Marco Antônio Alves Schetino

Tese de Doutorado

Análises moleculares em Xenarthra: contribuições para a sistemática filogenética de Tolypeutinae, taxonomia de *Cabassous* e filogeografia de *Bradypus torquatus*.

cipit vita ni

Tese apresentada ao curso de doutorado em Genética do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção de Título de Doutor em Genética.

Orientador: Fabrício Rodrigues dos Santos

Belo Horizonte

Fevereiro - 2017

Marco Antônio Alves Schetino

Tese de Doutorado

Análises moleculares em Xenarthra: contribuições para a sistemática filogenética de Tolypeutinae, taxonomia de *Cabassous* e filogeografia de *Bradypus torquatus*

> Tese apresentada ao curso de Pósgraduação em Genética do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção de Título de Doutor em Genética.

> Orientador: Fabrício Rodrigues dos Santos

Belo Horizonte Fevereiro - 2017

043 Schetino, Marco Antônio Alves.

 Análises moleculares em Xenarthra: contribuições para a sistemática filogenética de Tolypeutinae, taxonomia de Cabassous e filogeografia de Bradypus torquatus [manuscrito] / Marco Antônio Alves Schetino. – 2017.

100 f. : il. ; 29,5 cm.

Orientador: Fabrício Rodrigues dos Santos.

 Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas.

 1. Filogenia - Teses. 2. Filogeografia - Teses. 3. Biologia - Classificação. 4. Xenartros. 5. Preguiça (Zoologia). I. Santos, Fabrício Rodrigues dos. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 575

Ficha elaborada pela Biblioteca do Instituto de Ciências Biológicas da UFMG

Universidade Federal de Minas Gerais Instituto de Ciências Biológicas Programa de Pós-Graduação em Genética

ATA DA DEFESA DE TESE

92/2017 entrada $2^{\circ}/2012$ $CPF:$ 012.507.786-62

Marco Antônio Alves Schetino

As quatorze horas do dia 23 de fevereiro de 2017, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: "Análises moleculares em Xenarthra: contribuições para a sistemática filogenética de Tolypeutinae, taxonomia de Cabassous e filogeografia de Bradypus torquatus. ", requisito para obtenção do grau de Doutor em Genética. Abrindo a sessão, o Presidente da Comissão, Fabrício Rodrigues dos Santos, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Pelas indicações, o candidato foi considerado:

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

APROVADO

10 rues

Belo Horizonte, 23 de fevereiro de 2017.

Fabrício Rodrigues dos Santos - Orientador

Almir Rogério Pepato

Flávio Henrique Guimarães Rodrigues

Anderson Vieira Chaves

Fernando Araújo Perini

"I, I can remember Standing, by the wall And the guns, shot above our heads And we kissed, as though nothing could fall And the shame, was on the other side Oh we can beat them, for ever and ever Then we could be Heroes, just for one day" David Bowie

À Luana e Matheus.

Agradecimentos

Agradeço ao Professor Fabrício Rodrigues dos Santos pela oportunidade, paciência e compreensão. Sem sua inteligência, visão e capacidade de resolução de cada imprevisto que aconteceu, nada disso seria possível. Meu mais profundo obrigado.

Agradeço à FAPEMIG, CNPq, Capes e Fundação o Boticário pelos fundos disponibilizados para a execução do meu doutorado.

À Teresa Anacleto: obrigado por me ensinar até mais do que deveria. Muito do que sei devo a você.

Aos membros da banca, meus agradecimentos pela avaliação e sugestões.

A todos do LBEM, sem exceções. De cada um, uma experiência dividida pois cada um carrega o peso de seu próprio mundo nas costas: Meu sincero obrigado.

Ao Anderson Feijó, Flávia Miranda, Arnaud Debiez, Victor Yunes (Biota), Luiz Cesca (Biota) e participantes do "PAN Tatu-bola": Obrigado pelas dicas e amostras doadas. Sem a contribuição de vocês a tese não teria tantos frutos.

Aos amigos Babi, Anderson, Ana Carolina, Davidson, Patrízzia, Camilla e Thaís: vocês fizeram a diferença e muito.

Aos amigos companheiros de república Raphael e Samuel: Valeu pelas risadas e absurdos.

Aos antigos amigos "Coiotes" e de infância: Obrigado pelos conselhos.

Aos meus pais, irmã, sobrinha, sobrinho e agregados: Obrigado pelo carinho e paciência. O exemplo de vocês sempre me faz seguir adiante.

À tia Mirinha e tio Zé (*in memorian*): sem vocês eu não teria chegado nem na metade do caminho. Muito obrigado.

À minha esposa: só tenho a agradecer... por tudo. Obrigado por ser minha base nos momentos de desespero e por ser forte nos momentos em que estivemos longe. Te amo!

Ao meu filho: desculpa o papai. Sei que foi difícil este tempo longe. Você foi minha força para continuar. Por você e para você. Te amo!

Ao meu sogro, sogra e cunhados: obrigado por cuidar bem de minha família na minha ausência e pela força nesta reta final.

A todos que contribuíram diretamente ou indiretamente que aqui não foram citados. Obrigado, minha cabeça falha, mas vocês não são menos importantes.

A Deus em todas suas formas de manifestações.

Índice

Lista de Figuras

Introdução

Figura 1: Hipóteses sobre a posição da raiz da árvore filogenética de Eutérios **.....................5**

Capítulo1

Figure 1: Map of geographic distribuition of Tolypeutinae. Species distribuition based in IUCN (2016). Legends: **A-B** = *Cabassous*; **C** = *Tolypeutes;* **D** = *Priodontes* **………………………...23**

Figure 2: Phylogenetic relationships between Tolypeutinae's genera. The actual tree (left) was taken from the last classification suggested for this subfamily (Gibb *et al.* 2016). The lines illustrate previous classifications of the authors represented above. *a =* Family Tolypeutidae; *b =* Family Dasypodidae; *c* = Tribe Tolypeutini; *d* = Tribe Priodontini; *e* = Subfamily Priodontinae; *f* = Subfamily Tolypeutinae **…………………………………………………………24**

Figure 3: Molecular time scale for Tolypeutinae nodes inferred with a Bayesian phylogeny obtained in BEAST. Node ages were obtained under the reversible-jump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a birth-death tree prior with soft fossil calibrations. Node bars indicate the 95% HPD intervals for age estimates in million years ago (MYA). All nodes not shown were supported by maximum posterior probabilities **……29**

Capítulo 2

Figure 1: Sampling localities of *Cabassous* species in South and Central America. Star = *C. chacoensis*; Circle = *C. u. unicinctus*; Asterisk = *C. u. unicinctus squamicaudis*; Triangle = *C. tatouay*; Square = *C. centralis* **……………………………………………………………………..48**

Figure 2: Haplotype networks for mitochondrial genes of *Cabassous* genus. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the lines **……………………………………………………………………………………………...……52**

Figure 3: Haplotype networks for six nuclear genes of *Cabassous* genus. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the line **……………………………………………………………………….……………………………53**

Figure 4: Molecular timescale for *Cabassous* nodes inferred with a Bayesian phylogeny obtained in *BEAST. Node ages were obtained under the reversible-jump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a Yule tree prior. Node bars indicate the 95% HPD intervals for age estimates in million years ago (mya). All nodes with the values not shown were supported by maximum posterior probabilities **..………………………………………………………………………………………………………..54**

Capítulo 3

Figure 1: Sampling localities of *B. torquatus* and the mitochondrial haplotype network constructed with the median-joining algorithm. Black haplotipes $=$ Bahia; White haplotipes $=$ Espírito Santo; and gray haplotipes = Rio de Janeiro. The letters refer to the localities in table 1 **...72**

Figure 2: Molecular time scale for *Bradypus torquatus* and Pilosa nodes inferred with a Bayesian phylogeny obtained in BEAST. Node ages were obtained under the reversible-jump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a birthdeath tree prior with soft fossil calibrations. Node bars indicate the 95% HPD intervals for age estimates in million years ago (MYA). All nodes were supported by maximum posterior probabilities **..79**

Figure 3: The divergence lineages *B. torquatus* tree. The biogeographical range estimation was inferred under the S-DIVA model with the RASP software. All nodes were supported by maximum posterior probabilities. The letters A, B and C represent the ancestral range estimate. Legends: $A = BA + ES + RJ$ range populations: $B = ES + RJ$ range populations: $C = BA$ range population; 1 = Dispersion event; 2 = Vicariant event**………………………………….…79**

Figure S1: (A) Population estimation: Index of MCMC iterations and probability density of number of clusters. (B), (C) and (D) Area estimation *of B. torquatus* BA, ES and RJ populations. Lines represent posterior probability. The lighter area indicates the region with the most probability to belong to the same population **…………………………………………………..…97**

Figure S2: Haplotype networks for tree nuclear genes showing the BA, ES and RJ populations. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the lines. Black = Bahia (BA); white = Espírito Santo (ES); and gray = Rio de Janeiro (RJ) **..98**

Lista de tabelas

Capítulo 1

Table 1: Sample details of individuals of Tolypeutinae used in this work. LBEM = Laboratório de Biodiversidade e Evolução Molecular; UFMG = Universidade Federal de Minas Gerais **..16**

Table 2: Primers used for partial amplification of Tolypeutinae genes. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular **..17**

Table 3: Approximated body mass, head-body length and tail length of the species of Tolypeutinae **…………………………………………………………………………….…………...19**

Table 4: Chromosome patterns of Tolypeutinae species. Legends: 2n = diploid number; Fn = Fundamental number; $X = X$ chromosome; $Y = Y$ chromosome; $M =$ Metacentric; SM= Submetacentric; A= Acrocentric **…….……………………………………………………………..21**

Table 5: Divergence time estimates with 95% confidence intervals for all nodes **…..29**

Table S1: Accession numbers of sequences retrieved from Genbank **……………………….43**

Capítulo 2

Table 1: Sampling localities and number of samples of *Cabassous* used in this work. Legend: G = Genbank. Legend of Brazilian states: GO = Goiás; MG = Minas Gerais; MS = Mato Grosso do Sul; MT = Mato Grosso; PA = Pará; PB = Paraíba; RO = Rondônia; SC = Santa Catarina **..49**

Table 2: Primers used for partial amplification of *Cabassous* genes. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular **..50**

Table 3: Divergence times estimates of *Cabassous* clades with 95% confidence intervals for all node **………………………………………………………………………..................................55**

Table S1: Accession numbers of sequences retrieved from Genbank **…………………….…..64**

Capítulo 3

Table 1: Sampling localities, geographic coordinates (decimal degrees) and number of samples (N) **…..………………………………………………………………..………………..…71**

Table 2: Primers used for partial amplification of *B. torquatus* genes, PCR annealing temperatures and respective references. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular **...........................74**

Table 3: Genetic and molecular diversity indices of population of *B. torquatus*. Number of polymorphic sites (S), number of haplotypes (NH), haplotype diversity (Hd) and nucleotide diversity (π) …**…………………………………………………………….………………………..77**

Table 4: Percentage of molecular variation among and within populations of *B. torquatus* obtained in the AMOVA (mtDNA/nuDNA) **…….……………………………............................78**

Table 5: Divergence time estimates with 95% confidence intervals for all Pilosa nodes. **……………………………………………………………………………………….......................80 Table S1**: List of genes, species, haplotypes, population of *Bradypus torquatus***…………..95**

Table S2: Pairwise φ_{ST} matrix obtained with Arlequin 3.5 (mtDNA/nuDNA)96

Lista de Abreviaturas

- **AMOVA:** Analysis of molecular variance
- **Cu1:** *Cabassous unicinctus unicinctus 1*
- **Cu2:** Cabassous unicinctus unicinctus 2
- **ddNTP:** dideoxiribonucleotides
- **DNA:** Deoxyribonucleic acid
- **GABI:** Great American Biotic Interchange
- **HPD:** Highest posterior density
- **IUCN:** International Union for Conservation of Nature
- **KYA:** Thousand years ago
- **LBEM:** Laboratório de Biodiversidade e Evolução Molecular
- **mtDNA:** Mitochondrial DNA
- **MYA:** Million years ago
- **NaCl:** Sodium chloride
- **nuDNA:** Nuclear DNA
- **PCR:** Polymerase chain reaction
- **UFPB:** Universidade Federal da Paraíba
- **UFMG:** Universidade Federal de Minas Gerais

Resumo

A Magnaordem Xenarthra compreende as ordens Pilosa (Tamanduás e preguiças) e Cingulata (Tatus). Distribuem-se principalmente desde o México até o sul da América do Sul com exceção de *Dasypus novemcinctus* que tem sua distribuição estendida até os Estados Unidos. Apesar de haver a compreensão de alguns aspectos sistemáticos e taxonômicos de Xenarthra poucos estudos foram totalmente direcionados para suas subfamílias ou dentro de uma espécie em particular. A subfamília Tolypeutinae é composta pelos gêneros *Cabassous*, *Priodontes* e *Tolypeutes* e, desde a descrição de suas espécies, sua sistemática tem passado por várias mudanças e ainda não existe um consenso entre trabalhos morfológicos e moleculares sobre a filogenia dentro desta subfamília. O gênero *Cabassous* é composto por quatro espécies: *Cabassous centralis*; *Cabassous chacoensis*; *Cabassous tatouay* e; *Cabassous unicinctus*, este último dividido em duas subespécies que são *C. u. unicinctus* e *C. u. squamicaudis* que ainda restam muitas duvidas quanto à sua classificação taxonômica. *Bradypus torquatus*, também conhecido como "preguiça de coleira" é uma espécie exclusivamente brasileira e sofre consideravelmente os efeitos da redução de seu habitat. Até o momento foram três os principais estudos sobre a filogeografia de *B. torquatus* onde todos demonstraram alto nível de estruturação genética nas regiões amostradas e isolamento entre as populações estudadas. Porém estas análises foram feitas utilizando-se apenas marcadores mitocondriais (mtDNA) que representam somente a história matriarcal de suas linhagens o que pode não reconstruir efetivamente a história demográfica da espécie. Com o objetivo aumentar o conhecimento sobre a genética de xenartros, três trabalhos foram realizados. O primeiro foi a revisão bibliografica de Tolypeutinae e a proposta de uma nova filogenia devido a encontrarmos relações de parentesco entre as espécies de *Cabassous* diferente do que proposto atualmente por outros autores. No segundo trabalho, com marcadores moleculares, análises de delimitação de espécies em *Cabassous* foram realizadas onde obtivemos dados que corroboram com a hipótese de que *Cabassous unicinctus squamicaudis* e *Cabassous unicinctus unicinctus* são duas espécies além de sugerirmos novos estudos para entender as relações entre *C. u. unicinctus* e *C. centralis.* Por fim, no terceiro trabalho. Adicionalmente outro estudo utilizando marcadores moleculares nucleares e mitocondriais de *B. torquatus* com inferências filogeográficas e populacionais foi realizado e se evidenciou um alto nível de estruturação entre as populações analisadas e sua reconstrução filogeográfica remete a um padrão de dispersão direcionado por eventos ocorridos no Plioceno e Pleistoceno além de propor uma nova região geográfica para a linhagem ancestral desta espécie.

Abstract

The Magnaordem Xenarthra comprises the orders Pilosa (anteaters and sloths) and Cingulata (armadillos). They are distributed from Mexico to the south of South America with the exception of *Dasypus novemcinctus* that has its distribution extended to the United States. Despite the understanding of some systematic and taxonomic aspects of Xenarthra, few studies have been totally focused on their subfamilies or within a particular species. The subfamily Tolypeutinae is composed of the genera *Cabassous*, *Priodontes* and *Tolypeutes* and, since the description of its species, its systematics has several changes and there is not yet a consensus between morphological and molecular works about the phylogeny within this subfamily. *Cabassous* is the genus of most species of family Tolypeutinae composed of four species: *Cabassous centralis*; *Cabassous chacoensis*; *Cabassous tatouay* and; *Cabassous unicinctus*, the latter divided into two subspecies, C. u. *unicinctus* and *C. u. squamicaudis*, that there are still many doubts as to its taxonomic classification. *Bradypus torquatus*, also known as "Maned sloth" is an exclusively Brazilian species and suffers considerably the effects of the reduction of its habitat. To date, there have been three main studies on the phylogeography of *B. torquatus* where all showed high level of genetic structure in the sampled regions and isolation among the studied populations. However, these analyzes were done using only mitochondrial markers (mtDNA) that represent only the matriarchal history of their lineages which may not effectively reconstruct the demographic history of the species. In order to increase the knowledge about xenarthrans genetics, three studies were carried out. The first one is a bibliographical review of the Tolypeutinae systematics and the proposal of a new phylogeny due to the relationship between *Cabassous* species different from that currently proposed by other authors. In the second work, with molecular markers, analyzes of species delimitation in Cabassous was performed and our results corroborate the hypothesis that *Cabassous unicinctus squamicaudis* and *Cabassous unicinctus unicinctus* are two species. Furthermore, we suggesting new studies to understand the relations between *C. u. unicinctus* and *C. centralis*. Finally, in the third work, another study using nuclear and mitochondrial molecular markers of *B. torquatus* with time scaled phylogeography and population inferences was performed and a high level of structuring between the analyzed populations was evidenced and its phylogeographic reconstruction refers to a dispersion pattern directed by events occurring in the Pliocene and Pleistocene. Besides, we propose a new geographic region for the ancestral lineage of this species.

Introdução Geral

A Magnaordem Xenarthra compreende as ordens Pilosa (tamanduás e preguiças) e Cingulata (tatus) (McKenna & Bell 1997; Wetzel *et al.* 2007). Em conjunto com Afrotheria, Euarchontoglires e Laurasiatheria compõem a supraordem Eutheria dos mamíferos (Murphy *et al.* 2001a; b; Delsuc *et al.* 2002; Springer 2004; Kriegs *et al.* 2006). Apesar da morfologia distinta que caracteriza estas ordens, existem sinapomorfias que unem-as em um único clado monofilético como, por exemplo, o processo xenartrico, que são articulações adicionais entre as vértebras lombar e dorsal (Glass 1985; Gaudin 1999) e a ausência de três códons no gene alpha-cristalino (Van Dijk *et al.* 1999). Adicionalmente são caracterizados por: sua temperatura que varia entre 32,7º C e 35,5º C devido ao baixo metabolismo basal que corresponde de 30% a 60% de um mamífero não Xenarthra do mesmo tamanho; ausência de uma clara diferenciação entre útero e vagina; testículos intra-abdominais e; dentição ausente ou ausência de incisivos e caninos e simplificação dos molares e pré-molares (Gregory 1910; McNab 1980; Glass 1985; Cetica *et al.* 2005; Gardner 2007a).

A ordem Cingulata é caracterizada pela presença de placas ou escudos ósseos cobrindo o corpo de seus indivíduos. É composta por 21 espécies de tatus distribuídas em uma única família chamada Dasypodidae e quatro subfamílias: Dasypodinae com o diversificado gênero *Dasypus*; Euphractinae com os gêneros *Euphractus* e *Chaetophractus* e *Zaedyus*; Tolypeutinae que engloba os gêneros *Cabassous*, *Priodontes* e *Tolypeutes* e; Chlamiphorinae que compreende os gêneros *Calyptophractus* e *Chlamyphorus* (Wetzel *et al.* 2007; Delsuc *et al.* 2012). Os tatus possuem hábitos terrestres e fossoriais e possuem dieta carnívora-onívora sendo extremamente dependentes de sua exímia capacidade de escavar o solo atrás do próprio alimento (McDonough & Loughry 2008).

A ordem Pilosa não possui escudos ou placas cobrindo o corpo de seus indivíduos e sim são cobertos de pêlos. Possui 10 espécies e é dividida nas subordens Vermilingua e Folívora. A primeira é composta por duas famílias de tamanduás: Cyclopedidae com uma única espécie, *Cyclopes didactyla*, e Myrmecophagidae com os gêneros *Tamandua* e *Myrmecophaga*. A segunda reúne as famílias de preguiças Megalonychidae representada pelo gênero *Choloepus* e Bradypodidae pelo gênero *Bradypus* (Gardner 2007b). As preguiças são mamíferos herbívoros, solitários e estritamente arborícolas enquanto os tamanduás são terrestres ou arborícolas e são especialistas em se alimentar de cupins e formigas (Chiarello 2008; Rodrigues *et al.* 2008).

Os xenartros atuais se distribuem principalmente desde o México até o sul da América do Sul com exceção de *Dasypus novemcinctus* que tem sua distribuição estendida até os Estados Unidos (Gardner 2007a; Wetzel *et al.* 2007). São resquícios, de acordo com os mais de 200 fósseis já registrados, do que foi antes uma fauna altamente diversificada que dominou a América do Sul do fim do Cretáceo até o fim do terciário, período em que o continente era isolado dos outros restantes (Patterson & Pascual 1968; Simpson 1980).

Xenarthra era originalmente considerado uma subordem de Edentata incluindo os aardvarks e pangolins (Engelmann 1985). Posteriormente Simpson (1945) reconhece Xenarthra como um clado distinto dos outros dentro da ordem Edentatas (Glass 1985). Dados moleculares confirmam a monofilia do clado Xenarthra tanto quanto a de suas ordens, famílias e subfamílias, porém as relações filogenéticas entre alguns gêneros e espécies como, por exemplo, as de Tolypeutinae e Euphractinae, não foram totalmente elucidadas (Delsuc *et al.* 2001, 2003, 2008, 2012, 2016; Gibb *et al.* 2016). Ainda de acordo com dados moleculares este grupo surgiu há cerca de 105 MA e sua radiação ocorreu cerca de 70 milhões de anos atrás com a divergência entre Cingulata e Pilosa e posteriormente, há 60 MA, houve a divergência entre preguiças e tamanduás (Delsuc *et al.* 2012). No Cenozóico, mudanças paleoclimáticas e alterações drásticas de relevo possibilitaram o surgimento de novos nichos e favoreceram a diversificação dos Xenarthras, porém, sua diversidade começou a reduzir consideravelmente há 10000 anos no pleistoceno (Patterson & Pascual 1968; Delsuc *et al.* 2004, 2012).

Existem 3 hipóteses que tentam elucidar sua possível posição filogenética dentro da supraordem Eutheria (Figura 1). A primeira situa Xenarthra em uma posição basal as outras superordens que, juntas, compõe a magnaordem Epitheria (Shoshani & McKenna 1998; Kriegs *et al.* 2006; O'Leary *et al.* 2013). A segunda, chamada Exafroplacentalia, insere Afrotheria na posição mais basal (Murphy *et al.* 2001a; Scally *et al.* 2001; Amrine-Madsen *et al.* 2003; Nikolaev *et al.* 2007; Nishihara *et al.* 2009). E, por fim, a terceira hipótese considera Xenarthra e Afrotheria como grupos irmãos formando o clado Atlantogenata que compõe a base da árvore dos eutérios (Madsen *et al.* 2001; Delsuc *et al.* 2002; Hallström *et al.* 2007; Waters *et al.* 2007; Murphy *et al.* 2007).

4

Figura 1: Hipóteses sobre a posição da raiz da árvore filogenética de Eutérios.

Apesar do umento no número de trabalhos publicados de xenartros, poucos estudos são realizados exclusivamente para alguma de suas subfamílias ou dentro de uma espécie em particular (Superina *et al.* 2014) pois, existe a escassez de coleta destes indivíduos que é relacionada a dificuldade de se encontrá-los no meio selvagem além do armazenamento correto do material biológico para o uso em análises moleculares e identificações errôneas de indivíduos depositados em museus (Wetzel *et al.* 2007; Abba & Vizcaíno 2008; Loughry *et al.* 2015). Por consequência, existem lacunas do conhecimento do status taxômico de algumas espécies, sistemática, história evolutiva e estruturação populacional que podem afetar consideravelmente, por exemplo, no planejamento de medidas necessárias à sua conservação (Frankham 2003).

