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DNA sequencing of topotypes helps delineate species distributions in the *Ischnocnema verrucosa* complex (Anura, Brachycephalidae)

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Abstract

As many new evolutionary lineages are being discovered and formally named, sequencing topotypes when holotypes are not available becomes essential for taxonomy. This study uses a DNA-taxonomy approach to sequence new populations of the *Ischnocnema verrucosa* species complex (Brazilian Wart Frogs) from different locations, including, for the first time, individuals from the type localities. Phylogenetic analysis of the mitochondrial *16S* gene recovered a monophyletic *Ischnocnema verrucosa* species series composed of three main clades. The most recent common ancestor was estimated to be 33.76 million years ago, and diversification within the three main clades occurred primarily during the Miocene. We delimited eight species-level lineages with high levels of sequence divergence (7% to 16%). Our study highlights the importance of DNA taxonomy and the necessity of protecting and sequencing topotypes in taxonomic studies. Our study also contributes to the conservation and understanding of the genus *Ischnocnema* and the biodiversity of the Brazilian Atlantic Forest region.

Key Words

Brazilian Atlantic Forest, DNA taxonomy, Eocene, Miocene, phylogeny, taxonomy, type locality

Introduction

In the last 300 years, taxonomists have served the purpose of describing, classifying, and naming organisms based, exceptionally, on morphological characters (Linnaeus 1750; Haeckel 1866; Hennig 1966). At present, integrative taxonomic approaches incorporating molecular data have revolutionized the field of biodiversity research, providing a powerful tool for uncovering cryptic diversity (Padial et al. 2010; Pilgrim and Darling 2010). This

† In Memoriam.

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is particularly important because cryptic diversity, which refers to distinct species erroneously classified under one species name due to morphological similarities, is a significant contributor to the Linnean shortfall, where the number of known species falls short of the actual number of existing species (Hortal et al. 2015; Fišer et al. 2018; Struck et al. 2018). This has implications for our understanding of global biodiversity, highlighting the need for continued efforts to uncover and document new species.

In recent years, many species previously thought to be a single taxon have been revealed to be species complexes (e.g., Suatoni et al. 2006; Guimarães et al. 2022; Moraes et al. 2022; Araujo-Vieira et al. 2023; Brunes et al. 2023), with potential candidate species or known nominal species having small distributions and being under threat (Gehara et al. 2013). This trend is particularly frequent in anurans in several ecoregions across the globe (Hasan et al. 2012; Guarnizo et al. 2015; Cryer et al. 2019). The Neotropics harbor the largest amphibian diversity in the world, and many new species continue to be discovered, especially in tropical forest ecoregions such as the Amazon and Brazilian Atlantic Forest (Moura et al. 2018; Vacher et al. 2020).

The Brazilian Atlantic Forest (BAF), a biodiversity hotspot (Ribeiro et al. 2011), is characterized by a complex topography that has driven amphibian diversification (Paz et al. 2021). Among the many anuran groups that have evolved in the BAF, direct-developing frogs of the genus *Ischnocnema* Reinhardt & Lütken, 1862 are strongly associated with the biome (Hedges et al. 2008; Canedo and Haddad 2012). This genus comprises 39 species (Frost 2024), six of which have been described using integrative taxonomic approaches with molecular data (Brusquetti et al. 2013; Taucce et al. 2018a, 2018b, 2019; Silva-Soares et al. 2021), but many identified species complexes remain unresolved taxonomically (Gehara et al. 2013, 2017; Thomé et al. 2020; Oswald et al. 2023). If the nomenclatural history of a taxon is not assessed properly, the number of taxonomic inconsistencies tends to increase (e.g., Tobias et al. 2010; Mângia et al. 2014; Yodthong et al. 2019; Mângia et al. 2020), and even the resolutions and descriptions may not be effectively implemented from a nomenclatural standpoint (Leaché and Fujita 2010; Lourenço et al. 2015; Guayasamin and Trueb 2020). The act of naming species removes independent lineages from anonymity and highlights their evolutionary historical importance (Hillis 2007; Vences et al. 2013), while also improving the evaluation of conservation status (Mace 2004).

A lack of type specimens can create problems for taxonomic decision-making, just as the difficulty (and still high cost) of sequencing genetic material from century-old, fluid-preserved specimens brings challenges for proper nomenclature of cryptic species (Bell et al. 2020). However, this bias can often be overcome through the collection of recent topotypes, that is, new specimens collected from the type locality, which provide genetic material (Cacciali et al. 2017; Murphy et al. 2017). Over the last two decades, several molecular studies have focused on the brachycephalid frog genus *Ischnocnema*, defining and redelimiting the genus and its main clades, which currently include the *I. guentheri*, *I. lactea*, *I. parva*, *I. verrucosa*, and *I. venancioi* species series (*sensu* Hedges et al. 2008) (Canedo and Haddad 2012; Taucce et al. 2018b). The *Ischnocnema verrucosa* series comprises eight species (Canedo and Haddad 2012; Taucce et al. 2018b): *I. abdita* Canedo & Pimenta, 2010; *I. bolbodactyla* (Lutz, 1925); *I. juipoca* (Sazima & Cardoso, 1978); *I. karst* Canedo, Targino, Leite, & Haddad, 2012; *I. octavioi* (Bokermann, 1965); *I. penaxavantinho* Giaretta, Toffoli, & Oliveira, 2007; *I. surda* Canedo, Pimenta, Leite, & Caramaschi, 2010; and *I. verrucosa* (Reinhardt & Lütken, 1862). The placements of *I. surda* and *I. karst* in this group are not verified by genetic data because tissue samples were not available (Canedo and Haddad 2012).

