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Hidden diversity within the broadly distributed Amazonian giant monkey frog (*Phyllomedusa bicolor*: Phyllomedusidae)

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Abstract. *Phyllomedusa bicolor* is a large-sized nocturnal tree frog found in tropical rainforests throughout much of the Amazonian region of Brazil, Colombia, Bolivia, Peru, Venezuela, and the Guianas. Very little is known about *P. bicolor* genetic diversity and genealogical history of its natural populations. Here, using a sampling design that included populations covering most of its distributional range, we investigated the spatial distribution of genetic variability of this species, and we tested the hypothesis that *P. bicolor* is composed of deeply structured genetic groups, constituting more than one lineage across the Brazilian Amazonia. The results suggested two main lineages in two geographic mega-regions: Western and Eastern Amazonia, the latter consisting of three population groups distributed in the Guiana and Brazilian Shields. The present findings have implications to taxonomy, to understanding the processes that lead to diversification, and to defining strategies of conservation and medicinal use of the species.

Keywords: cryptic diversity, genetic diversity, Phyllomedusidae, phylogeography, tropical rainforest.

Introduction

Surveys of molecular biodiversity suggest high levels of intraspecific diversity within anurans from the Neotropics (Fouquet et al., 2007; Funk, Caminer and Ron, 2012; Motta et al., 2018) which often upon further examination translate to species diversity (e.g. Caminer et al., 2017; Rojas et al., 2018). The nominal species to present such a pattern are often categorized as complexes of cryptic species, being generally those with wide geographical distribution, small body size and low dispersal abilities (Gehara et al., 2014); however, species with restricted distribution cannot be discarded either (Guayasamin et al., 2017). Also, such evolutionary differentiation is likely favored by habitat specificity (Vences and Wake, 2007; Kaefer et al., 2013; Maia, Lima and Kaefer, 2017), i.e. in general, forest specialist species and species with low dispersal capacity tend to be genetically structured (Smith and Green, 2005; Rodríguez et al., 2015). In summary, trait, distribution, and life history can influence genetic divergence between populations and may, ultimately, influence the phylogeographic patterns of the species. Besides, the taxonomic limits within Amazonian species complexes as well as the geographic factors promoting and maintaining differentiation among populations remain poorly understood (Wesselingh et al., 2010; Kaefer et al., 2013; Fouquet et al., 2014).

In this context, several species of Amazonian anurans with a wide distribution turn out to be geographically structured in many evolutionary lineages, some of which deserving specific status (Kaefer et al., 2013; Rojas et al., 2018). *Phyllomedusa bicolor* (Boddaert, 1772) (Anura, Phyllomedusidae) is a relatively large-sized nocturnal tree frog found in tropical rainforests throughout a broad geographic area, occurring in the Amazonian region of Brazil, Colombia, Bolivia, Peru, Venezuela, and the Guianas; it also occurs in the Cerrado habitat of Maranhão state, Brazil (Frost, 2019). The currently accepted hypothesis that individuals of

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P. bicolor from geographically distant locations represent the same taxon awaits support from genetic data. Skin secretions from this frog have been used by indigenous people from Brazil and Peru for centuries as a ritualistic healing substance, and its use has spread via urban expansion into alternative therapy clinics (Silva, Monteiro and Bernarde, 2019). Even though *Phyllomedusa bicolor* is a pharmacologically important taxon, no study has assessed the genetic diversity and genealogical history of natural populations of this species, and many of its few published DNA sequences are not assigned to any specific geographic location, given that they have been obtained from pet trade specimens. Therefore, we assess the genetic diversity of *Phyllomedusa bicolor* populations by sequencing mitochondrial and nuclear genetic markers. Assuming its large geographical distribution, we aim to test whether populations of *P. bicolor* constitute the same evolutionary unit throughout its range.

Materials and methods

Sampling and data collection

Sampling occurred in the years 2008 to 2009 and was performed in sampling sites across five states in the Brazilian Amazonia. A total of 117 individuals were obtained from 11 different sampling sites (supplementary table S1), which include populations that cover a large part of the species distribution in the Amazonian biome.

