypic intergradation that will have to be further studied at the population level to examine the spatial diversification process and species limits.

Our results suggest that both the Tertiary and Quaternary were important for the diversification of species of the *P. burmeisteri* group which is in agreement with other molecular studies in BAF organisms (e.g. Lara and Patton, 2000; Ribas and Miyaki, 2004; Wüster et al., 2005; Grazziotin et al., 2006). While a pattern of association of phylogeographic breaks to river/tectonic barriers is emerging in BAF, the challenge will be to undertake studies designed to distinguish between the alternative, but non-exclusive, forest refugia and barrier hypotheses to explain diversification patterns of BAF organisms.

Acknowledgments

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Appendix 1

Population number (code), taxon, voucher (see text for institution information) and mitochondrial haplotypes of *Phyllomedusa burmeisteri* species group. "*", tissue samples without vouchers.

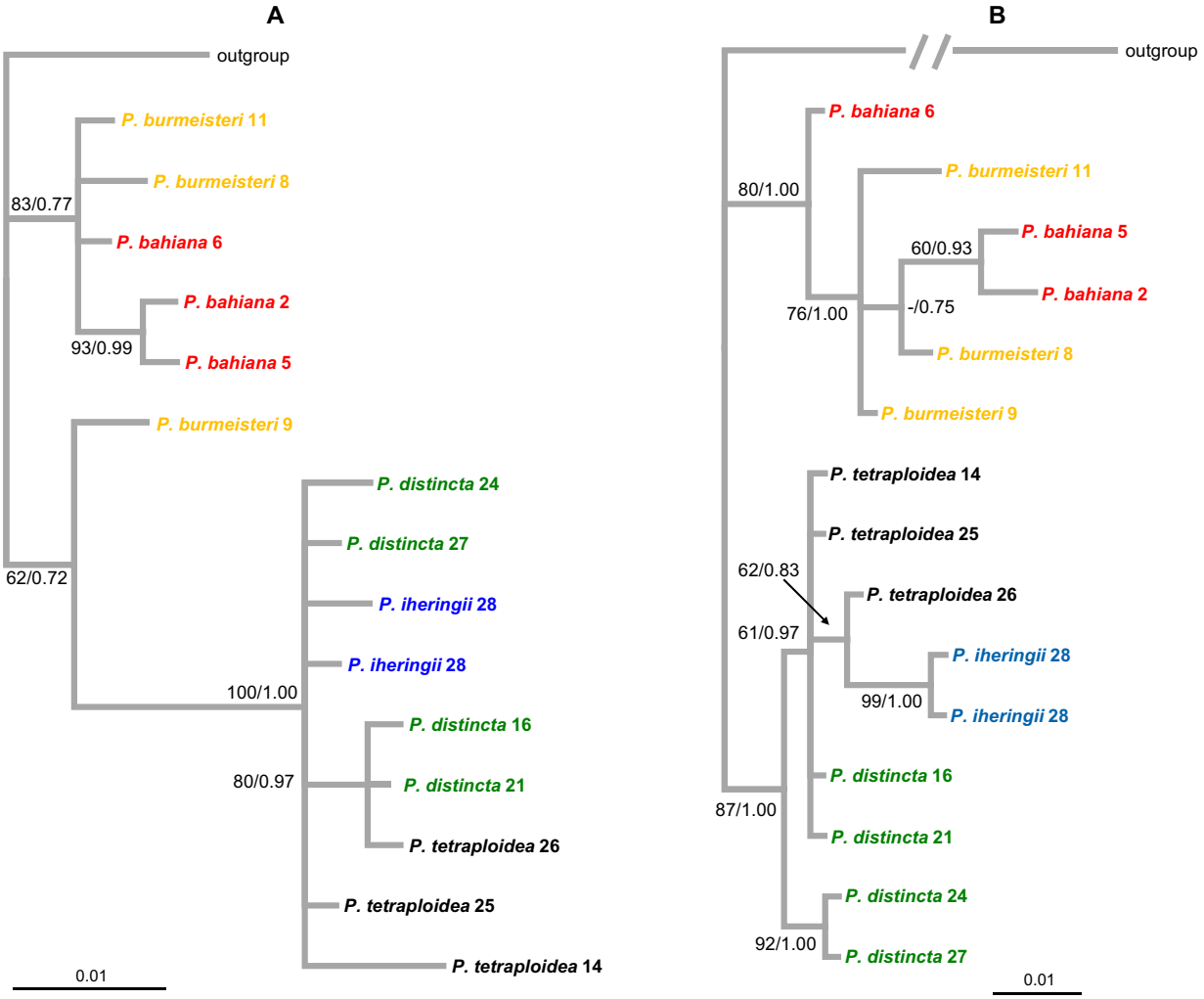
Code	Taxon	Voucher	*h*
1	*P. bahiana*	CFBH 2596	1
2	*P. bahiana*	CFBH 19514, 19524	2
2	*P. bahiana*	CFBH 19525, 19526	3
3,4	*P. bahiana*	CFBH 18741, 18773, 13355,13357, 13359, 13360	4
3	*P. bahiana*	CFBH 18747	5
3,4	*P. bahiana*	CFBH 18772, 13358	6
5	*P. bahiana*	CFBH *	7
6	*P. bahiana*	CFBH *	8
7	*P. bahiana*	CFBH 10207, 10209, 10210	9
8	*P. burmeisteri*	CFBH *	10
9	*P. burmeisteri*	CFBH 10207, 10209, 10210	11
10	*P. burmeisteri*	CFBH 14428	12
11	*P. burmeisteri*	CFBH *	13
12,15, 18,21	*P. distincta*	CFBH 8334, 8246, 2657, 2659, MTR *	14
13	*P. tetraploidea*	MZUSP A 134841, 134839	15
13	*P. tetraploidea*	MZUSP A 134838	16
13,15	*P. tetraploidea, distincta*	MZUSP A 134840, CFBH 2125, 8249	17
14	*P. tetraploidea*	CFBH 18829	18
15,19	*P. distincta, tetraploidea*	CFBH 8245, *, MZUSP A 129325, CFBH 2096	19
15	*P. distincta*	CFBH 8250, 8251, 8252, 2047	20
15	*P. tetraploidea*	CFBH *	21
15	*P. tetraploidea*	CFBH 2126	22
15,17	*P. distincta*	CFBH 2114, *	23
16	*P. distincta*	CFBH 13868	24

Appendix 1 (*continued*)

Code	Taxon	Voucher	*h*
20	*P. distincta*	CFBH 11067	25
21	*P. distincta*	CFBH 2658	26
22,24, 27	*P. distincta*	CFBH 10998, 10999, 10990, 11007, 10321, 12408, 12409, 12410, 12412	27
23	*P. tetraploidea*	CFBH 18251, 18253, 18254	28
23	*P. tetraploidea*	CFBH 18258	29
25	*P. tetraploidea*	CFBH *	30
26	*P. tetraploidea*	CFBH 6949, 6950	31
28,29	*P. iheringii*	CFBH *	32
28	*P. iheringii*	CFBH 3894	33
29	*P. iheringii*	CFBH *	34
30	*P. iheringii*	MCT/PUCRS 4238	35
–	*P. boliviana*	CFBH 2570	–

Appendix 2

Tree derived from Bayesian analysis of (data set 1) 454 of the C-myc2 (A) and 604 bp β-fibint7 (B) nuclear sequences in the *Phyllomedusa burmeisteri* species group. Bootstrap values ML and Bayesian posterior probabilities are given near the branches, respectively. Values under 50% are represented by "–".



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