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CELLULAR AND MOLECULAR BIOLOGY

Molecular data reveal multiple lineages of *Scinax nebulosus* (Spix, 1824) (Anura: Hylidae) with Plio-Pleistocene diversification in different Brazilian regions

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Abstract: To understand the organism's history, we can start assessing the complexity of the biome where they occur. In this study, we used a region of the mitochondrial genome, the rRNA 16S, to evaluate the genetic differentiation in *Scinax nebulosus* along with its geographical range highlighting important Brazilian biomes as Restinga, Cerrado, Amazon, and Atlantic Forest. Geographically structured genetic divergence was observed within the species *S. nebulosus*. The values of the fixation index (Φ_{st}) and the pairwise F*st* index were high and significant regarding this structuring. Besides, the haplotype network corroborates these results with the haplotypes arrangement found by separating the *S. nebulosus* populations in two major groups: North and Northeast. The lineage delimitation analyses indicate the occurrence of several lineages with divergence mainly between the samples from the Northeast group. Thus, we can suggest that *S. nebulosus* may present itself as a group of cryptic species due to the genetic characteristics found. The existence of a mosaic of heterogeneous habitats may explain the genetic divergence found, which justifies the existence of cryptic species in this group. However, this hypothesis needs more detail in molecular studies, including large sample sizes and other population and demographic analyses.

Key words: Anurans, Biomes, Molecular biology, Population structuring.

INTRODUCTION

The genus *Scinax* Wagler, 1830 (Anura: Hylidae) was included in the Scinaxinae subfamily after a new taxonomic and phylogenetic revision of the Hylidae family (Duellman et al. 2016). The genus comprises 72 species distributed throughout the American continent, occurring from southern Mexico to Argentina, Uruguay, Trinidad and Tobago, and Saint Lucia (Nunes et al. 2012, Duellman et al. 2016, Frost 2020). Its wide distribution and diversity are associated with different habitats where these species can be found, e.g. open areas of scarce vegetation, deciduous, semi-deciduous and ombrophilous forests, gallery and riparian forest (Faivovich 2002).

Some *Scinax* species are not yet well defined given the possibility of cryptic complexes among them (Fouquet et al. 2007, Ferrão et al. 2016, 2017), especially those with a wide range as *Scinax nebulosus* (Spix, 1824). Since its discovery, the species has been characterized and named several times. First, recognized as *Hyla egleri* (Lutz, 1968) for specimens collected in Belém, state of Pará (Lutz, 1968). Later, it was redescribed as *Ololygon egleri* (Fouquette & Delahoussaye 1977), then *O. nebulosus* (Hoogmoed & Gruber 1983), after that *S. nebulosa* (Duellman &

Wiens 1992), until the current designation of *S. nebulosus* (Köhler & Böhme 1996).

Scinax nebulosus shows an arboreal lifestyle and wide geographical distribution. It is found in Brazil, Bolivia, Guianas, Suriname, and Venezuela. In Brazil, *S. nebulosus* is found in the Amazon Basin, Center-West, and in the Northeast (Dias et al. 2015). In general, the species is associated with temporary water bodies in the tropical forest. It is also found in open areas of Cerrado as well as in anthropogenic habitats like pastures and gardens (La Marca et al. 2004, Dias et al. 2015). These associations may drive the distribution of species with limited mobility characteristics such as amphibians (Lourenço et al. 2009, Sturaro & Peloso 2014).

Lutz (1973) morphologically described *S. nebulosus* as a species with a great number of glands all over the dorsum (in the head, upper eyelids, limb margins). However, these features are not enough to differentiate *S. nebulosus* from other species in the genus, such as *S. pedromedinae* (Henle 1991) occurring in the Brazilian Amazon and northern Peru. It is worth mentioning that specimens of *S. nebulosus* from the Center-West Brazil and Bolivia may be specimens of *S. pedromedinae* (Henle, 1991) (Hoogmoed & Avila-Pires 2011). Moreover, comparisons of *S. nebulosus* vocal repertory record with other sympatric congeners suggests the need for a careful assessment of this species taxonomic status along with its geographical distribution (Lima et al. 2004).

