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Lineages of Tuco-Tucos (Ctenomyidae: Rodentia) from Midwest and Northern Brazil: Late Irradiations of Subterranean Rodents Towards the Amazon Forest

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Abstract

The genus *Ctenomys* comprises approximately 70 recognized living species of subterranean rodents endemic to South America. Phylogenetic studies to date, based on mitochondrial DNA data, place 44 recognized species in eight species groups and provide evidence for a burst of speciation early in the history of the genus. Species from Argentina, Uruguay, Bolivia, and southern Brazil are well studied at the phylogenetic level. However, the taxonomic status of the species inhabiting midwest and northern Brazil remains poorly understood. In this study, we construct phylogenies based on maximum likelihood and Bayesian inference methods with cytochrome b gene haplotypes of *Ctenomys* from midwest and northern Brazil and with haplotypes representative of the genus *Ctenomys* to place the sampled haplotypes into a phylogenetic framework; we also evaluate skull geometric morphometrics data among sampling sites to assess whether skull morphology corroborates the phylogenetic patterns observed. The results show that the sampling sites used in this study are represented by two species, namely, *Ctenomys bicolor*, which is present in the state of Rondônia, and *Ctenomys nattereri*, which is present in Mato Grosso and Bolivia. The results also reveal two lineages of *Ctenomys* distinct from *C. bicolor* and *C. nattereri*, henceforth called *Ctenomys* sp. "xingu" and *Ctenomys* sp. "central." Both the species and lineages share a most recent common ancestor with *C. boliviensis* and are part of the *boliviensis* species group.

Keywords *Ctenomys* · Cytochrome b · Phylogeny · Species diversity

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Introduction

The order Rodentia is the most speciose order of mammals, with approximately 2277 species described and distributed in 34 families (Patton et al. 2015). These mammals occur in almost every continent, except for Antarctica, occupying arboreal, aquatic, terrestrial, or subterranean habitats (Nowak 1999). Subterranean rodents of the genus *Ctenomys* Blainville, 1826, are characterized by a dependency on tunnel systems, leaving the tunnels only for foraging, mating, or dispersing; they are also generally solitary and territorial, with each individual occupying its own tunnel system (Reig et al. 1990; Lacey et al. 2000).

The most speciose family of subterranean rodents, Ctenomyidae, comprises one extant genus, *Ctenomys,* which is distributed throughout the southern half of the Neotropical region (Fig. 1a) and contains approximately 70 valid species based on morphological, karyotypic, and molecular data (Bidau 2015; Patton et al. 2015; Freitas 2016). In the first
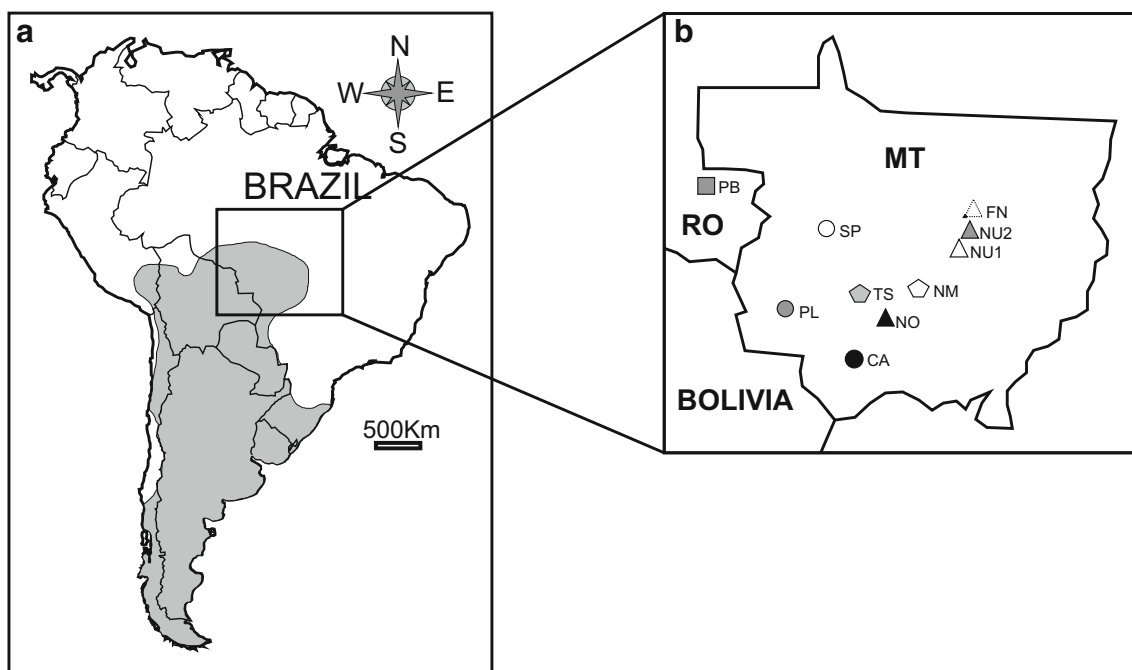
Fig. 1 **a** Distribution of the genus *Ctenomys* throughout southern South America (light gray area). **b** Detailed distribution of sampled localities in the states of Mato Grosso (MT) and Rondônia (RO). Pimenta Bueno – PB, Pontes e Lacerda – PL, Cáceres – CA, Sapezal – SP, Nova Olímpia – NO, Tangará da Serra – TS, Nova Mutum – NM, Nova Ubiratã 1 – NU1, Nova Ubiratã 2 – NU2, Feliz Natal – FN

phylogenetic study of the genus based on molecular data, Lessa and Cook (1998) identified strong monophyly between Octodontidae and Ctenomyidae in a cytochrome b (cyt b) phylogeny as well as evidence of polytomies in basal nodes of the phylogeny for *Ctenomys*, indicating a rapid radiation in the early history of the genus. Other studies included species from Uruguay and southern Brazil (D'Elía et al. 1999), compared chromosomal rearrangements with phylogenetic structuring (Slamovits et al. 2001), and provided phylogenetic estimates based on nuclear noncoding DNA markers (Castillo et al. 2005). Parada et al. (2011) identified eight monophyletic species groups with strong to moderate statistical support and estimated that most of the species groups originated approximately 3 million years ago, an estimate that is supported by the minimum estimated age of the oldest fossil known for the genus (~3.5 mya; Verzi et al. 2009). Upham and Patterson (2012) estimated that the genus *Ctenomys* originated approximately 4.3 mya, based on a nuclear and a mitochondrial gene. Finally, Gardner et al. (2014), based on cyt b sequences, provided the most comprehensive phylogenetic study to date for the genus *Ctenomys*, including 44 recognized species, four of which were described as new Bolivian species.

In Brazil, eight species of *Ctenomys* are currently known: five in southern Brazil (Rio Grande do Sul State – RS), namely, *Ctenomys torquatus* Lichtenstein, 1830, *Ctenomys minutus* Nehring, 1887, *Ctenomys lami* Freitas, 2001, *Ctenomys flamarioni* Travi, 1981, and *Ctenomys ibicuiensis* Freitas et al., 2012; one in the state of Mato Grosso (MT), namely, *Ctenomys nattereri* Wagner, 1848; and two species, namely,

Ctenomys rondoni Miranda Ribeiro, 1914, and *Ctenomys bicolor* Miranda Ribeiro, 1914, in the state of Rondônia (RO) (Bidau and Ávila-Pires 2009). While species from southern Brazil, Argentina, Uruguay, and Bolivia are considerably well studied at the phylogenetic level (Lessa and Cook 1998; D'Elía et al. 1999; Slamovits et al. 2001; Castillo et al. 2005; Parada et al. 2011; Freitas et al. 2012; Gardner et al. 2014), the taxonomic status for the genus *Ctenomys* in midwest and northern Brazil remains poorly resolved. A revalidation of *C. bicolor* as a species based on molecular, karyotypic and morphological evidence (Stolz et al. 2013) is the only study published to date that involves individuals from these regions of Brazil. Considering the species distributed in midwest and northern Brazil, *C. bicolor* and *C. nattereri* are included in the *boliviensis* species group and are sister taxa to the Bolivian species *Ctenomys boliviensis* Waterhouse, 1848, and *Ctenomys steinbachi* Thomas, 1907 (Parada et al. 2011; Stolz et al. 2013; Gardner et al. 2014).