We collected samples at night around permanent and semi-permanent puddles as well as around ponds near streams. At least seven individuals were collected from each area. The sampling was conducted through auditory and visual searches with a headlamp. Tissue samples were collected from one of the toes, preserved in 95% ethanol and deposited in the Animal Genetics Tissue Collection – CTGA-ICB/UFAM Campus (CGEN, Resolution No. 75 of August 26, 2004) Laboratory of Animal Genetics and Evolution of the Federal University of Amazonas, Manaus, Brazil.

Genomic DNA extraction was performed according to the protocol described by Sambrook, Fritsch and Maniatis (1989). We used sets of primers to amplify two regions of the mitochondrial DNA (mtDNA) and one nuclear gene. Amplification was performed via polymerase chain reaction (PCR) using primers for 16S rRNA mitochondrial gene (Palumbi, 1996), Cytochrome b (Cyt*b*) specific primers developed for *Phyllomedusa bicolor* using amplifications obtained from the primers MZV15-L (Moritz, Wright and Brown, 1992) and Control-IP-H (Goebel, Donnelly and Atz, 1999) to amplify a portion of the Cyt*b* gene, and primers for the nuclear gene RAG1 (Rag1_phyFL and Rag1_phyR).

The amplification of the 16S and Cyt*b* was performed in a final volume of 15 μl containing 5.5 μl of ddH2O, 1.5 μl of dNTP (1.5 mM), 1.5 μl of 10X buffer (Tris-HCl 100 mM, KCl 500 mM), 1.5 ml of each primer (0.2 mM), 1.5 μl of $MgCl_2$ (25 mM), 1 μl of DNA (approx. 10 ng) and 0.3 μl of Taq DNA polymerase. The amplification cycles were performed using the following conditions: (1) initial denaturation at 92°C for 1 minute, (2) 35 cycles consisting of denaturation at 92°C for 1 minute, annealing of primers at 50°C for 40 seconds, and extension at 72°C for 1.5 minutes, as well as (3) final extension at 72°C for 5 minutes.

The PCR products were purified with the exonuclease and alkaline phosphatase enzymes, following the manufacturer's protocol (Fermentas). Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit, following the manufacturer's protocol (Life Technologies). Products were resolved on the ABI3130xl automatic sequencer (Life Technologies) and edited and aligned using the software BioEdit (Hall, 1999).

Data analysis

A network of haplotypes was generated using a concatenated data of all molecular markers (16S rRNA+ Cyt*b*+RAG1) in the program HAPLOVIEWER for the reconstruction of the genealogical relations between individuals (Salzburger, Ewing and von Haeseler, 2011).

Single locus discovery and phylogeography

To test whether populations of *Phyllomedusa bicolor* comprise just one evolutionary unit, we used the ultrametric consensus tree topology generated in BEAST as input for single-locus species discovery analyses: bGMYC, a Bayesian implementation of the GMYC (Reid and Carstens, 2012), and the Poisson tree process (PTP) model for species delimitation (Zhang et al., 2013) as implemented in mPTP (Kapli et al., 2017). For these analyses, we used only unique haplotypes from the concatenated data set. The phylogeographic analysis in BEAST was conducted using concatenated data from all sampled individuals. Clade support was assessed with 1,000 non-parametric bootstraps. For these analyses, we used the TIM+G+I model of molecular evolution selected by the program jMODELTEST (Posada, 2008).

Using the concatenated data we calculated the time of most recent common ancestor (TMRCA) for each group of haplotypes using a coalescence history approach investigated by Markov Chain Monte Carlo (MCMC) using the BEAST v1.8 program (Drummond and Rambaut, 2007). The analyses were performed assuming a relaxed molecular clock lognormal type, which assumes independent rates on different branches (Drummond et al., 2006). Prior trees were modeled according to the Yule process of speciation. The chain (MCMC) used in the BEAST analysis of divergence had a size of 10 million steps, with a consensus