Inserido neste contexto podemos citar 3 situações. A última revisão taxonômica do gênero *Cabassous* foi realizada cerca de 4 décadas atrás (Wetzel 1980) e, após isso, pouca informação foi inserida em relação ao status taxonômico de suas espécies sendo a maior parte das publicações relacionadas a sua ecologia e distribuição geográfica (Superina *et al.* 2014). Adicionalmente em sua revisão, (Wetzel 1980, 1982, 1985) sugere que ainda existe dúvidas relacionadas a classificação taxonômica de *Cabassous centralis* e as subespécies de *Cabassous unicinctus*.

Como mencionado anteriormente, a sistemática da subfamília Tolypeutinae tem sofrido várias mudanças e ainda não existe um consenso entre trabalhos morfológicos e moleculares sobre sua filogenia, a mais recente possui alto índice de valores estatísticos de confiança (Gibb *et al.* 2016), porém foi realizada apenas com dados mitocondriais, carente de indivíduos de diferentes regiões geográficas e sem adicionar indivíduos da subespécie *Cabassous unicinctus squamicaudis*, o que pode interferir consideravelmente em suas análises.

Bradypus torquatus devido a constante degradação de seu habitat é uma das espécies mais ameaçadas dentre os xenartros e a compreensão de sua estruturação populacional e história demográfica é imprescindível para a formulação de melhores

estratégias de conservação (Crandall *et al.* 2000; Frankham 2003; Lara-Ruiz *et al.* 2008). Até o momento foram três os principais estudos *Bradypus torquatus* onde todos demonstraram alto nível de estruturação genética nas regiões amostradas e isolamento entre as populações estudadas (Moraes *et al.* 2002; Moraes-Barros *et al.* 2006; Lara-Ruiz *et al.* 2008; Moraes-Barros & Arteaga 2015). Porém algumas destas análises foram feitas utilizando-se um número reduzido de indivíduos apenas com marcadores mitocondriais (mtDNA) que representam somente a história matriarcal de suas linhagens o que pode não reconstruir efetivamente a história demográfica da espécie. Um dos motivos, por exemplo, pode ser a seleção indireta de moléculas de mtDNA que estão em desequilíbrio com outros genes transmitido maternalmente (Ballard & Whitlock 2004; Hurst & Jiggins 2005).

No intuito de melhor elucidar as situações acimas citadas, esta tese apresenta três capítulos, sendo eles: a delimitação de espécies do gênero *Cabassous* com o uso de marcadores moleculares; uma nova proposta filogenética para a subfamília Tolypeutinae e; a filogeografia de *Bradypus torquatus* abordando sua história demográfica e evolutiva. Todos os capítulos estão em formato de artigo e em inglês.

Referências

- Abba AM, Vizcaíno SF (2008*) Los xenartros (Mammalia: Xenarthra) del Museo Argentino de Ciencias Naturales" Bernardino Rivadavia" y del Museo de La Plata (Argentina)*. Museo argentino de ciencias naturales.
- Amrine-Madsen H, Koepfli K-P, Wayne RK, Springer MS (2003) A new phylogenetic marker, apolipoprotein B, provides compelling evidence for eutherian relationships. *Molecular Phylogenetics and Evolution*, **28**, 225–240.
- Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729–744.
- Cetica PD, Aldana Marcos HJ, Merani MS (2005) Morphology of female genital tracts in Dasypodidae (Xenarthra, Mammalia): a comparative survey. *Zoomorphology*, **124**, 57–65.
- Chiarello AG (2008) Sloth ecology: an overview of field studies. *The biology of the Xenarthra*, 269–280.
- Crandall KA, Bininda-Emonds OR, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. *Trends in ecology & evolution*, **15**, 290–295.
- Delsuc F, Ctzeflis FM, Stanhope MJ, Douzery EJ (2001) The evolution of armadillos, anteaters and sloths depicted by nuclear and mitochondrial phylogenies: implications for the status of the enigmatic fossil Eurotamandua*. Proceedings of the Royal Society of London B: Biological Sciences*, **268**, 1605–1615.
- Delsuc F, Douzery EJP, Vizcaíno SF, Loughry WJ (2008) Recent advances and future prospects in xenarthran molecular phylogenetics. *The biology of the xenarthra*.
- Delsuc F, Gibb GC, Kuch M et al. (2016) The phylogenetic affinities of the extinct glyptodonts. *Current Biology*, **26**, R155–R156.
- Delsuc F, Scally M, Madsen O et al. (2002) Molecular phylogeny of living xenarthrans and the impact of character and taxon sampling on the placental tree rooting. *Molecular Biology and Evolution*, **19**, 1656–1671.
- Delsuc F, Stanhope MJ, Douzery EJ. (2003) Molecular systematics of armadillos (Xenarthra, Dasypodidae): contribution of maximum likelihood and Bayesian analyses of mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, **28**, 261–275.
- Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A (2012) Molecular phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. *Molecular Phylogenetics and Evolution*, **62**, 673–680.
- Delsuc F, Vizcaíno SF, Douzery EJ (2004) Influence of Tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. *BMC Evolutionary Biology*, **4**, 1.
- Engelmann GF (1985) The phylogeny of the Xenarthra*. The evolution and ecology of armadillos, sloths, and vermilinguas*. Smithsonian Institution Press, Washington, DC, 51–64.
- Frankham R (2003) Genetics and conservation biology. *Comptes Rendus Biologies*, **326**, 22–29.
- Gardner AL (2007a) Cohort placentalia owen, 1837. Magnorder Xenarthra Cope, 1889. ______. *Mammals of South America, Marsupials, Xenarthrans, Shrews, and Bats*. Chicago: University of Chicago, **1**, 127–177.
- Gardner AL (2007b) Order Pilosa Flower, 1883. In: *Mammals of South America*. University of Chicago Press, Chicago.
- Gaudin TJ (1999) *The morphology of xenarthrous vertebrae (Mammalia: Xenarthra)*. Field Museum of Natural History.
- Gibb GC, Condamine FL, Kuch M et al. (2016) Shotgun Mitogenomics Provides a Reference Phylogenetic Framework and Timescale for Living Xenarthrans. *Molecular Biology and Evolution*, **33**, 621–642.
- Glass BP (1985) History of classification and nomenclature in Xenarthra (Edentata). *The evolution and ecology of armadillos, sloths, and vermilinguas*, 51–64.
- Gregory WK (1910) *The orders of mammals. order of the Trustees.* American Museum of Natural History.
- Hallström BM, Kullberg M, Nilsson MA, Janke A (2007) Phylogenomic data analyses provide evidence that Xenarthra and Afrotheria are sister groups. *Molecular biology and evolution*, **24**, 2059–2068.
- Hurst GD., Jiggins FM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1525– 1534.
- Kriegs JO, Churakov G, Kiefmann M et al. (2006) Retroposed Elements as Archives for the Evolutionary History of Placental Mammals (D Penny, Ed,). *PLoS Biology*, **4**, e91.
- Lara-Ruiz P, Chiarello AG, Santos FR (2008) Extreme population divergence and conservation implications for the rare endangered Atlantic Forest sloth, *Bradypus torquatus* (Pilosa: Bradypodidae). *Biological Conservation*, **141**, 1332–1342.
- Loughry WJ, Superina M, McDonough CM, Abba AM (2015) Research on armadillos: a review and prospectus. *Journal of Mammalogy*, **96**, 635–644.
- Madsen O, Scally M, Douady CJ et al. (2001) Parallel adaptive radiations in two major clades of placental mammals. *Nature*, **409**, 610–614.
- McDonough CM, Loughry WJ (2008) Behavioral ecology of armadillos. *The biology of the Xenarthra*, 281–293.
- McKenna MC, Bell SK (1997) *Classification of mammals: above the species level*. Columbia University Press.
- McNab BK (1980) Energetics and the Limits to a Temperate Distribution in Armadillos. *Journal of Mammalogy*, **61**, 606–627.
- Moraes N, Morgante JS, Miyaki CY (2002) Genetic diversity in different populations of sloths assessed by DNA fingerprinting. *Brazilian Journal of Biology*, **62**, 503–508.
- Moraes-Barros N, Arteaga MC (2015) Genetic diversity in Xenarthra and its relevance to patterns of neotropical biodiversity. *Journal of Mammalogy*, **96**, 690–702.
- Moraes-Barros N, Silva JAB, Miyaki CY, Morgante JS (2006) Comparative Phylogeography of the Atlantic Forest Endemic Sloth (*Bradypus torquatus*) and the Widespread Three-toed Sloth (*Bradypus variegatus*) (Bradypodidae, Xenarthra). *Genetica*, **126**, 189–198.
- Murphy WJ, Eizirik E, Johnson WE et al. (2001a) Molecular phylogenetics and the origins of placental mammals. *Nature*, **409**, 614–618.
- Murphy WJ, Pringle TH, Crider TA, Springer MS, Miller W (2007) Using genomic data to unravel the root of the placental mammal phylogeny. *Genome Research*, **17**, 413–421.
- Murphy WJ, Stanyon R, O'Brien SJ (2001b) Evolution of mammalian genome organization inferred from comparative gene mapping. *Genome biology*, **2**, 1.
- Nikolaev S, Montoya-Burgos JI, Margulies EH et al. (2007) Early History of Mammals Is Elucidated with the ENCODE Multiple Species Sequencing Data. *PLoS Genetics*, **3**, e2.
- Nishihara H, Maruyama S, Okada N (2009) Retroposon analysis and recent geological data suggest near-simultaneous divergence of the three superorders of mammals. *Proceedings of the National Academy of Sciences*, **106**, 5235–5240.
- O'Leary MA, Bloch JI, Flynn JJ et al. (2013) The Placental Mammal Ancestor and the Post-K-Pg Radiation of Placentals. *Science*, **339**, 662–667.
- Patterson B, Pascual R (1968) The fossil mammal fauna of South America. *Quarterly Review of Biology*, 409–451.
- Rodrigues FHG, Medri IM, De Miranda GHB et al. (2008) Anteater behavior and ecology. *The biology of the Xenarthra*, 257–268.
- Scally M, Madsen O, Douady CJ et al. (2001) Molecular evidence for the major clades of placental mammals. *Journal of Mammalian Evolution*, **8**, 239–277.
- Shoshani J, McKenna MC (1998) Higher taxonomic relationships among extant mammals based on morphology, with selected comparisons of results from molecular data. *Molecular phylogenetics and evolution*, **9**, 572–584.
- Simpson GG (1945) The principles of classification and a classification of mammals. *Bull. Amer. Museum Nat. History*., **85**.

Simpson GG (1980) Splendid isolation: the curious history of South American mammals.

- Springer M (2004) Molecules consolidate the placental mammal tree. *Trends in Ecology & Evolution*, **19**, 430–438.
- Superina M, Pagnutti N, Abba AM (2014) What do we know about armadillos? An analysis of four centuries of knowledge about a group of South American mammals, with emphasis on their conservation: Four centuries of knowledge about armadillos. *Mammal Review*, **44**, 69–80.
- Van Dijk MA, Paradis E, Catzeflis F, De Jong WW (1999) The virtues of gaps: Xenarthran (Edentate) monophyly supported by a unique deletion in αA-crystallin. *Systematic biology*, **48**, 94–106.
- Waters PD, Dobigny G, Waddell PJ, Robinson TJ (2007) Evolutionary History of LINE-1 in the Major Clades of Placental Mammals (J Fraser, Ed,). *PLoS ONE*, **2**, e158.
- Wetzel RM (1980) *Revision of the naked-tailed armadillos, genus Cabassous McMurtrie*. Carnegie Museum of Natural History.
- Wetzel RM (1982) Systematics, distribution, ecology, and conservation of South American Edentates. In: *Mammalian biology in South America, special publication series, Pymatuning laboratory of ecology*., pp. 345–375. M. A. Mares & H. H. Genoways, University of Pitsburgh, Linesville.
- Wetzel RM (1985) The identification and distribution of recent Xenarthra (= Edentata). *The evolution and ecology of armadillos, sloths, and vermilinguas*, 5–21.
- Wetzel RM, Gardner AL, Redford KH, Eisenberg JF (2007) Order Cingulata Illiger, 1811. In: *Mammals of South America*. University of Chicago Press, Chicago.

Capítulo 1

The Tolypeutinae subfamily and a molecular insight about its phylogeny.

Marco Antônio Alves Schetino, Teresa Cristina da Silveira Anacleto, and Fabrício Rodrigues Santos*

Universidade Federal de Minas Gerais, Avenida Antonio Carlos 6627, Belo Horizonte, Minas Gerais, Brazil (MAAS, FRS)

Universidade do Estado de Mato Grosso, Br 158, Km 655, Nova Xavantina, Mato Grosso, Brazil (TCSA)

Abstract

The Tolypeutinae subfamily Gray, 1865, is one of the four subfamilies of Dasypodidae (Xenarthra: Cingulata), and comprises two tribes Tolypeutini and Priodontini with four genera. This article aims to provide a brief description about this subfamily and provide an updated view of its systematics. Information was gathered about morphological, molecular traits, geographical distribution, systematics, taxonomy, phylogeny, fossils and molecular dating estimates of *Tolypeutes tricinctus*, *T. matacus, Priodontes maximus, Cabassous tatouay*, *C. unicinctus, C. centralis* and *C. chacoensis*. We performed a phylogenetic analysis with 7 nuclear and 2 mitochondrial genes using all data available for this subfamily. The resulting topology shows relationships between the genera that corroborates those of recent molecular works, and we point out the main climatic and geological events that occurred according to the divergence times among Tolypeutinae lineages, and propose a new topology for the genus *Cabassous*.

Running title: Filogeny of the Tolypeutinae subfamily

Key words: armadillo, Tolypeutinae, morphological traits, molecular traits, geographical distribution, systematics, molecular dating, biogeography.

Introduction

Tolypeutinae (Gray 1865) is one of the four subfamilies of Dasypodidae (Xenarthra: Cingulata), with species extending from southern Mexico to Argentina. This subfamily comprises three extant genera: *Tolypeutes* (Illiger 1811), which belongs to the Tolypeutini tribe; and *Cabassous* (McMurtrie 1831) and *Priodontes* (Cuvier 1825), which belong to the Priodontini tribe (McKenna & Bell 1997; Gardner 2005; Wetzel *et al.* 2007). According to Billet *et al.* (2011), considering also the extinct Tolypeutinae genus *Kuntinaru*, two apomorphies can be used to identiify this subfamily: the vertical anterior edge of the orbit and a surface anterior to the postglenoid foramen elongated anteroposteriorly, which is not delimited posteriorly by a continuous line with the lateral edge of the zygomatic arch. In addition, the monophyly of the group is corroborated by several molecular analyzes (Delsuc *et al.* 2002, 2003, 2012; Moller-Krull *et al.* 2007; Gibb *et al.* 2016).

There are few scientific publications related to Tolypeutinae and a relatively low number of works directed to the taxonomy and systematics of this subfamily (Superina *et al.* 2014). For example, the taxonomy of the genus *Cabassous* was revised more than 3 decades ago (Wetzel 1980), an analysis that at the time was hampered by the scarcity of specimens. The current taxonomy of *Tolypeutes* and *Priodontes* genera appears to be no better than *Cabassous* (Superina *et al.* 2014). Although there is an increasing number of publications about the molecular systematics of xenarthrans, the best phylogeny until now is based only in mitochondrial DNA and no phylogeographic studies have been published about any Tolypeutinae species so far (Delsuc *et al.* 2002, 2003, 2012, 2016; Gibb *et al.* 2016). This lack of knowledge considerably interferes with the conservation status of their species. For example, according to the International Union for Conservation of Nature and Natural Resources – IUCN (IUCN 2016), *Cabassous centralis, C. tatouay* and *Tolypeutes matacus* are categorized as data deficient (DD). *Tolypeutes tricinctus* is considered endangered (EN), *Priodontes maximus* is vulnerable (VU), *C. chacoensis* is near threatened (NT) an *C. unicinctus* is least concern (LC). However, the threat level may be seriously underestimated and requires attention.

In order to evaluate the knowledge gaps in evolution, taxonomic and systematics within this subfamily of the order Cingulata, this article aims to make a brief historical survey of what is known so far about Tolypeutinae species traits and your systematics and constructed a dated molecular phylogeny with nuclear and mitochondrial genes of species of the *Cabassous*, *Priodontes* and *Tolypeutes* to elucidate the systematics relationship among Tolypeutinae taxa.

Material and Methods

Tolypeutinae bibliographic survey.

Our survey is based in online scientific databases using the combination of keywords like 'armadillo', 'Cingulata', 'Tolypeutinae', 'systematics', 'taxonomy', and the names of all species belonging to the Tolypeutinae subfamily. We also retrieved citations in the armadillo bibliography database maintained by Mariella Superina [\(http://www.xenarthrans.org/bibliography/armadillo\)](http://www.xenarthrans.org/bibliography/armadillo)) and personal bibliographic lists of references obtained from armadillo researchers. Later, we selected the works that covered the objectives of our review divided into four topics: Morphological and molecular traits; Geographical distribution; Systematics and taxonomy of Tolypeutinae and; Fossils and molecular dating estimates. The content of the last two topics are presented chronologically in a way that demonstrates the major changes over time and the divergent and complementary results between morphological and molecular data.

Molecular analysis

Sampling, DNA extraction, PCR and sequencing: The total DNA of liver, muscle or blood samples of 15 individuals of the genus *Cabassous*, *Priodontes* and *Tolypeutes* were extracted using standard phenol-chloroform protocol (Sambrook & Russel 2001). A list of sampled species is available in Table 1. Fragments of two mitochondrial genes (mtDNA) and 7 nuclear genes (nuDNA) were amplified with Platinum *Taq* DNA Polymerase kit (Thermo Fisher Scientific) using specific primers and annealing temperatures for each fragment (Table 2) following the touchdown PCR techniques (Korbie & Mattick 2008). The primers of gene ADRB2 and vWF, ADRB2F = $5'$ -ATCGTCCTGGCCATCGTGTT-3' and ADRB2R = 5'-CTCCTGGAAGGCAATCCTGA,- 3', and vWF1F = 5'-TGTCAACCTCACCTGTGAAGCCTG-3', vWF1R = 5'- TGCAGGACCAGGTCAGGAGCCTCTC-3', $VWF2F = 5'$ -TCAAGCAGATCCGCTTCATCGAG-3' and vWF2R = 5'- GTGGAGAAGCTGTGCTCCGAGAC-3' were designed by our laboratory. PCR efficiency was confirmed by electrophoresis on 1% agarose gel and purified with 20% polyethylene glycol in 2.5 M NaCl (Santos Júnior *et al.* 2015). The sequencing was carried out according to the chain termination method with the BigDye Terminator Cycle Sequencing kit in a 3130xl ABI Genetic Analyzer system (Thermo Fisher Scientific). The chromatograms were analyzed in the SeqScape 2.6 software (Applied Biosystems, Foster City, CA) and the consensus sequences were aligned using the Clustal W algorithm implemented in the MEGA 7 software (Thompson *et al.* 1994; Kumar *et al.* 2016). The identification of the gametic phases for subjects with multiple heterozygous sites was performed with the PHASE 2.0 program (Stephens *et al.* 2001; Stephens & Donnelly 2003) included in the software DNAsp 5.1 (Librado & Rozas 2009).

Phylogenetic analysis and divergence time estimates: The analysis was performed using the sequences obtained here and others of the genera *Priodontes, Tolypeutes* and *Euphractus* (external group) retrieved from Genbank (Table S1, Supporting information). The phylogenetic reconstruction was performed in the *BEAST 2.3 program (Bouckaert *et al.* 2014) using the reversible-jump based substitution model implemented in this software through the RBS 1.3.1 package (Bouckaert *et al.* 2013), allowing gamma rate heterogeneity and invariant sites. We chose a birth-death tree prior combined with soft fossil calibration constraints (Yang 2005) and the *Kuntinaru* fossil calibration interval (maximum age 37.8 MYA, minimum age 23 MYA) for Tolypeutinae diversification (Billet *et al.* 2011; Gibb *et al.* 2016). Three independent MCMC chains of 100,000,000 generations were carried out on CIPRES Science Gateway v.3.3 (Miller *et al.* 2010) and sampled every 5,000 generations. The effective sample sizes (ESS) for each run was checked in Tracer v. 1.6 (Rambaut *et al.* 2014). The tree files were combined in LogCombiner and summarized in TreeAnnotator, both available in the BEAST 2.3 package, to obtain the best 10,000 trees.

Table 1: Sample details of individuals of Tolypeutinae used in this work. LBEM = Laboratório de Biodiversidade e Evolução Molecular; UFMG = Universidade Federal de Minas Gerais.

Table 2: Primers used for partial amplification of Tolypeutinae genes. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular.

Results

Subfamily Tolypeutinae.

Morphological and molecular traits

Among Tolypeutinae, the genera *Cabassous* and *Priodontes* are apparently the most similar, sharing morphological (Wetzel 1982; Engelmann 1985; Wetzel 1985a), ecological, behavioral (Wetzel 1982, 1985b; Meritt 2006), and spermatozoa similarities (Cetica *et al.* 1998). *Tolypeutes* presents distinctive external characteristics and genitalia in comparison to *Cabassous* and *Priodontes* (Cetica *et al.* 2005), beyond the anatomical peculiarity consisting in the ability to completely roll its carapace into a ball (Wetzel 1985a), which is reflected in their common name in many languages like "tatu-bola" in Brazil (Superina & Aguiar 2006). Some basic morphological measures of the Tolypeutinae species are shown in table 3.

Cabassous and *Priodontes* present the snout blunt and rounded, relatively large eyes, and thick and fleshy ears. Their carapaces are ovoid, dome-shaped and separated from the head shield by three short rows of nuchal scutes. These species have between 11 and 14 movable bands separating the pelvic and scapular shields. In addition, their forefeet have five toes, and the third digit bears a long and sickle-shaped claw that facilitates digging (Wetzel 1985a; Wetzel *et al.* 2007). These genera present very similar sperm shapes, the heads are very large, with a extremely thin profile and a central concavity, giving them a shovel-like shape (Cetica & Merani 2008).

Cabassous currently of four species: *C. centralis* (Miller 1899), *C. chacoensis* (Wetzel 1980), *C. tatouay* (Desmarest 1804), and *C. unicinctus* (Linnaeus 1758). Members of this genus have a very flexible carapace with a variable number of movable bands (usually eleven) and a rounded tail which can be unarmored or partially armored with scattered, small scutes and scales (Wetzel 1980, 1985a; b). This naked tail distinguishes this genus from all other armadillos, which also recalls their common name in Brazil as "tatus-do-rabo-mole", or soft-tail armadillos (Superina & Aguiar 2006). *Cabassous centralis* is morphologically similar to *C. unicinctus,* but it is smaller and have the external surface of pinnae without scales (Wetzel 1980; Hayssen *et al.* 2013; Hayssen 2014a). *C. chacoensis* is the smallest less known species of this genus and is best identified by its well-separated smaller pinnae which has a fleshy expansion in its anterior margin (Wetzel 1980; Smith 2008; Hayssen 2014b). *Cabassous unicinctus* has two recognized subspecies: *Cabassous unicinctus unicinctus* and *Cabassous unicinctus squamicaudis*. This latter is proportionally smaller and have a greater number of scutes on the cephalic shield, wider teeth and shorter rostrum (Wetzel 1980; Hayssen 2014a). *Cabassous tatouay* is the largest of *Cabassous* and has the palate and rostrum proportionally more elongate than other species of this genus (Wetzel *et al.* 2007; Hayssen 2014c).

