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Original Article

Identification of the taxonomic status of *Scinax nebulosus* and *Scinax constrictus* (Scinaxinae, Anura) based on molecular markers

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(With 4 figures)

Abstract

The validation of many anuran species is based on a strictly descriptive, morphological analysis of a small number of specimens with a limited geographic distribution. The *Scinax* Wagler, 1830 genus is a controversial group with many doubtful taxa and taxonomic uncertainties, due a high number of cryptic species. One example is the pair of species *Scinax constrictus* and *Scinax nebulosus*, which share a similar morphology. *Scinax constrictus* is restricted to the Brazilian Cerrado savanna, while *S. nebulosus* is widely distributed throughout northern South America. Despite the validation of many anuran species, discriminations based only on morphological traits is quite difficult due to the high conservative morphology of some groups. In this context, the present study uses mitochondrial and nuclear genes to provide a more consistent diagnosis and test the validity of *S. constrictus* as a distinct species from *S. nebulosus*, as well as evaluate the position of these taxa within the *Scinax* genus. The topologies obtained herein uphold the monophyletic status of *Scinax* based on all molecular markers assessed in this study, in all analytical approaches, with high levels of statistical support.

Keywords: anurans, cryptic species, molecular markers, phylogeny, *Scinax.*

Identificação do status taxonômico de *Scinax nebulosus* e *Scinax constrictus* (Scinaxinae, Anura), baseado em marcadores moleculares

Resumo

A validação de muitas espécies de anuros é baseada em uma análise morfológica e descritiva de um pequeno número de espécimes com uma distribuição geográfica limitada. O gênero *Scinax* Wagler, 1830 é um grupo controverso com muitos táxons duvidosos e incertezas taxonômicas devido ao grande número de espécies crípticas. Um exemplo são as espécies, *Scinax constrictus* e *Scinax nebulosus*, que compartilham uma morfologia similar. *Scinax constrictus* é restrito à savana do Cerrado brasileiro, enquanto *S. nebulosus* é amplamente distribuído pelo norte da América do Sul. Apesar da validação de muitas espécies de anuros, a discriminação baseada apenas em características morfológicas é bastante difícil, devido à alta morfologia conservadora de alguns grupos. Neste contexto, o presente estudo utiliza genes mitocondriais e nucleares para fornecer um diagnóstico mais consistente e para testar a validade de *S. constrictus* como uma espécie distinta de *S. nebulosus*, bem como avaliar a posição destes táxons dentro do gênero *Scinax*. As topologias obtidas confirmaram o status monofilético de *Scinax* com base em todos os marcadores moleculares, em todas as abordagens analíticas, com altos níveis de suporte estatístico.

Palavras-chave: anuros, espécies crípticas, marcadores moleculares, filogenia, *Scinax*.

1. Introduction

Phylogenetic systematics seeks evidence on the evolutionary relationships among species in order to comprehend diversity patterns (Lourenço et al., 2015). Neotropical amphibians are characterized by considerable phylogeographic structuring and profound levels of genetic divergence, which require extended periods of time for the evolution of phenotypical differences (Sá et al., 2014; Suk et al., 2020).

As a group, Neotropical amphibians present relatively conserved morphological characteristics and, in many cases, reliable diagnostic traits are difficult to identify (Nogueira et al., 2016), which often implies the existence of cryptic species, which have been increasingly identified in recent years (Guarnizo et al., 2015; Pinto et al., 2019). This means that the species-level diversity of many genera has long been underestimated. In this context, molecular analyses can be extremely useful for the identification of cryptic variations in morphologically identical populations (Lima, 2011; Takazone, 2015; Bessa-Silva et al., 2016).

The Hylidae family is one of the most diverse anuran groups, found in Europe, northern Africa and Asia, South and Central America, western India, Australia, and New Guinea (Salducci et al., 2002). The Hylidae group is currently one of the most diverse anuran families, comprising about 701 species, around 14.5% of all anurans. Almost half of these species (338 species in 27 genera) are found in Brazil (Frost, 2017).

In the recently proposed arrangement by Duellman et al. (2016), the *Scinax* (Wagler, 1830) genus was excluded from the Hylinae subfamily and assigned to a new subfamily, Scinaxinae, which contains 70 described species (Frost, 2017). This analysis also supported a new arrangement for species belonging to the *Scinax* genus, which were originally categorized into two groups, the *Scinax ruber* and *Scinax catharinae* clades. In this new classification, the entire *catharinae* clade was reclassified into the *Ololygon* genus (Duellman et al., 2016).

Despite the scope of the review by Duellman et al. (2016), the validity of some taxa and their position in the *Scinax* genus remain unresolved. *Scinax nebulosus* Spix, 1824 and *Scinax constrictus* Lima et al., 2004 are part of the *rostratus* group, as well as *Scinax rostratus* Peters, 1863, *Scinax boulengeri* Cope, 1887, *Scinax garbei* Miranda-Ribeiro, 1926, *Scinax kennedyi* Pyburn, 1973, *Scinax jolyi* Lescure & Marty, 2000, *Scinax pedromedinae* Henle, 1991, *Scinax proboscideus* Brongersma, 1933, and *Scinax sugillatus* Duellman, 1973. *Scinax nebulosus* ranges from southeastern Venezuela, through the Guianas and lower Amazon basin to the state of Alagoas, in northeastern Brazil, with some occurrence records for the Bolivian Amazon (Faivovich et al., 2005). *Scinax constrictus* is found exclusively in Cerrado savanna areas, and is amply distributed in the Brazilian state of Goiás.