tree recorded every 10,000 steps and a burn-in of 5%. The TREEANNOTATOR was used to summarize the sampled trees after discarding the initial burn-in and FIGTREE 1.4.2 (Rambaut, 2014) was used to view and edit the tree chronogram. We used a secondary calibration point obtained from Wiens, Pyron and Moen (2011), since it was based on several genes and probably reflected the likely true phylogeny of the species tree. To apply the time calibration, we added GenBank sequences of 16S rRNA and RAG1 from *Phyllomedusa vaillanti* (AY549363, AY844498, Faivovich et al., 2005), which is the sister species of *Phylomedusa bicolor* (Wiens, Pyron and Moen, 2011). We also included in the 16S rRNA data set sequences of *Phyllomedusa trinitatis* (GQ366287, Faivovich et al., 2010), *Phyllomedusa boliviana* (GQ366253, Faivovich et al., 2010), *Phyllomedusa tetraploidea* (GQ366284, Faivovich et al., 2010), *Phyllomedusa tarsius* (AY843726, Faivovich et al., 2005), which are all closely related to the *P. bicolor* − *P. vaillantii* clade. The secondary calibration points were the *P. bicolor* + *P. vaillantii* (17.37 My) clade, the *P. tarsius* + *P. trinitatus* clade (3.98 My) and the age of all species included in this present analysis (28.14 My). We set age priors to be normally distributed with the mean and 95% CIs reported by Wiens, Pyron and Moen (2011).

Population genetic structure

The level of genetic diversity within population groups was measured by the nucleotide (π) and gene (h) diversities. Both genetic parameters were calculated using the program ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) tests were used to testing for selective neutrality of the DNA sequences. Significant values for these tests could indicate that the sequences are not evolving according to the hypothesis of selective neutrality or that the populations were previously subdivided and/or experienced fluctuations in the past (i.e. they are not in migration-drift equilibrium; Hartl and Clark, 2006). The significance of both statistics was tested by comparisons of the statistics against a distribution generated from 10,000 random samples on the assumptions of selective neutrality and population equilibrium. The tests were implemented with the program Arlequin v3.5 (Excoffier and Lischer, 2010).

The presence of population subdivisions was tested using analysis of molecular variance (AMOVA) (Excoffier, Smouse and Quattro, 1992) implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010). The AMOVA components of covariance estimate and calculate Φ*ST*, which is analogous to *FST* (Weir and Cockerham, 1984). The pairwise Φ*ST* values between individuals from each sampling site were also calculated to estimate the population structure of *Phyllomedusa bicolor*. Additionally, we used the spatial analysis of molecular variance (SAMOVA) to estimate the formation of biological groups by the best grouping option with the highest *FCT* value (Dupanloup, Schneider and Excoffier, 2002). Several SAMOVA runs were computed by varying the number of K (number of groups tested) from 2 to 10 until the *FCT* value remained relatively constant. For each K value, 10,000 simulations were carried out for each group. Additionally, the presence of genetic structure was also assessed using the Bayesian Analysis of Population Genetic Structure (BAPS) program, version 4.14. (http://web.abo.fi/fak/mnf/mate/jc/software/baps.html) developed by Corander, Waldmann and Sillanpää (2003), in which the number of populations was treated as "not known" and estimated *a posteriori*.

The correlation between genetic (Φ*ST*) and geographical distances was performed using the Mantel test (Mantel, 1967) in the ARLEQUIN 3.5 (Excoffier and Lischer, 2010). The significance of the correlation was assessed by permutation tests (10,000 replicates).

Pairwise genetic distances between groups were calculated using the Kimura two-parameter evolutionary model in the MEGA 6.0 software (Tamura et al., 2013) in order to check the rate of divergence between groups of populations of *Phyllomedusa bicolor*.

Results

One hundred seventeen sequences representing 1,373 nucleotides were obtained from all the individuals of *Phyllomedusa bicolor*: 520, 517 and 336 nucleotides for the genes 16S, Cytb and RAG-1, respectively. Of these, seven, 21 and one were parsimony informative for 16S, the Cyt*b* and RAG-1, respectively. Despite the low mutation rate, we decided to maintain the RAG-1 gene because the single parsimony-informative site separates the population groups from eastern and western Amazonia. All *P. bicolor* sequences were deposited in the GenBank under accession numbers KY752833 – KY752920, KY752921 – KY753008, KY753009 – KY753096.

The results of haplotype network (fig. 1A) show haplogroups within two Amazonian regions: The western and eastern Amazonia. The western Amazonia includes a monophyletic group of individuals from Acre, Santa Isabel do Rio Negro, Tabatinga and Atalaia. The eastern Amazonia comprises a monophyletic group of individuals from the Guiana Shield sampling sites (Adolpho Ducke Forest Reserve, REBio Uatumã, UFAM Campus, Barcelos, PARNA of Viruá, and FLONA of Amapá) and individuals from the Brazilian Shield (Altamira).

