








New records and a new mtDNA lineage of *Cryptonanus agricolai* (Moojen, 1943) (Didelphimorphia, Didelphidae) from Maranhão state, northeastern Brazil

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Abstract

Cryptonanus agricolai (Moojen, 1943) is a small didelphid widely distributed across of central and northeastern Brazil and in the Brazilian Atlantic Forest. A recent phylogeographic study of this species identified three major mtDNA clades with strong geographic structure. Here, we present new records of *C. agricolai* in northeastern Brazil, including the first report for Maranhão state. We also identify a novel mtDNA lineage, sister to one of the three major clades, in some specimens from Maranhão, indicating that *C. agricolai* is morphologically and genetically more variable than previously recognized.

Keywords

Caatinga, Cerrado, genetic diversity, geographic distribution, marsupials, mitochondrial DNA

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Introduction

Cryptonanus Voss, Lunde & Jansa, 2005 is a genus of marsupial didelphids that contains four valid species distributed throughout South America: *C. agricolai* (Moojen, 1943), *C. chacoensis* (Tate, 1931), *C. guahybae* (Tate, 1931), and *C. unduaviensis* (Tate, 1931). A

fifth species, *C. ignitus* (Díaz, Flores & Barquez, 2002), which was known from a single specimen and considered extinct by the IUCN, has been synonymized with *C. chacoensis* by Teta and Díaz-Nieto (2019). Until the revision by Voss et al. (2005), specimens belonging to

Cryptonanus were assigned to *Gracilinanus* Gardner & Creighton, 1989. This represents a challenge for retrieving records of *Cryptonanus* species from museum specimens, which must undergo a detailed investigation for a correct identification. *Cryptonanus* is distinguished from *Gracilinanus* by some craniodental characters, including: the lack of maxillary palatal vacuities, the presence of a secondary foramen ovale and a rostral process on the premaxillae, a P3 that is taller than P2, and accessory cusps that are often present on C1 (Voss et al. 2005). Despite previously having been allocated together, specimens from both genera belong to non-sister monophyletic clades with high support in the molecular phylogeny of Voss and Jansa (2009).

Cryptonanus is mostly known from tropical and subtropical biomes south of the Amazon River and east of the Andes (Fig. 1), including the Caatinga, Cerrado, Chaco, the northern Pampas, and the Brazilian Atlantic Forest (in the Parana dominion; sensu Morrone 2014) (Voss et al. 2005; Voss and Jansa 2009; De La Sancha and D'Elia 2015). Two species have relatively small geographical distributions: *C. guahybae*, which occurs in the Brazilian state of Rio Grande Sul in the boundary region between the Pampas province and the Parana dominion towards Santa Catarina and Paraná states (Dias et al. 2016; Fegies

et al. 2021), and *C. unduaviensis*, which is restricted to relatively close localities in Bolivia, Paraguay, and Brazil (De La Sancha and D'Elia 2015; Fegies et al. 2021). On the other hand, *C. chacoensis* and *C. agricolai*, are widely distributed in a region with considerable ecologic diversity. In particular, *C. agricolai*, which presents the most equatorial distribution of the genus, has been reported exclusively from Brazil. This species seems to be more widely distributed in the open areas of central Brazil, but recently it has been recorded in the Brazilian Atlantic Forest in the Brazilian Southeast (Bezerra et al. 2009, 2014; Souza et al. 2010; Carmignotto and Aires 2011; Bonvicino et al. 2012; Gomes et al. 2015; Fegies et al. 2021; Guerra and Costa 2021).

Traditionally, discrete craniodental characters, measurements, and skin color patterns have been used for identification of *Cryptonanus* species (Voss et al. 2005). However, some of these characters, such as color patterns, may be subjective, especially when it is not possible to compare specimens side-by-side (Voss et al. 2005). Indeed, studies reporting new records of *Cryptonanus*, including the unexpected find of this genus in the Amazon region of French Guiana (Kocher et al. 2016), have failed to provide definitive taxonomic identifications to species (Garcia et al. 2010; Lóss et al. 2011; Vivo 2011;