Table 3: Approximated body mass, head-body length and tail length of the species of Tolypeutinae (Wetzel 1980; 1985a,1985b; Hayssen et al. 2013; Hayssen 2014a, 2014b, 2014c; Superina et al. 2014; Carter et al. 2016)

Priodontes maximus (Kerr 1792) is the largest species of living armadillos. While the shells of other armadillos seem to envelop their sides and flanks, the *Priodontes* carapace extends only halfway down their sides. Its forefeet bear well-developed claws, which can reach up to 200 mm long at the middle toe. This species has about 100 teeth, the largest number among armadillos (Nowak 1999; Wetzel *et al.* 2007; Carter *et al.* 2016).

Tolypeutes comprises two species: *T. tricinctus* (Linnaeus 1758) and *T. matacus* (Desmarest 1804). These armadillos are the most distinctive because they can roll their carapaces into a ball. They have a rigid shield, which is relatively thicker than any other armadillo (Wetzel 1982), with three to four movable bands (Nowak 1999). The forefeet have four toes in *T. matacus*, and five toes in *T. tricinctus*, and the hindfeet have five toes, with the second, third and fourth digits almost unified, flattened and hoof-like, while the first and fifth digits present "normal" claws (Wetzel 1985a; Smith 2007; Wetzel *et al.* 2007). They barely dig their own burrows, but their main strategy of escape is to curl up into a ball (Attias *et al.* 2016). Other peculiarities concern the genital traits of males and females. The *Tolypeutes*' penis is extremely long, with 100-150 mm (Herrick *et al.* 2002) with a particular extension that provides an outstanding asymmetry (Cetica & Merani 2008). The females present a true vagina, the cervix ends abruptly in the dorsal middle region of a slightly arched tubular structure lined by a non-keratinized stratified squamous epithelium. In other armadillos, the cervix leads to a tubular structure where the columnar epithelium changes abruptly to a transitional epithelium, forming a urogenital sinus rather than a true vagina (Cetica *et al.* 2005).

Few cytogenetic studies were done with different Tolypeutinae species (Table 4). *Tolypeutes matacus* presents the lowest diploid number (2n=38) of all Xenarthra (Jorge *et al.* 1977), while the largest diploid number in Tolypeutinae belongs to the species *C. centralis*, with 2n=62 (Benirschke *et al.* 1969). Banding techniques were performed only for three species: *T. matacus*, *C. chacoensis* and *C. unicinctus* (Benirschke 2006; Jacintho *et al.* 2009; Luaces *et al.* 2010). So far there is no karyotype information available only for *T. tricinctus*.

The first molecular studies using Tolypeutinae species was performed by Sarich (1985) with the use of immunoproteins for the inference of phylogenetic relationships among Xenarthra. Posteriorly, Van Dijk *et al.* (1999) used short DNA sequences of the αA-Crystallin gene of many species, and identified a three amino acids motif that was only found in Xenarthra. The mitochondrial genes 12S, 16S, and ND1, and nuclear sequences of transposable elements and their flanking regions, and the three genes vWF, BRCA1 and ADRA2B were used to elucidate the position of Xenarthra within the Eutheria clade, to characterize the monophyly of the Cingulata and Pilosa orders and, also to infer the relationships between families, subfamilies, and genera (Delsuc *et al.* 2001, 2002, 2003, 2012; Moller-Krull *et al.* 2007). The first well-resolved phylogeny of Tolypeutinae genera using the total mitogenome was recently presented by Gibb *et al.* (2016).

Geographical distribution

The Tolypeutinae are predominantly found in South America, with exception of *Cabassous centralis*, whose distribution ranges from Mexico, towards Central America and northwestern South America, including western Colombia, northwestern Ecuador, and northwestern Venezuela (Fig. 1) (Abba & Superina 2011; Hayssen *et al.* 2013).

Cabassous unicinctus occurs from Venezuela and Guyanas (French Guiana, Guyana, and Suriname) to southern Brazil, Bolivia, and Paraguay (Fonseca & Aguiar 2004; Wetzel *et al.* 2007; Smith *et al.* 2011; Hayssen 2014a). The *C. u. unicinctus* subspecies is distributed in northern South America, in the Amazon and Cerrado biomes, and *C. u. squamicaudis* is found in the Cerrado and Atlantic Forest biomes (Anacleto *et al.* 2013). *Cabassous tatouay* is found in Uruguay, Argentina, Paraguay and Brazil (Wetzel *et al.* 2007; Feijó & Langguth 2013; Hayssen 2014c). *Cabassous chacoensis* occupies a narrow range in the Chaco of western Paraguay and northern Argentina, but no animals were reported in Bolivia (Abba & Superina 2011). The possible occurrence in southwestern Brazil is doubtful, as the only report was based on a skull whose label indicates "Brazil" (Wetzel 1980), which was identified as *C. unicinctus* by Abba & Vizcaíno (2008).

Priodontes maximus occurs in most countries of South America, except Chile and Uruguay (Fallabrino & Castiñeira 2006; Wetzel *et al.* 2007), but it is not truly abundant in any region. In Brazil, it has a wide distribution, except in the Northeast region (Anacleto & Marinho-Filho 2001; Srbek-Araujo *et al.* 2009) and in the South region, where the only record from Passo Fundo city, Rio Grande do Sul state (AMNH 1308), is a mistake (Anacleto 2013).

Tolypeutes matacus occurs in the southwest of South America, including areas of Bolivia, Paraguay, Argentina and Brazil (Wetzel 1985b). In Argentina, south of Buenos Aires city, recent surveys suggest that it is locally extinct (Abba & Vizcaíno 2008), and in Brazil, it is threatened in the Upper Paraguay River Basin (Coutinho *et al.* 1997). *Tolypeutes tricinctus* is endemic to Brazil, mostly associated with the Caatinga biome in the Northeast (Feijó *et al.* 2015). Since the first record for the Brazilian Cerrado (Marinho-Filho *et al.* 1997), other findings confirm that the species occurs in the region of Posse/Jaborandi/Correntina, on the border of Goiás, Bahia and Minas Gerais states, and further north in the Cerrado regions of Piauí and Tocantins states (Reis 2002).

Figure 1: Map of geographic distribuition of Tolypeutinae. Species distribuition based in IUCN (2016). Legends: A-B = *Cabassous*; C = *Tolypeutes;* D = *Priodontes*.

Systematics of Tolypeutinae

Since the 19th century there were different attempts to generate a phylogenetic consensus (Fig. 2). In 1865, John Edward Gray added *Prionodos* Gray, 1865 (*Priodontes*) and *Xenurus* Lund, 1843 (*Cabassous*) genera to the Dasypodina tribe, and *Tolypeutes* in the Tolypeutina tribe. Subsequently, in 1873, the same author grouped *Prionodos* and *Xenurus* in the Priodontina tribe, and *Tolypeutes* became part of a family called Tolypeutidae.

Figure 2: Phylogenetic relationships between Tolypeutinae's genera. The actual tree (left) was taken from the last classification suggested for this subfamily (Gibb *et al.* 2016). The lines illustrate previous classifications of the authors represented above. *a =* Family Tolypeutidae; *b =* Family Dasypodidae; *c* = Tribe Tolypeutini; *d* = Tribe Priodontini; *e* = Subfamily Priodontinae; *f* = Subfamily Tolypeutinae.

Max Weber (1928) defined the current nomenclature of the tribes: Tolypeutini and Priodontini. Later, Patterson & Pascual (1968) recognized these two tribes in a subfamily Priodontinae.

Engelmann (1985) analyzed morphological characters and proposed a dichotomy between *Tolypeutes* and Dasypodinae, within the Dasypodidae. The Dasypodinae included Priodontini (with *Cabassous* and *Priodontes* genera) and Dasypodini (modern genus *Dasypus*) as sister groups, which shared the occurrence of an optic foramen virtually inside the sphenorbital fissure, and the placement position of the infraorbital canal below the zygomatic process of the maxilla.

An important fact that groups the three living Tolypeutinae genera was demonstrated in a publication of Patterson *et al.* (1989). Differing from Engelman (1985), this author analyzed the degree of ossification of the entotympanic bone and concluded that *Tolypeutes* is intermediate between those of *Cabassous* and *Priodontes*. He also recorded transitional shapes in the degree of enclosure of the venous canal between squamosal and *processus cristae facialis*, where *Priodontes*, *Cabassous* and *Tolypeutes* exhibit this structure ranging from an open gap seen in the former genus to a closed canal in the latter genus. In most of other structures, *Tolypeutes* was found to be similar to *Cabassous*.

Finally, a classic taxonomic review by McKenna & Bell (1997) included both fossils and extant species, and grouped *Tolypeutes, Cabassous* and *Priodontes* in the subfamily Tolypeutinae, instead of Priodontinae as suggested by Pattersson & Pascual (1968). This classification is maintained today by many authors (Wetzel *et al.* 2007; Delsuc *et al.* 2008; Billet *et al.* 2011).

Cetica *et al.* (1998), in a study of spermatozoa morphology, grouped *Priodontes* and *Cabassous* due to their close similarities in shape and size. Because the spermatozoa of *Tolypeutes* slightly differ from the others in shape and, this would suggest, according to the authors, an older divergence from the other two genera of the Tolypeutinae subfamily. However, molecular studies (see below) suggests it to be an autapomorphic change (as many other particularities of *Tolypeutes*) that may be not informative to suggest any particular branching in the tree.

Delsuc *et al.* (2001), using three genes, two mitochondrial and one nuclear, suggested the Priodontini tribe (only *Cabassous* was used) was a sister-group of Euphractini (*Chaetophractus*), instead of Dasypodini (*Dasypus*), contradicting Engelman (1985). However, their data did not show high confidence values to corroborate their phylogenetic conclusions.

In 2002, Delsuc *et al.* presented another phylogenetic tree built with three nuclear genes and using more taxa than their previous work in 2001. Their results identified, with high confidence degree, the monophyly of the subfamilies Dasypodinae, Euphractinae and Tolypeutinae, besides putting the latter two as sister groups, and the first as the most basal, contradicting the morphological analysis of Engelman (1985), but agreeing with the spermatozoa analysis (Cetica et al., 1998). Within the clade Tolypeutinae, *Cabassous* and *Tolypeutes* genera formed a sister-group, and *Priodontes* was basal, contrary to studies that put *Tolypeutes* as basal in the subfamily, where *Cabassous* and *Priodontes* would belong to the Priodontini tribe (Wetzel 1985b; McKenna & Bell 1997; Cetica et al. 1998). This discrepancy, according to Delsuc et al. (2002), could be due to the distinctive anatomical features of the genus *Tolypeutes*, making it difficult to identify postcranial morphological traits. Anyway, the relationship among these three genera was unclear because there was a low support for the basal separation of *Priodontes* (Delsuc et al. 2002).

In 2003, Delsuc *et al.* attempted to resolve the inconsistencies in the Tolypeutinae subfamily through the analysis of three mitochondrial and two nuclear genes. The results obtained for each gene or using a concatenation (a combination of all genes) produced different trees for the genera of this subfamily. The concatenation of all the genes produced a tree where *Cabassous* and *Priodontes* were sister-taxa, but with low confidence support.

Gaudin & Wible (2006), using morphological data of skull and teeth, argued against all molecular data produced until then (Delsuc *et al.* 2001, 2002, 2003). In the analyses with extinct and extant individuals, *Priodontes* appeared as sister-group of *Cabassous* among all other Cingulata genera (including *Tolypeutes*). Further, when the analysis was done only with extant individuals, two maximum parsimony trees were generated, and the first formed a monophyletic clade of *Priodontes* with *Cabassous*, and *Tolypeutes* was a sister-group of Euphractinae. In the second tree, *Priodontes + Dasypus* and *Cabassous* were grouped, and *Tolypeutes* was the most basal.

In another analysis with skull, teeth, skeletal, and carapace characters of 28 fossil and extant species of Dasypodidae (Abrantes & Bergqvist 2006), the phylogeny of *Cabassous* and *Priodontes* as a sister-group of *Tolypeutes* was maintained. Moreover, the *Pampatherium* genus was added within the Dasypodidae as the most basal taxon of the Tolypeutinae subfamily. There was no subsequent study that kept this extinct genus within Tolypeutinae, although Wolf *et al.* (2012) have observed a similarity in the osteoderm histological organization of the pampatheres, *Tolypeutes* and the basal glyptodont cingulates.

In a molecular analysis of transposable elements and their flanking regions, Möller-Krull *et al.* (2007) observed, with a higher degree of confidence, the same results as previous molecular phylogenetic studies (Delsuc *et al.* 2002, 2003), where *Cabassous* and *Tolypeutes* formed a sister-group, arguing against the morphological data that suggested *Priodontes* and *Cabassous* were closely related (Gaudin & Wible 2006; Abrantes & Bergqvinst 2006).

Galliari *et al.* (2010) analyzed the vertebrae of Tolypeutinae taxa and showed results that were more congruent with Gaudin and Wible (2006) than with Delsuc *et al.* (2002, 2003). The genus *Tolypeutes* presents a combination of character states found in both Tolypeutinae and Euphractinae subfamilies. Furthermore, this genus is the only one that presents a fusion of the seventh cervical vertebra with the first thoracic one in Dasypodidae.

Billet *et al.* (2011) added 13 characters and a new fossil taxon in the data from Gauldin & Wible (2006) to build a tree where the extinct and recently discovered genus *Kuntinaru* was added to Tolypeutinae. This study also corroborated the monophyly of the subfamily and grouped *Cabassous* and *Priodontes*.

With the inclusion of two living genera, *Chlamyphorus* Harlan, 1825 and *Calyptophractus* Fitzinger, 1871, which were not previously used in phylogenetic studies of the Cingulata, Delsuc *et al.* (2012) added a new subfamily Chlamyphorinae in Dasypodidae. This altered the phylogenetic relationships among the subfamilies because this new clade became the sister-group of Tolypeutinae, and another subfamily, Euphractinae, appeared as the most basal of these three subfamilies (Fig. 2). Subsequently, the relationship between Chlamyphorinae and Tolypeutinae is corroborated by Billet *et al.* (2015) by identifying a synapomorphy represented by a large lateral semicircular canal of the inner ear between these two subfamilies.

With the advent of new sequencing techniques, the complete mitochondrial genomes of all living and some extinct armadillos were obtained, which allowed the construction of trees with higher confidence values than the previous ones. Gibb *et al*. (2016) places *Priodontes* as the most basal of Tolypeutinae, maintaining the proposal of Delsuc *et al.* (2012). In addition, this article proposes that the subfamily Chlamyphorinae should be elevated to the family Chlamyphoridae including the Chlamyphorinae, Tolypeutinae and Euphractinae. This assumption is based in the old divergence between the Dasypodinae and the other subfamilies.

Fossils and molecular dating estimates

The oldest well-preserved cranial structure of the Cingulata (and Xenarthra as a whole) is the Tolypeutinae species *Kuntinaru boliviensis*, which is a fossil found in the Salla Beds of Bolivia with occurrence estimated at 23 million years ago (mya) in the late Oligocene (Billet *et al.* 2011). Fossil records for *Tolypeutes* and *Cabassous* are estimated respectively to Pliocene and Middle Pleistocene (Paula-Couto 1979; McKenna & Bell 1997), but no fossil data is available for the *Priodontes* genus.

The first attempt to estimate molecular divergence times between armadillos was done by Sarich (1985). In this work the author estimated the divergence time between *Cabassous* and *Dasypus* at around 40 mya. In 2001, Delsuc *et al.* used mitochondrial and nuclear data to estimate the divergence between *Cabassous* and *Chaetophractus* at about 21 mya.

With the use of a relaxed molecular clock in a Bayesian inference, Delsuc *et al.* (2004) estimated the time for the separation event between subfamilies Euphractinae and Tolypeutinae at about 33 mya (42-25), and also estimated the diversification among genera of Tolypeutinae in a short period between 20 to 22 mya. This time appears in synchrony with other diversification events within Xenarthra, like the lineage split between the sloths genera *Bradypus* and *Choloepus*, which occurred between 21 and 22 mya in the early Miocene (Delsuc *et al.* 2004). Posteriorly, Delsuc *et al.* (2012) estimated the divergence times between Tolypeutinae and Chlamyphorinae at around 32 mya (35-29), and recalibrated the molecular clock for the divergence of Tolypeutinae + Chlamyphorinae and Euphractinae to about 33 mya (37-29). More recently, Gibb *et al.* (2016) found values of divergence between clades close to those found in previous studies, where Euphractinae diverged from Tolypeutinae and Chlamyphorinae at about 37 mya, and the latter two diverged at 33 mya.

The estimated divergence times between these three subfamilies match the transition of the Eocene to Oligocene, when the temperature fell dramatically, and there was a change from a tropical-temperate environment to a cold and dry habitat dominated by grasslands and savannas (Pascual & Ortiz-Jaureguizar 1990; Zachos 2001; Delsuc *et al.* 2004).

Additionally, one of the factors that may have influenced the beginning of Tolypeutinae divergence may be the climate changes in the Late Oligocene or middle of Early Miocene (Delsuc *et al.* 2004, 2012), which are related to the first major "Bolivian crisis" when the Andes became the main relief of the west coast of South America, dramatically changing both temperature and rainfall regimes of the continent (Marshall & Sempere 1993; Zachos 2001; Delsuc *et al.* 2004; Ortiz-Jaureguizar & Cladera 2006).

Tolypeutinae Phylogeny

A total of 6952 base pairs (bp) of 9 genes of 15 Tolypeutinae samples (Table 2) was obtained in this study. The phylogenetic relationships between genera were similar to those found in the most recent xenarthran studies (Delsuc *et al.* 2012; Gibb *et al.* 2016). Additionally, within the *Cabassous* genus, two main clades were found, one containing the taxa *C. tatouay*, *C. chacoensis* and *C. u. squamicaudis* and another with the taxa *C. u. unicinctus* and *C. centralis* (Fig. 3).

The periods of divergence between clades are shown in Table 5, where the first split of this subfamily is dated in the Late Oligocene (*Priodontes* and others) and, later, in the Miocene, occurs the divergence between *Cabassous* and *Tolypeutes* genera, and between *T. matacus* and *T. tricinctus* species. The divergence between the *Cabassous* species dates from the Miocene to the Pliocene, and to the Middle Pleistocene.

Figure 3: Molecular time scale for Tolypeutinae nodes inferred with a Bayesian phylogeny obtained in BEAST. Node ages were obtained under the reversible-jump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a birth-death tree prior with soft fossil calibrations. Node bars indicate the 95% HPD intervals for age estimates in million years ago (MYA). All nodes not shown were supported by maximum posterior probabilities.

Table 5: Divergence time estimates with 95% confidence intervals for all nodes.

Discussion

Even though genera belonging to the Tolypeutinae subfamily are not much differentiated in morphology from other armadillos, they are quite discrepant among them, varying mainly in size and carapace forms. They are derived from deeply divergent evolutionary lineages, which due to the absence of fossils of this subfamily, few evidence is available to understand these morphological changes. Delsuc *et al.* (2016) precisely presented molecular evidence that puts the subfamily Glyptodontinae as sister group of Tolypeutinae and Chlamyphorinae, within the new family Chlamyphoridae. Still, according to these authors, the common ancestor of these three subfamilies should be a taxon of about 6 kg, which indicates that the extinct Glyptodonts underwent a significant increase of body mass during the Neogene. This increase in body mass seems to be related to the appearance of ecomorphological characteristics in Glyptodonts, such as teeth resistant to abrasive particles in their environment, which favored the better exploitation of their food (Vizcaíno 2009; Vizcaíno *et al.* 2011, 2012). Perhaps, similar to the Glyptodonts, there was an ancestral of Tolypeutinae with similar adaptive structures, which favored the emergence of lineages with large body mass within this subfamily, represented by the genus *Priodontes*.

Despite the increasing number of publications involving xenarthrans, this is still not enough to elucidate the real geographic distribution of their taxa (Superina et al., 2014). In the case of Tolypeutinae, there are several factors that hinder the accuracy of these estimates. Many museum records have erroneous identification of the specific epithet (T. Anacleto; A. Feijó, personal communication) as well as doubts about the merits of holotypes (Wetzel 1980), and the originality of lectotypes (Wetzel *et al.* 2007). *Priodontes maximus* seems to cover the largest geographic area among the Tolypeutinae, but occurs in low densities and with an apparently discontinuous distribution, which makes it difficult to evaluate their conservation status (Meritt 2006; Carter *et al.* 2016). With respect to *Cabassous unicinctus*, few studies have plotted the distribution records of the two subspecies on maps, and their taxonomic status as belonging to a single species is uncertain (Wetzel 1980, 1982, 1985a,b; Anacleto *et al.* 2013). *Tolypeutes* has the most recent and complete study of geographic distribution within the Tolypeutinae (Feijó *et al.* 2015). With well-defined geographical areas and no overlaps, with the exception of the northeastern limits of the distribution of *T. matacus* (Anacleto *et al.* 2006), the two species (*T. matacus* and *T. tricinctus*) present different diets adapted to the characteristics of each biome. While *T. matacus* (Chaco) has a more widespread insectivorous diet (Bolkovi *et al.* 1995), *T. tricinctus* (Caatinga) seems to have a more specialized diet of termites and ants (Guimarães 1997).

Although there are recent advances in the systematics of Tolypeutinae, there are

still some inconsistencies in the phylogenetic relationships between their genera when compared to the more recent analyzes using molecular data (Delsuc *et al.* 2012, 2016; Gibb *et al.* 2016) and morphological data (Gaudin & Wible, 2006; Abrantes & Bergqvinst 2006; Galliari *et al.* 2010). This can be due to the differences in the unique morphological structures found in *Tolypeutes,* compared to the other genera, which makes it difficult to find characters that can define the relationships between them. Fortunately, molecular analyses with lots of genomic data, and also extinct individuals (Delsuc *et al.* 2016) are now being produced with more advanced sequencing techniques, which can be used to revise the actual phylogeny. Just as there is a new family proposal in which Tolypeutinae is present (Chlamyphoriadae, Gibbs *et al.* 2016), it is still necessary to review the Priodontini tribe that, according to the current data, is paraphyletic.

In our phylogenetic analysis with mtDNA and nuDNA, we obtained a tree that showed phylogenetic relationship between genera and divergence dates between clades similar to the most recent molecular analyzes (Delsuc *et al.* 2012, 2016, Gibbs *et al.* 2016). However, our tree shows an increased taxon resolution when comparing the topology and time estimates of divergences within the *Cabassous*. This can be explained because previous studies have not used individuals of *C. u. squamicaudis* in their analyzes which, consequently, altered the entire topology of the genus. This new tree groups the *Cabassous* species in clades with taxa sharing more similar external morphologies (Wetzel, 1980, 1985; Hayssen *et al.* 2013; Hayssen 2014a, b, c), dividing the two recognized *C. unicinctus* subspecies in each of two major clades. Indeed, previous studies have already addressed the possibility of *C. u. unicinctus* and *C. u. squamicaudis* to be different species (Wetzel, 1980; Anacleto *et al.* 2013). The tree presents a highly confident topology, where the only clade with low posterior value (0.85) was *C. chacoensis* + *C.u.squamicaudis* possibly because there were no nuclear DNA sequences available for *C. chacoensis* at Genbank. Furthermore, the phylogenetic analysis performed without *C chacoensis* does not change the general *Cabassous* topology (data not shown).

