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

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. guahybæ* (Tate, 1931), and *C. unduavensis* (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 craniomandibular 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’Eliá 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’Eliá 2015; Fegies et al. 2021). On the other hand, C. chacoensis and C. agricolai, are widely distributed in a region with considerable ecological 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; De La Sancha and D’Eliá 2015).
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’Eliá 2015; Dias et al. 2016; Guerra and Costa 2021). Fegies et al. (2021) were able to identify two major lineages for *C. guayhybae*, 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 2013). 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* species were included for comparison (Table 1).

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

| *Cryptonanus* sp. | Field no. | Voucher | Locality | Latitude | Longitude | Morphological data | Cytb (H3) | COI (H1) | HB | TL | EL | HL | Sex |
|-------------------|-----------|---------|----------|----------|-----------|--------------------|----------|---------|-----|-----|----|----|-----|
| *C. agricolai*    | BC397     | BR: PE | Arecóverde | −08.41   | −037.05   | Skin               | H33      | —       | 95  | 105 | 16 | 14/15 | F   |
| *C. agricolai*    | T35       | UFPB10941 | BR: CE | Quixadá | −04.95   | −039.01 | Skin, skull | H32      | —       | 76  | 96  | 16 | 13/13 | M   |
| *C. agricolai*    | T30       | UFPB9343 | BR: MA | Carurubá | —       | —       | Skin               | —        | —       | 120 | 93  | 19 | 17/16 | M   |
| *G. agilis*       | UFPB9373  | BR: MA | Carurubá | —       | —       | 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
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 Fegie 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
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 cerraden sis Gonçalves, Almeida & Bonvicino, 2005 (Olímpio et al. 2014), and the occurrence of rare species of herpeto fauna (Barros et al. 2008).

In summary, we expand the distribution of C. agricolai northward by 4° latitude, from −09.15 (Estação Ecológica de Uruçuí-Unu, 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 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.
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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 Brasilia; UFMG = 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.

| C. agricolai mtDNA lineages | Field/tissue no. | Voucher no./institution | Hap Cytb | Hap COI | Locality no. | Locality | State | Genbank COI |
|-----------------------------|-----------------|------------------------|----------|--------|--------------|----------|-------|-------------|
| A                            | BC397           | H33                    | 1        | Areia   | PE           | OM223080 |
| A                            | T35             | H32                    | 2        | Quixadá | CE           | OM223079 |
| A                            | TG09            | H31                    | 3        | Guadalupe | PI          | OM232078 |
| A                            | DCH18           | H29                    | 4        | Caxias / APA Municipal do Inhambum | MA | OM232077 | OM236543 |
| A                            | DCH14           | H30                    | 4        | Caxias / APA Municipal do Inhambum | MA | OM232076 | OM236542 |
| A                            | DCH30           | H22                    | 4        | Caxias / APA Municipal do Inhambum | MA | OM236444 |
| A                            | PCM02           | H20                    | 5        | Barra do Camaragipe | AL | MW208246 | MW208203 |
| A                            | MTR 13508       | H15                    | 6        | Trancoso | BA | MW208241 | MW208195 |
| A                            | MTR 13510       | H14                    | 6        | Trancoso | BA | MW208240 | MW208198 |
| A                            | AP 889          | H21                    | 7        | REBIO Guaribas | PB | MW208247 | MW208201 |
| A                            | AP 919          | H24                    | 7        | REBIO Guaribas | PB | MW208248 | MW208202 |
| A                            | DAM 141         | H23                    | 8        | Igarassu | PE | MW208249 | MW208200 |
| A                            | UU 178          | H25                    | 9        | ESEC Urucuí-Uma | PI | MW208251 | MW208206 |
| A                            | UUP1 134        | H27                    | 9        | ESEC Urucuí-Uma | PI | MW208253 | MW208207 |
| A                            | UUP1 167        | H29                    | 9        | ESEC Urucuí-Uma | PI | MW208255 | MW208208 |
| A                            | UUP1 419        | H28                    | 9        | ESEC Urucuí-Uma | PI | MW208254 | MW208205 |
| A                            | UUP1 440        | H26                    | 9        | ESEC Urucuí-Uma | PI | MW208252 | MW208204 |
| A                            | ARB 819         | H18                    | 10       | Monte Alegre do Sergipe | SE | MW208244 | MW208196 |
| A                            | ARB 820         | H16                    | 10       | Monte Alegre do Sergipe | SE | MW208242 | MW208194 |
| A                            | ARB 821         | H19                    | 10       | Monte Alegre do Sergipe | SE | MW208245 | MW208199 |
| A                            | ARB 832         | H17                    | 11       | Porto da Folha | SE | MW208243 | MW208197 |
| A                            | ARB 473         | H15                    | 12       | Paraná | TO | MW208193 |
| A                            | APC 1351        | H24                    | 13       | ESEC Serra Geral do Tocantins | TO | MW208259 | MW208192 |
| B                            | MNT-09          | H6                     | 14       | Campo Verde / LT Madeira | MT | MW208232 | MW208176 |
| B                            | LAB061          | H10                    | 15       | Cuiabá | MT | MW208236 | MW208179 |
| B                            | M 111           | H9                     | 16       | Guaíba do Norte | MT | MW208235 | MW208178 |
| B                            | MNG-42          | H12                    | 17       | Jaguari / Rosário d’Oeste / LT Madeira | MT | MW208238 | MW208184 |
| B                            | ECO 1           | H11                    | 17       | Nova Xavantina | MT | MW208237 | MW208180 |
| B                            | ECO 11          | H8                     | 17       | Nova Xavantina | MT | MW208234 | MW208182 |
| B                            | ECO 9           | H7                     | 17       | Nova Xavantina | MT | MW208234 | MW208182 |
| B                            | RMM 220         | H7                     | 19       | Novo São Joaquim | MT | MW208233 | MW208177 |
| B                            | RMM 224         | H13                    | 19       | Novo São Joaquim | MT | MW208239 | MW208183 |
| C                            | CAP 033         | H4                     | 20       | Poços de Caldas | MG | MW208228 | MW208187 |
| C                            | DOM 013         | H1                     | 21       | PARNIA Serra da Canastra | MG | MW208225 | MW208185 |
| C                            | MZUSP 15409     | H5                     | 22       | Pirai | RJ | KT341295 |
| C                            | APC 1165        | H5                     | 23       | ESEC Santa Bárbara | SP | MW208229 | MW208189 |
| C                            | FU 60/30        | H5                     | 24       | Caucaia do Alto | SP | MW208231 | MW208186 |
| C                            | FB 103          | H2                     | 25       | Taparai | SP | MW208226 | MW208188 |
| C                            | FB 982          | H5                     | 25       | Taparai | SP | MW208230 | MW208191 |
| C                            | ITM 165         | H3                     | 26       | Riacho Grande | SP | MW208227 | MW208190 |

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