The diagnostic characters traditionally used to identify *S. nebulosus* and *S. constrictus* are inadequate for the reliable differentiation of these species, leading to the uncertain taxonomic status of some specimens from populations historically identified as belonging to one of the two species. The increasing use of molecular data has reinforced the often enigmatic nature of amphibian morphology evolution (Menezes et al., 2016; Ferrão et al., 2016), and has revitalized the study of amphibian taxonomy. Many groups of amphibians are morphologically conservative and this, alongside their high convergence degree, has resulted in numerous erroneous interpretations of anuran phylogeny when based solely on morphological characteristics (Bossuyt and Milinkovitch, 2000; Guarnizo et al., 2015).

As morphological characters are not adequate to reliably distinguish these species, the genetic validity of populations diagnosed as *S. constrictus* or *S. nebulosus* was tested in the present study using both nuclear and mitochondrial data, as the lack of genetic data for these species reinforces the need for molecular analyses, allowing for assessments concerning the species arrangement within the *Scinax* genus.

2. Material and Methods

2.1. Sample collection and molecular procedures

S. nebulosus and *S. constrictus* specimens were obtained through donations and exchanges with scientific institutions, and new specimens were also sampled in the field (Figure 1, Table 1). Their collection was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), through permanent license number 52593–3. The sampling covers a wide range of localities within the geographic distribution of the species (Figure 1). The samples were complemented by *Scinax* sequences available in GenBank. It is important to note that this study does not aim to generate threats of extinction.

In the case of the newly collected specimens, muscle tissue samples were extracted from the posterior members and conserved in absolute alcohol in 1.5 mL microtubes. The specimens themselves were fixed in 10% formalin and stored in 70% ethanol and subsequently catalogued and deposited at the Federal University of Maranhão (UFMA) scientific collection.

Pseudis paradoxa, representing a closely-related hylid genus, was used as the outgroup, based on the recent phylogenies published for the genus (Faivovich et al., 2005; Pyron and Wiens, 2011; Duellman et al., 2016). Genomic DNA was isolated from muscle tissue using a phenol-chloroform protocol modified from Sambrook and Russel (2001). As negative amplification of some samples was noted, a Wizard® Genomic DNA Purification kit was applied, using the mouse tail protocol. DNA extraction confirmation and the quantification and quality determination of the extracted material was based on electrophoresis runs in 1% agarose gels.

2.2. Amplification and sequencing

Two mitochondrial genes (16S rRNA and Cytochrome Oxidase I (COI)) and the nuclear gene Rhodopsin were analyzed. Each gene was amplified with a specific pair

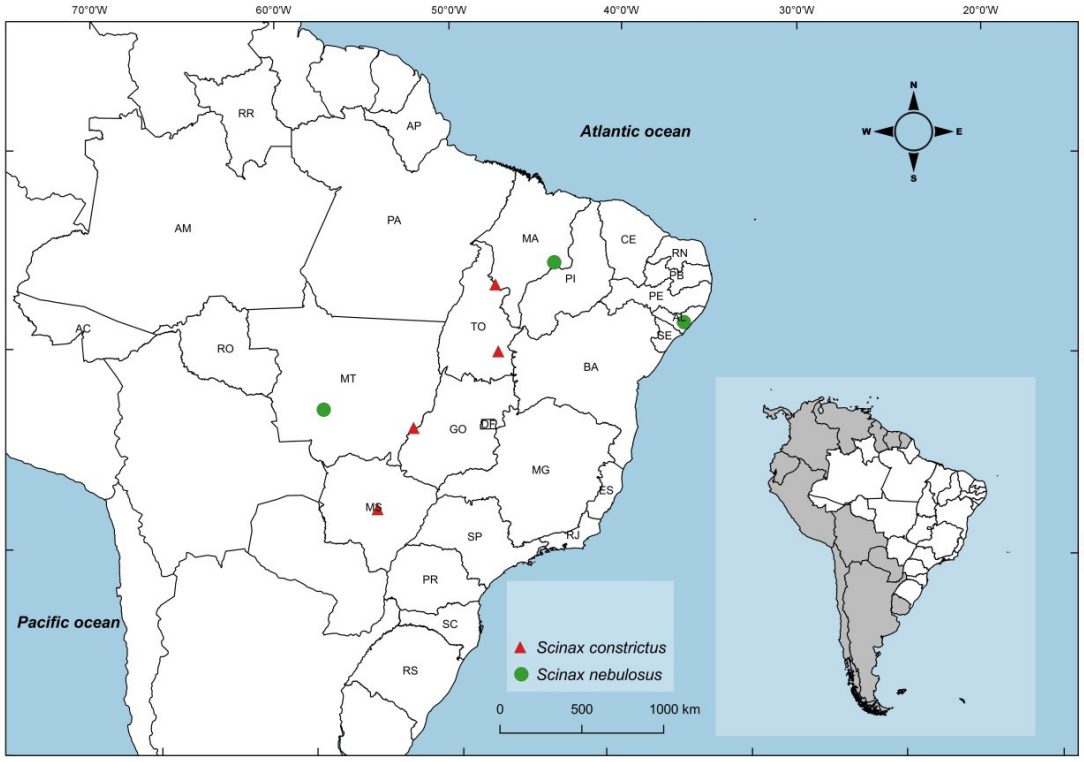

Figure 1. Sampling localities of the *S. nebulosus* and *S. constrictus* specimens included in the phylogenetic analyses.