Within the *I. verrucosa* species series, there is an unresolved taxonomic puzzle regarding *Ischnocnema verrucosa*, *I. octavioi*, and *I. surda*, three very similar-looking species (Fig. 1) not easily distinguished from each other (Canedo et al. 2010; Dantas and Ferreira 2010; Silva et al. 2013; Holer et al. 2017; Araújo et al. 2023). *Ischnocnema verrucosa* was described as *Leiuperus verrucosus* from 'Omegnen af Byen Juiz de Fora i Minas Geraes (altsaa fra Urskovsregionen)' (Reinhardt and Lütken 1862), i.e., 'the surroundings of the municipality of Juiz de Fora, state of Minas Gerais (from the primeval forest region)'. Currently, its distribution includes localities in the Brazilian states of Espírito Santo, Minas Gerais (Caramaschi and Canedo 2006; Silva et al. 2013), and Bahia (Orrico 2010; Freitas et al. 2011; Rojas-Padilla et al. 2020; Bastos and Zina 2022). *Ischnocnema octavioi* was described from the Tijuca Mountains in the state of Rio de Janeiro, Brazil (Bokermann 1965); currently, it is recorded in the Brazilian states of São Paulo (Holer et al. 2017), Espírito Santo (Dantas and Ferreira 2010), and Rio de Janeiro (Vrcibradic et al. 2008). Canedo et al. (2010) could not diagnose *I. octavioi* and *I. verrucosa* using morphological features. Finally, *I. surda* was described from Estação Ecológica do Tripuí, in the municipality of Ouro Preto, in the state of Minas Gerais, and shortly after its distribution was expanded to include other localities of this state (Silva et al. 2013; Lacerda et al. 2014). Silva et al. (2013) suggest clinal variation in the diagnostic characteristic between *I. surda* and *I. verrucosa*, indicating the need for studies to better elucidate the taxonomic identity of both species. Furthermore, topotype specimens of *I. verrucosa* and *I. octavioi* have never been included in molecular analyses, and populations identified as *I. verrucosa* have not been recovered as monophyletic (Canedo and Haddad 2012). Thus, whether species diversity is correctly delimited has not been adequately evaluated. Here, we use a DNA-taxonomy approach with new populations of the *Ischnocnema verrucosa* species complex from different locations, including, for the first time, individuals from the type localities of *I. verrucosa* and *I. octavioi* and individuals from the same municipality, close to the type locality of *I. surda*. We demonstrate the importance of sampling topotypes to accurately delineate distributions and properly name species in these complexes.