Recently, we sampled new sampling sites for *Ctenomys* in midwest and northern Brazil, further expanding the known distribution for the genus towards the Brazilian Amazon forest. Indeed, some of the individuals we sampled in the northernmost sampling sites were located in forests in areas of transition between the Amazonia and Cerrado (savanna) biomes of Brazil (IBGE 2004). As species within the genus *Ctenomys* are primarily known for inhabiting open areas covered mostly by grassy vegetation, sampling individuals in Mato Grosso is interesting because the southernmost parts of Amazon – roughly, western Rondônia and northern/central

Mato Grosso – present vegetation characteristic of both the "evergreen forests" of the Amazon to the north and the Cerrado (Savanna), dominated by smaller trees and bush-like vegetation, to the south, the same region where our sampling sites are located. The convergence of the two biomes forms a zone of ecological tension (ZOT) that spans hundreds of kilometers at the southern border of the Amazon (Ackerly et al. 1989; Marimon et al. 2013). This ZOT is likely the product of continuous alternation of temperature and precipitation throughout the Quaternary and is dominated by vegetation characteristic of the Amazon during wet events and by vegetation characteristic of the Cerrado during dry events (Marimon et al. 2013 and references therein). Such dynamics may affect the dispersion of *Ctenomys* throughout midwest and northern Brazil, if the changes in vegetation composition represent geographical barriers. In addition, some sampling sites were located near the type locality for the species *C. nattereri* (Cáceres – MT; Miranda Ribeiro, 1914) and *C. bicolor* (Pimenta Bueno – RO, Stolz et al. 2013).

Therefore, these new sampling sites provide an opportunity to clarify the taxonomic status of poorly known species of *Ctenomys* located at the northern end of the distribution of the genus, making way for future population genetics and ecological studies. Herein, we build on previous phylogenetic studies and characterize the known localities of *Ctenomys* from midwest Brazil based on cyt *b* sequences and geometric morphometrics of the skull, placing the haplotypes representative of the localities sampled into a phylogenetic framework and defining, when possible, taxonomic units.

Material and Methods

Sample Collection and DNA Extraction from Field Specimens

We discovered and sampled ten localities for the genus *Ctenomys* between the years 2007 and 2017, totaling 92 specimens (Table 1). We captured the specimens using Oneida

Victor n° 0 Snap Traps (Zenuto and Busch 1998) under a license from the Brazilian government (IBAMA, Authorization No. 14690–1). We conducted at least one field trip per year, with a minimum sampling effort of 14 days per field trip. All procedures involving the captured animals were allowed by IBAMA (Brazil's environmental agency) and followed the guidelines of the American Society of Mammalogists (Sikes et al. 2016). Liver samples were collected from 92 individuals distributed among 10 sampling sites in the states of Mato Grosso (MT) and Rondônia (RO) (individuals per locality and geographic coordinates of the sampling sites are detailed in Table 1). Tissue samples were housed at the Laboratory of Cytogenetics and Evolution in the Department of Genetics at Universidade Federal do Rio Grande do Sul (UFRGS) and stored in 96° GL alcohol at −20 °C. We extracted DNA from the samples following the CTAB DNA extraction protocol (Doyle and Doyle 1987), with modifications. All DNA extractions were stored at −20 °C.

Mitochondrial DNA Primers We amplified partial coding sequences – CDS (1087 bp) –of the cyt b gene (complete CDS: 1146 bp) from 92 individuals using the primer pairs MVZ05 and Tuco06 (Smith and Patton 1999) and Tuco07 and Tuco14 (Wlasiuk et al. 2003). Only forward primers were used for sequencing PCR products.

Cytochrome b PCR conditions and sequencing We conducted PCR using 100 ng of purified genomic DNA, 0.4 μL of 10 mM forward and reverse primers, 0.4 μL of 10 mM deoxynucleotide triphosphates, 2.0 μL of 10X PCR buffer, 1.6 μL of 50 mM $MgCl_2$ polymerase cofactor, and 0.2 μL of 5 U/uL DNA Taq polymerase (Ludwig Biotec), totaling 20 μL of reaction per sample. PCR cycling conditions were as follows: initial denaturation at 94 °C for 1 minute, followed by 30 cycles of denaturation at 93 °C for 1 minute, primer annealing at 45 °C for 1 minute,

Table 1 Individuals sampled for DNA and skull samples and haplotypes by locality. PL, Pontes e Lacerda; CA, Cáceres; SP, Sapezal; NO, Nova Olímpia; NU1, Nova Ubiratã 1; NU2, Nova Ubiratã 2; FN, Feliz Natal; NM, Nova Mutum; TS, Tangará da Serra; PB, Pimenta Bueno

Locality	Sample Size - DNA	Sample Size - Skulls	Coordinates	Haplotypes by locality
PL	$n = 10$	$n = 9$	15°09′31.38"S, 59°13′47.54"W	H1, H2, H3, H4
CA	$n = 11$	$n = 9$	15°58′11.91"S, 57°45′58.13"W	H5
SP	$n = 7$	$n = 4$	13°02′11.94"S, 58°48′56.89"W	H4, H6
NO	$n = 11$	$n = 6$	14°52′17.32S, 57°17′25.96"W	H7, H8
NU1	$n = 9$	$n = 8$	12°57′29.78"S, 54°55′39.84"W	H9, H10
NU2	$n = 13$	$n = 7$	12°54′44.16"S, 54°50′25.14"W	H10
FN	$n = 9$	$n = 5$	12°35′5.40"S, 54°48′51.95"W	H10, H12, H13
NM	$n = 11$	$n = 10$	13°51′54.44"S, 55°16′34.47"W	H14
TS	$n = 1$	–	14°13′36.09"S, 57°41′15.69"W	H15
PB	$n = 10$	$n = 9$	12°13′31.84"S, 60°40′47.36"W	H11
Total	$n = 92$	$n = 67$		

and fragment extension at 72 °C for 1.5 min, ending with a final extension at 72 °C for 10 min. To confirm amplification, the PCR products were visualized in 1.5% agarose gel prior to sequencing. Sequencing was conducted abroad (Macrogen Inc., Seoul, Korea).

Mitochondrial DNA Data Sets We created two cyt b data sets. The global data set comprises all unique haplotypes representative of midwest and northern Brazil, 69 sequences representative of the genus *Ctenomys*, and two Octodontidae haplotypes downloaded from GenBank (accession numbers: Table S1) used as outgroups; we used this data set to reconstruct a complete phylogeny for *Ctenomys,* placing Brazilian haplotypes into species groups, and to estimate the time of origin of the most recent common ancestor (MRCA) between taxa of interest. The local data set comprises all haplotypes representative of the sampled localities, ten sequences representative of the *boliviensis* species group (sensu Parada et al. 2011), and six sequences representative of the species *Ctenomys andersoni* Gardner et al., 2014, *Ctenomys erikacuellarae* Gardner et al., 2014, and *Ctenomys yatesi* Gardner et al., 2014, which form a clade sister to *boliviensis* (Gardner et al. 2014). We calculated partial phylogenetic reconstructions to assess intragroup phylogenetic relationships and divergence times between taxa belonging to the *boliviensis* species group; we assumed it was plausible to calculate partial reconstructions for this data set because all haplotypes representative of known sampling sites clustered within the *boliviensis* species group for all phylogenies constructed using the global data set (see Results). In addition, we also compared estimates obtained through the partial phylogenetic reconstruction with those obtained through the complete phylogenetic reconstruction to assess whether the estimates corroborate each other.

Mitochondrial DNA Analysis We inspected chromatograms and trimmed sequences using Chromas Lite 2.1.1 (Technelysium Inc.). We aligned the sequences used in both data sets independently through the MUSCLE algorithm (Edgar 2004) implemented in MEGA 6.0 (Tamura et al. 2013) using the default parameters. Best-fit models of molecular evolution for each data set were calculated using JModelTest2 (Guindon and Gascuel 2003; Darriba et al. 2012). The model selected was based on the smallest variation in the logarithm of the Bayesian Information Criteria (BIC). The haplotype (Hd) and nucleotide diversity (Pi) indexes and number of segregating (S) and invariant (I) sites obtained were estimated using DnaSP 5.0 (Librado and Rozas 2009). We used the Kimura-2-parameters (K2P) substitution model with pairwise deletion of gaps and missing data to estimate percentages of sequence divergence between haplotypes for the cyt b gene, with 1000 bootstrap replicates and considering the 1st, 2nd, and 3rd codon positions, in MEGA 6.0. We performed a

nonspatial genetic mixture analysis in BAPS 6 (Corander et al. 2006, 2008; Cheng et al. 2011) to estimate the genetic clusters formed by individuals of each locality analyzed with the complete data set for the cyt b gene (92 sequences, 1087 bp), using five iterations and a maximum number of clusters of 20 (K = 20); all other parameters were left as default.

