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ARTICLE

DNA barcoding reveals a new population of the threatened Atlantic Forest frog *Sphaenorhynchus canga*

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Abstract: Species identification plays a significant role in biodiversity conservation. As many species remain unrecognized, particularly in neotropical hotspots like the Brazilian Atlantic Forest (AF), novel molecular techniques are being widely employed to bridge this gap. In this study, we used DNA barcoding and phylogenetic tools to identify a new population of *Sphaenorynchus canga* in the central region of the Brazilian AF. Our results extend the species' known distribution by approximately 200 km to the south, encompassing a different mountain range than its type locality (Serra do Espinhaço). This disjunct distribution, while not uncommon among amphibians, suggests a historical connection between these two mountain complexes as a biogeographic explanation. Despite the discovery of a new *S. canga* population, the species continues to face numerous anthropogenic threats such as mining, land use, and cattle ranching. Urgent conservation and research efforts are warranted to ensure the survival of *S. canga* populations across these habitats.

Keywords: 16S mtDNA, Hylidae, Mantiqueira mountain range, Minas Gerais, species identification.

Abbreviations: ASAP – Assemble Species by Automatic Partitioning | CAUFJF – Coleção de Anfíbios da Universidade Federal de Juiz de Fora | ICMBIO – Instituto Chico Mendes de Conservação da Biodiversidade | IUCN – International Union for Conservation of Nature | MCMC – Markov chain Monte Carlo | ML – Maximum Likelihood | mtDNA – mitochondrial DNA | PCR – Polymerase chain reaction | SISBio – Sistema de Autorização e Informação em Biodiversidade | ZUFMS-AMP – Coleção Zoológica da Universidade Federal de Mato Grosso do Sul.

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Species identification is a crucial component of biodiversity research and conservation (Delić et al. 2017; Lyra et al. 2017; Sheth & Thaker 2017). To this end, DNA barcoding has become a widely used molecular technique for identifying species. This approach relies on sequencing a standardized fragment of DNA that can be compared to reference databases to accurately identify species (Gehara et al. 2013; Koroiva & Santana 2022). DNA barcoding has also proven to be effective in delimiting species, and it has been applied across a wide range of taxa, including amphibians (Jansen et al. 2011; Koroiva et al. 2020; Koroiva & Santana 2022).

Delimitation and identification of amphibians using robust methods is paramount, given that they are the most threatened group of terrestrial vertebrates worldwide (Howard & Bickford 2014; Cox et al. 2022; Toledo et al. 2023). Many species of amphibians are being classified under the IUCN Red List categories at the same time they are being formally named (Brasileiro et al. 2007; Caramaschi & Cruz 2011; Assis et al. 2013). Atlantic Forest, a biodiversity hotspot (Myers et al. 2000; Ribeiro et al. 2011; Zachos & Habel 2011), harbors more than 625 amphibian species, 77% of them endemic, and many with very narrow distributions (Rossa-Feres et al. 2017). Since the arrival of the first European colonizers in the early 16th century, the Atlantic Forest has lost most of its original cover, and the remaining is heavily fragmented (Ribeiro et al. 2009, 2011). In Brazil, habitat loss is the main threat to amphibians living in this rainforest (ICMBio 2018); 41 species are in peril, and two are already declared extinct (Ministério do Meio Ambiente 2022). The Atlantic Forest is also the region with the highest amphibian population declines reported worldwide (Toledo et al. 2023). Therefore, identifying and describing the amphibian diversity of the Atlantic Forest is crucial for its conservation and for developing targeted conservation strategies.

The landscape of the southeastern portion of the Atlantic Forest includes many mountain ranges that are considered cradles of amphibian diversity (Leite et al. 2008; Neves et al. 2018; Silva et al. 2018). These mountain chains harbor most of the endemic amphibian species from the Atlantic Forest (Guedes et al. 2020), and many are threatened (Pontes & Guidorizzi 2023). One such species is the Hatchet-faced Canga Lime Treefrog, *Sphaenorhynchus canga*, first described in 2015 (Araujo-Vieira et al. 2015) and known only from a small area in the southern portion of the Espinhaço Mountain range in Minas Gerais (Silveira et al. 2020). The species is classified by the Brazilian Ministry of Environment as Critically Endangered (Ministério do Meio Ambiente 2022; Pontes & Guidorizzi 2023).