Genetic distance values higher than 1% were found among individuals occupying Eastern and

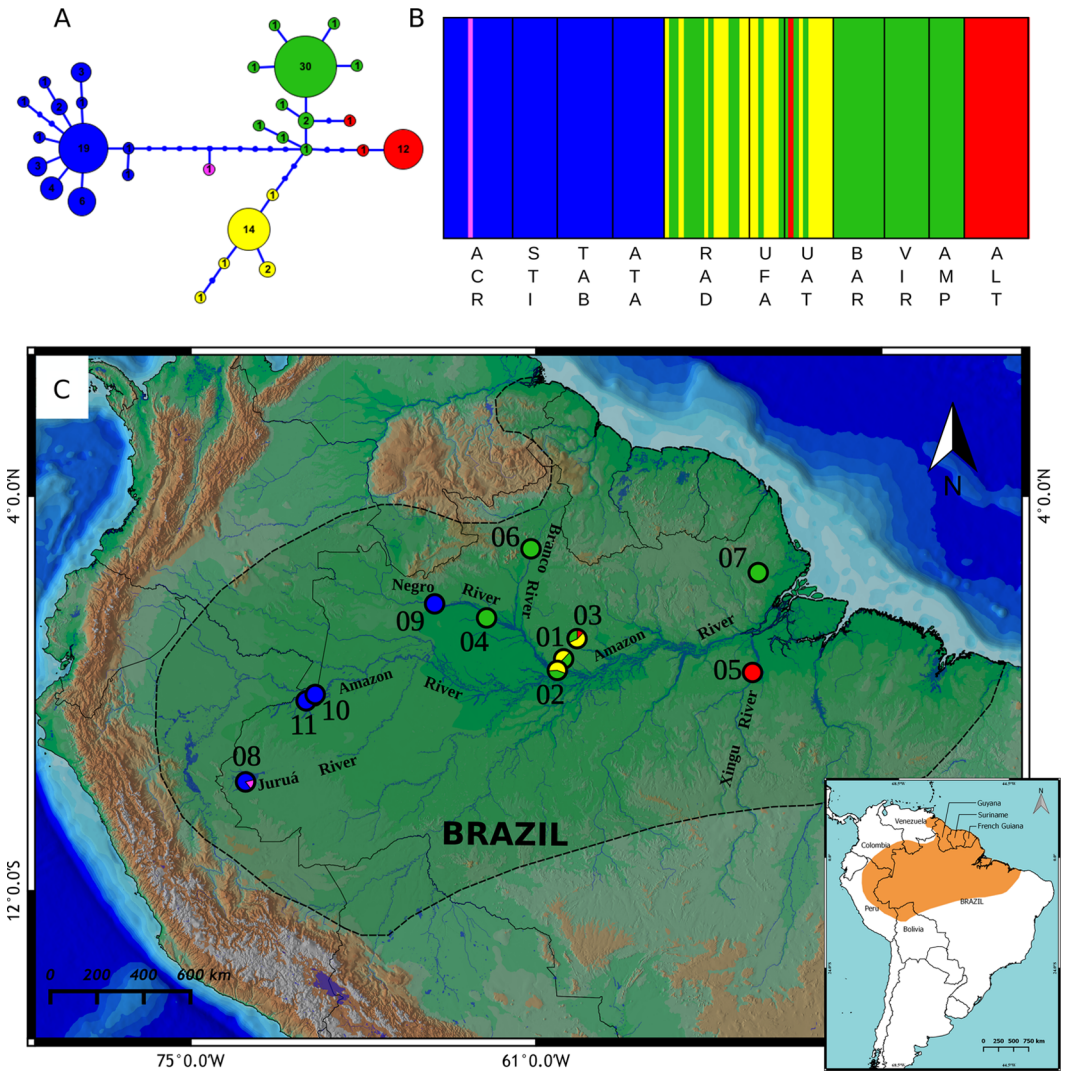

Figure 1. Map showing the distribution and sampling localities of *Phyllomedusa bicolor* in the Amazon basin. Sampling site codes follow supplementary table S1. The shading indicates the geographic distribution of the species according to IUCN (accessed October 8, 2019). A) Haplotype network of *Phyllomedusa bicolor* and the distribution of the haplotypes according to the sampling sites. Circle sizes correspond to the number of observations, and missing haplotypes remain unfilled. The colors represent the geographical haplogroups to which the individuals belong. B) Bar graph generated by Bayesian analysis implemented in BAPS program, which estimates the posterior probability of the formation of groups that are represented by color, showing a total of 4 groups of individuals: blue = western Amazon; yellow/green = Guiana Shield; red = Brazilian Shield. C) Distribution of the haplotypes of *Phyllomedusa bicolor* according to the sampling sites.