The time of divergence between the genera of Tolypeutinae is within the Patagonian Faunistic cycle, which is marked by the adaptive radiation of a large number of xenarthrans (Pascual and Ortiz Jareziguar, 1990; Ortiz-cladera 2006). It coincides with the transition between Oligocene and Miocene, in which the Andes consolidated as the main South American relief and caused a great impact on the continental climate (Delsuc et al., 2004; Sepulcher et al., 2010). In addition, the Mi1 glaciation (circa 24 Mya) contributed to the increase of aridity in the southern part of the continent and to the progressive increase of woodland savannas, possibly favoring the dispersion of these species to other regions of the continent (Zachos, 2001; Ortiz-Cladera, 2006).

In the Middle Miocene, there was the divergence of the lineages that originated the current species of *Tolypeutes*. In this period, there was an Atlantic marine transgression forming the "Paranean Sea" (Pascual, 1996; Donato et al., 2003; Ortiz-Cladera, 2006), which probably was the main vicariant event that separated to the west the ancestral population that originated *T. matacus*, and to the east the population that originated *T. tricinctus*, corroborating the hypothesis previously raised by Feijó *et al.* (2015).

At the transition between the Miocene and the Pliocene, the northern (*C. centralis* and C*. u. unicinctus*) and southern (*C. tatouay, C. chacoensis* and *C. u. squamicaudis*) *Cabassous* lineages of the continent diverged. During this period, in the north of South America, the Andes continued to uplift, the Amazon River became one of the main barriers isolating the northern part of the continent, and neotectonic processes promoted the creation of geological arches, reshaping the landscape of the Amazonian relief and hydrography (Hoorn et al., 1995, 2010; Costa et al., 2001, Toivonen et al.,2007). In the south of the continent, the expansion of grassland areas reached their apogee and new habitats arose as a result of the retreat of the Atlantic marine transgressions. These open areas expanded also with climate changes caused by the increase towards the west of the Antarctic cap, and also as a consequence of the Quechua phase initiated in the Miocene (Pascual & Ortiz, 1999; Zachos, 2001; Ortiz & Cladera, 2006; Pascual, 2006).

According to our results, the lineages that originated the living species of the genus *Cabassous* diversified in the Pleistocene, a period of several glaciations where the cyclical advance and regression of the glaciers, and corresponding expansion and contraction of the arid and humid biomes of the continent strongly influenced the local biota (Marshall & Cifelli, 1990; Hewitt, 2000; Zachos, 2001). In addition, the continent still suffered a great ecological impact resulting from the GABI (Great American Biota Interchange) initiated in the Pliocene (Woodborne, 2010, Bacon 2015). These factors may have considerably influenced the divergence between the ancestral lineages of *C. tatouay* and the clade *C. u. squamicaudis* + *C. chacoensis* at the beginning of the Pleistocene. Additionally, the divergence between *C. chacoensis* and *C. u. squamicaudis* may have occurred due to the geographic isolation of their ancestral population, possibly due to some marine incursion in the interglacial periods of the Pleistocene (Short, 1975; Werneck, 2011), and to the uplifts occurred in the Brazilian plateau at the end of the Pliocene (Silva, 1997; Coli, 2005). Alternatively, the ancestral lineage of these species, may have a population dispersed across the Paraguay River in glaciation periods of extremely arid conditions, founding populations isolated eastern and western of this river. Studies with the Didelphidae genus *Thylamis* demonstrate in this same period an event which has the Paraguay River as the main barrier separating *T. macrurus* from other species (Giarla & Jansa, 2014). The Paraguay River has a Mesozoic origin (Potter, 1997) and currently splits the distribution of *C. chacoensis* to its western margin and *C. u. squamicaudis* to its eastern margin (Hayssen, 2013a, c). In addition, the Paraguay River basin serves as an ecotone region that separates distinct faunas, vegetation and soil patterns between east and west sides (Carlini, 2004; Spichiger et al., 2004; Ortiz Cladera, 2006; Werneck, 2011), which is also corroborated by several studies of mammals and birds (Short, 1975; Willig et al., 2000; Lópes-Gonzales, 2004; Giarla & Jansa, 2014).

The divergence between the ancestral lineages of *C. centralis* and *C. u. unicinctus* appears to be the most recent of the genus, occurring in the transition from Early to Middle Pleistocene. *Cabassous centralis* is the only species of the genus with distribution reaching Central America, and the dates indicate that its northwards dispersal may have occurred in GABI2 (Woodburne, 2010). It is possible that the ancestral lineage of *C. centralis* in this period took advantage of factors such as the expansion of savannas in northern South America and Central America (Andreisssen et al., 1993; Piperno, 2006; Woodburne, 2010), and the reduction of about $50 - 60$ m of sea level (Sosdian & Rosental, 2009)), associated with a glaciation cycle, where the Andes was a partial barrier to the dispersion of this species (Wetzel, 1985).

The systematics of the subfamily Tolypeutinae underwent several modifications over time. Many changes were due to the use of different methodologies of classification, where molecular and morphological data did not reach a consensus. However, as new data and studies emerge, the understanding of this subfamily becomes better. The addition of new taxa and genes of this study corroborated the relationship between the genera of Tolypeutinae obtained by more recent studies, but also demonstrated a new topology within the genus *Cabassous*. In no phylogenetic study done so far, individuals of the *C. u. squamicaudis* subspecies were included, which changed remarkably the species diversification scenario within the genus. The most direct conclusion of the available phylogenetic evidence is that C*. unicinctus* is not single species, which is also corroborated by the known geographic distribution of *Cabassous* lineages in South America. In view of this fact, more detailed studies of the taxonomic status of *C. unicinctus* with an accurate species delimitation analyses and larger geographic sampling are needed.

References

Abba AM, Superina M (2011) The 2009/2010 armadillo red list assessment.

- Abba AM, Vizcaíno SF (2008) *Los xenartros (Mammalia: Xenarthra) del Museo Argentino de Ciencias Naturales" Bernardino Rivadavia" y del Museo de La Plata (Argentina)*. Museo argentino de ciencias naturales.
- Abrantes EAL, Bergqvist LP (2006) Proposta filogenética para os Dasypodidae (Mammalia: Cingulata). *Paleontologia de Vertebrados: Grandes Temas e Contribuições Científicas, Interciência*, 261–274.
- Anacleto TCS (2013) Cingulata e Pilosa. *Mamíferos do Brasil. Santa Maria: Editora UFSM*, 81–105.
- Anacleto TCS, Diniz-Filho JAF, Vital MVC (2006) Estimating potential geographic ranges of armadillos (Xenarthra, Dasypodidae) in Brazil under niche-based models / Estimation de la distribution géographique potentielle des tatous (Xenarthra, Dasypodidae) au Brésil à partir de modèles basés sur les niches écologiques. *mammalia*, **70**.
- Anacleto TC da S, Godoy LP, Tubelis DP (2013) New records of the southern nakedtailed armadillo Cabassous unicinctus unicinctus Linnaeus, 1758 (Cingulata: Dasypodidae) in Brazil. *Biota Neotropica*, **13**, 293–296.
- Anacleto TCS, Marinho-Filho J (2001) Hábito alimentar do tatu-canastra (Xenarthra, Dasypodidae) em uma área de cerrado do Brasil Central. *Revista Brasileira de Zoologia*, **18**, 681–688.
- Andriessen, P. A. M., Helmens, K. F., Hooghiemstra, H., Riezebos, P. A., & Van der Hammen, T. (1993). Absolute chronology of the Pliocene-Quaternary sediment sequence of the Bogota area, Colombia. *Quaternary Science Reviews*, *12*(7), 483–501.
- Attias N, Miranda FR, Sena LMM, Tomas WM, Mourão GM (2016) Yes, they can! Threebanded armadillos Tolypeutes sp. (Cingulata: Dasypodidae) dig their own burrows. *Zoologia (Curitiba)*, **33**.
- Bacon, C. D., Molnar, P., Antonelli, A., Crawford, A. J., Montes, C., & Vallejo-Pareja, M. C. (2016). Quaternary glaciation and the Great American Biotic Interchange. *Geology*, *44*(5), 375–378. doi:10.1130/G37624.1

Benirschke K (2006) Xenarthra. *Atlas of Mammalian Chromosomes*, 81–93.

Benirschke K, Low RJ, Ferm VH (1969) Cytogenetic studies of some armadillos. In: *Comparative mammalian cytogenetics*, pp. 330–345. Springer.

Billet G, Hautier L, Lebrun R (2015) Morphological diversity of the bony labyrinth (inner ear) in extant Xenarthrans and its relation to phylogeny. *Journal of Mammalogy*, **96**, 658–672.

- Billet G, Hautier L, de Muizon C, Valentin X (2011) Oldest cingulate skulls provide congruence between morphological and molecular scenarios of armadillo evolution. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 2791– 2797.
- Bolkovi ML, Caziani SM, Protomastro JJ (1995) Food Habits of the Three-Banded Armadillo (Xenarthra: Dasypodidae) in the Dry Chaco, Argentina. *Journal of Mammalogy*, **76**, 1199–1204.
- Bouckaert R, Alvarado-Mora MV, Pinho JR (2013) Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. *Antivir Ther*, **18**, 497–503.
- Bouckaert R, Heled J, Kühnert D *et al.* (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis (A Prlic, Ed,). *PLoS Computational Biology*, **10**, e1003537.
- Carlini, A. A., Zurita, A. E., Gasparini, G., & Noriega, J. I. (2004). Los Mamíferos del Pleistoceno de la Mesopotamia argentina y su relación con los del Centro Norte de la Argentina, Paraguay y Sur de Bolivia, y los del Sur de Brasil y Oeste de Uruguay: Paleobiogeografía y Paleoambientes. *Miscelánea*, *12*(8).
- Carter TS, Superina M, Leslie DM (2016) *Priodontes maximus* (Cingulata: Chlamyphoridae). *Mammalian Species*, **48**, 21–34.
- Cetica PD, Aldana Marcos HJ, Merani MS (2005) Morphology of female genital tracts in Dasypodidae (Xenarthra, Mammalia): a comparative survey. *Zoomorphology*, **124**, 57–65.
- Cetica PD, Merani MS (2008) Sperm evolution in dasypodids. *The biology of the Xenarthra (SF Vizcaíno and WJ Loughry, eds.). University Press of Florida, Gainesville*, 143–150.
- Cetica PD, Solari AJ, Merani MS, De Rosas JC, Burgos MH (1998) Evolutionary sperm morphology and morphometry in armadillos. *Journal of submicroscopic cytology and pathology*, **30**, 309–314.
- Colli, GR (2005) As origens ea diversificação da herpetofauna do Cerrado. Cerrado: ecologia, biodiversidade e conservação, p. 247-264.
- Costa, L. P. (2003). The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography*, *30*(1), 71–86.
- Coutinho M, Campos Z, Mourão G, Mauro R (1997) Aspectos ecológicos dos vertebrados terrestres e semi-aquáticos no Pantanal. *BRASIL. Ministério do Meio Ambiente, dos Recursos Hídricos e da Amazônia Legal. Plano de conservação da Bacia do Alto Paraguai (Pantanal): diagnóstico dos meios físicos e bióticos*, **2**, 183–322.
- Cuvier FG (1825) *Des dents des mammiferes, considérées comme caracteres zoologiques*. Levrault; Normant.
- Delsuc F, Ctzeflis FM, Stanhope MJ, Douzery EJ (2001) The evolution of armadillos, anteaters and sloths depicted by nuclear and mitochondrial phylogenies: implications for the status of the enigmatic fossil Eurotamandua. *Proceedings of the Royal Society of London B: Biological Sciences*, **268**, 1605–1615.
- Delsuc F, Douzery EJP, Vizcaíno SF, Loughry WJ (2008) Recent advances and future prospects in xenarthran molecular phylogenetics. *The biology of the xenarthra*, **11**.
- Delsuc F, Gibb GC, Kuch M *et al.* (2016) The phylogenetic affinities of the extinct glyptodonts. *Current Biology*, **26**, R155–R156.
- Delsuc F, Scally M, Madsen O *et al.* (2002) Molecular phylogeny of living xenarthrans and the impact of character and taxon sampling on the placental tree rooting. *Molecular Biology and Evolution*, **19**, 1656–1671.
- Delsuc F, Stanhope MJ, Douzery EJ. (2003) Molecular systematics of armadillos (Xenarthra, Dasypodidae): contribution of maximum likelihood and Bayesian analyses of mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, **28**, 261–275.
- Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A (2012) Molecular phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. *Molecular Phylogenetics and Evolution*, **62**, 673–680.
- Delsuc F, Vizcaíno SF, Douzery EJ (2004) Influence of Tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. *BMC Evolutionary Biology*, **4**, 1.
- Desmarest AG (1804) *Tableau Méthodique des Mammifères in Nouveau Dictionnaire dHistoire Naturelle, Appliquée aux Arts, Principalement à lAgriculture, à lÉconomie Rurale et Domestique: Par une Société de Naturalistes et dAgriculteurs: Avec des Figures Tirées des Trois Règnes de la Nature*. Deterville, Paris, France.
- Donato, M., Posadas, P., MIRANDA-ESQUIVEL, D. R., Jaureguizar, E. O., & Cladera, G. (2003). Historical biogeography of the Andean region: evidence from Listroderina (Coleoptera: Curculionidae: Rhytirrhinini) in the context of the South American geobiotic scenario. *Biological Journal of the Linnean Society*, *80*(2), 339–352.
- Engelmann GF (1985) The phylogeny of the Xenarthra. *The evolution and ecology of armadillos, sloths, and vermilinguas. Smithsonian Institution Press, Washington, DC*, 51–64.
- Fallabrino A, Castiñeira E (2006) Situación de los edentados en Uruguay. *Edentata*, 1– 3.
- Feijó A, Garbino GST, Campos BATP *et al.* (2015) Distribution of *Tolypeutes* Illiger, 1811 (Xenarthra: Cingulata) with Comments on Its Biogeography and Conservation. *Zoological Science*, **32**, 77–87.
- Feijó A, Langguth A (2013) Mamíferos de médio e grande porte do Nordeste do Brasil: distribuição e taxonomia, com descrição de novas espécies. *Revista Nordestina de Biologia*, **22**, 3–225.
- Fonseca GAB, Aguiar JM (2004) The 2004 Edentate species assentment workshop. *Edentata*, **6**, 1.
- Galliari FC, Carlini AA, Sánchez-Villagra MR (2010) Evolution of the axial skeleton in armadillos (Mammalia, Dasypodidae). *Mammalian Biology - Zeitschrift für Säugetierkunde*, **75**, 326–333.
- Gardner AL (2005) Order Cingulata. *Mammal species of the world: a taxonomic and geographic reference*, **1**, 94–103.
- Gaudin TJ, Wible JR (2006) 6 The Phylogeny of Living and Extinct Armadillos (Mammalia, Xenarthra, Cingulata): A Craniodental Analysis. *Amniote Paleobiology: Perspectives on the Evolution of Mammals, Birds, and Reptiles: University of Chicago Press, Chicago, IL*, 153–198.
- Giarla, T. C., & Jansa, S. A. (2014). The role of physical geography and habitat type in shaping the biogeographical history of a recent radiation of Neotropical marsupials (*Thylamys* : Didelphidae). *Journal of Biogeography*, *41*(8), 1547–1558.
- Gibb GC, Condamine FL, Kuch M *et al.* (2016) Shotgun Mitogenomics Provides a Reference Phylogenetic Framework and Timescale for Living Xenarthrans. *Molecular Biology and Evolution*, **33**, 621–642.
- Gray JE (1865) Revision of the genera and species of entomophagous edentata, found on the examination of the specimens in the Britsh museum. In: *Proceedings of the Zoological Society of London*, pp. 359–386. Wiley Online Library.
- Gray JE (1873) *Hand-list of the edentate, thick-skinned and ruminant mammals in the British Museum*. order of the Trustees of the British Museum.
- Guimarães MM (1997) Área de vida, territorialidade e dieta do tatu-bola. Universidade de Brasilia.
- Hayssen V (2014a) *Cabassous unicinctus* (Cingulata: Dasypodidae). *Mammalian Species*, **907**, 16–23.
- Hayssen V (2014b) *Cabassous chacoensis* (Cingulata: Dasypodidae). *Mammalian Species*, **908**, 24–27.
- Hayssen V (2014c) *Cabassous tatouay* (Cingulata: Dasypodidae). *Mammalian Species*, **909**, 28–32.
- Hayssen V, Ortega J, Morales-Leyva A, Martínez-Mendez N (2013) *Cabassous centralis* (Cingulata: Dasypodidae). *Mammalian Species*, **898**, 12–17.
- Herrick JR, Campbell MK, Swanson WF (2002) Electroejaculation and semen analysis in the La Plata three-banded armadillo (Tolypeutes matacus). *Zoo Biology*, **21**, 481–487.
- Hoorn, C., Guerrero, J., Sarmiento, G. A., & Lorente, M. A. (1995). Andean tectonics as a cause for changing drainage patterns in Miocene northern South America. *Geology*, *23*(3), 237. doi:10.1130/0091- 7613(1995)023<0237:ATAACF>2.3.CO;2
- Hoorn, C., Wesselingh, F. P., ter Steege, H., Bermudez, M. A., Mora, A., Sevink, J., … Antonelli, A. (2010). Amazonia Through Time: Andean Uplift, Climate Change, Landscape Evolution, and Biodiversity. *Science*, *330*(6006), 927–931. doi:10.1126/science.1194585
- Illiger JKW (1811) *Prodomus systematis mammalium et avium additis terminis zoographicii utriusque classis eorumque versione germanica*. sumptibus C. Salfeld.
- IUCN (2016) The IUCN Red List of Threatened Species. Version 2016-3. <www.iucnredlist.org>. Downloaded on 29 January 2017.
- Jacintho PJHR, Santiloni V, Rosa PS, da Mota LSLS, Jorge W (2009) The karyotype of Cabassous unicinctus (Dasypodidae, Xenar-thra). *Caryologia*, **62**, 24–29.
- Jorge W, Meritt Jr DA, Benirschke K (1977) Chromosome studies in Edentata. *Cytobios*, **18**, 157–172.
- Kerr R (1792) *The animal kingdom, or zoological system, of the celebrated Sir Charles Linnæus. containing a complete systematic description, arrangement, and nomenclature, of all the known species and varieties of the mammalia, or animals which give suck to their young /*. Printed for A. Strahan, and T. Cadell, London, and W. Creech, Edinburgh, Edinburgh :
- Korbie DJ, Mattick JS (2008) Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nature Protocols*, **3**, 1452–1456.
- Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, msw054.
- Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.
- Linnaeus C v (1758) Systema Naturae, edition X, vol. 1 (Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata). *Holmiae Salvii*, **1**, 824.
- López-González, C. (2004). Ecological zoogeography of the bats of Paraguay. *Journal of Biogeography*, *31*(1), 33–45.
- Luaces JP, Rossi L, Castillo LF, Merani MS (2010) Citogenética de Cabassous chacoensis Wetzel, 1980.
- Marinho-Filho J, Guimarães MM, Reis ML *et al.* (1997) The discovery of the Brazilian three banded armadillo in the Cerrado of Central Brazil. *Edentata*, **3**, 11–13.
- Marshall LG, Sempere T (1993) Evolution of the Neotropical Cenozoic land mammal fauna in its geochronologic, stratigraphic, and tectonic context. *Biological relationships between Africa and South America*, 329–392.
- McKenna MC, Bell SK (1997) *Classification of mammals: above the species level*. Columbia University Press.
- McMurtrie H (1831) *The animal kingdom arranged in conformity with its organization, by the Baron Cuvier, The Crustacea, Arachnides and Insecta, by P. A. Latreille. , Translated from the French with notes and additions by H. M'Murtrie.* G. and C. and H. Carvill, New York.
- Meritt DA (2006) Research Questions on the Behavior and Ecology of the Giant Armadillo (Priodontes maximus). *Edentata*, **7**, 30.
- Miller GS (1899) *Notes on the naked-tailed armadillos*.
- Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Gateway Computing Environments Workshop (GCE), 2010*, pp. 1–8. IEEE.
- Moller-Krull M, Delsuc F, Churakov G *et al.* (2007) Retroposed Elements and Their Flanking Regions Resolve the Evolutionary History of Xenarthran Mammals (Armadillos, Anteaters, and Sloths). *Molecular Biology and Evolution*, **24**, 2573– 2582.
- Nowak RM (1999) *Walker's Mammals of the World*. JHU Press.
- Ortiz-Jaureguizar E, Cladera GA (2006) Paleoenvironmental evolution of southern South America during the Cenozoic. *Journal of Arid Environments*, **66**, 498–532.
- Pascual R, Ortiz-Jaureguizar E (1990) Evolving climates and mammal faunas in cenozoic south America. *The Platyrrhine Fossil Record*, **19**, 23–60.
- Patterson B, Pascual R (1968) The fossil mammal fauna of South America. *Quarterly Review of Biology*, 409–451.
- Patterson B, Segall W, Turnbull WD (1989) *The ear region in Xenarthrans (: Cingulates*. Field Museum of Natural History.
- Piperno, D. R. (2006). Quaternary environmental history and agricultural impact on vegetation in Central America. *Annals of the Missouri Botanical Garden*, *93*(2), 274–296.

Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) *Tracer v1. 6*.

- Reis ML (2002) Relatório de Fauna. In: *Jalapão, Expedição Científica e Conservacionista* (eds Arruda MB, von Berh, M), pp. 29–44. IBAMA, Brasília - DF.
- Sambrook J, Russel DW (2001) *Molecular Cloning: a laboratory manual, Cold Spring Hrbour Laboratory Press*. Cold Spring Harbour.
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, **74**, 5463–5467.
- Santos Júnior JE, Santos FR, Silveira FA (2015) Hitting an Unintended Target: Phylogeography of Bombus brasiliensis Lepeletier, 1836 and the First New Brazilian Bumblebee Species in a Century (Hymenoptera: Apidae) (S Brady, Ed,). *PLOS ONE*, **10**, e0125847.

Sarich VM (1985) Xenarthran systematics: albumin immunological evidence. *The Evolution and Ecology of Armadillos, Sloths, and Vermilinguas*, 77–81.

- Short, L. L. (1975). Zoogeographic analysis of the South American chaco avifauna. *Bulletin of the American Museum of Natural History*, *154*.
- Silva, J. M. C. D. (1997). Endemic bird species and conservation in the Cerrado Region, South America. *Biodiversity & Conservation*, *6*, 435–450.

Smith P (2007) Southern tree banded armadillo: Tolypeutes matacus.

