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Hidden diversity of the genus *Trinomys* (Rodentia: Echimyidae): phylogenetic and populational structure analyses uncover putative new lineages

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Trinomys, one of the most species-rich spiny rat genera in Brazil, is widely distributed in Caatinga, Cerrado and Atlantic Forest biomes, and currently includes ten recognized species, three of which are polytypic. Although some studies employing molecular data have been conducted to better characterize phylogenetic relationships among species, 19 nominal taxa have been suggested, implying considerable incongruence regarding species boundaries. We addressed this incongruence by intensively sampling all species across the geographic distribution of the genus. In addition to publicly available data, we generated 182 *mt-Cytb* gene sequences, and employed phylogenetic and computational species delimitation methods to obtain a clearer picture of the genus diversity. Moreover, we evaluated populational diversity within each accepted species, considering their geographical distribution and a timescale for the evolution of the genus. Beyond confirming the general patterns described for the evolution of the group, this new analysis suggests that *Trinomys* is comprised of at least 16 evolutionary lineages, 13 of them recognized as species or subspecies, and three never before characterized. This study highlights the importance of increased sample sizes and computational species delimitation methods in uncovering hidden diversity in *Trinomys*.

ADDITIONAL KEYWORDS: biogeography – computational species delimitation – *Cytb* – rodents – species boundaries.

INTRODUCTION

Species are the fundamental unit by which taxonomic diversity is measured, and recognizing them is not only a taxonomic challenge, but also essential for conservation, biogeography, ecology and evolutionary biology (Sites & Marshall, 2003; Fujita *et al.* 2012). Depending on the operational criteria used to recognize species, vastly different numbers can be identified (de Queiroz, 2007). This variation emerges from the complexity of identifying discrete boundaries among natural populations that exist along a continuum of divergence (Seehausen *et al.*, 2014). Modern systematists integrate different kinds of data (genetic,

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morphological and ecological) in consistent analytic frameworks to more clearly depict such boundaries and, hence, the diversity of life on Earth (Dayrat, 2005; Carstens *et al.*, 2013).

In this context, computational species delimitation methods have arisen as a tool for identifying evolutionarily distinct lineages that could not be validated under other operational criteria (e.g. morphological). These methods employ molecular sequence data and ad hoc mathematical models developed in a variety of ways to estimate species richness across datasets. Importantly, some methods, mainly those based on just one locus, are used as tools to delimit entities, be they populations, subspecies or species (Fujita *et al.*, 2012; Fujisawa & Barraclough, 2013). This limitation is due to the fact that the models may not reflect the divergence at the entire genome level, being thus a proxy for studying diversity rather than a hard-bound species delimiter (Fujita *et al.*, 2012). Nevertheless, these methods are useful for conducting exploratory diversity research, which further allows novel species to be discovered (Pavón-Vázquez *et al.*, 2018; Hurtado & D'Elía, 2019).

In this work, we used computational species delimitation methods, in addition to phylogeny and populational structure analyses, to address species diversity in the genus *Trinomys* Thomas, 1921, one of the most species-rich genera of echimyid rodents endemic to Brazil (Lara & Patton, 2000; Tavares *et al.*, 2016). This genus is comprised of ten recognized species, three of which are polytypic, occurring in Caatinga, Cerrado and Atlantic Forest biomes (Moojen, 1948; Pessôa *et al.*, 2015). Due to the remarkable morphological variation and few synapomorphies, *Trinomys* is a complex echimyid genus (Lara *et al.*, 1996; Lara & Patton, 2000). The currently recognized diversity of this genus is a result of several morphological (Moojen, 1948; Pessôa & Reis, 1992; Pessôa *et al.*, 1996, Tavares & Pessôa, 2020), karyological (Yonenaga-Yassuda *et al.*, 1985; Leal-Mesquita *et al.*, 1992; Fagundes *et al.* 2004; Corrêa *et al.* 2005; Pessôa *et al.*, 2005; Souza *et al.*, 2006; Lazar *et al.*, 2017; Araújo *et al.*, 2018) and molecular studies (e.g. Lara & Patton, 2000; Fabre *et al.*, 2013, Galewski *et al.*, 2005, Tavares *et al.*, 2015, 2016, Courcelle *et al.*, 2019). Among the molecular studies, only one focused on the systematics of the genus (Lara & Patton, 2000), changing the taxonomic arrangement proposed by Moojen (1948), who recognized four species, three of them polytypic based on morphological data (Supporting Information, Tables S1, S2). Subsequently, other molecular studies have been conducted, focusing on only a few species of *Trinomys* (Tavares *et al.*, 2015, 2016) or on the relationships between the genera of Echimyidae (e.g. Fabre *et al.*, 2013, Galewski *et al.*, 2005, Courcelle *et al.*, 2019), yet still answering some questions about the relationships within *Trinomys*. At present, morphological and molecular evidence supports the recognition of *Trinomys albispinus albispinus* (Geoffroy, 1838), *T. albispinus minor* (Reis & Pessôa, 1995), *T. gratiosus gratiosus* (Moojen, 1948), *T. gratiosus bonafidei* (Moojen, 1948), *T. setosus setosus* (Desmarest, 1817), *T. setosus elegans* (Lund, 1839), *T. dimitiatus* (Günther, 1876), *T. iheringi* (Thomas, 1911), *T. moojeni* (Pessôa *et al.* 1992), *T. mirapitanga* (Lara *et al.*, 2002), *T. eliasi* (Pessôa & Reis, 1993), *T. paratus* (Moojen, 1948) and *T. yonenagae* (Rocha, 1995). However, given the lack of molecular studies conducted on a comprehensive set of samples obtained throughout the wide geographical distribution described for *Trinomys*, we suggest that the number of species in this genus is underestimated.

Although considerable understanding of the phylogenetic placement of *Trinomys* in Echimyidae has been developed (Lara *et al.*, 1996; Leite & Patton, 2002; Galewski *et al.*, 2005; Patterson & Velazco, 2008; Upham & Patterson, 2012, 2015; Fabre *et al.*, 2013; Voloch *et al.*, 2013; Fabre *et al.*, 2016; Courcelle *et al.* 2019), the relationships and species boundaries in the genus are not entirely clear. Accordingly, we analyse the phylogenetic relationships among *Trinomys* species, and estimate species boundaries and evolutionary timescales. Through the combination of intensive sampling of molecular data, phylogeny, populational structure analysis and computational species delimitation methods, we characterize diversity previously unrecognized in this genus.

MATERIAL AND METHODS

Taxon sampling, sequencing and phylogenetic analysis

We assembled a dataset comprising samples collected across the entire range occupied by *Trinomys* in Brazil (Fig. 1). Our sample included specimens of *T. albispinus* ($N = 22$), *T. dimidiatus* ($N = 79$), *T. eliasi* ($N = 9$), *T. gratiosus* ($N = 20$), *T. iheringi* ($N = 40$), *T. mirapitanga* ($N = 2$), *T. paratus* ($N = 5$) and *T. setosus* ($N = 6$) (Table 1). We isolated DNA from thigh muscle and liver tissue samples stored in absolute ethanol following the phenol-chloroform protocol (Sambrook *et al.* 1989). We amplified fragments containing the full-length cytochrome *b* gene (*mt-Cytb*; genes' acronyms following *Mus musculus* nomenclature of Eppig *et al.* 2015) for 181 individuals (Table 1) and the exon 28 of the von Willebrand factor (*e28-vWF*) for 88 individuals (Supporting Information, Table S3). The cycling conditions for *mt-Cytb* PCR reaction was as follows: initial denaturation at 94 °C for 2 min, four cycles of 94 °C for 30 s, 42 °C for 30 s and 72 °C for 90 s, four cycles of 94 °C for 30 s, 40 °C for 30 s and