Table 1. Sample origins and collection localities of the specimens analyzed in the present study.

Collection/Locality	Number
Goeldi Museum (MPEG) herpetological collection, Belém	14 samples
University of São Paulo (USP) herpetological collection	16 samples
USP Museum of Zoology (MZUSP)	15 samples
National Museum of Rio de Janeiro (MNRJ)	7 samples
UFRN Herpetological Collection	32 samples
São Luís, Maranhão	17 specimens
Raposa, Maranhão	12 specimens
Mirador, Maranhão	7 specimens
Itapiracó, Maranhão	1 specimen
Parauapebas, Pará	3 specimens
Total	92 samples

of primers. The primers described by Palumbi et al. (1991) were used for the 16S region, namelu L1987–5' GCCTCGCCTGTTTACCAAAAAC 3' and H2609–5'-CCGGTCTGAACTCAGATCACGT 3'. In the case of COI, the primers LCO-1490 5'GGTCAACAAATCATAAAGATATTGG 3' and HCO-2198 5'TAAACTTCAGGGTGACCAAAAAATCA 3' were used (Folmer et al., 1994), and in the case of Rhodopsin, the primers consisted of Rhod1A 5'ACCATGAACGGAACAGAAGGYCC 3' and Rhod1C 5' CCAAGGGTAGCGAAGAARCCTTC 3', described by Bossuyt and Milinkovitch (2000).

The sequencing reactions were prepared using the ABI Dye Terminator kit (Applied Biosystems), following the manufacturer's recommendations. Once amplified, the samples were injected into an ABI 3500XL automatic sequencer, resulting in a file with base sequences and the chromatogram.

It is important to note that, while an attempt was made to extract genomic DNA from all the samples and amplify all three markers, in some cases specimen conditions or tissue storage altered the quality of the extracted DNA, which was reflected in the amplification resolution.

2.3. Phylogenetic analyses

The DNA sequences were visualized using the Codoncode 3.0 (Codoncode Corporation), and aligned in MAFFT (Katoh and Standley, 2013), followed by visual

inspection to detect possible base identification errors. Variable sites were checked using the MEGA 7.0 software (Kumar et al., 2016), and genetic distance values were also calculated using this program

Two different phylogenetic inferences were applied herein, namely Bayesian Inferences (BI), performed using the Mr. Bayes v.3.2 software (Ronquist et al., 2012) and a Maximum Likelihood (ML) analysis, using the PhyML v.3.0 program (Guindon et al., 2010). The most appropriate evolutionary model for each database was identified using jModeltest 2 (Darriba et al., 2012). For the ML analysis, the reliability of each node was estimated using a nonparametric bootstrap procedure comprising 1000 replicates (Felsenstein, 1985). The Bayesian Inferences were based on MCMC sampling in four simultaneous runs, each with four chains (one hot and three heated) with a total run of 10 million generations. The Bayesian probabilities were selected based on a consensus of 60%. As a conservative measure, 25% of the initial trees were discarded as burn-in. The log-likelihood scores were plotted in the Tracer v.1.5 program (Rambaut and Drummond, 2009) to confirm the validity of the initial burn-in period used in Mr. Bayes. The post burn-in samples were used to build the trees used in the present study.

As the number of sequences obtained for the different markers varied, the results were presented in two different ways: (i) mitochondrial gene trees and (ii) a tree combining the 16S and Rhodopsin genes. In the latter case, only *S. constrictus* and *S. nebulosus* specimens in which both the 16S and Rhodopsin genes were amplified were included in the analyses.

Inconsistencies among gene trees and in species analyses based on multiple loci are relatively common in evolutionary studies (Nichols, 2001). In the present study, the phylogenetic reconstructions were compared using the BEAST v. 1.6.2 software (Rambaut and Drummond, 2007), in order to evaluate inter-specimen relationships (*BEAST) (Heled and Drummond, 2010). Runs comprising 80 million generations were conducted, with samples being retrieved every 10,000 generations, of which 10% were discarded as burn-in. The runs were analyzed for possible cuts, using Tracer v.1.6, available in the BEAST program (Rambaut and Drummond, 2007), to verify whether the Effective Sample Size (ESS) was adequate, in all cases. All the trees generated in the present study were edited in Figtree v.1.3.1.

3. Results

The database included 477 base pairs (bps) for the 16S gene, 615 bps for COI, and 295 bps for Rhodopsin. The concatenated 16S+Rhod database contained 772 bps.

The greatest genetic distance was recorded for the COI marker (Table 2), with a divergence of 12.7% between *S. nebulosus* and *S. constrictus*. In the case of the 16S gene (Table 3), the divergence was of 4.7%, whereas for the Rhodopsin gene, the genetic distance between the two species was of only 1% (Table 4).

The same evolutionary model was selected by jModelTest 2 for the COI and Rhodopsin genes, with a different model selected for the 16S gene (Table 5). As the topologies retrieved by both analytical approaches (BI and ML) were highly consistent and very similar to one another, only the BI trees are presented herein. The ML trees can be provided upon request.

