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# TUCO-TUCOS DO PAMPA RIO-GRANDENSE: A FILOGEOGRAFIA DE *Ctenomys torquatus* (RODENTIA - CTENOMYIDAE) E A DESCRIÇÃO DE UMA NOVA ESPÉCIE.

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O presente trabalho foi desenvolvido junto ao Laboratório de Citogenética e Evolução de Mamíferos da Universidade Federal do Rio Grande do Sul e, em parte, no Laboratório de Diversidade Genética da Universidade Federal de Santa Maria, contando com o auxílio financeiro de:

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*Assim como a fertilidade do solo no vale do Nilo criou o Egito; assim como a posição à beira do Mediterrâneo deu ocasião à soberania marítima dos fenícios; assim como as montanhas azuis da Hélada se tornaram o berço da cultura helênica: da mesma maneira, a Campanha do sudoeste deu origem à cultura rio-grandense. Em toda parte, o verdadeiro criador da cultura não é o ambiente físico, é o homem com seus valores indestrutíveis; mas a roupagem externa da cultura, sua expressão regional, esta sim, é determinada pela paisagem natural.*

(Balduíno Rambo)

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**SUMÁRIO**

**RESUMO**

O tuco-tuco de colar, *Ctenomys torquatus*, possui uma das maiores distribuições geográficas entre estes roedores, habitando os campos de baixa altitude em toda metade norte do Uruguai e na porção sul do estado do Rio Grande do Sul, Brasil. A variabilidade cromossômica, comum em espécies deste roedor subterrâneo, ocorre em apenas algumas populações periféricas de *C. torquatus*, particularmente em regiões na quais grandes cursos d'água separam formas cromossômicas distintas.

Com os objetivos de descrever os padrões filogeográficos de *C. torquatus*, bem como o efeito de grandes rios na estruturação de populações e diferentes cariótipos, o presente estudo fez uso de três marcadores mitocondriais (sequência hiper variável 1 da região controle e os genes citocromo-*c* oxidase I e citocromo-*b*), além do desenvolvimento e aplicação de locos de microssatélites.

A filogenia obtida com o gene citocromo-*b*, juntamente com evidências morfológicas e cariotípicas, claramente demonstraram a singularidade de uma nova espécie, denominada *Ctenomys ibicuiensis*. Esta espécie possui apenas um cariótipo (2n = 50) e não compartilha um ancestral comum direto recente com o tuco-tuco de colar, apesar da proximidade geográfica. Este trabalho apresenta a posição filogenética da nova espécie e sua relação filogeográfica com o tuco-tuco de colar. Análises filogenéticas também foram empregadas com o objetivo de estimar uma taxa de evolução molecular para o DNA mitocondrial dos ctenomídeos, a qual foi posteriormente aplicada em inferências demográficas considerando uma escala de tempo.

Associada a estreita área de ocorrência e pequena amostragem, as populações de *C. ibicuiensis* apresentaram baixa variabilidade genética em comparação com as demais espécies do gênero *Ctenomys*, embora não tenha havido evidências da ocorrência de processos de redução populacional (*bottleneck*) nem índices de endogamia singificativos.

Análises de estruturação genética para dados de microssatélite e DNA mitocondrial revelaram diferenciação significativa entre as populações, não havendo situações de clados ou grupos de haplótipos altamente divergentes, relacionados a linhagens regionais, para nenhuma das espécies. Testes de neutralidade, *mismatch distribution* e duas análises demográficas baseadas em coalescência sinalizaram a ocorrência de expansão populacional recente para *C. torquatus*,

amplamente distribuída; ao passo que um padrão de estabilidade populacional foi observado para a espécie *C. ibicuiensis*, geograficamente restrita.

A região central do Rio Grande do Sul é a provável origem da expansão populacional recente descrita para *C. torquatus*. O cariótipo mais comum e amplamente distribuído 2n = 44, considerado plesiomórfico, e os registros pontuais e periféricos de variações cariotípicas, corroboram este cenário de expansão.