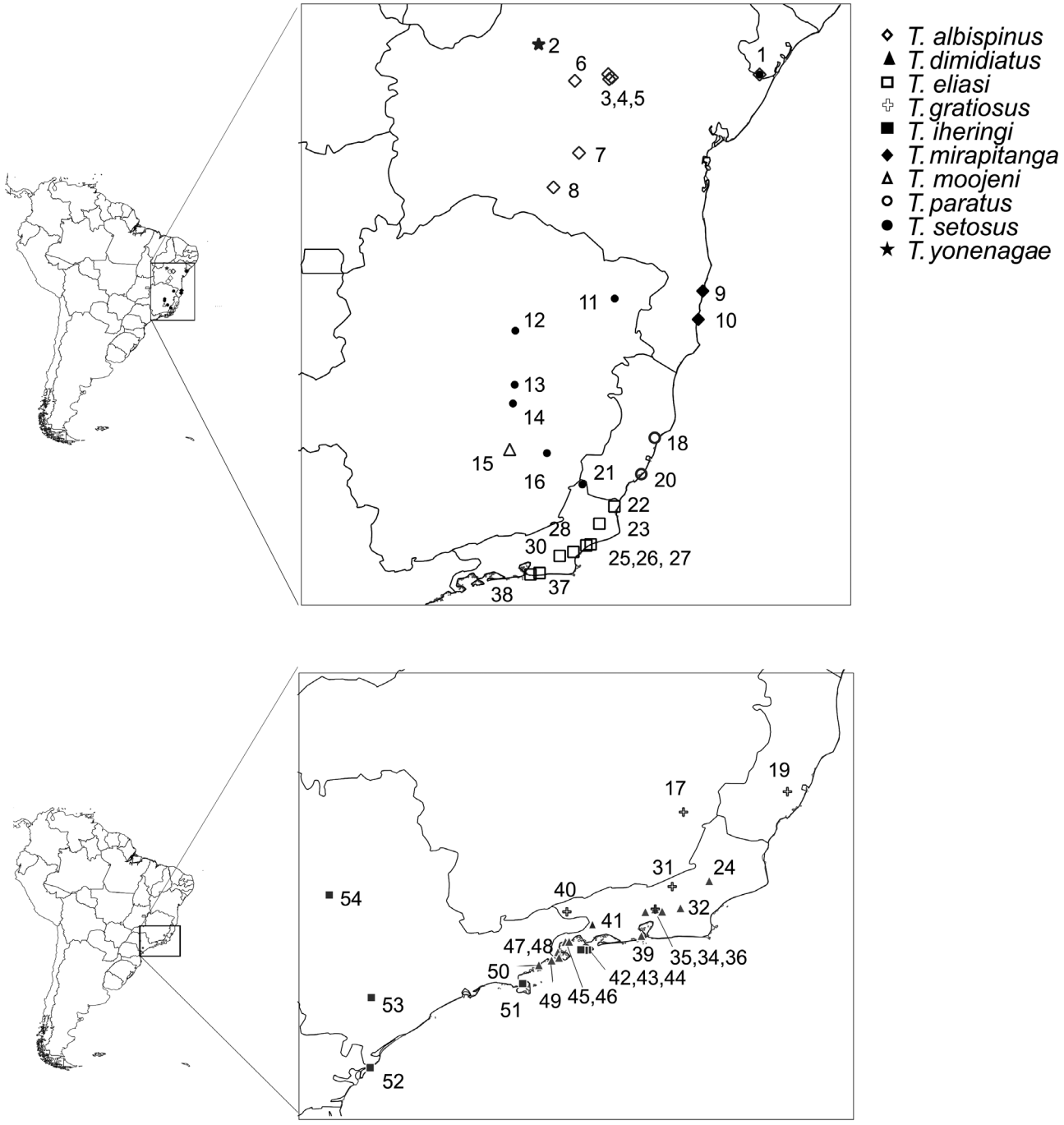

Figure 1. Map with localities of analysed sampled for each species of *Trinomys*. Sergipe: (1) Fazenda Cruzeiro; Bahia: (2) Ibiraba, (3) Faz. Jaboticaba, (4) Morro do Chapéu, (5) Morrão, (6) Ibipeba, (7) Abaíra, (8) Caetité, (9) Porto Seguro, (10) Cumuruxatiba; Minas Gerais: (11) Joaíma, (12) Turmalina (13) Serro, (14) Conceição do Mato Dentro, (15) Catas Altas, (16) Faz. Esmeralda, (17) Serra do Brigadeiro; Espírito Santo: (18) Aracruz, (19) Domingo Martins, (20) Guarapari; Rio de Janeiro: (21) Cambucí, (22) São Francisco de Itabapoana, (23) Campos dos Goytacazes, (24) Santa Maria Magdalena, (25) Conceição de Macabu, (26) Carapebus, (27) Cabiúnas, (28) Rio das Ostras, (29) Correntezas, (30) Silva Jardim, (31) Sumidouro, (32) Nova Friburgo, (33) Cachoeira de Macacu, (34) PN Serra dos Órgãos, (35) Petrópolis, (36) Guapimirim, (37) Restinga de Maricá, (38) Itaipuaçu, (39) PN da Tijuca, (40) Itatiaia, (41) Rio Claro, (42) Ilha Grande, Vila Dois Rios, (43) Ilha Grande, Parnaióca, (44) Ilha Grande, Aventureiro, (45) Angra dos Reis, (46) Tarituba, (47) Paraty, (48) Trindade, São Paulo: (49) Picinguaba, (50) Ubatuba, (51) Ilha de São Sebastião, (52) Ilha do Cardoso, (53) Boracéia, (54) Carlos Botelho.

Table 1. List of analysed species with *mt-Cytb* with haplotype (H), locality (Brazilian state, municipality and/or locality), field or museum number, GenBank accession number and reference (Ref). PS = present study, see footnotes for numbered references and abbreviations

Species	H	Locality	Field or museum no.	GenBank	Ref
T. a. albispinus	H1	SE, Cristinápolis	AL3054	KM014003	1
T. a. albispinus	H2	BA, Cachoeira do Ferro Doido	MN-JAO1474		PS
T. a. minor	H2	BA, Morro do Chapéu	MN-LMP297, LMP300–303, LMP327		PS
T. a. minor	H3	BA, Morro do Chapéu	MN-LMP258		PS
T. a. minor	H4	BA, Morro do Chapéu	MN-LMP289, LMP291, LMP295–296, LMP298–299, LMP315		PS
T. a. minor	H5	BA, Morro do Chapéu	MN-LMP294		PS
T. a. minor	H6	BA, Morro do Chapéu	MN-LMP324		PS
T. a. minor	H7	BA, Mata do Tijuquinha	MN-JAO1385		PS
T. a. minor	H8	BA, Caetité	MN63343, MN63386, MN63418		PS
T. a. minor	H9	BA, Caetité	MN63406		PS
T. dimidiatus	H10	RJ, Tijuca	MN-CRB1733		PS
T. dimidiatus	H11	RJ, Tijuca	MN-CRB1748		PS
T. dimidiatus	H12	RJ, Teresópolis	RF07, RF15, RF21, RF56-57, RF61, RF85, LBCE7092, LBCE7108		PS
T. dimidiatus	H13	RJ, Teresópolis	RF44, RF59		PS
T. dimidiatus	H14	RJ, Teresópolis	FS1022, MN75822–823		PS
T. dimidiatus	H15	RJ, Teresópolis	MN75830–831		PS
T. dimidiatus	H16	RJ, Teresópolis	RF50, RF58, FS1035, MN75809-820, MN75824, MN75829, MN75832, LBCE7095–96, LBCE7107–08, LBCE7117–20, LBCE7123–24		PS
T. dimidiatus	H16	RJ, Guapimirim	MN42798		PS
T. dimidiatus	H16	RJ, Cachoeira de Macacu	FS1151		PS
T. dimidiatus	H17	RJ, Guapimirim	MN48011		PS
T. dimidiatus	H18	RJ, Rio Claro	PCHB21		PS
T. dimidiatus	H19	RJ, Petrópolis	LBCE19061		PS
T. dimidiatus	H20	RJ, Petrópolis	LBCE19085		PS
T. dimidiatus	H21	RJ, Petrópolis	LBCE19100, LBCE19109		PS
T. dimidiatus	H22	RJ, Petrópolis	LBCE19107		PS
T. dimidiatus	H23	RJ, Petrópolis	LBCE19129		PS
T. dimidiatus	H24	RJ, Petrópolis	LBCE19138		PS
T. dimidiatus	H25	RJ, Petrópolis	LBCE19150		PS
T. dimidiatus	H26	RJ, Petrópolis	LBCE19157		PS
T. dimidiatus	H27	RJ, Petrópolis	LBCE19158		PS
T. dimidiatus	H28	RJ, Petrópolis	JDM 3	U35170	2
T. dimidiatus	H29	RJ, Santa Maria Magdalena	MN31413	U35168	2
T. dimidiatus	H30	RJ, Nova Friburgo	MN42796		PS
T. dimidiatus	H31	RJ, Nova Friburgo	MN42797–798		PS
T. dimidiatus	H32	RJ, Angra dos Reis	MN42763	U35169	2
T. dimidiatus	H33	RJ, Paraty	LBCE18353, LBCE18360–18363		PS
T. dimidiatus	H34	RJ, Paraty	MN62295, LG164, LG169		PS
T. dimidiatus	H35	RJ, Paraty	MN62296		PS
T. dimidiatus	H36	RJ, Paraty	MN62298		PS
T. dimidiatus	H37	RJ, Paraty	MN62299		PS
T. dimidiatus	H38	RJ, Paraty	MN62294		PS
T. dimidiatus	H39	SP, Picinguaba	MN-JAO766		PS
T. dimidiatus	H40	SP, Picinguaba	MN69871		PS
T. dimidiatus	H41	SP, Picinguaba	MN69873, MN69878		PS
T. eliasi	H42	RJ, Maricá	LBCE18125, LBCE18129, LBCE18131, LBCE19639, LBCE19640,U35166		PS