However, *S. nebulosus* is still considered as valid species with distribution in central and north of South America (Dias et al. 2015). In general, terrestrial organisms spread over this area may have discontinuities in its distribution in response to climate change and geological events that occurred during the Plio-Pleistocene (Bell et al. 2012, Menezes et al. 2016).

Bell et al. (2012) investigated how Pleistocene refugia interfered with genetic differentiation in continental and island populations, and the role of forest fragmentation in this differentiation. Pleistocene biogeographic events have been traditionally related to the process of population structuring and vertebrate speciation (Avise et al. 1998). Menezes et al. (2016) evaluated the genetic structure of *Scinax eurydice* (Bokermann, 1968) and they observed events of lineage segregation that occurred at the end of Pliocene to Pleistocene. Pleistocene climate fluctuations played a key role in the distribution of diversity and endemism in Brazilian biomes. For amphibians, these fluctuations may indicate new lineages spanning climatically distinct regions.

The wide geographical distribution of *S. nebulosus* is a constant subject of discussion, questioning the correct identification of populations, which has been made only from vocalization and morphological data (Duellman et al. 2016). Thus, this species may present inter-population variations associated with Plio-Pleistocene climate events. In this context, we tested this hypothesis using molecular data by performing an analysis of the genetic diversity of *S. nebulosus* from mitochondrial markers to increase the level of knowledge about its taxonomic status.

MATERIALS AND METHODS

Specimens sampling and DNA extraction, PCR and sequencing

The specimens of *S. nebulosus* were collected in fieldwork and obtained from zoological collections, totaling 52 specimens analyzed from 13 localities (Figure 1, Table I, Table SI – Supplementary Material). The collections of this project were carried out under authorization from the Instituto Chico Mendes de Conservação