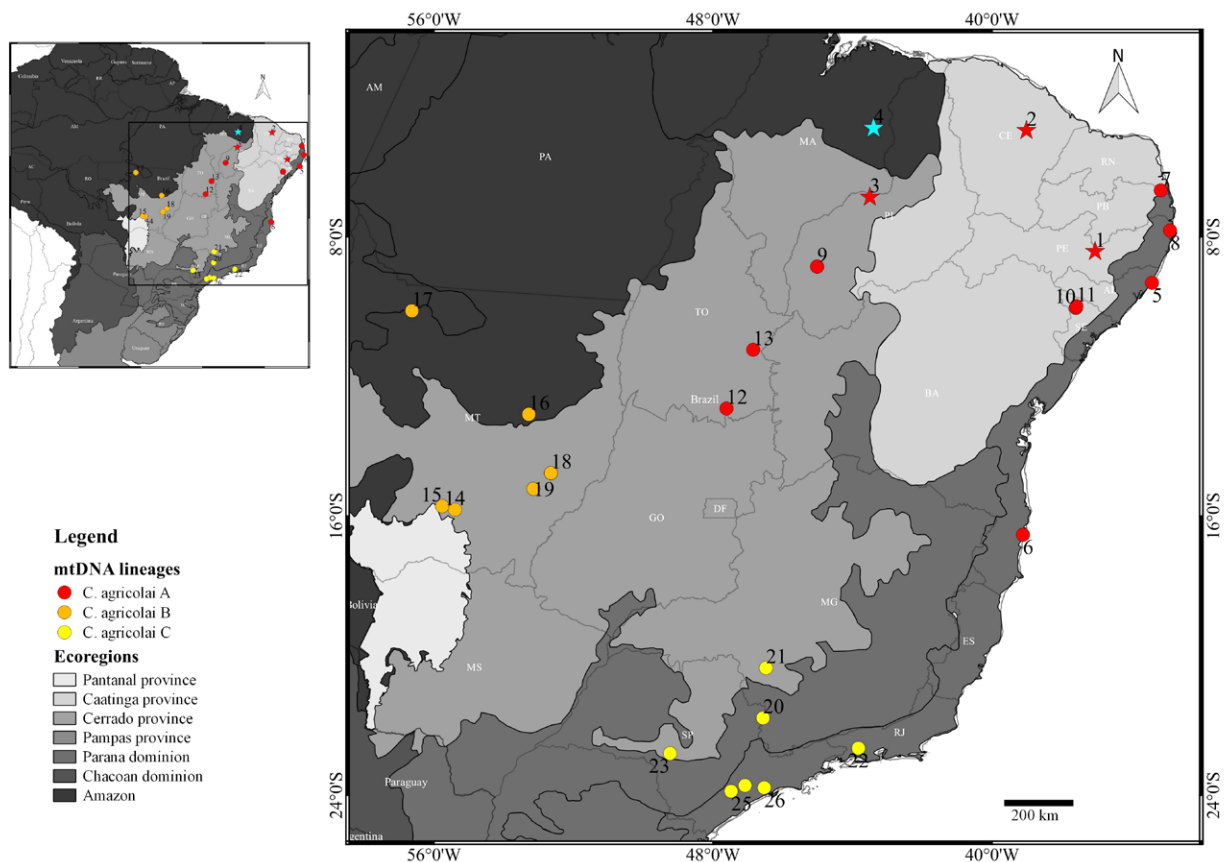


Figure 1. Species distribution for the genus *Cryptonanus agricolai*. The numbers refer to all localities detailed in Appendix Table A1. New records from this study are represented with a star (northeastern Brazil), and the location showing the new mtDNA lineage is shown in light blue. The ecoregions follow the patterns described by Morrone (2014). For the Amazon ecoregion we group the Boreal and South Brazilian domains and Xingu-Tapajós Province. Note: although the point located in the state of Maranhão is shown on the map within the Amazon ecoregion, this location does not belong to the Amazon biome, but to the Cerrado biome within the Pará province by Morrone (2014).

Carmignotto and Bezerra 2014). These taxonomic issues are a challenge for the better understanding of the natural history and ecology of *Cryptonanus* and, in particular, for the correct interpretation of new geographical records (e.g., Garcia et al. 2010; Vivo 2011; Delciellos et al. 2016; Dias et al. 2016; Fegies et al. 2021) that may represent new taxa or novel phylogeographic lineages.

A recent study using molecular markers has shown a clear separation among *Cryptonanus* species (Fegies et al. 2021), reinforcing the usefulness of these markers for the identification of species (De La Sancha and D'Elia 2015; Dias et al. 2016; Guerra and Costa 2021). Fegies et al. (2021) were able to identify two major lineages for *C. guahybae*, four lineages of *C. chacoensis*, and three lineages for *C. agricolai*. Here, we present new records of *C. agricolai*, including the first report from Maranhão state, in addition to other locations, in northeastern Brazil. Importantly, we also identify a novel mtDNA lineage in two specimens from Maranhão, indicating that *C. agricolai* is morphologically and genetically more variable than previously recognized.

Methods

Data collection and morphological analysis. We analyzed six specimens of *Cryptonanus* from four states in northeastern Brazil (Table 1). The material is deposited in the Mammal Collection of the Federal University of Paraíba (UFPB). Craniodental and skin characters were analyzed following Voss et al. (2005) and Voss and Jansa (2009). The diagnostic characters for *Cryptonanus* species were compared with Voss et al.'s (2005) descriptions and figures. For the morphological analysis, two specimens of the genus *Gracilinanus* were included for comparison (Table 1).

Molecular analysis. DNA extraction was performed using Promega's Wizard Genomic DNA Purification Kit. We sequenced a fragment of the cytochrome b (Cytb) and cytochrome oxidase I (COI) genes in the mtDNA. Cytb was amplified by polymerase chain reaction (PCR) using primers MVZ5 and MVZ16 (Smith and Patton 1993) with an initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 72 °C for 10 min. The

COI gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al. 1994) with an initial denaturation (94 °C for 3 min), 40 cycles of denaturation (94 °C for 45 s), annealing (48 °C for 45 s), and extension (72 °C for 1 min and 30 s), and a final extension at 72 °C for 3 min. PCR products were checked in agarose gel, purified enzymatically using the kit ExoProStar 1-Step (GE Healthcare), and sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturers' protocols. We used primers MVZ5 (Cytb) and HCO2198 (COI) for sequencing. The sequences were obtained using an ABI 3500 Automatic DNA sequencer with Associated Sequencing Analysis software (Applied Biosystems).