AMOVA e outras análises de estruturação não demonstraram substancial partição da variação genética associada à presença dos rios. O efeito do rio atuando como barreira ao fluxo gênico foi verificado apenas pela comparação da baixa estruturação genética entre as localidades da nascente em relação aos demais pontos do rio onde a largura e a vazão são maiores. A expansão demográfica do tuco-tuco de colar e diversos eventos de dispersão cruzando o rio, evidenciados pelo compartilhamento de haplótipos em localidades de ambas as margens, podem estar vinculados as condições de clima seco e frio durante o período glacial pleistocênico, caracterizado pelo domínio dos campos, ausência de mata ciliar e rios com cursos d'água bastante reduzidos. Considerando as condições de baixa vagilidade, territorialidade e organização em pequenos demes, os efeitos de isolamento pela distância e deriva genética parecem prevalecer sobre as descontinuidades do habitat, a exemplo da alta diferenciação populacional encontrada.

**ABSTRACT**

The collared tuco-tuco, *Ctenomys torquatus*, has one of the widest ranges among tuco-tucos, inhabiting the grassed lowlands from the half north of Uruguay to the southern part of the Rio Grande do Sul State, Brazil. Chromosomic variability, common for these subterranean rodents, is showed in punctual and peripherical populations of *C. torquatus*, with particular reference for two situations were a great river separating distinct cariotípica forms.

With the aims of describing the phylogeographic patterns of *C. torquatus*, as well as the effects of major rivers on structuration of its populations and karyomorphs, the present study made use of three mitochondrial markers (the hyper variable sequence 1 of the control region, the cytochrome *c* oxidase I and the cytochrome *b* genes), besides the development and application of microsatellite loci.

Phylogeny of the cytochrome *b* gene, associated with morphological and karyotypic evidences, unambiguously demonstrated the uniqueness of a new species, denominated *Ctenomys ibicuiensis*. This species is karyotypicaly monomorphic (2n = 50) and do not share a direct and recent common ancestor with the collared tuco-tuco, despite ranges neighborhood. The present work shows the phylogenetic position of the new species and its phylogeographic relationships with the collared tuco-tuco. Phylogenetic analysis was also approached in order to estimate molecular rates for the mitochondrial DNA of tuco-tucos, which subsequently were applied for demographic inferences in a timescale framework.

Associated with its small geographic range and sampling, *C. ibicuiensis* populations showed low genetic variability in comparison with other tuco-tucos, but no evidences of bottlenecks or endogamy were reported.

Analysis of genetic structure for microsatellite loci and mitochondrial DNA revealed strong differentiation among populations, anyone deeply divergent clade associated with regional lineages was found for both species. Neutrality tests, mismatch distributions and two demographic analyses based in coalescence showed a pattern of recent population expansion for the widespread *C. torquatus*, whereas a long time population stability was recovered for the highly restrict *C. ibicuiensis*.

The recently expanded populations of *C. torquatus* probably originated from the central region of the Rio Grande do Sul State. The widespread karyotype 2n = 44, previously cited as plesiomorphic,

and the few chromosomic variations reported on the periphery of this species, are in agreement with such scenery of expansion.

The AMOVA analyses, and other structuration assays, did not show a substantial relationship between the genetic variation partitioning and the presence of great rivers. The effect of river as a barrier to gene flow was reported only comparing the low genetic structuration between headwater sites in comparison with all the other wider sites along the river. Expansion of the collared tuco-tuco and several events of crossing the river, as indicated by haplotype sharing, can be related with dry and cold climatic conditions during the glaciations of the Pleistocene, when grasslands dominated, gallery forest did not occur and water courses probably were reduced. As a low vagile and territorial subterranean rodent, living in small demes, geographic distances and genetic drift seem let a stronger signature of population differentiation for tuco-tucos, rather than habitat discontinuities.

# INTRODUÇÃO

## O bioma Pampa

De acordo com o Instituto Brasileiro de Geografia e Estatística (IBGE, 2004), o Brasil possui seis biomas continentais: Amazônia, Caatinga, Cerrado, Mata Atlântica, Pampa e Pantanal (Figura 1). O bioma Pampa é um dos menores em extensão, com uma área de aproximadamente 176 $km^2$, representando cerca de 2,07% do território nacional. Gramíneas e ervas compõem a vegetação predominante, com ocorrência esparsa de arbustos e formações arbóreas (IBGE, 2004).