- Smith P (2008) Chaco naked-tailed armadillo Cabasssous chacoensis Wetzel, 1980. *Fauna Paraguay Handbook of the Mammals of Paraguay*.
- Smith P, Owen RD, Atkinson K, Castillo HD, Northcote-Smith E (2011) First Records of the Southern Naked-Tailed Armadillo *Cabassous unicinctus* (Cingulata: Dasypodidae) in Paraguay. *Edentata*, **12**, 53–57.
- Sosdian, S., & Rosenthal, Y. (2009). Deep-Sea Temperature and Ice Volume Changes Across the Pliocene-Pleistocene Climate Transitions. *Science*, *325*(5938), 306– 310. doi:10.1126/science.1169938
- Spichiger, R., Calenge, C., & Bise, B. (2004). Geographical zonation in the Neotropics of tree species characteristic of the Paraguay-Paraná Basin. *Journal of Biogeography*, *31*(9), 1489–1501.
- Srbek-Araujo AC, Scoss LM, Hirsch A, Chiarello AG (2009) Records of the giantarmadillo Priodontes maximus (Cingulata: Dasypodidae) in the Atlantic Forest: are Minas Gerais and Espírito Santo the last strongholds of the species? *Zoologia (Curitiba)*, **26**, 461–468.
- Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, **73**, 1162–1169.
- Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**, 978–989.
- Superina M, Aguiar JM (2006) A Reference List of Common Names for the Edentates. *Edentata*, **7**, 33.
- Superina M, Pagnutti N, Abba AM (2014) What do we know about armadillos? An analysis of four centuries of knowledge about a group of South American mammals, with emphasis on their conservation: Four centuries of knowledge about armadillos. *Mammal Review*, **44**, 69–80.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weight matrix choice. *Nucleic acids research*, **22**, 4673– 4680.
- Toivonen, T., Mäki, S., & Kalliola, R. (2007). The riverscape of Western Amazonia a quantitative approach to the fluvial biogeography of the region: The riverscape of Western Amazonia. *Journal of Biogeography*, *34*(8), 1374–1387.
- Van Dijk MA, Paradis E, Catzeflis F, De Jong WW (1999) The virtues of gaps: Xenarthran (Edentate) monophyly supported by a unique deletion in αA-crystallin. *Systematic biology*, **48**, 94–106.
- Vizcaíno SF (2009) The teeth of the "toothless": novelties and key innovations in the evolution of xenarthrans (Mammalia, Xenarthra). *Paleobiology*, **35**, 343–366.
- Vizcaíno SF, Cassini GH, Fernicola JC, Bargo MS (2011) Evaluating Habitats and Feeding Habits Through Ecomorphological Features in Glyptodonts (Mammalia, Xenarthra). *Ameghiniana*, 305–319.
- Vizcaíno SF, Cassini GH, Toledo N, Bargo MS (2012) On the evolution of large size in mammalian herbivores of Cenozoic. *Bones, clones and biomes: an*, 76–101.
- Werneck, F. P. (2011). The diversification of eastern South American open vegetation biomes: Historical biogeography and perspectives. *Quaternary Science Reviews*, *30*(13–14), 1630–1648.
- Wetzel RM (1980) *Revision of the naked-tailed armadillos, genus Cabassous McMurtrie*. Carnegie Museum of Natural History.
- Wetzel RM (1982) Systematics, distribution, ecology, and conservation of South American Edentates. In: *Mammalian biology in South America, special publication series, Pymatuning laboratory of ecology.*, pp. 345–375. M. A. Mares & H. H. Genoways, University of Pitsburgh, Linesville.
- Wetzel RM (1985a) The identification and distribution of recent Xenarthra (= Edentata). *The evolution and ecology of armadillos, sloths, and vermilinguas*, 5–21.
- Wetzel RM (1985b) Taxonomy and distribution of armadillos, Dasypodidae. *The evolution and ecology of armadillos, sloths, and vermilinguas*, 23–46.
- Wetzel RM, Gardner AL, Redford KH, Eisenberg JF (2007) Order Cingulata Illiger, 1811. In: *Mammals of South America*, University of Chicago Press, Chicago.
- Willig, M. R., Presley, S. J., Owen, R. D., & López-González, C. (2000). Composition and structure of bat assemblages in Paraguay: a subtropical–temperate interface. *Journal of Mammalogy*, *81*(2), 386–401.
- Wolf D, Kalthoff DC, Sander PM (2012) Osteoderm histology of the Pampatheriidae (Cingulata, Xenarthra, Mammalia): Implications for systematics, osteoderm growth, and biomechanical adaptation. *Journal of Morphology*, **273**, 388–404.
- Woodburne, M. O., Goin, F. J., Bond, M., Carlini, A. A., Gelfo, J. N., López, G. M., Zimicz, A. N. (2014). Paleogene Land Mammal Faunas of South America; a Response to Global Climatic Changes and Indigenous Floral Diversity. *Journal of Mammalian Evolution*, *21*(1), 1–73.
- Yang Z (2005) Bayesian Estimation of Species Divergence Times Under a Molecular Clock Using Multiple Fossil Calibrations with Soft Bounds. *Molecular Biology and Evolution*, **23**, 212–226.
- Zachos J (2001) Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science*, **292**, 686–693.

Supporting information

Species	Genbank accession numbers				
Euphractus sexinctus	AY011193.1, AY011254.1, AY011379.1,				
	AY011443.1, AY011688.1,				
	NC 028571.1, AY011867.1 and				
	AJ427364.1				
Priodontes maximus	NC 028573.1 and AJ427363.1				
Tolypeutes matacus	NC 028575.1 and AJ427362.1				
Tolypeutes tricinctus	AJ427362.1				
Cabassous centralis	NC 028556.1				
Cabassous chacoensis	NC 028557.1				
Cabassous tatouay	NC 028558.1				
Cabassous unicinctus	NC_028559.1, KT818530.1				

Table S1: Accession numbers of sequences retrieved from Genbank.

Capítulo 2

Molecular taxonomy of the genus *Cabassous* **(Cingulata: Tolypeutinae) and its implications on the taxonomic status of** *Cabassous unicinctus***.**

*Marco Antônio Alves Schetino, Teresa Cristina da Silveira Anacleto, and Fabrício Rodrigues Santos**

Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Belo Horizonte, Minas Gerais, Brazil (MAAS, FRS)

Universidade do Estado de Mato Grosso, Br 158, Km 655, Nova Xavantina, Mato Grosso, Brazil (TCSA)

Abstract

Cabassous McMurtrie, 1831 is a genus of armadillos belonging to the family Tolypeutinae and its last taxonomic revision was carried out almost four decades ago. Due to the small number of characters analyzed, the lack of specimens from other regions in which the species of this genus are found and the possible misclassification of some epithets, the taxonomic status of some species is still uncertain. Species delimitation analyzes were performed with sequences obtained from six nuclear and two mitochondrial genes of *Cabassous centralis*, *Cabassous tatouay* and *Cabassous unicinctus* species. The analyzes suggest that the subspecies *Cabassous unicinctus squamicaudis* and *Cabassous unicinctus unicinctus* are species and there are morphological differences that corroborate this hypothesis. Two clades of distinct geographic regions of *C. u. unicintus* were identified as species and one of them is sister group of *Cabassous centralis*, which suggests new analyzes for a better understanding of their respective taxonomic status.

Running title: Molecular taxonomy of the genus *Cabassous*

Key words: armadillo, delimitation species, systematics, molecular dating, Biogeography.

Introduction

Cabassous McMurtrie, 1831 is a genus of armadillos belonging to the subfamily Tolypeutinae. Its main feature is the absence of dermal shield on its tail which makes it commonly known as "Naked-tailed armadillo". The last taxonomic review of this genus was carried out almost four decades ago by Wetzel (1980) in which the author cites four species: *Cabassous centralis* Miller, 1899; *Cabassous chacoensis* Wetzel, 1980; *Cabassous tatouay* Desmarest, 1804 and; *Cabassous unicinctus* Linnaeus, 1758, the latter divided into two subspecies: *C. u. unicinctus* and *C. u. squamicaudis.*

This genus is found predominantly in South America, with the exception of *Cabassous centralis* which has its distribution from Mexico through Central America to the north of South America, including western Colombia, northwestern Ecuador and northwestern Venezuela (Abba & Superina 2010; Hayssen *et al.* 2013). *Cabassous chacoensis* occupies a narrow portion of Chaco in the regions of western Paraguay, northern Argentina (Abba & Superina 2010) and there is a possible occurrence in southern Bolivia (Hayssen 2014b). Its distribution in Brazil is doubtful because it comes from a skull deposited in the Museum of Buenos Aires that probably is *C. unicinctus* (Abba & Vizcaíno 2008). *Cabassous tatouay* is found in Uruguay, Argentina, Paraguay and Brazil (Wetzel *et al.* 2007). *Cabassous unicinctus* occurs in great part of South America in the countries of Venezuela, Guyana, French Guiana, Suriname, Brazil, Peru, Bolivia, and Paraguay (Fonseca & Aguiar 2004; Wetzel *et al.* 2007; Smith *et al.* 2011; Hayssen 2014a). Its subspecies *C. u. unicinctus* is distributed in the Amazon and Cerrado biomes while *C. u. squamicaudis* is distributed in the Amazon, Cerrado and Atlantic Forest biomes (Wetzel 1982; Anacleto *et al.* 2013). According to the International Union for Conservation of Nature and Natural Resources - IUCN - (2016), *Cabassous chacoensis* falls into the category "Near Threatned" (NT), *C. tatouay* and *C. unicinctus* are classified as "least concern" (LC) and *C. centralis* as "Data deficient" (DD).

The taxonomic status of some *Cabassous* species has uncertain and this can be attributed mainly to the small number of characters studied and the lack of sampling in certain localities (Anacleto *et al.* 2013). Wetzel (1980, 1982, 1985) comments about the scarcity of specimens in museums and possible errors in the identification of their epithets. In this same studies the author suggests that there are still doubts about the taxonomic classification of *C. centralis*, *C. u. unicinctus* and *C. u. squamicaudis*. In addition to distinct geographic distributions, these subspecies have different morphological characteristics. *C. u. unicinctus* is larger, has fewer scutes on the cephalic shield and less or no scales in cheek bellow eye and has a distinct white band around the flanks of your carapace while *C. u. squamicaudis* is smaller, has more scutes on the cephalic shield, more scales on cheek, tail and posterior side of the pinna (Wetzel 1980).

In addition, despite the difference between sizes, *C. centralis* and *C. unicinctus* have very similar structures (Hayssen 2014a). Although studies with molecular markers performed in an attempt to elucidate the phylogeny of the genus (Delsuc *et al.* 2012; Gibb *et al.* 2016), no studies related to species taxonomy, population genetics or phylogeography are available.

This article aims, using mitochondrial and nuclear markers, to contribute to the updating of the taxonomic status of the *Cabassous* species and its systematics. This knowledge directly interferes with the reassessment of the conservation status of each species (Frankham 2003), which lack of data to assist in their allocation to the most appropriate threat categories according to IUCN. In addition, this information precedes the best design for future phylogeographic studies.

Material and methods

Sampling & DNA extraction

We extracted, by standard phenol-chloroform protocol (Sambrook & Russel 2001), total DNA of collected liver, muscle or blood samples of 55 individuals of *Cabassous* from 23 localities of the Brazil, French Guiana and Guatemala (Fig. 1; Table 1). Fragments of two mitochondrial genes (mtDNA) and 6 nuclear genes (nuDNA) were amplified with Platinum *Taq* DNA Polymerase (ThermoFisher) using specific primers and annealing temperatures for each fragment (Table 2) following the touchdown PCR techniques described by Korbie & Mattick (2008). We designed in our laboratory the primers of genes ADRB2 (ADRB2F = 5'-ATCGTCCTGGCCATCGTGTT-3' and ADRB2R = 5'- CTCCTGGAAGGCAATCCTGA,-3') and vWF (vWF1F = 5'- TGTCAACCTCACCTGTGAAGCCTG-3', vWF1R = 5'- TGCAGGACCAGGTCAGGAGCCTCTC-3', vWF2F = 5'- TCAAGCAGATCCGCTTCATCGAG-3' and vWF2R = 5'- GTGGAGAAGCTGTGCTCCGAGAC-3'). PCR efficiency was confirmed by electrophoresis on 1% agarose gel and purified with 20% polyethylene glycol in 2.5 M NaCl (Santos Júnior *et al.* 2015). The sequencing was carried out according to the chain termination through the incorporation of dideoxiribonucleotides (ddNTPs) technique (Sanger *et al.* 1977) in a 3130xl ABI Genetic Analyzer system (Applied Biosystems, Foster City, CA). The chromatograms were analyzed in the SeqScape 2.6 software (Applied Biosystems, Foster City, CA) and the consensus sequences were aligned using the Clustal W algorithm implemented in the MEGA 7 software (Thompson *et al.* 1994; Kumar *et al.* 2016). The identification of the gametic phases for subjects with multiple heterozygous sites was performed with the PHASE 2.0 program

(Stephens *et al.* 2001; Stephens & Donnelly 2003) included in the software DNAsp 5.1 (Librado & Rozas 2009).

Figure 1: Sampling localities of *Cabassous* species in South and Central America. Star = *C. chacoensis*; Circle = *C. u. unicinctus*; Asterisk = *C. u. squamicaudis*; Triangle = *C. tatouay*; Square = *C. centralis*.

					Number of Samples			
Country	Locality	Map Number	C. centralis	C. chacoensis	C. tatouay	C. u. squamicaudis	C. u. unicinctus	
Argentina	Presidencia Roque Sáenz Peña	1G						
Brazil	Cachoeira Alta - GO	$\overline{2}$						
	Araxá - MG	3						
	Brasilândia de Minas - MG							
	Catas Altas - MG	5						
	Chapada Gaúcha - MG	6						
	Quartel Geral - MG							
	Unaí - MG	8						
	Baía das Pedras - MS	9						
	Miranda - MS	10			2			
	Nova Alvorada do Sul - MS	11						
	Nova Andradina - MS	12						
	Três Lagoas - MS	13						
	Agua Boa - MT	14						
	Confresa - MT	15						
	Lucas do Rio Verde - MT	16						
	Nova Xavantina - MT	17						
	Ribeirão Cascalheira - MT	18				3		
	Belo Monte - PA	19						
	Juriti - PA	20						
	Cruz do Espírito Santo - PB	21						
	Porto Velho - RO	22					3	
	Paranaguá - SC	23						
	Unknown							
Costa Rica	Siquirres	24G						
French Guianna	Cayenne	25G					4+2(Genbank)	
Guatemala	Ikcán	26						
Uruguay	Unknow	27G						

Table 1: Sampling localities and number of samples of *Cabassous* used in this work. Legend: G = Genbank. Legend of Brazilian states: GO = Goiás; MG = Minas Gerais; MS = Mato Grosso do Sul; MT = Mato Grosso; PA = Pará; PB = Paraíba; RO = Rondônia; SC = Santa Catarina.

Table 2: Primers used for partial amplification of *Cabassous* genes. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular.

Phylogeny and Delimitation of species.

Median-joining haplotype networks (Bandelt *et al.* 1999) were constructed using the sequences obtained here for each one of the nuDNA and additional sequences of *C. centralis*, *C. chacoensis*, *C. tatouay* e *C. unicinctus,* obtained in Genbank (Fig.1; Table S1), were used to performed this analysis based on the concatenated sequences of mtDNA using the software NETWORK 5.0 (Fluxus Engineering) as an alternative to visualize parsimony relationships between haplotypes and the *Cabassous* species (Bandelt *et al.* 1995).

To estimate the number of species and to delimit them, the 55 sequences obtained here were analyzed by the STACEY 1.2.2 package (Jones 2016) included in BEAST 2.4 software (Bouckaert *et al.* 2014), using the reversible-jump based substitution model implemented through the RBS 1.3.1 package in this same software (Bouckaert *et al.* 2013) and allowing gamma rate heterogeneity and invariant sites. We chose a Yule tree prior, Collapse Height was set by 1.0E-4 and, the Birth Diff Rate, Collapse Weight and Origin Height STACEY priors were estimated by the program itself. Three independent MCMC chains of 300,000,000 generations were carried out and sampled every 10,000,000 generations. The effective sample sizes (ESS) for each run was checked in Tracer 1.6 (Rambaut *et al.* 2014). The log files were combined in LogCombiner and summarized in TreeAnnotator, both available in the BEAST 2.3 package, to obtain the best 10,000 trees. The final result was processed by the software SpeciesDelimitationAnalyser (Jones *et al.* 2014). To verify the relationships and divergence time between the species of *Cabassous* the phylogenetic reconstruction was performed in the *BEAST 2.4 program (Bouckaert *et al.* 2014) using the same basic conditions described to STACEY package and a calibration constraint correspondent to the divergence time of the two main clades of *Cabassous* obtained in the previous Tolypeutinae tree (Chapter 1) with three independent MCMC chains of 300,000,000 generations sampled every 5,000 generations.

The validation of the species found was conducted using the BPP 3.1 software (Yang 2015) where was performed an analysis of delimitation species under the "Multi Species Coalescent" (MSC) model using a *BEAST phylogenetic tree has a guide (Yang & Rannala 2010; Rannala & Yang 2013). The population size parameters (θs) are assigned the gamma prior G(2, 2000). The divergence time at the root of the species tree (T0) is assigned the gamma prior G(5, 1000), while the other divergence time parameters are assigned the Dirichlet prior (Yang & Rannala 2010). This analysis was performed two times to confirm consistency of the results and the ESS for each run was checked in Tracer 1.6.

Results

According to the mtDNA network, all haplotypes are unique to each species (Fig. 2), whereas in most networks produced with nuDNA there are haplotypes shared mainly among the taxa of *C. tatouay* and *C. u. squamicaudis* and among the species of *C. centralis* and *C. u. Unicinctus* (Fig. 3). Furthermore*,* the mtDNA network shows two distinct groups of *C. u.unicinctus* which, one of them, composed of individuals from French Guiana, is close to *C. centralis*. The subspecies *C. u. squamicaudis*, is closest *C. chacoensis* than *C. u. unicinctus* (Fig. 2).

Figure 2: Haplotype networks for mitochondrial genes of *Cabassous* genus. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the lines.

Figure 3: Haplotype networks for six nuclear genes of *Cabassous* genus. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the lines.

The phylogenetic relationships between *Cabassous* shown two main clades: *C. u. squamicaudis* + *C. tatouay* and *C. u. unicinctus* + *C. centralis* (Fig. 4). Additionally, the clade of *C. u. unicinctus* composed of individuals from south of the Amazon river (Cu1) form a sister group of *C. u. unicinctus* composed of individuals from north of Amazon river (Cu2) and *C. centralis*. (Fig. 4). The divergence between the *Cabassous* species dates from Pliocene and Pleistocene (Table 3)

The analyzes performed by STACEY indicated the presence of five clusters in 89.8% of their trees (Appendix 1) referring to *C. centralis*, *C. tatouay*, *C. u. Squamicaudis* and the two clades found of *C. u. Unicinctus*. With a posterior value of 99.9% the analyzes performed by BPP also indicated that the best model, given the proposed tree, is that of 5 species (model: 7 1111) similar to those obtained by the STACEY program (Appendix 2).

Figure 4: Molecular timescale for *Cabassous* nodes inferred with a Bayesian phylogeny obtained in *BEAST. Node ages were obtained under the reversiblejump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a Yule tree prior. Node bars indicate the 95% HPD intervals for age estimates in million years ago (mya). All nodes with the values not shown were supported by maximum posterior probabilities.

Table 3: Divergence times estimates of *Cabassous* clades with 95% confidence intervals for all nodes.

Discussion

Until now, these are the first molecular analyzes (included Chapter 1) conducted using individuals of *Cabassous* species or subspecies from different regions of the South American continent. Even in the more detailed review of the genus made by Wetzel (1980), only individuals of *C. u. unicictus* from the north of the continent were analyzed due their distribution to the south, until then, was not known (Anacleto *et al.* 2013). The most recent phylogenetic analyzes (Delsuc *et al.* 2012; Gibb *et al.* 2016), although presenting a great advance in understanding the genus, used only local specimens of each species, such as *C. u. unicinctus* of French Guiana and *C. tatouay* of Uruguay, which limits the understanding of their real diversity and evolutionary history given their vast geographic distribution and different habitats where they live (Wetzel 1982; Anacleto *et al.* 2013). Furthermore, there is the difficulty of collecting these individuals due to their largely nocturnal and fossorial way of life (Hayssen *et al.* 2013; Loughry & McDonough 2013; Hayssen 2014a; b; c). Only with the collaboration of armadillo specialists was possible to obtain this number of samples, even so, we were get only one individual from *C. centralis* for nuDNA analysis and any *C. chacoenis* samples.

Our phylogenetic analysis shows that the subspecies of *C. unicinctus* are not grouped in a single monophyletic clade but rather they are divided in two large clades: one have two distinct clades of *Cabassous unicintcus unicinctus* where one of these is the sister group of *C. centralis*; and another where *C. u. squamicaudis* is the sister group of *C. tatouay*. Similar pattern is found in network analyzes. In addition, the analyzes of species delimitation suggest that the individuals used in this work are divided into five possible species.

Besides the morphological differences mentioned above, Wetzel (1980) showed in his canonical analyzes only a small overlap between the clusters of *C. u. unicinctus* and *C. u. squamicaudis*. However, grouped them in the same species due to similarities in characteristics as mean interorbital width and mean zygomatic width. Perhaps these characteristics do not serve as diagnostic for this taxon since their mean values may have undergone interference of sampling deviation considering that several regions of occurrences of these individuals had not yet been discovered or, these are plesiomorphic characteristics maintained in these two subspecies or adaptive convergences to the habitat in which they are found, such as forests (Bonato *et al.* 2008; Abba & Superina 2010; Anacleto *et al.* 2013; Hayssen 2014a).

In the case of *C. u.unicinctus* we observed a clear division between the clades to the south (Cu1) and north (Cu2 + *C. Centralis*) of the Amazon river. Despite the hypothesis that Amazonian rivers as geographic barriers do not serve all species (Leite & Rogers 2013), it is known that regions of great extension of the Amazon River can limit the gene flow between its margins (Hayes & Sewlal 2004). Our results raise four hypotheses. The first one considers that the three groups of this clade are different species as determined by the programs of delimitation of species used in this work and that Cu1 would be a new species. There is still no morphological description in the literature which corroborates that individuals of the south of the Amazon River may be a new species, but there are characteristics of *C. centralis* that differentiates it from *C. u. unicinctus* as its smaller size and external surface of pinnae without scales plus distinct geographical distribution (Wetzel 1980, 1982; Wetzel *et al.* 2007; Hayssen *et al.* 2013). The second and third hypotheses take into account that species delimitation programs may have identified only different genetic structures and not species (Sukumaran & Knowles 2017). Therefore, it is possible to be a single species with several ESUs (Crandall *et al.* 2000), with a wide geographic distribution and regional morphological variations or, that Cu1 is one species and Cu2 and *C. centralis* is another. Finally, the fourth hypothesis takes into account that only one individual of C. centralis was sequenced and that this may have influenced the phylogeny of the group. The *posteriori* value which groups Cu2 with *C. centralis* is only 0.74, which indicates that there is a possibility that *C. centralis* could be the Cu1 + Cu2 or Cu1 sister group.

Although not the main focus to be covered in this chapter, our results showed mean node ages slightly different for some of the clades of *Cabassous* when compared to chapter 1 Tolypeutinae tree, mainly in relation to the first cladogenesis within the genus, although 95% HPD intervals for each estimated time were similar and overlapped. These discrepancies may be related to differences in the molecular dating method, sample set and size used in each work. Even so, the geological periods and biogeographic events that possibly explain the divergence between the clades are similar to those mentioned in Chapter 1. In Pliocene, the northern (*C. centralis* and C*. u. unicinctus*) and southern (*C. tatouay, C. chacoensis* and *C. u. squamicaudis*) lineages diverged. During this period, in the south of the continent, there was the expansion of open areas and the grasslands reached their apogee, while to the north the tropical forests were relatively less affected (Pascual & Ortiz-Jaureguizar 1990; Pascual 2006; Hoorn *et al.* 2010), which may justify the current northern species being strictly to the forest habitats (Anacleto *et al.* 2013; Hayssen *et al.* 2013), while those with southern distribution may also be found in open areas of Chaco and Cerrado (Wetzel *et al.* 2007; Hayssen 2014a, c; b). The distribution of *C. u. squamicaudis* in the north of the continent (Wetzel 1982) and *C. u. unicinctus* to the south may be related to later events that favored its dispersion, such as the changes in continental vegetation occurred in Late Quaternary and Holocene (Behling 2002; De Vivo & Carmignotto 2004).