Data analysis. All chromatograms were checked in Bioedit v. 7.0.8.0 (Hall 1999), and the regions of low quality were manually removed from the final sequence. For the complete dataset, we included in the analysis 92 Cytb (MW208219–MW208298, KT334295, KM188469, KM188470–KM188476, KM188479–KM188481, KM188484, KR190438, KF313984) and 68 COI (MW208149–MW208216) sequences previously reported for *Cryptonanus* (Fegies et al. 2021), which were concatenated totaling 108 individuals and 67 different haplotypes. We also included sequences from other species as outgroups, including *Chacodelphys formosa* (KU171186), *Gracilinanus agilis* (AJ508401), and *Thylamys velutinus* (NC054268).

The sequences were aligned using the ClustalW (Thompson et al. 1994) algorithm implemented in Bioedit v. 7.0.8.0 (Hall 1999) using standard parameters, and then converted into the fasta format. We used DAMBE (Xia 2013) to evaluate the saturation curve of base replacements and DNASP v. 5.10.01 (Rozas et al. 2017) to identify the different haplotypes. The median-joining network (Bandelt et al. 1999) was separately estimated in Network v. 10.2.0 (fluxus-engineering.com) for each gene. The phylogenetic relationship among *Cryptonanus* genetic lineages were reconstructed for the concatenated Cytb/COI alignment using the maximum-likelihood (ML) method in IQTree v. 1.6.12 (Nguyen et al. 2015). For estimating the best molecular evolutionary model we created different partitions for each gene and used IQTree to select the best partitioning scheme

Table 1. Details of the *Cryptonanus agricolai* and *Gracilinanus agilis* specimen analyzed in the present study.

<i>Cryptonanus</i> sp.	Field no.	Voucher	Locality	Latitude	Longitude	Morphological data	Cytb	COI	HB	TL	EL	HL	Sex
<i>C. agricolai</i>	BC397	—	BR: PE: Arcoverde	−08.41	−037.05	Skin	H33	—	95	105	16	14/15	F
<i>C. agricolai</i>	T35	UFPB10943	BR: CE: Quixadá	−04.95	−039.01	Skin, skull	H32	—	76	96	16	13/13	M
<i>C. agricolai</i>	TG09	—	BR: PI: Guadalupe	−06.85	−043.50	—	H31	—	—	—	—	—	—
<i>C. agricolai</i>	DCN18	—	BR: MA: Caxias	−04.88	−043.40	—	H29	H23	—	—	—	—	—
<i>C. agricolai</i>	DCN14	UFPB9082	BR: MA: Caxias	−04.88	−043.40	Skin, skull	H30	H22	85	107	15	13	M
<i>C. agricolai</i>	DCN30	UFPB9047	BR: MA: Caxias	−04.88	−043.40	Skin, skull	—	H22	—	67	11	10	M
<i>G. agilis</i>	—	UFPB9343	BR: MS: Corumbá	—	—	Skin	—	—	120	93	19	17/16	M
<i>G. agilis</i>	—	UFPB9373	BR: MS: Corumbá	—	—	Skin	—	—	139	108	21	12/12	F

BR = Brazil; PE = Pernambuco; CE = Ceará; PI = Piauí; MA = Maranhão; HB = head-to-body length; TL = tail length; EL = ear length; HL = hindfoot length.

and the best model for each partition using the Akaike information criterion (AIC). The best partition scheme merged the two genes in the same partition, and the best evolutionary model was TIM2+F+I+G4. We used the default settings for the tree search and 1,000 ultrafast bootstrap replicates to evaluate node support. The trees were visualized and edited in Figtree v. 1.4.2 (<http://tree.bio.ed.ac.uk/software/fig-tree/>).

Results

Cryptonanus agricolai (Moojen, 1943)

New records. BRAZIL – Maranhão • Caxias, Área de Proteção Ambiental (APA) Municipal do Inhamum; -04.88, -043.4; 66 m alt.; 7.III.2011; D.C. Nascimento leg.; Genbank OM223076 and OM236542; ♂; UFPB9082 • Caxias, APA Municipal do Inhamum; -04.88, -043.4; 66 m alt.; 10.IV.2011; D.C. Nascimento leg.; Genbank OM223077 and OM236543; undetermined sex; DCN18 • Caxias, APA Municipal do Inhamum; -04.88, -043.4; 66 m alt.; 16.VII.2011; D.C. Nascimento leg.; Genbank OM236544; ♂; UFPB 9047 – Ceará • Quixadá; -04.95, -039.01; 189 m alt.; 23.VII.2009; G. Toledo leg.; Genbank OM223079; undetermined sex, UFPB10943 – Pernambuco • Arcoverde; -08.42, -037.05; 663 m alt.; 30.VI.2009; B.A.T.P. Campos; Genbank OM223080; ♀, BC397 – Piauí • Guadalupe; -06.85, -043.50; 177 m alt.; 21.III.2015; G. Toledo; Genbank OM223078; undetermined sex, TG09.