During field expeditions in the northern portion of the Mantiqueira Mountain range in southern Minas Gerais in December 2015, January 2020, and November 2021, a series of specimens of *Sphaenorhynchus* were collected. We collected five adult male specimens during visual and acoustic searches in one pond in the countryside of Bom Jardim de Minas, Minas Gerais (-22.004, -44.180; 1,210 m; datum = SAD69). Specimens were euthanized in a 2% lidocaine chlorhydrate solution (MCTIC 2018), fixed in 10% formalin, and preserved in 70% alcohol. Prior to fixation, we collected tissue samples (muscle and liver) and stored them in cryotubes filled with 100% ethanol. Voucher specimens and tissues were deposited in the Coleção de Anfíbios da Universidade Federal de Juiz de Fora (CAUFJF), Juiz de Fora municipality, Minas Gerais, and in the Coleção Zoológica da Universidade Federal de Mato Grosso do Sul (ZUFMS-AMP), Campo Grande municipality, Mato Grosso do Sul, Brazil. Collection permits for this study were issued by ICMBIO (SISBio 73975-1 and 72874-1).

DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (Valencia, California, USA) following the manufacturer's protocol. Next, a fragment of the mitochondrial 16S gene was amplified using primers 16Sar and 16Sbr (Palumbi et al. 2002). The PCR protocol was configured with one initial phase of 94°C for 3 min, followed by 35 cycles of 94°C for 20s, 50°C for 20s, 72°C for 60s, and a final extension phase of 72°C for 5 min. Purification of PCR products and sequencing were performed by Eurofins Genomics Inc. (Louisville, Kentucky, USA). Comparable 16S sequences of *Sphaenorhynchus* from GenBank and one sequence of *Scinax fuscovarius* to use as an outgroup were downloaded (Supplementary Table 1). All 16S mtDNA gene fragments were aligned using the MAFFT algorithm (Katoh & Toh 2008) in Geneious v9.0.5 with default settings. The final dataset comprised 53 sequences of a 515 base-pair (bp) fragment of the 16S gene. A maximum likelihood tree was inferred in RAxML (Stamatakis 2014) via raxmlGUI 2.0 (Edler et al. 2021). The analysis was conducted using a ML + rapid bootstrap setting with a GTR+I+G substitution model and 1,000 bootstrap replicates. The appropriate substitution model was confirmed with Modeltest (Darriba et al. 2020) in raxmlGUI 2.0. Additionally, PTP and bPTP species delimitation analyses were conducted (Zhang et al. 2013) using the ML Tree. Calculations were performed on PTP webserver (http: //species.h-its.org/ptp/) with 500,000 MCMC generations, thinning set at 100, and burn-in at 10%. In addition, we performed the

delimitation method Assemble Species by Automatic Partitioning (ASAP) on the online server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) using a simple distance model to compute distances between samples and default parameters (Puillandre et al. 2021). To explore relationships among mtDNA haplotypes, we estimated a 16S haplotype network among species closely related to *S. canga*—*S. botocudo*, *S. cammaeus*, *S. caramaschii*, *S. platycephalus*, and *S. surdus* (Pereira et al. 2022)—in POPART (Leigh & Bryant 2015) using the median-joining network method. We depict each species using different colors in the haplotype network. Lastly, we calculated sequence divergence (uncorrected *p*-distance) among species/individuals using MEGA v10.1.1 (Kumar et al. 2018).