Scinax was monophyletic in all topologies derived from all different genetic markers, and in all analytical approaches. Regarding the BI phylogenetic tree obtained for the 16S marker, a number of the *Scinax* species also formed well-supported monophyletic groups. In this topology, *S. nebulosus* and *S. constrictus* form a well-supported clade in which both species tend to form natural groups (Figure 2).

Table 2. Genetic distances between pairs of *Scinax* species, based on COI.

	Sco	**Sne**	**Sru**	**Swa**	**Sal**	**Scfke**	**Sro**	**Sac**	**Sna**	**Sfv**	**Sfm**	**Scr**	**Seu**	**Sma**	**Ssq**
Sne	**0.127**														
Sru	0.266	0.292													
Swa	0.260	0.262	0.248												
Sal	0.309	0.311	0.249	0.237											
Scfke	0.234	0.252	0.270	0.258	0.263										
Sro	0.222	0.227	0.262	0.239	0.235	0.079									
Sac	0.239	0.251	0.241	0.215	0.262	0.180	0.179								
Sna	0.252	0.295	0.186	0.224	0.238	0.266	0.272	0.273							
Sfv	0.264	0.294	0.209	0.227	0.264	0.267	0.252	0.238	0.220						
Sfm	0.280	0.310	0.236	0.231	0.213	0.265	0.252	0.229	0.264	0.256					
Scr	0.277	0.275	0.258	0.237	0.196	0.292	0.264	0.262	0.245	0.260	0.235				
Seu	0.283	0.304	0.257	0.224	0.227	0.245	0.238	0.227	0.234	0.213	0.224	0.224			
Sma	0.302	0.308	0.252	0.224	0.233	0.263	0.263	0.263	0.239	0.280	0.195	0.274	0.212		
Ssq	0.264	0.279	0.254	0.219	0.218	0.273	0.267	0.267	0.255	0.254	0.230	0.271	0.222	0.246	
Sxs	0.230	0.273	0.217	0.230	0.240	0.242	0.222	0.222	0.214	0.150	0.237	0.262	0.211	0.281	0.250

Species codes are as follows: Sco (*Scinax constrictus*), Sne (*S. nebulosus*), Sru (*S. ruber*), Swa (*S. wandae*), Sal (*S. alter*), Scfke (*S. cf. kennedyi*), Sro (*S. rostratus*), Sac (*S. acuminatus*), Sna (*S. nasicus*), Sfv (*S. fuscivarius*), Sfm (*S. fuscomarginatus*), Scr (*S crospedospilus*), Seu (*S. eurydice*), Sma (*S. maderae*), Ssq (*S. squalirostris*), Sxs (*S. x-signatus*).

Table 3. Genetic distances between pairs of *Scinax* species, based on 16S.

	Sne	**Sco**	**Scr**	**Sel**	**Swa**	**Siq**	**Sch**	**Ske**	**Sro**	**Sac**	**Sna**	**Sfm**	**Sbo**	**Sbou**	**Sjo**	**Scro**	**Seu**	**Sga**	**Sfv**	**Spro**	**Spe**	**Sru**	**Sxs**	**Sst**
Sco	**0.047**																							
Scr	0.197	0.187																						
Sel	0.16	0.165	0.154																					
Swa	0.175	0.172	0.167	0.102																				
Siq	0.157	0.156	0.107	0.104	0.12																			
Sch	0.198	0.207	0.179	0.146	0.151	0.114																		
Ske	0.126	0.11	0.179	0.198	0.21	0.189	0.214																	
Sro	0.141	0.12	0.197	0.228	0.218	0.213	0.239	0.048																
Sac	0.096	0.096	0.155	0.137	0.149	0.146	0.16	0.13	0.151															
Sna	0.199	0.199	0.161	0.115	0.122	0.139	0.175	0.236	0.253	0.17														
Sfm	0.193	0.201	0.203	0.113	0.157	0.164	0.209	0.231	0.269	0.172	0.179													
Sbo	0.197	0.198	0.183	0.109	0.154	0.155	0.211	0.208	0.22	0.175	0.141	0.169												
Sbou	0.145	0.14	0.179	0.145	0.157	0.158	0.181	0.135	0.151	0.113	0.193	0.176	0.178											
Sjo	0.098	0.097	0.136	0.13	0.159	0.132	0.155	0.089	0.119	0.081	0.193	0.196	0.165	0.09										
Scro	0.161	0.157	0.146	0.143	0.154	0.131	0.174	0.188	0.206	0.125	0.17	0.167	0.172	0.157	0.145									
Seu	0.179	0.176	0.136	0.12	0.122	0.138	0.162	0.167	0.186	0.125	0.125	0.169	0.148	0.149	0.141	0.144								
Sga	0.117	0.113	0.156	0.145	0.173	0.135	0.165	0.113	0.131	0.103	0.209	0.204	0.175	0.097	0.043	0.153	0.165							
Sfv	0.187	0.188	0.194	0.117	0.119	0.127	0.163	0.215	0.25	0.16	0.119	0.167	0.154	0.165	0.171	0.171	0.136	0.178						
Spro	0.117	0.113	0.156	0.14	0.175	0.132	0.159	0.117	0.149	0.1	0.208	0.188	0.167	0.099	0.034	0.16	0.162	0.046	0.182					
Spe	0.192	0.186	0.132	0.105	0.137	0.104	0.169	0.23	0.242	0.164	0.128	0.186	0.122	0.181	0.169	0.162	0.113	0.181	0.12	0.17				
Sru	0.22	0.226	0.156	0.137	0.139	0.141	0.148	0.237	0.257	0.17	0.103	0.18	0.16	0.186	0.183	0.184	0.133	0.199	0.146	0.192	0.141			
Sxs	0.213	0.215	0.186	0.145	0.152	0.131	0.201	0.26	0.27	0.2	0.129	0.18	0.154	0.197	0.203	0.206	0.16	0.209	0.106	0.214	0.142	0.142		
Sst	0.151	0.163	0.168	0.09	0.114	0.125	0.153	0.187	0.211	0.122	0.141	0.135	0.137	0.131	0.126	0.139	0.107	0.146	0.135	0.146	0.118	0.155	0.138	
Ssq	0.143	0.148	0.159	0.081	0.132	0.106	0.145	0.175	0.209	0.089	0.143	0.121	0.113	0.135	0.115	0.122	0.121	0.122	0.139	0.104	0.123	0.148	0.175	0.092