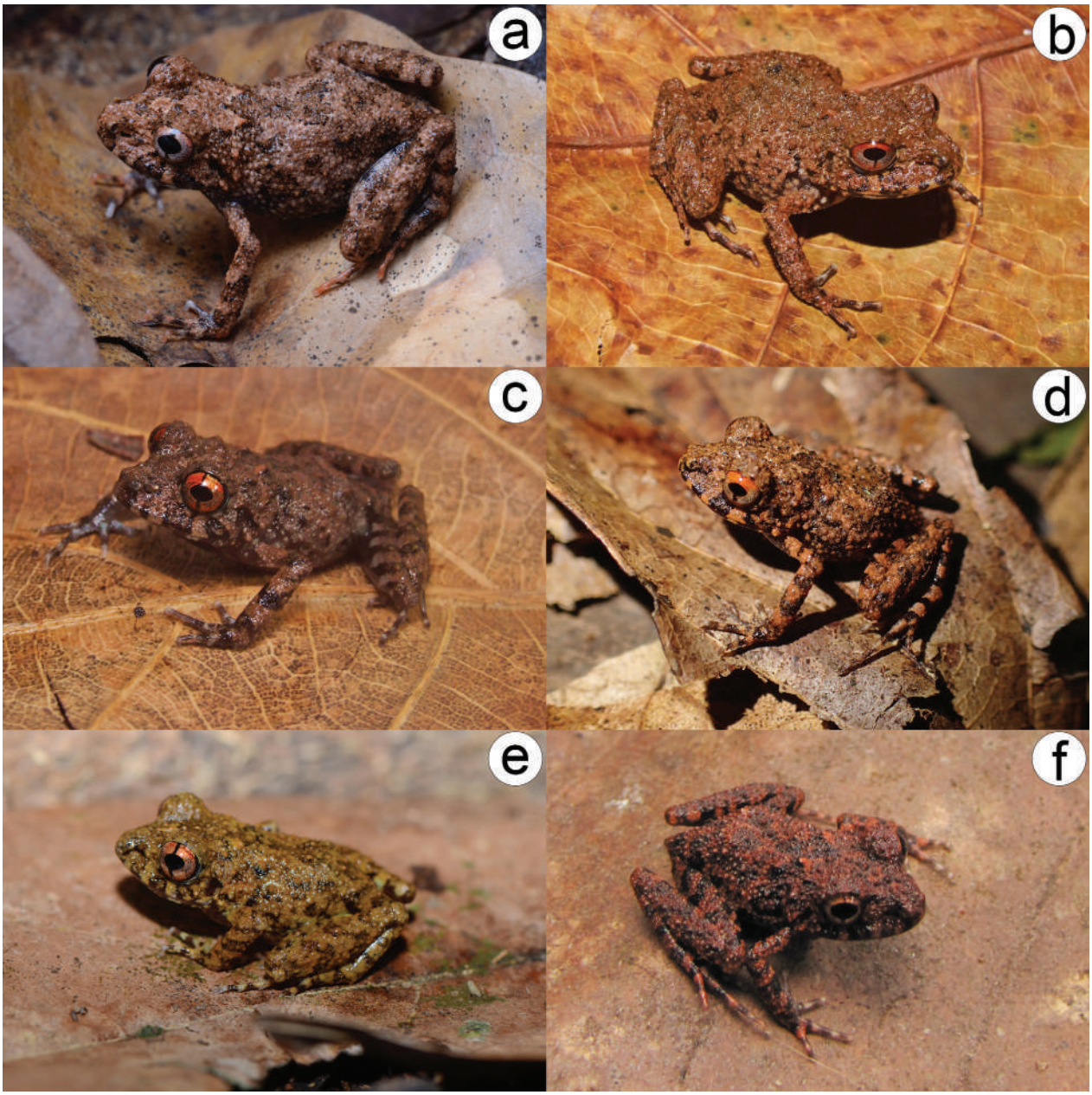

Figure 1. Live specimens in the *Ischnocnema verrucosa* species complex. **a.** Topotype of *Ischnocnema verrucosa* (MAP6749) from Parque Natural Municipal da Lajinha, municipality of Juiz de Fora, state of Minas Gerais; **b.** *I. verrucosa* (MZUESC8829) from Serra Bonita, municipality of Camacan, state of Bahia; **c.** *I. verrucosa* (MZUESC23730) from municipality of Wenceslau Guimarães, state of Bahia; **d.** *I.* aff. *verrucosa* (unvouchered specimen) from municipality of Santa Teresa, state of Espírito Santo; **e.** Topotype of *I. octavioi* (MNRJ94357) from Parque Nacional da Tijuca, municipality of Rio de Janeiro, state of Rio de Janeiro; and **f.** *I. surda* (UFMG-A17166) from municipality of Ouro Preto, state of Minas Gerais. (Photos by D.J. Santana [**a**], H.C. Costa [**f**], P.H. Pinna [**e**], I. Dias [**b, c**], J.V.A. Lacerda [**d**]).

Materials and methods

Whole genomic DNA was extracted from muscle or liver tissues of 11 specimens from the *Ischnocnema verrucosa* series using a Qiagen DNeasy kit (Valencia, California, USA) following the manufacturer's protocol. Next, we amplified a fragment of the mitochondrial *16S* gene using primers 16Sar and 16Sbr (Palumbi et al. 2002) or 16S-AR and 16S-Wilk2 (Wilkinson et al. 1996). Reactions contained 10 µl of GoTaq(R) G2 Green Master Mix, 6 µl of H_2O, 1 µl of each primer (10 µM), and 2 µl of template DNA (≥ 2 ng/µl). The PCR protocol was configured with one initial phase of 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 40 s, with a final extension phase of 72 °C for 5 min. Purification of PCR products and DNA sequencing were performed by Eurofins Genomics Inc. (Louisville, Kentucky, USA) and Centro de Pesquisa Sobre o Genoma Humano e Células Tronco (Universidade de São Paulo, Brazil).

We combined our newly generated *16S* sequences with all comparable *16S* sequences of the *Ischnocnema verrucosa* series available on GenBank. In addition, we downloaded from GenBank one *16S* sequence for each *Ischnocnema* species series and one *Brachycephalus* for use as outgroups, following Taucce et al. (2018b). Chromatogram sequences were visualized in Chromas and Geneious v.9.0.5 (Biomatters Ltd., Auckland, New Zealand). We aligned the *16S* gene fragments using MAFFT (Katoh et al. 2002) implemented in Geneious v.9.0.5 with the algorithm set as auto, which selects an appropriate strategy from L-INS-i, FFT-NS-I, and FFT-NS-2. The final alignment comprised 39 sequences of a 560 base-pair (bp) fragment of the mitochondrial *16S* gene. All GenBank accession numbers and genetic vouchers used here are listed in Table 1.

Table 1. Species, GenBank accession numbers for sequence data, specimen voucher numbers, collecting locality information, GPS coordinates, and references for all samples included in molecular analyses. * Topotypes.