Table 1. Continued

Species	H	Locality	Field or museum no.	GenBank	Ref
T. eliasi	H43	RJ, Maricá	LBCE19550		PS
T. eliasi	H44	RJ, Conceição de Macabu	WCT96		PS
T. eliasi	H45	RJ, Rio das Ostras	MN80960–961	KF562084, KF562085	3
T. eliasi	H46	RJ, Silva Jardim	MN80982	KF562079	3
T. eliasi	H46	RJ, Rio das Ostras	MN80955	KF562083, KF562084	3
T. eliasi	H47	RJ, Cabiúnas	NPM314, NPM329, NPM387, NPM438	KF562086-87 KJ707244-45	3
T. eliasi	H48	RJ, Carapebus	MN73194, NPM444, NPM466	KF562088, KJ707246-47	3
T. eliasi	H49	RJ, Campo dos Goytacazes	MN73246–248, MN73263	KF562089, KF562090-92	3
T. eliasi	H50	RJ, Rio das Ostras	MN80950	KF562082	3
T. eliasi	H51	RJ, Rio das Ostras	MN80955	KF562083	3
T. eliasi	H52	RJ, São Francisco de Itabapoana	PSP58		PS
T. g. bonafidei	H53	RJ, Teresópolis	LBCE7385, LBCE7915, LBCE7917, MN75828		PS
T. g. bonafidei	H54	RJ, Teresópolis	MN75825, LBCE7092		PS
T. g. bonafidei	H55	RJ, Teresópolis	MN75826, LBCE7447–48, LBCE7955		PS
T. g. bonafidei	H56	RJ, Teresópolis	LBCE7920, LBCE7939		PS
T. g. gratiosus	H57	RJ, Sumidouro	MN61806		PS
T. g. gratiosus	H58	RJ, Itatiaia		JX312694	4
T. g. gratiosus	H59	RJ, Itatiaia	HGBDB24		PS
T. g. gratiosus	H60	MG, Serra do Brigadeiro	PAB21		PS
T. g. gratiosus	H61	MG, Serra do Brigadeiro	PAB23		PS
T. g. gratiosus	H62	MG, Serra do Brigadeiro	GL113		PS
T. g. gratiosus	H63	MG, Serra do Brigadeiro	BRG144		PS
T. g. gratiosus	H64	ES, Domingo Martins	NPM227	KJ707248	3
T. iheringi	H65	SP, Carlos Botelho	MZUSP-EDH278, -EDH294, -EDH300, UFES-ROD156		PS
T. iheringi	H66	SP, Boracéia	CIT1616	EU544664	5
T. iheringi	H67	SP, Ilha de São Sebastião	MAMME 55	U35171	2
T. iheringi	H68	SP, Ilha do Cardoso	FMNH141668	EU313255	6
T. iheringi	H69	SP, Ilha do Cardoso	FMNH141667	EU313254	7
T. iheringi	H70	RJ, Ilha Grande	MN62266–267, MN62284–285, MN62288–289, MN62279, MN66188, LBCE16101, LBCE16121, LBCE16165		PS
T. iheringi	H71	RJ, Ilha Grande	LBCE16100, LBCE16106, LBCE16110, LBCE16127, LBCE16130, LBCE16134, LBCE16137-138, MN62280, MN66188, MN62277, MN62287, MN62302		PS
T. iheringi	H72	RJ, Ilha Grande	LBCE16122, MN62290		PS
T. iheringi	H73	RJ, Ilha Grande	LBCE16131, LBCE16170, LBCE16172		PS
T. iheringi	H74	RJ, Ilha Grande	LBCE16133		PS
T. iheringi	H75	RJ, Ilha Grande	LBCE16136, LBCE16158		PS
T. iheringi	H76	RJ, Ilha Grande	LBCE16171, LBCE16175		PS
T. iheringi	H77	RJ, Ilha Grande	MN62278		PS
T. mirapitanga	H78	BA, Cumuruxatiba	MN48012–013		PS
T. mirapitanga	H79	BA, Porto Seguro	MN31459	U35173	2
T. moojeni	H80	MG, Serra do Caraça	MCN1037	KF562097	3
T. moojeni	H80	MG, Catas Altas		KX650080	8
T. paratus	H81	ES, Guarapari	LBCE17652, LBCE17662		PS

Table 1. Continued

Species	H	Locality	Field or museum no.	GenBank	Ref
T. paratus	H82	ES, Guarapari	LBCE17653–654, LBCE17658	KY553152-KY553154	PS
T. paratus	H83	ES, Aracruz	MBML3033	KF562094	3
T. paratus	H84	ES, Aracruz	UFES- CTA588	KU892763	9
T. s. elegans	H85	RJ, Cambuci	LBCE18108, LBCE18120–121		PS
T. s. elegans	H86	RJ, Cambuci	LBCE18112, LBCE18119		PS
T. s. elegans	H87	MG, Fazenda Esmeralda	MN31448	AF422924	10
T. s. setosus	H88	MG, Morro do Pilar	AMOPI01	KF562096	3
T. s. setosus	H89	SE, Cristinápolis	AL3072	U34856	PS
T. s. setosus	H90	MG, Joaíma	MN82936		PS
T. s. setosus	H91	MG, Turmalina	YL710	KU892764	9
T. s. setosus	H92	MG, Serro		KX655539	8
T. s. setosus	H93	MG, Conceição do Mato Dentro	MN31441	AF422923	10
T. yonenagae	H94	BA, Ibiraba	PEU880027	U35172	10

Locality abbreviations: (BA) Bahia state, (ES) Espírito Santo state, (MG) Minas Gerais state, (RJ) Rio de Janeiro state, (SP) São Paulo state. Field and museum number abbreviations: (AL) A Langguth, (BRG-GL-PAB) G Lessa, (EDH) E. Hingst-Zaher, (HGBDB) HG Bergallo, (LG-PCHB-PSP) L Geise, (JAO) JA de Oliveira, (LMP) LM Pessôa, (WCT) WC Tavares, (LBCE) Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios, (MN) Museu Nacional. References: (1) Fabre *et al.*, 2014, (2) Lara *et al.*, 1996, (3) Tavares *et al.*, 2015, (4) Voloch *et al.*, 2013, (5) Vilela *et al.*, 2009, (6) Patterson & Velazco, 2008, (7) Leite & Patton, 2002, (8) Araújo *et al.*, 2016, (9) Fabre *et al.*, 2016, (10) Leite & Patton, 2002. (PS) present study.