Species codes are as follows: Sne (*S. nebulosus*), Sco (*Scinax constrictus*), Scr (*S. crospedospilus*), Sel (*S. elaeochroa*), Swa (*S. wandae*), Siq (*S. iquitorum*), Sch (*S. chiquitanus*), Ske (*S. kennedyi*), Sro (*S. rostratus*), Sac (*S. acuminatus*), Sna (*S. nasicus*), Sfm (*S. fuscomarginatus*), Sbo (S. boesemani), Sbou (*S. boulengeri*), Sjo (S. *jolyi*), Scro (*S. crospedospilus*), Seu (*S. eurydice*), Sga (*S. garbei*), Sfv (S. fuscivarius), Spro (*S. proboscideus*), Spe (*S. perereca*), Sru (*S. ruber*), Sxs (*S. x-signatus*), Sst (*S. staufferi*), Ssq (*S. squalirostris*).

Table 4. Genetic distances between pairs of *Scinax* species, based on Rhodopsin.

	Sne	Sco	Sru	Sel	Sga	Sst	Ssq	Sna	Sfv	Sbou
Sco	**0.01**									
Sru	0.02	0.02								
Sel	0.02	0.02	0.01							
Sga	0.02	0.02	0.02	0.02						
Sst	0.02	0.03	0.02	0.01	0.02					
Ssq	0.01	0.02	0.01	0.01	0.01	0.01				
Sna	0.02	0.03	0.01	0.02	0.02	0.01	0.01			
Sfv	0.03	0.03	0.01	0.02	0.02	0.02	0.02	0.02		
Sbou	0.01	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.02	
Sac	0.02	0.02	0.01	0.01	0.01	0.01	0	0.01	0.02	0.01

Species codes are as follows: Sne (*S. nebulosus*), Sco (*Scinax constrictus*), Sru (*S. ruber*), Sel (*S. elaeochroa*), (*S. eurydice*), Sga (*S. garbei*), Sst (*S. staufferi*), Ssq (*S. squalirostris*), Sna (*S. nasicus*), Sfv (*S. fuscovarius*), Sbou (*S. boulengeri*), Sac (*S. acuminatus*).

Table 5. Evolutionary models used for the phylogenetic reconstructions applying the BI and ML methods.

Marker	AIC Evolutionary model (ML)	BIC Evolutionary model (BI)	–lnL values (ML/IB)
16S	TIM2+I+G	TIM2+I+G	4014.5271/ 4014.5271
COI	TrN+I+G	TrN+I+G	6761.4570 /6761.4570
Rhod	TrN+I+G	TrNef+G	791.6834/ 798.3193

Poorly-identified specimens (labeled as *S*. gr. *nebulosus*) *were* grouped with either *S. nebulosus* or *S. constrictus*. Some structuring was observed for *S. nebulosus* samples. Specimens from northeastern (Alagoas and Maranhão) and central (Goiás) Brazil were separated with high support from both the BI (0.97) and ML (98%) analyses, as were those from two central Brazilian Cerrado portions, namely Mato Grosso and Tocantins (BI = 1.00, ML = 99%) and Mato Grosso alone (BI = 1.00, ML = 99%).

Scinax constrictus appears as a sister species for *S. nebulosus* in both the BI and ML analyses. Similar findings were obtained in the COI trees (Figure 3), in which *S. constrictus* and *S. nebulosus* appear as genetically distinct species, with high support values for the phylogenetic analyses (BI = 0.98, ML = 100%). As in the 16S trees, the COI tree recovered *S. nebulosus* as a polyphyletic species, formed by at least three groups, with *S. constrictus* as a sister group. One group contained the specimen from Roraima, the second group encompassed the specimens from Mato Grosso, while the third group included the sequences from Alagoas, Pará, and Maranhão.

The species tree obtained from BEAST also indicates that *S. constrictus* and *S. nebulosus* are separate genetic lineages, with high support values (1). The analysis nevertheless confirmed the close phylogenetic relationship between both species (Figure 4).

4. Discussion

The genetic distances obtained from all three markers indicate that *S. constrictus* and *S. nebulosus* are distinct species. The divergence value recorded for the COI gene was of over 10%, a comparatively high value for differences between amphibian species (Vences et al., 2005a; Guarnizo et al., 2015). This differentiation was also supported by more conservative markers used in other studies (Nair et al., 2012; Nogueira et al., 2016) and in the present study, i.e., 16S and Rhodopsin.