Identification. The four specimens for which morphological data were available (BC 397 and UFPB9082) were identified as *Cryptonanus* because, in comparison with *Gracilinanus* specimens, we observed narrower circular masks, smaller ears, shorter mystical vibrissas, and less dense and woolly dorsal hairs (Table 1). A short,

dark midrostral band was present in both *Cryptonanus* specimens. Although Voss et al. (2005) considered this character absent in *Cryptonanus*, Dias et al. (2016) found the same pattern in *C. guahybae*. The cranial data from UFPB9082 corroborate this specimen's assignment to *Cryptonanus*, as it shows an absence of maxillary fenestration, the presence of both the secondary oval foramen, formed by a fine antero-medial process of the alisphenoid tympanic wing, and accessory cusps in the canines, characters that distinguish *Cryptonanus* from *Gracilinanus* (Fig. 2).

The dorsal fur pattern of BC 397, UFPB9047, UFPB9082, and UFPB10943 varies from brown to grayish, differing from *C. guahybae* which has distinctly reddish dorsal fur (Voss et al. 2005) (Fig. 3). However, the ventral portion of the fur was variable in our samples; while UFPB9082 had a grey-based fur color pattern, BC 397 had a more whitish color, without difference between the base and apex of the hair. Specimens UFPB 9047 and UFPB10943 were observed only in photographs, and therefore it was not possible to determine the color of the base of the ventral fur. A grey-based ventral color pattern is considered typical of *C. guahybae*, while in *C. agricolai* most specimens show a more whitish pattern (with the exception of a grey-based ventral fur sample of *C. agricolai* from Lagoa Santa, Minas Gerais) (Voss et al. 2005). Therefore, these four specimens are consistent with *C. agricolai* from the type locality in the northeastern Brazil.

Molecular identification. We obtained an 801-bp and 659-bp fragment of Cytb and COI, respectively, totaling 1524-bp for the six specimens collected in our study. We did not find any evidence of substitution saturation in the dataset (Fig. 4). The phylogeny of mtDNA lineages revealed that *Cryptonanus* formed a moderately supported

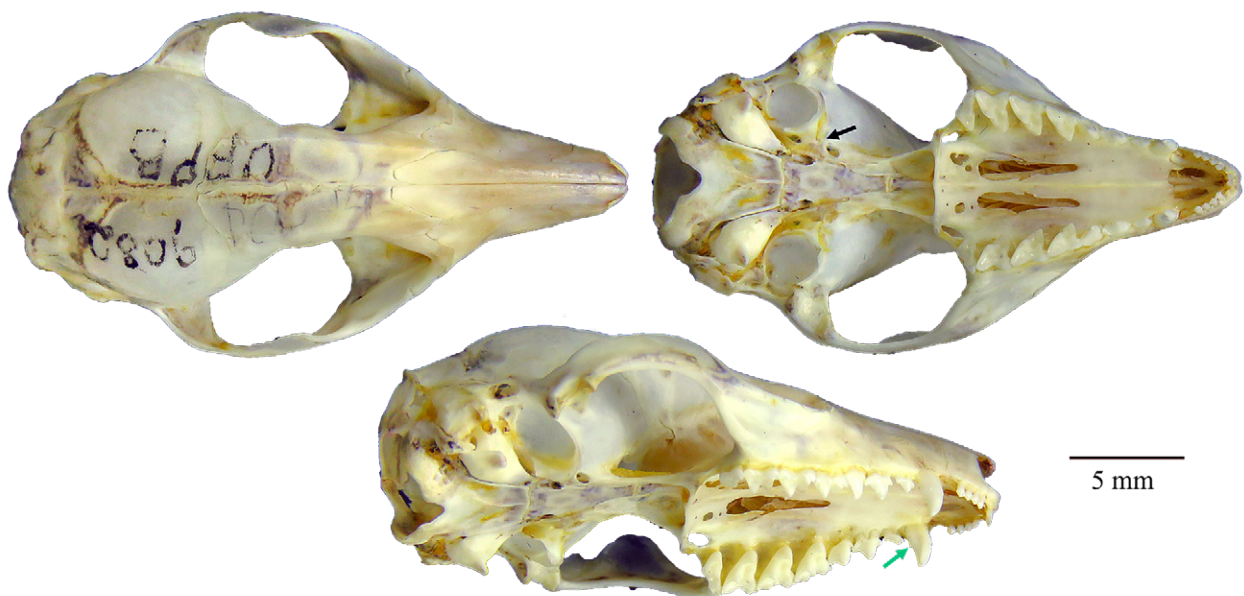


Figure 2. Cranium of *C. agricolai*, UFPB 9082, an adult male from Caxias, Maranhão, Brazil. The black arrow points to the antero-medial tympanic wing alisphenoid process; the green arrow points to accessory cusps in the canines.