We constructed maximum likelihood (ML) phylogenies (global data set) in MEGA 6.0 using 1000 bootstrap replicates, considering the 1st, 2nd, and 3rd codon positions; all other parameters were left as default. We constructed Bayesian inference (BI)-based phylogenies for both the global and local data sets in BEAST v2.4.0 (Bouckaert et al. 2014) with empirical base frequencies, four gamma categories and data partitioned into the 1st, 2nd, and 3rd codon positions. As the data are not clock-like, a relaxed uncorrelated lognormal clock (Drummond et al. 2006) was used to allow different mutation rates for each branch in the phylogeny while preserving the time-dependent nature of the evolutionary process. A calibrated Yule speciation prior (Heled and Drummond 2011) was implemented to allow calibration of trees calculated with the global data set for both the Ctenomyidae/Octodontidae split (Marshall and Sempere 1993; Vucetich et al. 1999; Verzi 2002; Table 4) and the MRCA for the genus *Ctenomys* (Verzi et al. 2009; Table 4); we calibrated the local data set with the MRCA estimates for the *boliviensis* species group obtained through the complete phylogenetic reconstruction (see Table 5). Twenty million iterations in a Markov chain Monte Carlo (MCMC) process were used in each run, sampling a tree every 1000 steps in both the global and local analyses; all other parameters were left as default. Six independent runs were calculated for each data set, and the outputs of the runs were submitted to a 25% burn-in and combined into a single tree file using LogCombiner v2.4.3 (Rambaut and Drummond 2015a). The consensus tree was chosen using TreeAnnotator v.1.8.2 (Rambaut and Drummond 2015b). The log files of all runs were inspected in Tracer v1.6 (Rambaut et al. 2013), to ensure that all parameters had estimated sample size (ESS) values higher than 200.

Skull Geometric Morphometric Analysis The sample consisted of 67 skulls of adult specimens of *Ctenomys* from nine sampling sites in midwest and northern Brazil (see Table 1). Skulls from all individuals are housed at the Laboratory of Cytogenetics and Evolution in the Department of Genetics at UFRGS (archive numbers, Table S4). Dorsal, ventral, and lateral left sides of the skulls were photographed with a Canon G10 digital camera at maximum resolution (4416 × 3312) with the macro function activated and without flash. We digitized 29, 30, and 21 morphological landmarks for each individual for the dorsal, ventral and lateral views, respectively (sensu Fernandes et al. 2009), with TPSDig 2.16 (Rohlf 2010). All landmarks plotted were assigned to

each individual by the same person (L.E.J.R.). Coordinates were superimposed by the generalized Procrustes analysis (GPA) algorithm (Dryden and Mardia 1998). We symmetrized landmarks in dorsal and ventral views of the skull and used the symmetric component of variation in each view for our analysis (Kent and Mardia 2001; Klingenberg et al. 2002). We used the residuals of GPA-like shape variables as the shape, and we used the centroid size (the square root of the sum of distances of each landmark from the centroid; Bookstein 1991) as a measure of overall skull size. To avoid redundancy of the three views, we summed the centroid sizes of the three views of the skull and used the obtained mean for the analysis.

We tested skull centroid size for normality with Shapiro's test, sexual dimorphism for size using Student's t test, and sexual dimorphism for skull shape with multivariate analysis of variance (MANOVA). We tested differences in size among sampling sites with analysis of variance (ANOVA) and used Tukey's test for pairwise comparisons. We implemented principal component analysis (PCA) to explore shape variation for each view of the skull and multivariate analysis of variance (MANOVA) to test for differences in skull shape between sampling sites. We calculated jackknife cross-validation to generate percentages of correct classification among sampling sites and within hypothesized molecular clades (see Results). We conducted all aforementioned analyses in R software version 3.2.1 (R Core Team 2017) with the *geomorph* package (Adams and Otárola-Castillo 2013). We used canonical variate analysis (CVA) in MorphoJ (Klingenberg 2011) to generate scatterplots and wireframes to discriminate the individuals and localities sampled in shape space.

Data Availability All sequences from the localities studied used in the analyses present in this manuscript are available at GenBank (https://www.ncbi.nlm.nih.gov/genbank) under accession numbers MG065617 to MG065634. Detailed individual information on the origin of each sequence we downloaded from GenBank and on the sequences we published is available as supplemental material (Tables S1 and S2, respectively).

Results

Cytochrome b diversity indexes for sampled localities Partial coding sequences for the cyt b gene (1087 bp) were obtained for 92 individuals. A total of 1083 bp were used in the analysis, excluding 4 bp of missing data. The haplotype diversity (Hd) and nucleotide diversity (Pi) indexes were equal to 0.8830 and 0.01737, respectively. The total number of segregating sites (S) was 69, while 1014 sites were invariant (I). Eighteen individual sequences corresponded to 15 haplotypes representative of the genetic variability of the sampled localities. Out of these 18

sequences, 15 corresponded to exclusive haplotypes, and three were shared among sampling sites (H4: PL and SP; H10: NU1, NU2, and FN; Fig. 1 and Table S2). Therefore, we included 15 sequences representing the total haplotype variability of the sampling sites in the phylogenetic analyses (GenBank accession numbers, Table S2).

Cytochrome b diversity indexes for the global data set A total of 86 sequences were used in the complete phylogenetic reconstruction analyses with the global data set: 15 haplotype sequences representing the sampling sites of *Ctenomys* from midwest and northern Brazil, 69 sequences representative of the genus *Ctenomys*, and two Octodontidae sequences as outgroups. The sequences from GenBank were mostly complete (1140 bp), but some were shorter; a few sequences contained small nucleotide gaps or undetermined nucleotides ('N'). Overall, 1042 bp were used in the phylogenetic analyses, given that there were 45 sites with missing data. From a total of 84 haplotypes, 577 were variable sites, and 445 were parsimony informative (S) sites. Out of the 1042 sites analyzed, 465 were invariant (I).

Genetic Distances between Sampled Localities from Midwest and Northern Brazil Genetic distances based on the Kimura-2-parameters model for the partial CDS of the cyt b gene (1087 bp) presented mean values of 0.2% between PL and SP, 0.1% between NU1 and NU2, and 0.7% between NM and TS. The maximum distance reached was 3.8%, between PB and CA. Some subsets of localities had lower sequence divergence between haplotypes within subsets than other subsets of localities: there was a maximum of 1.3% divergence between sequences from the sampling sites PL, CA, and SP, but a maximum of over 2.0% when compared against any other haplotypes. Sequence divergence between the localities NO, NU1, NU2, and FN presented a mean of 0.6 and 1.6% when compared against NM and TS and a mean of over 2.2% when compared to PL, CA, SP, and PB. PB, the only locality known to represent the species *C. bicolor* (Stolz et al. 2013), diverges from the other localities by a mean of 2.9% (Table 2). Groups of localities, therefore, seem to cluster into clades when the genetic distances between them are considered. We name such clusters by their relative geographic positioning (PL, CA, and SP: Western Clade; NO, NU1, NU2, and FN: Eastern Clade; and NM and TS: Central Clade; Table 3).