We identified the *Sphaenorhynchus* from Bom Jardim de Minas as *Sphaenorhynchus canga* (Image 1). Our maximum likelihood tree (Figure 1) of the mitochondrial 16S gene confidently (bootstrap = 0.98) placed the sequenced specimens with *Sphaenorhynchus canga*, sister to a clade formed by *S. botocudo* and *S. surdus*. The three species delimitation methods we used yielded the same results, recovering one evolutionary entity for each known species (Figure 1). All three analyses confidently recovered all populations of *Sphaenorhynchus canga* as a single evolutionary lineage. Our haplotype network (Figure 2) shows a clear separation between all species of *Sphaenorhynchus*. The genetic distance between *S. canga* from Bom Jardim de Minas and *S. canga* from the type locality was 0.4% (Supplementary Table 2). Overall, the morphology of *S. canga* from Bom Jardim de Minas also have the standard diagnosis of the species presented in its original description, such as the lack of tympanic membrane, the snout protruding in profile, the presence of a canthal white line, a dorsolateral white line from the eye to sacral region, and a dorsolateral black line from the tip of snout extending beyond the eye and disappearing up to the flanks (Araujo-Vieira et al. 2015). The newly discovered population of *S. canga* in Bom Jardim de Minas extends the distribution of the species by about 200 km southward to a different mountain range, Serra da Mantiqueira (Image 2).

The distribution of *S. canga* in both the southern Espinhaço and the northern Mantiqueira mountain ranges is a pattern observed in other anuran species as well, including *Bokermannohyla feioi*, *Pithecopus ayeaye*, *Physalaemus maximus*, and *Scinax tripui* (Baêta et al. 2007; Magalhães et al. 2017; Silveira et al. 2019; Brunes et al. 2023). This shared distribution pattern has led biogeographers to hypothesize a historical connection between the Espinhaço and Mantiqueira mountain ranges (Magalhães et al. 2017; Neves et al. 2018; Brunes et al. 2023). The discovery of *S. canga* in the Mantiqueira Mountains adds additional support for a historical connection between these mountain ranges and increases the potential area where this species could occur, particularly in protected areas in the northern portion of Serra da Mantiqueira.

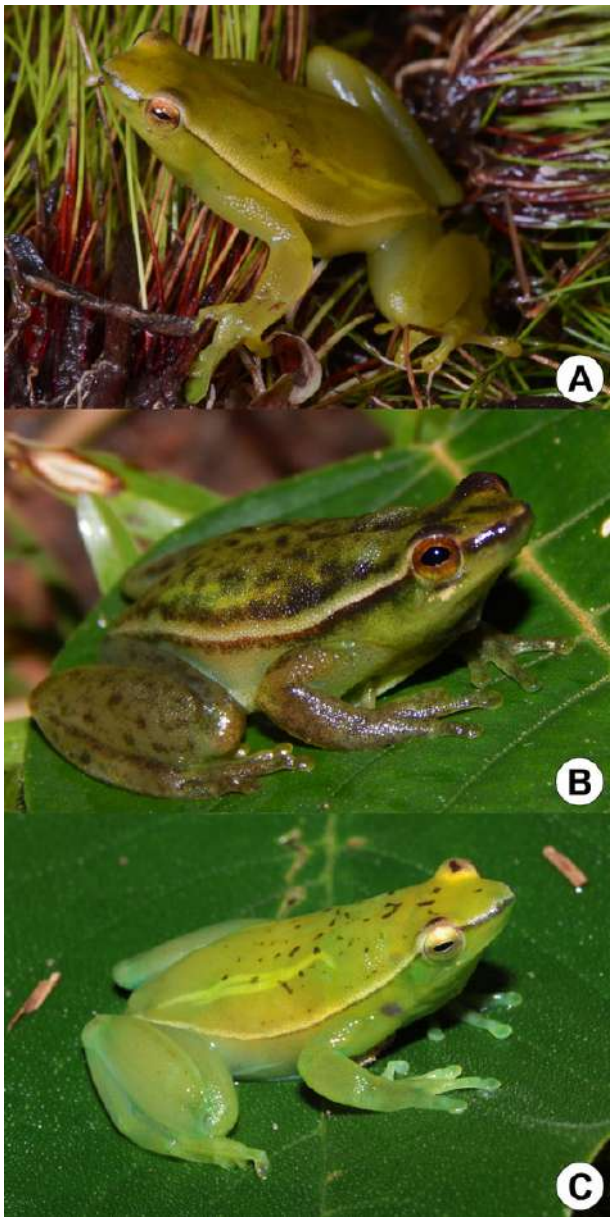

Image 1. Adult males of *Sphaenorhynchus canga* documented in Bom Jardim de Minas, Minas Gerais, Brazil. A—MAP6807 collected on 1 November 2021 | B–C—unvouchered specimens photographed on 9 December 2015. © A—DJ Santana, B&C—EA Pereira.