The Pleistocene is a period marked by several glaciations that constantly caused the expansion and contraction of dry and humid biomes and directly affecting the local biota (Marshall & Cifelli 1990; Hewitt 2000; Zachos 2001). In addition, the continent suffered a great ecological impact from the arrival of new species from North America in the Great American Biota Interchange (GABI) initiated in the Pliocene (Woodburne *et al.* 2014; Bacon *et al.* 2016). These changes may have great influence on the divergence between the ancestral lineages of *C. tatouay* and *C. u. squamicaudis* at the beginning of the Pleistocene. In addition, these same glaciations may have favored the dispersion of the ancestral lineage of Cu2 and *C. centralis* in periods of dry climates to the north of the Amazon River around 1.24 mya. Posteriorly, the divergence between the ancestral lineages of *C. centralis* and Cu2 occurring in the transition from Early to Middle Pleistocene. The dates indicate that the dispersion of *C. centralis* to the Central America possibly occurred in GABI2 (Woodburne *et al.* 2014). It is possible that the expansion of savannas in the north of South America and Central America (Andriessen *et al.* 1993; Piperno 2006; Woodburne *et al.* 2014) and the reduction of sea level (Sosdian & Rosenthal 2009) favored this northward dispersion and, posteriorly, the Andes was a partial barrier to the dispersion of this species ((Wetzel 1985).

It is not unusual new species to be suggested with genetic data, as in the case of elephants *Loxodonta africana* and *Loxodonta cyclotis* (Roca 2001; Roca *et al.* 2004). However, other analyzes are necessary and, in this case, integrative taxonomy would link these molecular data to geographic, ecological and morphological data for the validation of their respective taxonomic status (Padial *et al.* 2010). At the same time, morphometric studies have been carried out by our collaborators in order to achieve this objective (Anacleto, personal communication).

The recognition of new species directly affects the conservation of these taxa, as implies the re-evaluation of their geographic distributions and population data, which are important parameters for the elaboration of conservation plans (Williams *et al.* 2002; Rondinini *et al.* 2006). It is likely that if subspecies of *C. unicinctus* are recognized as species, the IUCN threat level categorization and other conservation agencies data are underestimated and a new assessment is needed.
References

- Abba AM, Superina M (2010) The 2009/2010 armadillo red list assessment. *Edentata*, **11(2)**.
- Abba AM, Vizcaíno SF (2008) *Los xenartros (Mammalia: Xenarthra) del Museo Argentino de Ciencias Naturales" Bernardino Rivadavia" y del Museo de La Plata (Argentina)*. Museo argentino de ciencias naturales.
- Anacleto TC da S, Godoy LP, Tubelis DP (2013) New records of the southern nakedtailed armadillo *Cabassous unicinctus unicinctus* Linnaeus, 1758 (Cingulata: Dasypodidae) in Brazil. *Biota Neotropica*, **13**, 293–296.
- Andriessen PAM, Helmens KF, Hooghiemstra H, Riezebos PA, Van der Hammen T (1993) Absolute chronology of the Pliocene-Quaternary sediment sequence of the Bogota area, Colombia. *Quaternary Science Reviews*, **12**, 483–501.
- Bacon CD, Molnar P, Antonelli A *et al.* (2016) Quaternary glaciation and the Great American Biotic Interchange. *Geology*, **44**, 375–378.
- Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, **16**, 37–48.
- Bandelt H-J, Forster P, Sykes BC, Richards MB (1995) Mitochondrial portraits of human populations using median networks. *Genetics*, **141**, 743–753.
- Behling H (2002) South and southeast Brazilian grasslands during Late Quaternary times: a synthesis. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **177**, 19–27.
- Bonato V, Martins EG, Machado G, da-Silva CQ, dos Reis SF (2008) Ecology of the Armadillos *Cabassous unicinctus* and *Euphractus sexcinctus* (Cingulata: Dasypodidae) in a Brazilian Cerrado. *Journal of Mammalogy*, **89**, 168–174.
- Bouckaert R, Alvarado-Mora MV, Pinho JR (2013) Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. *Antivir Ther*, **18**, 497–503.
- Bouckaert R, Heled J, Kühnert D *et al.* (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis (A Prlic, Ed,). *PLoS Computational Biology*, **10**, e1003537.
- Caballero S, Trujillo F, Vianna JA *et al.* (2007) Taxonomic status of the genus *Sotalia*: species level ranking for "tucuxi" (*Sotalia fluviatilis*) and "costero" (*Sotalia guianensis*) dolphins. *Marine Mammal Science*, **23**, 358–386.
- Crandall KA, Bininda-Emonds OR, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. *Trends in ecology & evolution*, **15**, 290–295.
- De Vivo M, Carmignotto AP (2004) Holocene vegetation change and the mammal faunas of South America and Africa. *Journal of Biogeography*, **31**, 943–957.
- Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A (2012) Molecular phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. *Molecular Phylogenetics and Evolution*, **62**, 673–680.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*, **3**, 294–299.
- Fonseca GAB, Aguiar JM (2004) The 2004 Edentate species assentment workshop. *Edentata*, **6**, 1.
- Frankham R (2003) Genetics and conservation biology. *Comptes Rendus Biologies*, **326**, 22–29.
- Gibb GC, Condamine FL, Kuch M *et al.* (2016) Shotgun Mitogenomics Provides a Reference Phylogenetic Framework and Timescale for Living Xenarthrans. *Molecular Biology and Evolution*, **33**, 621–642.
- Hayes FE, Sewlal J-AN (2004) The Amazon River as a dispersal barrier to passerine birds: effects of river width, habitat and taxonomy. *Journal of Biogeography*, **31**, 1809–1818.
- Hayssen V (2014a) *Cabassous unicinctus* (Cingulata: Dasypodidae). *Mammalian Species*, **907**, 16–23.
- Hayssen V (2014b) *Cabassous chacoensis* (Cingulata: Dasypodidae). *Mammalian Species*, **908**, 24–27.
- Hayssen V (2014c) *Cabassous tatouay* (Cingulata: Dasypodidae). *Mammalian Species*, **909**, 28–32.
- Hayssen V, Ortega J, Morales-Leyva A, Martínez-Mendez N (2013) *Cabassous centralis* (Cingulata: Dasypodidae). *Mammalian Species*, **898**, 12–17.
- Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.
- Hoorn C, Wesselingh FP, ter Steege H *et al.* (2010) Amazonia Through Time: Andean Uplift, Climate Change, Landscape Evolution, and Biodiversity. *Science*, **330**, 927–931.
- IUCN (2016) The IUCN Red List of Threatened Species. Version 2016-3. <www.iucnredlist.org>. Downloaded on 29 January 2017.
- Jones G (2016) Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. *Journal of Mathematical Biology*, **74**, 447–467.
- Jones G, Aydin Z, Oxelman B (2014) DISSECT: an assignment-free Bayesian discovery method for species delimitation under the multispecies coalescent. *Bioinformatics*, **31**, 991–998.
- Korbie DJ, Mattick JS (2008) Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nature Protocols*, **3**, 1452–1456.
- Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, msw054.
- Leite RN, Rogers DS (2013) Revisiting Amazonian phylogeography: insights into diversification hypotheses and novel perspectives. *Organisms Diversity & Evolution*, **13**, 639–664.
- Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.
- Loughry WJ, McDonough CM (2013) Beyond Natural History: Some Thoughts About Research Priorities in the Study of Xenarthrans. *Edentata*, **14**, 9–14.
- Marshall LG, Cifelli RL (1990) *Analysis of changing diversity patterns in Cenozoic land mammal age faunas, South America*. Laboratoire de Paleontologie des Vertébrés.
- Murphy WJ, Eizirik E, Johnson WE *et al.* (2001) Molecular phylogenetics and the origins of placental mammals. *Nature*, **409**, 614–618.
- Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. *Frontiers in zoology*, **7**, 16.
- Pascual R (2006) Evolution and geography: the biogeographic history of South America land mammals. *Annals of the Missouri Botanical Garden*, **93**, 209–230.
- Pascual R, Ortiz-Jaureguizar E (1990) Evolving climates and mammal faunas in cenozoic south America. *The Platyrrhine Fossil Record*, **19**, 23–60.
- Piperno DR (2006) Quaternary environmental history and agricultural impact on vegetation in Central America. *Annals of the Missouri Botanical Garden*, **93**, 274– 296.
- Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) *Tracer v1. 6*.
- Rannala B, Yang Z (2013) Improved Reversible Jump Algorithms for Bayesian Species Delimitation. *Genetics*, **194**, 245–253.
- Roca AL (2001) Genetic Evidence for Two Species of Elephant in Africa. *Science*, **293**, 1473–1477.
- Roca AL, Georgiadis N, O'Brien SJ (2004) Cytonuclear genomic dissociation in African elephant species. *Nature Genetics*.
- Rondinini C, Wilson KA, Boitani L, Grantham H, Possingham HP (2006) Tradeoffs of different types of species occurrence data for use in systematic conservation planning: Species data for conservation planning. *Ecology Letters*, **9**, 1136–1145.
- Sambrook J, Russel DW (2001) *Molecular Cloning: a laboratory manual, Cold Spring Hrbour Laboratory Press*. Cold Spring Harbour.
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, **74**, 5463–5467.
- Santos Júnior JE, Santos FR, Silveira FA (2015) Hitting an Unintended Target: Phylogeography of *Bombus brasiliensis* Lepeletier, 1836 and the First New Brazilian Bumblebee Species in a Century (Hymenoptera: Apidae) (S Brady, Ed,). *PLOS ONE*, **10**, e0125847.
- Smith P, Owen RD, Atkinson K, Castillo HD, Northcote-Smith E (2011) First Records of the Southern Naked-Tailed Armadillo *Cabassous unicinctus* (Cingulata: Dasypodidae) in Paraguay. *Edentata*, **12**, 53–57.
- Sosdian S, Rosenthal Y (2009) Deep-Sea Temperature and Ice Volume Changes Across the Pliocene-Pleistocene Climate Transitions. *Science*, **325**, 306–310.
- Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, **73**, 1162–1169.
- Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**, 978–989.
- Sukumaran J, Knowles LL (2017) Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences*, 201607921.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weight matrix choice. *Nucleic acids research*, **22**, 4673–4680.
- Vianna JA, Bonde RK, Caballero S *et al.* (2006) Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. *Molecular Ecology*, **15**, 433–447.
- Wetzel RM (1980) *Revision of the naked-tailed armadillos, genus Cabassous McMurtrie*. Carnegie Museum of Natural History.
- Wetzel RM (1982) Systematics, distribution, ecology, and conservation of South American Edentates. In: *Mammalian biology in South America, special publication series, Pymatuning laboratory of ecology.*, pp. 345–375. M. A. Mares & H. H. Genoways, University of Pitsburgh, Linesville.
- Wetzel RM (1985) Taxonomy and distribution of armadillos, Dasypodidae. *The evolution and ecology of armadillos, sloths, and vermilinguas*, 23–46.
- Wetzel RM, Gardner AL, Redford KH, Eisenberg JF (2007) Order Cingulata Illiger, 1811. In: *Mammals of South America*, University of Chicago Press, Chicago.
- Williams PH, Margules CR, Hilbert DW (2002) Data requirements and data sources for biodiversity priority area selection. *Journal of biosciences*, **27**, 327–338.
- Woodburne MO, Goin FJ, Bond M *et al.* (2014) Paleogene Land Mammal Faunas of South America; a Response to Global Climatic Changes and Indigenous Floral Diversity. *Journal of Mammalian Evolution*, **21**, 1–73.
- Yang Z (2015) The BPP program for species tree estimation and species delimitation. *Current Zoology*, **61**, 854–865.
- Yang Z, Rannala B (2010) Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences*, **107**, 9264–9269.
- Zachos J (2001) Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science*, **292**, 686–693.

Supporting information

Table S1: Accession numbers of sequences retrieved from Genbank.

Appendix

count	fraction	similarity	nclusters
44900	0.897982	44900	5
1191	0.02382	1191	6
709	0.01418	709	6
529	0.01058	529	6
429	0.00858	429	6
354	0.00708	354	6
203	0.00406	203	4
157	0.00314	157	6
131	0.00262	131	6
122	0.00244	122	6

Appendix 1: The 10 best results of STACEY.

Appendix 2: Results of BPP analysis.

Cu1 (34) Cta (10) Cus (56) Cu2 (8) Cc (2)

Current Pjump: 0.72535 0.00372 0.02120 0.00000 0.85700 0.92233 Current finetune: 0.01000 0.01000 0.01000 0.01000 0.01000 0.01000 New finetune: 0.04263 0.00011 0.00065 0.00010 0.08590 0.16007

Current Pjump: 0.67836 0.25038 0.26468 0.20358 0.35300 0.48740 Current finetune: 0.04263 0.00011 0.00065 0.00010 0.08590 0.16007 New finetune: 0.15128 0.00009 0.00057 0.00006 0.10441 0.30196

Current Pjump: 0.67312 0.30865 0.32233 0.57975 0.29700 0.29493 Current finetune: 0.15128 0.00009 0.00057 0.00006 0.10441 0.30196 New finetune: 0.52650 0.00010 0.00062 0.00016 0.10319 0.29605

Current Pjump: 0.67406 0.30522 0.30989 0.22975 0.25400 0.29773 Current finetune: 0.52650 0.00010 0.00062 0.00016 0.10319 0.29605 New finetune: 1.83878 0.00010 0.00064 0.00012 0.08538 0.29345

Summarizing the species-delimitation sample in file Cabassousmcmc.txt

[Note: Ancestral nodes in order: 6 Cu2CcCu1CtaCus 7 Cu2CcCu1 8 Cu2Cc 9 CtaCus]

Guide tree with posterior probability for presence of nodes (((Cu2, Cc)#0.99996, Cu1)#1, (Cta, Cus)#1)#1;

Capítulo 3

Time scaled phylogeography and demography of *Bradypus torquatus* **(Pilosa: Bradypodidae)**

*Marco Antônio Alves Schetino, Raphael Teodoro Franciscani Coimbra, and Fabrício Rodrigues Santos**

Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Belo Horizonte, Minas Gerais, Brazil (MAAS, RTFC, FRS)

Abstract

The maned sloth *Bradypus torquatus* (Bradypodidae:Pilosa) is an endemic species of the Atlantic Forest in Brazil, which is endangered particularly because its biome was anthropogenically reduced to about 7% of its original extent. Nowadays, an apparently decreasing population is restricted to remaining rainforest fragments. We analyzed nuclear and mitochondrial genes genes of 69 individuals from the states of Bahia, Espírito Santo and Rio de Janeiro to estimate their current and historical population dynamics. The history of the diversification of *B. torquatus* populations was mainly led by dispersal and vicariant events occurring during Pliocene and Pleistocene due to several climatic and vegetation changes. Besides, the current distribution of the three identified populations was also affected by recent anthropogenic deforestation occurring in the last five centuries in Brazil, which likely resulted in local extinction of many Atlantic Forest populations. We suggest that in the Pliocene, an ancestral population of *B. torquatus* was originally located in the central region of the current Atlantic Forest between BA and ES states and dispersed northwards and southwards to its current range. Our results suggest that the northern and southern lineages of the Atlantic Forest should have particular management plans and conservation policies due to their history of ancient isolation and evolutionary independency.

Running title: Phylogeography of *Bradypus torquatus*.

Keywords: Atlantic Forest, maned sloth, Evolutionary Significant Units, Phylogeny, Phylogeography, Population structure.

Introduction

The maned sloth *Bradypus torquatus*, Illiger, 1811 (Pilosa: Bradypodidae) is endemic to the Atlantic Forest, occurring exclusively in northeastern and southeastern coastal rainforests of Brazil, between the states of Sergipe and Rio de Janeiro, although there are unconfirmed records of the species in the states of Minas Gerais and Pernambuco (Fonseca & Aguiar 2004; Gardner 2007; Hayssen 2009; Chagas *et al.* 2009; Hirsch & Chiarello 2012). *Bradypus torquatus* distinguishes from other sloths of the Bradypodidae family mainly by the absence of a speculum and a long dark coat in the base of the neck, which projects on the shoulders and stands out from the rest of the body coat (Wetzel & Avila-Pires 1980; Gardner 2007; Hayssen 2009). Because of its distinct anatomical and genetics features, some authors consider *B. torquatus* belonging to their own genus *Scaeopus* (Wetzel & Avila-Pires 1980; Barros *et al.* 2008), while others consider it as a subgenus of *Bradypus* (Wetzel 1982; Gardner 2007; Hayssen 2009). There are no fossil records for Bradypodidae, which makes hard dating phylogenetic reconstructions and understanding its origin and diversification. Anyway, current molecular phylogenies indicate that the *B. torquatus* lineage separated about 19 MYA from other species of the genus, which diversified into other three species at about 7.7 MYA (Gibb *et al.* 2016).

According to the IUCN (Chiarello & Moraes-Barros 2014), *B. torquatus* is the most endangered species among the continental sloths, with a risk of extinction categorized as vulnerable (VU). Because of hunting pressure and the constant deforestation and fragmentation to make way for cattle pasture and plantations of sugar cane, cocoa, coffee and eucalyptus, the Atlantic Forest biome was reduced to about 7% of its original range, and *B. torquatus* was considerably affected by the reduction of their habitat, mainly by loss of the original forest cover, and increase in exposure to hunters, predators and diseases (Thornback & Jenkins 1982; Oliver & Santos 1991; Chiarello *et al.* 2004). Currently, the remaining coastal forest fragments where *B. torquatus* individuals occur are extremely isolated. For example, the rainforests of southern Bahia and central Espírito Santo states are disconnected between the valleys of the rivers Mucuri and Doce, as well as between Espírito Santo and Rio de Janeiro states (Oliveira-Filho *et al.* 2005; Carnaval & Moritz 2008).

This lack of connectivity between forest fragments associated with *B. torquatus* habits like solitary life, diet of specific plants and low dispersion capacity (Chiarello 1998a; b; Chiarello *et al.* 2004; Lara-Ruiz & Chiarello 2005) should result in significant changes in the population dynamics of the maned sloth, leading to loss of genetic diversity and increased inbreeding (Lara-Ruiz *et al.* 2008). In medium and long term, these factors associated to the lack of habitat may reduce the reproductive success and capacity of the populations to adapt to environmental changes (Lynch & Lande 1998; Frankham 2003; Tabarelli *et al.* 2005).

Understanding the degree of genetic differentiation of these populations can be critical to determine the practice of conservation and which measures should be taken in favor of their "in situ" management (Chiarello 1999; Crandall *et al.* 2000; Moritz 2002; Lara-Ruiz *et al.* 2008). Therefore, it is necessary to conduct studies to broaden our knowledge on the population dynamics and the historical and anthropogenic factors behind their current diversity and distribution. In this regard, population genetics and phylogeographic analyses allow us to understand the processes that govern the geographical distribution and mold the evolutionary history of these lineages over time (Avise *et al.* 1987; Frankham 2003).

A detailed mtDNA study (Lara-Ruiz *et al.* 2008) in *B. torquatus* showed a population structure divided into three lineages occupying disjunct segments of the Atlantic Forest: Northern (Bahia State, BA) and Southern (Espírito Santo, ES and Rio de Janeiro, RJ states). These mtDNA lineages showed high genetic divergence among regions but low intrapopulation diversity, with a regional divergence between RJ and ES states (Moraes-Barros *et al.* 2006; Lara-Ruiz *et al.* 2008; Moraes-Barros & Arteaga 2015). Also, according to the molecular data, independent of human actions on the biome, this high divergence between different regions appears to be resulted from past dispersal events associated to historical vicariance, likely due to climatic and vegetation changes that took place during the Pleistocene (Lara-Ruiz *et al.* 2008). Although enlightening, previous analyses used only mitochondrial markers (mtDNA) without a proper phylogenetic timeframe approach, which may not reconstruct effectively the demographic history of the species (Ballard & Whitlock 2004; Hurst & Jiggins 2005).

In order to expand our knowledge on the historical population dynamics of *B. torquatus* we analyzed a new set of mitochondrial and nuclear genes in a time scaled phylogeographic analysis. Results allowed to estimate their population structure and dynamics through time, to date divergence of inter and intraspecific lineages and, finally, to reconstruct their ancestral range based on the phylogeny obtained here.

Methods

Sampling & DNA extraction

We extracted DNA of previously collected (Lara-Ruiz *et al.* 2008) liver, muscle or blood samples of 69 individuals of *B. torquatus* from nine counties (Fig. 1) of the Brazilian states of Bahia (BA, $n = 20$), Espírito Santo (ES, $n = 46$) and Rio de Janeiro (RJ, $n =$ 3). We also extracted the genomic DNA of two individuals of *B. variegatus* from the state of Pará (Altamira) by standard phenol-chloroform protocol (Sambrook & Russel 2001). The samples are deposited in the Centro de Coleções Taxonômicas of the Universidade Federal de Minas Gerais (CCT-UFMG). A list of sampling localities is available in Table 1.

Table 1: Sampling localities, geographic coordinates (decimal degrees) and number of samples (N).

County	Map Localities	State	Latitude	Longitude	N
Mata de São João	A	BA	-12.50	-38.05	6
Pratigi	B	BA	-13.65	-39.04	1
Una	C	BA	-15.14	-39.08	13
Itarana	D	ES	-19.87	-40.87	1
Santa Maria de Jetibá	Е	ES	-20.03	-40.70	18
Santa Teresa	F	ES	-19.93	-40.59	13
Aracruz	G	ES	-19.79	-40.20	14
Silva Jardim	н	RJ	-22.62	-42.43	1
Cassimiro de Abreu		RJ	-22.55	-42.28	2

Figure 1: Sampling localities of *B. torquatus* and the mitochondrial haplotype network constructed with the median-joining algorithm. Black haplotipes = Bahia; White haplotipes = Espírito Santo; and gray haplotipes = Rio de Janeiro. The letters refer to the localities in table 1.

PCR & DNA sequences

We analyzed the mitochondrial genes (mtDNA) Cytochrome b (*Cyt-b*), Cytochrome c Oxidase subunit I (*COI*) and Control region (CR), and three nuclear genes (nuDNA): von Willebrand Factor (*vWF*, exon 28), Apolipoprotein B (*ApoB*, exon 26) and Adrenoceptor Beta 2 (*ADRB2*). All loci were amplified with Platinum *Taq* DNA Polymerase kit using specific primers and annealing temperatures for each PCR fragment (Table 2) following the manufacturer's recommendations (Thermo Fisher Scientific). PCR products were confirmed by electrophoresis in 1% agarose gel and purified with 20% polyethylene glycol in 2.5 M NaCl (Santos Júnior *et al.* 2015). The sequencing was carried out with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in an ABI 3130xl Genetic Analyzer system (Applied Biosystems, Foster City, CA). The fluorograms were analyzed in the SeqScape 2.6 software (Applied Biosystems, Foster City, CA). The consensus sequences were aligned using the Clustal W algorithm (Thompson *et al.* 1994) implemented in the MEGA 7 software (Kumar *et al.* 2016). The identification of the haplotype phases for subjects with multiple heterozygous sites was performed with the PHASE 2.0 program (Stephens *et al.* 2001; Stephens & Donnelly 2003), which is a Bayesian method for inferring haplotypes through coalescence included in the software DNAsp 5.1 (Librado & Rozas 2009).

