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TAXONOMIA E SISTEMÁTICA DOS TUCO-TUCOS DO NOROESTE DO BRASIL (RODENTIA - CTENOMYIDAE) E A DESCRIÇÃO DE UMA NOVA ESPÉCIE DA AMAZÔNIA BRASILEIRA

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SUMÁRIO

Resumo

Existem atualmente oito espécies de tuco-tucos, roedores subterrâneos do gênero *Ctenomys* descritas para o Brasil. Quatro delas, que habitam a região sul, são bem conhecidas e estudadas: *C. flamarioni, C. minutus, C. lami e C. torquatus*. As outras: *C. brasiliensis, C. nattereri, C. rondoni e C. bicolor*, que habitam a região noroeste possuem como conhecimento científico apenas a descrição de cada uma delas. Pouco ou nenhum material testemunho de suas descrições e um abandono por parte da ciência em uma lacuna de tempo de mais de cem anos sem que seu status taxonômico, evolutivo ou qualquer tipo de conhecimento tenha sido pesquisado levaram ao questionamento destas entidades como espécies válidas.

Sabendo da existência de populações de tuco-tucos habitando os estados de Mato-Grosso e Rondônia, foram realizadas expedições de levantamento e coleta de dados objetivando abranger a distribuição noroeste brasileira do gênero *Ctenomys* tratando de elucidar as questões evolutivas e taxonômicas existentes.

Foram coletados cinquenta indivíduos em cinco populações distintas e analisados quanto ao cariótipo, através de análises moleculares utilizando o DNA mitocondrial, quanto a morfologia externa e a morfometria geométrica craniana dos espécimes encontrados.

Com base nos resultados obtidos este trabalho eleva novamente ao nível de espécie *C. nattereri*, que havia sido sinonimizado a *C. boliviensis* por falta de trabalhos mais consistentes de comparação entre as duas espécies. Também redescobrimos e validamos como espécie *C. bicolor*, a espécie que habita mais ao norte dentre as 60 espécies conhecidas do gênero. Propões-se a descrição de uma nova espécie, *C. amazonicus*, habitando o interior da floresta amazônica

na sua porção leste, o que é uma novidade para o gênero, que habitualmente ocupa áreas de vegetação aberta.

O presente trabalho aduda a resolver questões importantes ao entendimento de parte da biodiversidade brasileira, que só pode ser realizado com o uso de diferentes abordagens, que permitiram um parecer irrevogável da situação destas entidades biológicas e traz a luz do conhecimento científico um novo grupo de espécies que possui características evolutivas únicas.

Abstract

There are currently eight species of tuco-tucos, subterranean rodents of the genus *Ctenomys* described for Brazil. Four of them, who inhabit the southern region, are well known and studied: *C. flamarioni C. minutus, C. lami* and *C. torquatus*. The others: *C. brasiliensis, C. nattereri, C. rondoni* and *C. bicolor*, inhabiting the northwest have only the description of each as scientific knowledge. Little or no material witness to their description and abandonment by science in a time gap of more than one hundred years without their taxonomic status, evolution or any kind of knowledge has been investigated led to the questioning of these entities as valid species.

Knowing the existence of populations of tuco-tucos inhabiting the states of Mato Grosso and Rondonia, expeditions were carried out to survey and collect data aiming to cover the distribution of the genus *Ctenomys* in the northwestern Brazil trying to clarify the existing taxonomic and evolutionary questions.

Fifty individuals were collected in five distinct populations and analyzed for karyotype, molecular analysis using mitochondrial DNA, plus external morphology and cranial geometric morphometrics of specimens.

This work elevates to the level of species *C. nattereri*, which had been synonymized to *C. boliviensis* for lack of more consistent comparison between the two species. Also rediscovered and validated as a species *C. bicolor*, the species that inhabits farther north among the 60 known species of the genus. A new species was discovered, *C. amazonicus*, inhabiting the interior of the Amazon rainforest in its eastern portion, which is new to the genus, which usually occupies open vegetated areas.

Necessary issues were resolved to the understanding of Brazilian biodiversity, which can only be achieved by using different approaches, which allowed an irrevocable opinion of the situation of these biological entities and bring the light of scientific knowledge to a new group of species that have exclusive evolutionary characteristics.

Introdução

O gênero *Ctenomys*

O gênero *Ctenomys* compreende um grupo de aproximadamente 60 espécies (Woods e Kilpatrick, 2005), que atendem por diversos nomes comuns, dependendo da sua área de ocorrência, sendo o mais comum tuco-tuco, ou a variante espanhola tucu-tucu, bem como rato-cururu, ratão e rato-paca. Este gênero está incluso na família monogenérica Ctenomyidae, subordem Hystricognathi, ordem Rodentia (Woods, 1982). A especiação do gênero *Ctenomys* é considerada uma das mais explosivas entre os gêneros de mamíferos (Cook & Lessa, 1998; Lessa & Cook, 1998), ocasionando no surgimento de aproximadamente 60 espécies descritas atualmente, das quais algumas necessitam revisão taxonômica (Reig et al., 1990). Os tuco-tucos são exclusivos da América do Sul, ocupando boa parte das áreas de solo bem drenados e arenosos deste continente, ocorrendo desde a Terra do Fogo, na Argentina, até o Sul da Bolívia e Peru, e desde o nível do mar, em algumas regiões litorâneas, até mais de 4.000m de altitude nos Andes peruanos (Pearson, 1959; Novak, 1999). O surgimento do gênero *Ctenomys* foi atribuído ao centro da Argentina, durante o Plioceno Tardio – Pleistoceno (Contreras et al.,1987; Reig et al., 1990; Lessa & Cook, 1998; Verzi et al., 1999). Existem registros fósseis de *Ctenomys* presentes também em formações do Terciário (Formação Chapadmalalal, Plioceno Superior, Argentina), implicando uma antiguidade para o gênero maior do que três milhões de anos (3Ma). Subsequente ao surgimento do gênero, houve uma explosiva cladogênese, produzindo um grande número de espécies que tornou-se dominante na exploração do nicho subterrâneo na Região Neotropical (Reig et al., 1990; Cook & Lessa, 1998; Lessa & Cook, 1998; Maschereti et al., 2000).

Estes roedores passam a maior parte de suas vidas abaixo da superfície do solo, em sistemas de túneis escavados por indivíduos solitários, na maioria absoluta das espécies, ou dividindo os túneis em sociedade, como no caso de *Ctenomys sociabilis* (Nowak, 1999; Lacey et al., 1998; Lacey, 2000). A estrutura destes túneis consiste basicamente em uma galeria principal, com diversos apêndices onde estão localizados pequenos túneis e câmaras que dão acesso ao exterior, a partes subterrâneas de plantas, ou a câmaras de estocagem de material vegetal e latrinas (Nevo, 1979; Gallardo & Anrique, 1991; Altuna et al., 1999; Busch et al., 2000). Os túneis são mantidos fechados ativamente, com tampões de solo e algumas vezes material vegetal, o que proporciona não apenas proteção contra predadores mas também condições físico químicas mais estáveis do que o meio externo, como menores flutuações de temperatura, alto grau de umidade relativa, concentrações de O_2 menores (de 15 a 21%) e de CO_2 maiores (de 0,5 a 2%) do que o ambiente externo (McNab, 1966). A constatação da presença destes animais no ambiente costuma ser relativamente fácil, pela presença de amontoados de areia que correspondem aos tampões e deposição do material escavado logo acima do nível do solo (Pearson et al., 1968).

A ampla presença de tuco-tucos na América do Sul de fato é reflexo da estabilidade conferida pelo tipo de vida subterrâneo, apropriando as condições locais a níveis mais confortáveis para a presença das espécies. Contudo, ao analisarmos mais detalhadamente sua distribuição, os tuco-tucos apresentam forte tendência a viver em solos arenosos e bem arejados, e em micro escala, bem drenados (Contreras, 1973). Esta característica não está restringida apenas pela atividade escavatória dos indivíduos, mas também por restrições relacionadas com a manutenção do calor e o intercâmbio de gases através do solo (McNab, 1966; 1979; Contreras & McNab, 1990).

Relacionado às atividades subterrâneas, os tuco-tucos apresentam adaptações morfológicas características, como corpo fusiforme, pernas curtas, cabeça e pescoço robustos, cauda achatada lateralmente, pavilhão auditivo reduzido, patas grandes e dedos longos, com presença de pelos rígidos em forma de pente, que aumentam a área de contato com o solo, aumentando a capacidade de manipulação escavatória e dão nome ao gênero (do grego, ktenes = pente; *mys* = rato), além de uma profunda adaptação bucal, na qual os incisivos encontram-se projetados para fora da boca, mesmo com os lábios fechados por trás destes, o que permite a escavação sem a ingestão de solo (Nevo, 1979; Reig et al. 1990; Nowak, 1999).

Alimentam-se principalmente de gramíneas, tanto das partes aéreas como das partes subterrâneas, sendo generalistas na maioria dos casos e têm grande influência sobre as comunidades de plantas das regiões onde habitam (Nevo, 1979; Gallardo & Anrique, 1991; Zenuto & Busch, 1995; Borrel et al., 1998; Altuna et al., 1999; Busch et al., 2000).

Os tuco-tucos apresentam uma grande diversidade cariotípica, variando desde 2n=10 em *C. steinbachi*, a 2n=70 em *C. pearsoni* (Reig & Kiblisky, 1969; Kiblisky et al.,1977; Gallardo, 1979; Lessa & Langguth, 1983; Freitas & Lessa, 1984; Ortells et al., 1990; Ortells, 1995; Massarini et al., 1991; Freitas, 1990; 1994; 1997; Giménez et al., 1997; 1999; Mascheretti et al. 2000; Garcia et al. 2000; Slamovits et al. 2001). Esta grande amplitude cariotípica tem sido motivo de especulações a respeito dos mecanismos promotores de tal diversificação (Nevo 1999). Reig & Kiblisky (1969) foram os primeiros a propor o modelo de especiação cromossômica para este clado de mamíferos. A diversificação do grupo estaria facilitada pela formação de pequenos demes isolados, característica da estrutura populacional da maioria das espécies de tuco-tucos (Reig et al., 1990; Busch et al; 2000), e a ação da deriva genética que permitiu a fixação rápida de rearranjos cromossômicos. Esta deriva, associada à estrutura

populacional da maioria das espécies, explicaria esta grande variabilidade tanto inter como intraespecífica que muitas vezes se observa para espécies do gênero.

A alta velocidade de diversificação no clado tem sido sugerida através de estudos filogenéticos, pela persistência de politomias em filogenias obtidas tanto a partir de sequências do citocromos-b mitocondrial (Cook & Lesssa, 1998; Lessa & Cook, 1998; Mascheretti et al., 2000; Slamovits et al.,2001; Parada et al., 2011) como a partir de dados provenientes do DNA nuclear (fragmentos dos genes da rodopsina e vimentina; Castilho et al., 2005).

Histórico das espécies do noroeste brasileiro

No ano de 1848, Waterhouse, naturalista do British Museum de Londres, recebeu uma pele de mamífero oriunda da Bolivia, a qual, após uma série de comparações morfotípicas, como coloração, padrões de mancha, proporções corporais e medidas biométricas externas, com exemplares das apenas três espécies conhecidas para o gênero *Ctenomys* naquele momento, vindo a tornar-se o holótipo de *Ctenomys boliviensis*. A descrição formal da espécie contempla apenas caracteres discretos, muitas vezes comuns a mais de uma espécie do gênero, como orelhas curtas e patas com cerdas enrijecidas, mas define em sua publicação a localidade tipo de *C. boliviensis* como sendo as planícies próximas a cidade de Santa Cruz de la Sierra.

Neste mesmo ano, de 1848, A.Wagner, naturalista alemão recebeu dois indivíduos taxidermizados para que pudesse apreciar, oriundos de Caissora (MT). Comparou-os também com as três espécies descritas até aquele momento através da morfologia externa, principalmente coloração e tamanho geral, e preferiu chama-los de *Ctenomys nattereri* por estar convencido de que se tratava de uma espécie diferente, descrevendo assim uma nova espécie para o Brasil.

Em 1914, o naturalista Alípio de Miranda Ribeiro, que acompanhava a expedição do Marechal Rondon da Comissão de Linhas Telegraphicas Estratégicas de Matto Grosso ao Amazonas, que visava desbravar, demarcar e mapear o território oeste brasileiro tombou em coleção dois indivíduos do gênero *Ctenomys* aos quais ele nomeou *Ctenomys rondoni* (Museu Nacional: MN2048) e *Ctenomys bicolor* (MN2025), provindos de Juruena (MT) e José Bonifácio (RO) respectivamente. As descrições apresentadas nos textos baseiam-se em apenas um indivíduo de cada suposta espécie, as quais foram feitas sob a pele já preparada e danificada, como citado ao longo do texto, que permitiram realizar apenas medidas externas muito rudimentares em relação a estes indivíduos. As descrições dos espécimes também é bastante superficial, sendo formalizada em alguns poucos parágrafos, a título de história natural dos espécimes, e não apresenta características diagnósticas em relação a outras espécies do gênero, ou quando apresenta, compara com espécimes mal identificados, como no caso de *Ctenomys minutus* coletado na Bolívia, sendo esta uma espécie de distribuição conhecida apenas para o sul do Brasil, ou da comparação com *Ctenomys brasiliensis*, considerada atualmente um equívoco taxonômico. De maneira geral, percebe-se que com as condições dadas naquele momento, Ribeiro tentou comparar o material com aquilo que possuía ao seu dispor, não realizando porém uma descrição formal de acordo com as normas internacionais de nomenclatura zoológica.

No ano de 1916, J.A. Allen, mastozoólogo que acompanhou Rondon em uma de suas expedições, publica no boletim do Museu Americano de História Natural (American Museum of Natural History - AMNH), uma lista de mamíferos coletados durante esta excursão, onde consta um indivíduo coletado com a ajuda de uma tribo indígena em José Bonifácio (RO) o qual chamou de *Ctenomys nattereri* (AMNH 37121).

Somente em 1987, quando Sydney Anderson e colaboradores fizeram o inventariamento dos ctenomídeos da Bolívia, foram realizados os primeiros cariótipos conhecidos para esse conjunto de espécies que ocupam as partes baixas da Bolívia e parte da região noroeste do Brasil. Neste trabalho, Anderson chama a atenção para um conjunto de pequenas diferenças cariotípicas e craniométricas de dois indivíduos oriundos de Roboré (Bolívia) cidade próxima da fronteira com o Brasil, mas por falta de uma diferenciação morfológica externa mais evidente, sinonimiza os indivíduos do Brasil como *Ctenomys boliviensis* subespécie roboré. Neste trabalho, 4 diferentes cariótipos são assumidos para boliviensis: 46, 44, 42 e 36. O cariótipo 2n=36 foi encontrado em indivíduos de Roboré (330km de Cáceres e a população mais fronteiriça de boliviensis analisada). A descrição original de *C. nattereri*, baseada em dois indivíduos de "Caissora" = Cáceres (MT, Brasil), não apresenta caracteres que distinguam as duas formas. *Ctenomys rondoni* (Ribeiro, 1914), passa a ser assumido a partir daqui como sinonímia de *Ctenomys nattereri*, como proposto por Cabrera (1961:553) em vista da sobreposição da distribuição das espécies.

Joseph Cook e Terry L. Yates (1994) realizam uma análise de aloenzimas e afirmam que *Ctenomys boliviensis* é um táxon com possivelmente mais de uma espécie e chamam atenção para os indivíduos de Roboré. *Ctenomys minutus* de Anderson 1987 é tratado como espécie nova e necessita de revisão.

Anderson (1997) no seu trabalho de revisão dos mamíferos da Bolívia afirma que *C. boliviensis* é uma possível superespecie, embora não seja separável pela morfologia externa. Ele afirma ainda que o suposto *C. minutus* (que deve ser revisado segundo resultados prévios de seus outros trabalhos) está na fronteira, perto de roboré, e possui duas subespecies segundo Cabrera (1961), *C. m. minutus e C. m. bicolor*.

Enrique Lessa e Joseph Cook (1998) publicam a primeira analise molecular utilizando o marcador citocromo-b, e apesar da distinção sempre presente dos indivíduos provindos de Roboré, que ficam como grupo irmão de *C. boliviensis* e *C. goodfellowi*, os autores não entram no mérito taxonômico da análise.

Mascheretti e colaboradores (2000), realizaram outra análise molecular mais abrangente em indivíduos com o marcador citocromo-b, sugere que a entidade *Ctenomys nattereri* seja uma espécie válida, junto com os espécimes de *C. boliviensis robo* que possivelmente estejam inclusas erroneamente junto com *C. boliviensis*, já que estes indivíduos possuem afinidade no ramo filogenético obtido, e compartilham o cariótipo 2n=36.

Cook e Jorge Salazar-Bravo (2004), realizando um trabalho com heterocromatina, não confirmam, mas comentam em separado na análise os indivíduos considerados *C. nattereri*, vindos de locais próximos à fronteira com o Brasil e que apresentam número cariotípico 2n=36.

Andrés Parada e colaboradores (2011) realizam a mais completa análise molecular através do citocromo-b já realizada com o gênero, e evidenciam um clado evolutivo monofilético, bem definido, juntando as espécies *C. boliviensis*, *C. goodfellowi* e os indivíduos de Roboré, nomeando como grupo boliviensis na análise filogenética, novamente sem fazer menção ao *status* taxonômico dos indivíduos.

Novos métodos de taxonomia e sistemática de roedores

Métodos de avaliação cariotípica

Cromossomos são unidades de herança genética que estão localizadass nos núcleos das células dos organismos eucariotos. Estas estruturas podem diferir em tamanho, número e forma.

Estas modificações estão sujeitas a alterações evolutivas, podendo variar entre indivíduos e/ou organismos, como por exemplo: diferenças no número cromossômico de diferentes espécies (Schubert, 2007).

Estas variações cariotípicas, tanto em relação ao número diploide, morfologia cromossômica, ou ambos, não são raras de ser encontradas em espécies de mamíferos. Estas mutações normalmente não produzem efeitos fenotípicos significativos, embora possam estar associadas na redução da fertilidade dos heterozigotos ou facilitar a fixação de homozigotos, tornando-se uma barreira reprodutiva, isolando diferentes raças cromossômicas, possibilitando em alguns casos, conduzir à especiação Este tipo de rearranjos podem sofrer pressões de seleção negativas ou positivas, ou ainda serem fixados por ação de deriva genética, principalmente em pequenas subpopulações divididas em demes (White, 1978; Lande, 1984, King, 1993).

Alguns rearranjos podem ter papel preponderante em processos de cladogênese, principalmente aqueles que podem ou causam segregação diferencial durante a meiose dos híbridos, como: fusões Robertsonianas, inversões paracentricas e pericentricas, fusões em *tandem*, e translocações recíprocas. Outros rearranjos podem ser considerados neutros ou adaptativos, pois não estão envolvidos na especiação, tais como variações na quantidade de heterocromatina constitutiva e rearranjos neutros ou com heterose positiva, que ocorrem como polimorfismos balanceados (King, 1993).

Cenário cariotípico em *Ctenomys*

O gênero *Ctenomys* apresenta uma das maiores variações cariotípicas, tanto intra como interespecíficas conhecida para os mamíferos, com números cromossômicos variando desde 2n=10, em *C. steinbachi*, até 2n=70, em *C. pearsoni* e *C. dorbigny*, e com números fundamentais

variando desde NF=16 até NF=84 (Reig et al., 1992; Cook and Bravo, 2004; Mascheretti et al. 2000; Villar et al. 2005). Este gênero é um exemplo típico de cladogênese explosiva, compreendendo mais de 60 espécies atualmente descritas e originadas a partir do Plioceno (Verzi, 2002), apresentando características ecológicas e comportamentais próprias, tais como baixa mobilidade, distribuição em *patches* e número populacional efetivo geralmente pequeno (Reig et al., 1990; Villar et al., 2005). Além disso, a presença de barreiras ecológicas e geográficas ao fluxo gênico tem sido apontada como uma das principais responsável pelas divergências encontradas entre as populações e as espécies (Patton e Smith, 1990).

Tendo isto em vista, supõe-se que os rearranjos cromossômicos desempenham importante papel no processo de diferenciação populacional, possivelmente sendo a força principal na especiação deste gênero, sendo as fusões Robertsonianas ou fissões apontadas como o mecanismo ativo operando no processo evolutivo dos tuco-tucos (Reig e Kiblisky, 1969; Reig et al.,1990; Freitas, 1997).

Uma analise filogenética baseada em cariótipos com bandas G sugeriu que os números diploides e números fundamentais baixos parecem ser caracteres plesiomórficos dentro do gênero *Ctenomys*, possuindo provavelmente o ancestral deste gênero um numero diploide entre 2n=18 e 2n=36, e numero fundamental entre FN=30 e FN=48 (Ortells, 1995). Através desta hipótese pode-se dimensionar a importância dos rearranjos na variabilidade cromossômica atual deste gênero (Fernandes, 2008).

Métodos de avaliação molecular

O genoma mitocondrial de animais é haploide e está formado por uma dupla fita circular entre 15.000 e 17.000 pares de bases (pb) de comprimento, estando presente desde centenas a milhares de cópias por célula (Meyer, 1993; Li & Graur, 2000).

Tipicamente, cada genoma de DNA mitocondrial (mtDNA) consiste em 37 genes funcionais sem longos espaços intergênicos. Estes loci codificam para 22 ribonucleotídeos (RNAs) de transferência, dois RNAs ribossômicos e 13 RNAs mensageiros para a síntese de subunidades de proteínas específicas envolvidas na fosforilação oxidativa e no transporte de elétrons (Avise, 2000).

O mtDNA é simples em estrutura e econômico em tamanho, sendo que o único grande fragmento não codificante (de aproximadamente 1.000pb) é a região controladora (control region, CR) que tem função regulatória na dinâmica da molécula e é predominantemente rica em bases AT (Brown, 1985). Nos vertebrados esta região é referida como D-loop (displacement-loop), devido a formação de uma estrutura de fita tripla que gera deslocamentos dinâmicos no começo da replicação do mtDNA (Brown et al.1986).