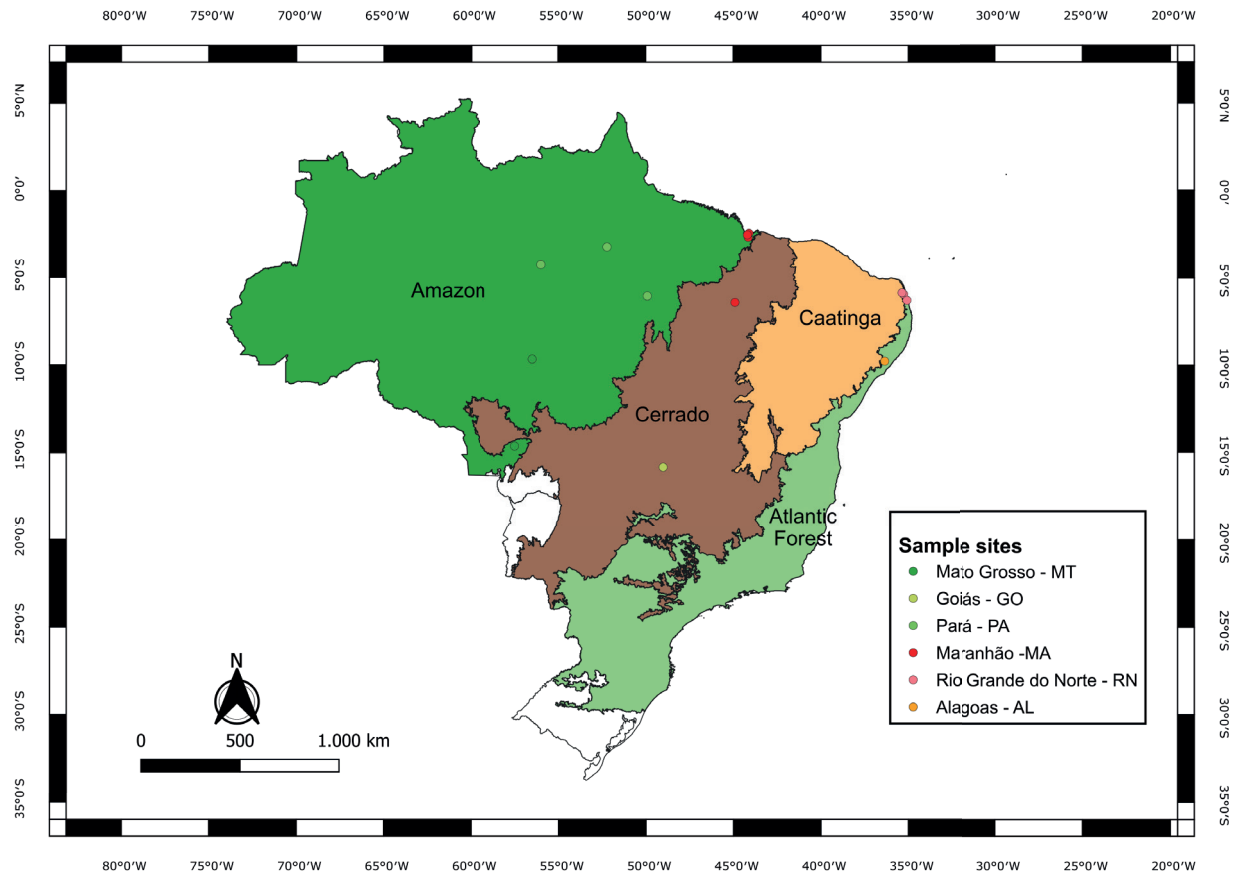

Figure 1. Map identifying the locations of origin of the *Scinax nebulosus* specimens used in this study.

da Biodiversidade (ICMBio), through permanent license number 52593-3. Subsequently, we removed muscle tissue samples from hind limbs, placed in 1.5 ml microtubes, and stored in 100% ethanol. The specimens were fixed in 10% formaldehyde solution, stored in 70% ethanol, and deposited in the Herpetological Collection of the Universidade Federal do Maranhão (CHUFMA).

The genomic DNA isolation followed Sambrook & Russel (2001) protocol. We utilized the rRNA 16S marker in the analyses; we amplified it by PCR, using the primers described by Palumbi et al. (1991): L1987–5' GCCTCGCCTGTTTACCAAAAAC 3' e H2609–5'-CCGGTCTGAACTCAGATCACGT 3', under the followed amplification conditions: initial denaturation at 94°C for 5 min, 25 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C and final extension at 72°C for 5 min. After amplification, the samples were subjected to sequencing reactions using the Big Dye Terminator Kit (Applied Biosystems), following the manufacturer's recommendations, and then injected into the ABI 3500 automated sequencer.

Data analyses

We aligned the sequences in BioEdit (Hall 1999) using the automatic alignment tool ClustalW (Thompson et al. 1994), followed by visual inspection to detect identification base errors. To estimate the genetic variability of the possible populations, the pairwise genetic differentiation indices (Fst), fixation index (Φ_{st}), Analysis of Molecular Variance (AMOVA) and the number of haplotypes were estimated in the software Arquelin v. 3.5 (Excoffier &

Table I. Genetic diversity indices obtained for the 16S gene from *Scinax nebulosus* populations used in the present study. N = number of individuals, Hap = number of haplotypes, h = haplotype diversity, π = nucleotide diversity, sd = standard deviation.

Population	N	Hap	(h) (sd)	(π) (sd)	D Tajima	Fs Fu
Pará	7	4	0.809 (0.8 ± 0.1)	0.028 (0.006)	0.069	4.467
Mato Grosso	5	4	0.900 (0.8 ± 0.1)	0.018 (0.007)	-1.239	1.537
Maranhão	29	6	0.756 (0.8 ± 0.1)	0.003 (0.000)	0.499	0.018
Alagoas	2	1	-	-	-	-
Rio Grande do Norte	7	2	0.285 (0.8 ± 0.1)	0.045 (0.007)	-1.748	13.294
Goiás	2	1	-	-	-	-
Total	52	18	0.863 (0.8 ± 0.1)	0.027 (0.003)	-1.823	3.143

Lischer 2010). Haplotype diversity index (h) and nucleotide diversity (π) were estimated in DnaSP v.5 (Librado & Rozas 2009). We tested the hypotheses of selective neutrality using the D tests (Tajima 1989) and demographic expansion through Fs-statistics (Fu 1997).