72 °C for 90 s, 24 cycles of 94 °C for 30 s, 38 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 15 min. Amplification of *mt-Cytb* was conducted with the primers L14724 (Irwin *et al.*, 1991) and CitBREV (Casado *et al.*, 2010). The cycling conditions for e*28-vWF* PCR were initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 60–66 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Two pairs of primers were used: V10/W1 (Galewski *et al.*, 2005) and V2/W1 (Huchon *et al.*, 1999). Sanger sequencing was conducted on an Applied Biosystems 3130XL sequencer. *mt-Cytb* PCR products were sequenced with two additional internal primers (Sotin1 and Sotin2; Cassens *et al.*, 2003). Generated sequences were assembled and edited in Chromas Pro.

Consensus sequences were aligned in MEGAX v.10.2.6 software (Kumar *et al.*, 2016), using the MUSCLE algorithm (Edgar, 2004). Added to the alignment were 43 sequences of *mt-Cytb* (Table 1) and five of *e28-vWF* (Supporting Information, Table S3) of *Trinomys* available on GenBank. The alignment for *mt-Cytb* comprised 226 *Trinomys* sequences and 1140 sites and for *e28-vWF* comprised 94 sequences and 1142 sites. The presence of frameshift mutations and stop codons was verified visually. Both alignments were submitted to DNAsp 6 (Rozas *et al.* 2017) to determine unique haplotypes, which were used to construct non-redundant datasets.

Phylogenetic inference with *mt-Cytb* was performed for the non-redundant alignment by both maximum likelihood and Bayesian inference, using a representative of each genus in Echimyidae, in addition to *Octodon* Bennett, 1823 and *Ctenomys* Blainville, 1826 (Table 2) as outgroup sequences, following previous publications (Verzi *et al.*, 2016; Olivares *et al.*, 2017; Courcelle *et al.*, 2019). Maximum likelihood was also performed with *e28-vWF*, using *Clyomys laticeps* Thomas, 1909 (AJ849306), *Euryzygomatomys spinosus* (Fischer, 1814) (AJ849319), *Capromys pilorides* Say,

Table 2. List of outgroup genera used in the *mt-Cytb* analysis with the GenBank accession number

Genus	Accession number
Euryzygomatomys	EU544667.1
Clyomys	AF422918
Thrichomys	JX459852.1
Proechimys	NC_039544
Hoplomys	KU892779.1
Myocastor	LC257678.1
Lonchothrix	KU892786.1
Mesomys	KU892787.1
Isothrix	KU892785.1
Echimys	EU302690
Phyllomys	KU756488.1
Toromys	NC_037782.1
Makalata	NC_037779.1
Olallamys	KU892774.1
Dactylomys	NC_029876.1
Kannabateomys	EU544665.1
Octodon	KJ742651.1
Ctenomys	MT787172.1

1822 (AJ251142) and *Makalata macrura* (Wagner, 1842) (AJ849312) as outgroup sequences. The estimates for *mt-Cytb* marker were conducted under the GTR+I+G nucleotide substitution model and the estimates for *e28-vWF* under HKY+G+I nucleotide substitution model (Hasegawa *et al.*, 1985), which was selected by ModelFinder (Kalyaanamoorthy *et al.*, 2017) as having the best fit to the dataset. The maximum likelihood tree was reconstructed with IQ-TREE (Nguyen *et al.*, 2015), using 1000 replicates of SH-aLRT to access branch support levels (Guindon *et al.*, 2010). Bayesian inference was conducted with BEAST 2 (Bouckaert *et al.*, 2014), applying a birth and death tree prior and the uncorrelated relaxed clock model with log-normal distribution for evolutionary rates (Drummond *et al.*, 2005, 2006). Four calibration points were used for molecular dating analysis. The first calibration point referred to the *Octodon* + *Ctenomys* root, with minimum age of 25 Myr [Mean (M) = 0; Sigma (S) = 1]. The second calibration point corresponded to the fossil *Paradelphomys* Patterson & Pascual, 1968, with a minimum age of 20 Myr (M = 0; S = 1). The third and fourth calibration points corresponded to the fossils *Pampamys* Verzi Vucetich & Montalvo, 1995 and *Theridomysops* Vucetich, 1995, respectively, both with a minimum age of 6 Myr (M = 0.01; S = 0.6) (Tavares *et al.*, 2015; Courcelle *et al.*, 2019). The prior on root age was expressed as log-normal distribution with mean 0.01, standard deviation 0.6 and offset 6 Myr.

The analysis was performed with two MCMC of 100 million generations, sampling every 5000 steps, which were combined using the LogCombiner. Subsequently, parameter mixing (ESS > 200) was verified with TRACER 1.5 (Rambaut *et al.*, 2018) and a maximum clade credibility tree was compiled on TreeAnnotator v.1.7.5, discarding the initial 10% sample trees as burn-in. All inferred trees were visualized on FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Outgroup taxa were removed from all phylogenies using the software ARCHAEOPTERYX (Han & Zmasek, 2009), to proceed with species delimitation analysis.

Species delimitation and population analysis

We tested species boundaries in *Trinomys* by employing two independent single locus-based species delimitation methods for *mt-Cytb*: bPTP (Bayesian Poisson tree processes; Zhang *et al.*, 2013) and GMYC (generalized mixed Yule coalescent model; Fujisawa & Barraclough, 2013). bPTP models Yule-coalescent transition points based on changes in substitution rates on the maximum likelihood phylogenetic input tree. The analyses were run on the bPTP web server (http://species.h-its.org/ptp/), using 400 000 MCMC generations, with thinning value = 100 and burn-in = 0.25, using the maximum likelihood tree estimated in IQ-TREE. We visually confirmed the convergence of the MCMC chains, as recommended by Zhang *et al.* (2013). GMYC species delimitation analysis was also performed on the webserver (http://species.h-its.org/gmyc/). Based on an ultrametric input tree, GMYC estimated the speciation–coalescent threshold, choosing the transition point between speciation and coalescent processes (Pons *et al.*, 2006; Puillandre *et al.*, 2012). This analysis was performed with default settings using the time tree estimated in BEAST.

Estimates of *p*-distances were carried out with MEGAX v.10.2.6 for each lineage recognized by bPTP and GMYC, separately. NETWORK v.4.5.1.6 (https://www.fluxus-engineering.com/; Bandelt *et al.*, 1999) was used to reconstruct median-joining networks for *T. albispinus*, *T. dimidiatus*, *T. iheringi* and *T. setosus*. These species were selected in view of better sampling of sequences, geographical distribution completeness and results of previous analyses. Population structure and patterns of geographic distribution were characterized from the *p*-distance results and network reconstructions.

RESULTS

Phylogenetic analyses

Overall, maximum likelihood (Supporting Information, Fig. S1) and Bayesian inference (Fig. 2) with *mt-Cytb* recovered nearly identical phylogenetic relationships, but different from the one with *e28-vWF* (Supporting Information, Fig. S1). The topology for *mt-Cytb* separated the genus into three main clades: A, B and C (Fig. 1). Clade A is comprised of *T. dimidiatus*, *T. iheringi*, *T. mirapitanga*, *T. gratiosus* and *T. moojeni*. This clade is divided into two subclades, one with *T. dimidiatus* and *T. iheringi* as sister-groups and *T. mirapitanga* as their closest species. The other clade within Clade A is comprised of *T. gratiosus* and *T. moojeni*. Clade B is comprised of *T. yonenagae*, *T. setosus*, *T. paratus* and *T. eliasi*. Clade B is divided into three subclades based on maximum likelihood: *T. setosus*, *T. yonenagae* and a subclade comprised of *T. paratus* and *T. eliasi*, and two subclades from Bayesian inference: *T. setosus* + *T. yonenagae* and *T. eliasi* + *T.paratus*. In both analyses, *T. eliasi* was inferred as a paraphyletic species (due to the haplotype H52, sample PSP58, phylogenetic position; Fig. 1). Clade C is comprised only of *T. albispinus* and was the first one to diverge within *Trinomys*. All relationships recovered for the genus were supported by SH-aLRT values higher than 80, and posterior probability (PP) > 0.72, except for the clade comprised of *T. setosus* + *T. paratus* / *T. eliasi*