Devido a sua rápida evolução, o mtDNA torna-se um bom marcador para o estudo de diferenciações genéticas recentes por acumular substituições de base, inserções e deleções com uma taxa media de cinco a dez vezes mais rápida que o DNA nuclear copia simples (Brown et al. 1979). Várias hipóteses tem sido levantadas na tentativa de explicar a rápida evolução do mtDNA: (1) o relaxamento das limitações funcionais; (2) a taxa de mutação devido a ineficiência dos mecanismos de reparo de DNA, alta exposição aos radicais livres (mutagênicos) produzidos no interior da mitocôndria ou ao rápido turnover dentro das linhagens celulares; (3) o fato de

estar livre de histomas, que são evolutivamente conservadas e poderiam limitar a taxa evolutiva do DNA nuclear (Avise, 2000).

Outra vantagem do mtDNA, como marcador para o estudo de diferenciações genéticas recentes é a frequência extremamente baixa de transposições, inversões , rearranjos e recombinação (Brown, 1985). Isto permite a caracterização de linhagens filogenéticas sem a ambiguidade causada pela recombinação meiótica que ocorre nos genes nucleares.

Analises de sequencias desta região tem gerado resultados com boa resolução em estudos populacionais e evolutivos, em diferentes grupos taxonômicos de mamíferos, como primatas, ursos, baleias, manatis, lêmures, marsupiais e roedores (Fernández-Stolz, 2007).

O citocromo b é uma das proteínas envolvidas na cadeia transportadora de eletros. Embora evolua lentamente em termos de substituição não-sinônimas, a taxa de evolução em posições silenciosas é relativamente rápida (Irwin et al., 1991). O amplo uso do citocromo b gerou o *status* de molécula universal, possibilitando que os estudos possam ser facilmente comparados.

Métodos de avaliação da forma

A forma dos organismos é um dos temas mais antigos investigados dentro das ciências da vida (Monteiro e Reis, 1999), porém a palavra morfologia só começou a ser utilizada no século XVIII pelo naturalista alemão Goethe (1749-1832), que cunhou o termo como sendo o estudo das formas orgânicas (Kardong, 1995). Já a palavra morfometria começou a ser usada por Blakith em 1965 (Monteiro e Reis, 1999) para determinar estudos que visavam quantificar a

forma dos organismos. Nos anos 30, os estudos em morfometria tiveram um significativo avanço graças ao surgimento das análises de componentes principais, análise da variância, análise multivariada da variância e a análise discriminante (Marcus et al., 1996; Monteiro e Reis, 1999).

O surgimento da morfometria geométrica ocorreu com a criação de um método para estudar diferenças de forma dentro de um espaço multivariado de análises morfométricas em configurações de marcos anatômicos (Bookstein, 1991; Bookstein, 1996a; Bookstein, 1996b) e cunhou o termo forma como sendo "todas as propriedades de uma configuração de pontos que não se alteram por efeitos de tamanho, posição e orientação".

A utilização da morfometria tem especial aplicação nos métodos de descrição, na análise estatística da variação da forma entre organismos e na análise das mudanças na forma em função do crescimento, bem como de tratamentos experimentais e evolução (Rohlf e Marcus, 1993). Diferenças em morfologias complexas podem ser comparadas e testadas estatisticamente (Marroig e Cheverud, 2004; Marroig et al.,2004). A morfometria tradicional, que aplica métodos de estatística multivariada em um conjunto de medidas tomadas de um organismo, principalmente comprimentos e larguras de estruturas, distâncias entre certos pontos de referência, ângulos, áreas e razões entre distâncias (Monteiro e Reis, 1999) e é utilizada pricipalmente para estudos de alometria. Esta abordagem não possibilita a recuperação da forma original da estrutura avaliadas, mas pode indicar regiões de possível variação (Rohlf e Marcus, 1993; Marcus et al.,1996). Já a morfometria geométrica captura a geometria da estrutura em estudo através das coordenadas de marcos anatômicos, que podem ser bi ou tridimensionais e permite a separação dos componentes forma e tamanho (Rohlf e Marcus, 1993; Monteiro e Reis, 1999). Através do uso de marcos anatômicos (pontos homólogos em estruturas), essa técnica permite localizar e descrever regiões de mudanças na forma.

Utilizam-se coordenadas cartesianas nessas configurações de marcos anatômicos, que são as variáveis utilizadas no estudo geométrico das estruturas de interesse. A estatística da nova morfometria é a mesma estatística multivariada usada nas medidas lineares, só que agora aplicada a coordenadas de marcos anatômicos alinhados adequadamente. Então, as mesmas análises de componentes principais, análise fatorial e analise de variáveis canônicas podem ser aplicadas a dados de pontos de referência. Estas coordenadas incluem informações sobre sua posição relativa, permitindo assim a reconstrução da forma após as análises uni e multivariadas (Fornel, 2010). A morfometria geométrica tem maior robustez na analise integrada e exclui fatores de posição, orientação e tamanho na analise da forma (Bookstein, 1991, Rohlf e Marcus, 1993, Marcus et al., 1996; Monteiro e Reis, 1999; Adams et al. 2004, Fornel, 2010).

Apenas para o gênero *Ctenomys*, podemos citar várias abordagens que o uso das técnicas de morfometria geométrica tem propiciado ao longo das últimas décadas, como avaliações taxonômicas (Fernandes, et al., 2012), evolutivas (D'Anatro e Lessa, 2006; Fernandes, 2008; Fernandes et al., 2009; Fornel, 2010; Fornel *et al*., 2010), fisiologia (Souza, et al. 2010).

Objetivos

Objetivo geral

Compreender o panorama evolutivo das espécies de ctenomídeos brasileiros presentes na região noroeste do país, que se encontravam em abandono científico desde suas breves descrições, no século XIX.

Objetivos específicos

1. Analisar as características cariotípicas, moleculares e morfométricas dos indivíduos a fim de compreender as diferenças intra e interespecíficas, comparando com espécies já descritas que possuem estes atributos mensurados.

2. Analisar o processo evolutivo ocorrido através de uma abordagem filogenética nas espécies encontradas.

3. Descrever ou redescrever adequadamente, de acordo com as normas taxonômicas vigentes, a condição taxonômica dos espécimes encontrados.

4. Traçar o panorama evolutivo e o papel dos refúgios amazônicos no processo de especiação das entidades taxonômicas encontradas na região centro-oeste brasileira.

Capítulo I

Rediscovering and validating *Ctenomys bicolor*, with designation of a neotype

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The taxonomy of the genus *Ctenomys* (Rodentia: Ctenomyidae) shows several inconsistencies in some entities, in part because of a lack of information and material. We rediscovered a species of *Ctenomys* from northwestern Brazil, in the Amazon ecoregion, and now provide more reliable taxonomic information and material, with the designation of a neotype. Phylogenetic relationships among *Ctenomys bicolor* and eight other species of *Ctenomys* were examined using nucleotide sequence data from the entire 1,140 base pairs of the mitochondrial cytochrome-b gene. Maximum-likelihood and Bayesian analyses found the new species to be distinct from the others, and sister to *Ctenomys boliviensis robore*. We present analyses of morphological data and qualitative descriptions of the external and cranial morphology, and of its karyotype (2n=40, FN=64). This species is important for the South American fauna because its distribution is the

most northerly of *Ctenomys*, among the nearly 60 recognized species, and the first that is known to occur in the Amazon forest.

Keyword: *Ctenomys bicolor*, new species, Amazon forest, neotype

INTRODUCTION

The South American rodent genus *Ctenomys* (popularly known as tuco-tucos) includes 60 recognized species and a group of morphotypes of uncertain taxonomic position (Bidau, 2006; Woods and Kilpatrick, 2005). Among these morphotypes of uncertain taxonomic position is *Ctenomys bicolor*, which was described by Miranda Ribeiro (1914), based on the external morphology of one individual. This type specimen, deposited in the Museu Nacional do Rio de Janeiro (specimen MNRJ-2052) (Langguth et al., 1997) was collected on October 9, 1912 by the Comissão Rondon (probably by Alípio de Miranda Ribeiro, the expedition's zoologist) (Avila-Pires, 1968: 182). The description of this new species, based on this single individual of unknown sex, appeared two years later (Miranda Ribeiro, 1914: 41), with no mention of the type locality, which was later indicated by Miranda Ribeiro (1955) and Avila-Pires (1968) as Mato Grosso. This was interpreted as meaning that the type locality of *C. bicolor* is somewhere in the present-day state of Mato Grosso, Brazil (Cabrera, 1961). Bidau and Avila-Pires (2009), in a historical review, redefined the type locality as a location in the present Brazilian state of Rondônia (coordinates: 11º50'10"S and 12º00'00"S, and 60º5'35"W and 61º19'29"W) (Fig. 1). Subsequently to the capture of this single specimen, very few other individuals of *Ctenomys* have been collected in northwest Brazil; Allen (1916) reported one individual captured with the help of the indigenous people. The skin and holotype skull deposited in the MNRJ are now damaged (Miranda Ribeiro, 1914; Langguth et al., 1997) and because some bones and teeth are missing,

do not allow precise verification of the species classification and comparisons. There is also some inconsistency in regard to the specimen number, which was cited both as MNRJ-2052 (Langguth et al., 1997) and as MNRJ-2025 (Miranda Ribeiro, 1955).

There is a clear lack of information about what species of ctenomyids inhabit this part of Brazil, and their distribution, taxonomy and systematics. Defining these entities is the first step in providing a solid basis for needed future studies on their ecology and conservation, because these species inhabit a landscape that is being dramatically changed by the human presence in Amazonia (Fearnside, 2005).

MATERIAL AND METHODS

The specimens were collected in the field, by means of Oneida Victor no. "0" traps, with rubber padding to avoid injuring the rodents, from the banks of the Barão de Melgaço River (8°38'50"S 35°10'15"W), the type locality indicated for the species, in the municipality of Pimenta Bueno, state of Rondônia, Brazil (Fig. 1). The search for a population was carried out by means of interviews with local residents, and also directly on the ground, looking for tunnel entrances, in an area covering 20 km² around the reported locality of the original population. External body measurements (total length, body length, hind foot) were taken in the field, using a digital caliper (Mitutoyo®; 0.01 mm), and mass with a dynamometer. Linear cranial measurements (Leite, 2003) were taken in the laboratory with the same digital caliper.

The entire genomic DNA was isolated from liver, kidney, and heart tissue, following standard protocols (Longmire et al., 1997) from 10 individuals collected from the type locality. For the outgroup taxa, we obtained from GenBank the entire cytochrome-b sequences from 15 *Ctenomys* samples (8 sepcies) from Bolivia and Peru and one from Brazil, which represent the closest distributions to the point of collection of *C. bicolor*; one sequence of *Spalacopus cyanus;* and

one of *Octodon degus* (GenBank access numbers: AF007055 *C. conoveri*; AF007051 *C. goodfellowi*; AF007050 *C. goodfellowi*; AF007056 *C. leucodon*; AF007042 *C. opimus*; AF007041 *C. opimus*; AF007044 *C. steinbachi*; AF007043 *C. steinbachi*; AF007040 *C. boliviensis*; AF007039 *C. boliviensis*; AF007038 *C. boliviensis*; AF007037 *C. boliviensis*; AF007049 *C. lewisi*; AF007046 *C. frater*; AF007045 *C. frater*; AF119107 *C. flamarioni*; AF007058 *Octodon degus*; AF007061 *Spalacopus cyanus*). These specimens were selected by taking into account their historical use in phylogenetic analyses in recent decades. The aligned sequences were inspected visually in ClustalX2 (Larkin et al., 2007).

The entire cytochrome b gene (1,140 bp) was amplified by the polymerase chain reaction (PCR); usually 30 cycles, alternating denaturation at 93°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min, using combinations of the primers MVZ5 and MVZ14 (Smith and Patton, 1993) as external primers. All PCR experiments included negative controls.

Amplifications were performed in 20-*M*l reaction volumes, each containing 25-100 ng DNA, 1.0 unit of Taq DNA polymerase, 0.2 m*M* of each external primer, 1.5 m*M* $MgCl_2$, 1.0 mM of 10X buffer, and water to complete the volume. The thermal profile consisted of an initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, and 45ºC for 30 s, extension at 72 ºC for 45 s, and a final extension at 72 ºC for 5 min. Double-stranded polymerase chain reaction products were purified using the EXO-SAP PCR system. Purified products were sequenced in a ABI 3730XL DNA analyzer (MACROGEN, Korea). Mitochondrial cytochrome-b gene sequences for the specimens examined in this study have been submitted to GenBank).

Phylogenetic relationships among *Ctenomys* species were estimated under the criteria of maximum likelihood using MEGA version 5 (Tamura et al., 2011) and Bayesian phylogenetics using MRBAYES (Huelsenbeck and Ronquist, 2001). We considered clades receiving bootstrap

support >= 50% or Bayesian probabilities >=0.95%, or both. Maximum-likelihood approximation (Tamura et al., 2011) was used before maximum-likelihood analysis to determine the model of DNA sequence evolution that best fit to our data. The TN93+G model of evolution was chosen, along with the following parameters: base frequencies = 0.2978, 0.3096, 0.1040, 0.2886; nst = 2; t ratio = 8.9256; rates = gamma with shape parameter (a) 5 1.8156; and proportion of invariant sites = 0.5688.

Bayesian analysis was used to obtain an independent measure of phylogenetic relationships (Huelsenbeck and Ronquist, 2001). The HKY+G model of DNA sequence evolution was used, based on a preview analysis using jModelTest (Posada, 2008) along with site-specific rates of variation calculated for each of the 3 positions of the codon via the ‘‘ssgamma’’ option in MRBAYES. Four simultaneous Markov chains were run for 2,000,000 generations, starting with random, unconstrained, starting trees. Temperature was set at 0.02 to facilitate greater movement between the 4 Markov chains, and trees were sampled every 10 generations. Resulting burn-in values (the point at which the model parameters and tree scores became stationary) were determined empirically by evaluating likelihood scores. Three independent runs of MRBAYES were performed, using a different outgroup taxon, to ensure that the final trees converged upon the same topology.

Percent sequence divergence within and among clades was computed based upon Kimura 2-parameter corrected distances (Kimura, 1980). The Kimura 2-parameter model of sequence evolution was chosen to allow for comparison of the percent sequence divergence among taxa in this study with the recommendations of Bradley and Baker (2001) and Baker and Bradley (2006) regarding the cytochrome-b gene sequence difference, to evaluate cryptic genetic species. The

percent sequence divergence based on 3rd codon position transversion substitutions was calculated using MEGA5 (Tamura et al., 2011).

Two males and two females were karyotyped in the field, following the general procedure of Baker et al. (1982). Mitotic metaphases from bone marrow were stained using phosphate-buffered Giemsa stain to determine the diploid number (2n) and fundamental number (FN). Ten metaphases per animal were observed.

The geometry of the cranium shape was captured by a configuration of topographically corresponding landmarks modified from D'Anatro and Lessa (2006) and Fernandes et al., 2009; Fornel et al., 2010. Each cranium was photographed in dorsal, ventral, and left lateral views of the skull, with a digital camera with 3.1 megapixels (2048 X 1536) of resolution, with macro function and without zoom or flash. We used 15 two-dimensional landmarks for dorsal, 13 for ventral, and 12 for lateral views of the skull, as follows. Fifteen landmarks, assumed to be homologous among all specimens analyzed, were chosen in each individual skull, as follows: Dorsal view: 1 – anterior extremity of suture between nasals, 2 - anterolateral extremity of incisor alveolus, 3 - anteriormost point of root of zygomatic arch, 4 - externalmost point of orbit in zygomatic arch foramen, 5 – suture between nasals and frontals, 6 - tip of extremity of superior jugal process, 7 - lateral extremity of suture between jugal and squamosal in the zygomatic arch, 8 – suture between squamosal and jugal, 9 – suture between frontals and parietals, 10 – suture between frontal, parietal and squamosal, 11 – tip of posterior process of jugal, 12 – internalmost contact between squamosal and tympanic bulla, 13 - anterior tip of external auditory meatus, 14 - point of maximum curvature on mastoid apophysis, and 15 - posteriormost point of occipital along the midsagittal plane. Ventral view: 1 – anterior extremity of suture between premaxillaries, 2 – anterolateral extremity of incisive alveolus, 3 – suture

between premaxillary and maxillary in the external outline of the skull (on the photographic plane), 4 – tip of suture between premaxillaries in the incisive foramen, 5 - externalmost point of orbit in zygomatic arch foramen, 6 – anteriormost point of first molar alveolus, 7 - anteriormost point of intersection between jugal and squamosal, 8 – posteriormost point of fourth molar alveolus, 9 – anteriormost point in mesopterigoid fossa, 10 – anterior extremity of tympanic bulla, 11 – internalmost contact between squamosal and tympanic bulla, 12 – posterior extremity of mastoid apophysis, and 13 – posteriormost point of foramen magnum along midsagittal plane. Lateral view: 1 – point of intersection between premaxillary and posterior end of incisor, 2 – anteriormost point of suture between nasals and premaxillary, 3 – anterior extremity of suture between nasals, 4 – suture between premaxilla, maxilla and frontal in superior zygomatic root, 5 – suture between premaxillary and maxillary in the outline of the skull (on the photographic plane), 6 – anteriormost point of premolar alveolus, 7 – inferior end of suture between maxillary and jugal in zygomatic arch, 8 – extremity of superior jugal process, 9 – tip of posterior jugal process, 10 – extremity of inferior jugal process, 11 – superior extremity of lambdoidal crest, and 12 – anteriormost margin of paraoccipital apophysis. For the dorsal and ventral illustrations, skulls were digitized only on the left side to avoid redundant information in symmetrical structures, following Cardini and O'Higgins (2004). The anatomical landmarks were digitized by the same individual (J.F.B.S.) for each specimen, using TPSDig2, version 2.16 (Rohlf, 2004; http://life.bio.sunysb.edu/morph). Coordinates were superimposed using a generalized Procrustes analysis (GPA) algorithm (Dryden and Mardia, 1998). GPA removes differences unrelated to shape, such as scale, position, and orientation (Rohlf and Slice, 1990; Rohlf and Marcus, 1993; Bookstein, 1996a, b; Adams and Rohlf, 2004). The error in landmark acquisition (operator variance) was evaluated through a one-way analysis of variance of centroid size for the repeated

landmark acquisition of one image for each species. The mean estimated measurement error was 0.08%. The size of each skull was estimated using its centroid size, namely the square root of the sum of the squares of the distance of each landmark from the centroid (mean of all coordinates) of the configuration (Bookstein, 1991). Because each skull had three separate centroid sizes for each view, we calculated a single value by summing the logarithms of the dorsal, ventral, and lateral centroid sizes. We also used form (size plus shape), using log-transformed centroid size plus the principal components matrix of shape variables. Differences in the shape of the skull inferred from statistical analyses were visualized through multivariate regression of shape variables on discriminant axes.

Differences in the log of centroid size of taxa or populations were tested with analysis of variance (ANOVA)] and pairwise comparisons using Tukey test. Differences in shape were explored by canonical variate analyses (CVA) and multivariate ANOVA. To visualize the shape differences, deformations along factorial axes were calculated by multivariate regressions (Monteiro et al., 2003). To test the validity of the a priori taxonomic assignments, classification percentages were estimated by multiple discriminant functions, using shape and form (size plus shape) parameters and leave-one-out cross-validations (Ripley, 1996). Because of the relatively small sample sizes and the large number of variables (40 bidimensional landmarks), statistical analyses of shape were performed using the dimension-reduction approach advocated by Baylac and Friess (2005): we used the smallest first PC set that maximizes the discrimination values. The overall phenotypic similarities between taxa were depicted using a Neighbor-joining tree computed from the matrix of Mahalanobis' D2 distances. All morphometric calculations were performed using the 'R' language, version 2.0 for Linux (R Development Core Team, 2004). Morphometric procedures were carried out with the 'Rmorph' library for R (Baylac, 2007).

The sample of skulls consisted of 10 specimens (TR1462, TR1463, TR1464, TR1465, TR1466, TR1467, TR1468, TR1469, TR1470, TR1471) collected in Pimenta Bueno municipality, Rondônia, Brazil (Fig. 1), plus three museum specimens including the species' historic type (Miranda Ribeiro, 1914) (MNRJ-2052 - holotype; MNRJ-2050) and (AMNH-37121). Specimen AMNH-37121 is presently designated as *C. nattereri*, but we presumed that this was a misidentification because the collection point was José Bonifácio, RO, and we suspected that it was actually a specimen of *C. bicolor*, to be confirmed in the morphometric analyses. We compared these specimens with skulls from two different species of the boliviensis group (Parada et al., 2011), *C. boliviensis* (N = 52) (all specimens from AMNH: 256008, 260804, 260805, 260806, 260808, 260810, 260811, 260814, 260815, 260820, 260821, 260822, 260824, 260825, 260826, 260827, 260828, 260829, 260830, 260831, 260832, 260834, 264503, 264504, 264505, 264506, 264507, 264508, 264509, 264510, 264511, 264513, 264515, 264516, 264517, 264518, 264519, 264520, 264521, 264522, 264523, 264524, 264525, 264527, 264528, 264530, 264531, 264533, 264534, 264535, 264536, 264537), and *C. steinbachi* (N = 12) (AMNH-260851, AMNH-260853, AMNH-260856, AMNH-262293, AMNH-262294, AMNH-262295, AMNH-262296, AMNH-262297, AMNH-75339, AMNH-75340, FMNH-51894, FMNH-51895). All these specimens were previously deposited in collections or museums, acronyms as follows: AMNH (American Museum of Natural History, New York City, USA); FMNH (Field Museum of Natural History, Chicago, USA), MNRJ (Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil), and TR (Mammal Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil). A single skull (FMNH-28358) from Mato Grosso, Brazil, was used for descriptive comparisons between *C. bicolor* and *C. boliviensis (robore)*. The latter is presently recognized as a distinct species from *C. boliviensis* from the

Santa Cruz region in Bolivia. The field collections of specimens followed the guidelines of the American Society of Mammalogists for animal care and use (Gannon et al., 2007) and were approved by the Brazilian government (IBAMA) under authorization number 14690-1.