We estimated genetic distance within and between populations in MEGA v. 7 (Kumar et al. 2016) using the evolutionary model K2P (Kimura 1980). Later, the genealogical relationship between haplotypes was obtained through a haplotype network created in the Haploviewer software (Salzburger et al. 2011) using the maximum likelihood algorithm determined in the jModelTest 2 (Darriba et al. 2012).

Bayesian Inference tree was constructed in Mr. Bayers on the CIPRES Science Gateway (Miller et al. 2010). The nucleotide substitution model was chosen from the analysis performed in jModeltest2 (Darriba et al. 2012), in which the GTR+G was indicated as the best model to fit the data set. Because *S. nebulosus* has doubts about its correct identification and geographical distribution, in which there are more than one species or population misidentification based on morphological and vocalization data (Lima et al. 2004, Hoogmoed & Avila-Pires 2011, Dias et al. 2015), we chose two analysis using genetic distance to classification and identification of lineage: Automatic Barcoding Gap Discovery (ABGD) (Puillandre et al. 2012) and Generalized Mixed Yule Coalescent (GMYC) (Pons et al. 2006).

The ABGD analysis (Puillandre et al. 2012) was performed on the web interface (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) following the priors, according to Ferrão et al. (2016), Kimura 2 parameter (K2P) nucleotide substitution model, ten recursive steps, gap width of 1.0 and value of intraspecific divergence of 0.03 (3%). In general, a divergence of 3% in the 16S gene in neotropical frogs, in this analysis, is recommended to identify different lineages (Vences et al. 2005, Fouquet et al. 2007). GMYC analysis (Pons et al. 2006) was implemented in the SPLITS package in R (R Core Team 2019). This analysis requires an ultrametric genealogical tree, estimated in BEAST 2.3.6. (Bouckaret et al. 2014), available in CIPRES Science Gateway (Miller et al. 2010). To obtain the ultrametric genealogical tree, a lognormal relaxed clock with a substitution rate of 0.00735 was constructed (Ferrão et al. 2016), with 50 million generations and burn-in of 10%.

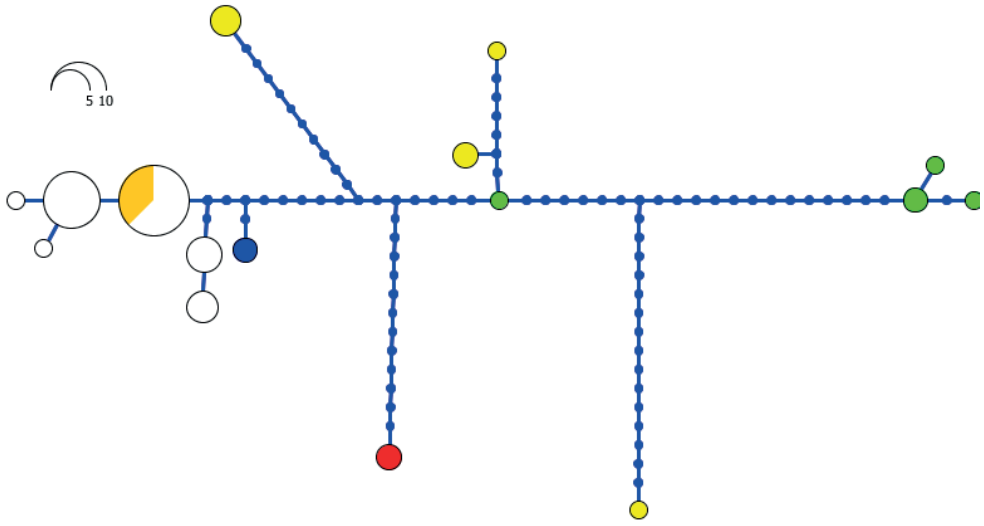

Figure 2. Haplotype network for *Scinax nebulosus* populations. The colors represent the following populations: White: Maranhão, Blue: Alagoas, Yellow: Pará; Green: Mato Grosso, Red: Goiás, Orange: Rio Grande do Norte.