Apesar da baixa representatividade em extensão, em relação aos demais biomas brasileiros, o Pampa representa 63% da área do estado do Rio Grande do Sul (RS) (IBGE, 2004) e, juntamente com as áreas campestres adjacentes do Uruguai e Argentina, compõe a Província Pampeana, uma das maiores áreas de campo em clima subtropical (AABA, 2010; Cabrera e Willink, 1980).

Ao contrário de biomas florestais, o Pampa tem uma vocação histórica para pastagens e produção de grãos, sendo essas as principais atividades econômicas desenvolvidas na região. Entretanto, o avanço da fronteira agrícola nas últimas décadas, a pecuária intensiva com substituição das pastagens naturais por cultivadas, bem como o crescente cultivo de árvores exóticas como *Pinus* sp. e *Eucalyptus* sp. tem promovido o desgaste do solo e degradação ambiental (Overbeck e cols., 2007; Roesch e cols., 2009). Além da perda de biodiversidade pela fragmentação de habitat, espécies exóticas são introduzidas com o objetivo de aumentar a produtividade das pastagens. Carvalho e Batello (2009) abordaram a questão da produtividade nos campos sulinos, sugerindo ações para remediar o conflito entre a demanda mundial pela produção de grãos e carne e a necessidade emergente de medidas de conservação dos campos nativos.

Esta preocupação se deve a drástica redução de áreas cobertas por vegetação nativa, comprometendo uma das regiões mais ricas do mundo em gramíneas. A diversidade da vegetação campestre do RS é da ordem 2.200 espécies (Boldrini, 2009), sendo que há um número expressivo de espécies citadas como ameaçadas ou com distribuição restrita (Deble, 2011).

Embora as condições climáticas atuais sejam propícias à formações florestais na região sul do Brasil, o estabelecimento e a diversificação da vegetação campestre estão associados ao longo período de clima seco e frio durante o último máximo glacial. Análises de paleovegetação utilizando grãos de pólen confirmam o domínio de gramíneas na metade sul do RS durante todo o último

período glacial. O surgimento e a expansão de florestas de galeria aparecem nos registros apenas nos últimos 5.000 anos, refletindo a mudança para um clima mais quente e úmido no Holoceno, à medida que o período glacial finalizava (Behling e cols., 2005).


**Figura 1**: Mapa do Brasil destacando os seis biomas, de acordo com o IBGE, 2004. (Imagem modificada a partir do site http://naturezaepaz.blogspot.com/2010/11/em-defesa-do-bioma-pampa.html).

A retração das florestas e a expansão da planície costeira são eventos bem documentados de comportamento da cobertura vegetal e do nível dos oceanos frente às alterações climáticas drásticas atribuídas ao período glacial no sul do Brasil (Behling e cols., 2004; 2005; Tomazelli e cols., 2000). Em se tratando de sistemas extremamente dinâmicos, é plausível considerar também uma redução significativa no volume de água pertinente às bacias hidrográficas durante este período seco e frio. Behling e cols. (2005) reportaram a ocorrência de águas rasas em tempos glaciais para a bacia do rio Ibicuí, no oeste do estado do RS.

A história geológica e climática do bioma Pampa fornece um cenário ainda pouco explorado, mas extremamente convidativo e promissor para estudos filogeográficos. A forma intensiva como esta paisagem natural vem se modificando e se descaracterizando, torna ainda mais emergente a necessidade deste tipo de estudo na região. O conhecimento básico a respeito dos processos e padrões associados à biodiversidade de um bioma, sem dúvida são as principais ferramentas utilizadas para o planejamento de ações conservacionistas.

**O gênero Ctenomys**

Roedores do gênero *Ctenomys* são popularmente conhecidos como tuco-tucos, nome este que representa uma aproximação onomatopéica da vocalização deste pequeno mamífero em seu habitat

subterrâneo. Pertencentes à família Ctenomyidae, subordem Hystricognathi, as cerca de 60 espécies deste gênero representam uma das maiores taxas de especiação e evolução cromossômica entre os mamíferos, considerando a sua origem extremamente recente, datando do Plioceno (Verzi e cols., 2010) e a extraordinária diversidade cariotípica, com números diplóides variando de 2n=10 a 2n=70 (Reig e cols., 1990). De acordo com Nevo (1979), a diversificação de roedores fossoriais como os tuco-tucos pode estar associada às condições climáticas mais secas em meados do Cenozóico que proporcionaram a retração das florestas e a expansão de ambientes campestre, preferencialmente habitados por estas espécies para escavarem seus sistemas de túneis subterrâneos.