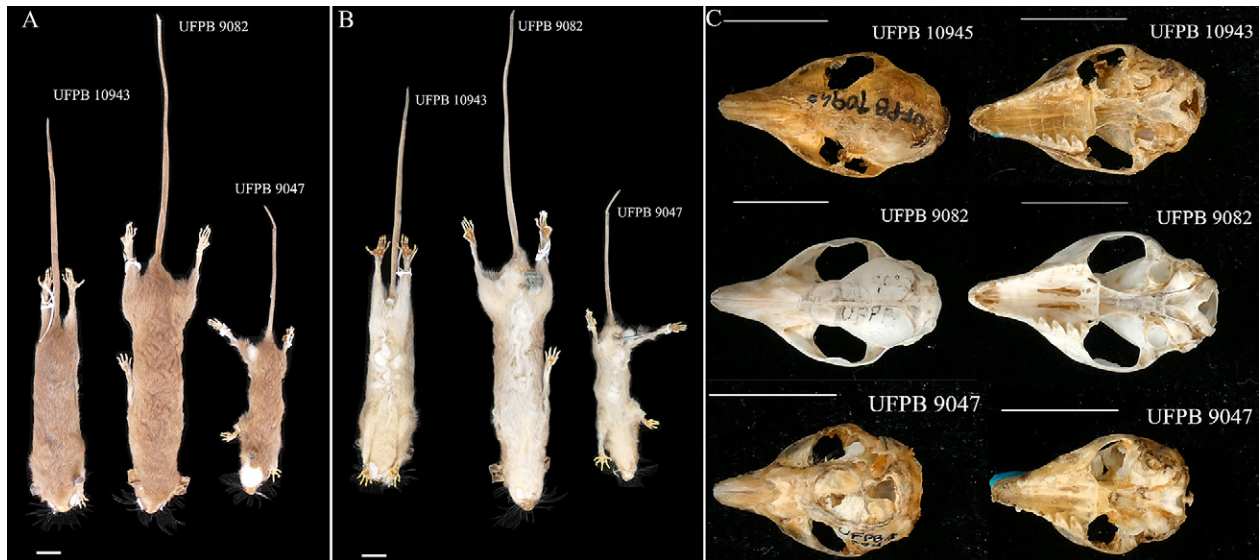


Figure 3. *Cryptonanus agricolai* from this study in dorsal and ventral view. **A, B.** Fur. **C.** Skull. Scale bars = 1 cm.

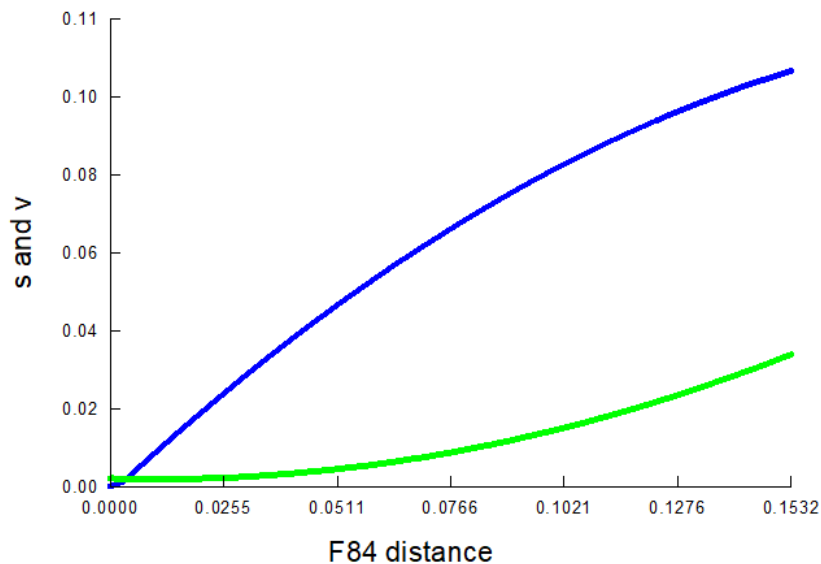


Figure 4. Saturation plot for the mtDNA data analyzed here considering both transitions (s) and transversions (v).

clade (86% bootstrap support; BS) (Fig. 5). In agreement with the phylogeny of Fegies et al. (2021), we retrieved all major lineages for all species of *Cryptonanus*. Four specimens from this study, all from northeastern Brazil, fell within *C. agricolai* A (sensu Fegies et al. 2021), with high support (96% BS). However, two specimens from this study, all from Maranhão state, were retrieved as the most divergent within the *C. agricolai* clade A (98% BS). The haplotype network for both genes (H30 for Cytb; H22 for COI) corroborate the high differentiation of this lineage within *C. agricolai* clade A (Fig. 6).

Discussion

Based on the morphological data, we were able to clearly discriminate between *Cryptonanus* and *Gracilinanus*. However, the precise species identifications were difficult due to the lack of craniodental characters which could be used to distinguish all species (Voss et al. 2005)

and due to the polymorphic fur color pattern in *C. agricolai*, as shown by this and other studies (Voss et al. 2005; Dias et al. 2016). In our study, both analyzed specimens had the canonical patterns of *C. agricolai* in the dorsal region, but not in the ventral region, for which the specimen from Maranhão (UFPB9082) showed a gray-based pattern that has been associated with *C. guahybae* (contrary to the whitish pattern typical of *C. agricolai*; Gurgel-Filho et al. 2015). Dias et al. (2016) reported that fur color variation in *C. guahybae* may be due to age, but it is not clear if this could also be the case in *C. agricolai*, as both individuals were adults.