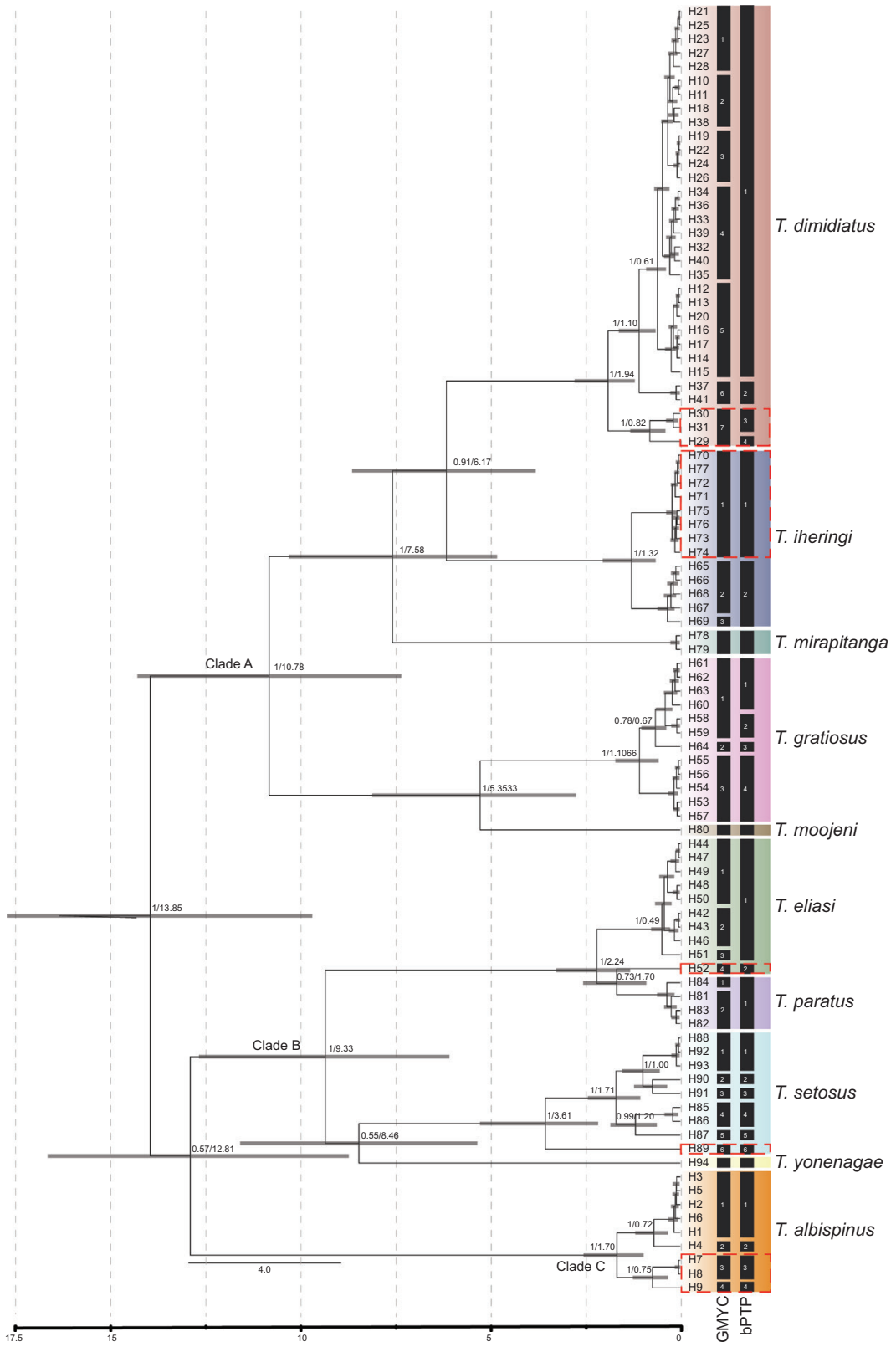

Figure 2. *mt-Cytb* gene time-tree reconstructed by Bayesian analysis. Horizontal bar on the nodes indicates 95% HPD. The values at the nodes represent the posterior probability and the height of the molecular dating analysis, respectively. Vertical bars indicate the ESUS by bPTP and GMYC, respectively. Red dashed rectangles indicate possible new lineages.

(SH-aLRT = 31.6) recovered in maximum likelihood, the clade *T. setosus* + *T. yonenagae* (PP = 0.55) recovered in Bayesian inference and the root of Clade B + C (SH-aLRT = 56.4; PP = 0.57), recovered in both analyses.

Maximum likelihood analysis with *e28-vWF* was performed without samples from *T. mirapitanga* and *T. moojeni* and also recovered three clades: A, B and C. Clade A showed a composition similar to that recovered by *mt-Cytb* (*T. dimidiatus* + *T. iheringi* + *T. g. bonafidei*). Clade C, comprising *T. albispinus* was recovered as a sister-group to Clade A with high support. Clade B was recovered with the same relationships as observed in the analysis conducted with *mt-Cytb*, with the exception of *T. eliasi* and *T. paratus*, which were recovered as belonging to the same lineage. The support for the relationships between *T. setosus* + *T. paratus*/*T. eliasi* were low (SH-aLRT = 49.6), but all other nodes had SH-aLRT support higher than 90.

Bayesian inference was also used to estimate the divergence times of *Trinomys* species. According to the molecular dating, the genus began to diversify approximately 13.8 Mya [highest posterior density (HPD) 95% = 9.6–17.6 Mya; Fig. 2], in the Middle Miocene. The first divergence of Clade A occurred approximately 10.7 Mya (HPD 95% = 7.3–14.2 Mya). The separation between *T. moojeni* and *T. gratiosus* occurred approximately 5.35 Mya (HPD 95% = 2.7–8.0 Mya), between *T. mirapitanga* and (*T. dimidiatus* + *T. iheringi*) approximately 7.5 Mya (HPD 95% = 4.8–10.2 Mya) and between *T. dimidiatus* and *T. iheringi* approximately at 6.2 Mya (HPD 95% = 3.8–8.6 Mya). Clades B and C diverged *c*. 12.8 Mya (HPD 95% = 8.7–16.6 Mya). The first divergence of Clade B occurred approximately 9.33 Mya (HPD 95% = 6.0–12.6 Mya) near the end of the Miocene. The separation between *T. setosus* and *T. yonenagae* occurred approximately 8.4 Mya (HPD 95% = 5.3–11.5 Mya) and the split between *T. eliasi* and *T. paratus*, except for the haplotype H52 from São Francisco de Itabapoana (specimen PSP58), approximately 2.2 Mya (HPD 95% = 1.3–3.2 Mya) and between H52 and *T. paratus* approximately 1.7 Mya (HPD 95% = 0.8–2.5 Mya).

Computational species delimitation and populational analyses

The analysis of *mt-Cytb* recovered more biological entities than the ten currently recognized species. GMYC species delimitation results for *mt-Cytb* recovered 32 evolutionarily significant units (ESU), in which only the species statuses of *T. mirapitanga*, *T. moojeni* and *T. yonenagae* were recovered (Fig. 1). The analysis for *T. paratus* recovered two ESUs, without geographic structure. The analysis for *T. dimidiatus* recovered seven ESUs, with noticeable geographic structure only between the Nova Friburgo ESU and the other ESUs recovered. The analysis for *T. iheringi* recovered three ESUs, one from São Paulo state and two from Ilha Grande. The analysis of *T. gratiosus* recovered three ESUs that match the subspecies already described for the species. The analysis for *T. eliasi* recovered four ESUs with some degree of geographic structure only between the sample PSP58 from São Francisco de Itabapoana municipality and others from Rio de Janeiro state. For *T. setosus*, six ESUs were recovered, two of them in accordance with the subspecies described for the species and one (H89) very distinct, probably belonging to another lineage. The lineages *T. setosus* 1–3 probably correspond to the subspecies *T. s. setosus*, occurring in Sergipe, Bahia, Espirito Santo and Minas Gerais states and the lineages *T. setosus* 4–5 to the subspecies *T. s. elegans*, occurring in Minas Gerais and Rio de Janeiro states. For *T. albispinus* several distinct evolutionary lineages with no detectable geographic structure were recovered.