O registro fóssil mais antigo de um ctenomídeo (~3,5 milhões de anos) provém de sedimentos do noroeste da Argentina (Verzi e cols., 2010) e atualmente as espécies de *Ctenomys* ocorrem em toda a metade sul da América do Sul, desde a Terra do Fogo, na Argentina, até o sul da Bolívia e Peru (Reig e cols., 1990).

A cladogênese recente e explosiva atribuída ao gênero *Ctenomys* se reflete em estudos filogenéticos utilizando marcadores moleculares mitocondriais e nucleares, os quais costumam resultar em uma politomia basal (D'Elía e cols., 1999; Castillo e cols., 2005; Lessa e Cook 1998; Mascheretti e cols., 2000; Parada e cols., 2011; Slamovits e cols., 2001). O estudo filogenético mais recente para o gênero *Ctenomys* é também o mais completo, considerando o maior número de espécies reconhecidas e com ampla cobertura geográfica (Parada e cols., 2011). Os resultados permitiram o reconhecimento de 8 clados bem suportados, compondo grupos de espécies de tuco-tucos, apesar de algumas espécies não pertencerem a nenhum grupo e as relações entre elas e os demais grupos não estarem bem resolvidas na base da filogenia.

Entre os grupos de espécies recuperados pela análise filogenética de Parada e cols. (2011), o grupo *torquatus* é composto por espécies de tuco-tuco que além de compartilharem um ancestral comum, habitam a mesma região no sul do Brasil, Uruguai e nordeste da Argentina (Figura 2). O complexo de espécies deste roedor que habita as províncias de Corrientes e Entre Ríos na Argentina é composta por populações de *Ctenomys dorbignyi*, *C. roigi*, *C. perrensi* e diversas formas de status taxonômico indefinido, os quais apresentam uma dinâmica de diferenciação e especiação relacionada à instabilidade do habitat, frequentemente inundável (Mirol e cols., 2010).

No litoral dos estados do RS e parte de Santa Catarina, sul do Brasil, populações de *C. minutus* ocorrem ao longo da planície costeira, uma região de evolução geológica dinâmica, extremamente afetada pelas transgressões marinhas do Quaternário. Com um total de 45 cariótipos descritos até então, *C. minutus* representa a espécie com maior variabilidade cromossômica entre os roedores subterrâneos (Castilho, 2004; Freitas 1997; Freygang e cols., 2004; Gava e Freitas, 2002; 2003; Lopes, 2011). A variação genética do DNA mitocondrial (mtDNA) e as variações cariotípicas descritas para esta espécie foram associados às descontinuidades do ambiente costeiro que habita, bem como à características intrínsecas dos ctenomídeos, como baixa dispersão e distribuição em pequenos demes isolados (Lopes, 2011).