While the International Union for Conservation of Nature (IUCN) Red List of Threatened Species classifies this species as of Least Concern (IUCN & Boitatá 2023),

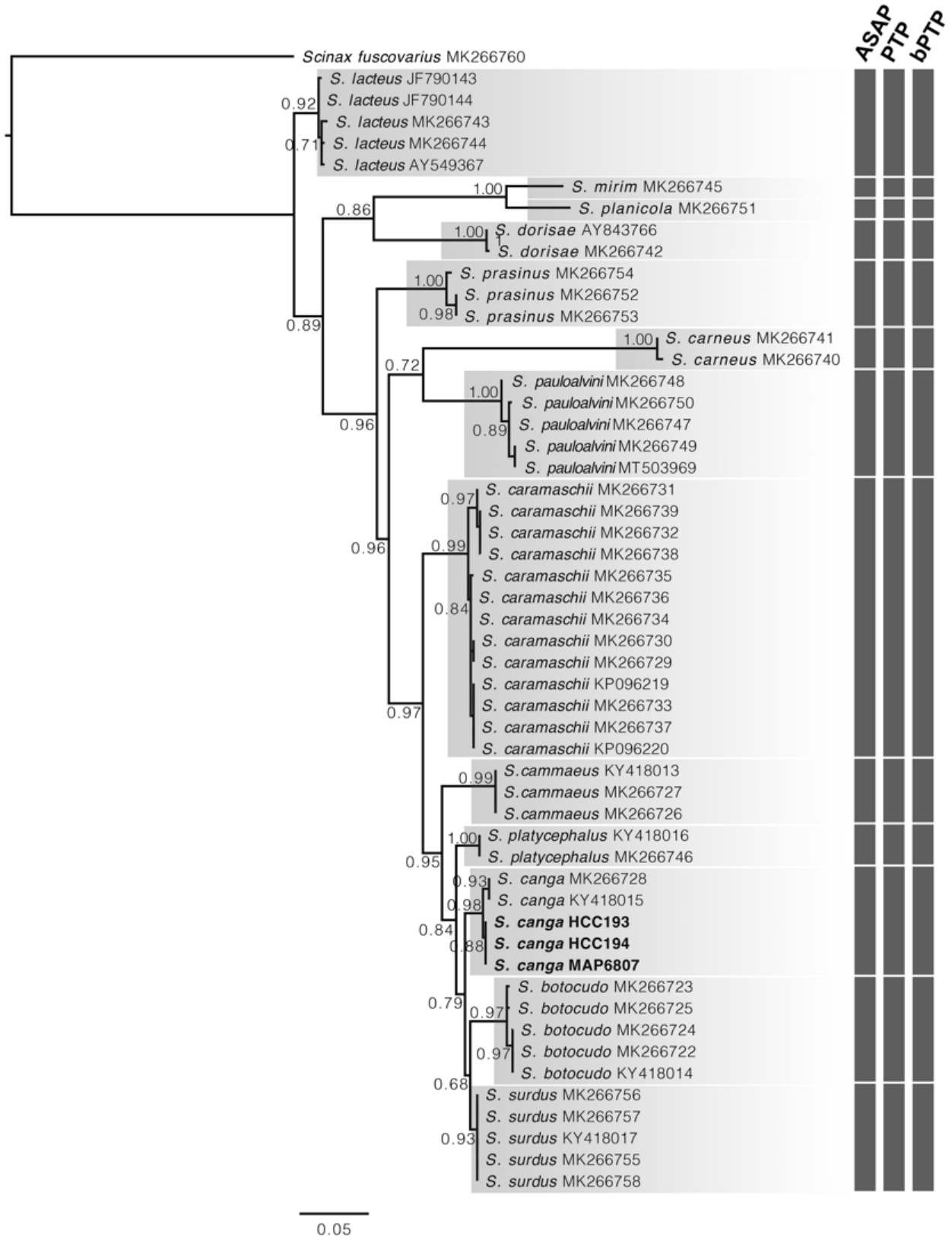

Figure 1. Maximum likelihood tree for *Sphaenorhynchus* based on the mitochondrial 16S gene. Nodes are labeled with Bootstrap values. Vertical grey bars on the right represent each evolutionary entity delimited by ASAP (Assemble Species by Automatic Partitioning), PTP (Poisson Tree Process), and bPTP (Bayesian Poisson Tree Process).

the latest update of the Brazilian Red List, which is based on IUCN criteria, classifies *Sphaenorhynchus canga* as Critically Endangered (Pontes & Guidorizzi 2023) due to its limited geographic distribution and restriction to ponds in ironstone outcrops, a habitat severely impacted by mining (Bastos et al. 2022). The IUCN assessment states that 'there are no ongoing major threats, the species is a habitat generalist occurring even in modified areas, and it is presumed to have a large and stable population' (IUCN & Boitatá 2023). However, mining activity poses a significant threat to *S. canga*. Five out of six ponds where the type series was collected are influenced by mining activities (Pena et al. 2017). Mining activity has resulted in a continuous decline in both the area and quality of *S. canga*'s habitat due to the suppression of ironstone outcrops and vegetation (Bastos et al. 2022). Recent surveys have had some success in finding the species in additional localities and habitats, including perennial small dams and anthropogenic swamps inside or on the edge of semi-deciduous seasonal forests, suggesting that it may have some degree of ecological plasticity; nonetheless, the species' spatial extent has only slightly increased because of these discoveries (Silveira et al. 2020). The discovery of *S. canga* in Bom Jardim de Minas is an important contribution to the conservation of this species, as research on its geographic distribution is among the main priorities (Bastos et al. 2022). Although our discovery has increased the species' distribution by more than 200 km (straight-line distance), it is important to note that is still restricted to high-elevation areas, reproducing in ponds (Araujo-Vieira et al. 2015; Silveira et al. 2020). Furthermore, no known populations of the species occur in protected areas (Bastos et al. 2022; this study).

While this discovery provides a glimmer of hope for the species, it is essential to note that the new population was found adjacent to a dirt road and in areas designated for cattle ranching, where vegetation around marshes is typically burned annually by local farmers. Moreover, the region is experiencing an increase in real estate speculation for allotments, and a proposal is currently under consideration for the installation