The bPTP analysis recovered 26 ESUs from the *mt-Cytb* dataset (Fig. 1). Only *T. paratus*, *T. mirapitanga*, *T. moojeni* and *T. yonenagae* were recovered as single species. The results for *T. iheringi*, *T. setosus* and *T. albispinus* were identical to those reported for GMYC. The analysis for *T. dimidiatus* and *T. gratiosus* recovered four ESUs, and for *T. eliasi* two ESUs were recovered, all of them with the same geographic structure as that recovered for GMYC.

The mean genetic *p*-distance estimates were derived from *mt-Cytb* for each lineage recovered by the bPTP and GMYC analyses (Supporting Information, Tables S4, S5). Estimates of genetic distance derived from the strains recovered by bPTP and GMYC showed a mean genetic distance between clades of 15%. Within *T. albispinus*, the same strains were recovered in the bPTP and GMYC analyses, showing a lowest distance of 2% and a highest of 3.9%. The most distant lineage from the others is *T. albispinus* 2 (~3.75% distance to the others). Among the lineages recovered by these analyses for *T. setosus*, the closest genetic distance was 2% and the greatest was 7.4%. *Trinomys setosus* strain 1, represented by H89 from Cristianápolis, is the most differentiated *T. setosus* lineage respective to the remaining ones (~6.9%). The genetic distance between the two strains recovered by GMYC for *T. paratus* was 0.9%. While bPTP recovered four lineages for *T. gratiosus*, GMYC recovered three. Among the lineages recovered by bPTP the maximum genetic distance was 2.9% and the minimum 0.9% and GMYC recovered the maximum genetic distance of 2.8% and the minimum 1.7%. Among *T. iheringi*, the distance between *T. iheringi* 1 and *T. iheringi* 2,

the former representing Ilha Grande individuals, is 3.6%, in the putative lineages recovered by bPTP. With respect to the lineages recovered in GMYC, the *T. iheringi* 2 line was split into two (*T. iheringi* 2 and *T. iheringi* 3), with a genetic distance of 0.08%. Among the lineages recovered in bPTP for *T. dimidiatus*, the closest distance recovered was 2.2% and the greatest was 4.7%. The most distantly related lineages are *T. dimidiatus* 3 and 4, representing samples from Santa Maria Magdalena and Nova Friburgo (~4%). For lineages recovered for *T. dimidiatus* in GMYC, the mean distance between them was *c*. 1%, being greater only between *T. dimidiatus* 7 and the others, showing a genetic distance of 4.6%. The bPTP mean distance between lineages *T. eliasi* 1, represented by H52 from São Franscisco de Itabapoana and *T. eliasi* 2, is 4%. Although *T. eliasi* 1 was recovered phylogenetically closer to *T. paratus*, the genetic distance between them is 5.1%. In the GMYC analysis the mean distance between the lineages is *c*. 3%, except for lineage H52 defined as *T. eliasi* 4, which shows a genetic distance of 6.1%.

Taking species delimitation and populational structure results added to sampling distribution and current taxonomic knowledge into account, haplotype networks were reconstructed to detect geographic structure in the presently recognized species. This analysis was performed on cases indicating there is a possibility of a cluster of species being recognized by a single name (e.g. *T. iheringi)*.

Among the 23 sequences of *T. albispinus*, nine haplotypes were identified and recovered into two haplogroups, the Southern Bahia haplogroup (SB) and the Central North Bahia-Sergipe haplogroup (CNBS). The maximum number of substitutions that unite two different haplotypes within a haplogroup was 21 substitutions plus a median vector, present on haplogroup CNBS. The two haplogroups (SB and CNBS) were separated by at least 42 nucleotide substitutions and one median vector, which also split CNBS haplotype H3 from the others. Samples morphologically identified as *T. a. minor* were present in both haplogroups (Fig. 3). Regarding *T. dimidiatus*, 32 haplotypes, from 82 sequences, were recovered in two main haplogroups, corresponding to the Central and Southern Serra do Mar haplogroup (CSSM) and the northern Serra do Mar haplogroup (NSM). Such haplogroups are separated by at least 30 nucleotide substitutions and three medium vectors (Fig. 3), the same number of maximum substitutions that occur within the CSSM haplogroup. Nevertheless, we recognized two main groups, congruent with the remaining analyses performed (phylogenetic inference and *p*-distance). With respect to *T. iheringi*, 13 out of 43 sequences were classified into distinct haplotypes, which also split into two groups separated by at least 25 substitutions and one medium vector, the Ilha Grande (IG) and São Paulo (SP) haplogroups (Fig. 3). Within these haplogroups the maximum number of substitutions is seven, occurring in the SP haplogroup. For *T. setosus*, nine haplotypes were assigned to 12 sequences. The median-joining analysis recovered two groups: one comprising H89 from Cristianápolis (SE) and another comprised of all the other haplotypes, distributed among Minas Gerais and Rio de Janeiro (MGRJ). These two groups are separated by at least 68 nucleotide substitutions (Fig. 3), less than the maximum number of substitutions present within the haplogroup MGRJ, which is 75 nucleotide substitutions. Results are summarized in Table 1.

DISCUSSION

Molecular clock analysis estimated the origin of *Trinomys* in the Middle Miocene (Fig. 1), similar to previous studies, which suggested Early and Middle Miocene (Lara & Patton, 2000; Tavares *et al.* 2015; Fabre *et al.* 2016, Álvarez *et al.* 2017, Olivares *et al.* 2020) and rather differently from a study with multigene approach based on several echimyid species and five fossil constraints (Courcelle *et al.*, 2019). The predicted date of divergence between *mt-Cytb* Clades A and B was recovered in the Miocene, similar to that already found in the literature (Tavares *et al.*, 2016). The dates of divergence between species within Clades A and B indicate that *T. moojeni* and *T. gratiosus*, *T. iheringi* and *T. dimidiatus*, and *T. setosus* and *T. yonenagae* diverged in the Miocene, while *T. paratus* and *T. eliasi* diverged in the Pleistocene, consistent with the literature for mitochondrial genes (Fabre *et al*, 2016; Tavares *et al.*, 2016). The dates estimated in this work for the divergence between species in each clade are older when compared to the dates estimated with genomic data (Courcelle *et al.*, 2019). For populational divergence within each species, we recovered estimates that support the hypothesis that the last event of great diversification of South American hystricognath rodents occurred at the end of the Miocene and beginning of the Pliocene (Vucetich *et al.*, 1995). This hypothesis, which states that these diversification processes were partially driven by the disappearance of South American palaeogenic fauna and the rise of the Andes, is also consistent with the origins of other hystricognath rodent genera such as *Proechimys* J.A.Allen, 1899 and *Thrichomys* Trouessart, 1880 (Da Silva & Patton, 1998; Nascimento *et al.*, 2013).