RESULTS

The sequences from the rRNA 16S marker were 543 pb. The generated haplotype network (Figure 2) showed a sub-structure of the sample in two large groups: North and Northeast. The presence of exclusives haplotypes corroborates the high haplotypic (h) and nucleotide (π) diversity (Table I). The selective neutrality tests (Fs and D) were not significant for the sampled populations. Populations from the Alagoas and Goiás showed only one haplotype, which was not enough to make inferences of any indices.

The genetic distances obtained indicate the structure of the sample with a high divergence index between North and Northeast groups as well as the haplotype network. The largest genetic distances were calculated between the samples from the Rio Grande do Norte (RN) x Mato Grosso (MT), RN x Pará (PA), and RN x Goiás (GO) (6.9%, 5.9%, and 5.9% from divergence, respectively), GO and MT (5.7%) and MT and Maranhão (MA) (5.1%), while the distance between the samples of Alagoas (AL) and MA was only 1% (Table II). Rio Grande do Norte presented greater genetic distance when compared to MA and AL.

The AMOVA results (Table III) showed that the accumulated genetic variance between the groups was greater than within the groups. Nevertheless, a significant structure (Φ_{st}= 0.62) was observed in the sample. The comparison of F*st* values (Table IV) maintained the high values among all population pairs, being smaller only between the RN samples and the other localities.

Lineage delimitation analyses indicate the occurrence of 7 (ABGD) and 12 (GMYC) lineages, with divergences mainly between the samples from the Northeast group (Figure 3). In ABGD, samples from AL, MA, and RN are considered to belong to a single lineage, while GMYC delimited 4 lineages for these samples. Since *S. nebulosus*

Table II. Estimated genetic distance (p-distance) for the 16S gene for *Scinax nebulosus* populations at the locations listed below. PA = Pará, MT = Mato Grosso, MA = Maranhão, AL = Alagoas, RN = Rio Grande do Norte, GO = Goiás.

	MA	AL	PA	MT	GO
MA					
AL	0.010				
PA	0.041	0.037			
MT	0.051	0.046	0.048		
GO	0.043	0.040	0.045	0.057	
RN	0.028	0.032	0.059	0.069	0.059

Table III. AMOVA values of *Scinax nebulosus* populations used in the present study. Φ_{st} = Fixation index.

Source of variation	% of Variation	Φ_{st}
Among populations	62.50	0.62503
Within populations	37.50	

is a group with doubts about the correct identification along with its distribution and we do not have morphological data or vocalization to compare with our results, we chose to follow the most conservative result of these analyses, considering that we have 7 lineages in our sample.

Molecular clock analysis (Figure 4) identified that a single sample from RN presents an 8.7 Ma divergence from the other samples and the GO samples presented 4.3 Ma divergence. Among the other samples, we identified a separation between the North and Northeast with 2.7 Ma groups, and within these groups, subsequent most recent separations.

DISCUSSION

Scinax nebulosus is a species with wide geographical distribution, ranging from Venezuela to Guiana, Suriname, Bolivia, and Brazil (Central-West, North, and Northeast) (Dias et al. 2015, Frost 2020). This wide geographical distribution has been a source of controversy over the correct identification of populations of this species, where some authors believe the populations of northeastern Brazil constitute a different species from the populations of northern and western Brazil and Bolivia, based on morphological and vocalization data (Lima et al. 2004, Hoogmoed & Avila-Pires 2011, Dias et al. 2015).

Our results indicated the occurrence of genetic structuring in the sampled populations,

Table IV. Estimated pairwise *Fst* for the 16S gene for the *Scinax nebulosus* populations used in the present study. PA = Pará, MT = Mato Grosso, MA = Maranhão, AL = Alagoas, RN = Rio Grande do Norte, GO = Goiás.