Genetic Distances for the Global Data Set We estimated genetic distances between haplotypes based on the Kimura-2-parameters model (Table 3). Mean genetic distances within clades are lower (Western Clade: 0.7%; Eastern Clade: 0.6%; Central Clade: 0.7%) than those between clades (Western vs. Eastern: 2.7%; Western vs. Central: 2.2%; Eastern vs. Central: 1.6%). The genetic distances between *C. bicolor* (PB) and all other clades are higher than the

Table 2 Cytochrome b (1,087 bp) genetic distances between haplotypes sequenced in this study based on the Kimura-2-Parameters Model. PL, Pontes e Lacerda; CA, Cáceres; SP, Sapezal; NO, Nova Olímpia; NU1, Nova Ubiratã 1; NU2, Nova Ubiratã 2; FN, Feliz Natal; PB, Pimenta Bueno; NM, Nova Mutum; TS, Tangará da Serra

	PL	CA	SP	NO	NU1	NU2	FN	PB	NM	TS
PL	0.1–0.3									
CA	1.1–1.3	–								
SP	0.0–0.4	1.1–1.3	0.2							
NO	2.2–2.5	2.5–2.7	2.4–2.5	0.1						
NU1	2.4–2.8	2.8–3.0	2.3–2.7	0.5–0.8	0.2					
NU2	2.6–2.8	2.9	2.6–2.8	0.7–0.8	0.0–0.2	–				
FN	2.6–3.1	2.8–3.2	2.5–2.9	0.7–1.1	0.0–0.7	0.0–0.7	0.3–0.7			
PB	3.3–3.4	3.8	3.3–3.5	2.2–2.3	2.3–2.6	2.6	2.6–2.8	–		
NM	2.1–2.2	2.4	2.1–2.3	1.4–1.5	1.5	1.5	1.5–1.9	2.3	–	
TS	2.0–2.1	2.3	2.0–2.2	1.3–1.4	1.4–1.6	1.6	1.6–1.8	2.3	0.7	–

distances between clades (Table 3). Genetic distances between the Western Clade and a haplotype representative of *C. nattereri* from Bolivia (HM777484) present a mean of 1.0%; *C. nattereri* from Bolivia diverges in over 1.9% of the sequence when compared against the Eastern and Central Clades (*C. nattereri* vs. Eastern: mean of 2.4%; *C. nattereri* vs. Central: 2.0%) and in 3.2% of the sequence compared to *C. bicolor*. Sequences identified as *C. boliviensis* differ widely from each other (AF007039 and AF007040 compared to AF007037, AF007038, and KJ778554: 5.3%; AF007037 and AF007038 compared to KJ778554: 1.4%) and when compared to *C. nattereri*, *C. bicolor*, and the clades specified above (Table 3). However, the haplotypes AF007039 and AF007040 are known to have been misidentified as *C. boliviensis*, because individuals had a diploid number of $2n = 36$, which is distinct from that known from *C. boliviensis* localities ($2n = 42$, 44, and 46) and did not cluster with actual *C. boliviensis* haplotypes in past studies (Lessa and Cook 1998; Mascheretti et al. 2000). Haplotypes of *C. goodfellowi* differ by a mean of 1.3% from true *C. boliviensis* (AF007037, AF007038, and KJ778554) but by over 5.0% from the haplotypes AF007039 and AF007040, which is consistent with the findings of Gardner et al. (2014), who synonymized *C. goodfellowi* with *C. boliviensis* (Table 3). All other haplotypes representative of *Ctenomys* diverge in over 6.0% of their sequences compared to the Western, Eastern, and Central Clades; *C. nattereri* from Bolivia; and *C. bicolor* (PB) (Table 3).

Nonspatial Genetic Mixture Analysis The Bayesian inference of hierarchical clustering of sampling sites estimated in BAPS 6 (Corander et al. 2006, 2008; Cheng et al. 2011) yielded six genetic clusters (K = 6), which resemble the clades inferred by genetic distances between sampling sites (PL and SP; CA; NO; NU1, NU2 and FN; PB; and NM and TS; Fig. 2).

Table 3 Cytchrome b (1,087 bp) genetic distances between clades of localities sampled in this study and sequences representative of the *boliviensis* group of species based on the Kimura-2-Parameters Model in percentage points. Western Clade: PL, CA, SP; Eastern Clade: NO, NU1, NU2, FN; Central Clade: NM, TS; *Ctenomys bicolor*: PB

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
1.Western Clade	0.0–1.3												
2.Eastern Clade	2.3–3.2	0.0–1.1											
3.*C. bicolor*	3.3–3.8	2.2–2.8	–										
4.Central Clade	2.0–2.4	1.3–1.9	2.3	0.7									
5.HM777484.1_C._nattereri	0.9–1.1	2.1–2.7	3.2	1.9–2.0	–								
6.AF007039.1_C._boliviensis	1.6–2.1	2.2–2.9	3.4	2.2–2.3	1.9	–							
7.AF007040.1_C._boliviensis	1.6–2.1	2.2–2.9	3.4	2.2–2.3	1.9	0.0	–						
8.AF007037.1_C._boliviensis	5.0–5.3	5.8–6.2	6.8	5.2–5.6	4.9	5.6	5.6	–					
9.AF007038.1_C._boliviensis	5.0–5.3	5.8–6.2	6.8	5.2–5.6	4.9	5.6	5.6	0.0	–				
10.KJ778554.1_C._boliviensis	5.2–5.5	5.9–6.5	6.6	5.0–5.3	5.3	5.6	5.6	1.4	1.4	–			
11.AF007043.1_C._steinbachi	6.4–6.7	7.4–8.1	8.4	7.0–7.5	6.7	6.7	6.6	6.6	7.2	7.0	–		
12.AF007044.1_C._steinbachi	6.3–6.6	7.4–7.9	8.3	6.9–7.4	6.6	6.6	6.5	6.5	7.0	7.9	0.1	–	
13.Other *Ctenomys* spp.	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0

Fig. 2 Non-spatial genetic mixture analysis implemented in BAPS 6. Localities sampled in this study structure into six genetic clusters (K = 6). Colors from white to dark grey indicate each genetic genetic cluster. From left to right: PL/SP; CA; NO; NU1 NU2, FN; PB; NM, TS