RESULTS

Four males and six females were collected in the field. Their external measurements include the external body measurements reported for the type species by Miranda Ribeiro (total length = 403 ± 23 mm, body length =314 ± 17 mm, hind foot with nail = 45 ± 1.8 mm, hind foot without nail = 39.9 ± 1.27 mm, mass = 418 ± 100 g). This population of *Ctenomys* seemed to be very sparse in the forest, and was only aggregated in the manioc (*Manihot esculenta*) fields. All the individuals captured inhabited the same manioc field, each in its own individual tunnel.

Karyotype - The diploid number (2n) is 40 and the fundamental number (FN) is 64. The chromosomal complement consists of 5 pairs of submetacentric pairs, ranging from medium to large, 6 pairs of metacentrics pairs from small to large, and 8 pairs of acrocentrics from small to large. The X is a large metacentric (Fig. 2). This chromosomal composition is exclusive to these specimens, and does not correspond to that of any other known *Ctenomys* species.

Description of nucleotide data. - Of the 1,140 sites resulting from alignment of cytochrome-b sequences of *Ctenomys* (excluding the outgroup), 841 (73.78%) were invariant. Of the 299 (26.2%) variable sites that were potentially phylogenetically informative, 44 (15.9%) were at 1st codon positions, 11 (4.0%) at 2nd codon positions, and 150 (80.1%) at 3rd codon positions. All ten individuals of *C. bicolor* showed exactly the same mitochondrial sequence.

Phylogenetic analyses - In the composite tree (Fig. 3), a clade composed of *Octodon degus* and *Spalacopus cyanus*, with *Ctenomys flamarioni* is basal to all other taxa examined, as expected because these are the most divergent species in relation to the boliviensis, opimus and frater

species groups analyzed (sensu Parada, 2011). The topology is consistent with previous analyses (Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004; Parada et al., 2011), including a monophyletic branch that joins *C. boliviensis* and *C. goodfellowi* with low support to separate this species through mitochondrial analysis. This species is considered differentiated because of its karyotype and morphology (Anderson et al., 1987).

The branch containing the *C. bicolor* sequence is monophyletic, showing a well-supported sister group formed by two sequences of *C. boliviensis*. These GenBank sequences are actually from two individuals captured near the Bolivian border, and misidentified as *C. boliviensis*, when they are actually *C. boliviensis robore*, as presently considered for individuals living near Robore (Bolivia), which have karyotype 2n=36 (Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004). Correction of this misidentification exclude the observed paraphyly of *C. boliviensis*, and reorganizes *C. bicolor* as a sister species of *C. boliviensis robore*, assuming that there is a differentiation between specimens with karyotype 2n=36 and individuals that are broadly recognized as *C. boliviensis* with karyotype numbers 2n=42, 44 and 46 (Lessa and Cook, 1998; Parada et al., 2011).

Percent sequence divergences among species (Table 2) were calculated using Kimura 2-parameter corrected distances. Comparisons among the currently recognized species of *Ctenomys* averaged 0.097 and ranged from 0.014 to 0.141. Percent sequence divergence was lowest between *C. boliviensis* and *C. goodfellowi* (0.014), and highest (0.141) between *C. conoveri* and *C. leucodon*. Percent sequence divergences between *C. bicolor* and other species of tuco-tucos were lowest with *C. boliviensis (robore*) (0.037) and *C. boliviensis* and *C. goodfellowi* (0.071).

Geometric morphometric analysis of the skull revealed significant differences in centroid size between the species for the dorsal (ANOVA: $P < 0.001$; $F = 17.28$; df=2), lateral (ANOVA: $P < 0.001$; $F = 27.24$, df=2) and ventral views (ANOVA: $P < 0.001$; $F = 29.09$, df=2). The Tukey pairwise comparison showed significant differences in size between *C. bicolor* and *C. boliviensis* ($P<0.001$) and between *C. bicolor* and *C. steinbachi* ($P<0.001$), but not between *C. boliviensis* and *C. steinbachi* ($P=0.974$), being bicolor smaller in all comparisons. The results of the MANOVA showed significant differences in shape for all skull views, both separately and pooled ($P < 0.01$; dorsal: λWilks = 0.38, $F = 22.29$; lateral: λWilks = 0.43, $F = 19.08$; ventral: λWilks = 0.08, $F = 9.32$; and the three views pooled: λWilks = 0.36, $F = 10.35$). The percentage of correct classification using form (size plus shape) provided the highest value (100%) for the three species analyzed, for the three views of the skull separately and pooled (Table 1), with the exception of the lateral view of *C. boliviensis*, reaching a 92.3% correct reclassification, and includes the correct reclassification of AMNH-37121 as a *C. bicolor* form.

Canonical variate analyses (CVA) in the three views combined with Mahalanobis distances (Fig. 4) showed a clear separation in form among the three species analyzed, indicating that *C. bicolor* is more distinct in form among these species than *C. boliviensis* and *C. steinbachi* are from each other. Specimen AMNH-37121 grouped inside the *C. bicolor* cloud, as presumed from its collection locality, and we assume that it pertains to this species. The shape differences among the species are shown in Figure 5.

DISCUSSION

The results of the chromosomal, molecular and morphological analyses indicate that the specimens of *Ctenomys* from Pimenta Bueno, state of Rondônia, examined in our study represent a single evolutionary lineage of *Ctenomys*, with no currently adequate museum type series. These animals are genetically distinct from other species of *Ctenomys* in the region, exhibit morphological characters that differentiate them from other species of *Ctenomys*, and are restricted in distribution to the sandy-soil portions of the southern Amazon basin. The levels of percent sequence divergence are comparable to values for recognized species of the genus *Ctenomys*, particularly from the boliviensis clade (Parada et al., 2011).

Based on the molecular, chromosomal and morphometric results, we propose the recognition of *Ctenomys bicolor* as a valid species. Because of the poor condition of the holotype deposited in the MNRJ, we designate a neotype herein.

Formal recognition of this species as a valid species is not completely defined, since the author himself recognizes the poor state of conservation of the voucher material deposited in the museum. Identification of the material deposited is doubtful, as the pertinent literature mentions different collection numbers in different articles (Miranda Ribeiro, 1955; Langguth et al., 1997), and a collection number is not provided in the original description, which raises the question of the correct designation of the material. Moreover, the origin of the material is uncertain, since it cites the state of Mato Grosso as the type locality, in an area that is recognized today as being in the state of Rondônia (Bidau and Avila-Pires, 2009), without mentioning a more precise location. Moreover, the original description of the species does not allow accurate recognition of it, since it is based on skull and external characters that fit the descriptions of other ctenomyids. The species is not recognized in databases (Wilson and Reeder, 2005) and has not been cited as a

species or included in any work in the almost one hundred years that have passed since its description.

This set of assumptions led us to question the true *status* of the species. The present analysis confirmed important differences for the ctenomyids from Pimenta Bueno, and with new information about the historical collection point, we presumed that our specimens are *C. bicolor*. Although the deposited specimen (MNRJ-2052) is damaged, the skull analysis placed the museum specimen within this population, reinforcing the presumption that it belongs to the same entity.

Based on Article 75 of the International Code of Zoological Nomenclature (ICZN 1999), we express the exceptional necessity of a better name-bearing specimen, in order to clarify the taxonomic status and the type locality of the species. In the absence of paratypes or paralectotypes, we designate a neotype, as follows:

Ctenomys bicolor (Miranda Ribeiro 1914)

Original description – The skull of this species closely resembles that of *Ctenomys minutus* Nehring, from which it is separated by the larger width of the diameter over the zygomatic arches, which are more strongly curved and larger; the post-orbital process on the frontal, which is lacking in *C. minutus*; by the narrower parietal and palatine, for which the outline of the front can be defined by a hexagon; and the weaker molars. It departs from *Ctenomys rondoni* in the shape of the occipital foramen, which lacks upper transverse processes; the wider frontal and interparietal; and the curvature of the zygomatic arches, which have the anterior border arched and not square. The hair on the upper body is dark-grayish, and on the flanks the tips of the hairs are sparsely brown, forming meshes of this color, which is dominant on the entire bottom. Tail whitish. The skin is imperfect, and the ends are badly damaged, which precludes taking

measurements. The skull also has the upper incisors broken. It is still possible to ascertain the width of the lower incisors, which is one-third less than in *C. rondoni*; and the color identity.

Neotype – Stuffed skin, tissue sample fixed in ethanol and skull number TR1466, of an adult female housed in the Mammal collection, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil, collected by José Stolz and Thales de Freitas on 12 May 2009.

Type locality – Amazon Forest, municipality of Pimenta Bueno, state of Rondônia, Brazil, 8°38'50"S 35°10'15"W, elevation 100 m (Fig. 1). This area seems to be in the middle of the Amazon Forest, near the city of Pimenta Bueno and the Barão do Melgaço River, where Rondon's expedition crossed during his journey through western Brazil, according to Bidau and Avila-Pires (2009). The specific habitat where the animals were trapped appears to be a recently planted residential garden, with a mix of corn, manioc and fruits planted in a recently cleared area of forest in this small farm. However, the entire area is presently surrounded in all directions by forest that is impacted to different degrees. The individuals appeared to be very sparsely distributed within the forest, but occurred in high density in the garden plantings.

Distribution –All individuals collected were trapped at this same site. The historical reference suggests that this species occurs along the sandy banks of the Barão de Melgaço River, and perhaps also along the banks of its tributaries. The collectors searched for any sign of the presence of tuco-tucos in the surrounding area, and found them to be distributed very specifically near the population sampled, and very sparse in the forest where the soil is sandier. The nearest known colony of another species of *Ctenomys* is located at a distance of 405 km, in the Pontes e Lacerda municipality in Brazil. The entire area between these two populations was examined in the search for other populations, without success.

Diagnosis – A medium-sized *Ctenomys* with karyotype number 2n=40, FN= 64. The frontal bones have a triangular lateral expansion, with the outer point of the triangle directed toward the front of the cranium (Fig. 6).

External morphology – External measurements: total length = 395 mm, body length = 310 mm, hind foot = 41 mm, mass = 300 g. The coat is usually brown to chestnut, with each hair having the basal half dark gray and the distal half brown (Fig. 7). The hair is long, and varies from 10 to 15 mm under the body and on the belly. The general color is slightly darker on the back and lightens toward the belly, with a marked color change on the ventral part, which is light brown and has long hair with a pale-gray base and a pale-brown distal part. On the midline of the head and on the back is a noticeable concentration of shorter hairs, forming a dark-brown band that fades in the region of the scapula. Beneath the ear is a small, distinct spot of slightly shorter and lighter-colored hairs. The inguinal and perianal regions have long whitish hair, making the region very pale. The vibrissae are long and light brown, and the longest reach the base of the ear. The ear is very short, extending only a few millimeters above the head. The eyes are small but quite visible, completely dark. The sole of the foot is hairless and the back has very pale, well spaced, stiff hairs. The legs are short and strong, with small feet compared to other ctenomyids. The nails are strong, flattened laterally, and slightly sharp at the bottom. The incisors are orange in the front, as in other species. The tail is laterally flattened and has dark-brown hairs above and lighter hairs below, ending with light-brown, slightly stiff hairs.

Cranium – The rostrum is short and narrow (Fig. 6). The nasal ends at the line of insertion of the zygomatic arch. The skull is large and rounded. The zygomatic arch is slightly narrower than the width of the auditory meatus, rounded, and narrows in the anterior half. The parietal and frontal are long, and the nose is proportionately short. Laterally, the skull is somewhat flattened, with a

very robust braincase and well-developed tympanic bulla, the diastema slightly spaced and incisors slightly procumbent. The molar series is short. The auditory bullae are large, long and robust. The frontals have a triangular protuberance on the lateral side, with the outer edge of the triangle directed toward the front of the skull (Fig. 6), which distinguishes it from other ctenomyids. In comparison with *Ctenomys boliviensis (robore)* (FMNH-28358), the sister species in the phylogenetic tree, the skull of this specimen has a much more rounded zygomatic arch, larger than the meatus width, with a proportionally longer rostrum; and does not have the triangular lateral expansion of the frontal bone, but rather an adorned quadrate structure that clearly does not fit within the diagnosis of *C. bicolor*.

Linear cranial measurements: greatest skull length = 48.76; nasal = 16.66; rostral = 18.77; orbital = 14.77; rostral breadth = 12.86; interorbital constriction = 13.05; mastoid breadth = 33.19; zygomatic breadth = 32.97; condyloincisive = 49.76; basilar = 42.46; diastema = 13.37; maxillary toothrow = 10; palatal a = 23.02; palatal b = 8.76; incisive foramina = 7.89; bullar = 14.45; post palatal = 18.68; mesopterygoid fossa width = 6.17; maxillary breadth = 10.15; occipital condyle width = 9.9; rostral depth = 10.22; cranial depth = 19.62; cranial depth at m1 = 17.92.

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FIGURE LEGENDS

Fig. 1 – Map showing the locations of points of capture of *Ctenomys bicolor*. The rectangle represents the historical limits of the original capture by Miranda-Ribeiro, as defined by Bidau and Avila-Pires (2009). The dot represents the exact point of capture of the present individuals, only 40 km distant from the southeast corner of the historically defined type locality.

Fig. 2.- *Ctenomys bicolor* female karyotype, showing the chromosome arms. 2n=40 and FN=64

Fig. 3 – Phylogenetic relationships for species of *Ctenomys,* based on the entire 1,140 base pairs of the cytochrome-b gene. Numbers at nodes are bootstrap support from maximum likelihood (left), followed by Bayesian posterior probabilities (right), in percentages.

Fig. 4. Canonical variate analysis of *Ctenomys* species form shape variables using three cranial views combined. The dotted line represents the Mahalanobis distance for the three species analyzed.

Fig. 5 - Skull shape differences for three *Ctenomys* species (*C. boliviensis, C. bicolor* and *C. steinbachi*), for dorsal (A), ventral (B), and lateral (C) views of the skull. Positive PC scores (solid lines), negative PC scores (dotted lines), PC1 (1), PC2 (2), in relation to analisys shown on figure 4.

Fig. 6. *Ctenomys bicolor* neotype (TR1466) cranium. Top to bottom: detail of the diagnostic triangular lateral expansion of the frontal bone, with the outer edge pointing toward the front of the skull; dorsal, ventral and lateral views, showing the mandible.

Fig. 7. *Ctenomys bicolor* neotype (TR1466). Upper left: dorsal view of stuffed skin. Lower left: ventral view of stuffed skin. Right: Live specimen in the field.

TABLES

Table. 1 – Percentage of correct classification from the linear discriminant analysis for previously recognized species of *Ctenomys*, for dorsal, lateral, ventral, and the three views pooled of the skull, using form (size plus shape).

	C. bicolor	*C. boliviensis*	*C. steinbachi*
dorsal	100	100	100
lateral	92.3	100	100
ventral	100	100	100
3 views	100	100	100

Table 2 – Estimates of mean Kimura 2-parameter distances between species. Estimated standard errors are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The analysis involved 11 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. There were a total of 1140 positions in the final dataset. The overall average is 0.113. The horizontal names are abbreviations of the same species names in the vertical column.

	bicol	conov	goodf	leuco	opimu	stein	robor	boliv	lewis	frate	flama
C. bicolor		0.011	0.008	0.011	0.009	0.008	0.006	0.008	0.012	0.011	0.009
C. conoveri	0.120		0.011	0.012	0.010	0.010	0.010	0.010	0.009	0.010	0.010
C. goodfellowi	0.071	0.114		0.010	0.008	0.008	0.007	0.004	0.011	0.010	0.009
C. leucodon	0.111	0.141	0.100		0.010	0.010	0.010	0.010	0.011	0.011	0.010
C. opimus	0.084	0.098	0.068	0.098		0.009	0.008	0.008	0.011	0.011	0.008
C. steinbachi	0.083	0.110	0.071	0.109	0.078		0.008	0.008	0.011	0.010	0.008
C. bol (robore)	0.037	0.112	0.060	0.097	0.074	0.069		0.008	0.011	0.011	0.008
C. boliviensis	0.071	0.107	0.014	0.101	0.067	0.066	0.060		0.011	0.010	0.008
C. lewisi	0.134	0.088	0.116	0.132	0.120	0.119	0.123	0.118		0.007	0.010
C. frater	0.134	0.100	0.121	0.136	0.126	0.121	0.129	0.115	0.054		0.010
C. flamarioni	0.088	0.106	0.081	0.108	0.075	0.086	0.081	0.077	0.116	0.117	

Figures:

Figure 1

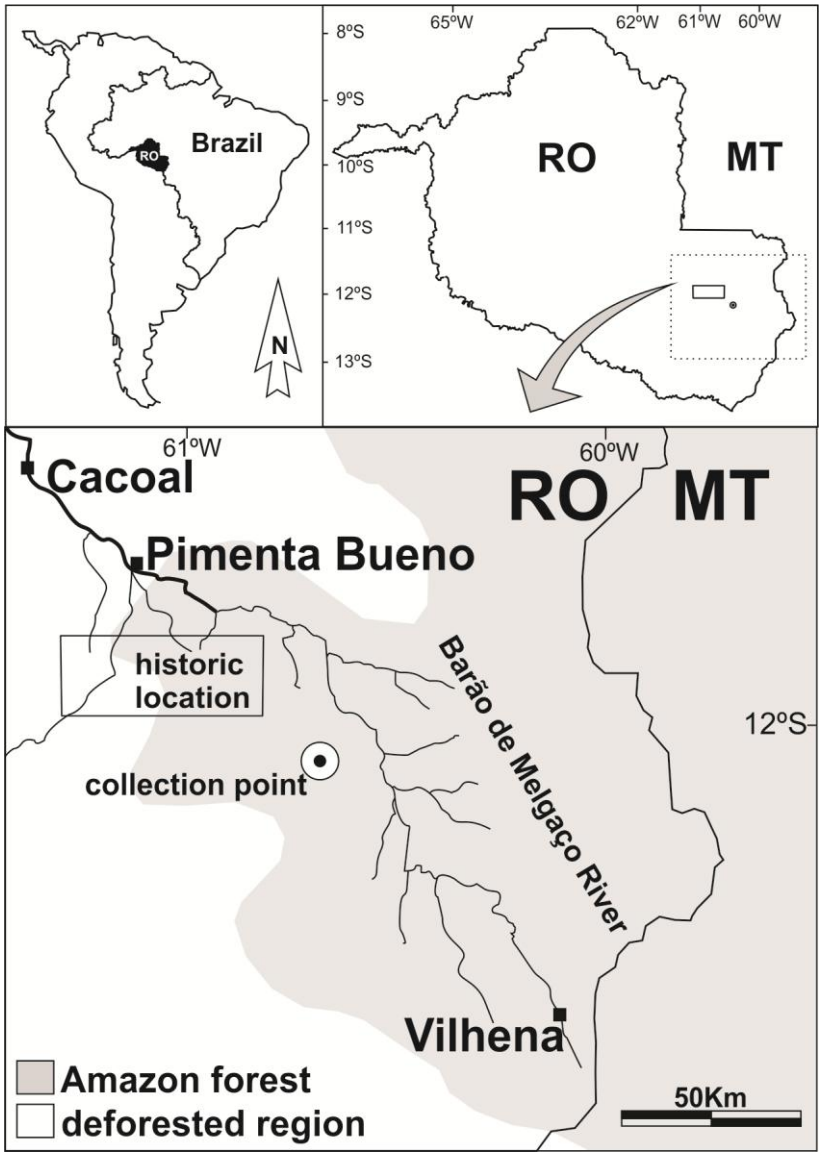

Figure 2

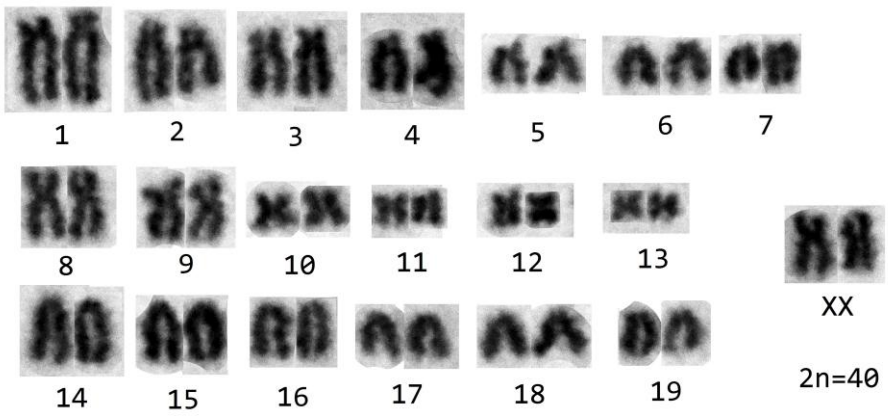

Figure 3

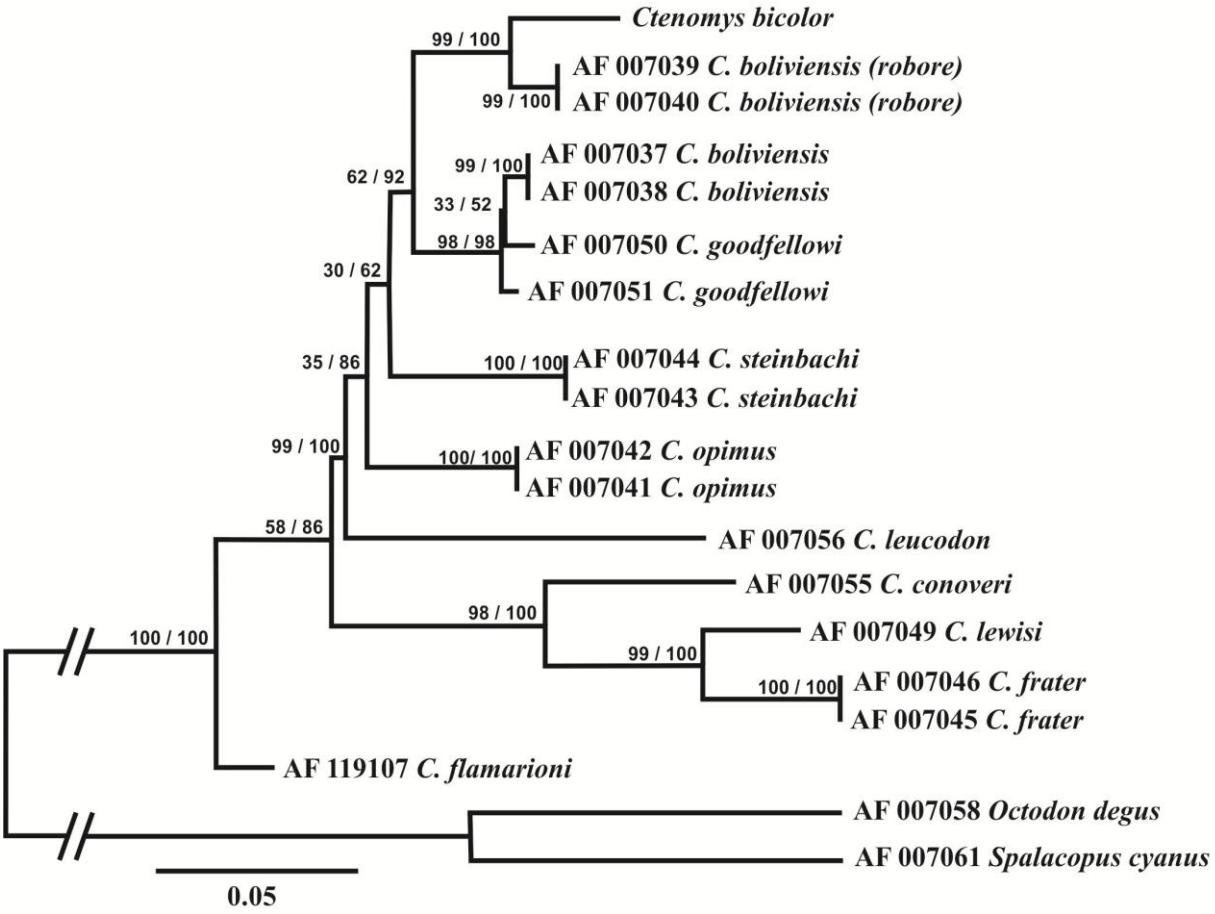

Figure 4

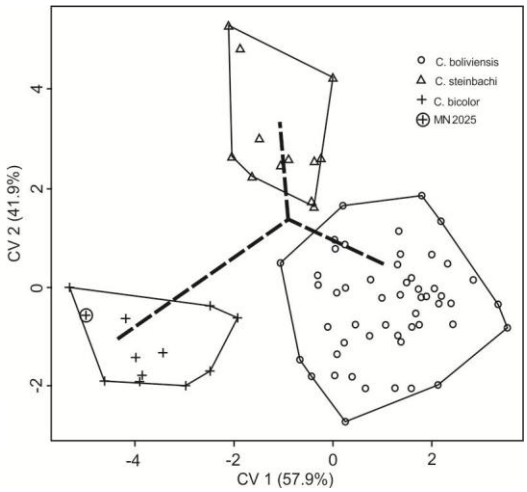

Figure 5

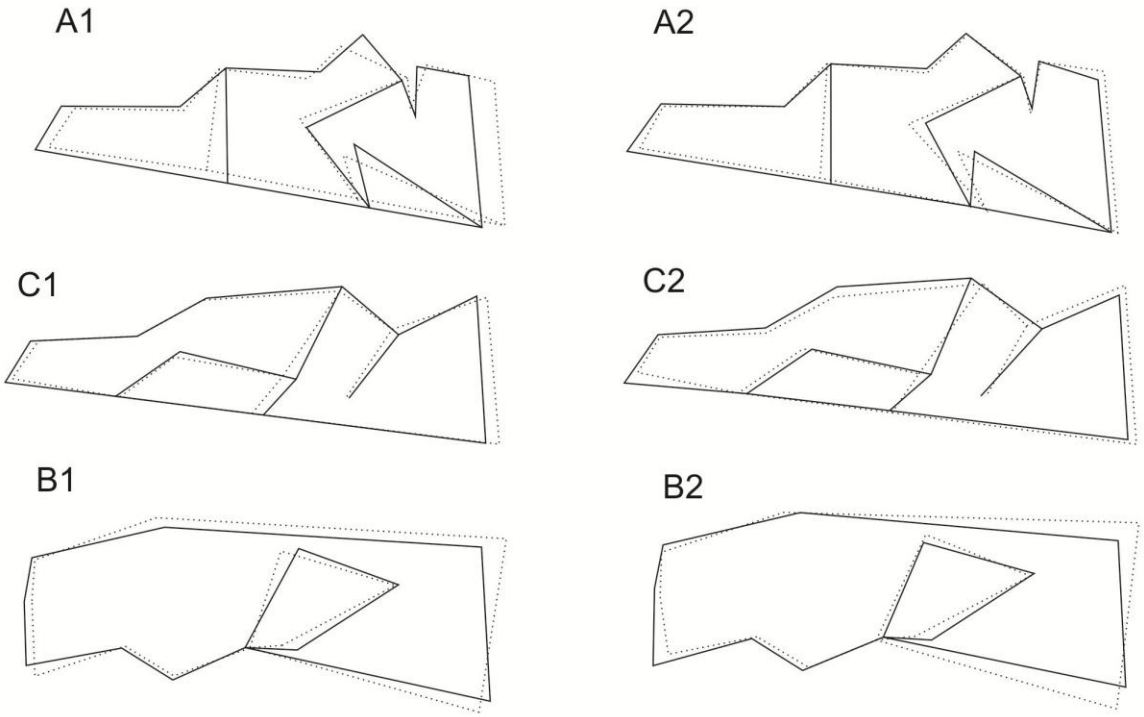

Figure 6

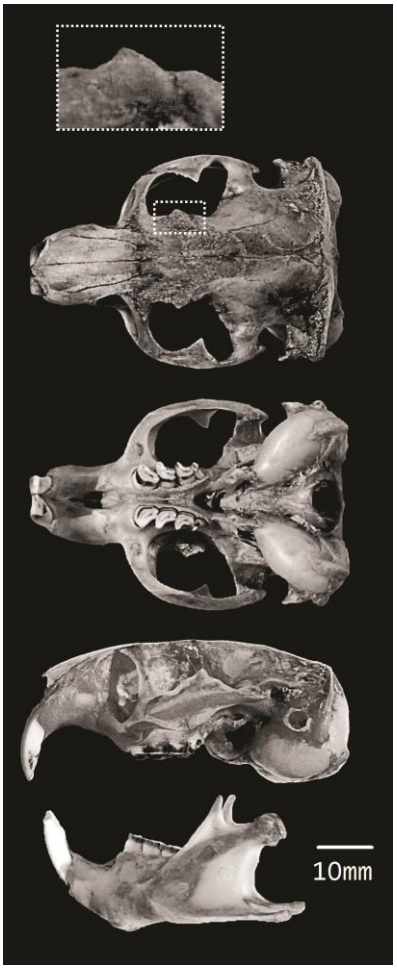

Figure 7

Capítulo II

Validating *Ctenomys nattereri* (Wagner, 1848) with designation of a neotype

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The taxonomy of the genus *Ctenomys* (Rodentia: Ctenomyidae) shows several inconsistencies in some entities, in part because of a lack of information and material. We validate a species status of *Ctenomys nattereri* from northwestern Brazil, providing more reliable taxonomic information and material, with the designation of a neotype. Phylogenetic relationships among this and eight other species of *Ctenomys* were examined using nucleotide sequence data from the entire 1,140 base pairs of the mitochondrial cytochrome-b gene. Maximum-likelihood and Bayesian analyses found the species to be distinct from the others, and sister to *Ctenomys boliviensis*. We present analyses of morphological data and qualitative descriptions of the external and cranial morphology, and of its karyotype. This species was treated as synonym to *Ctenomys boliviensis* even that some evidence of their distinctiveness had been discussed in some analyses in the past.

Keyword: *Ctenomys nattereri*, taxonomy, neotype, Amazon forest.

INTRODUCTION

The South American rodent genus *Ctenomys* (popularly known as tuco-tucos) includes almost 60 recognized species and a group of taxonomic inconsistencies (Bidau, 2006; Woods and Kilpatrick, 2005). This is the case of *Ctenomys nattereri*, described by Wagner (1848) based on just one specimen in the same year that Waterhouse described *Ctenomys boliviensis*. In that time, only two *Ctenomys* species had been recognized (Tate, 1935) and the differentiation among them were made in basis of external morphology and measurements. With the passage of time the type specimen of *Ctenomys nattereri* was lost in some German museum were the material collected in South America arrived for formal description. *C. boliviensis* and *C. nattereri* was posteriorly considered synonyms with Waterhouse as the first author (Anderson et al., 1987) because the very similar morphology. More recently, many works were realized including Bolivian ctenomyids (Anderson et al., 1987; Anderson, 1997; Cook and Yates, 1994; Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004; Castilho et al., 2005; Parada et al., 2011) and the most part of it indicates that there some level of differentiation between *Ctenomys boliviensis* from type locality, near Santa Cruz (Bolivia), and more Eastern populations, like Roboré region, near Brazilian border, with no taxonomic resolutions or formal descriptions. The most conspicuous distinction among this populations regards to karyotype, were populations from Roboré region presents karyotype number 2n=36 and the Santa Cruz region populations presents 2n=42, 44 and 46 (Cook and Bravo, 2004). It is recognized that Bolivian ctenomyids inhabits Brazilian northwestern (Anderson et al., 1987; Cook and Bravo, 2004) although Brazilian *Ctenomys* populations, including *C. nattereri* historical type locality has never been sampled since the original description. There is a clear lack of information about what species of ctenomyids inhabit this part of Brazil, and their distribution, taxonomy and systematics.

Defining these entities is the first step in providing a solid basis for needed future studies on their ecology and conservation, because these species inhabit a landscape that is being dramatically changed by the human presence in Amazonia (Fearnside, 2005).

MATERIAL AND METHODS

The specimens were collected in the field, by means of Oneida Victor no. "0" traps, with rubber padding to avoid injuring the rodents, from two populations: an Amazonian forest border in Pontes e Lacerda (PL - 15°9'22"S 59°13'41"W) and the type locality indicated for *C. nattereri*, in the municipality of Cáceres (CA - historically known as Caissora - 15°58'13"S 57°45'58"W), state of Mato Grosso, Brazil (Fig. 1). The search for populations was carried out by means of interviews with local residents, and also directly on the ground, looking for tunnel entrances. External body measurements (total length, body length, hind foot) were taken in the field, using a digital caliper (Mitutoyo®; 0.01 mm), and mass with a dynamometer. Linear cranial measurements (Leite, 2003) were taken in the laboratory with the same digital caliper.

The entire genomic DNA was isolated from liver, kidney, or heart tissue, following standard protocols (Longmire et al., 1997) from 20 individuals collected from the type locality. For the outgroup taxa, we obtained from GenBank the entire cytochrome-b sequences from 16 *Ctenomys* samples from 9 species, mostly from Bolivia and Peru, which represent the closest distributions to the point of collection of *C. nattereri*; one sequence of *Spalacopus cyanus;* and one of *Octodon degus* (GenBank access numbers: AF007055 *C. conoveri*; AF007051 *C. goodfellowi*; AF007050 *C. goodfellowi*; AF007056 *C. leucodon*; AF007042 *C. opimus*; AF007041 *C. opimus*; AF007044 *C. steinbachi*; AF007043 *C. steinbachi*; AF007040 *C. boliviensis*; AF007039 *C. boliviensis*; AF007038 *C. boliviensis*; AF007037 *C. boliviensis*; AF007049 *C. lewisi*; AF007046 *C. frater*; AF007045 *C. frater*; AF119107 *C. flamarioni*; AF007058 *Octodon degus*; AF007061

Spalacopus cyanus). These specimens were selected by taking into account their historical use in phylogenetic analyses in recent decades. The aligned sequences were inspected visually in ClustalX2 (Larkin et al., 2007).

The entire cytochrome b gene (1,140 bp) was amplified by the polymerase chain reaction (PCR); usually 30 cycles, alternating denaturation at 93°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min, using combinations of the primers MVZ5 and MVZ14 (Smith and Patton, 1993) as external primers. All PCR experiments included negative controls.

Amplifications were performed in 20-*M*l reaction volumes, each containing 25-100 ng DNA, 1.0 unit of Taq DNA polymerase, 0.2 m*M* of each external primer, 1.5 m*M* $MgCl_2$, 1.0 mM of 10X buffer, and water to complete the volume. The thermal profile consisted of an initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, and 45ºC for 30 s, extension at 72 ºC for 45 s, and a final extension at 72 ºC for 5 min. Double-stranded polymerase chain reaction products were purified using the EXO-SAP PCR system. Purified products were sequenced in a ABI 3730XL DNA analyzer (MACROGEN, Korea). Mitochondrial cytochrome-b gene sequences for the specimens examined in this study have been submitted to GenBank).

Phylogenetic relationships among *Ctenomys* species were estimated under the criteria of maximum likelihood using MEGA version 5 (Tamura et al., 2011) and Bayesian phylogenetics using MRBAYES (Huelsenbeck and Ronquist, 2001). We considered clades receiving bootstrap support >= 50% or Bayesian probabilities >=0.95%, or both. Maximum-likelihood approximation (Tamura et al., 2011) was used before maximum-likelihood analysis to determine the model of DNA sequence evolution that best fit to our data. The TN93+G model of evolution was chosen, along with the following parameters: base frequencies = 0.314, 0.304, 0.259, 0.124;

nst = 2; t ratio = 7.01; rates = gamma with shape parameter (a) 5 0.27; and proportion of invariant sites = 0.57.

Bayesian analysis was used to obtain an independent measure of phylogenetic relationships (Huelsenbeck and Ronquist, 2001). The HKY+G model of DNA sequence evolution was used, based on a preview analysis using jModelTest (Posada, 2008) along with site-specific rates of variation calculated for each of the 3 positions of the codon via the ‘‘ssgamma’’ option in MRBAYES. Four simultaneous Markov chains were run for 2,000,000 generations, starting with random, unconstrained, starting trees. Temperature was set at 0.02 to facilitate greater movement between the 4 Markov chains, and trees were sampled every 10 generations. Resulting burn-in values (the point at which the model parameters and tree scores became stationary) were determined empirically by evaluating likelihood scores. Three independent runs of MRBAYES were performed, using a different outgroup taxon, to ensure that the final trees converged upon the same topology.

Percent sequence divergence within and among clades was computed based upon Kimura 2-parameter corrected distances (Kimura, 1980). The Kimura 2-parameter model of sequence evolution was chosen to allow for comparison of the percent sequence divergence among taxa in this study with the recommendations of Bradley and Baker (2001) and Baker and Bradley (2006) regarding the cytochrome-b gene sequence difference, to evaluate cryptic genetic species. The percent sequence divergence based on 3rd codon position transversion substitutions was calculated using MEGA5 (Tamura et al., 2011).

Two males and two females were karyotyped in the field, following the general procedure of Baker et al. (1982). Mitotic metaphases from bone marrow were stained using phosphate-buffered Giemsa stain to determine the diploid number (2n) and fundamental number (FN). Ten

metaphases per animal were observed. The geometry of the cranium shape was captured by a configuration of topographically corresponding landmarks modified from D'Anatro and Lessa (2006) and Fernandes et al., 2009; Fornel et al., 2010. Each cranium was photographed in dorsal, ventral, and left lateral views of the skull, with a digital camera with 3.1 megapixels (2048 X 1536) of resolution, with macro function and without zoom or flash. We used 15 two-dimensional landmarks for dorsal, 13 for ventral, and 12 for lateral views of the skull, as follows. Fourteen landmarks, assumed to be homologous among all specimens analyzed, were chosen in each individual skull, as follows: Dorsal view: 1 – anterior extremity of suture between nasals, 2 - anterolateral extremity of incisor alveolus, 3 - anteriormost point of root of zygomatic arch, 4 - externalmost point of orbit in zygomatic arch foramen, 5 – suture between nasals and frontals, 6 - tip of extremity of superior jugal process, 7 - lateral extremity of suture between jugal and squamosal in the zygomatic arch, 8 – suture between squamosal and jugal, 9 – suture between frontals and parietals, 10 – suture between frontal, parietal and squamosal, 11 – tip of posterior process of jugal, 12 – internalmost contact between squamosal and tympanic bulla, 13 - anterior tip of external auditory meatus, 14 - point of maximum curvature on mastoid apophysis, and 15 - posteriormost point of occipital along the midsagittal plane. Ventral view: 1 – anterior extremity of suture between premaxillaries, 2 – anterolateral extremity of incisive alveolus, 3 – suture between premaxillary and maxillary in the external outline of the skull (on the photographic plane), 4 – tip of suture between premaxillaries in the incisive foramen, 5 - externalmost point of orbit in zygomatic arch foramen, 6 – anteriormost point of first molar alveolus, 7 - anteriormost point of intersection between jugal and squamosal, 8 – posteriormost point of fourth molar alveolus, 9 – anteriormost point in mesopterigoid fossa, 10 – anterior extremity of tympanic bulla, 11 – internalmost contact between squamosal and tympanic bulla, 12 – posterior extremity

of mastoid apophysis, and 13 – posteriormost point of foramen magnum along midsagittal plane. Lateral view: 1 – point of intersection between premaxillary and posterior end of incisor, 2 – anteriormost point of suture between nasals and premaxillary, 3 – anterior extremity of suture between nasals, 4 – suture between premaxilla, maxilla and frontal in superior zygomatic root, 5 – suture between premaxillary and maxillary in the outline of the skull (on the photographic plane), 6 – anteriormost point of premolar alveolus, 7 – inferior end of suture between maxillary and jugal in zygomatic arch, 8 – extremity of superior jugal process, 9 – tip of posterior jugal process, 10 – extremity of inferior jugal process, 11 – superior extremity of lambdoidal crest, and 12 – anteriormost margin of paraoccipital apophysis. For the dorsal and ventral illustrations, skulls were digitized only on the left side to avoid redundant information in symmetrical structures, following Cardini and O'Higgins (2004). The anatomical landmarks were digitized by the same individual (J.F.B.S.) for each specimen, using TPSDig2, version 2.16 (Rohlf, 2004; http://life.bio.sunysb.edu/morph). Coordinates were superimposed using a generalized Procrustes analysis (GPA) algorithm (Dryden and Mardia, 1998). GPA removes differences unrelated to shape, such as scale, position, and orientation (Rohlf and Slice, 1990; Rohlf and Marcus, 1993; Bookstein, 1996a, b; Adams and Rohlf, 2004). The error in landmark acquisition (operator variance) was evaluated through a one-way analysis of variance of centroid size for the repeated landmark acquisition of one image for each species. The mean estimated measurement error was 0.08%. The size of each skull was estimated using its centroid size, namely the square root of the sum of the squares of the distance of each landmark from the centroid (mean of all coordinates) of the configuration (Bookstein, 1991). Because each skull had three separate centroid sizes for each view, we calculated a single value by summing the logarithms of the dorsal, ventral, and lateral centroid sizes. We also used form (size plus shape), using log-transformed centroid size

plus the principal components matrix of shape variables. Differences in the shape of the skull inferred from statistical analyses were visualized through multivariate regression of shape variables on discriminant axes.

Differences in the log of centroid size of taxa or populations were tested with analysis of variance (ANOVA)] and pairwise comparisons using Tukey test. Differences in shape were explored by canonical variate analyses (CVA) and multivariate ANOVA. To visualize the shape differences, deformations along factorial axes were calculated by multivariate regressions (Monteiro et al., 2003). To test the validity of the a priori taxonomic assignments, classification percentages were estimated by multiple discriminant functions, using shape and form (size plus shape) parameters and leave-one-out cross-validations (Ripley, 1996). Because of the relatively small sample sizes and the large number of variables (40 bidimensional landmarks), statistical analyses of shape were performed using the dimension-reduction approach advocated by Baylac and Friess (2005): we used the smallest first PC set that maximizes the discrimination values. The overall phenotypic similarities between taxa were depicted using a Neighbor-joining tree computed from the matrix of Mahalanobis' D2 distances. All morphometric calculations were performed using the 'R' language, version 2.0 for Linux (R Development Core Team, 2004). Morphometric procedures were carried out with the 'Rmorph' library for R (Baylac, 2007).

The sample of skulls consisted of 9 specimens (TR1424, TR1425, TR1426, TR1427, TR1428, TR1438, TR1439, TR1440, TR1441) collected in Pontes e Lacerda municipality and 10 specimens (TR1429, TR1430, TR1431, TR1432, TR1433, TR1434, TR1435, TR1436, TR1446, TR1447) collected in Caceres, Mato Grosso, Brazil (Fig. 1), plus one museum specimen (FMNH-28358). We compared these specimens with skulls from two different species of the boliviensis group (Parada et al., 2011), *C. boliviensis* (N = 52) (all specimens from AMNH:

256008, 260804, 260805, 260806, 260808, 260810, 260811, 260814, 260815, 260820, 260821, 260822, 260824, 260825, 260826, 260827, 260828, 260829, 260830, 260831, 260832, 260834, 264503, 264504, 264505, 264506, 264507, 264508, 264509, 264510, 264511, 264513, 264515, 264516, 264517, 264518, 264519, 264520, 264521, 264522, 264523, 264524, 264525, 264527, 264528, 264530, 264531, 264533, 264534, 264535, 264536, 264537), and *C. steinbachi* (N = 12) (AMNH-260851, AMNH-260853, AMNH-260856, AMNH-262293, AMNH-262294, AMNH-262295, AMNH-262296, AMNH-262297, AMNH-75339, AMNH-75340, FMNH-51894, FMNH-51895). All these specimens were previously deposited in collections or museums, acronyms as follows: AMNH (American Museum of Natural History, New York City, USA); FMNH (Field Museum of Natural History, Chicago, USA) and TR (Mammal Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil). The field collections of specimens followed the guidelines of the American Society of Mammalogists for animal care and use (Gannon et al., 2007) and were approved by the Brazilian government (IBAMA) under authorization number 14690-1.