Species	Genbank Accession	Voucher	Locality	Latitude, Longitute	Reference	Lineage
Brachycephalus nodoterga	KJ649783	IB6311	Ilha de São Sebastião, SP	-23.827, -45.373	Clemente-Carvalho et al. (2016)	Outgroup
*I. abdita**	JX267471	MNRJ34903	Santa Teresa, ES	-19.919, -40.619	Canedo and Haddad (2012)	*I. abdita*
I. abdita	JX267472	MTR12625	Caparao, MG	-20.524, -41.906	Canedo and Haddad (2012)	*I. abdita*
I. abdita	KY646094	MZUFV15919	Espera Feliz, MG	-20.651, -41.908	Rocha et al. (2017)	*I. abdita*
I. abdita	KY646095	MZUFV15920	Espera Feliz, MG	-20.651, -41.908	Rocha et al. (2017)	*I. abdita*
I. abdita	KY646096	MZUFV15922	Espera Feliz, MG	-20.651, -41.908	Rocha et al. (2017)	*I. abdita*
I. abdita	KY646097	MZUFV15923	Espera Feliz, MG	-20.651, -41.908	Rocha et al. (2017)	*I. abdita*
I. abdita	MN450228	TLFT 2831	Ibitirama, Parque Nacional do Caparao ES	-20.502, -41.722	Zornosa-Torres et al. (2020)	*I. abdita*
I. bolbodactyla	JX267476	CFBH5785	Paraty, RJ	-23.219, -44.716	Canedo and Haddad (2012)	*I. bolbodactyla*
I. cf. *penaxavantinho*	JX267298	CFBH10230	Grao Mogol, MG	-16.565, -42.893	Canedo and Haddad (2012)	*I.* cf. *penaxavantinho*
I. guentheri	EF493533	NA	Estacao Ecologica de Juréia, SP	-24.530, -47.189	Heinicke et al. (2007)	*I. guentheri*
I. juipoca	DQ283093	CFBH4450	Poços de Caldas, MG	-21.785, -46.561	Frost et al. (2006)	*I. juipoca*
I. juipoca	JX267348	CFBH19697	Caieiras, SP	-23.362, -46.746	Canedo and Haddad (2012)	*I. juipoca*
I. juipoca	JX267349	CFBH9904	Campos de Jordao, SP	-22.740, -45.594	Canedo and Haddad (2012)	*I. juipoca*
I. juipoca	JX267373	AFlab#0963	Poços de Caldas, MG	-21.785, -46.561	Canedo and Haddad (2012)	*I. juipoca*
I. juipoca	JX267511	AFlab#0969	Poços de Caldas, MG	-21.785, -46.561	Canedo and Haddad (2012)	*I. juipoca*
I. juipoca	JX267512	MCLfield#0122	Estacao Biologica de Boraceia, Salesopolis, SP	-23.636, -45.946	Canedo and Haddad (2012)	*I. juipoca*
I. juipoca	JX267513	MCLfield#0069	Estacao Biologica de Boraceia, Salesopolis, SP	-23.636, -45.946	Canedo and Haddad (2012)	*I. juipoca*
I. lactea	JX267308	MTR10435	Paranapiacaba, Santo Andre, SP	-23.777, -46.299	Canedo and Haddad (2012)	*I. lactea*
I. octavioi	JX267312	MNRJ48752	Reserva Biologica Uniao, RJ	-22.378, -42.118	Canedo and Haddad (2012)	*I. verrucosa*
I. octavioi	JX267322	MNRJ42488	Parque Estadual do Desengano, Santa Maria Madalena, RJ	-21.904, -41.949	Canedo and Haddad (2012)	*I. verrucosa*
I. octavioi	JX267521	MNRJ62328	Reserva Ecológica de Guapiaçu, Cachoeira de Macacu, RJ	-22.449, -42.776	Canedo and Haddad (2012)	*I. verrucosa*
I. octavioi	MN450229	MN450229	Alto Caparao, Parque Nacional do Caparao, MG	-20.446, -41.847	Zornosa-Torres et al. (2020)	*I. verrucosa*
*I. octavioi**	PQ456291	MNRJ93604*	Parque Nacional da Tijuca, Rio de Janeiro, RJ	-22.950, -43.286	Present work	*I. octavioi*
I. octavioi	PQ456292	MNRJ92356	Parque Arqueológico e Ambiental de São João Marcos, Rio Claro, RJ	-22.800, -44.029	Present work	*I. octavioi*
I. octavioi	PQ456293	MNRJ92360	Parque Arqueológico e Ambiental de São João Marcos, Rio Claro, RJ	-22.800, -44.029	Present work	*I. octavioi*
I. parva	EF493532	NA	NA	NA, NA	Heinicke et al. (2007)	Outgroup
I. surda	PQ456283	UFMG-A17166	Samarco, Ouro Preto, MG	-20.188, -43.509	Present work	*I. surda*
I. surda	PQ456284	MNRJ77811	Reserva Particular do Patrimônio Natural Serra do Caraça, MG	-20.133, -43.500	Present work	*I. surda*
I. venancioi	JX267321	MNRJ44564	Parque Nacional da Serra dos Órgãos, RJ	-22.485, -43.067	Canedo and Haddad (2012)	Outgroup
I. verrucosa	JX267383	MNRJ34900	Santa Teresa, ES	-19.919, -40.619	Canedo and Haddad (2012)	*I.* aff. *verrucosa*
I. verrucosa	JX267537	MNRJ34899	Santa Teresa, ES	-19.919, -40.619	Canedo and Haddad (2012)	*I.* aff. *verrucosa*
I. verrucosa	JX267538	CFBH23685	RPPN Serra Bonita, Camacan, BA	-15.397, -39.572	Canedo and Haddad (2012)	*I. verrucosa*
*I. verrucosa**	PQ456285	MAP6749*	Parque Natural Municipal da Lajinha, Juiz de Fora, MG	-21.792, -43.366	Present work	*I. verrucosa*
*I. verrucosa**	PQ456286	MAP6751*	Parque Natural Municipal da Lajinha, Juiz de Fora, MG	-21.792, -43.366	Present work	*I. verrucosa*
*I. verrucosa**	PQ456287	MAP6750*	Parque Natural Municipal da Lajinha, Juiz de Fora, MG	-21.792, -43.366	Present work	*I. verrucosa*
I. verrucosa	PQ456288	MZUESC23732	Parque Nacional da Serra das Lontras, Arataca, BA	-15.262, -39.414	Present work	*I. verrucosa*
I. verrucosa	PQ456289	MZUESC15886	Serra do Mandim, Itarantim, BA	-15.654, -40.060	Present work	*I. verrucosa*
I. verrucosa	PQ456290	MZUESC23731	Estação Ecológica Wenceslau Guimarães, BA	-13.687, -39.478	Present work	*I. verrucosa*

We performed Bayesian phylogenetic inference in BEAST v.2.7.4 (Bouckaert et al. 2019) for 100 million generations, sampling every 10,000 steps, using a Yule Process tree prior and a relaxed-clock model rate of 0.006 substitutions/site/million years (Gehara et al. 2017) with uniform distribution. Additionally, we set a prior with all individuals belonging to the genus *Ischnocnema* as monophyletic. We used the default settings for all the other priors. We used the model-averaging method by running the analysis with bModelTest (Bouckaert and Drummond 2017). We performed two independent runs to check for convergence. We checked for stationarity by visually inspecting trace plots and ensuring all effective sample size values were > 200 in Tracer v.1.7.1 (Rambaut et al. 2018). The first 10% of sampled genealogies were discarded as burn-in, and the maximum clade credibility tree with median node ages was calculated with TreeAnnotator v.2.7.4 (Bouckaert et al. 2019).

We conducted the Generalized Mixed Yule Coalescent (GMYC; Pons et al. 2006; Fujisawa and Barraclough 2013) and the multi-rate Poisson Tree Processes (mPTP; Kapli et al. 2017) delimitation analyses. To perform these analyses, based on the topologies, we created a new alignment using unique haplotypes. To remove identical sequences, we used the Biostrings package (Pagès et al. 2024) in R. This reduced alignment had 32 sequences (including the same outgroups) with the same 560 bp. For both analyses, we used as input the tree obtained in a Bayesian analysis in BEAST with the same parameters mentioned earlier. We performed GMYC in R v.4.1.1 (R Core Team 2021) using the package *splits* v.1.0-20 (Ezard et al. 2017) and mPTP species delimitation analysis on the mPTP webserver (https://mptp.h-its.org/). Finally, we performed Assemble Species by Automatic Partitioning (ASAP; Puillandre et al. 2021), which is a distance-based method. We performed the ASAP delimitation on the webserver (https://bioinfo.mnhn.fr/abi/public/asap/asapweb), considering a simple distance model to compute the distances between samples and default parameters. We retained the delimitation scheme supported by the lowest ASAP score (Puillandre et al. 2021). Finally, we defined the lineages based on the combined evidence from these three delimitation methods, considering congruence of results when at least two of three agreed (Moraes et al. 2022). Lastly, we calculated pairwise-sequence divergences (uncorrected *p*-distances) among species/individuals using MEGA v.10.1.1 (Kumar et al. 2018), with pairwise deletion for gaps.