| PL | SP | CA | NO | NU 1 | NU 2 | FN | PB | NM TS |
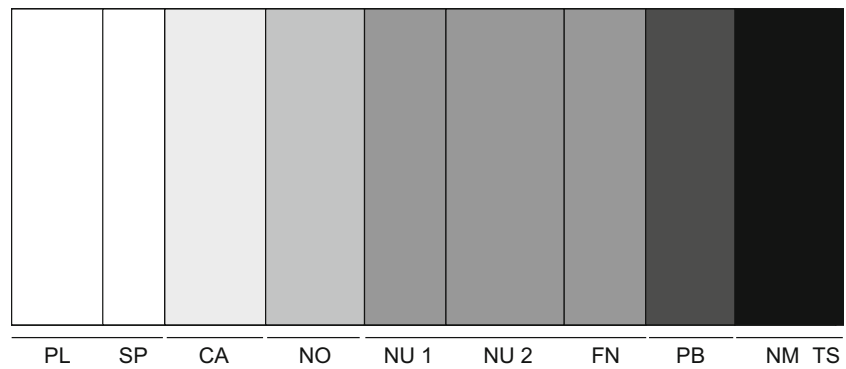
Cytochrome b phylogenies: global data set The best-fit model of molecular evolution calculated in JModelTest2 for the global data set was Hasegawa-Kishino-Yano (Hasegawa et al. 1985) + gamma + invariant sites (HKY + G + I). Most species groups were recovered with moderate to strong statistical support in both phylogenies, though the estimated times of divergence between most species groups had only moderate to low support (Figs. 3 and 4). All haplotypes from midwest and northern Brazil are included in the *boliviensis* species group, which is monophyletic, with strong statistical support (Figs. 3a and 4a). Haplotypes from the Western Clade and *C. nattereri* from Bolivia (HM777484) share an MRCA with moderate support in the ML phylogeny (Fig. 3) and strong support in the BI phylogeny (Fig. 4). Haplotypes from the Eastern Clade share an MRCA with *C. bicolor*, while haplotypes from the Central Clade are sister taxa to the Eastern Clade plus *C. bicolor*, with strong statistical support in both phylogenies. All haplotypes from midwest and northern Brazil; the two misidentified haplotypes representative of the species *C. boliviensis* (AF007039 and AF007040) and one haplotype representative of the species *C. nattereri* from Bolivia (HM777484) share an MRCA with five *C. boliviensis* haplotypes (AF007037, AF007038, AF007050, AF007051, and KJ778554) with strong statistical support in both trees. It is important to note that the haplotypes AF007050 and AF007051 are identified in data bases as *C. goodfellowi*, but since Gardner et al. (2014), this species is considered synonymous with *C. boliviensis*. Mean estimates for time of divergence and their 95% highest probability densities (HPD95%) between species groups, between the Ctenomyidae and Octodontidae split, and for the MRCA for the genus *Ctenomys* are summarized in Table 4. The MRCA for the genus *Ctenomys* was estimated at approximately 3.71 mya; the *frater* species group is estimated to be the first species group to have originated, approximately 2.18 mya, while the other species groups are more derived. Conversely, *talarum* and *mendocinus* are the most recently originated species groups (mean time estimates of 0.52 and 0.64 mya, respectively; Table 4; Figs. 3 and 4).
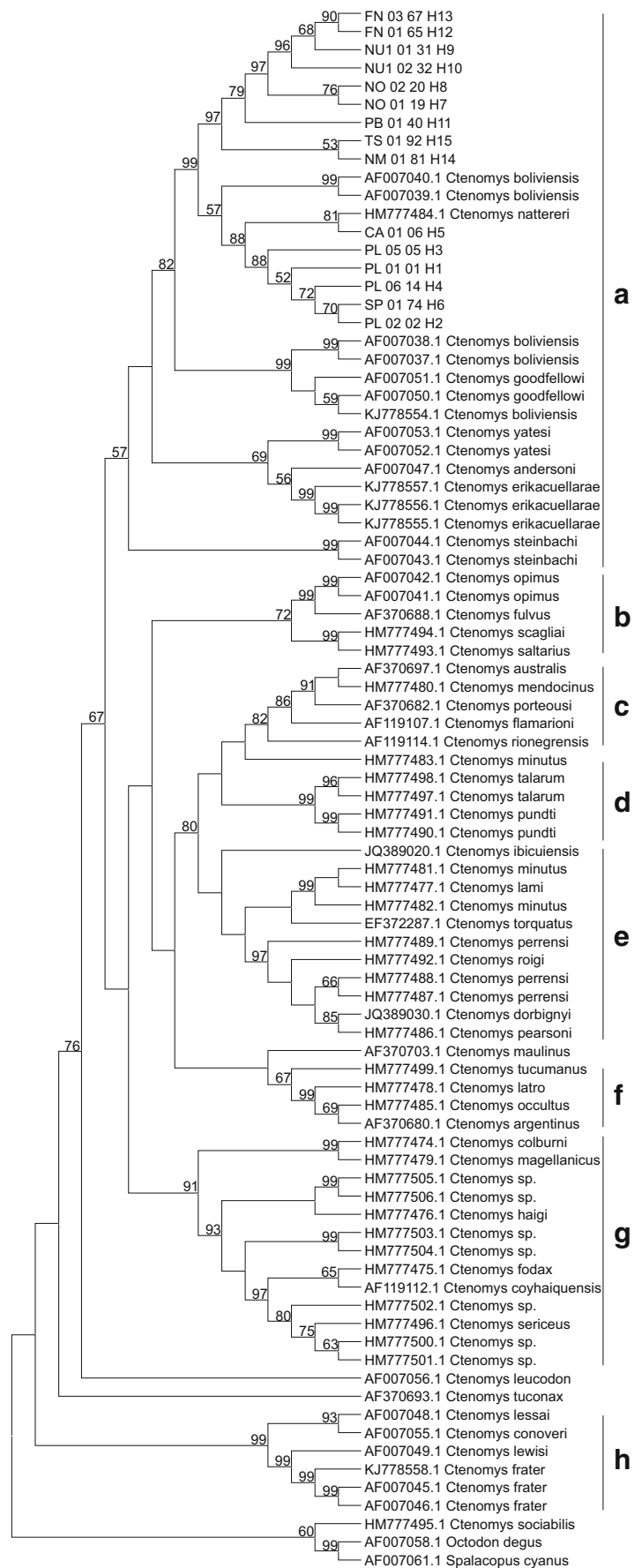
There is one incongruence between the topologies of the BI and ML phylogenies related to the *C. boliviensis* haplotypes

AF007039 and AF007040. When comparing these haplotypes to the others attributed to *C. boliviensis*, the haplotypes AF007039 and AF007040 share an MRCA with *C. nattereri* and the Western Clade but not with the other haplotypes of *C. bicolor*. In both cases, however, there is low statistical support for such positioning (Figs. 3 and 4). Statistical support for the topologies including *C. steinbachi*, *C. erikacuellarae*, *C. andersoni*, and *C. yatesi* as species either within or outside the *boliviensis* species group is equal to one in the BI phylogeny, while the bootstrap values for such clusters are low (57) and moderate (82) in the ML phylogeny for the more inclusive and exclusive groups, respectively (Figs. 3 and 4). Given the maximum support was obtained when including the species in a monophyletic species group (e.g., *boliviensis*) in the BI phylogeny, we chose to consider *boliviensis* as a more inclusive group (species group A; Figs. 3 and 4) as a means to estimate the times of origin for the MRCAs between the taxa of interest while not constraining the analyses to a smaller data set. Therefore, the mean time of divergence for the *boliviensis* species group was estimated at approximately 1.58 mya (Table 4).

Almost all clades within the *boliviensis* species group estimated through the complete phylogenetic reconstruction present moderate (0.70–0.89) to high (0.90–1) statistical support for the given best tree topology, except when considering the clustering of individuals within sampling sites and the positioning of *C. steinbachi* within the species group (posterior probability values less than 0.50). The localities from midwest and northern Brazil share an MRCA with *C. boliviensis* at approximately 1.17 mya. The MRCA between the localities was estimated to have originated 0.57 mya on average, while the clades were estimated to be somewhat more recent (*C. nattereri* and Western Clade, 0.50 mya; Eastern Clade, 0.17 mya; Central Clade, 0.20 mya) (Local group, Table 4).

Cytochrome b phylogenies: local data set Bayesian phylogenies for the local data set were calculated in BEAST v2.4.2 using the HKY + G model of nucleotide substitution with four gamma categories and empirical base frequencies. The mean estimated time of divergence for the *boliviensis* species group