Western Amazonia, with divergences ranging from 1.2 to 1.6%.

Phylogeography and timing of divergences

The dataset composed of the sequences of *Phyllomedusa bicolor* plus the outgroups resulted in 1379 nucleotides, of which 131 were variable and 82 were informative for parsimony.

For the two single-locus species discovery analyses performed, mPTP identified only one taxonomic entity, and bGMYC was congruent in identifying the two main lineages of *Phyllomedusa bicolor*, western and eastern Amazo-

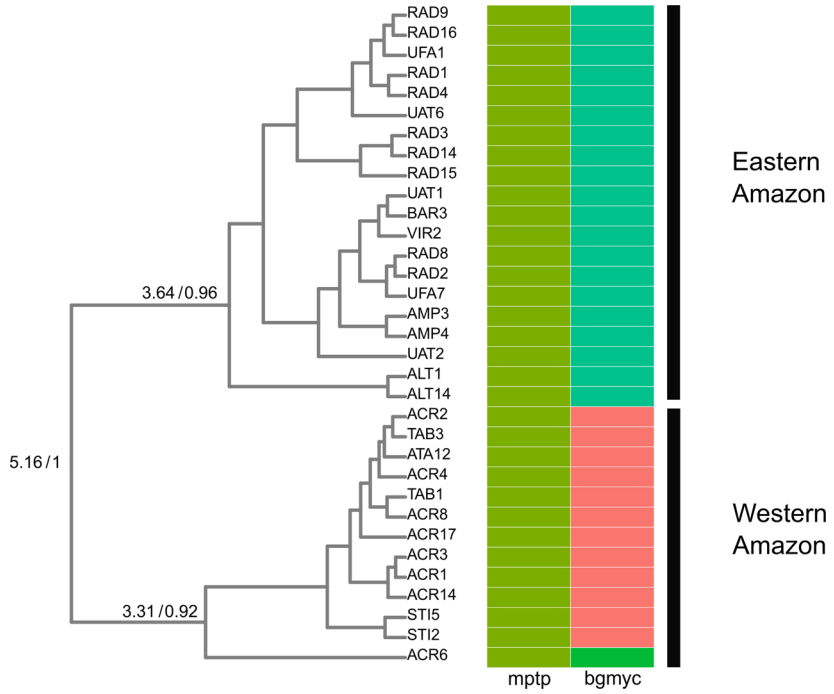

Figure 2. Maximum clade credibility chronogram from 1,000 posterior trees generated using BEAST. Data set is comprised of 33 unique haplotypes of *P. bicolor*. Numbers above the nodes represent estimates of divergence times and Bayesian posterior probabilities above 0.90 for the two main lineages, respectively. The mPTP single locus species discovery method resulted in only one lineage, while bGMYC method resulted in the discovery of three lineages.

nia groups, with one additional lineage related to a single individual from the Acre sampling site (fig. 2).

The phylogenetic tree revealed two well supported monophyletic groups (posterior probability = 1, fig. 2) (western and eastern Amazonia groups) within the geographical distribution of *Phyllomedusa bicolor*, which diverged approximately 5.16 Mya (95% HPD = 3.29-6.84 Mya). The divergence event of the western Amazonia group was estimated at 3.31 Mya (95% HPD = 3.27-6.84 Mya) and the eastern Amazonia groups estimated at 3.64 Mya (95% HPD = 2.08-4.79 Mya).

Population genetic structure

Based on the concatenated sequences of the three genes, total genetic diversity (h) was estimated to be 0.884, with a large variation among sampling sites. The nucleotide diversity index (π) was also calculated for all sampling sites, with a mean of 0.0083. For the selective mutation neutrality tests, the Tajima's *D* test (Tajima, 1989) and Fu's *Fs* test (Fu, 1997), did not show significant values, which suggests that *P. bicolor* populations are in genetic equilibrium, showing no evidence of population expansion or fluctuations in the past. Intra population genetic variability is summarized in table 1.