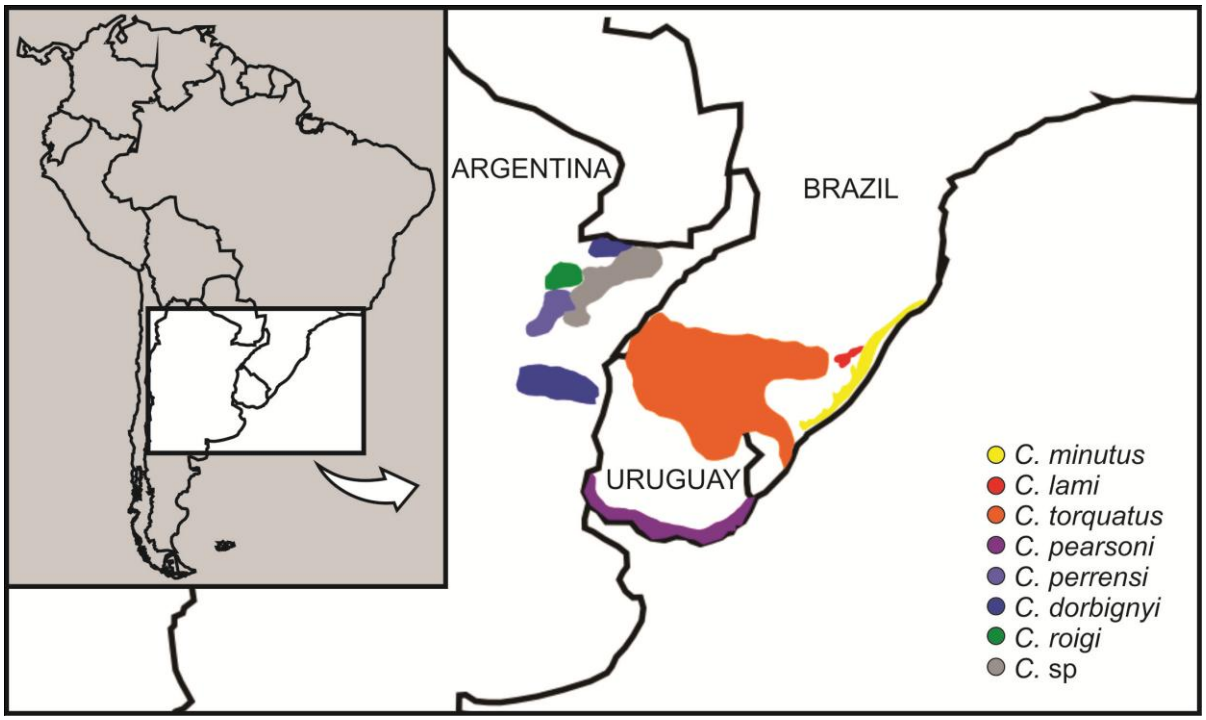


**Figura 2**: Áreas de distribuição das espécies de tuco-tuco do grupo *torquatus* no Uruguai, sul do Brasil e nordeste da Argentina.

*Ctenomys lami* é outra espécie sul brasileira do grupo *torquatus*, cuja história evolutiva é mais um exemplo de como alterações no ambiente afetam padrões filogenéticos e filogeográficos, especialmente relacionadas à hidrografia (áreas úmidas, inundáveis, rios) em se tratando de um organismo que ocupa o nicho subterrâneo e que, portanto, é extremamente vulnerável a instabilidade de cursos e corpos d'água. Além do expressivo polimorfismo cromossômico, com 25 cariótipos diferentes, *C. lami* é intimamente relacionado geográfica (Figura 2) e filogeneticamente com *C.*

*minutus* (Freitas, 2001; 2006; Lopes, 2011). O processo de diferenciação recente entre ambas as espécies era assegurado pela existência de um banhado (área úmida) atuando como barreira. Nas últimas décadas, a drenagem deste banhado em função do cultivo de arroz acabou expondo uma área de campo arenoso que permitiu o contato secundário entre populações de *C. lami* e *C. minutus*, propiciando a formação de uma zona híbrida interespecífica (Gava e Freitas, 2003) e com indício de introgressão (Lopes, 2011).

Na planície costeira do sul do Uruguai ocorre outra espécie cromossomicamente politípica do grupo *torquatus*, *C. pearsoni*. Assim como *C. minutus*, a região costeira habitada por *C. pearsoni* foi exposta a transgressões marinhas holocênicas. Embora um padrão de estabilidade demográfica seja descrito para a espécie em geral, a região leste de sua distribuição teria sido recentemente colonizada, em concordância com dados palegeográficos indicando que esta região foi mais intensamente afetada pelas perturbações do nível do mar (Tomasco e Lessa, 2007).

Populações de *C. torquatus* distribuem-se pelos campos de baixa altitude de toda a metade sul do RS e norte do Uruguai, distante das planícies costeiras afetadas por transgressões marinhas, bem como de outras regiões alagadas, exceto por uma pequena região ao sul de sua ampla distribuição geográfica (Figura 2). Esta espécie, popularmente conhecida como tuco-tuco de colar e que empresta seu nome ao grupo *torquatus*, possui uma das maiores áreas de ocorrência entre os tuco-tucos (Fernandes, 2008; Fernandes e cols., 2009; Freitas, 1995; Freitas e Lessa, 1984).