Based on an experimental analysis, Vences et al. (2005b) concluded that the mitochondrial 16S rRNA gene satisfies the prerequisites for a universal DNA barcode marker for amphibians and may be superior to COI sequences for the identification of vertebrate clades. This study observed that, while COI varied considerably in amphibians, 16S was highly conserved in these vertebrates. The authors thus recommend the use of 16S as an additional marker for vertebrate DNA barcoding.

Xia et al. (2012) also support the use of the 16S rRNA gene for amphibian DNA barcoding, given that the authors indicated that genetic distances based on the Kimura 2-parameter (K2P) model revealed that the mean intraspecific distance was 1.4% in the COI gene, but only 0.3% in the 16S rRNA. The level of 16S rRNA variation obtained in the present study was higher than this aforementioned value, indicating a high degree of genetic variation among the study species, reinforcing their taxonomic validity.

Since the description of *S. constrictus* (Lima et al., 2004), many specimens of both species have been difficult to classify reliably, given the lack of precision in the traditional morphological approach for taxa differentiation. In general, taxa with uncertain morphological variations are considered to be species complexes, with cryptic forms. This is probably the case for both *S. nebulosus* and *S. constrictus*, which present high levels of genetic differentiation in morphologically similar forms. In such

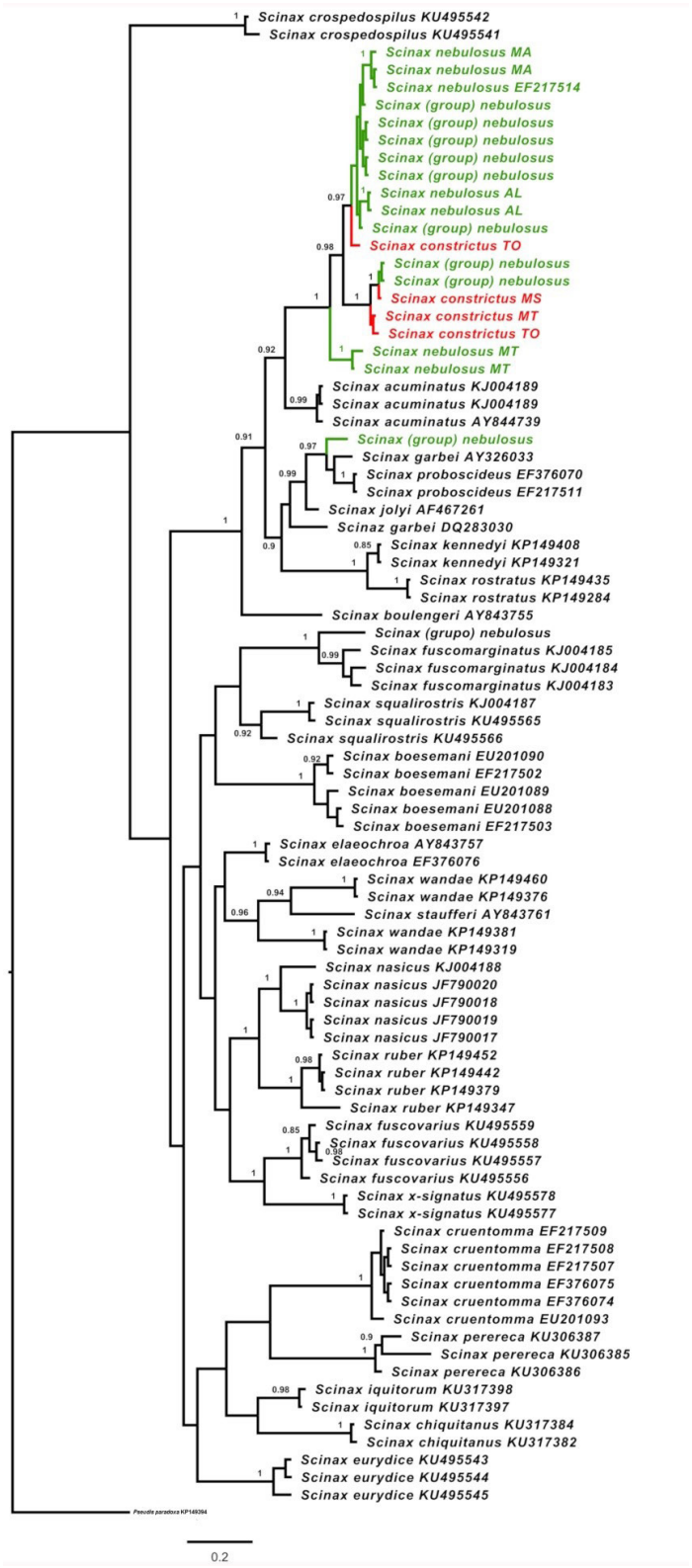

Figure 2. Tree obtained from the 16S gene analysis with significant bootstrap in the BI and ML analyses, respectively. Species name + GenBank voucher. The numbers on the branches represent the Bayesian (BI) support and bootstrap values (ML), respectively. Each color represents a monophyletic clade. The outgroups and paraphyletic groups are shown in black, with *S. nebulosus* in green and *S. constrictus* in red.