Results

Our gene tree recovered the *Ischnocnema verrucosa* species series as monophyletic (PP = 0.94), comprising eight lineages: *Ischnocnema* cf. *penaxavantinho*, *I. juipoca*, *I. bolbodactyla*, *I. abdita*, *I. verrucosa*, *I. octavioi*, *I. surda*, and *I.* aff. *verrucosa* (Fig. 2). The initial divergences at the base of the tree gave rise to three main clades. The first clade (PP = 1.0), composed of *I.* cf. *penaxavantinho* and *I. juipoca*, is the sister group of the other two clades. The second clade (PP = 1.0), composed of *I. bolbodactyla* and *I. abdita*, is the sister group of the third clade (PP = 1.0), which is composed of species in the *I. verrucosa* complex: *I. verrucosa*, *I. octavioi*, *I. surda*, and one allied lineage that we call here, *I.* aff. *verrucosa*. Additionally, populations from Reserva Biológica União (RJ), Parque Estadual do Desengano (RJ), Reserva Ecológica de Guapiaçu (RJ), and Parque Nacional do Caparaó (MG), previously identified as *I. octavioi*, were revealed to be *I. verrucosa*.

The most recent common ancestor (MRCA) of the *Ischnocnema verrucosa* species series was estimated to be 33.76 million years ago (mya; HPD 95%: 26.18–42.01) at the end of the Eocene, when the last common ancestor of *I.* cf. *penaxavantinho* and *I. juipoca* separated from the last common ancestor of the other two clades. Soon after, in the Oligocene, around 28.53 mya (HPD 95%: 26.18–42.01), the MRCA of the other two clades diverged. Finally, the *Ischnocnema verrucosa* species complex diversified throughout the Miocene with the divergence between *I. octavioi* from the others in the early Miocene, around 21.25 mya (HPD 95%: 16.5–26.51), and then, right after this separation, the lineage we called *I.* aff. *verrucosa* diverged from *I. surda–I. verrucosa* in the early Miocene around 20.38 mya (HPD 95%: 14.88–24.21). Finally, the most recent lineages, *I. verrucosa* and *I. surda*, diverged in the Miocene, around 16.28 mya (HPD 95%: 11.93–19.84).

The mPTP (Score Null Model: -178.0243) and ASAP (asap score: 4.00; threshold distance: 0.075) species delimitation methods recovered the same eight lineages for the *I. verrucosa* series, while the GMYC split the series into 12 lineages (confidence interval: 10–13; likelihood ratio test: 16.604; result of the LR test: < 0.001) (Fig. 2). The *p*-distances between lineages within the *I. verrucosa* complex show high levels of sequence divergence, ranging from 10% between *I. surda* and *I. verrucosa* to 13.1% between *I.* aff. *verrucosa* and *I. octavioi* (Table 2).

Table 2. Average pairwise uncorrected (*p*-distance) sequence divergence between different lineages within the *Ischnocnema verrucosa* species series. Data in bold on the diagonal are intraspecific divergences. n/c = Not Calculated.

	Lineage	1	2	3	4	5	6	7	8
1	*I. surda*	**0.004**							
2	*I. verrucosa*	0.100	**0.059**						
3	*I.* aff. *verrucosa*	0.128	0.111	**<0.001**					
4	*I. bolbodactyla*	0.148	0.136	0.158	**n/c**				
5	*I. abdita*	0.127	0.109	0.123	0.079	**0.004**			
6	*I.* cf. *penaxavantinho*	0.133	0.144	0.140	0.140	0.124	**n/c**		
7	*I. juipoca*	0.151	0.151	0.140	0.153	0.150	0.088	**0.007**	
8	*I. octavioi*	0.129	0.129	0.131	0.153	0.137	0.145	0.164	**0.012**