	MA	AL	PA	MT	GO
MA					
AL	0.722				
PA	0.761	0.363			
MT	0.891	0.699	0.458		
GO	0.938	1.000	0.534	0.780	
RN	0.222	-0.070	0.314	0.471	0.428

from these data is also possible to infer greater genetic proximity of the individuals from Northeast (MA, AL, and RN) concerning PA, GO, and MT populations. This corroborates the hypotheses that the Northeast and North groups constitute distinct species. São Pedro (2014) performing a phylogeographic analysis of *Phyllomedusa* (= Pithecopus) (Anura, Hylidae) with the objective of recognizing cryptic species, as well as the elements involved in the diversification processes, observed high haplotypic diversity, high F*st* index among lineages, and the genetic distance calculations indicated a difference between and within the lineages, which results are similar to those found by us.

High genetic diversity and population structure were also found in other Brazilian anuran species like *Proceratophrys boiei* (Lynch 1971), (Prado & Pombal Jr 2008) (Odontophrynidae) and *Ischnocnema* gr. *ramagii* (Boulenger, 1888) (Brachycephalidae) in the highlands enclave in Northeast (Carnaval & Bates 2007); species of *Phyllomedusa* gr. *burmeisteri* (Boulenger, 1882) (Phyllomedusidae) in Atlantic Forest Atlântica and Brazilian Pampas (Brunes et al. 2010), *Rhinella* gr. *crucifer* (Wied-Neuwied, 1821) (Bufonidae) in Atlantic Forest (Thomé et al.