Apesar de sua ampla distribuição geográfica e do histórico de polimorfismo cariotípico entre as demais espécies do grupo *torquatus*, as populações do tuco-tuco de colar apresentam o cariótipo predominante 2n = 44 e apenas alguns registros de variação cromossômica em populações periféricas.

O estudo de Kiblisky e cols. (1977) descreveu os primeiros cariótipos 2n = 44 de *C. torquatus* para amostras uruguaias de El Aguila, Guabiyú e Salto Nuevo. Posteriormente, Freitas e Lessa (1984) encontraram a mesma forma cromossômica em populações do sul do RS, às margens do Canal São Gonçalo, um grande curso de água que liga a Laguna dos Patos à Lagoa Mirim, no sul do RS. O cariótipo 2n = 44 foi amostrado na margem norte do Canal, ampliando a ocorrência desta forma cariotípica para mais de 500 km a partir dos registros uruguaios de Kiblisky e cols. (1977). Outras duas localidades, ao sul do referido Canal, apresentaram cariótipo 2n = 46. Estas duas localidades encontram-se envoltas pelo oceano Atlântico a leste, áreas alagadas ao sul, a Lagoa Mirim e o Canal

São Gonçalo a oeste e a Laguna dos Patos ao norte; constituindo uma variação cariotípica periférico ao restante da área de ocorrência do tuco-tuco de colar (Figura 3). Ainda segundo Freitas e Lessa (1984), a forma cariotípica 2n = 44 seria plesiomórfica, dada a sua ampla distribuição geográfica, e o Canal São Gonçalo que divide as formas 2n = 44 e 2n = 46 teria surgido recentemente, a cerca de 2.600 anos (Jost e cols., 1975).

Em estudos posteriores de biomonitoramento de regiões em torno de minas de extração de carvão, novos registros de ocorrência de *C. torquatus* foram mapeados para o estado do RS. No município de Candiota, ainda no sul do estado, e em Butiá, próximo a capital Porto Alegre, os espécimes apresentaram o cariótipo predominante 2n = 44 (Silva e cols., 2000a, b).

No outro extremo de sua distribuição, no oeste do RS, Fernandes e cols. (2009) descreveram outra variação cariotípica para o tuco-tuco de colar associada à presença de um curso de água, o Rio Ibicuí. O cariótipo 2n = 44 foi encontrado em uma população de *C. torquatus* na margem sul do rio Ibicuí, ao passo que ao cruzá-lo, a população cariotipada na margem norte apresentou 2n = 40 (Figura 3). Com exceção desta população, muito próxima a desembocadura do rio Ibicuí no rio Uruguai, não há nenhum outro registro de tuco-tuco de colar na margem norte, ao longo de toda a extensão do rio Ibicuí deste a região central do estado. Em contrapartida, espécimes de tuco-tuco encontrados no município de Manoel Viana, próximos ao rio Ibicuí (Figura 3), apresentaram características morfológicas diferenciadas de *C. torquatus*, tamanho menor e número cromossômico 2n = 50 (Freitas, dados não publicados), os quais provavelmente tratam-se de uma nova espécie do gênero *Ctenomys*.

Ao contrário das duas situações de variações cariotípicas periféricas associados à presença de um rio, citadas anteriormente para *C. torquatus*, populações com cariótipo 2n = 40 e 2n = 42 ocorrem próximas a registros de 2n = 44 em Alegrete, no oeste do estado (Figura 3), aparentemente sem nenhuma barreira geográfica (Gonçalves e Freitas, 2009). Todas as demais localidades amostradas para *C. torquatus* apresentaram o cariótipo mais freqüente 2n = 44 (Fernandes, 2009), inclusive populações ao longo do caudaloso rio Jacuí (Figura 3).

Além da existência de uma espécie nova, todos esses registros revelam o cenário de uma espécie de mamífero de habito subterrâneo, amplamente distribuída pelos campos do bioma Pampa sul brasileiro. A extensão de sua distribuição é dissecada por grandes bacias hidrográficas, entre elas as dos rios Jacuí e Ibicuí. Descrever os padrões filogeográficos desses roedores, bem como o papel

desses rios em sua diversificação, contribuirá não somente para a compreensão da história evolutiva deste grupo taxonômico, como também para a história e a valorização do bioma Pampa.