In contrast, the molecular analysis was conclusive in identifying these specimens as *C. agricolai* (Fig. 5). Interestingly, two specimens from Maranhão state showed a new mitochondrial lineage, which is sister to all previously reported lineages of *C. agricolai* clade A (sensu Fegies et al. 2021), while another individual collected from the same site showed a canonical *C. agricolai*

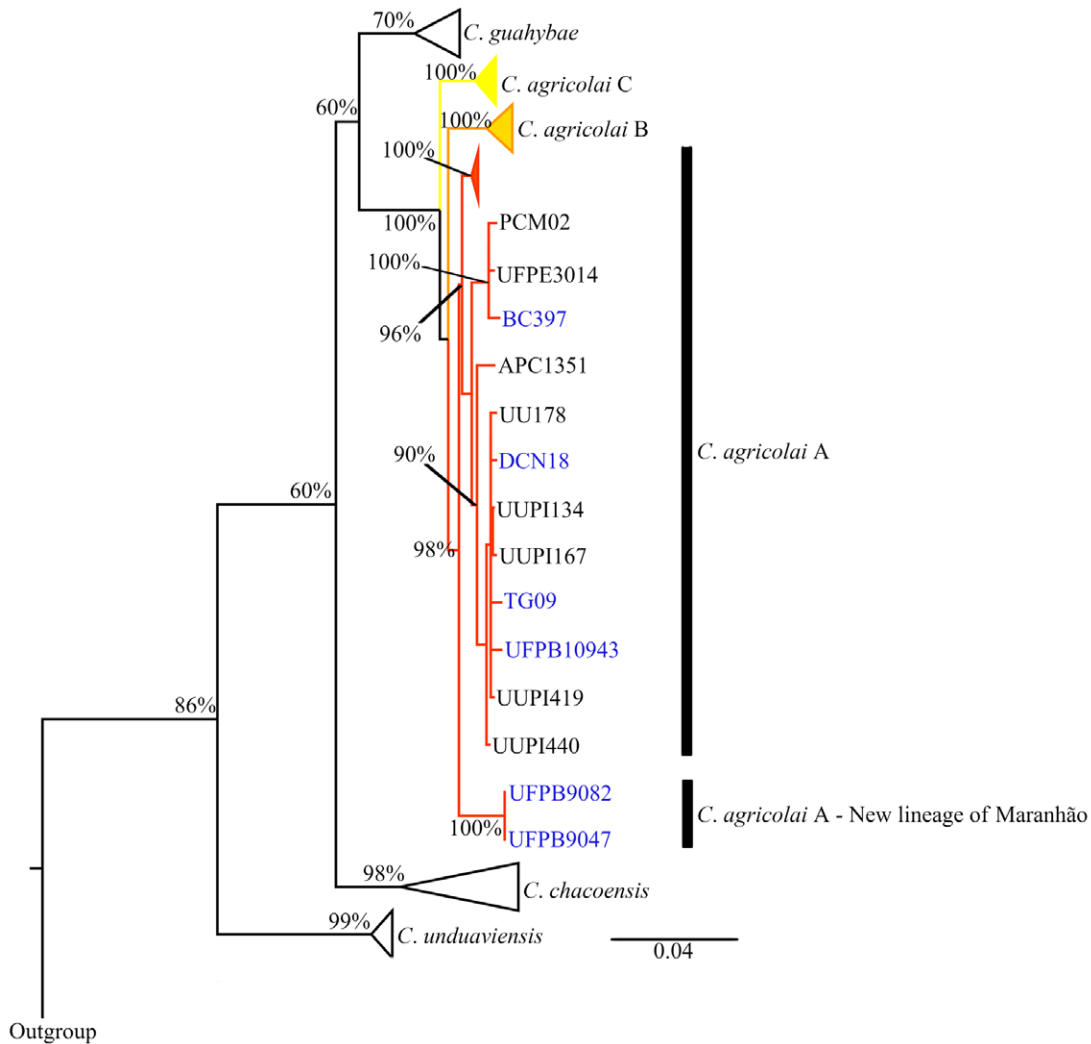


Figure 5. Maximum-likelihood phylogenetic tree for *Cryptonanus* specimens based on Cytb + COI. Numbers on branches correspond to bootstrap support values. Each terminal is identified by an alphanumeric specimen identifier and species as in Fegies et al. (2021) (see Table 1). New records are shown in blue.

clade A lineage. This finding highlights the phylogenetic diversity at a single site, and that indicates that genetic diversity in *Cryptonanus* may be underestimated in spite of recent and comprehensive studies (e.g., Fegies et al. 2021). An important caveat of this study is that we only used mtDNA markers for species (and lineage) identification. Because of its exclusive maternal inheritance, and because it represents a single genealogical event, it is not possible to evaluate how much of an independent evolutionary lineage this novel mtDNA lineage represents. Nonetheless, the novel lineage was identified in an ecotone between the Cerrado and Caatinga biomes, and other findings seem to suggest that ecotone regions in the area are associated with increased biodiversity, including the co-occurrence of two genetic populations of *Didelphis albiventris* Lund, 1840 (Nascimento et al. 2018), the first record of the rodent *Wiedomys cerradensis* Gonçalves, Almeida & Bonvicino, 2005 (Olimpio et al. 2014), and the occurrence of rare species of herpetofauna (Barros et al. 2008).