The topology of the *mt-Cytb* maximum likelihood tree is consistent with the results found in previous studies, recovering three major clades within *Trinomys* (Fig. 1; Leite & Patton, 2002; Galewski *et al.*, 2005; Tavares *et al.*, 2015, 2016, Álvarez *et al.*, 2017, Olivares

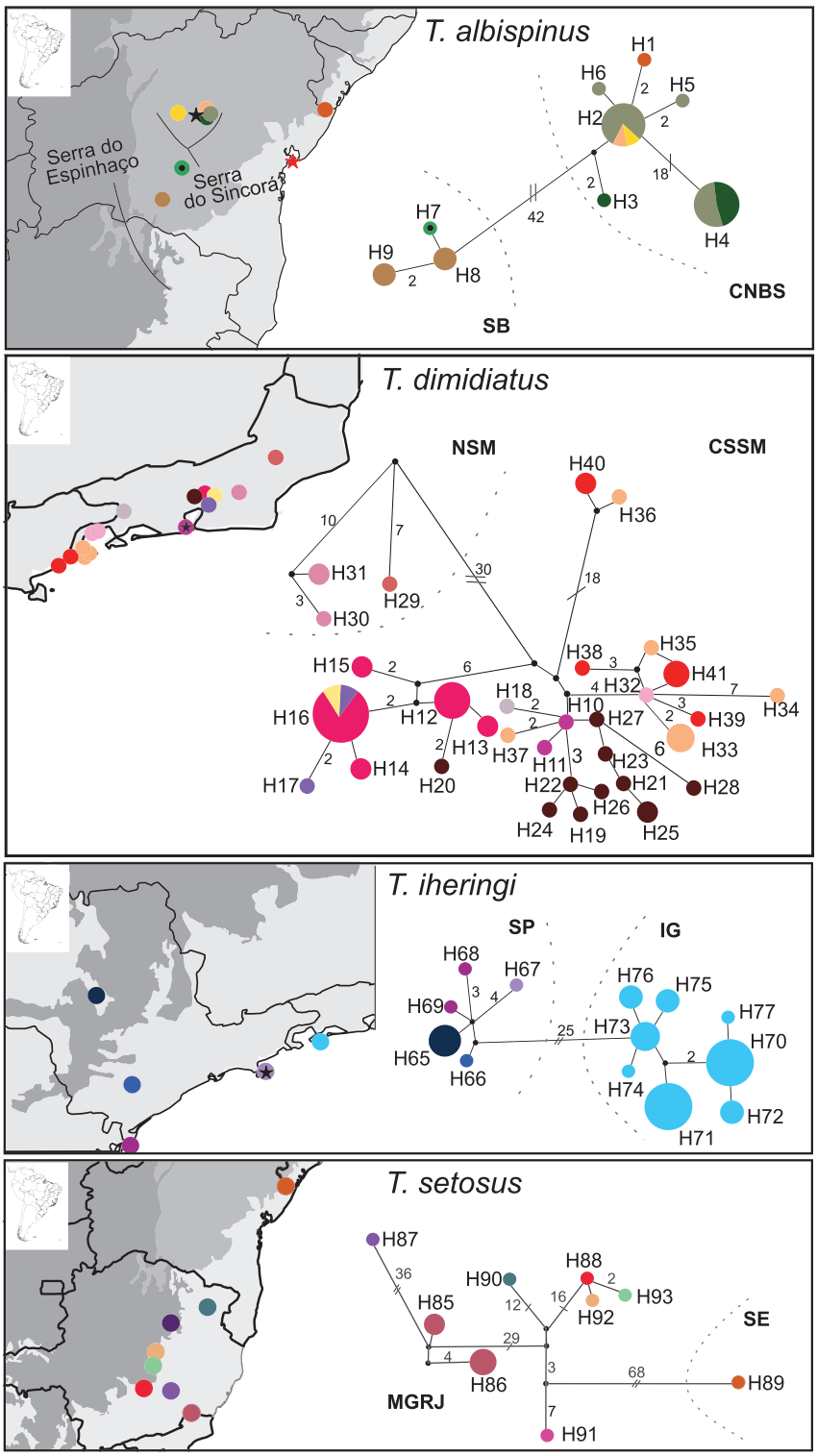

Figure 3. Maps indicating the type locality (star) and the collecting localities (circle) coloured according to the haplotype network. Circle size is proportional to the number of shared sequences, numbers refer to the haplotypes in Table 1, the small black circles represent the median vectors, and the numbers adjacent to the lines are nucleotide substitutions. SB = Southern Bahia, CNBS = Central North Bahia-Sergipe, NSM = Northern Serra do Mar, CSSM = Central and Southern Serra do Mar, IG = Ilha Grande, SP = São Paulo, SE = Sergipe, MGRJ = Minas Gerais and Rio de Janeiro.

et al., 2020). In this topology, *T. albispinus* belongs to Clade C, which is the sister-group to Clade B, and *T. paratus* and *T. eliasi* are recovered as distinct species and sister-group. The maximum likelihood topology recovered with *e28-vWF* shows *T. albispinus* as sister-group of Clade A, consistent with a previous study that used a multigene approach (Courcelle *et al.*, 2019). Another difference in the topology is the relationship between *T. paratus* and *T. eliasi*, which were recovered as the same lineage, showing that this exon is not useful in distinguishing clearly between groups that have diversified more recently. These results reflect the incongruence resulting from using few markers to infer the evolutionary history of a group (Rokas *et al.*, 2003). Although the sampling used for the analysis with *e28-vWF* was much smaller, this is not the only factor influencing the topological difference. Each gene tells its own evolutionary story and is subject to events such as incomplete lineage sorting and introgression and does not necessarily reflect the evolutionary history of the taxa (Rokas *et al.*, 2003).

Species delimitation and spatial population genetics

Beyond the characterization of diversification dynamics through molecular dating, two different methods of computational species delimitation were used to assess the number of ESUs across the phylogeny. Both methods employed to delimit ESUs recovered a higher diversity than that previously recognized for *Trinomys*. While the analysis with *mt-Cytb* on GMYC delimited 32 ESUs, the analysis of bPTP estimated 26 ESUs (Fig. 1). The main differences were observed in the diversity of the *T. dimidiatus* cluster (considered seven ESUs by GMYC and four by bPTP), *T. eliasi* (considered four ESUs by GMYC and two by bPTP), *T. gratiosus* (considered three ESUs by GMYC and four by bPTP), *T. iheringi* (considered three ESUs by GMYC and two by bPTP) and *T. paratus* (considered two ESUs by GMYC and one by bPTP).

Within *T. eliasi*, the only lineage recovered as a different ESU in all analyses was H52 (individual PSP58) from São Francisco de Itabapoana. This individual was more closely related to *T. paratus* than to *T. eliasi* in our analyses with *mt-Cytb*, although its morphological characteristics matched those of *T. eliasi*. The collection site of this individual is a region that has remained isolated from other areas of *T. eliasi* and *T. paratus* for a long period during the evolution of these groups, as suggested by Tavares *et al.* (2015). The genetic distance between H52 and the rest of *T. eliasi* and *T. paratus* is ~4.75%, one of the highest within the genus. Studies comparing genetic distances between rodent lineages suggest that genetic distances below 2% represent population divergence, between 2 and 5% represent subspecific divergence and above this value represent specific divergence (Bradley & Baker, 2001; Baker & Bradley, 2006). In this context, the individual PSP58 indicates a possible new lineage, but more individuals should be analysed for confirmation.

Trinomys setosus also contained several ESUs, one of which was highly corroborated by genetic distance (H89). The genetic distance of H89 to the other haplotypes of *T. setosus* is approximately 7% and is considered specific divergence (Bradley & Baker, 2001; Baker & Bradley, 2006). This result had already been reported by Lara & Patton (2000), but no statement was made. As with H52, this haplotype is comprised of only one individual, thereby limiting the validity of any conclusions. In addition, there is a wide sampling gap between the locality of this haplotype and the other localities used in this work. Nevertheless, there is evidence that this is a new lineage, but more extensive sampling studies should be conducted.

Disagreement between computational species delimitation methods has been observed in other studies with Neotropical rodents (Weksler & Bonvicino, 2015; Da Cruz & Weksler, 2018). GMYC and bPTP are sensitive to sample and population sizes and speciation rates of each group (Papadopoulou *et al.*, 2008; Esselstyn *et al.*, 2012; Fujisawa & Barraclough, 2013; Zhang *et al.*, 2013, Luo *et al.*, 2018). When variance associated with branch size estimation is large, GMYC can generate biased diversity estimates (Tang *et al.*, 2014). Meanwhile, bPTP is sensitive to heterogeneity in molecular evolutionary rates, where high mutations rates can generate oversplitting (Tang *et al.*, 2014; Dellicour & Flot, 2018). Although the GMYC method characteristically overestimates the true species diversity in a group (Fujisawa & Barraclough, 2013; Serrano *et al.*, 2019), it is sensitive to diagnosing species that are valid and present remarkably similar morphologies, which are not diagnosed by other methods (Nogueira *et al.*, 2020). Previous studies indicate that there are large differences between taxonomic estimates of diversity and those generated by single locus-based methods (Tang *et al.*, 2014), an observation also herein reported. Nonetheless, unilocus species delimitation has been used for suggesting new species in rodents (Da Cruz & Weksler, 2018; Pinto *et al.*, 2018; Hurtado & D'Elia, 2019; Thanou *et al.*, 2020; Ge *et al.*, 2021; Ojeda *et al.*, 2021).