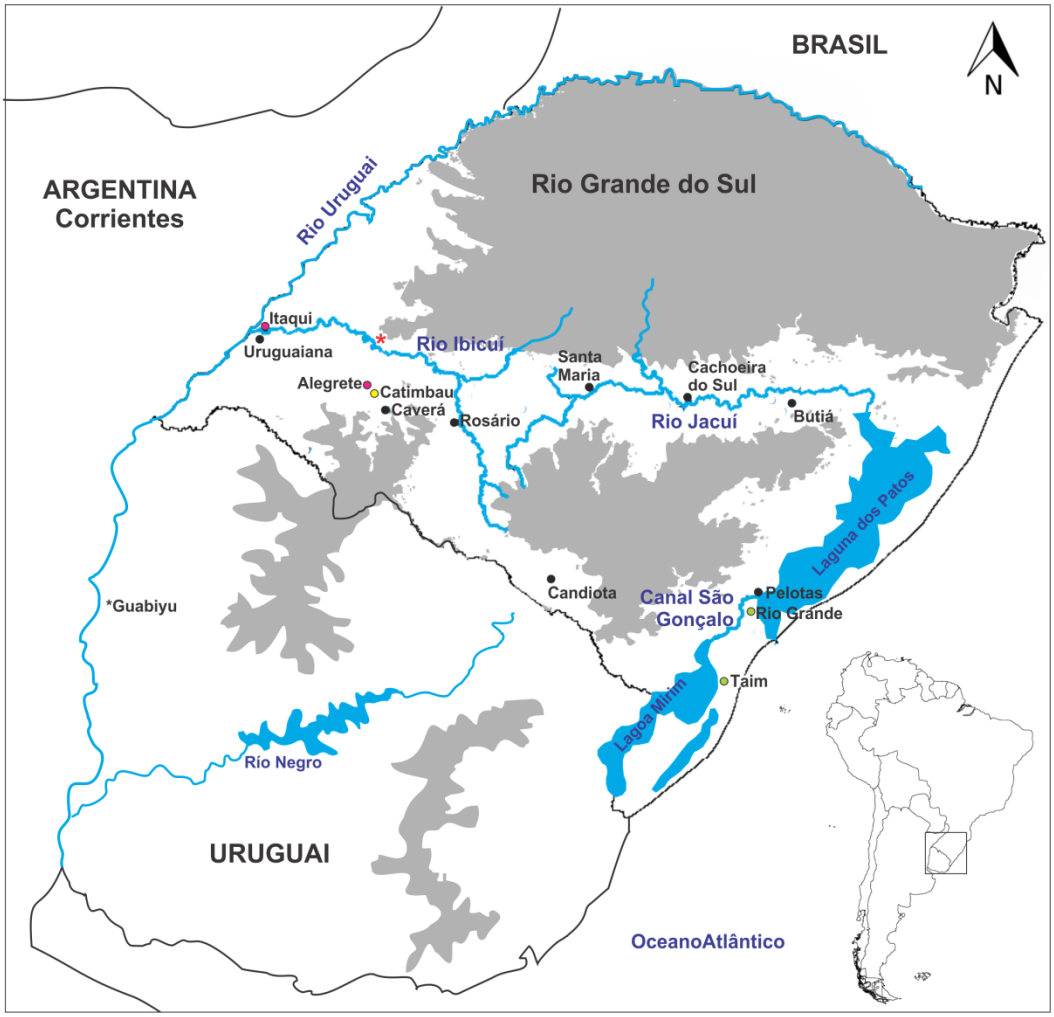


**Figura 3**: Mapa do estado do Rio Grande do Sul (Brasil) e Uruguai, indicando os registros de ocorrência de *Ctenomys torquatus* listados no texto. A coloração dos pontos refere-se ao número cromossômico: rosa para 2n = 40, amarelo para 2n = 42, preto para 2n = 44 e verde para 2n = 46. Áreas em cinza representam altitudes acima de 200 m. *Primeiro registro de cariótipo 2n = 44. O asterisco em vermelho indica a ocorrência da espécie nova.