In summary, we expand the distribution of *C. agricolai* northward by 4° latitude, from -09.15 (Estação

Ecológica de Uruçuí-Una, Piauí) to -04.88 (APA Municipal do Inhamum, Caxias, Maranhão), or 480 km. The new records of *C. agricolai* clade A are within the expected range for the species, as they mainly occur in the South American “dry diagonal” (Vanzolini 1963), where this species is already known. However, our findings indicate that genetic diversity in the Brazilian Northeast, and especially in Cerrado–Caatinga ecotones, may be higher than previously anticipated.

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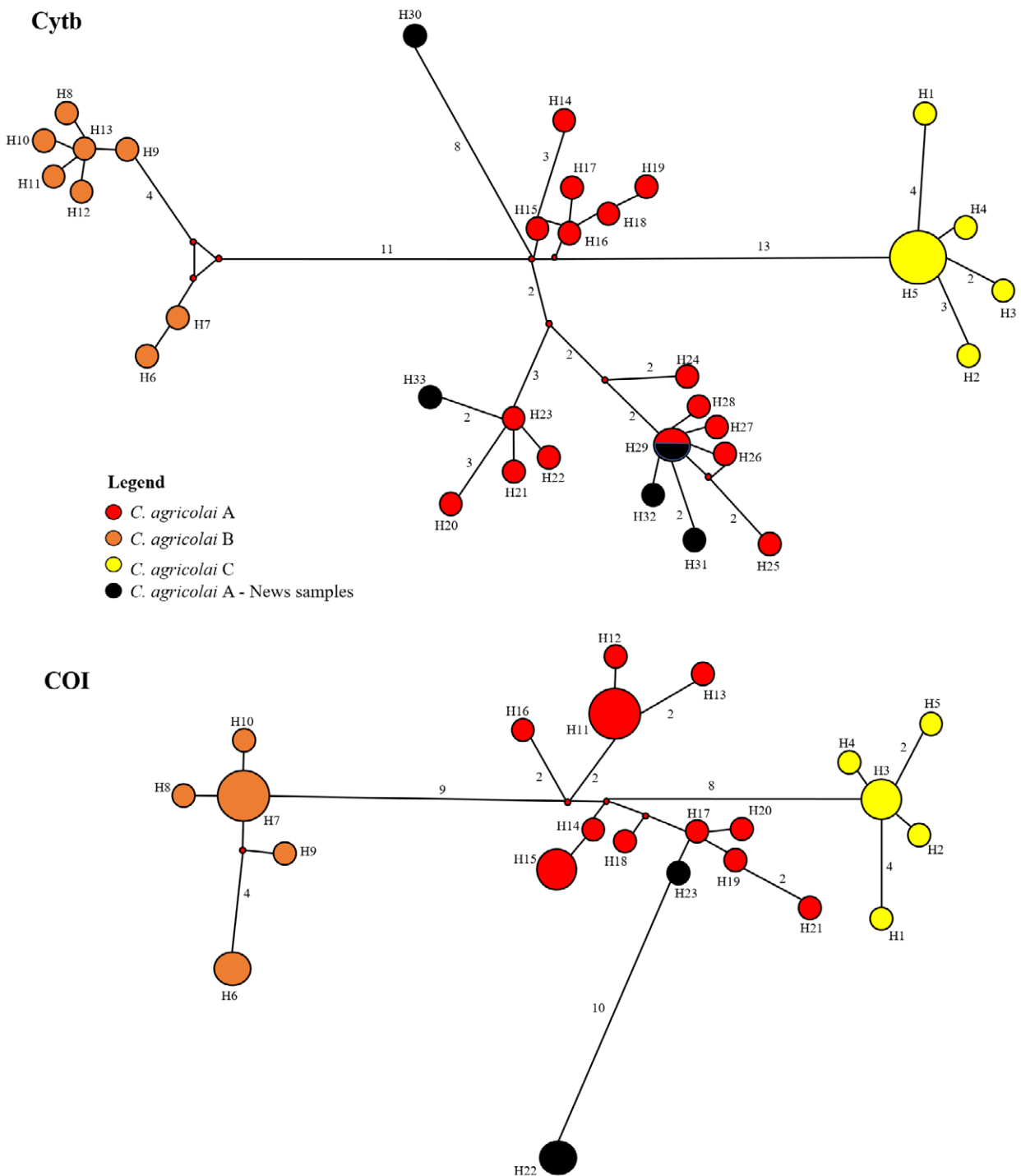


Figure 6. Haplotype network generated using the Cytb and COI genes for *Cryptonanus agricolai*. The number represents the mutational sites between the haplotypes (spaces without numbers corresponds to a mutation), while the size of the circles is proportional to the frequency of occurrence of the haplotypes.

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Authors' Contributions

Data curation: DCN, BATPC. Formal analysis: DCN.

Methodology: DCN. Validation: BATPC, MCB, ECF. Visualization: DCN, NJRF. Writing – original draft: DCN, NJRF, BATPC. Writing – review and editing: NJRF.