RESULTS

Eight males and twelve females were collected in the field. Their external measurements include the external body measurements reported for the original type species by Wagner, 1848 (total length = 372.8 ± 30.1 mm, body length = 292.3 ± 23.6 mm, hind foot with nail = 43.1 ± 2.7 mm, hind foot without nail = 37.8 ± 2.6 mm, mass = 265.7 ± 60 g). Pontes e Lacerda (PL) population seemed to live in the forest border division with rubber tree (*Hevea brasiliensis*) plantation, feeding entirely on this species root. Caceres (CA) population was very sparse in the forest and was only aggregated in the manioc (*Manihot esculenta*) fields. All the individuals captured inhabited their own individual tunnel.

Karyotype - The diploid number (2n) is 36 and the fundamental number (FN) is 64. The chromosomal complement consists of 8 pairs of submetacentrics, ranging from medium to large, 6 pairs of metacentrics from small to large and 3 pairs of acrocentrics from medium to large. The X is a large metacentric. This chromosomal composition is exactly the same founded to Bolivian Roboré region specimens (Fig. 2), and does not correspond to that of any other known *Ctenomys* species.

Description of nucleotide data. - Of the 1,140 sites resulting from alignment of cytochrome-b sequences of *Ctenomys* (excluding the outgroup), 728 (63.86%) were invariant. Of the 412 (36.14%) variable sites that were potentially phylogenetically informative, 108 (9.47%) were at 1st codon positions, 171 (15%) at 2nd codon positions, and 133 (11.67%) at 3rd codon positions. Five different haplotypes were found for Ponte e Lacerda and three for Caceres populations.

Phylogenetic analyses - In the composite tree (Fig. 3), a clade composed of *Octodon degus* and *Spalacopus cyanus*, with *Ctenomys flamarioni* is basal to all other taxa examined, as expected because these are the most divergent species in relation to the boliviensis, opimus and frater species groups analyzed (sensu Parada, 2011). The topology is consistent with previous analyses (Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004; Parada et al., 2011), including a monophyletic branch that joins *C. boliviensis* and *C. goodfellowi* with low support to separate this species through mitochondrial analysis. This species is considered differentiated because of its karyotype and morphology (Anderson et al., 1987).

The branch containing PL and CA specimens and two sequences of *C. boliviensis* is a well-supported monophyletic branch. These GenBank sequences are actually from two individuals captured near the Bolivian border, and misidentified as *C. boliviensis*, when they are actually *C. boliviensis robore*, as presently considered for individuals living near Roboré (Bolivia), which

have karyotype 2n=36 (Anderson et al., 1987; Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004). Correction of this misidentification exclude the observed paraphyly of *C. boliviensis*, and reorganizes the clade containing *C. boliviensis* and *C. goodfellowi* as a sister clade of *C. nattereri*, assuming that there is a differentiation between specimens with karyotype 2n=36 and individuals that are broadly recognized as *C. boliviensis* with karyotype numbers 2n=42, 44 and 46 (Lessa and Cook, 1998; Parada et al., 2011).

Percent sequence divergences among species (Table 2) were calculated using Kimura 2-parameter corrected distances. Comparisons among the currently recognized species of *Ctenomys* averaged 0.092 and ranged from 0.012 to 0.141. Percent sequence divergence was lowest between PL and CA (0.012), followed by divergence between this populations and *C. boliviensis robore* populations (0.020-0.021) and highest (0.141) between *C. conoveri* and *C. leucodon*. Percent sequence divergences of PL, CA and Roboré populations compared to other species of tuco-tucos were similar or higher to those found between recognized distinct species as *C. boliviensis* and *C. goodfellowi* (0.014).

For geometric morphometric analysis of the skull we considered CA and PL as the same species, what revealed no significant differences in centroid size between the species for the dorsal (ANOVA: P = 0.1928; F = 1.679) and ventral (ANOVA: P = 0.124; F = 2.1416), but significant differences were found in lateral view (ANOVA: P = 0.03174; F = 3.5995) with Tukey indicating significant differences between *C. boliviensis* and PL and CA populations, being *C nattereri* smaller. The results of the MANOVA showed significant differences in shape for all skull views, both separately and pooled ($P < 0.05$; dorsal: λWilks = 0.39507, F = 23.934; ventral: λWilks = 0.16185, F = 6.22; lateral: λWilks = 0.2302, F = 42.912; and the three views pooled: λWilks = 0.042, F = 10.601). The percentage of correct classification using form (size plus

shape) provided high values for the three species analyzed, for the three views of the skull separately and pooled (Table 1), with the highest value (100%) to the lateral view.

Canonical variate analyses (CVA) in each view and in the three views combined and Mahalanobis distances (Fig. 4) showed a clear separation in form among the three species analyzed, indicating that *C. nattereri* is more distinct in form among these species than *C. boliviensis* and *C. steinbachi* are from each other for lateral view of the skull. The shape differences among the species are shown in Figure 4.

DISCUSSION

Based on external morphology evaluation *C. boliviensis* and *C. nattereri*, the first described by Waterhouse (1848) and the second described by Wagner in the same year (Wagner, 1848), both species were synonymized (Anderson et al., 1987). In this same work, the authors showed a distinct karyotype for *C. boliviensis* from Roboré region with 2n=36. Lessa and Cook (1998) generated the *C. boliviensis robore* cytochrome-b sequence and based on phylogenetic analysis have already suggested that it may represent a different species from the typical *C. boliviensis*. A substantial nucleotide divergence between *C. boliviensis* and *C. boliviensis robore* haplotypes (approximately 5%) greater than *C. bo1iviensis robore* and *C. nattereri* haplotypes (approximately 2%) leads to a species separation of the individuals with karyotype 2n=36 (Mascheretti et al., 2000). Woods and Kilpatrick (in Wilson and Reeder, 2005) recognized it as a subspecies, *C. boliviensis nattereri*; however, no evidence or discussion supporting this opinion were presented.

The results of the chromosomal, molecular and morphological analyses indicate that the specimens of *Ctenomys* from Pontes e Lacerda and Caceres, state of Mato Grosso, plus specimens from Bolivia with karyotype 2n=36, FN=64, examined in our study represent a single

evolutionary lineage of *Ctenomys*, with no currently adequate museum type series. Because one of the populations contained in this species lives in the type locality of *Ctenomys nattereri* and there is no other species of *Ctenomys* known inhabiting the region and the external morphology is very similar to that original description we assumed that this is the historically described *Ctenomys nattereri* Wagner, 1848. These animals are genetically distinct from other species of *Ctenomys*, exhibit morphological characters that differentiate them in comparison with other species, inhabiting the sandy-soil portions of the Amazon forest border in Brazil and Bolivia. The levels of percent sequence divergence are comparable to values for recognized species of the genus *Ctenomys*, particularly from the boliviensis clade (Parada et al., 2011).

Unfortunately, no name-bearing specimen of *Ctenomys nattereri* is believed to be extant. Based on Article 75 of the International Code of Zoological Nomenclature (ICZN 1999), we express the exceptional necessity of a name-bearing specimen, in order to clarify the taxonomic status and the type locality of the species. In the absence of paratypes or paralectotypes, we designate a neotype, as follows:

Ctenomys nattereri (Wagner 1848)

Original description – The whole head and especially the muzzle is very broad and flat, the top of the nose is almost naked, with only a thin blur and high hairs, the eyes are small, incisors very broad and without slots. The cavity hearing is not involved by only a single strip, but by a true also very small, ear, which has the same shape as that of an otter and also to some extent resembles the human ear. The claws of the front legs are much larger than the hind legs, the former have their ends together and the latter did not. The soles are skinned and forepaw fingers as well as the back are stiffer in the nail root; directed towards the front, which however are arranged only in the hind legs as a comb, also the ends of the hands and soles the hind legs are

secured with hard. The tail is very thick and round at its base and laterally flattened toward the tip, is covered only by a few hairs, a little more numerous at the top. The hair on the head is very short, so that it cannot hide the ears, in the rest of the body is slightly longer, but not particularly abundant and is everywhere smooth, and bright. The color of the specimen is uniform and has a certain brightness rust-brown, with some black spots pending to some fine dark brown at the top, which lightly delimit tracks along the middle of the back and top of the head, gradually decreasing to finish in the sacral region. The bottom of the body have not black color, presenting a uniform rust color, interrupted only by some white deformed patches, found behind the front of the forelegs and hind legs. The isolated hairs present in the upper body is colored slate near their bases, which decreases in intensity and lateral extent, until it disappears completely at the bottom of the body, so that here are the monochrome light in the root. The whiskers are white, the incisors of a red-saffron alive, and the claws of a whitish horn color and also by the fingers of a dirty white. The soles of the feet are a light color of flesh. The same color also seems to be the skin of the fingers and tail, the latter are distributed hairs of a reddish brown. The female has two mammary glands between their hind legs, and the male does not have a scrotum pending. The following measures were taken in the specimen described: (total length = 331 mm, body length =257 mm, hind foot with nail = 47 mm, hind foot without nail = 33 mm).

Neotype – Stuffed skin, tissue sample fixed in ethanol and skull number TR1446, of an adult female housed in the Mammal collection, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil, collected by José Stolz and Thales de Freitas on 12 May 2009.

Type locality – Transition Cerrado/Amazon Forest, municipality of Cáceres, state of Mato Grosso, Brazil, 15°58'13"S 57°45'58"W, elevation 130 m (Fig. 1). This area seems to be the

transition of Amazon Forest, Cerrado grasslands and Pantanal wetland, near the Paraguai River. The specific habitat where the animals were trapped appears to be a manioc plantation in a cleared area of grassland in this small farm. The area comprehends the sandy alluvial deposits of several rivers.

Distribution – The species seems to be distributed in a sandy soil basin formed by several rivers that descend from the South Amazon forest border in direction to Pantanal wetland and Bolivian Chaco. The karyotype 2n=36, FN=64, that identifies it is present in Bolivian sandy soil along the Brazilian border, including Roboré (18°20'S 59°44'W), San Matias (16º22'S 58º24'W) and Cascabel, Bolivia (17º21'S 58º20'W), according to literature (Lessa and Cook, 1998; Mascheretti et al., 2000). The PL population were entirely captured in a rubber tree (*Hevea brasiliensis*) plantation, feeding entirely on this species root and is considered a plague, that feeds until the roots are gone, knocking down the trees leading to human conflicts.

Diagnosis – A medium-sized *Ctenomys* with karyotype number 2n=36, FN=64. The zygomatic arch is very rounded and the cranial depth at molar 1 very broad, premaxillary very short with nasal point reaching incisive insertion in premaxillary in lateral view of the skull (Fig.5). The fur is predominantly marbled brown and presents very dark hairs which delimit tracks along the middle of the back and top of the head, gradually decreasing to finish in the sacral region. The ventral hairs are pale brown unicolor (Fig.6).

External morphology – External measurements: total length = 398 mm, body length = 310 mm, hind foot = 38 mm, mass = 300 g. The coat is predominantly marbled brown. The head and back hairs are dark, forming a visible track that vanishes from head to sacral region. Lateral hairs have a dark gray base and a brown distal part, with visible abrupt transition to ventral hairs that are pale brown unicolor hairs. The inguinal and perianal regions have long whitish hair, making the

region very pale. The vibrissae are long and white, and the longest reach the base of the ear. The ear is very short, extending only a few millimeters above the head. The eyes are small but quite visible, completely dark. The sole of the foot is hairless and the back has very pale, well-spaced, stiff hairs. The legs are short and strong. The nails are strong, flattened laterally, and slightly sharp at the bottom. The incisors are orange in the front, as in other species. The tail is laterally flattened and has light-brown slightly stiff hairs.

Cranium – The rostrum is short, broad and robust (Fig. 5). The skull is large and rounded. The zygomatic arch is broader than the width of the auditory meatus and entirely rounded. Left auditory meatus is partially broken. The parietal and frontal are long, and the nose is proportionately short. The cranial depth at molar 1 is very broad, premaxillary very short with nasal point reaching incisive insertion in premaxillary in lateral view of the skull Laterally, the skull is broad, with a very robust braincase and a developed tympanic bulla, the diastema slightly spaced, incisors slightly procumbent and jugal projected toward front.

Linear cranial measurements: greatest skull length = 51.20; nasal = 16.88; rostral = 21.02; orbital = 15.41; rostral breadth = 12.66; interorbital constriction = 12.37; mastoid breadth = 33.33; zygomatic breadth = 34.73; condyloincisive = 48.17; basilar = 42.79; diastema = 14.35; maxillary toothrow = 10.09; palatal a = 23.78; palatal b = 11.03; incisive foramina = 6.58; bullar = 15.81; post palatal = 19.54; mesopterygoid fossa width = 5.43; maxillary breadth = 12.02; occipital condyle width = 10.57; rostral depth = 10.18; cranial depth = 22.24; cranial depth at m1 = 19.04.

Comparisons – In relation to *Ctenomys boliviensis* it is now clear that a karyotype difference separates this two entities, with *Ctenomys nattereri* presenting exclusively the 2n=36 number. The skull of the two species presents some evident differences, principally in lateral view, were

C. nattereri differentiates from *C. boliviensis* by having a much broader skull, a very short premaxillariy and jugal projected toward front.

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FIGURE LEGENDS

Fig. 1 – Map showing the locations of points of capture of *Ctenomys nattereri* populations.

Fig. 2 – *Ctenomys nattereri* female karyotype (upper line) and reorganized *Ctenomys boliviensis robore* (bottom line – from Cook and Bravo, 2004), showing the chromosome arms. 2n=36 and FN=64.

Fig. 3 – Phylogenetic relationships for species of *Ctenomys*, based on the entire 1,140 base pairs of the cytochrome-b gene. Numbers at nodes are bootstrap support from maximum likelihood (left), followed by Bayesian posterior probabilities (right), in percentages.

Fig. 4. Canonical variate analysis of *Ctenomys* species for shape variables using three cranial views combined (a1), dorsal (b1), ventral (c1) and lateral views (d1). Mahalanobis distance for the species analyzed for three views combined (a2) and lateral view (a3). Skull shape differences for dorsal (b2), ventral (c2), and lateral (d2) views of the skull, PC1; dorsal (b3), ventral (c3), and lateral (d3) views of the skull, PC2. Positive PC scores (solid lines), negative PC scores (dotted lines). Boliv= *C. boliviensis*; stein= *C. steinbachi* and natt= *C. nattereri.*

Fig. 6. *Ctenomys nattereri* neotype (TR1446) cranium. Top to bottom: dorsal, ventral and lateral views, showing the mandible.

Fig. 7. *Ctenomys nattereri* neotype (TR1446). Top to bottom: dorsal, ventral and lateral views of stuffed skin.

TABLES

Table. 1 – Percentage of correct classification from the linear discriminant analysis for previously recognized species of *Ctenomys*, for dorsal, lateral, ventral, and the three views pooled of the skull, using form (size plus shape).

	C. nattereri	*C. boliviensis*	*C. steinbachi*
dorsal	100	95	100
ventral	92.45	80	83.33
lateral	100	100	100
3 views	100	95	100

Table 2 – Estimates of mean Kimura 2-parameter distances between species. Estimated standard errors are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The analysis involved 12 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. There were a total of 1140 positions in the final dataset. The overall average is 0.092. The horizontal names are abbreviations of the same species names in the vertical column.

	PL	CA	conov	goodf	leuco	opim	stein	robor	boliv	lewisi	frater	flama
PL		0.003	0.010	0.007	0.011	0.009	0.008	0.004	0.007	0.011	0.011	0.008
CA	0.012		0.010	0.007	0.011	0.008	0.008	0.004	0.007	0.011	0.011	0.008
C. conoveri	0.112	0.115		0.011	0.011	0.010	0.010	0.010	0.010	0.009	0.010	0.010
C. goodfellowi	0.059	0.059	0.114		0.010	0.008	0.008	0.007	0.004	0.011	0.010	0.008
C. leucodon	0.103	0.100	0.141	0.100		0.010	0.010	0.010	0.010	0.011	0.011	0.010
C. opimus	0.081	0.077	0.100	0.070	0.099		0.009	0.008	0.008	0.011	0.011	0.008
C. steinbachi	0.070	0.070	0.110	0.071	0.109	0.080		0.008	0.008	0.011	0.010	0.008
C. bol (robore)	0.020	0.021	0.112	0.060	0.097	0.076	0.069		0.008	0.011	0.011	0.008
C. boliviensis	0.057	0.057	0.107	0.014	0.101	0.069	0.066	0.060		0.011	0.010	0.008
C. lewisi	0.126	0.131	0.088	0.116	0.132	0.118	0.119	0.123	0.118		0.007	0.010
C. frater	0.129	0.132	0.100	0.121	0.136	0.124	0.121	0.129	0.115	0.054		0.010
C. flamarioni	0.080	0.082	0.106	0.081	0.108	0.076	0.086	0.081	0.077	0.116	0.117	

Figures:

Figure 1

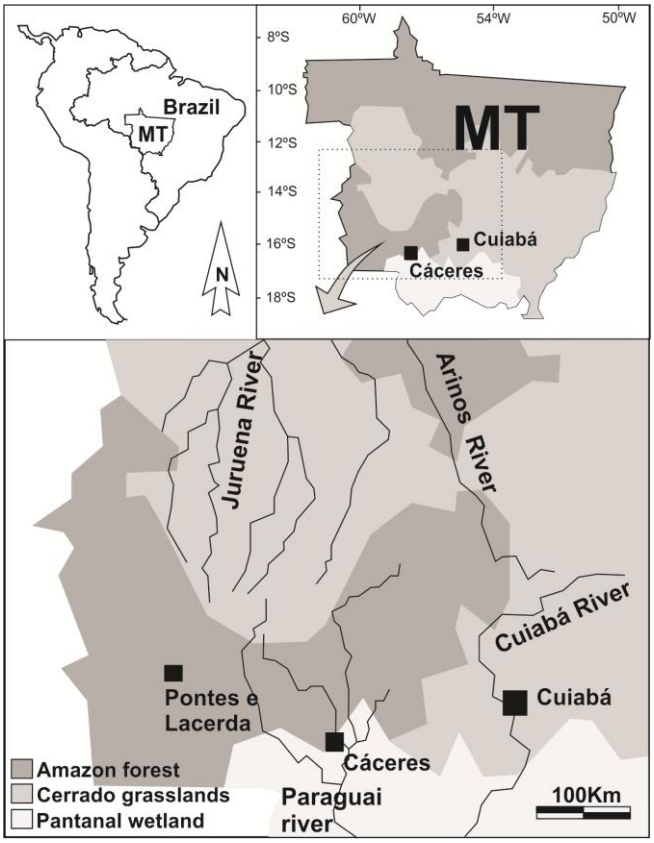


Figure 2

Figure 3

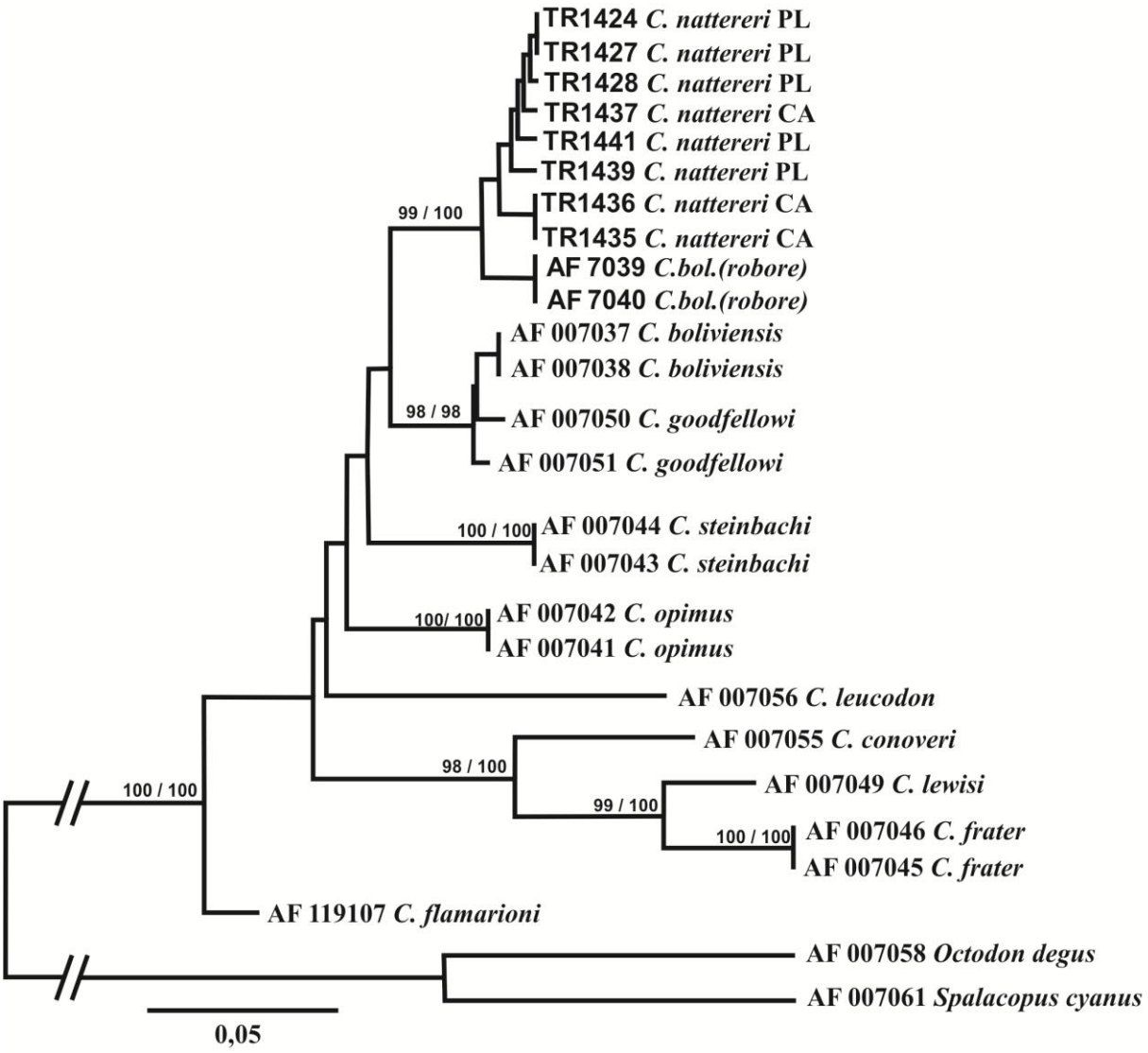

TR1424 C. nattereri PL
TR1427 C. nattereri PL
TR1428 C. nattereri PL
TR1437 C. nattereri CA
TR1441 C. nattereri PL
TR1439 C. nattereri PL
TR1436 C. nattereri CA
TR1435 C. nattereri CA
AF 7039 C.bol.(robore)
AF 7040 C.bol.(robore)
AF 007037 C. boliviensis
AF 007038 C. boliviensis
AF 007050 C. goodfellowi
AF 007051 C. goodfellowi
AF 007044 C. steinbachi
AF 007043 C. steinbachi
AF 007042 C. opimus
AF 007041 C. opimus
AF 007056 C. leucodon
AF 007055 C. conoveri
AF 007049 C. lewisi
AF 007046 C. frater
AF 007045 C. frater
AF 119107 C. flamarioni
AF 007058 Octodon degus
AF 007061 Spalacopus cyanus
99 / 100
98 / 98
100 / 100
100/ 100
98 / 100
99 / 100
100 / 100
100 / 100
0,05