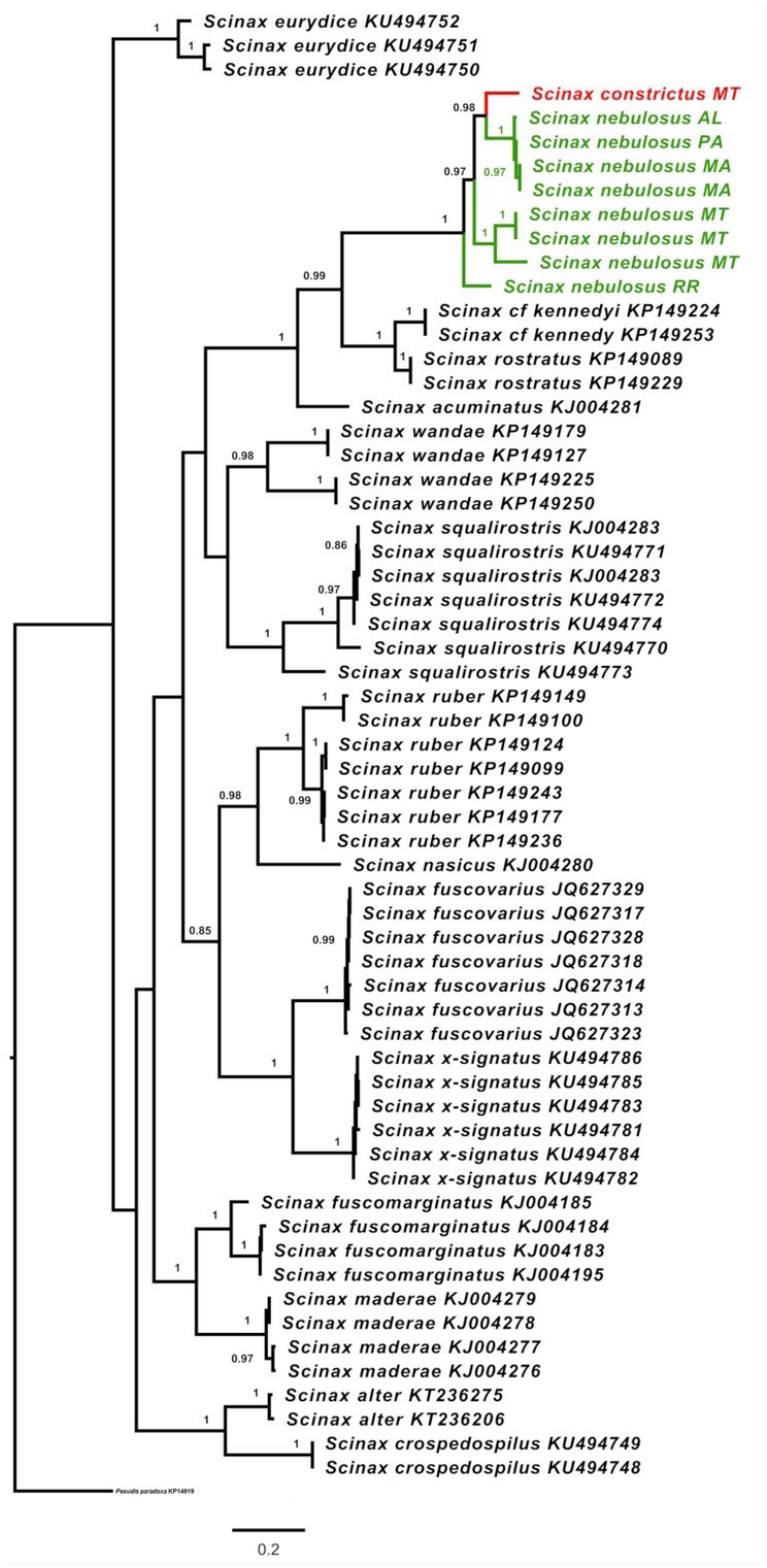

Figure 3. Tree obtained from the COI gene analysis with significant bootstrap in the BI and ML analyses, respectively. Species name + GenBank voucher. The numbers on the branches represent the Bayesian (BI) support and bootstrap values (ML), respectively. Each color represents a monophyletic clade. The outgroups and paraphyletic groups are shown in black, *S. nebulosus* in green and *S. constrictus* in red.

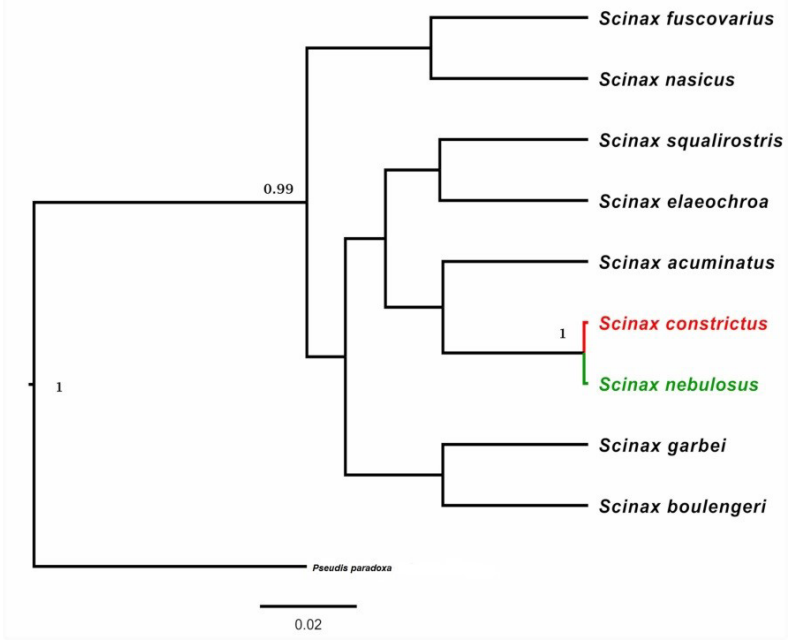

Figure 4. Species tree for the *Scinax* genus based on the concatenated data for the 16S and Rhodopsin genes. Values located at the nodes represent posterior probabilities, with only values equal to or above 0.99 shown.

cases, the molecular approach is essential for reliable species differentiation, as in the case of the *Rhinella marina* species complex, comprising distinct lineages composed of cryptic species (Bessa-Silva et al., 2016).