As with all studies employing computational species delimitation methods, confidence levels for the results are conditional on sampling strategy. Although we collated a comprehensive set of novel gene sequences throughout the distribution of *Trinomys*, the sampling intensity was uneven, ranging from 1 to 32 sequences for each species (Table 1). Additionally, some species have not been sampled across their entire geographic

distribution. In this sense, it is possible that diversity estimates for poorly sampled species and regions suffered from this bias. Nevertheless, the consistency between ESU boundaries and geographically structured subpopulations supports the efficiency of the methods in detecting populations experiencing discontinuities in gene flow. Moreover, several ESUs have been defined by samples from uniquely recognized species (e.g. *T. gratiosus*, *T. setosus*), indicating that the taxonomic units should be further evaluated.

Given the ESU composition detected throughout the *Trinomys* phylogeny, we analysed the geographic distribution of haplotypes contained in populations of *T. setosus*, *T. albispinus*, *T. dimidiatus* and *T. iheringi*. In this sense, we were able to detect whether different local subpopulations were defined as different ESUs and to evaluate the power of species delimitation methods in characterizing divergent lineages. Also, the spatial distribution of genetic diversity was interpreted chronologically following the dated tree. Integrating these approaches revealed greater diversity than that captured by the current taxonomic arrangement. In the following, we discuss results for all relevant populations and address cases where deeper taxonomic investigations could confirm novel species status.

TRINOMYS NOMINAL SPECIES WITH MORE THAN ONE LINEAGE

The last treatment of the genus *Trinomys* recognized two subspecies for *T. albispinus*: *T. a. albispinus* and *T. a. minor* (Pessôa *et al.*, 2015). Morphological analysis with samples from Chapada Diamantina, in Bahia, recovered both morphological forms occurring in Morro do Chapéu (Souza *et al.*, 2006). These morphological forms are classified as subspecies (Souza *et al.*, 2006) or species (Iack-Ximenes, 2005), with no consensus on their taxonomic status. Here, the *mt-Cytb* phylogeny and the haplotype network, with the greatest sampling of specimens and locations yet performed, divided *T. albispinus* into two groups: one formed by samples from south-central Bahia (SB) and another by samples from north-central Bahia and Sergipe (CNBS) that diverged approximately 0.75 Mya (2.00–0.43Mya) (Fig. 2A). From analysis of the divergence times and the geographic structure detected here, we suggest that the Serra do Sincorá may have acted as a geographical barrier between the two populations because its origin was 1.1 Mya (1.2–1.0 Mya) in the Pleistocene (Schobbenhaus, 1996). Estimates of genetic differentiation between populations separated by this mountain range suggest the presence of two evolutionary lineages, started by a process of vicariance associated with the emergence of Serra do Sincorá. This mountain range is comprised of deep valleys with steep cliffs and vast plateaus, with elevations that reach 2000 m (Funch, 1997). The division of *T. albispinus* into two lineages suggested by *mt-Cytb* sequence analysis did not match previous divisions based on morphological data (the two recognized subspecies, *minor* and *albispinus*), based mainly on body size (Pessôa & Strauss, 1999). However, our data clearly showed the presence of two lineages within *T. albispinus*, one lineage north of Serra do Sincorá and an undescribed taxon that is restricted to between South of Serra do Sincorá and Serra do Espinhaço, with a genetic distance of approximately 4% (Supporting Information, Tables S3, S4).

Trinomys dimidiatus is widely distributed, from São Paulo state to the north of Rio de Janeiro. Morphologically, there are qualitative and quantitative differences between specimens collected from São Paulo state to the southern part of Serra dos Órgãos (CSSM), in Rio de Janeiro state, and those collected in the north-central part of this mountain range (NSM; Iack-Ximenes, 2005) (Fig. 2B). The difference between them is not restricted to morphology but is also strongly reflected in the molecular data (Lara & Patton, 2000). One of the greatest genetic distances found here was within *T. dimidiatus*, between samples from Nova Friburgo and Teresópolis (separated by 100 km), showing up to 4.7% divergence. The samples from Picinguaba and Teresópolis, separated by about 250 km, showed 1.3% divergence. We suggest that the genetic distance reflects structuring between samples collected in Teresópolis and Nova Friburgo, implying the presence of two diverging lineages in this species, which is not correlated with geographic distance, and thereby conforming to the same conclusion reached by Lara and Patton (2000) (Supporting Information, Tables S3, S4).

Trinomys iheringi has a disjunct distribution, occurring in the coast and the centre of São Paulo and in Ilha Grande, Rio de Janeiro state (Moojen, 1948; Pêssoa *et al.* 2015). This is the first molecular study performed with samples of *T. iheringi* from Ilha Grande. Analysis with *mt-Cytb* supports *T. iheringi* being split into two clades: one from São Paulo (SP) and the other from Ilha Grande (IG). The estimated divergence time between these populations was approximately 0.6 Mya, a timeframe compatible with the diversification of this clade being affected by at least six glaciation events (Berggren *et al.*, 1972; Van-Husen, 2000) leading to expansion and contraction of the Atlantic forest (Grazziotin *et al.*, 2006). Furthermore, the São Paulo region (where one of the *T. iheringi* lineages occurs) was a refuge during the Pleistocene (Carnaval & Mortiz, 2008; Grazziotin *et al.*, 2006; Costa & Leite, 2012). Additionally, both species delimitation analyses recovered the two populations as two ESUs and the genetic distance estimate was 3.6%, compatible with subspecific divergence. Likewise, it has been observed that each population presents unique

qualitative and quantitative morphological characters (Iack-Ximenes, 2005). These different lines of evidence suggest that the two populations of *T. iheringi* are, in fact, different species. In this scenario, based on type locality (Ilha de São Sebastião), the population of São Paulo would represent *T. iheringi*. Nonetheless, additional morphological analyses are necessary to provide a robust species classification for the Ilha Grande population.

CONCLUSION

Through the course of this investigation, an array of molecular evolutionary methods, applied to a single locus of mtDNA, led to the conclusion that the species diversity currently recognized for the genus *Trinomys* is underestimated. Importantly, integrating these results implies that several putative novel species may exist, including diverse cases of diverged populations harbouring no morphological differences, calling for a complete systematic re-evaluation of the genus. Most notably, the convergence of this analysis with previous studies on *T. iheringi* suggests that this name includes more than one species. Our results emphasize the important role molecular data and computational species delimitation methods may hold on uncovering hidden biological diversity.

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DATA AVAILABILITY

The data underlying this article are available in the GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov, and can be accessed with accession numbers: OP277633 - OP277802; OP381023.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Maximum likelihood (ML) tree, with SH-alrt node supports > 90 represented by black circles and supports < 90 being displayed. Recognized species are indicated by dark-grey rectangles, with the currently recognized names. Potential new lineages are indicated with light-grey squares. A, result of ML with *mt-Cytb*, where haplotypes are according to those in Table 1. B, result of ML with *e28-vWF*.
Table S1. List of species and subspecies of *Trinomys* currently recognized and not recognized (Status) with corresponding type locality (municipality and/or locality and Brazilian state) and description date and author.
Table S2. Taxonomic rearrangements of *Trinomys*.
Table S3. List of analysed species with *e28-vWF* with haplotype (H), locality (Brazilian state, municipality and/or locality), field or museum number, GenBank accession number, and reference (Ref.)
Table S4. Average molecular distance (*p*) for each pair of lineages recognized for bPTP with *mt-Cytb* in Figure 2.
Table S5. Average molecular distance (*p*) for each pair of lineages recognized in GMYC with *mt-Cytb* in Figure 2.