Figure 4

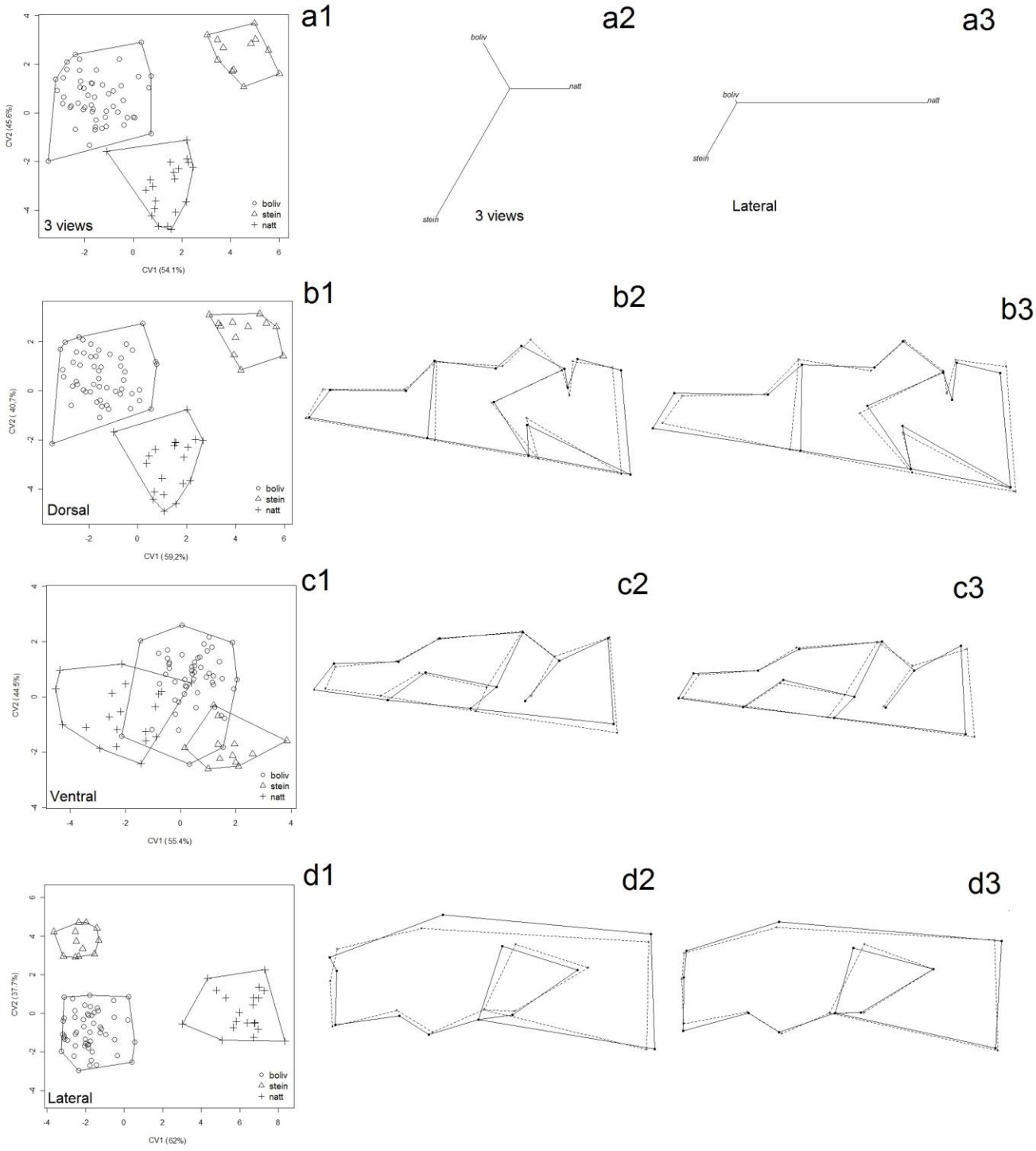

a1
a2
a3
3 views
boliv
stein
natt
CV1 (54.1%)
CV2 (45.6%)
3 views
Lateral
b1
b2
b3
Dorsal
CV1 (59,2%)
CV2 (40,7%)
c1
c2
c3
Ventral
CV1 (55.4%)
CV2 (44.5%)
d1
d2
d3
Lateral
CV1 (62%)
CV2 (37.7%)

Figure 5

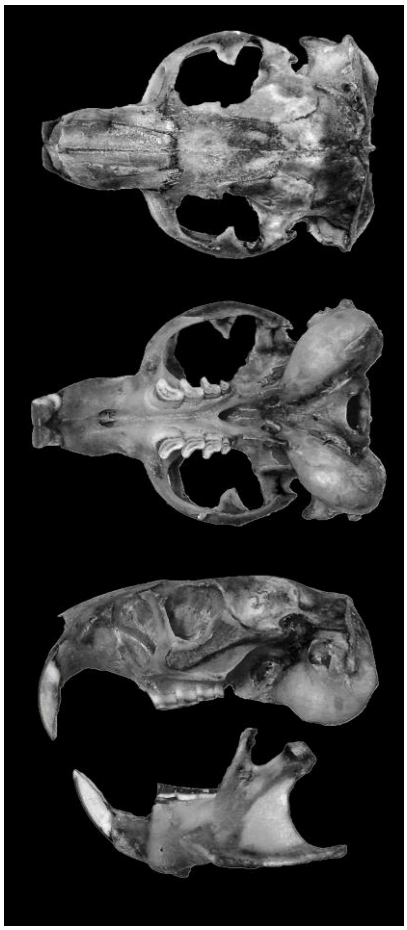
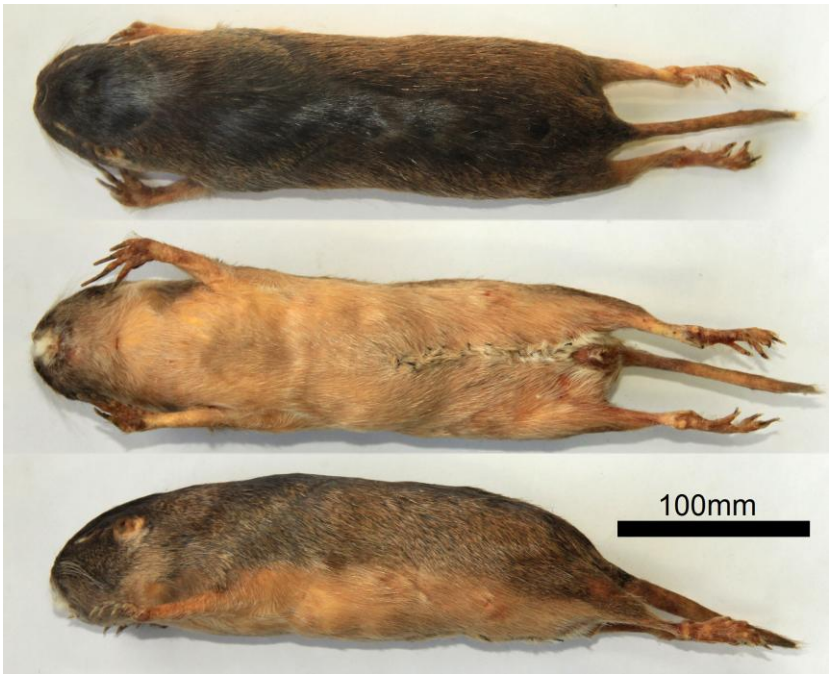


Figure 6



Capítulo III

An endemic new species of tuco-tuco, genus *Ctenomys* (Rodentia:Ctenomyidae) restricted to Amazon

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We present here a new species, *Ctenomys amazonicus*, from northwestern Brazil, in the Amazon ecoregion, and provide reliable taxonomic information and material. Phylogenetic relationships among *Ctenomys amazonicus* and ten other species of *Ctenomys* were examined using nucleotide sequence data from the entire 1,140 base pairs of the mitochondrial cytochrome-b gene. Maximum-likelihood and Bayesian analyses found the new species to be distinct from the others, and sister to *Ctenomys bicolor*. We present analyses of morphological data and qualitative descriptions of the external and cranial morphology, and of its karyotype. This species is important for the South American fauna because it is distributed in the East side of the Amazon forest, a novelty among the nearly 60 recognized species.

Keyword: *Ctenomys amazonicus*, taxonomy, new species, Amazon forest.

INTRODUCTION

The Neotropical subterranean rodents of the genus *Ctenomys* (tuco-tucos) comprise more than 60 described species. They are characterized by low vagility, patchy distribution, territoriality, and have been considered one of the most chromosomally variable mammals (2n = 10 – 70) and a model for studies of speciation and evolution (Reig et al. 1990; Freitas 2006). The relatively recent origin of the genus (Verzi et al. 2010) and the high species diversity suggest an explosive speciation event in tuco-tucos, which may be the reason for the recurrent poor resolution of basal relationships among species for all phylogenetic analyses performed with mitochondrial DNA (D'Elía et al. 1999; Lessa and Cook 1998; Mascheretti et al. 2000; Slamovits et al. 2001; Parada et al. 2011). In this case we can found *Ctenomys boliviensis* and *Ctenomys goodfellowi* clade that are undistinguishable by mitochondrial DNA although are recognized as distinct and valid species within boliviensis clade (Parada, 2011).

Taxonomy and systematics of the Brazilian ctenomyids is well known for the species that inhabit the southern region (Lichtenstein, 1830; Travi, 1981; Freitas and Lessa, 1984; Freitas, 1997; Freitas, 2001; Freitas, 2006; Fernandes et al., 2007). There are other species historically registered to Brazilian northwestern territory (Wagner, 1848; Miranda Ribeiro, 1914) although their taxonomy is not completely understood. This northwestern Brazilian species were discovered in expeditions trough the Brazilian wilderness like Rondon's expedition and naturalist John Natterer expeditions that focused on the west side of the Amazonian forest. There is a clear lack of information about what species of ctenomyids inhabit this part of Brazil, and their distribution, taxonomy and systematics. Defining these entities is the first step in providing a solid basis for needed future studies on their ecology and conservation, because these species

inhabit a landscape that is being dramatically changed by the human presence in Amazonia (Fearnside, 2005).

MATERIAL AND METHODS

The specimens were collected in the field, by means of Oneida Victor no. “0” traps, with rubber padding to avoid injuring the rodents, from two populations: Nova Ubiratã (NU - 12°57'13"S 54°55'0"W) and Nova Olímpia (NO - 14°52'14"S 57°17'22"W), state of Mato Grosso, Brazil (Fig. 1). The search for populations was carried out by means of interviews with local residents, and also directly on the ground, looking for tunnel entrances. External body measurements (total length, body length, hind foot) were taken in the field, using a digital caliper (Mitutoyo®; 0.01 mm), and mass with a dynamometer. Linear cranial measurements (Leite, 2003) were taken in the laboratory with the same digital caliper.

The entire genomic DNA was isolated from liver, kidney, or heart tissue, following standard protocols (Longmire et al., 1997) from 14 individuals collected from NO and NU municipalities and one individual of *Ctenomys bicolor* from Rondonia state, municipality of Pimenta Bueno, Brazil. For the outgroup taxa, we obtained from GenBank the entire cytochrome-b sequences from 14 *Ctenomys* samples (9 species), from Bolivia, Peru and Brazil, which represent the closest distributions and the most probable related evolutionary clade to this specimens; one sequence of *Spalacopus cyanus;* and one of *Octodon degus* (GenBank access numbers: AF007055 *C. conoveri*; AF007051 *C. goodfellowi*; AF007050 *C. goodfellowi*; AF007056 *C. leucodon*; AF007042 *C. opimus*; AF007041 *C. opimus*; AF007044 *C. steinbachi*; AF007043 *C. steinbachi*; AF007040 *C. boliviensis*; AF007039 *C. boliviensis*; AF007038 *C. boliviensis*; AF007037 *C. boliviensis*; AF007049 *C. lewisi*; AF007046 *C. frater*; AF007045 *C. frater*; AF119107 *C. flamarioni*; AF007058 *Octodon degus*; AF007061 *Spalacopus cyanus*) and two

species from Brazil: TR 1424 *C. nattereri*; TR1466 *C. bicolor* (Mammal Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil). These specimens were selected by taking into account their historical use in phylogenetic analyses in recent decades. The aligned sequences were inspected visually in ClustalX2 (Larkin et al., 2007). The entire cytochrome b gene (1,140 bp) was amplified by the polymerase chain reaction (PCR); usually 30 cycles, alternating denaturation at 93°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min, using combinations of the primers MVZ5 and MVZ14 (Smith and Patton, 1993) as external primers. All PCR experiments included negative controls.

Amplifications were performed in 20-*M*l reaction volumes, each containing 25-100 ng DNA, 1.0 unit of Taq DNA polymerase, 0.2 m*M* of each external primer, 1.5 m*M* $MgCl_2$, 1.0 mM of 10X buffer, and water to complete the volume. The thermal profile consisted of an initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, and 45ºC for 30 s, extension at 72 ºC for 45 s, and a final extension at 72 ºC for 5 min. Double-stranded polymerase chain reaction products were purified using the EXO-SAP PCR system. Purified products were sequenced in a ABI 3730XL DNA analyzer (MACROGEN, Korea). Mitochondrial cytochrome-b gene sequences for the specimens examined in this study have been submitted to GenBank).

Phylogenetic relationships among *Ctenomys* species were estimated under the criteria of maximum likelihood using MEGA version 5 (Tamura et al., 2011) and Bayesian phylogenetics using MRBAYES (Huelsenbeck and Ronquist, 2001). We considered clades receiving bootstrap support >= 50% or Bayesian probabilities >=0.95%, or both. Maximum-likelihood approximation (Tamura et al., 2011) was used before maximum-likelihood analysis to determine the model of DNA sequence evolution that best fit to our data. The TN93+G model of evolution was chosen, along with the following parameters: base frequencies = 0.314, 0.304, 0.259, 0.124;

nst = 2; t ratio = 7.01; rates = gamma with shape parameter (a) 5 0.27; and proportion of invariant sites = 0.57.

Bayesian analysis was used to obtain an independent measure of phylogenetic relationships (Huelsenbeck and Ronquist, 2001). The HKY+G model of DNA sequence evolution was used, based on a preview analysis using jModelTest (Posada, 2008) along with site-specific rates of variation calculated for each of the 3 positions of the codon via the ‘‘ssgamma’’ option in MRBAYES. Four simultaneous Markov chains were run for 2,000,000 generations, starting with random, unconstrained, starting trees. Temperature was set at 0.02 to facilitate greater movement between the 4 Markov chains, and trees were sampled every 10 generations. Resulting burn-in values (the point at which the model parameters and tree scores became stationary) were determined empirically by evaluating likelihood scores. Three independent runs of MRBAYES were performed, using a different outgroup taxon, to ensure that the final trees converged upon the same topology.

Percent sequence divergence within and among clades was computed based upon Kimura 2-parameter corrected distances (Kimura, 1980). The Kimura 2-parameter model of sequence evolution was chosen to allow for comparison of the percent sequence divergence among taxa in this study with the recommendations of Bradley and Baker (2001) and Baker and Bradley (2006) regarding the cytochrome-b gene sequence difference, to evaluate cryptic genetic species. The percent sequence divergence based on 3rd codon position transversion substitutions was calculated using MEGA5 (Tamura et al., 2011).

Two males and two females were karyotyped in the field, following the general procedure of Baker et al. (1982). Mitotic metaphases from bone marrow were stained using phosphate-

buffered Giemsa stain to determine the diploid number (2n) and fundamental number (FN). Ten metaphases per animal were observed.

The geometry of the cranium shape was captured by a configuration of topographically corresponding landmarks modified from D'Anatro and Lessa (2006) and Fernandes et al., 2009; Fornel et al., 2010. Each cranium was photographed in dorsal, ventral, and left lateral views of the skull, with a digital camera with 3.1 megapixels (2048 X 1536) of resolution, with macro function and without zoom or flash. We used 15 two-dimensional landmarks for dorsal, 13 for ventral, and 12 for lateral views of the skull, as follows. Fourteen landmarks, assumed to be homologous among all specimens analyzed, were chosen in each individual skull, as follows: Dorsal view: 1 – anterior extremity of suture between nasals, 2 - anterolateral extremity of incisor alveolus, 3 - anteriormost point of root of zygomatic arch, 4 - externalmost point of orbit in zygomatic arch foramen, 5 – suture between nasals and frontals, 6 - tip of extremity of superior jugal process, 7 - lateral extremity of suture between jugal and squamosal in the zygomatic arch, 8 – suture between squamosal and jugal, 9 – suture between frontals and parietals, 10 – suture between frontal, parietal and squamosal, 11 – tip of posterior process of jugal, 12 – internalmost contact between squamosal and tympanic bulla, 13 - anterior tip of external auditory meatus, 14 - point of maximum curvature on mastoid apophysis, and 15 - posteriormost point of occipital along the midsagittal plane. Ventral view: 1 – anterior extremity of suture between premaxillaries, 2 – anterolateral extremity of incisive alveolus, 3 – suture between premaxillary and maxillary in the external outline of the skull (on the photographic plane), 4 – tip of suture between premaxillaries in the incisive foramen, 5 - externalmost point of orbit in zygomatic arch foramen, 6 – anteriormost point of first molar alveolus, 7 - anteriormost point of intersection between jugal and squamosal, 8 – posteriormost point of fourth molar

alveolus, 9 – anteriormost point in mesopterigoid fossa, 10 – anterior extremity of tympanic bulla, 11 – internalmost contact between squamosal and tympanic bulla, 12 – posterior extremity of mastoid apophysis, and 13 – posteriormost point of foramen magnum along midsagittal plane. Lateral view: 1 – point of intersection between premaxillary and posterior end of incisor, 2 – anteriormost point of suture between nasals and premaxillary, 3 – anterior extremity of suture between nasals, 4 – suture between premaxilla, maxilla and frontal in superior zygomatic root, 5 – suture between premaxillary and maxillary in the outline of the skull (on the photographic plane), 6 – anteriormost point of premolar alveolus, 7 – inferior end of suture between maxillary and jugal in zygomatic arch, 8 – extremity of superior jugal process, 9 – tip of posterior jugal process, 10 – extremity of inferior jugal process, 11 – superior extremity of lambdoidal crest, and 12 – anteriormost margin of paraoccipital apophysis. For the dorsal and ventral illustrations, skulls were digitized only on the left side to avoid redundant information in symmetrical structures, following Cardini and O'Higgins (2004). The anatomical landmarks were digitized by the same individual (J.F.B.S.) for each specimen, using TPSDig2, version 2.16 (Rohlf, 2004; http://life.bio.sunysb.edu/morph). Coordinates were superimposed using a generalized Procrustes analysis (GPA) algorithm (Dryden and Mardia, 1998). GPA removes differences unrelated to shape, such as scale, position, and orientation (Rohlf and Slice, 1990; Rohlf and Marcus, 1993; Bookstein, 1996a, b; Adams and Rohlf, 2004). The error in landmark acquisition (operator variance) was evaluated through a one-way analysis of variance of centroid size for the repeated landmark acquisition of one image for each species. The mean estimated measurement error was 0.08%. The size of each skull was estimated using its centroid size, namely the square root of the sum of the squares of the distance of each landmark from the centroid (mean of all coordinates) of the configuration (Bookstein, 1991). Because each skull had three separate centroid sizes for

each view, we calculated a single value by summing the logarithms of the dorsal, ventral, and lateral centroid sizes. We also used form (size plus shape), using log-transformed centroid size plus the principal components matrix of shape variables. Differences in the shape of the skull inferred from statistical analyses were visualized through multivariate regression of shape variables on discriminant axes.

Differences in the log of centroid size of taxa or populations were tested with analysis of variance (ANOVA)] and pairwise comparisons using Tukey test. Differences in shape were explored by canonical variate analyses (CVA) and multivariate ANOVA. To visualize the shape differences, deformations along factorial axes were calculated by multivariate regressions (Monteiro et al., 2003). To test the validity of the a priori taxonomic assignments, classification percentages were estimated by multiple discriminant functions, using shape and form (size plus shape) parameters and leave-one-out cross-validations (Ripley, 1996). Because of the relatively small sample sizes and the large number of variables (40 bidimensional landmarks), statistical analyses of shape were performed using the dimension-reduction approach advocated by Baylac and Friess (2005): we used the smallest first PC set that maximizes the discrimination values. The overall phenotypic similarities between taxa were depicted using a Neighbor-joining tree computed from the matrix of Mahalanobis' D2 distances. All morphometric calculations were performed using the 'R' language, version 2.0 for Linux (R Development Core Team, 2004). Morphometric procedures were carried out with the 'Rmorph' library for R (Baylac, 2007).