Overall AMOVA revealed a strong genetic structuring ($\Phi ST = 0.8166$; $P < 0.001$). The results of this analysis suggest that a large portion of genetic variance (81.66%) is explained by among population differences with the remaining (18.34%) within populations. Analysing the ΦST values for pairs of populations, the majority of comparisons were significant ($P < 0.05$) (table 2), demonstrating a strong population structuring between the different sampling sites. The results of the pairwise comparisons of ΦST were also used for the indirect estimation of the effective number of mi-

Table 1. Parameters of genetic diversity of *Phyllomedusa bicolor*, where N = number of samples, NH = number of haplotypes, S = number of polymorphic sites, h = gene diversity, π = nucleotide diversity. Note: - mean calculation was not possible. Sampling sites are according to supplementary table S1.

Sampling sites	N	NH	S	h	π	Tajima's D test	Fu's Fs test
RAD (1)	17	09	10	0.875 ± 0.057	0.0037 ± 0.0021	1.10794	−1.44395
UFA (2)	07	03	07	0.523 ± 0.208	0.0030 ± 0.0020	0.51770	2.31363
UAT (3)	10	05	09	0.755 ± 0.129	0.0036 ± 0.0020	0.79029	0.85149
BAR (4)	10	01	-	0.000 ± 0.000	0.0000 ± 0.0000	-	-
ALT (5)	13	02	01	0.153 ± 0.126	0.0001 ± 0.0002	−1.14915	−0.53714
VIR (6)	09	02	01	0.222 ± 0.166	0.0002 ± 0.0003	−1.08823	−0.26348
AMP (7)	07	03	02	0.666 ± 0.159	0.0008 ± 0.0007	0.20619	−0.23726
ACR (8)	14	08	13	0.912 ± 0.048	0.0028 ± 0.0017	−1.12339	−1.99473
STI (9)	09	03	02	0.416 ± 0.190	0.0005 ± 0.0005	−0.58325	−0.53211
TAB (10)	11	03	02	0.690 ± 0.086	0.0010 ± 0.0008	1.66480	0.69375
ATA (11)	10	03	02	0.511 ± 0.164	0.0005 ± 0.0005	−0.69098	−0.59381
All	117	31	38	0.884 ± 0.017	0.0083 ± 0.0043	0.66285	−3.12573

Table 2. Pairwise comparison showing the ΦST values between pairs of populations of *Phyllomedusa bicolor* (below diagonal). Above the diagonal is the number of migrant females per generation (*Nemf*). Sampling site codes follow in supplementary table S1.

Localities	RAD (1)	UFA (2)	UAT (3)	BAR (4)	ALT (5)	VIR (6)	AMP (7)	ACR (8)	STI (9)	TAB (10)	ATA (11)
RAD (1)	-	Inf	inf	0.959	0.224	1.041	1.640	0.146	0.115	0.110	0.108
UFA (2)	−0.002	-	inf	0.214	0.096	0.253	0.418	0.116	0.064	0.066	0.058
UAT (3)	−0.041	−0.085	-	0.464	0.172	0.522	0.866	0.133	0.091	0.088	0.083
BAR (4)	0.420∗	0.990∗	0.518∗	-	0.007	40.00	1.055	0.075	0.011	0.022	0.011
ALT (5)	0.690∗	0.838∗	0.743∗	0.985∗	-	0.015	0.037	0.053	0.011	0.017	0.010
VIR (6)	0.324∗	0.663∗	0.488∗	0.012	0.969∗	-	1.608	0.083	0.017	0.027	0.016
AMP (7)	0.233∗	0.544∗	0.366∗	0.321∗	0.929∗	0.237∗	-	0.099	0.029	0.039	0.027
ACR (8)	0.773∗	0.810∗	0.788∗	0.868∗	0.903∗	0.857∗	0.834∗	-	5.928	2.067	4.309
STI (9)	0.812∗	0.886∗	0.845∗	0.976∗	0.978∗	0.966∗	0.943∗	0.077	-	1.365	5.779
TAB (10)	0.818∗	0.882∗	0.849∗	0.956∗	0.966∗	0.947∗	0.926∗	0.194∗	0.268∗	-	3.173
ATA (11)	0.821∗	0.894∗	0.856∗	0.977∗	0.979∗	0.967∗	0.947∗	0.103∗	0.079	0.136	-

Note: The numbers in parentheses are the numbers assigned for the sampling site in all analyses. * Indicates the significance level of $P < 0.05$, inf = infinite value of *Nemf*.

grant females per generation (*Nemf*), which revealed restricted gene flow for most of the comparisons (number of migrants less than 1). No significant correlation was found between genetic and geographic distances ($r = 0.26$, $P = 0.06$). Thus, the genetic variation among the *P. bicolor* populations cannot be explained simply by the geographic distribution of the samples.