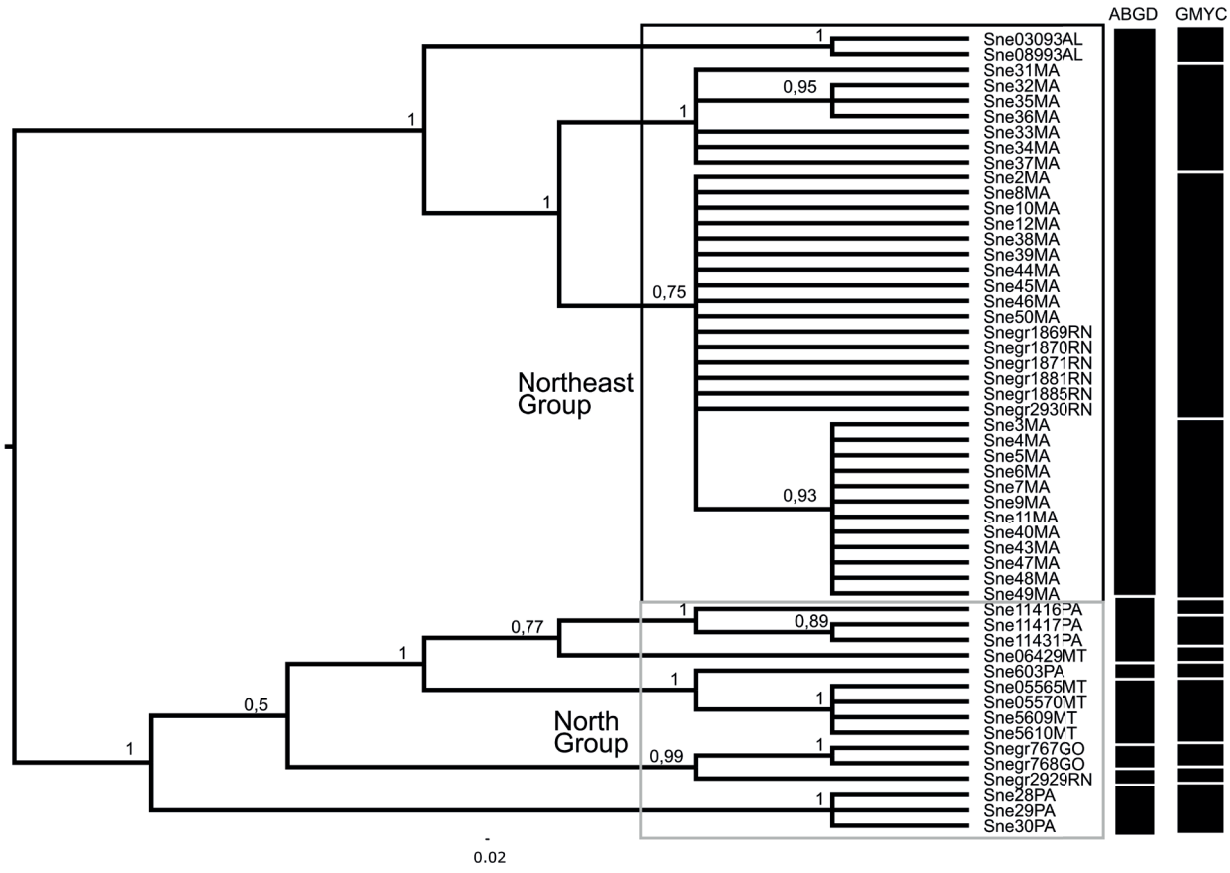

Figure 3. Bayesian Inference Tree for the 16S gene from the sampled populations of *Scinax nebulosus*. Black bars represent the results of the two lineages delimitation analyses. Numbers in branches indicate posterior probability values. ABGD= Automatic Barcode Gap Discovery, GMYC= Generalized Mixed Yule Coalescent.

2010), and *Hypsiboas albopunctatus* (Spix, 1824) (Hylidae) in Cerrado (Prado et al. 2012).

Brunes et al. (2014) and São Pedro (2014) highlight that genetic differentiation found in many studies about anurans has its origin related to vicariant events occurring in the Neogen and Quarternary periods, specifically since the end of the Pliocene and during the Pleistocene. Pleistocene refugia hypothesis, first proposed to explain the Amazon diversity, attributes the speciation process to the expansion and retraction biome dynamics during the Plio-Pleistocene transition period (Haffer 1969, Haffer & Prance 2002)

Although these events are not sufficient to explain the structure among populations of *S. nebulosus*, they seem to be the key to explaining the diversification of this species. For this Menezes et al. (2016), in an analysis of *S. eurydice* (Bokermann, 1968) (Anura, Hylidae), used molecular data to reveal the Plio-Pleistocene diversification proposed by the Pleistocene refugia theory. *Scinax eurydice* shows high genetic divergences in the lineages analyzed and the formation of two major clades corresponding to Northeast and Southeast groups (Menezes et al. 2016), as well as our populations of *S. nebulosus* also presented this structure to Northeast and North groups.

Bell et al. (2012) investigated how Pleistocene refugia influenced the genetic diversification in some species from *Scinax perpusillus* group. Through the analysis of mitochondrial markers, they obtained structured lineages