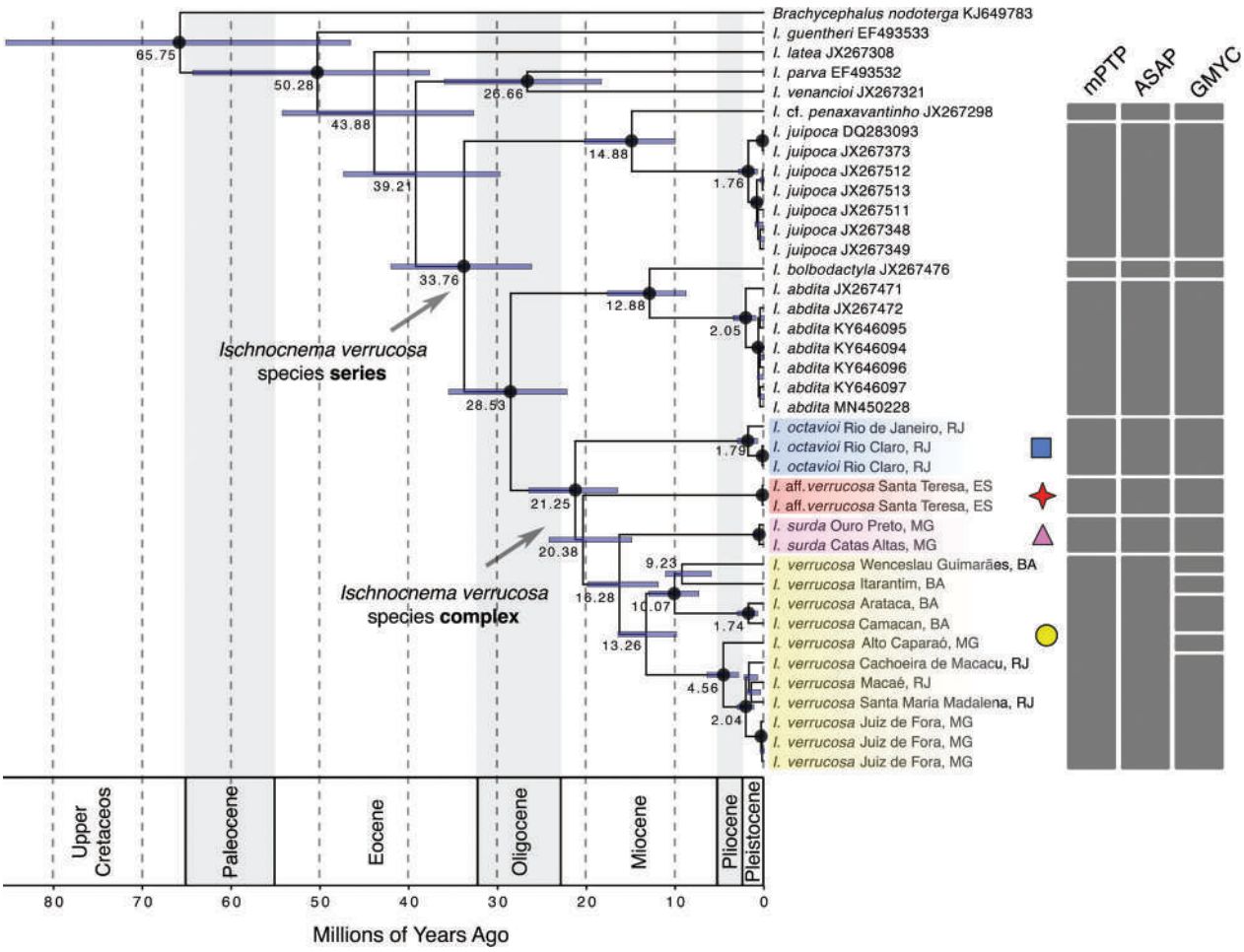

Figure 2. Bayesian chronogram for the *Ischnocnema verrucosa* series based on analysis of the mitochondrial *16S* gene. Circles on nodes denote significant posterior probability (pp = 0.95–1.0). Median ages in millions of years are shown on nodes, and 95% HPDs of node ages are indicated by horizontal blue bars. Vertical gray bars on the right represent the species delimited by GMYC (Generalized Mixed Yule Coalescent), mPTP (multi-rate Poisson Tree Processes), and Assemble Species by Automatic Partitioning (ASAP).

Discussion

In the present study on phylogenetic relationships of the *Ischnocnema verrucosa* species series, we found eight species-level lineages distributed in three main clades, with the most recent common ancestor estimated to have lived 33.76 mya (Fig. 2). The lineages were identified based on the congruence of two of the three species delimitation methods, revealing high levels of genetic divergence. Our study also identified diversification events, many of which originated in the Oligocene and Miocene, with some lineages experiencing diversification during the Pliocene. The estimated divergence times for the *Ischnocnema verrucosa* species complex suggest an ancient and gradual diversification. While mitochondrial data can sometimes overestimate divergence times (Duchêne et al. 2014), our results align with the broad timelines observed in other Terrarana (Gehara et al. 2017; Condez et al. 2020; Mônico et al. 2024). The ancient divergences indicate that the complex underwent significant diversification during a period of climatic shifts and landscape changes in the Brazilian Atlantic Forest (BAF) (Brown et al. 2020). Within Brachycephalidae, *Ischnocnema* has a complex evolutionary history (Heinicke et al. 2007; Canedo and Haddad 2012; Taucce et al. 2018a, 2018b), and many new species have recently been discovered (e.g., Taucce et al. 2018a; 2018b, 2019; Silva-Soares et al. 2021). Therefore, our finding of at least eight evolutionary lineages within the *I. verrucosa* series is expected.

Canedo and Haddad (2012) presented the first well-sampled, multi-locus phylogeny of *Ischnocnema*, finding the same relationships that we recovered in our tree. The authors also found *I. verrucosa* to be paraphyletic, but they did not indicate which group should be named as the nominal species as there was no topotype sampled. Nevertheless, this was an early indication of cryptic diversity within the *I. verrucosa* complex. In a later phylogeny of the Terrarana clade with emphasis on *Ischnocnema* (Taucce et al. 2018b), the topology of the *I. verrucosa* species series was the same as previously reported. However, the dataset used was also the same, with fewer samples of *I. verrucosa*, which disregarded its paraphyly. Here, using only the mitochondrial *16S* gene, a common marker for species identification and delimitation (Vences et al. 2012; Lyra et al. 2017; Koroi-

va and Santana 2022), we found the same topology for the main clades with high support, confirming the paraphyly of *I. verrucosa* and *I. octavioi*. In order to properly name the populations, we re-identified them based on our tree topology and the species delimitation methods, which yielded one candidate species (*I.* aff. *verrucosa*) in addition to the nominal ones.

In this study, we employed three delimitation methods, GMYC (General Mixed Yule-Coalescent), mPTP (multi-rate Poisson Tree Processes), and ASAP (Assemble Species by Automatic Partitioning), to analyze the species boundaries within the *Ischnocnema verrucosa* complex. While such methods have their strengths (Dellicour and Flot 2018), they also come with inherent limitations. The GMYC method, although effective in distinguishing between speciation and intraspecific variation, can sometimes oversplit (Dellicour and Flot 2018), particularly in cases of recent divergence or incomplete lineage sorting. On the other hand, the mPTP method, a more accurate implementation of PTP (Kapli et al. 2017), tends to be more conservative. Finally, ASAP is a distance-based method that tends to yield good results under a broad range of speciation conditions (Dellicour and Flot 2018; Puillandre et al. 2021). Future studies could benefit from integrating additional data types, such as bioacoustics and morphology, and using high-throughput sequencing to obtain a more comprehensive understanding of species boundaries.