The sample of skulls consisted of 6 specimens (TR1442, TR1443, TR1444, TR1449, TR1450, TR1452) collected in Nova Olímpia municipality and 8 specimens (TR1453, TR1454, TR1455, TR1456, TR1457, TR1458, TR1459, TR1460) collected in Nova Ubiratã, Mato Grosso, Brazil (Fig. 1). We compared these specimens with skulls from two different species of the

boliviensis group (Parada et al., 2011), *C. boliviensis* (N = 52) (all specimens from AMNH: 256008, 260804, 260805, 260806, 260808, 260810, 260811, 260814, 260815, 260820, 260821, 260822, 260824, 260825, 260826, 260827, 260828, 260829, 260830, 260831, 260832, 260834, 264503, 264504, 264505, 264506, 264507, 264508, 264509, 264510, 264511, 264513, 264515, 264516, 264517, 264518, 264519, 264520, 264521, 264522, 264523, 264524, 264525, 264527, 264528, 264530, 264531, 264533, 264534, 264535, 264536, 264537), and *C. steinbachi* (N = 12) (AMNH-260851, AMNH-260853, AMNH-260856, AMNH-262293, AMNH-262294, AMNH-262295, AMNH-262296, AMNH-262297, AMNH-75339, AMNH-75340, FMNH-51894, FMNH-51895). All these specimens were previously deposited in collections or museums, acronyms as follows: AMNH (American Museum of Natural History, New York City, USA); FMNH (Field Museum of Natural History, Chicago, USA) and TR (Mammal Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil). The field collections of specimens followed the guidelines of the American Society of Mammalogists for animal care and use (Gannon et al., 2007) and were approved by the Brazilian government (IBAMA) under authorization number 14690-1.

RESULTS

Seven males and seven females were collected in the field. Their external measurements are reported her: total length = 372.7 ± 93.4 mm, body length =310.5 ± 30.7 mm, hind foot with nail = 43.2 ± 4.1 mm, hind foot without nail = 37.8 ± 2.9 mm, mass = 343.9 ± 101.6 g. Karyotype - The diploid number (2n) is 34 and the fundamental number (FN) is 64. The chromosomal complement consists of 5 pairs of submetacentrics, ranging from medium to large, 9 pairs of metacentrics from small to large and 2 large acrocentrics pairs. The X is a large metacentric (Fig. 2), and this karyotype does not correspond to that of any other known *Ctenomys* species.

Description of nucleotide data. - Of the 1,140 sites resulting from alignment of cytochrome-b sequences of *Ctenomys* (excluding the outgroup), 728 (63.86%) were invariant. Of the 412 (36.14%) variable sites that were potentially phylogenetically informative, 108 (9.47%) were at 1st codon positions, 171 (15%) at 2nd codon positions, and 133 (11.67%) at 3rd codon positions. Two different haplotypes were found, one exclusive for NO and the other for NU populations.

Phylogenetic analyses - In the composite tree (Fig. 3), a clade composed of *Octodon degus* and *Spalacopus cyanus*, with *Ctenomys flamarioni* is basal to all other taxa examined, as expected because these are the most divergent species in relation to the "boliviensis, opimus and frater" species groups analyzed (sensu Parada, 2011). The topology is consistent with previous analyses (Lessa and Cook, 1998; Mascheretti, 2000; Cook and Bravo, 2004; Parada et al., 2011), including a monophyletic branch that joins *C. boliviensis* and *C. goodfellowi* with low support to separate this species through mitochondrial analysis. This species is considered differentiated because of its karyotype and morphology (Anderson et al., 1987).

The branch containing NO and NU specimens is a well-supported monophyletic sister branch of *C. bicolor*. The branch formed by this two species is sister of *C. nattereri* and this three species group are inserted within the boliviensis group (Parada, 2011). Percent sequence divergences among species (Table 2) were calculated using Kimura 2-parameter corrected distances. Comparisons among the currently recognized species of *Ctenomys* averaged 0.091 and ranged from 0.005 to 0.141. Percent sequence divergence was lowest between NO and NU (0.005), followed by divergence between this populations and *C. bicolor* (0.026) and highest (0.141) between *C. conoveri* and *C. leucodon*. Percent sequence divergences of NO and NU populations compared to other species of tuco-tucos were similar or higher to those found between recognized distinct species as *C. boliviensis* and *C. goodfellowi* (0.014).

Geometric morphometric analysis of the skull revealed no significant differences in centroid size between the species for the dorsal (ANOVA: P = 0.79; F = 0.225), ventral (ANOVA: P = 0.85; F = 0.162), and lateral view (ANOVA: P = 0.848; F = 0.1649). The results of the MANOVA showed significant differences in shape for all skull views, both separately and pooled ($P < 0.05$; dorsal: λWilks = 0.227, F = 40.593; ventral: λWilks = 0.03945, F = 15.13; lateral: λWilks = 0.17677, F = 51; and the three views pooled: λWilks = 0.0215, F = 14.286). The percentage of correct classification using form (size plus shape) provided high values for the three species analyzed, for the three views of the skull separately and pooled (Table 1), with the highest value (100%) to all views.

Canonical variate analyses (CVA) in each view and in the three views combined and Mahalanobis distances (Fig. 4) showed a clear separation in form among the three species analyzed, indicating that *C. amazonicus* is more distinct in form among these species than *C. boliviensis* and *C. steinbachi* are from each other for lateral view of the skull. The shape differences among the species are shown in Figure 4.

DISCUSSION

In Brazil, seven species of tuco-tucos have been described until now. Three of them, *Ctenomys rondoni*, *C. bicolor* and *C. nattereri*, are still little investigated; only a few records of these species have been reported in the state of Mato Grosso (Miranda Ribeiro 1914). The other four species of tuco-tuco, *C. torquatus*, *C. minutus*, *C. flamarioni* and *C. lami* occur in southern Brazil, in the states of RS and Santa Catarina (SC) (Fernandes et al. 2007). The type species of the genus, *Ctenomys brasiliensis*, until recently was presumed to have been described from Brazil, but, in fact, it was from Uruguay (Fernandes, personal communication). The levels of

percent sequence divergence are comparable to values for recognized species of the genus *Ctenomys*, particularly from the boliviensis clade (Parada et al., 2011).

The results of the chromosomal, molecular and morphological analyses indicate that the specimens of *Ctenomys* from NO and NU, state of Mato Grosso, represent a single evolutionary lineage of *Ctenomys*, adding one more species to the mammal diversity of Brazil with no currently adequate museum type series, whereby we present the holotype description as follows

Ctenomys amazonicus spec. nov.

Neotype – Stuffed skin, tissue sample fixed in ethanol and skull number TR1456, of an adult male housed in the Mammal collection, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil, collected by José Stolz and Thales de Freitas on May 2010.

Type locality –Amazon Forest, municipality of Nova Ubiratã, state of Mato Grosso, Brazil, 12°57'13"S 54°54'54"W, elevation 360 m (Fig. 1). This area is a mix of Amazon Forest and human deforested patches, near the Ronuro River. The specific habitat where the animals were trapped appears to be a recently cleared area of forest, including fired wood and fallen trees. The area comprehends the sandy alluvial deposits of Ronuro River.

Distribution – The species seems to be distributed in a sandy soil area that connects Amazon forest border from Serra dos Parecis at west to Xingu Amazon forest at east. The specimens were all collected in open areas on the forest border, where they were more easily sighted, but incursions into the forest (500m) could locate individual characteristic burrows and entrances on the forest floor. The collection points where entirely surrounded by forest by many kilometers of

extension, leading to believe that this is a species specialized or at least capable of leave inside the Amazon forest.

Diagnosis – A medium-sized *Ctenomys* with karyotype number 2n=34, NF=64. The fur is predominantly marbled nut brown and presents a very dark hair spot over the head. The ventral hairs are light brown with a pale-gray base and a light-brown distal part (Fig.6).

External morphology – External measurements: total length = 395 mm, body length = 315 mm, hind foot = 42 mm, mass = 300 g. The coat is predominantly marbled nut brown. A dark short hair spot is noticeable over the head. Lateral hairs have a dark gray base and a brown distal part, with soft transition to ventral hairs that have a light brown visual pattern but presents light gray base. The inguinal and perianal regions have long whitish hair, making the region very pale. The vibrissae are long and white, and the cheeks are light brown. The ear is very short, extending only a few millimeters above the head. The eyes are small but quite visible, completely dark. The sole of the foot is hairless and the back has very pale, well-spaced, stiff hairs. The legs are short and strong. The nails are strong, flattened laterally, and slightly sharp at the bottom. The incisors are orange in the front, as in other species. The tail is laterally flattened and has light-brown slightly stiff hairs.

Cranium – The rostrum is short, broad and robust (Fig. 5). The skull is large and rounded. The zygomatic arch is broader than the width of the auditory meatus and predominantly rounded. The parietal and frontal are long, and the nose is proportionately short. Laterally, the skull is broad, with a very robust braincase and well-developed tympanic bulla, the diastema slightly spaced, incisors slightly procumbent and jugal projected toward front. The base of nasal bones is very developed, forming an elevated area above the lacrimal insertion. The nasal front is projected and the premaxillary bone is noticeably very short.

Linear cranial measurements: greatest skull length = 54.69; nasal = 18.74; rostral = 25.43; orbital = 15.72; rostral breadth = 16.08; interorbital constriction = 12.27; mastoid breadth = 33.42; zygomatic breadth = 38.12; condyloincisive = 53.13; basilar = 46.08; diastema = 14.84; maxillary toothrow = 11.05; palatal a = 26.28; palatal b = 11.69; incisive foramina = 7.45; bullar = 14.21; post palatal = 19.82; mesopterygoid fossa width = 5.74; maxillary breadth = 12.22; occipital condyle width = 10.87; rostral depth = 11.29; cranial depth = 23.78; cranial depth at m1 = 20.38.

Comparisons – *Ctenomys amazonicus* differentiates from *C. boliviensis* by the pattern of fur, that has a dark spot over the head on the first and form a dark line over the back on the second. Although both species have a short premaxillary in lateral view, in *C. nattereri* the point of the nasal practically reaches the incisors insertion point in the bones, what do not happens in *C. amazonicus*. In comparison with C. bicolor, this species do not present the diagnostic triangular lateral expansion of the frontal bone.

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FIGURE LEGENDS

Fig. 1 – Map showing the locations of points of capture of *Ctenomys amazonicus* populations.

Fig. 2 – *Ctenomys amazonicus* male karyotype, showing the chromosome arms. 2n=34 and FN=64.

Fig. 3 – Phylogenetic relationships for species of *Ctenomys*, based on the entire 1,140 base pairs of the cytochrome-b gene. Numbers at nodes are bootstrap support from maximum likelihood (left), followed by Bayesian posterior probabilities (right), in percentages.

Fig. 4. Canonical variate analysis of *Ctenomys* species for shape variables using three cranial views combined (a1), dorsal (b1), ventral (c1) and lateral views (d1). Mahalanobis distance for the species analyzed for three views combined (a2) and lateral view (a3). Skull shape differences for dorsal (b2), ventral (c2), and lateral (d2) views of the skull, PC1; dorsal (b3), ventral (c3), and lateral (d3) views of the skull, PC2. Positive PC scores (solid lines), negative PC scores (dotted lines). boliv = *C. boliviensis*; stein = *C. steinbachi*; nova = *C. amazonicus*.

Fig. 5. *Ctenomys amazonicus* neotype (TR1456) cranium. Top to bottom: dorsal, ventral and lateral views, showing the mandible.

Fig. 6. *Ctenomys amazonicus* neotype (TR1456) stuffed skin. Top to bottom: dorsal, ventral and lateral views.

TABLES

Table. 1 – Percentage of correct classification from the linear discriminant analysis for previously recognized species of *Ctenomys*, for dorsal, lateral, ventral, and the three views pooled of the skull, using form (size plus shape).

	C. amazonicus	*C. boliviensis*	*C. steinbachi*
dorsal	100	100	100
ventral	100	100	100
lateral	100	100	100
3 views	100	100	100

Table 2 – Estimates of mean Kimura 2-parameter distances between species. Estimated standard errors are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The analysis involved 12 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. There were a total of 1140 positions in the final dataset. The overall average is 0.092. The horizontal names are abbreviations of the same species names in the vertical column.

	bicol	conov	goodf	leuco	opim	stein	boliv	lewisi	frater	flama	NU	NO	natter
C. bicolor		0.011	0.008	0.011	0.009	0.008	0.008	0.012	0.011	0.009	0.005	0.005	0.006
C. conoveri	0.120		0.011	0.012	0.010	0.010	0.010	0.009	0.010	0.010	0.011	0.011	0.010
C. goodfellowi	0.071	0.114		0.010	0.008	0.008	0.004	0.011	0.010	0.009	0.007	0.007	0.007
C. leucodon	0.111	0.141	0.100		0.010	0.010	0.010	0.011	0.011	0.010	0.010	0.010	0.011
C. opimus	0.086	0.100	0.070	0.099		0.009	0.008	0.011	0.011	0.008	0.009	0.009	0.009
C. steinbachi	0.083	0.110	0.071	0.109	0.080		0.008	0.011	0.011	0.008	0.008	0.008	0.008
C. boliviensis	0.071	0.107	0.014	0.101	0.069	0.066		0.011	0.010	0.008	0.008	0.008	0.007
C. lewisi	0.134	0.088	0.116	0.132	0.118	0.119	0.118		0.007	0.010	0.012	0.012	0.011
C. frater	0.134	0.100	0.121	0.136	0.124	0.121	0.115	0.054		0.010	0.011	0.011	0.011
C. flamarioni	0.088	0.106	0.081	0.108	0.076	0.086	0.077	0.116	0.117		0.008	0.009	0.008
NU	0.026	0.118	0.062	0.101	0.082	0.078	0.062	0.128	0.131	0.085		0.002	0.005
NO	0.026	0.117	0.062	0.097	0.082	0.076	0.060	0.125	0.128	0.085	0.005		0.005
C.nattereri	0.038	0.115	0.059	0.100	0.077	0.070	0.057	0.131	0.132	0.082	0.029	0.027	

Figures:

Figure 1

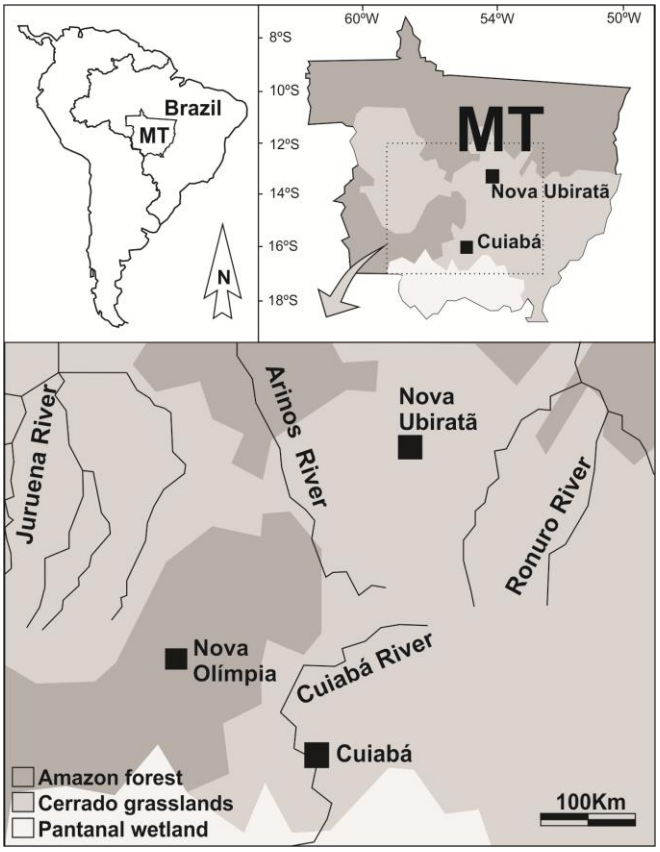


Figure 2

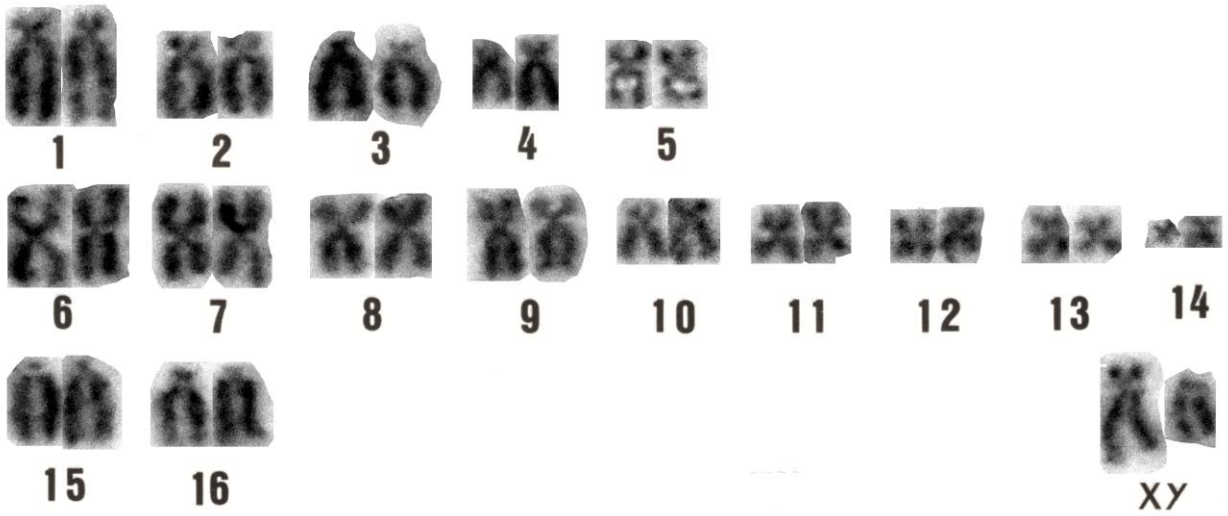


Figure 3

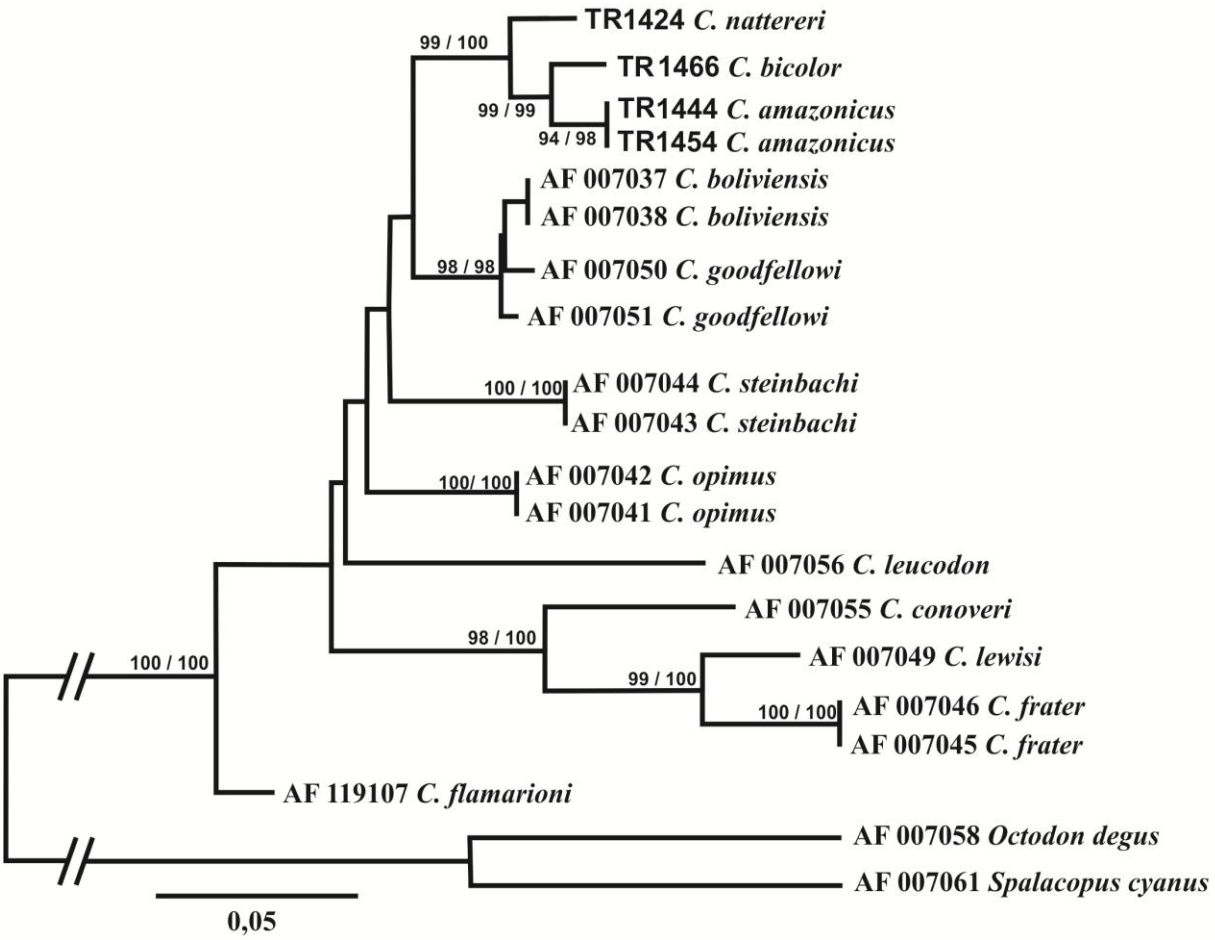

TR1424 C. nattereri
99 / 100
TR1466 C. bicolor
99 / 99
TR1444 C. amazonicus
94 / 98
TR1454 C. amazonicus
AF 007037 C. boliviensis
AF 007038 C. boliviensis
98 / 98
AF 007050 C. goodfellowi
AF 007051 C. goodfellowi
100 / 100
AF 007044 C. steinbachi
AF 007043 C. steinbachi
100/ 100
AF 007042 C. opimus
AF 007041 C. opimus
AF 007056 C. leucodon
AF 007055 C. conoveri
98 / 100
AF 007049 C. lewisi
100 / 100
99 / 100
AF 007046 C. frater
AF 007045 C. frater
AF 119107 C. flamarioni
AF 007058 Octodon degus
AF 007061 Spalacopus cyanus
0,05