Table 2: Primers used for partial amplification of *B. torquatus* genes, PCR annealing temperatures and respective references. Asterisks represent primers developed in our laboratory. LBEM = Laboratório de Biodiversidade e Evolução Molecular.

Population analyses

The population analyses were performed using all sequences of *B. torquatus* obtained in the previous article by Lara-Ruiz *et al.* (2008) and in the present work. To estimate the number of populations and their geographical boundaries we used a clustering approach of the Geneland 3.1.4 package (available for the R software) in two separate runs considering spatial and non-spatial model, with calculations performed through the Markov Chain Monte Carlo (MCMC) algorithm (Guillot *et al.* 2008). The statistics describing the genetic and molecular variability found among populations such as the haplotype diversity (Hd), nucleotide diversity (pi), haplotype number and number of polymorphic sites were calculated in the DNAsp 5.1 software (Librado & Rozas 2009), which was also used to perform the Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) neutrality tests. To assess past demographic patterns in the populations defined by the Geneland 3.1.4 we utilized the Extended Bayesian Skyline Plot (EBSP) method, which was performed in the *BEAST 2.3 program (Bouckaert *et al.* 2014) and visualized with the R software.

Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) and Mantel tests (Mantel 1967) were both performed in the Arlequin 3.5 software (Excoffier & Lischer 2010). They were used respectively to quantify the distribution of the genetic variation at different hierarchical levels and to test the hypothesis of isolation by distance. Medianjoining haplotype networks (Bandelt *et al.* 1999) were constructed based on the concatenated sequences of mtDNA and for each one of the nuDNA genes using the software NETWORK 5.0 (Fluxus Engineering) as an alternative to visualize parsimony relationships between haplotypes and their geographical regions (Bandelt *et al.* 1995).

Divergence time estimates and reconstruction of ancestral states

To obtain the divergence times of the Bradypodidae lineages and the main intraspecific *B. torquatus* lineages, two separate analyses were performed in the *BEAST 2.3 program, both of which did not include mtDNA CR sequences. In both analyses we used a reversible-jump based substitution model implemented in BEAST 2.3 through the RBS 1.3.1 package (Bouckaert *et al.* 2013), which allows gamma rate heterogeneity and invariant sites.

First, a Pilosa tree was constructed using individuals with unique combinations of the mtDNA and nuDNA haplotypes of *B. torquatus* and *B. variegatus*, and sequences of *B. tridactylus* (JN633662, JN413959, U31603 and KT818525), *Choloepus hoffmanni* (AY011252, JN413958, JN415027 and KR336793), *Choloepus didactylus* (AY011253, AJ278160 and KT818537), *Myrmecophaga tridactyla* (AY011257, JN413960, AJ278157

and NC_028572), *Tamandua tetradactyla* (AY011256, AF548426, AJ278161 and KT818552) and *Cyclopes didactylus* (JN633663, JN413961, AJ278156 and NC_028564) retrieved from Genbank. We have chosen a birth-death tree prior combined with soft fossil calibration constraints (Yang 2005) using three calibration intervals for Xenarthra: Pilosa (65.5–31.5 MYA), Folivora (40.6–15.97 MYA), and Vermilingua (61.1– 15.97 MYA) (Meredith *et al.* 2011; Gibb *et al.* 2016). Second, a new analysis was performed including only *B. torquatus* individuals with unique haplotypes from each sampling locality. For that, we used a Coalescent Constant Population tree prior and a calibration constraint correspondent to the divergence time of the two main lineages of *B. torquatus* obtained in the previous Pilosa tree. For both analyses for Pilosa and *B. torquatus* trees, three independent MCMC chains of 300,000,000 generations, sampled every 5,000 generations were carried out on the CIPRES Science Gateway v.3.3 (Miller *et al.* 2010). The effective sample sizes (ESS) for each run were checked in Tracer v. 1.6 (Rambaut *et al.* 2014). The tree files were combined in LogCombiner and summarized in TreeAnnotator, both available in the BEAST 2.3 package, to obtain the best 10,000 trees.

Furthermore, we used the *B. torquatus* trees to perform the ancestral range estimation in BioGeoBears (Matzke 2012) and RASP (Yu *et al.* 2015). BioGeoBears tests the best model of estimation only for the consensus tree, thus an additional analysis was performed in RASP, which uses multiple trees generated by *BEAST. We split our samples geographically in four areas: Espírito Santo, Rio de Janeiro, southern Bahia, and northern Bahia in order to test which biogeographical event would be related to the differences found in these regions. The best model selected by BioGeoBears to explain the historical biogeographic processes occurring among *B. torquatus* lineages was DIVALIKE, which reconstructs the ancestral distribution based on a three-dimensional cost matrix, where events such as dispersion and extinction have greater penalties than events of vicariance (Ronquist 1997). Because DIVALIKE model favors vicariance events, two analyses were performed in RASP and compared. The first used the S-DIVA model (Yu *et al.* 2010) and the second used the S-DEC model (Beaulieu *et al.* 2013), which differ between them about the probabilities of dispersion according to a certain period of time.

Results

A total of 4005 base pairs (bp) of 69 *B. torquatus* samples (630 bp of *COI*, 690 bp of *CytB*, 369 bp of *CR*, 996 bp of *ApoB*, 768 bp of *ADRB2* and 552 of *vWF*) was obtained. The sequences were deposited in Genbank (see Table S1 in Supporting Information). The Geneland analysis identified three populations in both spatial and non-spatial models, which we named BA (Bahia), ES (Espírito Santo) and RJ (Rio de Janeiro). The geographical regions estimated for each population in the spatial model presented posterior probabilities around 60% and none of them overlap (Fig. S1, Supporting information).

The number of polymorphic sites and haplotypes, and the values of haplotype and nucleotide diversities are shown in table 3. The ES population exhibited the largest number of nuclear haplotypes, while BA population had the highest number of mitochondrial haplotypes. Only one haplotype each of *ApoB* and *vWF* nuclear genes were shared between ES and BA, and ES and RJ, respectively (Fig. S2, Supporting Information). All mitochondrial haplotypes were unique to each population (Fig. 1).

Table 3: Genetic and molecular diversity indices of population of *B. torquatus*. Number of polymorphic sites (S), number of haplotypes (NH), haplotype diversity (Hd) and nucleotide diversity (π).

The results of Tajima's D and Fu's F_s neutrality tests were not significant and the EBSP did not detect any changes in effective population sizes for any identified population or the set of all of them during the last 5 MYA, suggesting a stationary effective population size over time (data not shown). The φ_{ST} analyses obtained with population pairwise matrices and AMOVA tests showed high levels of differentiation among the three populations in both mtDNA and nuDNA (Table 4 and S2, Supporting information) with significant *p-values* (p<0.01). Additionally, the genetic differences among populations were greater than within populations. Mantel's tests showed no significant results (p>0.05) indicating that geographic distance between populations is not the only factor to explain the pattern of genetic differentiation observed.

Table 4: Percentage of molecular variation among and within populations of *B. torquatus* obtained in the AMOVA (mtDNA/nuDNA).

Source of variation	Percentage of variation
Among populations	98.29/83.7
Within populations	1.71/16.3

The estimated divergence times between clades of Pilosa are shown in table 5 and figures 2 and 3. All major clades analyzed showed maximum posterior values in a topology similar to that found on others xenarthran studies (Lara-Ruiz *et al.* 2008; Delsuc *et al.* 2012; Moraes-Barros & Arteaga 2015; Gibb *et al.* 2016). The two major clades (BA and ES+RJ) of *B. torquatus* described by Lara-Ruiz *et al*. (2008) were maintained and had its divergence occuring between the Late Miocene and Early Pliocene. The *B. torquatus* populations of ES and RJ diverged some 390 KYA, while the northern and southern clades of the Bahia population diverged about 210 KYA, in the Late Pleistocene. The two analyses performed in RASP presented the same result and indicate that the cladogenetic events generating the four lineages observed were due to dispersal and vicariant processes (Fig. 3). Both programs for reconstruction of ancestral areas suggest that an ancestral lineage of *B. torquatus* occupied an area between southern Bahia and central region of Espírito Santo up to 5.36 MYA (Pliocene) and then spread northwards to northern Bahia (and likely Sergipe) and southwards to Rio de Janeiro. RASP results agree well with the phylogenetic reconstructions (Figs. 2 and 3), showing a first split of the maned sloth ancestral lineage into the Northern and Southern Atlantic Forest clades, which later subdivided in the ES and RJ lineages in the south, and the southern BA and northern BA lineages in the north (Fig. 3).

Figure 2: Molecular time scale for *Bradypus torquatus* and Pilosa nodes inferred with a Bayesian phylogeny obtained in BEAST. Node ages were obtained under the reversiblejump based substitution model, allowing gamma rate heterogeneity and invariant sites, and a birth-death tree prior with soft fossil calibrations. Node bars indicate the 95% HPD intervals for age estimates in million years ago (MYA). All nodes were supported by maximum posterior probabilities.

Figure 3: The divergence lineages *B. torquatus* tree. The biogeographical range estimation was inferred under the S-DIVA model with the RASP software. All nodes were supported by maximum posterior probabilities. The letters A, B and C represent the ancestral range estimate. Legends: $A = BA + ES + RJ$ range populations; $B = ES + RJ$ range populations; $C = BA$ range population; $1 = Disperson$ event; $2 = Vicariant$ event.

Table 5: Divergence time estimates with 95% confidence intervals for all Pilosa nodes.

Discussion

Population dynamics and lineage divergence in B. torquatus

Our molecular analyses reassure the distribution of *B. torquatus* individuals into two major genealogical lineages as suggested in Lara-Ruiz *et al.* (2008). These lineages correspond to populations occurring in RJ, BA and ES states, which exhibit a high degree of genetic differentiation between each other. AMOVA demonstrated that most part of the genetic variation is due to differences between populations. The φ_{ST} values obtained for the mtDNA and nuDNA were similar to those found in other studies of arboreal xenarthran species like other sloths of the genus *Bradypus* (Moraes-Barros *et al.* 2006, 2007; de Moraes-Barros *et al.* 2011) and the smallest of the anteaters *Cyclopes didactylus* (Coimbra *et al.* 2017). However, when compared to terrestrial dweller species of the same Magnorder (Arteaga *et al.* 2012; Nardelli *et al.* 2016; Clozato *et al.* 2017), the obtained values could be considered very high. This suggests a likely trend in which ecological habits strongly influence the population structure in Xenarthra.

While mtDNA lineages are exclusive of each population, some nuclear genes presented shared haplotypes between populations, which are likely explained by incomplete coalescence and maintenance of plesiomorphic characters due to higher effective sizes of nuclear diploid loci (Birky *et al.* 1989). Alternatively, males sharing the nuDNA haplotypes could have dispersed before the events that caused the split of the *B. torquatus* lineages promoting gene flow. Even though this later alternative is unlikely, a high dispersal for males is expected in sloths, as females remain longer periods in a given location to take care of their young and teach them how to obtain food (Montgomery & Sunquist 1978; Chiarello 1998a), which tends to maintain phylopatric mtDNA lineages (Lara-Ruiz *et al.* 2008). Anyway, when nuDNA data is analyzed together, all nuclear haplotype combinations become unique to each population, including the RJ population despite its scarce sample size.

The absence of a significant signal for population size change in the neutrality tests and EBSP analyses suggests that the *B. torquatus* populations were likely to have persisted stable until anthropogenic deforestation and fragmentation of the Atlantic Forest in the last five centuries, leading to a significant population decline of the species (Chiarello & Moraes-Barros 2014).

Divergence times in Pilosa and phylogeographic patterns in *B. torquatus*

Our results showed mean node ages slightly different for some of the clades within Pilosa when compared to previous works (Delsuc *et al.* 2001, 2004, 2012; Moraes-Barros & Arteaga 2015; Gibb *et al.* 2016), although 95% HPD intervals for each estimated time were similar and overlapped. These discrepancies may be related to differences in the molecular dating method, fossil calibration and sample set and size used in each work. The deep divergence times between clades are associated with major tectonic and climatic events that influenced the evolutionary processes of the South American fauna.

According to our estimated dates, the Folivora and Vermilingua orders became separated in the Middle Eocene. This was a period of sharp decrease in global temperature after the Eocene Climatic Optimum and intense orogenetic activity in the Andes (Incaic uplift event) that culminated in regional climatic changes, alteration of the vegetation of the south of the continent with the increase in grasslands, and restructuring of the watersheds in the northwest of the continent (Pascual & Ortiz-Jaureguizar 1990, 2007; Zachos 2001; Delsuc *et al.* 2004; Pascual 2006; Sepulchre *et al.* 2010; Hoorn *et al.* 2010). The Cyclopedidae and Myrmecophagidae families diverged in the Late Eocene–Early Oligocene boundary. At that time, the formation of the Antarctic ice cap and the complete opening of the Drake Passage contributed to the establishment of new ocean currents, which decreased the continental temperature and humidity resulting in the expansion of grasses to the tropical and subtropical regions and consequently the increase in open areas (Pascual & Ortiz-Jaureguizar 1990, 2007; Zachos 2001).

The two main lineages of extant sloths, *Choloepus* and *Bradypus*, diverged in the Late Oligocene–Early Miocene boundary, when the global temperature increased and the Andean Cordillera was becoming the major component of the South American landscape causing greater impact on the climate throughout the continent (Zachos 2001; Delsuc *et al.* 2004; Sepulchre *et al.* 2010). Furthermore, the drainage of the Amazon Basin was already in development in the east of the continent, shaping the precursor of the Lower Amazon River, while in intermediate altitudes, the savanna presented a balance between forests and open areas making it possible the emergence of new arboreal mammal species (Pascual & Ortiz-Jaureguizar 1990; Pascual 2006; Hoorn *et al.* 2010). During the Middle Miocene, two cladogenetic events occurred within Pilosa. The first separated the lineages that would originate the *Myrmecophaga* and *Tamandua* genera, whereas the second is related to the divergence between the ancestral lineages of *B. torquatus*, and *B. variegatus* and *B. tridactylus*. That period was marked by the occurrence of the Middle Miocene Climatic Optimum, the start of the aridification of the south of the continent, successive marine transgressions of the Atlantic, and the formation of the Pebas system (Pascual & Ortiz-Jaureguizar 1990; Zachos 2001; Wesselingh & Salo 2006; Ortiz-Jaureguizar & Cladera 2006; Hoorn *et al.* 2010). Our findings support the suggestion made by Moraes-Barros & Arteaga (2015) in which the Pebas system together with the Andean uplift favored the isolation and diversification of the *Bradypus* lineages in the northern Amazon region, while the remnants of land connections to the south of this system allowed for dispersion of the ancestral of *B. torquatus* to the east towards the Atlantic Forest. In addition, the antiquity of the divergence between *B. torquatus* lineages and other Bradypodidae are congruent with the idea that the former species can be categorized in its own genus, *Scaeopus*, as suggested by other molecular studies (Barros *et al.* 2008; Gibb *et al.* 2016). With the exception of *B. torquatus* and *B. pygmaeus* (not included in this work), at the end of the Miocene occurred the diversification of the lineages that originated the current species of sloths (*B. tridactylus* + *B. variegatus* and *Choloepus hoffmani* + *Choloepus didactylus*). In that period, the formation of lacustrine regions (Acre system), the onset of the Amazon River, the uplift of the Panamanian Isthmus increasing the biotic interchange between North and South Americas, the climatic influences from the second major Bolivian tectonic crisis, and the expansion of the Cerrado and formation of the Caatinga triggered great changes in the continental biota (Pascual & Ortiz-Jaureguizar 1990; Morley 2000; Costa 2003; Delsuc *et al.* 2004; Hoorn *et al.* 2010; Zanella 2011; Montes *et al.* 2015; Bacon *et al.* 2016).

Moreover, at the Late Miocene–Early Pliocene transition, the *B. torquatus* lineages from the north and south of the Atlantic Forest diverged. In this period, coastal mountain epirogenic events in eastern South America, associated with a decrease in global temperature and continental humidity has substantially altered the topography and climate of the region, which resulted in vegetation changes and fragmentation of the biome with the development of semiarid areas in between (Almeida 1976; Petri & Fúlfaro 1983; Vasconcelos *et al.* 1992; Morley 2000; Zachos 2001), favoring the diversification of various vertebrates (Lara *et al.* 1996; Mustrangi & Patton 1997; Lara & Patton 2000; Leite 2003; Grazziotin *et al.* 2006). This result is similar to those found by Moraes-Barros & Arteaga (2015) and indicates that the diversification of *B. torquatus* occurred before the date suggested by Lara-Ruiz *et al.* (2008) in which the first *Bradypus* split occurred during the Early Pleistocene.

Around 390 KYA, the *B. torquatus* lineage located in the southern Atlantic Forest diverged into two sublineages currently represented by the ES and RJ populations. More recently, around 210 KYA, two maned sloth sublineages became separated within the Atlantic Forest of Bahia, and we call them northern BA and southern BA sublineages. Pleistocene glaciation cycles occurring in a short time span mark this period and may have caused the expansion and contraction of forested areas in these regions (Hewitt 2000; Ortiz-Jaureguizar & Cladera 2006).

The large time interval found between the two main diversification moments in *B. torquatus* may be related to the different periods in which they occurred. When compared, the Pliocene had longer periods of climatic stability, whereas the Pleistocene had frequent glaciations that, around 900 KYA, had its cycle period increased from 40 KYA to 100 KYA, probably causing greater impact in biome and taxa distributions (Pascual & Ortiz-Jaureguizar 1990; Hewitt 2000; Zachos 2001; Ortiz-Jaureguizar & Cladera 2006). A similar diversification pattern in the Atlantic Forest is found for the frog species *Rhinella crucifer* (Thomé *et al.* 2010) and reinforces the idea that not only the Pleistocene, but also the Pliocene, was an important period for the diversification in this biome (Turchetto-Zolet *et al.* 2013). Although they occurred close in the Late Quaternary, the diversification events of *B. torquatus* in the Southern Atlantic Forest gave rise to two sublineages currently represented today by distinct populations (ES and RJ), while in the north of this biome we observed the divergence of other sublineages (northern BA and southern BA), which still remain as part of a single population defined by Geneland. This can be attributed to the fact that the Northern Atlantic Forest was climatically stable when compared to the Southern region, which faced perturbations that caused greater impact in the species distributions (Carnaval *et al.* 2009). Furthermore, between ES and RJ sublineages there are environmental gaps occupied by semideciduous forests (Oliveira-Filho *et al.* 2005), which may directly interfere with the presence of plants that constitute the restrict diet of *B. torquatus* (Chiarello 1998b; Hirsch & Chiarello 2012). Additionally, the valley of Paraíba do Sul River may further separate populations from RJ and ES, acting as a gene flow barrier as it was show in the delimitation of populations and species like the bird *Lepidocolaptes squamatus* (Silva & Straube 1996), the rodent *Euryoryzomys russatus* (Miranda *et al.* 2007; Libardi & Percequillo 2016) and the distribution of primates *Brachyteles hypoxantus* e *Brachyteles arachnoides* (Ingberman *et al.* 2016). On the Northern Atlantic Forest, the Paraguaçu River, one of the main rivers that flow into the Baía de Todos os Santos (Bahia state), separates the regions where northern BA and the southern BA lineages of *B. torquatus* are located. Indeed, this river barrier was also suggested to be a main cause structuring populations of a gecko *Gymnodactylus darwinii* (Pellegrino *et al.* 2005) and a plant species *Passiflora contracta* (Cazé *et al.* 2016).

Although the events previously cited may be associated with the population genetic structure of the main lineages of *B. torquatus*, we believe that the current distribution of the three observed populations is mainly related both to dispersal and vicariant events since the Late Pleistocene and the recent anthropogenic deforestation in the Holocene. The Atlantic Forest is thought to have suffered many retractions, particularly during a cold and dry period around 21 KYA, which corresponds to the Last Glacial Maximum (LGM). With the increase in temperature and humidity in the Holocene, the Atlantic Forest has expanded again (Behling 2002; De Vivo & Carmignotto 2004; Carnaval & Moritz 2008). Furthermore, it is also expected that many other *B. torquatus* lineages were recently extinct due to the unbridled deforestation of the Atlantic Forest since the 16th century. This likely resulted in the loss of a significant part of the evolutionary history of the species, increasing the vicariant effect observed among remaining populations. Anyway, new field expeditions are needed to increase knowledge on the species range.

In our reconstruction of the *B. torquatus* ancestral distribution area we did not find a pattern of historical distribution directed by dispersal towards the southern Atlantic Forest suggested by Lara-Ruiz *et al.* (2008). Our data suggest that a *B. torquatus* lineage was present at around 5 MYA in an area between southern Bahia and central the Espírito Santo states, from where there was dispersion towards the north and south of the Atlantic Forest. This range dispersal was also influenced by vicariant events promoted by climatic and geographic factors originating Southern and Northern Atlantic Forest lineages, and their current sublineages. These results are in agreement with the lack of significance of the Mantel test result, indicating that the observed genetic structure cannot be explained only by isolation due to geographic distance between the current representatives of each lineage.

Conservation and management of B. torquatus

Crandall *et al.* (2000) categorized various types of populations according to a scheme that considers genetic and ecologic processes (historical and recent) interfering directly in the populations' connectivity. Based on that scheme, our results suggest that at least the BA and ES+RJ maned sloth populations should be considered two ESUs (Evolutionary Significant Units; *sensu* Moritz 1994) and should have their management and conservation plans elaborated separately, as if they were different species (Case 1). Indeed, in a previous study based on *B. torquatus* habitats and climatic conditions, Moreira *et al.* (2014) also suggest that its populations should be divided for conservation purposes into southern (Rio de Janeiro and Espírito Santo) and northern (Bahia and Sergipe) units.

Considering the remarkable population genetic structure in *B. torquatus*, which is perhaps one of the most important aspects in population management for conservation purposes (Crandall *et al.* 2000), other population divisions should be also considered. Although the ES and RJ populations are included within this criterion, more individuals from the different areas of ES and RJ populations are needed to verify the boundaries of the genetic discontinuity. Even though Lara-Ruiz *et al*. (2008) indicate a possible ecological difference between high altitude and sea level population in the ES state, we suggest the maintenance of the conditions that allow for the current level of gene flow among the individuals of the ES population (Case 5). Such proposition is made considering the lack of evidence, both historical and recent, for disruption of gene flow and, even though there is a weight variation between the regions (Lara-Ruiz & Chiarello 2005), which may be most likely due to phenotypic plasticity and not a case of long term adaptation (Hammond *et al.* 2001). Currently we find maned sloths from elevated areas isolated from sea level populations (tabuleiro) because of recent anthropic deforestation. Even though our Geneland analysis does not identify northern and southern populations of Bahia as clearly separated units, phylogenetic data clearly indicates two sublineages, although not as differentiated as ES and RJ populations.