Fig. 3 Maximum Likelihood phylogeny for the Cytochrome b gene (1087 bp) calculated for the *global* data set. Haplotypes sampled in this study cluster with the *boliviensis* group of species and share a common ancestor with the Bolivian species *Ctenomys boliviensis*. Western Clade: PL, CA, SP; Eastern Clade: NO, NU1, NU2, FN; Central Clade: NM, TS; *C. bicolor*: PB. Species groups: (**a**) *boliviensis*; (**b**) *opimus*; (**c**) *mendocinus*; (**d**) *talarum*; (**e**) *torquatus*; (**f**) *tucumanus*; (**g**) *magellanicus*; (**h**) *frater*. Bootstrap values under 50 are ommited from the figure
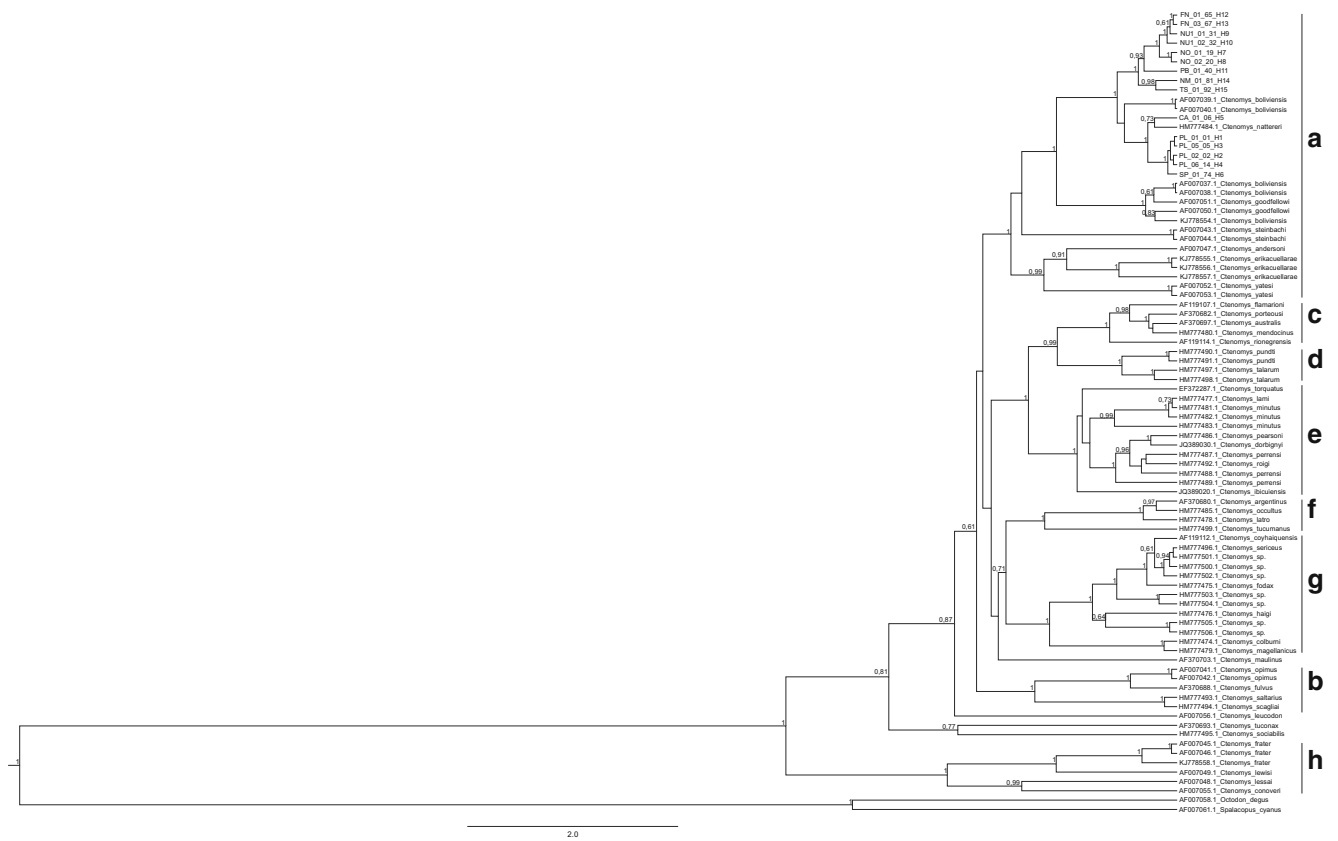
Fig. 4 Bayesian Inference based phylogeny for the Cytochrome b gene (1087 bp) for the global data set. Haplotypes sampled for the studied localities cluster within the *boliviensis* group of species and share a most recent common ancestor (MRCA) with the species *Ctenomys boliviensis*. Western clade: PL, CA and SP; Eastern Clade: NO, NU1, NU2 and FN; Central Clade: NM and TS; *Ctenomys bicolor*: PB. Species groups: (**a**) *boliviensis*; (**b**) *opimus*; (**c**) *mendocinus*; (**d**) *talarum*; (**e**) *torquatus*; (**f**) *tucumanus*; (**g**) *magellanicus*; (**h**) *frater*. Values of posterior probabilities lower than 0.5 were omitted from the figure

was 1.57 mya. *Ctenomys bicolor*, *C. nattereri*, and the Eastern, Western, and Central Clades share an MRCA with *C. boliviensis*, with a mean estimated time of origin of approximately 1.06 mya. The Eastern and Central Clades originated approximately 0.15 and 0.17 mya, respectively, while the Western Clade plus *C. nattereri* from Bolivia (HM777484) is estimated to be somewhat older on average (0.24 mya). The mean estimated times for common ancestors between the taxa in the local data set are summarized in Table 5.

The tree topology is also maintained in the phylogeny calculated with the local data set, with the exception of the positioning of *C. steinbachi*, which clusters as a sister taxon to *C. andersoni*, *C. erikacuellarae*, and *C. yatesi*, albeit with low statistical support (Fig. 5). Almost all nodes representative of valid species and lineages have strong statistical support (posterior probabilities ranging from 0.9 to 1), with the exception of the nodes that separate the species *C. steinbachi* from *C. andersoni*, *C. erikacuellarae*, and *C. yatesi* (0.45), the Western Clade and *C. nattereri* from Bolivia from the two haplotypes misidentified as *C. boliviensis* (AF007039 and AF007040; 0.56) and *C. bicolor* from the Eastern Clade (0.79).

Geometric morphometric analyses – Shapiro-Wilk's test indicated that the centroid sizes do not differ between localities (w = 0.97302, $P = 0.153$) for any of the three views (dorsal: $P = 0.153$; ventral: $P = 0.07$; lateral: $P = 0.233$), except for locality SP, the individuals of which are smaller on average than the ones in other localities ($F_{8;\ 58} = 6.489$; $P < 0.001$) (Fig. S1). We did not find significant sexual dimorphism for size or shape within or between localities ($t = 0.31798$, df = 32.362, $P = 0.7525$; Fig. S1). Therefore, the males and females were pooled together for further analyses.

The PCAs did not show structuring between localities for skull shape (data not shown). Cross-validation tests assigned on average 86%, 68%, and 67% of the individuals to their localities of origin (Table 6) and 91%, 83%, and 64% of the individuals to their respective molecular clade for the dorsal, ventral, and lateral views, respectively (Table 7). There are significant differences among localities for skull shape for the dorsal (Wilk's λ < 0.0001; $F_{8,\ 58} = 5.8889$; $P < 0.001$), lateral (Wilk's λ < 0.0001; $F_{8,\ 58} = 3.7815$; $P < 0.001$), and ventral (Wilk's λ < 0.0001; $F_{8,\ 58} = 3.3744$; $P < 0.001$) views. Pairwise MANOVA revealed significant differences in skull shape between the majority of comparisons for all views

Table 4 Calibration points used (Ctenomyidae/Octodontidae and genus *Ctenomys*), their associated times of divergence and estimated times of divergence obtained for the BI phylogeny between species groups of the genus *Ctenomys* (global data set). Clades: Western, Eastern and Central; *C. bicolor*: PB

Lineage	Calibration points – Mean (HPD95%)	Div. times – Mean (HPD95%)
Higher order groups and species groups		
Ctenomyidae/Octodontidae	10.65 (9.8–11.5)	10.98 (9.08–12.83)
genus *Ctenomys*	5.0 (3.5–6.5)	3.71 (2.38–5.14)
frater		2.18 (1.22–3.16)
All species groups minus *frater* and *C. sociabilis* and *C. tuconax*		2.74 (1.68–3.86)
opimus		1.35 (0.75–2.00)
mendocinus		0.64 (0.34–0.99)
talarum		0.52 (0.26–0.82)
torquatus		0.95 (0.55–1.38)
magellanicus		1.21 (0.71–1.77)
tucumanus		1.20 (0.73–1.83)
boliviensis		1.58 (0.98–2.24)
Local group (boliviensis)		
C. andersoni, C. erikacuellarae and *C. yatesi*		1.26 (0.74–1.84)
C. boliviensis, C. bicolor, C. nattereri and other clades		1.15 (0.67–1.65)
C, bicolor, C. nattereri and Clades		0.57 (0.33–0.84)
C. nattereri and Western Clade		0.50 (0.28–0.75)
C. bicolor and Eastern and Central Clades		0.37 (0.20–0.56)
C. bicolor and Eastern Clade		0.31 (0.17–0.47)
Eastern Clade		0.17 (0.08–0.27)
Central Clade		0.20 (0.06–0.37)

(Table S4). The scatterplot of the CVA for the three views of the skull showed different degrees of discrimination for the localities sampled (Fig. S2): in the dorsal view, the localities PB and CA overlap, with the highest negative values for CV1, with the skulls showing a proportionately more-elongated rostrum in positive scores (Fig. S2a). In contrast, NO presents the highest positive values for this axis. All other localities (PL, SP, NU1, NU2, FN, and NM) are differentiated from CA, PB, and NO, clustering with mostly positive values for CV2, though NM presents slightly negative values for CV1; locality FN is differentiated from all other localities by CV2,

presenting the highest positive values for that axis. In the ventral view, the localities PL, CA, and SP are differentiated from all other localities by CV1, where sampling sites with positive scores show skulls enlarged in the tympanic bullae (Fig. S2b). NM and NO present values closest to the mean skull shape for the ventral view, e.g., close to zero, while the NU1, NU2, and FN localities are differentiated by CV2. PB is separated from all other localities by CV1, presenting the most negative values for that CV. In the lateral view, the locality NU1 presents the shape closest to the mean shape, while all other sampling sites are separated from each other and from