Figure 4

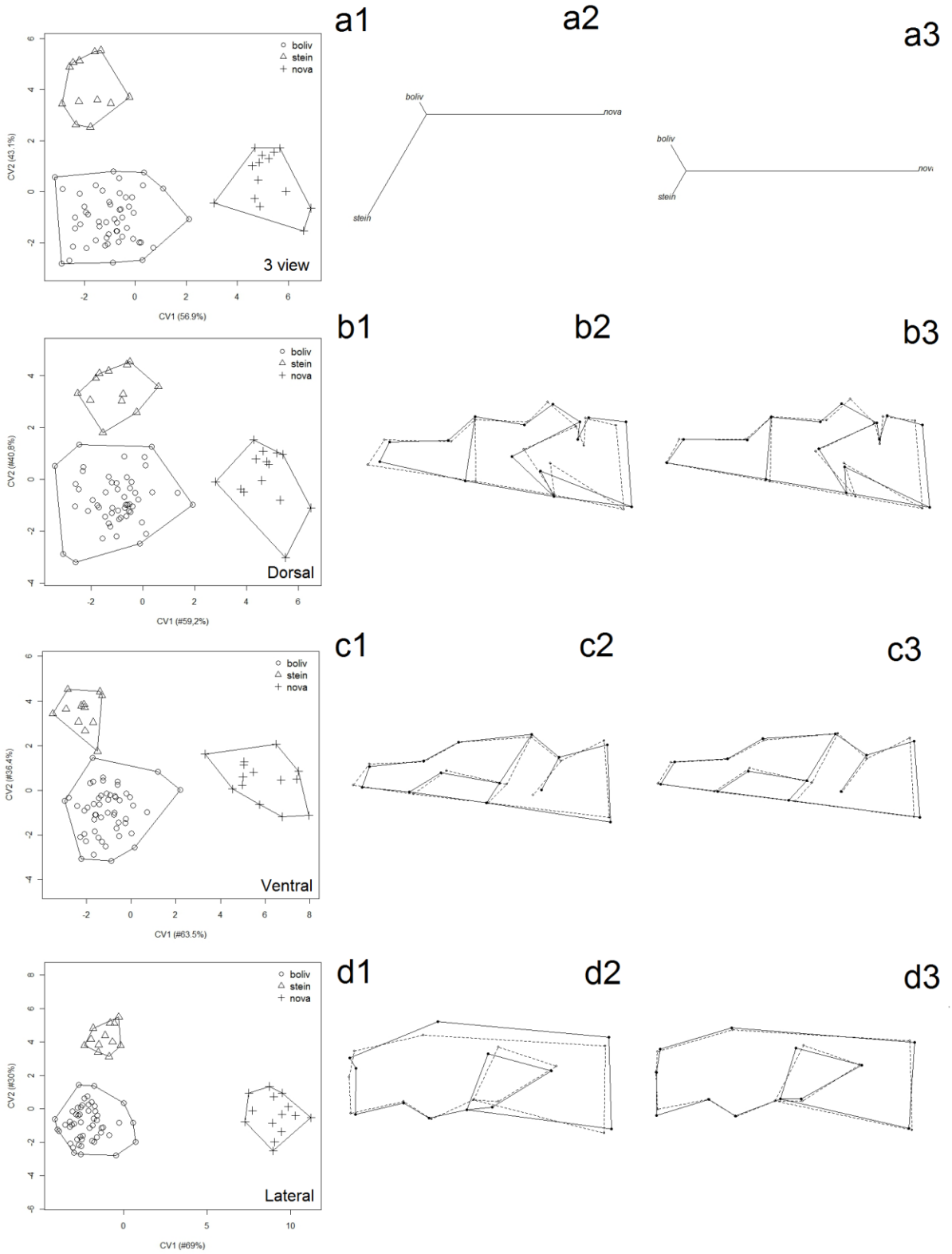
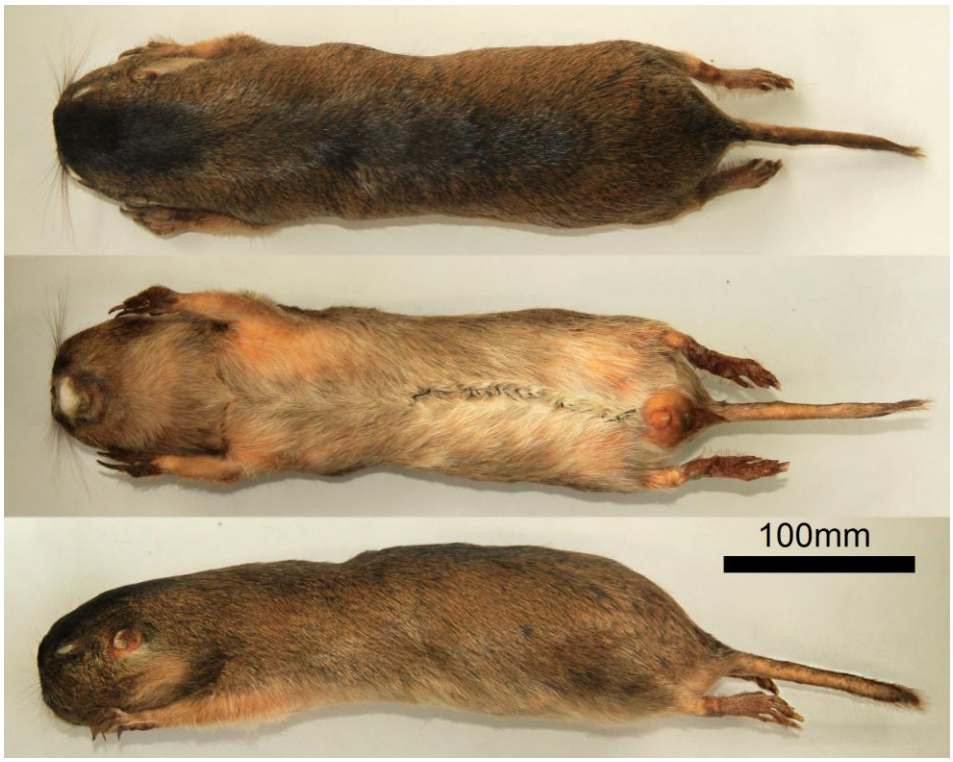

a1
a2
a3
boliv
stein
nova
3 view
CV1 (56.9%)
CV2 (43.1%)
b1
b2
b3
Dorsal
CV1 (#59,2%)
CV2 (#40,8%)
c1
c2
c3
Ventral
CV1 (#63.5%)
CV2 (#36.4%)
d1
d2
d3
Lateral
CV1 (#69%)
CV2 (#30%)

Figure 5

Figure 6



Capítulo IV

História evolutiva dos tuco-tucos do noroeste brasileiro e discussões gerais

Utilizando-se dos mesmos indivíduos e técnicas que compõem as análises por separado dos três primeiros capítulos desta tese, foram realizadas análises em conjunto buscando explicar um panorama evolutivo para estas espécies.

Para tanto, um indivíduo de cada espécie foi selecionado do GenBank e das três do presente trabalho para uma análise filogenética de máxima verossimilhança e também Bayesiana, com os mesmos parâmetros das análises dos capítulos anteriores (Fig.1). Utilizou-se também o método de datação molecular com calibração para o gênero *Ctenomys* (Roratto, 2012) para obterem-se os valores de divergência evolutiva em milhões de anos entre as espécies do grupo boliviensis que habitam a região amazônica. Foram realizados os testes de neutralidade D de Tajima (Tajima, 1989) e F de Fu (Fu, 1997) nas espécies de interesse com o intuito de verificar uma possível expansão populacional. Os valores dos testes de neutralidade indicam uma população estável ($p>0.1$; Tajima's D: -0,17570; Fu F: -0,14572).

Os mesmos crânios utilizados nas comparações interespecíficas foram utilizados em uma análise geométrica morfométrica global, usando a análise das variáveis canônicas (CVA) e MANOVA. Regressões multivariadas foram utilizadas para visualizar as deformações ao longo dos eixos fatoriais (Fig.2) e um teste de revalidação a priori global (Tab.1). Foi utilizada também uma análise de distância de Mahalanobis calculada através da árvore de Neighbor-joining para visualização das distâncias fenéticas entre as espécies avaliadas (Fig.2; a2, a3).

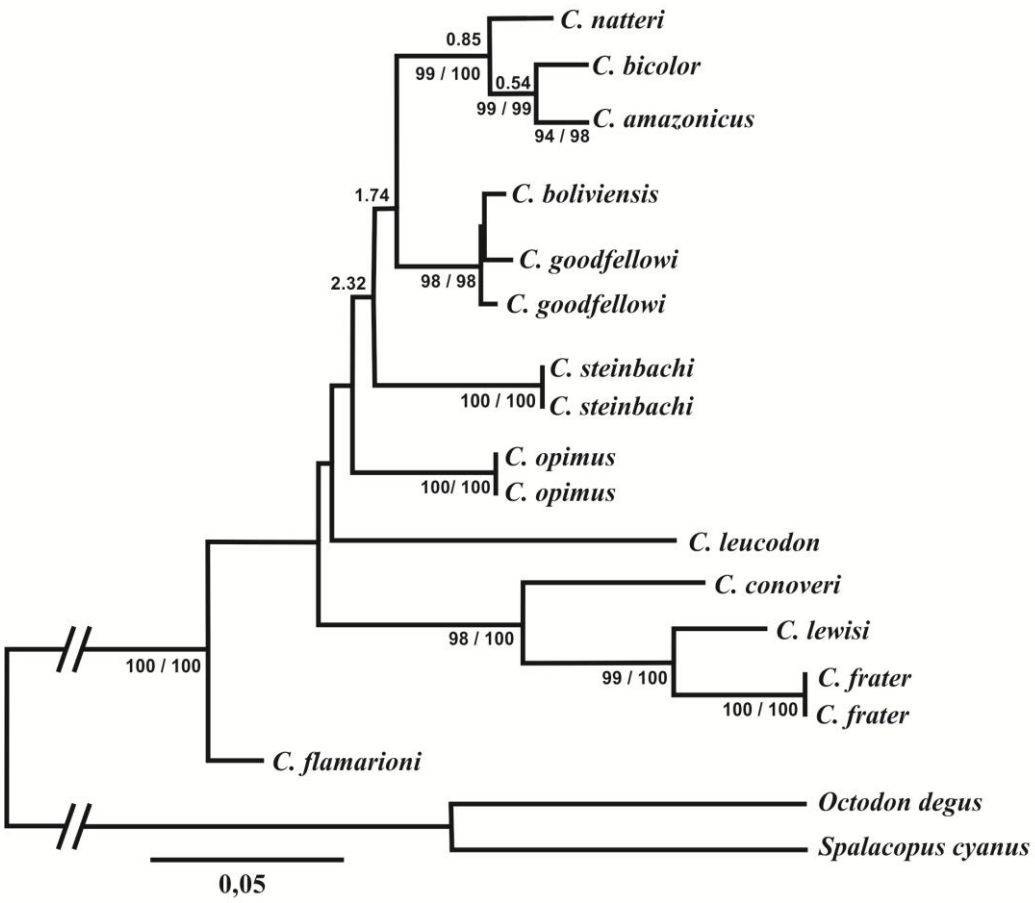


Fig. 1 – Relações filogenéticas para espécies de *Ctenomys*, baseadas na região completa do citocromo-b (1.140bp). Números abaixo dos nós são valores de suporte de bootstrap para máxima verossimilhança (esquerda) e probabilidades posteriores Bayesianas (direita), em percentagens. Números acima dos nós são os anos estimados através de datação molecular (em milhões de anos).

Tab. 1 – Percentagem de classificação correta da analise discriminante linear para espécies previamente identificadas de *Ctenomys* para visão dorsal, ventral, lateral e três vistas combinadas usando a forma do crânio.

	C. bicolor	*C. boliviensis*	*C. nattereri*	*C. amazonicus*	*C. steinbachi*
dorsal	92.30	98.07	100	100	100
ventral	69.23	90.38	60	92.85	83.3
lateral	84.61538	100	95	100	100
3 views	92.30	96.15	85	100	100

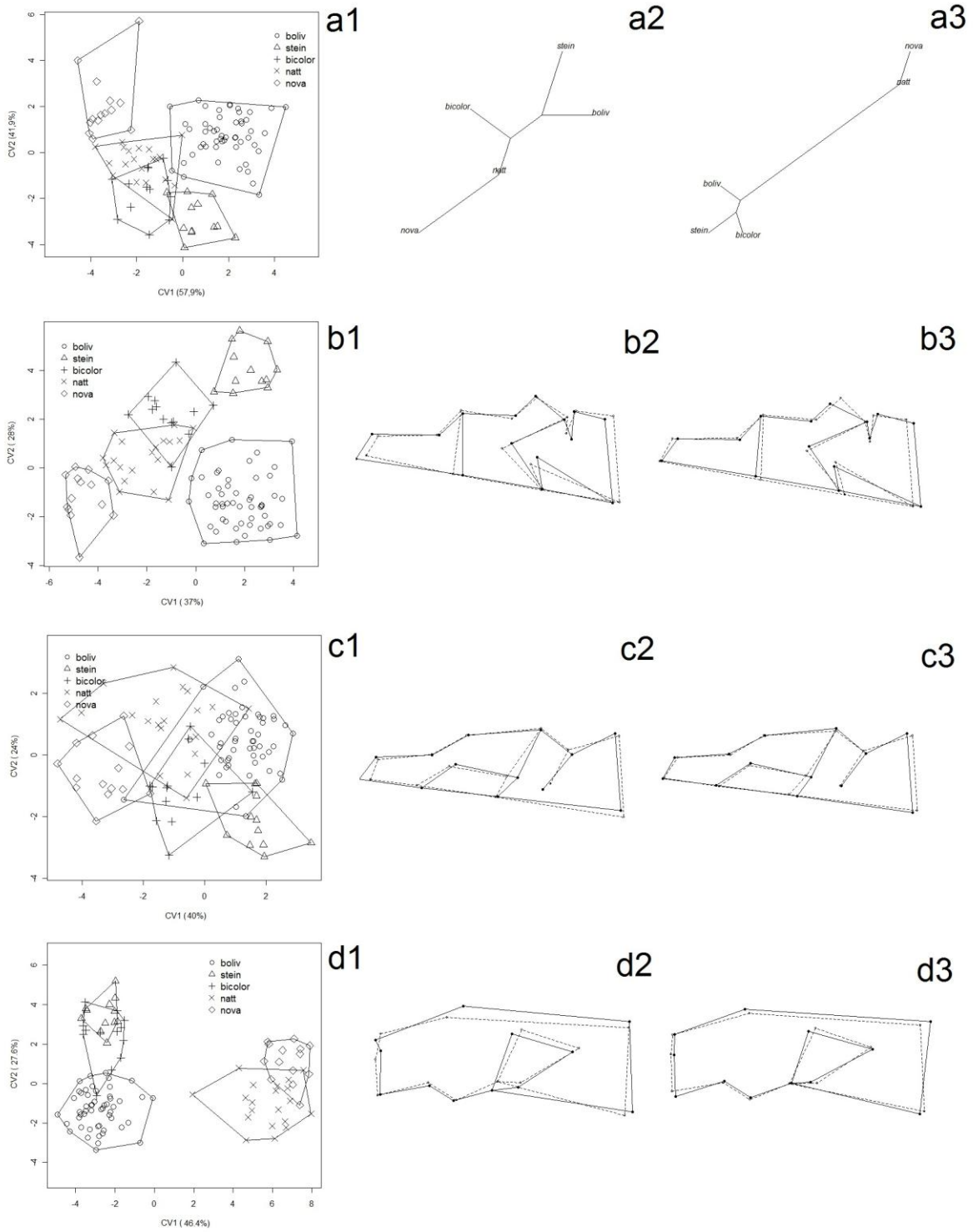


Fig. 2. Análise das variáves canonicas de espécies de *Ctenomys* para variáveis da forma usando vistas: dorsal (a1), ventral (b1), lateral (c1) e combinação das três vistas (d1). Distância de Mahalanobis para as espécies analisadas nas três vistas combinadas (a2) e vista lateral (a3). Diferenças na forma do crânio para vista dorsal (b2), ventral (c2), e lateral (d2), PC1; dorsal (b3), ventral (c3), e lateral (d3), PC2. Valores positivos de CVA (linhas sólidas), valores negativos de CVA (linhas pontilhadas. boliv= *C. boliviensis*; stein = *C. steinbachi*, bicolor = *C. bicolor*, natt = *C. nattereri e* nova = *C. amazonicus.* Os números entre parênteses representam o percentual de variação explicado para cada eixo.

Filogeneticamente, foram obtidos resultados inéditos para as espécies, uma vez *que C. bicolor e C. amazonicus* jamais haviam estado em uma análise molecular. Coincidentemente, estas duas espécies são irmãs, com um ancestral comum existente a cerca de 540 mil anos do presente, e estão separadas de um ancestral comum com *C. nattereri* a aproximadamente 850 mil anos do presente (Fig.1).

Morfologicamente, *C. nattereri* parece apresentar uma forma intermediária entre *C. bicolor e C. amazonicus* (Fig.2, a2), mas aproxima-se mais na forma de *C. amazonicus* por possuir um crânio mais robusto, mais alto na altura do molar 1, e uma pré-maxila mais curta, o que se destaca em visão lateral do crânio (Fig.2, a3,d1,d2). Talvez o encurtamento do rostro esteja sendo selecionado por questões ambientais ou devido aos hábitos alimentares, já que ambas as espécies supõe-se alimentarem-se de raízes de plantas lenhosas, já que foram capturados sob plantações de árvores ou no meio da floresta densa. Apesar das similaridades, todas as espécies analisadas apresentam altos valores de reclassificação específica (Tab.1), o que corrobora com a validação de diferentes entidades evolutivas.

Assim como foram publicadas originalmente cada uma das duas espécies, *Ctenomys boliviensis*, com localidade tipo próximo a cidade de Santa Cruz de la Sierra (Waterhouse, 1848) e *Ctenomys nattereri*, com localidade tipo na cidade de Caceres, MT, Brazil (Wagner, 1848) estavam corretamente válidas, apesar da posterior sinonimização proposta por Anderson (1987) que por falta de uma diferenciação morfológica externa mais evidente, sinonimiza os indivíduos do Brasil como *Ctenomys boliviensis* subespécie roboré. Realmente, a descrição original de *C. nattereri*, baseada em dois indivíduos de Cáceres não apresenta caracteres que distinguam as duas formas. Apesar disso, diversos autores chamaram a atenção para alguma característica que

distinguia os indivíduos da fronteira da Bolivia com o Brasil (Lessa e Cook, 1998; Mascheretti, 2000; Cook e Bravo, 2004). Somente agora podemos afirmar tratar-se de duas espécies distintas com linhagens evolutivas independentes.

A invasão do grupo amazônico na área florestal parece haver ocorrido em um tempo (anterior a 850 mil anos atrás) em que grandes extensões de savana tomaram lugar onde hoje está localizada a floresta, no quaternário (Haffer e Prance, 2002). Esse ancestral comum às três espécies fora depois pressionado pelo avanço das florestas em um período mais recente de temperaturas elevadas e mais úmido, onde a floresta voltou a expandir-se. Neste ambiente de mudanças, foram selecionononados indivíduos que puderam aproveitar o ambiente florestal para sobreviverem e diferenciaram-se em um novo morfotipo, adaptado à vida e à alimentação no solo das florestas, vide diferenciações morfológicas.

O isolamento entre as espécies parece haver ocorrido há muito tempo (aproximadamente 540 mil anos) e a diferenciação das mesmas parece ser antiga, já que os resultados dos testes de neutralidade não detectaram uma expansão recente. Uma vez invadida a floresta, as populações devem ter sido isoladas em ilhas florestais pelas sucessivas progressões e regressões do ambiente florestal sujeito às condições climáticas (Haffer e Prance, 2002; Freitas et al., 2001) dos últimos milhares de anos, levando ao isolamento reprodutivo. A diferenciação cromossômica estaria facilitada pela ação de demes isolados (Reig et al., 1990; Busch et al; 2000), e a ação da deriva genética que permite a fixação rápida de rearranjos cromossômicos. No conjunto dos dados, fica clara a diferenciação entre as espécies e intriga essa capacidade adaptativa para a vida florestal, que deve ser estudada ainda em minúcia no futuro.

Conclusões gerais

Existem de fato não somente as espécies já descritas de ctenomideos para a região noroeste do Brasil, como também uma espécie jamais coletada e identificada que habita o interior da floresta amazônica, o que é uma novidade evolutiva para o gênero, considerado ocupante de áreas de vegetação gramíneo-arbustiva.

Apesar do uso de diferentes técnicas na avaliação da variabilidade genética e morfológica dos indivíduos capturados, técnicas consagradas como morfologia externa e cariotipagem demonstraram-se eficientes para a identificação das espécies.

O uso da morfometria geométrica do crânio demonstrou-se muito eficiente na identificação de padrões de forma dificilmente visualizados sem o uso desta técnica, mesmo utilizando um número consideravelmente menor de marcos anatômicos marcados em cada indivíduo, o que demonstra ainda mais a robustez da técnica.

Somente o uso de diferentes técnicas de abordagem, tanto genéticas quanto morfológicas, podem prover uma análise realmente confiável das características evolutivas das espécies analisadas, em vista da diferenciação de resultados apresentada, uma vez que a avaliação de cada método em separado pode levar a conclusões distintas daquelas alcançadas com a observação das outras, como diferentes agrupamentos morfológicos e genéticos a sua vez.

O grupo de espécies em questão é bastante recente e parece haver surgido nas últimas variações climáticas que atingiram e modificaram a estrutura vegetal da região amazônica, fazendo surgir diferentes barreiras ambientais e o isolamento das populações em diferentes áreas deste bioma.

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