Spatial molecular analysis of variance (SAMOVA) was employed to reveal the formation of population groups *a posteriori*. Several analyses were computed varying the K number (number of groups tested). SAMOVA identified four population groups (K = 4) as the most significant genetic structure ($P < 0.001$), in the following configuration: Group 1) UFAM Campus, Adolfo Ducke Forest Reserve and Rebio Uatumã; Group 2) Barcelos, PARNA of Viruá and FLONA of Amapá; Group 3) Altamira; and Group 4) Cruzeiro do Sul, Santa Isabel do Rio Negro, Tabatinga and Atalaia do Norte.

The population structure results obtained using the program BAPS 5.1 suggest that the best *a posteriori* probability value (ln likelihood = 0.918) corresponded to five clusters (fig. 1B). Cluster 1 was represented only by individuals in the state of Acre. Cluster 2 was represented by individuals from Acre, Santa Isabel do Rio Negro, Tabatinga and Atalaia. Clusters 1 and 2 belong to the western Amazon groups of individ-

uals. Cluster 3 was represented by individuals in the Adolpho Ducke Forest Reserve, UFAM Campus and Rebio Uatumã. Cluster 4 was represented by individuals from Barcelos, PARNA of Viruá and FLONA of Amapá, few individuals from Adolpho Ducke Forest Reserve and UFAM Campus. Clusters 3 and 4 belong to the Guiana Shield group. All the individuals from Altamira site appear in the cluster analysis together with only one single individual from Rebio Uatumã in Cluster 5 (denominated herein as the Brazilian Shield group).

Discussion

Diversification and insights for biogeography

Except for the mPTP results, all analyses corroborate the presence of two main evolutionarily lineages in *Phyllomedusa bicolor*. The lineages found correspond to a geographic division in the east-west of Amazonia, with the large eastern Amazonia group composed of the Brazilian Shield and Guiana Shield haplogroups (fig. 1). Analysing the topology of the Bayesian chronogram (fig. 2), the first cladogenetic event led to the separation of the eastern and western lineages, which took place during the Pliocene (5.16 Mya). The age of these diversification times are in the range of speciation of various other taxa of *Phyllomedusa*, number of which showed divergences of less than 5 Mya (Wiens, Pyron and Moen, 2011; Duellman, Marion and Hedges, 2016). The present findings lend support to the increasing number of studies that show that many anuran species, widely distributed in Amazonia, contain evolutionary lineages with a distinctiveness that might be worthy of species status (Angulo and Icochea, 2010; Simões, Lima and Farias, 2010; Gehara et al., 2014; Ferrão et al., 2016; Rojas et al., 2016; Caminer et al., 2017; Rojas et al., 2018).

This eastern-western pattern has been observed in other anurans such as within *Adenomera* (Fouquet et al., 2014), and more recently for *Amazophrynella* (Rojas et al., 2018). This scenario has also been suggested to explain diversification in other taxa such as squamates (Avila-Pires, 1995; Gamble et al., 2008; Miralles and Carranza, 2010), and endemic vertebrate fauna (Sales et al., 2017), and even at the population level of a widespread Neotropical dwarf gecko (Pinto et al., 2019). The estimated time of divergence agrees with the conclusions of Turchetto-Zolet et al. (2013), which found that most herpetofauna (amphibians and reptiles) in South America underwent intraspecific lineage divergences mainly during the Pliocene and/or Miocene. In our results, there is a lack of obvious geographical barriers to explain the east-west Amazonia diversification. Recently Godinho and da Silva (2018) tested different hypotheses that could explain the biogeography patterns of anuran distributions, suggesting that major rivers in the Amazon basin strongly contributed to explaining the diversity in anuran biogeographic regions, followed by climate and topography. In the case of *Phyllomedusa bicolor*, rivers do not appear to be barriers to the distribution of the lineages, as both lineages are distributed across the large Amazonas and Negro rivers. One possible explanation could be that climatic factors influenced the distribution of these two lineages since the Amazon presents a well-defined pattern of climate gradient. Indeed, Tuomisto et al. (2019) based on analysis of field plot data to assess main ecological gradients across the Amazonia, mapped the edaphic, floristic, geoecological and climatic factors and corroborated the classical division of Amazonia proposed by Fittkau et al. (1975) into four geochemically defined regions: western Amazonia, central Amazonia, Brazilian and Guyana Shields. Finally, we are aware that all these explanations are speculative, since the observed pattern of diversification in *P. bicolor* must be analyzed and tested with an adequate sampling for these hypotheses to be tested.