In addition to confirming *Scinax* as a monophyletic group, the topologies obtained herein support the validation of *S. constrictus* and *S. nebulosus* as distinct species. Some aspects of the arrangement within some of the clades were unexpected, however, which could invalidate the species as a monophyletic group. Most of the incongruent samples were specimens recently assigned to the *S. nebulosus* group, probably as a consequence of the unreliable morphological definition of the species. *Scinax constrictus* can be distinguished from *S. nebulosus* by its smaller size, the presence of a discrete anal flap in males (absent in *S. nebulosus*), and the presence of a row of small tubercles on the lower jaw margin, which is absent or indistinct in *S. nebulosus* (Lima et al., 2004). The advertisement call of *S. constrictus* is also quite distinct concerning dominant frequency and number of notes (Lima et al., 2004), even though these traits vary considerably in *S. nebulosus*. Despite these diagnoses, the analysis of specimens diagnosed previously as *S. nebulosus* or *S. constrictus* indicate that these traits are not adequate for reliable species distinction (Weber, L.N. personal communication).

It is, therefore, clear, that morphological analyses are extremely vulnerable to error and taxa misidentification, made apparent by molecular tools. The geographic distributions of both taxa appear to support the molecular diagnosis, given that *S. constrictus* appears to be restricted to the Cerrado savanna of central Brazil, whereas *S. nebulosus* is more widely distributed, and may even be sympatric with *S. constrictus*.

Genetic analyses have been widely applied to validate anuran species described on the basis of morphological characteristics. Bruschi et al. (2013), for example, evaluated the taxonomic status of *Phyllomedusa hypochondrialis* (Anura, Hylidae, Phyllomedusinae) applying cytogenetic and molecular data to differentiate this species from *Phyllomedusa azurea*. Their results contributed to the extension of the geographic range of *P. hypochondrialis*, and confirmed its sympatry with *P. azurea*, given that incorrect specimen identification appeared to have resulted in inconsistencies in the original *P. azurea* geographic distribution definition. In the present study, the analysis of the 16S gene (Figure 2) indicated the grouping of *S. nebulosus* and *S. constrictus* specimens with *S. constrictus* specimens originally assigned to the *nebulosus* group, which appears to have been the result of species misidentification. In the case of both *Phyllomedusa* Bruschi et al. (2013) and *Scinax* (present study), molecular analyses indicated clearly a monophyletic position within the Hylidae family.

Brusquetti et al. (2014) reviewed the taxonomy of *Scinax fuscomarginatus* (Lutz, 1925), and evaluated morphologically similar species exhibiting controversial taxonomy, such as *S. fuscumarginatus*, *S. parkeri*, *S. trilineatus*, *S. lutzorum* and *S. pusillus*, by molecular analyses, with paraphyletic clades being recuperated for *S. fuscomarginatus* in comparison to the other species, similar to the paraphyletism between *S. nebulosus* and *S. constrictus* reported in the present study (Figures 2 and 3). Duellman et al. (2016) subsequently redistributed *Scinax* species, and among the species analyzed

by Brusquetti et al. (2014), only *S. fuscomarginatus* was maintained in the genus.

In their phylogenetic and biogeographic analyses of the Hylidae family, Duellman et al. (2016) proposed major changes in the *Scinax* genus arrangement, ranging from a new subfamily to species redistribution. *Scinax nebulosus* and *S. constrictus* remained in the genus, and the results of the present study confirm their status as valid *Scinax* genus lineages.

A number of the species described in the *Scinax* Wagler (Anura: Hylidae) genus require genetic validation, and the application of molecular markers has not only contributed to the identification of new species, but also in the differentiation of cryptic lineages (Nunes et al., 2012; Lourenço et al., 2013; Sturaro and Peloso, 2014; Nogueira et al., 2016).

The molecular phylogeny of Fouquet et al. (2007) indicates that *Scinax ruber*, found in the French Guiana region, is represented by six divergent lineages. The molecular phylogeny obtained by Lourenço et al. (2013) confirmed that the *Scinax* specimens collected at the Chapada dos Guimarães National Park, in central Brazil, which are morphologically similar to species from the *S. catharinae* group (*sensu* Faivovich et al., 2005), do, in fact, represent a new species. Nogueira et al. (2016) emphasize the need for further comparative analyses between these species, integrating morphological and bioacoustics data.

In this context, many researchers have highlighted the importance of a multiple approach, i.e., integrating molecular, morphological, bioacoustic, and ecological data, in taxonomic anuran assessments, in facilitating species identification (Padial et al., 2014; Silva, 2017; Pinto et al., 2019). Overall, molecular analyses aided in differentiating populations diagnosed as *S. constrictus* and *S. nebulosus*, despite the morphological similarities of both species.

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