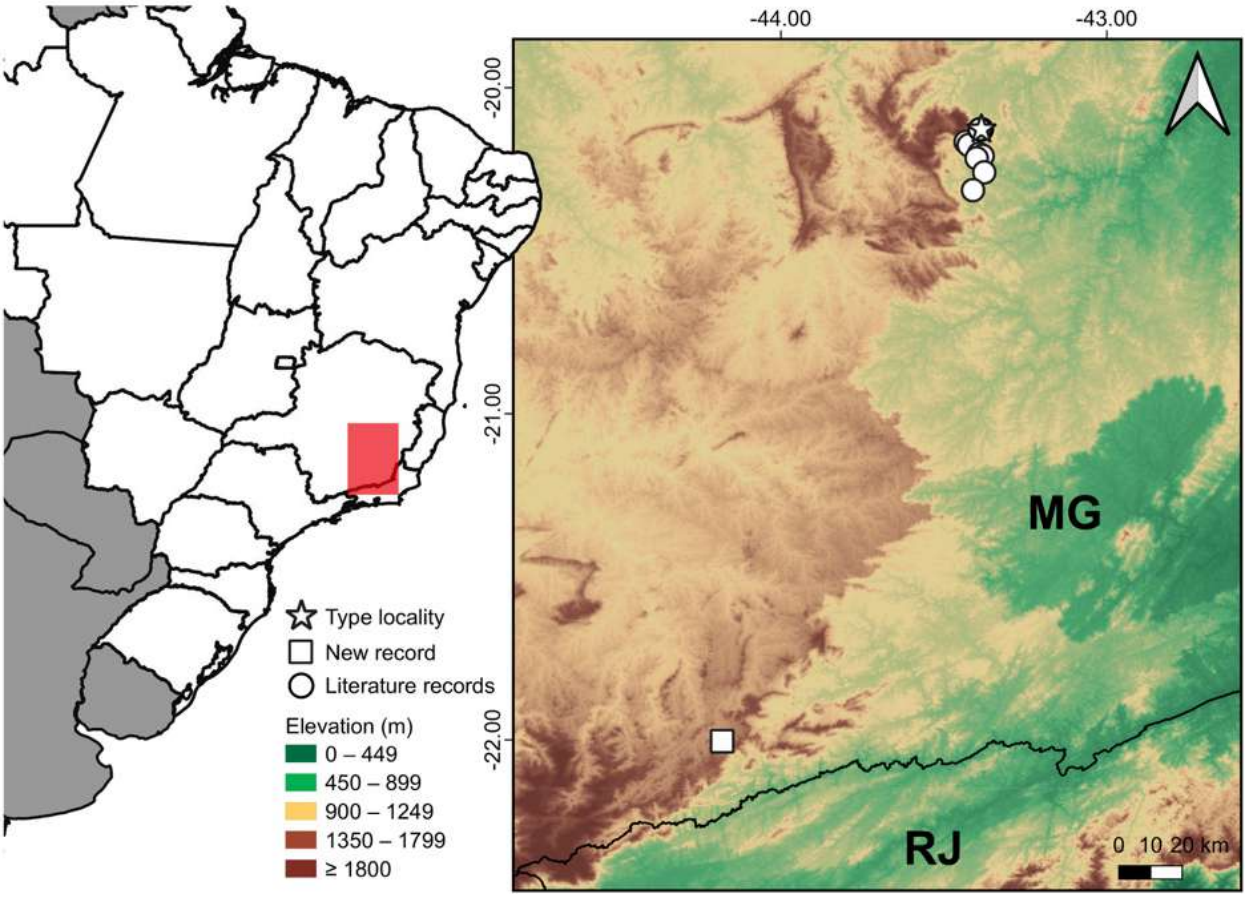

Image 2. Brazil state and elevation map showing the known geographic distribution of *Sphaenorhynchus canga* in the Espinhaço and Mantiqueira mountain ranges. State abbreviations: MG—Minas Gerais | RJ—Rio de Janeiro.