Table 5 Calibration point used (*boliviensis* species group), its associated time of divergence obtained for the local phylogeny and estimated times of MRCAs between species and Clades within the *boliviensis* species group

| Lineage	Calibration point – Mean (HPD95%)	Div. times – Mean (HPD95%)
boliviensis species group	1.48 (0.92–2.06)	1.57 (1.37–1.77)
C. steinbachi, C. andersoni, C. erikacuellarae and *C.yatesi*		1.26 (0.72–1.68)
C. boliviensis, C. bicolor, C. nattereri and other clades		1.06 (0.59–1.51)
C. bicolor, C. nattereri and Clades		0.51 (0.28–0.75)
C. nattereri and Western Clade		0.24 (0.11–0.39)
C. bicolor, Eastern and Central Clades		0.33 (0.17–0.49)
C. bicolor and Eastern Clade		0.28 (0.14–0.43)
Eastern Clade		0.15 (0.07–0.24)
Central Clade		0.17 (0.04–0.32)
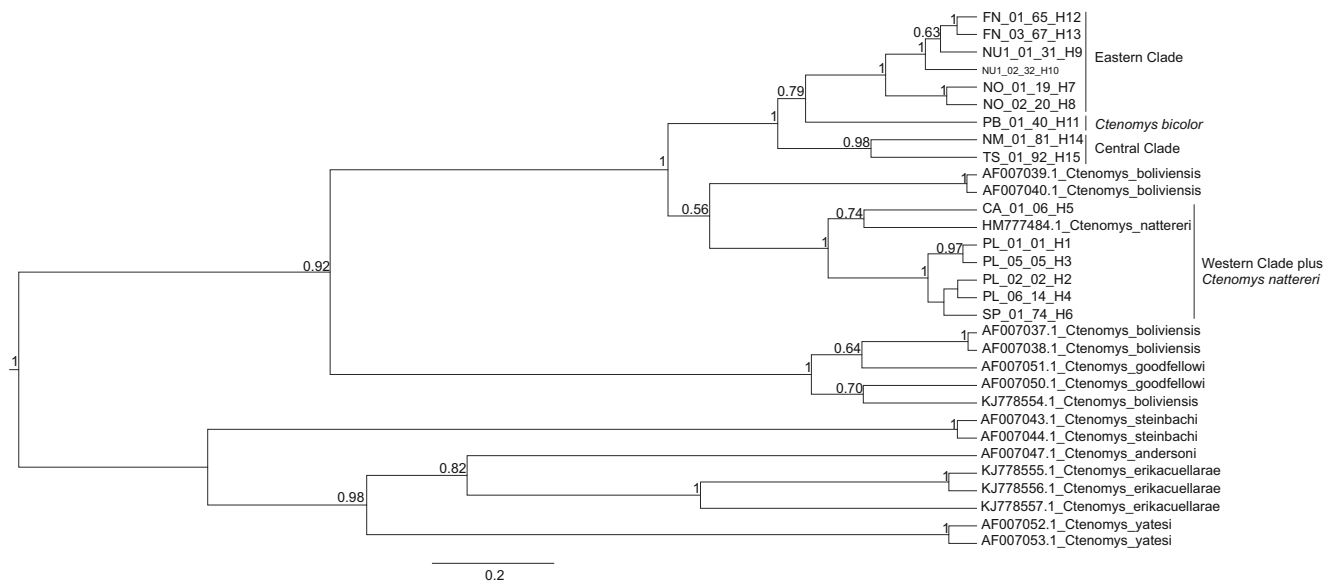
Fig. 5 Bayesian Inference based phylogeny for the Cytochrome b gene (1087 bp) for the local data set. Clades of localities are indicated at the bars to the right. The *boliviensis* species group is monophyletic with strong statistical support. Posterior probability values lower than 0.5 are ommited from the figure

NU1, with values distant from zero for both CVs. PL, FN, and PB are closely distributed in shape-space, presenting the most negative values for CV1 and little variation in skull shape (Fig. S2c).

Discussion

Diversity indices and general patterns of genetic structuring in Ctenomys *from midwest and northern Brazil*

Two haplotypes are shared between localities within clades (PL and SP: H4; NU1, NU2, and FN: H10), which were in turn proposed based on the genetic distances between haplotypes. In every case, the pairwise genetic distances between haplotypes in a clade were lower than those in any other comparison between haplotypes. Indeed, the clades specified are monophyletic with strong statistical support in all phylogenetic analyses and cluster within the *boliviensis* species group (group A; Figs. 3, 4 and 5). In addition, the Bayesian mixture analysis structures localities into six genetic groups, consistent with the groups formed when considering the observed shared

haplotypes, genetic distances, and tree topologies (Fig. 2). The clustering of the haplotypes from midwest and northern Brazil within the *boliviensis* group of species was expected, given that *C. bicolor* (Stolz et al. 2013) and *C. nattereri* from Bolivia (Parada et al. 2011; Gardner et al. 2014) are known to cluster within this species group.

The mean genetic distances observed between *C. nattereri* from Bolivia and the Western Clade (1.0%) are similar to the ones observed within the Western Clade localities (0.7%) and within the Eastern Clade localities (0.6%), which we consider intraclade values, as opposed to the values observed between the Eastern and Western Clades (mean of 2.8%), which can be two to three times larger. In contrast, *C. nattereri* and the Eastern Clade present a mean sequence divergence of 1.9%, while *C. bicolor* diverges in over 2.2% of the sequence when compared to the Western (mean of 3.5%), Eastern (mean of 2.5%), and Central (2.3%) Clades. Such genetic divergence between the haplotypes sampled, combined with the evidence of shared haplotypes, which is reflected by the structuring among haplotypes of the sampled localities, and with the phylogenetic structuring of clades into monophyletic groups with

Table 6 Percentage of correct cross-validation classification for skull shape for nine localities of *Ctenomys* from midwest and northern Brazil based in the skull shape for each of the three views. PL, Pontes e Lacerda; CA, Cáceres; SP, Sapezal; NO, Nova Olímpia; NU1, Nova Ubiratã 1; NU2, Nova Ubiratã 2; FN, Feliz Natal; PB, Pimenta Bueno; NM, Nova Mutum

	CA	FN	NM	NO	NU1	NU2	PB	PL	SP	Average
Dorsal	66.67	60.00	90.00	100.00	100.00	85.71	100.00	77.78	100.00	86.57
Ventral	66.67	80.00	66.67	66.67	62.50	57.14	77.77	55.55	75.00	68.66
Lateral	100.00	60.00	70.00	50.00	50.00	71.43	66.67	60.67	59.00	67.16

Table 7 Percentage of correct cross-validation classification for skull shape for clades of localities as proposed by molecular analysis for the dorsal, ventral and lateral views of the skull, and its associated average values. *C. bicolor*, PB; Western Clade: PL, CA and SP; Eastern Clade NO, NU1, NU2 and FN; Central Clade, NM

	C. bicolor	Western Clade	Eastern Clade	Central Clade	Average
Dorsal	100.00	86.36	96.15	80.00	91.04
Ventral	77.78	81.82	88.46	80.00	83.58
Lateral	66.67	77.27	61.54	40.00	64.10

strong statistical support indicate that the clades are representative of unique lineages of *Ctenomys* in midwest Brazil. However, proposing thresholds for sequence divergence to delimitate intra- and interspecific genetic distances is difficult, as the same marker may evolve at different rates in different taxa (Avise 1994). Few studies have estimated sequence divergence thresholds for mitochondrial genes. Tobe et al. (2010) compared cytochrome oxidase I (COI) and cytochrome b (cyt b) sequences for 217 mammalian taxa and concluded that at the specific level, cyt b estimates generally correctly assign individuals to their species better than do COI estimates, especially when considering 1.5–2.5% sequence divergence as the threshold to distinguish taxa. Such estimates would imply that our proposed clades present genetic distances within them below the 1.5–2.5% threshold, while the distances between clades can reach values that surpass this threshold, meaning that the clades may represent different species or, at least, represent lineages in the process of speciation. Thus, the distances between *C. bicolor* and the clades are either near or above 2.5%, which is expected because *C. bicolor* is considered a valid species (see Table 3; Figs. 3, 4 and 5).