Genetic diversity and population structure

Phyllomedusa bicolor exhibited relatively heterogeneous levels of genetic diversity through-

out its distribution in the Amazon (table 1). The broad distribution of the species does not appear to have been the result of demographic expansions in the past, as neutrality tests were not indicative of population expansion. At a population level, the results support high levels of genetic structure between pairs of populations, as a consequence of very restricted gene flow in almost all comparisons. Similar level of structuring is also observed in other Amazonian anurans such as *Allobates* (Kaefer et al., 2013), and *Osteocephalus* species (Ortiz et al., 2018). Like other species of the genus *Phyllomedusa*, this species is arboreal and lays eggs in a jelly-like mass on leaves or in leaves wrapped in twigs hanging branches above lentic water; although they can jump, individuals have a slow, elegant walk over branches and leaves (Caramaschi and Cruz, 2002). Their life history as the choice of wet habitats, relatively low movement patterns, breeding site fidelity and low dispersal (Neckel-Oliveira, 1996) may influence microevolutionary processes such as drift, immigration, and selection, which ultimately exert influences on the genetic diversity and population structure within a species (Fouquet et al., 2015; Ellegren and Galtier, 2016). Indeed, the results show high levels of genetic differentiation among populations with gene flow of less than one individual per generation for most population comparisons, which is very low, suggesting low dispersal ability between populations. Both SAMOVA and BAPS analyses identified one main population group for western Amazon. On the other hand, eastern Amazonia was composed of three population groups in which two were distributed in the Guyana Shield and one on the Brazilian Shield (fig. 1). Unlike western Amazonia, the population structure observed in the eastern maybe being shaped by the river barrier once the Amazon River is between Guiana and Brazilian shield haplogroups. Additionally, these three populations coincide with the three geographic regions established by Tuomisto et al. (2019) for the large area of eastern Amazonia.

Implications for conservation and medicinal use

Our result showing different evolutionary lineages in *Phyllomedusa bicolor* is one more example confirming that widely distributed species of anurans in Amazonia are likely to present cryptic diversity (Gehara et al., 2014). But, why should we care about cryptic diversity observed in *P. bicolor*? Cryptic diversity is characterized by deep genetic divergence within a nominal species, but not without clearly established phenotypic differences within or between populations (Pfenninger and Schwenk, 2007). Delimiting and identifying evolutionary independent lineages is of fundamental importance to taxonomy, to understanding the processes that lead to diversification, and to defining conservation strategies on a regional scale, since richness, endemism, and cryptic diversity are commonly used to estimate these parameters (Espíndola et al., 2016). However, the importance of cryptic lineages for conservation is still poorly understood, and many of them may be seriously threatened because they are not yet species formally described and therefore not included in conservation programs, as most of them remain unknown to science. The present study provides evidence of the existence of two profoundly divergent *P. bicolor* lineages and, therefore, it is important to recognize these lineages for specific conservation and management measures. The results highlight the need for more fine-scale studies in the populations of *P. bicolor*, which must include at least acoustic and morphological data to test if these groups are distinct species, and then be properly assessed for their conservation status.

Last but not least, the recognition of these lineages in *Phyllomedusa bicolor* has implications related to its pharmacologically important skin secretions. Different taxa within *Phyllomedusa* have exclusive peptides (Calderon et al., 2011; Pescatore et al., 2015) and geographic differences in frog skin peptide composition can be verified even on the intraspecific level (Samgina

et al., 2011). Therefore, the molecular characterization of populations within the range of *P. bicolor* presented here is crucial to guide future bioprospective research on this species, since different lineages could potentially harbour different and new important skin peptides.

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