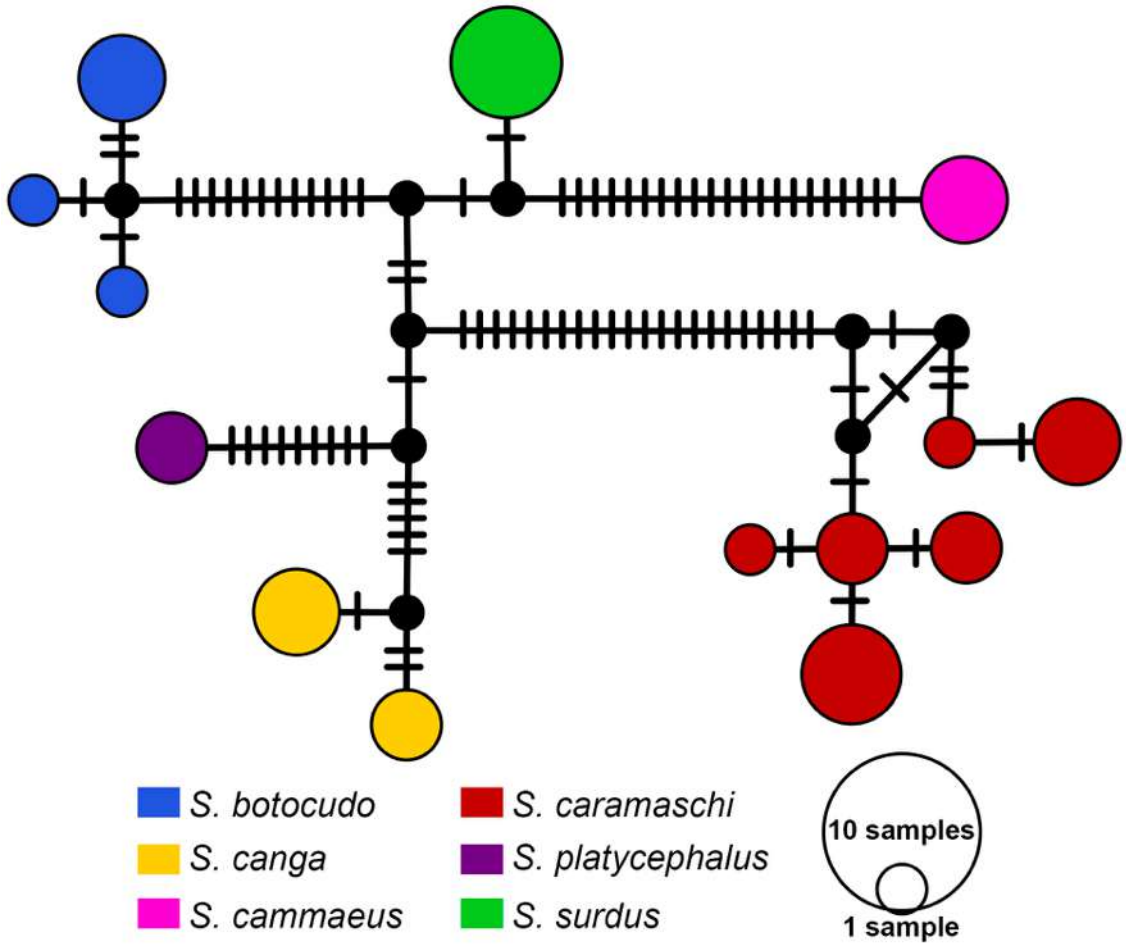

Figure 2. Median-joining haplotype network of *Sphaenorhynchus canga* and closely related species based on 16S mtDNA. Each haplotype is represented by a circle whose area is proportional to its frequency. Traits indicate additional mutational steps for branches with more than one mutation. Different colors indicate species-level units. The black dots are median vectors (hypothesized sequences).

of a hydroelectric power plant at the Pacau waterfall (Cachoeira do Pacau), just 5 km from the discovered population. Therefore, future visits to the locality are of utmost importance to monitor this population and to search for additional areas where the species may be present. A reevaluation of the conservation status of *S. canga* based on this discovery is beyond the scope of this work. Nonetheless, it is evident that this finding underscores the urgent need for further research, conservation measures, and advocacy efforts to ensure the survival of this critically endangered species.

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Supplementary Table 1. GenBank accession numbers for mitochondrial 16S gene sequences of specimens of Sphaenorhynchini (*Sphaenorhynchus* and *Gabohyla*) and the outgroup *Scinax fuscovarius* included in the molecular analyses.