Phylogenetic trees, skull geometric morphometrics, and diversity of *Ctenomys* in midwest and northern Brazil

The phylogenies calculated using BI and ML methods yield very similar topologies and recover all eight species groups proposed by Parada et al. (2011) with moderate to strong statistical support (Figs. 3, 4 and 5). There are minor incongruences between the phylogenies estimated with the two methods, but they are restricted to the species positioning within species groups, and none of them directly relate to our taxa of interest. Our analyses show that the *boliviensis* species group is one of the oldest species groups to have originated (mean estimate of 1.57 mya; Table 5), considering the estimate generated through the local phylogeny. The lack of resolution for the basal nodes on the phylogenies (bootstrap values under 50 and posterior probabilities under 0.5) demonstrates the rapid radiation of *Ctenomys* throughout South America, as has been pointed out by other authors (Lessa and Cook 1998; Castillo et al. 2005; Parada et al. 2011). The

estimates of origin of MRCAs for the *boliviensis* species group obtained from both the complete and partial phylogenetic reconstructions indicate that the mean and highest probability density (HPD95%) values obtained are consistent between analyses, with minor differences. This demonstrates that the results are reproducible and that the parameters used to reconstruct phylogenies, most notably the calibration points and the enforcing of monophyly of the genus *Ctenomys*, were adequate.

The CVAs for dorsal and ventral views of the skull more closely differentiate the localities in a phylogeny-like pattern: the CV1 for the ventral view of the skull segregates the sampling sites into groups resembling the molecular clades, while in the dorsal view, CV2 also discriminates the sampling sites in a geographical pattern (Fig. S2a and S2b). The CVA for the lateral view of the skull differentiates the localities into single-locality groups, with low variance for skull shape within them (Fig. S2c). The percentages of individuals correctly assigned to their localities of origin through jackknife cross-validation analyses suggest that differences in the dorsal view of the skull best differentiate the sampling sites from one another (86%), while the ventral and lateral views correctly differentiate the sampling sites less often (68 and 67%, respectively; Table 6). However, testing the assignment of individuals based on the proposed phylogenetic clades increased the frequency of correct classification for both the dorsal (91%) and ventral (83%) views, suggesting that skull shape is influenced partially by demography (Table 7), because the increase in correct classification reflects the common origin of the individuals of the localities within a clade. Patterns of discrimination between sampling sites through the CVA analysis corroborate the results obtained through the MANOVA analysis, in which there are significant differences in skull shape for most pairwise comparisons in all three views analyzed (Table S4).

Therefore, the geometric morphometric analysis of the skulls of individuals from our sampling sites demonstrates that variation in skull shape may contain both environmental and phylogenetic signals. Because mitochondrial genes are considered to evolve under near neutrality, and thus are widely used as molecular markers for estimating evolutionary processes dependent on geographical barriers, gene flow and migration (e.g., demographic effects), environmental pressures selecting for a given cranial structure may not reflect a pattern of cranial structure in which variation is caused by demographic effects but rather a pattern that reflects adaption or both adaption and demography. Indeed, the structure inferred through the morphometric analysis for both the ventral and dorsal views of the skull closely resembles the structure found in the phylogenetic analysis, while the morphometric analysis with the lateral view of the skull does not, indicating the influence of selection on skull shape.

There are many possible explanations for the differences in skull shape that do not match the patterns of structure inferred from the phylogenies as well as for the lack of differences

between individuals in localities that do belong to different phylogenetic clades; for example, convergence in the feeding apparatus in rodents inhabiting similar habitats is common (Samuels 2009). Therefore, if the habitats present similar ecological pressures, then the differences in skull shape between individuals of localities representative of distinct phylogenetic lineages can be more influenced by environmental pressures than by demography alone. Environmental pressures, such as the hardness of soil, can select for skull shape in fossorial rodents (Marcy et al. 2016), which requires a derived skull morphology with procumbent incisors for digging (Stein 2000); indeed, the bulk density of soils is correlated with bite force in *Ctenomys*, with species that inhabit denser soils generally exhibiting higher bite force measurements (Borges et al. 2017). Alternatively, allometry effects can influence intraspecific skull shape in conjunction with environmental effects, such as primary production and precipitation, or demographic effects, including restricted gene flow and reduced population size (Maestri et al. 2016). Conversely, the times of origin for the Brazilian clades appear to be recent (Table 5), in which case it is possible that not enough time has passed for the lineages to accumulate differences in skull shape. This demographic hypothesis could explain the subtle structural differences found between the three views of the skull. Alternatively, environmental pressures may be selecting for skull shape.

Overall, patterns of phylogenetic structure, sequence divergence between haplotypes, and skull shape differences between individuals within the sampled localities reveal two major lineages in midwest Brazil: i) a lineage including the haplotypes from the Western Clade plus *C. nattereri* from Bolivia (HM777484), and ii) a lineage including all haplotypes from the Central and Eastern Clades and *C. bicolor.* Given that *C. bicolor* is a valid species and is positioned intermediately relative to the Eastern and Central Clades in all phylogenetic analyses, both clades can be considered independent lineages. As the Eastern Clade sampling sites NU1, NU2, and FN are located near Xingu National Park, one of the largest and most conserved protected areas in Brazil, and the Central Clade sampling sites are central to the known distribution of *Ctenomys* in midwest and northern Brazil, we name the lineages *C.* sp. "xingu" and *C.* sp. "central," respectively.

Potential Shortcomings of the Analyses and Future Directions
Mitochondrial genes have been used extensively in phylogenetic studies due to their mode of inheritance, lack of recombination, and reduced effective sample size, which facilitate coalescence in a phylogeny (Neigel and Avise 1987; Avise 2000). In the case of *Ctenomys*, mitochondrial genes have been instrumental for defining species and confirming the monophyly of the genus. However, time estimates using mitochondrial markers should be interpreted with caution, because these markers may have broad confidence intervals that

may limit inference of the ages of phylogenetic clades (Ballard and Whitlock, 2004 and references therein). In any case, phylogenetic approaches in *Ctenomys* using the cytochrome b gene have yielded more reliable estimates of the origin of both the genus and its species groups than the nuclear markers that have been tested, based on the estimated age of the oldest known fossil (Lessa and Cook 1998; Castillo et al. 2005; Parada et al. 2011).

Ctenomys presents high levels of karyotype heterogeneity, with diploid numbers ranging from $2n = 10$ in *C. steinbachi* to $2n = 70$ in *Ctenomys dorbignyi* Contreras and Contreras, 1984 (Anderson et al. 1987; Woods and Kilpatrick 2005), although the species generally have species-specific karyotypes (Reig et al. 1990, but see Lopes et al. 2013). Therefore, knowing how localities structure karyotype variability is essential to corroborate molecular and morphological data to define and describe species within the genus *Ctenomys*. We concur with Parada et al. (2011) and Freitas (2005) in that additional nuclear markers, such as the ones used by Castillo et al. (2005) and Upham and Patterson (2012), should be sequenced genuswide to corroborate the karyotype, morphological, and mitochondrial DNA data. Additionally, sequencing additional genes can better estimate species trees, thus mitigating possible incongruences between species and gene trees, and can provide more reliable evidence for dating times of divergence between lineages with narrower confidence intervals.

Conclusions

Molecular phylogenetics and geometric morphometrics approaches have allowed us to resolve phylogenetic lineages and corroborate the presence of *C. bicolor* and *C. nattereri* in midwest and northern Brazil. We identify two independent lineages: *C.* sp. "xingu" and *C.* sp. "central." At the moment, a lack of karyotype data does not allow us to resolve lineages into defined species; therefore, sampling additional localities of *Ctenomys* from midwest and northern Brazil, and thus increasing the sample size for molecular and morphometric analysis and allowing for the karyotyping of individuals is essential to accomplish this task. In addition to increasing sample size, future population genetics and ecological studies involving *Ctenomys* in Brazil will require more molecular markers, both those that are neutral and those under selection, to estimate population parameters, understand genetic mechanisms of local adaption, and define evolutionary significant units sensu Moritz (1994). For example, next-generation sequencing (NGS) methods coupled with restriction enzyme digestion (Davey et al. 2011 and references therein), such as RADseq (Baird et al. 2008) and its derivatives (ddRADseq, Peterson et al. 2012), could be employed to generate sets of hundreds to thousands of neutral and potentially adaptive loci, allowing for the fine-tuning of phylogenies (Cariou et al.

2013; Emerson et al. 2010) and identification of polymorphisms that could account for the genetic basis of local adaption (Manthey and Moyle 2015).

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