Despite the names used to identify samples, the taxonomic complexity in the *Ischnocnema verrucosa* species complex has always been attributed to their conservative external morphology, which hampers the diagnosis of species (Canedo et al. 2010; Silva et al. 2013; Holer et al. 2017; Araújo et al. 2023). Therefore, finding distinct characters to better diagnose evolutionary lineages would help to resolve taxonomic issues. Since morphological characteristics alone have proven to be confusing for species identification, it is crucial to prioritize bioacoustics, osteological, and even ecological differences when describing new species (Carvalho and Giaretta 2013; Martins and Giaretta 2013; Hamdan et al. 2024). In today's taxonomic world, we are racing against time to describe new species before they disappear (Moura and Jetz 2021). Therefore, adopting integrative methods for delimiting species is essential for accurately describing new taxa (Fujita et al. 2012).

Ischnocnema verrucosa complex is endemic to the BAF, and it is found in the mountain ranges of Serra do Mar, Serra da Mantiqueira, southern Serra do Espinhaço, eastern Minas Gerais in the Rio Doce valley, and southern Bahia (Fig. 3). This distribution pattern is common among many amphibian groups, with closely related species or divergent populations of the same species occurring in distinct mountain ranges in southeastern Brazil (Cruz and Feio 2007; Cassini et al. 2013; Magalhães et al. 2020). Certain geographic congruences are observed in the phylogenetic relationships of lineages. Two samples of *I. verrucosa* from the Serra da Mantiqueira form a well-supported clade with samples from three other localities in Serra do Mar. Additionally, along the coastal mountains of the BAF in the states of Rio de Janeiro, Espírito Santo, and southern Bahia, the diversification of lineages within the *I. verrucosa* complex mainly occurred during the Miocene. Model-based analyses revealed population diversification and cryptic species in the *Ischnocnema parva* complex in the BAF (Gehara et al. 2017). Divergences between high-altitude lineages may indicate an influence of climatic cycles and mountains in diversification of *I. parva* (Gehara et al. 2017) and *I. verrucosa* species complexes.

Cryptic diversity appears to be common within *Ischnocnema* (Oswald et al. 2023); however, describing new species without evaluating topotypes of the closest-related species is problematic. Our work included, for the first time, samples of individuals from the type localities of the two nominal species within the *I. verrucosa* complex (*I. verrucosa* and *I. octavioi*) and populations attributed to *I. surda* close to its type locality. An important case to highlight is the population from the municipality of Santa Teresa, in the state of Espírito Santo, previously identified as *I. verrucosa* (see Rödder et al. 2007; Almeida et al. 2011; Canedo and Haddad 2012; Ferreira et al. 2019). The advertisement call of this population was recently described (Araújo et al. 2023). However, our samples from the municipality of Santa Teresa were recovered as a different species (named *I.* aff. *verrucosa*) (Figs 1, 2). Consequently, the advertisement call of the nominal population of *I. verrucosa* and the other lineages in the complex remain undescribed for more than 160 years. Nonetheless, there is a synonym for *I. verrucosa* from the municipality of Colatina, Espírito Santo state, *Eupsophus versus* Gorham, 1966 (a replacement name for the homonym *Eupsophus verrucosus* Miranda-Ribeiro, 1937). Our findings challenge the notion that geographic distance is the most effective criterion for delineating named species. Despite the proximity (~45 km) of the populations in Santa Teresa, we refrain from assigning the name *Eupsophus versus* to them. Vocalizations may prove critical for resolving taxonomic questions in these complexes still without morphological diagnosis, as demonstrated for the related species *I. oea* (Heyer, 1984), *I. garciai* Taucce, Canedo, & Haddad, 2018, and *I. feioi* Taucce, Canedo, & Haddad, 2018 (Taucce et al. 2018b), or even for the *I. guentheri* complex, which, although unnamed mainly because of sympatry of lineages, has candidate species with acoustic diagnoses (Gehara et al. 2013).

We emphasize that including samples from the type localities of the two named species in the *I. verrucosa* complex was crucial for defining to which evolutionary lineages these names correspond. In some other groups, potential new species have been and are frequently revealed, but no taxonomic decision can be made because of the troublesome taxonomic history of the type specimen or because the type locality is unknown or imprecise, leaving the group unresolved (e.g., Cassini et al. 2010). In the case of the *I. verrucosa* complex,