Some of the main goals of conservation are the maintenance of the evolutionary processes and viability of the species together with the preservation of functional landscapes (Moritz 2002). To achieve this, it is fundamental the maintenance of fitness and adaptive capacity of local populations to environmental changes (Moritz 1994; Lynch & Lande 1998). Thus, the most important measures to be taken involve the preservation of the *B. torquatus* occurrence areas (Moreira *et al.* 2014) and the use of management actions regarding the observed genetic structure of populations. A substantial parcel of the species individuals is found within Conservation Units (Chiarello 1998a), but the deforested area between the fragments of Atlantic Forest leave them exposed to various threats in the moment they move from one region to the other (Chiarello *et al.* 2004). Considering these facts, expanding the areas of existing Conservation Units or creating new ones, and establishing favorable conditions for the dispersion between landscapes (corridors) are necessary (Moreira *et al.* 2014). One example is the introduction of agroforestry systems in farming areas. The *cabrucas* system from southern Bahia, aside from mitigating the effects of fragmentation, could act as important wildlife corridors for *B. torquatus* in already deforested areas (Cassano *et al.* 2011).

Future management practices like translocation or reintroduction must only be attempted if regarding the local evolutionary history and the origin of each individual, promoting the conservation of population dynamics that has driven the evolution of the maned sloths for millions of years.

References

- Almeida F (1976) System of continental rifts bordering Santos Basin, Brazil. *Anais da Academia Brasileira de Ciências*, **48**, 15–26.
- Arteaga MC, Piñero D, Eguiarte LE, Gasca J, Medellín RA (2012) Genetic structure and diversity of the nine-banded armadillo in Mexico. *Journal of Mammalogy*, **93**, 547–559.
- Avise JC, Arnold J, Ball RM *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual review of ecology and systematics*, 489–522.
- Bacon CD, Molnar P, Antonelli A *et al.* (2016) Quaternary glaciation and the Great American Biotic Interchange. *Geology*, **44**, 375–378.
- Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729–744.
- Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, **16**, 37–48.
- Bandelt H-J, Forster P, Sykes BC, Richards MB (1995) Mitochondrial portraits of human populations using median networks. *Genetics*, **141**, 743–753.
- Barros MC, Sampaio I, Schneider H (2008) Novel 12S mtDNA findings in sloths (Pilosa, Folivora) and anteaters (Pilosa, Vermilingua) suggest a true case of long branch attraction. *Genetics and Molecular Biology*, **31**, 793–799.
- Beaulieu JM, Tank DC, Donoghue MJ (2013) A Southern Hemisphere origin for campanulid angiosperms, with traces of the break-up of Gondwana. *BMC evolutionary biology*, **13**, 80.
- Behling H (2002) South and southeast Brazilian grasslands during Late Quaternary times: a synthesis. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **177**, 19–27.
- Birky CW, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics*, **121**, 613–627.
- Bouckaert R, Alvarado-Mora MV, Pinho JR (2013) Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. *Antivir Ther*, **18**, 497–503.
- Bouckaert R, Heled J, Kühnert D *et al.* (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis (A Prlic, Ed,). *PLoS Computational Biology*, **10**, e1003537.
- Carnaval AC, Hickerson MJ, Haddad CFB, Rodrigues MT, Moritz C (2009) Stability Predicts Genetic Diversity in the Brazilian Atlantic Forest Hotspot. *Science*, **323**, 785-789.
- Carnaval AC, Moritz C (2008) Historical climate modelling predicts patterns of current biodiversity in the Brazilian Atlantic forest. *Journal of Biogeography*, **35**, 1187– 1201.
- Cassano CR, Kierulff MCM, Chiarello AG (2011) The cacao agroforests of the Brazilian Atlantic forest as habitat for the endangered maned sloth *Bradypus torquatus*. *Mammalian Biology - Zeitschrift für Säugetierkunde*, **76**, 243–250.
- Cazé ALR, Mäder G, Nunes TS *et al.* (2016) Could refuge theory and rivers acting as barriers explain the genetic variability distribution in the Atlantic Forest? *Molecular Phylogenetics and Evolution*, **101**, 242–251.
- Chagas RRD, Souza-Alves JP, Jerusalinsky L, Ferrari SF (2009) New Records of *Bradypus torquatus* (Pilosa: Bradypodidae) from Southern Sergipe, Brazil. *Edentata*, **8**–**10**, 21–24.
- Chiarello AG (1998a) Activity budgets and ranging patterns of the Atlantic forest maned sloth *Bradypus torquatus* (Xenarthra: Bradypodidae). *Journal of Zoology*, **246**, 1– 10.
- Chiarello AG (1998b) Diet of the Atlantic forest maned sloth *Bradypus torquatus* (Xenarthra: Bradypodidae). *Journal of Zoology*, **246**, 11–19.
- Chiarello AG (1999) Effects of fragmentation of the Atlantic forest on mammal communities in south-eastern Brazil. *Biological Conservation*, **89**, 71–82.
- Chiarello AG, Chivers DJ, Bassi C *et al.* (2004) A translocation experiment for the conservation of maned sloths, *Bradypus torquatus* (Xenarthra, Bradypodidae). *Biological Conservation*, **118**, 421–430.
- Chiarello AG, Moraes-Barros N (2014) Bradypus torquatus. The IUCN Red List of Threatened Species 2014: e.T3036A47436575. http://dx.doi.org/10.2305/IUCN.UK.2014-1.RLTS.T3036A47436575.en. Downloaded on 06 December 2016.
- Clozato CL, Miranda FR, Collevati RG, Santos FR (2017) Population structure and genetic diversity of the giant anteater (*Myrmecophaga tridactyla*: Myrmecophagidae, Pilosa) in Brazil. *Genetics and Molecular Biology*. In press
- Coimbra R, Miranda FR, Clozato CL, Schetino MAA, Santos FR (2017) Phylogeographic history of South American populations of the silky anteater *Cyclopes didactylus* (Pilosa: Cyclopedidae). *Genetics and Molecular Biology*. In press
- Costa LP (2003) The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography*, **30**, 71–86.
- Crandall KA, Bininda-Emonds OR, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. *Trends in ecology & evolution*, **15**, 290–295.
- De Vivo M, Carmignotto AP (2004) Holocene vegetation change and the mammal faunas of South America and Africa. *Journal of Biogeography*, **31**, 943–957.
- Delsuc F, Ctzeflis FM, Stanhope MJ, Douzery EJ (2001) The evolution of armadillos, anteaters and sloths depicted by nuclear and mitochondrial phylogenies: implications for the status of the enigmatic fossil *Eurotamandua*. *Proceedings of the Royal Society of London B: Biological Sciences*, **268**, 1605–1615.
- Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A (2012) Molecular phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. *Molecular Phylogenetics and Evolution*, **62**, 673–680.
- Delsuc F, Vizcaíno SF, Douzery EJ (2004) Influence of Tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. *BMC Evolutionary Biology*, **4**, 1.
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564–567.
- Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.
- Fonseca GAB, Aguiar JM (2004) The 2004 Edentate species assentment workshop. *Edentata*, **6**, 1.
- Frankham R (2003) Genetics and conservation biology. *Comptes Rendus Biologies*, **326**, 22–29.
- Fu Y-X (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**, 915–925.
- Gardner AL (2007) Order Pilosa Flower, 1883. In: *Mammals of South America*, University of Chicago Press, Chicago.
- Gibb GC, Condamine FL, Kuch M *et al.* (2016) Shotgun Mitogenomics Provides a Reference Phylogenetic Framework and Timescale for Living Xenarthrans. *Molecular Biology and Evolution*, **33**, 621–642.
- Grazziotin FG, Monzel M, Echeverrigaray S, Bonatto SL (2006) Phylogeography of the *Bothrops jararaca* complex (Serpentes: Viperidae): past fragmentation and island colonization in the Brazilian Atlantic Forest. *Molecular Ecology*, **15**, 3969–3982.
- Guillot G, Santos F, Estoup A (2008) Analysing georeferenced population genetics data with Geneland: a new algorithm to deal with null alleles and a friendly graphical user interface. *Bioinformatics*, **24**, 1406–1407.
- Hammond KA, Szewczak J, Król E (2001) Effects of altitude and temperature on organ phenotypic plasticity along an altitudinal gradient. *Journal of Experimental Biology*, **204**, 1991–2000.
- Hayssen V (2009) *Bradypus torquatus* (Pilosa: Bradypodidae). *Mammalian Species*, **829**, 1–5.
- Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.
- Hirsch A, Chiarello AG (2012) The endangered maned sloth *Bradypus torquatus* of the Brazilian Atlantic forest: a review and update of geographical distribution and habitat preferences. *Mammal Review*, **42**, 35–54.
- Hoorn C, Wesselingh FP, ter Steege H *et al.* (2010) Amazonia Through Time: Andean Uplift, Climate Change, Landscape Evolution, and Biodiversity. *Science*, **330**, 927–931.
- Hurst GD., Jiggins FM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1525– 1534.
- Ingberman B, Fusco-Costa R, de Araujo Monteiro-Filho EL (2016) A Current Perspective on the Historical Geographic Distribution of the Endangered Muriquis (*Brachyteles* spp.): Implications for Conservation. *PloS one*, **11**, e0150906.
- Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, msw054.
- Lara M, Patton JL (2000) Evolutionary diversification of spiny rats (genus *Trinomys*, Rodentia: Echimyidae) in the Atlantic Forest of Brazil. *Zoological Journal of the Linnean Society*, **130**, 661–686.
- Lara MC, Patton JL, da Silva MNF (1996) The simultaneous diversification of South American echimyid rodents (Hystricognathi) based on complete cytochrome b sequences. *Molecular phylogenetics and evolution*, **5**, 403–413.
- Lara-Ruiz P, Chiarello AG (2005) Life-history traits and sexual dimorphism of the Atlantic forest maned sloth *Bradypus torquatus* (Xenarthra: Bradypodidae). *Journal of Zoology*, **267**, 63.
- Lara-Ruiz P, Chiarello AG, Santos FR (2008) Extreme population divergence and conservation implications for the rare endangered Atlantic Forest sloth, *Bradypus torquatus* (Pilosa: Bradypodidae). *Biological Conservation*, **141**, 1332–1342.
- Leite YLR (2003) *Evolution and systematics of the Atlantic Tree Rats, genus Phyllomys (Rodentia, Echimyidae), with description of two new species*. University of California Press, Berkeley.
- Libardi GS, Percequillo AR (2016) Variation of craniodental traits in russet rats *Euryoryzomys russatus* (Wagner, 1848)(Rodentia: Cricetidae: Sigmodontinae) from Eastern Atlantic Forest. *Zoologischer Anzeiger-A Journal of Comparative Zoology*, **262**, 57–74.
- Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.
- Lynch M, Lande R (1998) The critical effective size for a genetically secure population. *Animal Conservation*, **1**, 70–72.
- Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer research*, **27**, 209–220.
- Matzke NJ (2012) Founder-event speciation in BioGeoBEARS package dramatically improves likelihoods and alters parameter inference in dispersal–extinction– cladogenesis DEC analyses. *Front. Biogeogr*, **4**, 210.
- Meredith RW, Janecka JE, Gatesy J *et al.* (2011) Impacts of the Cretaceous Terrestrial Revolution and KPg Extinction on Mammal Diversification. *Science*, **334**, 521– 524.
- Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Gateway Computing Environments Workshop (GCE), 2010*, pp. 1–8. IEEE.
- Miranda GB, Andrades-Miranda J, Oliveira LF, Langguth A, Mattevi MS (2007) Geographic patterns of genetic variation and conservation consequences in three South American rodents. *Biochemical genetics*, **45**, 839–856.
- Montes C, Cardona A, Jaramillo C *et al.* (2015) Middle Miocene closure of the Central American Seaway. *Science*, **348**, 226.
- Montgomery GG, Sunquist ME (1978) Habitat selection and use by two-toed and threetoed sloths. *The ecology of arboreal folivores*, 329–359.
- de Moraes-Barros N, Silva JAB, Morgante JS (2011) Morphology, molecular phylogeny, and taxonomic inconsistencies in the study of *Bradypus* sloths (Pilosa: Bradypodidae). *Journal of Mammalogy*, **92**, 86–100.
- Moraes-Barros N, Arteaga MC (2015) Genetic diversity in Xenarthra and its relevance to patterns of neotropical biodiversity. *Journal of Mammalogy*, **96**, 690–702.
- Moraes-Barros N, Miyaki CY, Morgante JS (2007) Identifying management units in nonendangered species: the example of the sloth *Bradypus variegatus* Schinz, 1825. *Brazilian Journal of Biology*, **67**, 829–837.
- Moraes-Barros N, Silva JAB, Miyaki CY, Morgante JS (2006) Comparative Phylogeography of the Atlantic Forest Endemic Sloth (*Bradypus torquatus*) and the Widespread Three-toed Sloth (*Bradypus variegatus*) (Bradypodidae, Xenarthra). *Genetica*, **126**, 189–198.
- Moreira D de O, Leite GR, Siqueira MF de *et al.* (2014) The Distributional Ecology of the Maned Sloth: Environmental Influences on Its Distribution and Gaps in Knowledge (B Fenton, Ed,). *PLoS ONE*, **9**, e110929.
- Moritz C (1994) Defining "Evolutionarily Significant Units" for conservation. *Trends in Ecology & Evolution*, **9**, 373–375.
- Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic biology*, **51**, 238–254.

Morley RJ (2000) *Origin and evolution of tropical rain forests.* John Wiley & Sons.

- Mustrangi MA, Patton JL (1997) *Phylogeography and systematics of the slender mouse opossum Marmosops (Marsupialia, Didelphidae)*. University of California Press, Berkeley.
- Nardelli M, Ibáñez EA, Dobler D *et al.* (2016) Genetic structuring in a relictual population of screaming hairy armadillo (*Chaetophractus vellerosus*) in Argentina revealed by a set of novel microsatellite loci. *Genetica*, **144**, 469–476.
- Oliveira-Filho AT, Tameirão-Neto E, Carvalho WAC *et al.* (2005) Análise florística do compartimento arbóreo de áreas de floresta atlântica sensu lato na região das Bacias do Leste (Bahia, Minas Gerais, Espírito Santo e Rio de Janeiro). *Rodriguésia*, **56**, 185–235.
- Oliver WLR, Santos IB (1991) *Threatened endemic mammals of the Atlantic forest region of south-eastern Brazil*.
- Ortiz-Jaureguizar E, Cladera GA (2006) Paleoenvironmental evolution of southern South America during the Cenozoic. *Journal of Arid Environments*, **66**, 498–532.
- Pascual R (2006) Evolution and geography: the biogeographic history of South American land Mammals. *Annals of the Missouri Botanical Garden*, **93**, 209–230.
- Pascual R, Ortiz-Jaureguizar E (1990) Evolving climates and mammal faunas in cenozoic south America. *The Platyrrhine Fossil Record*, **19**, 23–60.
- Pascual R, Ortiz-Jaureguizar E (2007) The Gondwanan and South American Episodes: Two Major and Unrelated Moments in the History of the South American Mammals. *Journal of Mammalian Evolution*, **14**, 75–137.

Pellegrino K, Rodrigues MT, Waite AN *et al.* (2005) Phylogeography and species limits in the *Gymnodactylus darwinii* complex (Gekkonidae, Squamata): genetic structure coincides with river systems in the Brazilian Atlantic Forest. *Biological Journal of the Linnean Society*, **85**, 13–26.

Petri S, Fúlfaro VJ (1983) *Geologia do Brasil: Fanerozóico*. T.A. Queiroz, Editor.

Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) *Tracer v1. 6*.

- Ronquist F (1997) Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. *Systematic Biology*, **46**, 195–203.
- Sambrook J, Russel DW (2001) *Molecular Cloning: a laboratory manual, Cold Spring Hrbour Laboratory Press*. Cold Spring Harbour.
- Santos Júnior JE, Santos FR, Silveira FA (2015) Hitting an Unintended Target: Phylogeography of *Bombus brasiliensis* Lepeletier, 1836 and the First New Brazilian Bumblebee Species in a Century (Hymenoptera: Apidae) (S Brady, Ed,). *PLOS ONE*, **10**, e0125847.
- Sepulchre P, Sloan LC, Fluteau F (2010) Modelling the response of Amazonian climate to the uplift of the Andean mountain range. In: *Amazonia: Landscape and Species Evolution: A look into the past*, pp. 211–222.
- Silva JMC, Straube FC (1996) Systematics and biogeography of scaled woodcreepers (Aves: Dendrocolaptidae). *Studies on Neotropical Fauna and Environment*, **31**, $3 - 10$.
- Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, **73**, 1162–1169.
- Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**, 978–989.
- Tabarelli M, Pinto LP, SILVA J, Hirota M, Bede L (2005) Challenges and opportunities for biodiversity conservation in the Brazilian Atlantic Forest. *Conservation Biology*, **19**, 695–700.
- Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.
- Thomé MTC, Zamudio KR, Giovanelli JGR *et al.* (2010) Phylogeography of endemic toads and post-Pliocene persistence of the Brazilian Atlantic Forest. *Molecular Phylogenetics and Evolution*, **55**, 1018–1031.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-

specific gap penalties and weight matrix choice. *Nucleic acids research*, **22**, 4673–4680.

- Thornback J, Jenkins M (1982) *The IUCN Mammal Red Data Book: Threatened mammalian taxa of the Americas and the Australasian zoogeographic region (excluding Cetacea)*. IUCN.
- Turchetto-Zolet AC, Pinheiro F, Salgueiro F, Palma-Silva C (2013) Phylogeographical patterns shed light on evolutionary process in South America. *Molecular Ecology*, **22**, 1193–1213.
- Vasconcelos PM, Becker TA, Renne PR, Brimhall GH (1992) Age and duration of weathering by 40K-40Ar and 40Ar/39Ar analysis of potassium-manganese oxides. *Science*, **258**, 451–455.
- Wesselingh FP, Salo JA (2006) A Miocene perspective on the evolution of the Amazonian biota. *Scripta Geologica*, **133**, 439–458.
- Wetzel RM (1982) Systematics, distribution, ecology, and conservation of South American Edentates. In: *Mammalian biology in South America, special publication series, Pymatuning laboratory of ecology.*, pp. 345–375. M. A. Mares & H. H. Genoways, University of Pitsburgh, Linesville.
- Wetzel RM, Avila-Pires F de (1980) Identification and distribution of the recent sloths of Brazil (Edentata). *Revista Brasileira de Biologia*, **40**, 831–836.
- Yang Z (2005) Bayesian Estimation of Species Divergence Times Under a Molecular Clock Using Multiple Fossil Calibrations with Soft Bounds. *Molecular Biology and Evolution*, **23**, 212–226.
- Yu Y, Harris AJ, Blair C, He X (2015) RASP (Reconstruct Ancestral State in Phylogenies): A tool for historical biogeography. *Molecular Phylogenetics and Evolution*, **87**, 46–49.
- Yu Y, Harris AJ, He X (2010) S-DIVA (Statistical Dispersal-Vicariance Analysis): A tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution*, **56**, 848–850.
- Zachos J (2001) Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science*, **292**, 686–693.
- Zanella FCV (2011) Evolução da biota da diagonal de formações abertas secas da América do Sul. *Biogeografia da América do Sul: padrões e processos*, 198–220.
Supporting information.

Table S1: List of genes, species, haplotypes, population of *Bradypus torquatus*.

QST	BA	ES	RJ
BA	0/0		
ES	0.98149/0.82919	0/0	-
RJ	0.98418/1.0000	0.96128/0.78955	0/0

Table S2: Pairwise φ_{ST} matrix obtained with Arlequin 3.5 (mtDNA/nuDNA).

Figure S1: (A) Population estimation: Index of MCMC iterations and probability density of number of clusters. (B), (C) and (D) Area estimation *of B. torquatus* BA, ES and RJ populations. Lines represent posterior probability. The lighter area indicates the region with the most probability to belong to the same population.

Figure S2: Haplotype networks for tree nuclear genes showing the BA, ES and RJ populations. Circle sizes are proportional to frequencies, and mutation step numbers greater than one are indicated on the lines. Black = Bahia (BA); white = E spírito Santo (ES) ; and gray = Rio de Janeiro (RJ).

Considerações finais

O presente estudo traz importantes informações relacionadas a subfamília Tolypeutinae e a *Bradypus torquatus* além de demonstrar a importância de aumentar o número de genes em uma análise molecular e de também considerar as regiões geográficas e quantidade de indivíduos a serem amostrados. Este trabalho, de maneira alguma desmerece os estudos anteriores e sim, colabora com a melhor compreensão dos táxons aqui estudados visto que a ciência é um processo contínuo e progressista.

Em Tolypeutinae foi observada uma grande diversidade morfológica entre os gêneros, variando desde o tamanho de *Priodontes maximus* em relação as outras espécies até a capacidade das espécies do gênero *Tolypeutes* em se curvar até ao formato de uma bola para se proteger dos predadores. Talvez por isso essa subfamília passou por tantas classificações e mesmo hoje não se consiga verificar sua sistemática apenas com caracteres morfológicos. Por outro lado, durante um bom tempo, as análises moleculares também não foram o suficiente para definir uma filogenia confiável que explicasse a relação de parentesco entre as espécies. Nosso trabalho demonstrou que a adição de novos indivíduos, principalmente da subespécie *Cabassous unicinctus squamicaudis*, alterou completamente a filogenia do gênero *Cabassous* possibilitando novas interpretações da história evolutiva de suas espécies.

A delimitação das espécies do gênero *Cabassous* com dados moleculares nos mostra a possibilidade de *Cabassous unicinctus unicintus* e *Cabassous unicinctus squamicaudis* serem diferentes espécies, o que é fortemente corroborado pelas filogenias apresentadas nos capítulos 1 e 2 deste trabalho. Adicionalmente o clado formado por *Cabassous unicinctus unicinctus* e *Cabassous centralis* nos mostra a possibilidade de existir um complexo de espécies onde são necessários maiores estudos para elucidar o status taxonômico de cada uma das espécies identificadas em nossas análises. É importante ressaltar que as análises moleculares por si só não são suficientes para classificar uma espécie em definitivo, mas sim são uma parte essencial da taxonomia integrativa onde quanto maior o acúmulo de informações mais robusta será a definição do status taxonômico das espécies do gênero estudado. Os dados aqui obtidos são de extremo valor para a conservação e manejo das espécies. Ao elevar as subespécies de *Cabassous unicinctus* ao nível de espécie, novos levantamentos como a área de abrangência, censo e habitats terão que ser revistos assim como novas análises como modelagem, filogeografia e genética de populações.

Com a inclusão de novos marcadores moleculares nas análises demográficas e filogeograficas de *Bradypus torquatus* foi possível propor uma nova história demográfica e evolutiva para a espécie. Com a adição de um novo gene mitocondrial e do DNA nuclear nas análises foi possível estabelecer uma nova estimativa de tempo de divergência entre suas principais linhagens e compreender os principais fatores no decorrer dos períodos geológicos que favoreceram a estruturação desta populações e atual distribuição geográfica. Dados importantes como a estimativa da região geográfica habitada pelas linhagens ancestrais de *B. torquatus* colaboram não só com a história da espécie, mas também com o entendimento das mudanças sofridas no continente durante este período. Adicionalmente nossos dados também possibilitaram uma nova visão dos eventos que delinearam não somente as linhagens da espécie foco do estudo, mas também da ordem Pilosa ao descrever desde sua primeira grande divergência no Eoceno até o período atual.

Apesar das novas perspectivas propostas nesse trabalho, para melhor robustez dos resultados aqui encontrados, ainda são necessários novos estudos com a adição de novos dados, principalmente de indivíduos de *Cabassous centralis* e *Cabassous chacoensis*. Além do mais, com o advento de novas técnicas de sequenciamento e com a descoberta de novos fósseis de Tolypeutinae e Bradypodidae será possível estimar com maior acurácia a história evolutiva de suas espécies.