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Appendix

Table A1. Background data for generating the distributional map and additional information about the specimens analyzed in the present study. Institutions: UFPB = State Universities of Maranhão; MZUSP = USP Museum of Zoology; UFPE = Federal University of Pernambuco; UNB = National University of Brasília; UFMT = Federal University of Mato Grosso; IBUSP = Institute of Biosciences of the São Paulo. Locality number correspond to those in the map (Fig. 1). Brazilian states: PE = Pernambuco; CE = Ceará; PI = Piauí; MA = Maranhão; AL = Alagoas; BA = Bahia; SE = Sergipe; TO = Tocantins; MT = Mato Grosso; MG = Minas Gerais; RJ = Rio de Janeiro; SP = São Paulo.

<i>C. agricolai</i> mtDNA lineages	Field/tissue no.	Voucher no./ institution	Hap Cytb	Hap COI	Locality no.	Locality	State	Genbank CYTB	Genbank COI
A	BC397		H33		1	Arcoverde	PE	OM223080	
A	T35	UFPB10943	H32		2	Quixadá	CE	OM223079	
A	TG09		H31		3	Guadalupe	PI	OM223078	
A	DCN18		H29	H23	4	Caxias / APA Municipal do Inhamum	MA	OM223077	OM236543
A	DCN14	UFPB 9082	H30	H22	4	Caxias / APA Municipal do Inhamum	MA	OM223076	OM236542
A	DCN30	UFPB 9047		H22	4	Caxias / APA Municipal do Inhamum	MA		OM236544
A	PCM 02	MZUSP	H20	H14	5	Barra do Camaragipe	AL	MW208246	MW208203
A	MTR 13508	MZUSP	H15	H12	6	Trancoso	BA	MW208241	MW208195
A	MTR 13510	MZUSP	H14	H11	6	Trancoso	BA	MW208240	MW208198
A	AP 889	UFPE 3014	H21	H13	7	REBIO Guaribas	PB	MW208247	MW208201
A	AP 919	UFPE 3016	H22	H14	7	REBIO Guaribas	PB	MW208248	MW208202
A	DAM 141	UFPE 2403	H23	H15	8	Igarassú	PE	MW208249	MW208200
A	UU 178	MZUSP 30516	H25	H17	9	ESEC Uruçuí-Una	PI	MW208251	MW208206
A	UUPI 134	MZUSP 30509	H27	H19	9	ESEC Uruçuí-Una	PI	MW208253	MW208207
A	UUPI 167	MZUSP 30511	H29	H21	9	ESEC Uruçuí-Una	PI	MW208255	MW208208
A	UUPI 419	MZUSP 30513	H28	H20	9	ESEC Uruçuí-Una	PI	MW208254	MW208205
A	UUPI 440	MZUSP 30514	H26	H18	9	ESEC Uruçuí-Una	PI	MW208252	MW208204
A	ARB 819	UFPB	H18	H11	10	Monte Alegre do Sergipe	SE	MW208244	MW208196
A	ARB 820	UFPB	H16	H13	10	Monte Alegre do Sergipe	SE	MW208242	MW208194
A	ARB 821	UFPB	H19	H11	10	Monte Alegre do Sergipe	SE	MW208245	MW208199
A	ARB 832	UFPB	H17	H11	11	Porto da Folha	SE	MW208243	MW208197
A	ARB 473	UNB 2588		H16	12	Paraná	TO		MW208193
A	APC 1351	MZUSP	H24	H16	13	ESEC Serra Geral do Tocantins	TO	MW208250	MW208192
B	MN7-09	MZUSP	H6	H6	14	Campo Verde / São Vicente - LT Madeira	MT	MW208232	MW208176
B	LAB061	UFMT 4023	H10	H9	15	Cuiabá	MT	MW208236	MW208179
B	M 111	MZUSP	H9	H8	16	Gaúcha do Norte	MT	MW208235	MW208178
B	MN6-42	MZUSP	H12	H10	17	Jangada / Rosário d'Oeste - LT Madeira	MT	MW208238	MW208184
B	ECO 1	MZUSP	H11	H7	18	Nova Xavantina	MT	MW208237	MW208180
B	ECO 11	MZUSP	H8	H7	18	Nova Xavantina	MT	MW208234	MW208182
B	ECO 9	MZUSP		H7	18	Nova Xavantina	MT		MW208181
B	RMM 220	MZUSP	H7	H16	19	Novo São Joaquim	MT	MW208233	MW208177
B	RMM 224	MZUSP	H13	H17	19	Novo São Joaquim	MT	MW208239	MW208183
C	CAP 033	MZUSP	H4	H4	20	Poços de Caldas	MG	MW208228	MW208187
C	DQM 013	MZUSP	H1	H1	21	PARNA Serra da Canastra	MG	MW208225	MW208185
C		MZUSP 35409	H5		22	Pirai	RJ	KT334295	
C	APC 1165	MZUSP	H5	H3	23	ESEC Santa Bárbara	SP	MW208229	MW208189
C	FU 60/30	IBUSP	H5	H5	24	Caucaia do Alto	SP	MW208231	MW208186
C	FB 103	IBUSP	H2	H2	25	Tapiraí	SP	MW208226	MW208188
C	FB 982	IBUSP	H5	H3	25	Tapiraí	SP	MW208230	MW208191
C	ITM 165	MZUSP 30767	H3	H3	26	Riacho Grande	SP	MW208227	MW208190