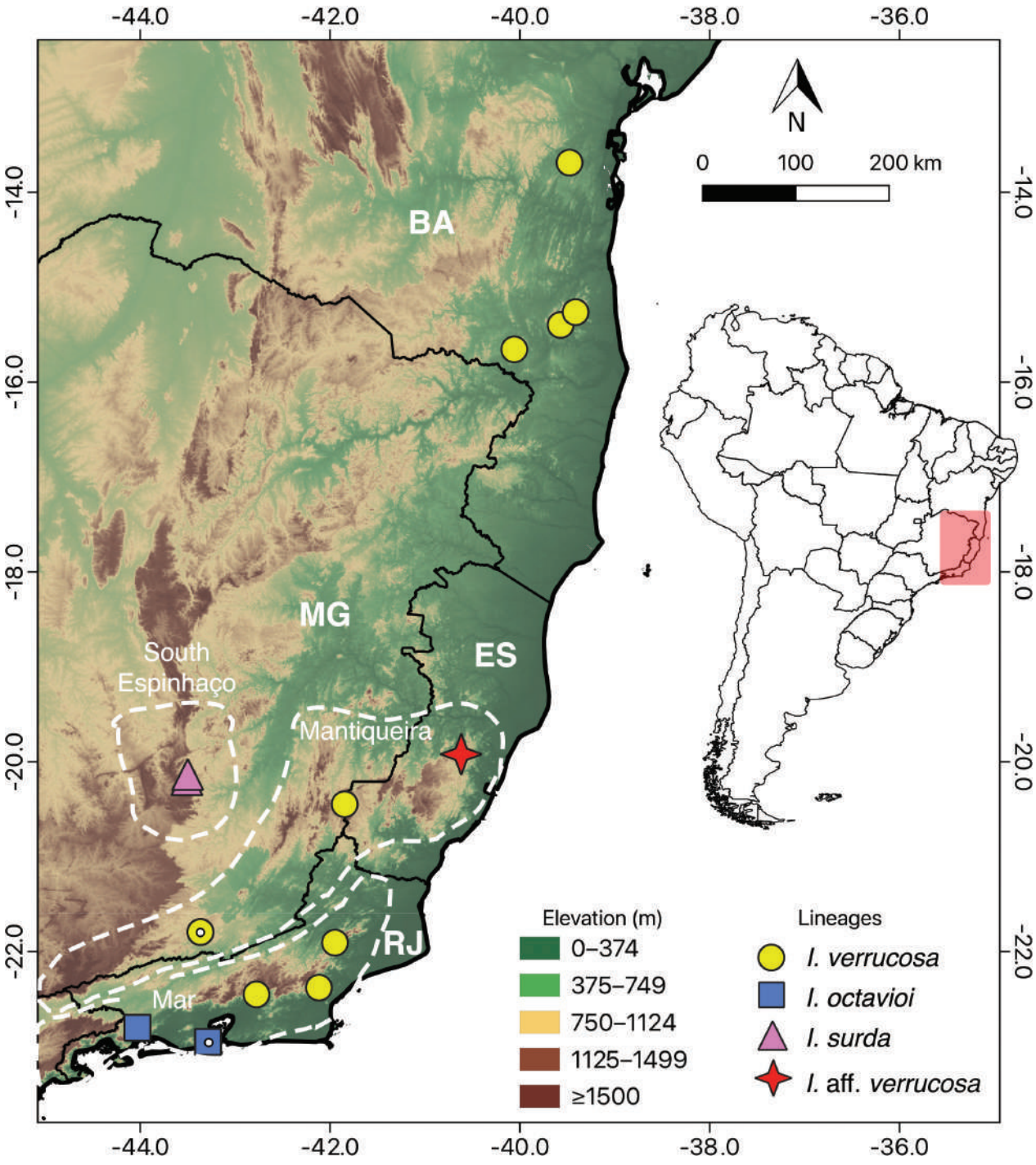

Figure 3. Geographic locations of samples of the *Ischnocnema verrucosa* complex used in this study. White dots on symbols indicate type localities. White dashed areas correspond to the mountain complexes.

I. surda occurs in the southern region of the Espinhaço mountain range, specifically within the Iron Quadrangle (Quadrilátero Ferrífero), also cited for the northern Mantiqueira range and across the Rio Doce valley (Silva et al. 2013). Unfortunately, we were unable to include samples from the formerly cited populations in our analysis. Consequently, in relation to the distribution of *I. surda*, we are refraining from definitively stating its precise geographic distribution. *Ischnocnema octavioi* occurs in the portion closest to the coast of the Serra do Mar, in the south of Rio de Janeiro state. *Ischnocnema verrucosa* occurs in the Serra da Mantiqueira, Serra do Mar (in the Serra dos Órgãos portion), and southern Bahia, making it the species with the broadest distribution of the complex. However, genetic divergence within *I. verrucosa* is high (5.9%; Table 2), higher than the average *16S* divergence usually found among anuran species (Fouquet et al. 2007; Vences et al. 2012; Lyra et al. 2017; Koroiva and Santana 2022). Nonetheless, our value is consistent with previous studies on the genus *Ischnocnema*. For instance, Gehara et al. (2013) reported a *16S p*-distance of 6% between lineages of *I. guentheri*, while Taucce et al. (2018a) found a 7.3% distance between lineages of *Ischnocnema*.

One possible limitation of our work and similar attempts relates to the sampling scope and the possibility of sympatry between cryptic lineages. Sympatry between cryptic lineages of *Ischnocnema* is known from previous studies (Gehara et al. 2013, 2017; Taucce et al. 2018b; Thomé et al. 2020; Oswald et al. 2023). Our sampling, for example, in the municipality of Juiz de Fora was restricted to a single area (Parque Natural Municipal de Lajinha), which may not fully represent the species' diversity in the region. Without extensive sampling at various points in the municipality, there remains doubt as to whether our samples accurately represent the nominal species. While we did not find any sympatric species in our samples, the possibility of their presence cannot be ruled out, as has been documented in other *Ischnocnema* complexes. Future research should aim to include broader sampling across different localities to reveal potential sympatric species.

Taxonomy, particularly when employing molecular approaches, plays a crucial role in identifying key areas for research and conservation efforts since it highlights the presence of endemic species and/or high-diversity hotspots (Baker et al. 2003; Frankham 2010; Gehara et al. 2013). Given the current biodiversity crisis and its increasingly accelerated loss, more support for taxonomy studies should be considered. Recently, the IUCN Red List of Threatened Species was re-evaluated, and *I. verrucosa* and *I. surda* were listed as Least Concern (LC), based on their wide geographic ranges (IUCN SSC Amphibian Specialist Group, Instituto Boitatá de Etnobiologia e Conservação da Fauna 2023a, 2023b). However, *I. surda* was sampled only in the southern Espinhaço, a region heavily impacted by mining activities and home to other threatened species (e.g., Bastos et al. 2023; Santana et al. 2024). It is crucial to search for new populations of *I. surda* to gauge the urgency of conservation efforts needed for this species, especially if it is confirmed that it occurs solely in this region. It is common to find microendemic species in Brachycephalidae frogs (Gehara et al. 2013; Pie et al. 2013; Thomé et al. 2020; Taucce et al. 2022), and small distributions are associated with increased extinction risk because they make species more prone to negative effects of habitat loss (Ficetola et al. 2014), climate change (Li et al. 2013), wildfires (Anjos et al. 2021), and emerging diseases (Kilpatrick et al. 2010). However, facing historical taxonomic difficulties, such as in the *I. verrucosa* species complex, we emphasize the importance of using a molecular delimitation approach that includes samples from type localities of these populations before using these occurrences to delimit species, delineate distributions, and revise taxonomy, all of which will aid in future evaluations of species' conservation status.

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