Species	Genbank accession number	Reference
S. botocudo	KY418014	Roberto et al. (2017)
S. botocudo	MK266722	Araujo-Vieira et al. (2019)
S. botocudo	MK266723	Araujo-Vieira et al. (2019)
S. botocudo	MK266724	Araujo-Vieira et al. (2019)
S. botocudo	MK266725	Araujo-Vieira et al. (2019)
S. cammaeus	KY418013	Roberto et al. (2017)
S. cammaeus	MK266726	Araujo-Vieira et al. (2019)
S. cammaeus	MK266727	Araujo-Vieira et al. (2019)
S. canga	KY418015	Roberto et al. (2017)
S. canga	**HCC193**	**Present Work**
S. canga	**HCC194**	**Present Work**
S. canga	**MAP6807**	**Present Work**
S. canga	MK266728	Araujo-Vieira et al. (2019)
S. caramaschii	KP096219	Araujo-Vieira et al. (2015)
S. caramaschii	KP096220	Araujo-Vieira et al. (2015)
S. caramaschii	MK266729	Araujo-Vieira et al. (2019)
S. caramaschii	MK266730	Araujo-Vieira et al. (2019)
S. caramaschii	MK266731	Araujo-Vieira et al. (2019)
S. caramaschii	MK266732	Araujo-Vieira et al. (2019)
S. caramaschii	MK266733	Araujo-Vieira et al. (2019)
S. caramaschii	MK266734	Araujo-Vieira et al. (2019)
S. caramaschii	MK266735	Araujo-Vieira et al. (2019)
S. caramaschii	MK266736	Araujo-Vieira et al. (2019)
S. caramaschii	MK266737	Araujo-Vieira et al. (2019)
S. caramaschii	MK266738	Araujo-Vieira et al. (2019)
S. caramaschii	MK266739	Araujo-Vieira et al. (2019)
S. carneus	MK266740	Araujo-Vieira et al. (2019)
S. carneus	MK266741	Araujo-Vieira et al. (2019)
S. dorisae	AY843766	Faivovich et al. (2005)
S. dorisae	MK266742	Araujo-Vieira et al. (2019)
S. lacteus	AY549367	Faivovich et al. (2004)
S. lacteus	JF790143	Jansen et al. (2011)
S. lacteus	JF790144	Jansen et al. (2011)
S. lacteus	MK266743	Araujo-Vieira et al. (2019)
S. lacteus	MK266744	Araujo-Vieira et al. (2019)
S. mirim	MK266745	Araujo-Vieira et al. (2019)
G. pauloalvini	MK266747	Araujo-Vieira et al. (2019)
G. pauloalvini	MK266748	Araujo-Vieira et al. (2019)
G. pauloalvini	MK266749	Araujo-Vieira et al. (2019)
G. pauloalvini	MK266750	Araujo-Vieira et al. (2019)
G. pauloalvini	MT503969	Orrico et al. (2021)
S. planicola	MK266751	Araujo-Vieira et al. (2019)
S. platycephalus	KY418016	Roberto et al. (2017)
S. platycephalus	MK266746	Araujo-Vieira et al. (2019)
S. prasinus	MK266752	Araujo-Vieira et al. (2019)
S. prasinus	MK266753	Araujo-Vieira et al. (2019)
S. prasinus	MK266754	Araujo-Vieira et al. (2019)
S. surdus	KY418017	Roberto et al. (2017)
S. surdus	MK266755	Araujo-Vieira et al. (2019)
S. surdus	MK266756	Araujo-Vieira et al. (2019)
S. surdus	MK266757	Araujo-Vieira et al. (2019)
S. surdus	MK266758	Araujo-Vieira et al. (2019)
Scinax fuscovarius	MK266760	Araujo-Vieira et al. (2019)

Supplementary Table 2. Average uncorrected (*p*-distance) sequence divergence between different species of *Sphaenorhynchus*. Values in bold along the diagonal are intraspecific divergences. n/c= not calculated.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*S. botocudo*	**0.004**												
2	*S. cammaeus*	0.067	**0.000**											
3	*S. canga*	0.039	0.055	**0.004**										
4	*S. caramaschi*	0.073	0.073	0.059	**0.006**									
5	*S. carneus*	0.151	0.140	0.143	0.137	**0.004**								
6	*S. dorisae*	0.133	0.140	0.121	0.137	0.167	**0.002**							
7	*S. lacteus*	0.107	0.102	0.106	0.099	0.137	0.090	**0.005**						
8	*S. mirim*	0.166	0.157	0.149	0.156	0.183	0.126	0.127	**n/c**					
9	*S. platycephalus*	0.053	0.054	0.032	0.060	0.140	0.132	0.102	0.135	**0.000**				
10	*S. pauloalvini*	0.114	0.092	0.107	0.097	0.130	0.128	0.107	0.149	0.096	**0.006**			
11	*S. planicola*	0.147	0.139	0.127	0.135	0.162	0.131	0.122	0.065	0.112	0.132	**n/c**		
12	*S. prasinus*	0.110	0.089	0.087	0.090	0.137	0.125	0.091	0.142	0.090	0.096	0.144	**0.007**	
13	*S. surdus*	0.032	0.045	0.023	0.060	0.140	0.135	0.107	0.157	0.028	0.105	0.139	0.092	**0.000**