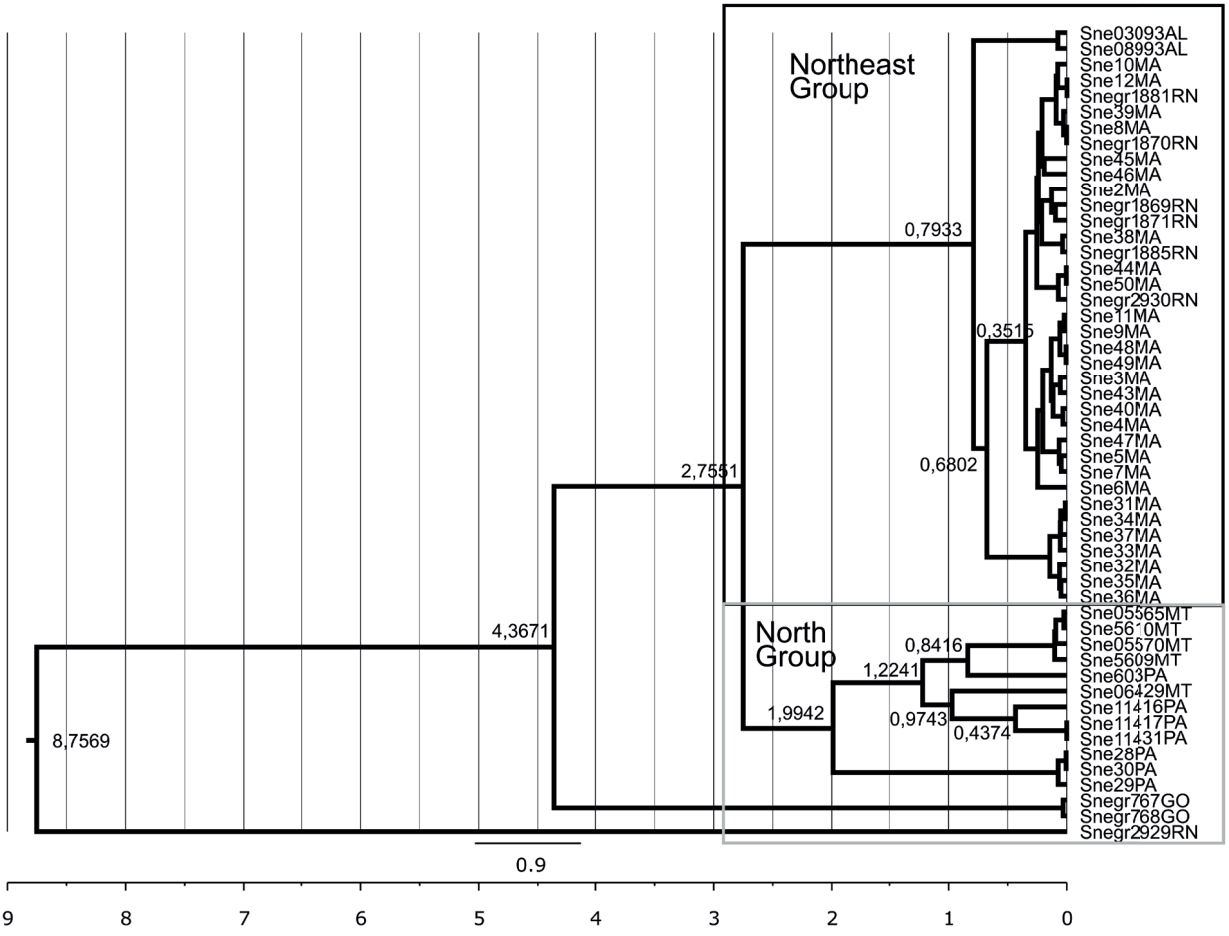

Figure 4. Relaxed molecular clock for the 16S gene from the sampled populations of *Scinax nebulosus*.

and high genetic diversity in the populations of the *S. perpusillus* species group. According to these authors, this structure is explained by the separation of populations between island and continent, since it deals with a fragment of Atlantic Forest. In addition, they also emphasize that this phylogeographic barrier is not known, but it occurs consistently. As presented by Bell et al. (2012), we could not identify the barrier responsible for the structuring found in the present study within *S. nebulosus*. These differences only demonstrate that species variation can occur in response to a shared climate history and indicate that further studies are needed to understand the roles of Pleistocene refugia and biogeographic barriers in anuran amphibian diversification.

Thus, we may suggest the existence of a mosaic of heterogeneous habitats that may be of great importance to anuran species, although this hypothesis needs further detail in molecular studies, including large sample sizes and other population and demographic analyses.

Besides, amphibians may be of particular interest in the investigation of diversification processes due to a tendency to present a strong genetic structure (Johns & Avise 1998), thus being considered excellent models in phylogeographic studies (Zeisset & Beebee 2008, Nuñez et al. 2011). Furthermore, they are more prone to the existence of cryptic species (Bickford et al. 2007, Fouquet et al. 2007), as already noted for some taxa in the Cerrado, Caatinga, and Atlantic Forest

(Prado et al. 2012, Fouquet et al. 2013, Viega-Menoncello et al. 2014).

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SUPPLEMENTARY MATERIAL

Table SI

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TMBF designed the experiments and collected the samples. TMBF, NMP, LNW, and JMSA conducted the analyses. IS provided reagents, materials, and analytical tools. TMBF, NMP, LNW and JMSA wrote the manuscript.