**Hipótese de rios como barreiras**

Grandes cursos d'água são descontinuidades do habitat apelativas como barreira ao fluxo gênico para espécies terrestres, especialmente em se tratando de roedores fossoriais como os tuco-tucos que além da especificidade do habitat possuem baixa vagilidade (Busch e cols., 2000).

A concepção de rios atuando como barreira surgiu das observações de Alfred Russel Wallace (1852) de que as fronteiras da distribuição de espécies de primatas intimamente relacionadas geralmente coincidiam com os grandes rios da bacia amazônica.

> "*Durante minha estadia no estado do Amazonas, eu tive a oportunidade de determinar os limites das espécies, e logo constatei que os rios Amazonas, Negro e Madeira formavam limites além dos quais certas espécies jamais ultrapassaram... À medida que se aproxima das nascentes dos rios, eles deixam de ser uma barreira e a maioria das espécies é encontrada em ambas as margens dos rios*". (Wallace, 1852).

Além de propor a hipótese de divergência causada por rios – *Riverine barrier hypothesis* (RBH) em inglês, Wallace sugeriu também que a força de um rio atuando como barreira diminui da foz em direção à nascente, como consequência da diminuição da largura do mesmo e do volume de água. As observações de Wallace foram posteriormente quantificadas e confirmadas por Ayres e Clutton-Brock (1992) analisando comunidades de primatas de ambos os lados dos rios da bacia Amazônica.

As espécies de tuco-tuco que compõem o grupo *torquatus*, citadas anteriormente, constituem um modelo interessante para avaliar a RBH, considerando o fato de compartilharem ancestralidade e distribuírem-se às margens de grandes cursos d'água como o Rio Uruguai e o Río de La Plata. Entretanto, uma das principais dificuldades em se testar a RBH é distinguir o efeito do rio como causador primário da divergência (efeito vicariante) ou apenas como um ponto de encontro secundário entre linhagens ou espécies que divergiram por outra razão, em outras regiões (Patton e cols., 2000). Outra questão é que, embora apelativos, mesmo os rios caudalosos são extremamente dinâmicos ao longo do tempo, podendo sofrer variações de volume de água e até mesmo mudança de curso (Moritz e cols., 2000; Patton e cols., 2000).

Patton e cols. (2000) realizaram um amplo estudo de avaliação da RBH na bacia amazônica, utilizando o rio Juruá como modelo de barreira para diversas espécies de mamíferos terrestres na década de 90. Os autores não encontraram evidência de estruturação das comunidades analisadas em função da presença do rio. A grande motivação do trabalho, no entanto, foi a abordagem filogeográfica utilizando marcadores moleculares em análises em nível de espécie. A estruturação de comunidades avaliando composição de espécies foi adaptada para uma avaliação da estruturação de populações – ou linhagens – utilizando sequências do gene citocromo-*b*.

De acordo com Patton e cols. (2000), análises filogeográficas podem distinguir hipóteses históricas alternativas que fundamentam diferentes padrões de diversificação. A hipótese de **Diversificação Primária** é a expressão máxima do rio atuando como uma barreira. Neste caso, um curso d'água é imposto à distribuição de um táxon, dividindo-a em duas partes (Figura 4A). Se este rio passa a atuar como uma barreira efetiva ao fluxo gênico ao longo do tempo, haverá monofilia recíproca entre as linhagens de cada lado do rio que passarão a ser clados irmão em relação qualquer linhagem fora deste contexto.

Alternativamente, o efeito de um rio atuando como barreira pode ser caracterizado como um **Contato Secundário** entre linhagens de um determinado taxon que divergiram em outros locais, e por migração ou expansão populacional acabaram encontrando-se em margens opostas do rio. Neste caso, espera-se encontrar monofilia recíproca entre os espécimes de cada margem do rio, mas as linhagens dos lados opostos não formariam clados irmãos entre si e provavelmente estariam mais relacionados com diferentes linhagens (Figura 4B).

Análises filogeográficas podem revelar também se houve um evento de **Dispersão** através do rio. Em algum momento, por algum motivo, linhagens que habitavam uma margem passaram a colonizar a margem oposta após um evento de cruzar o curso d'água. Supondo que a colonização tenha se dado da margem direita para a margem esquerda do rio, espera-se observar uma relação parafilética entre as linhagens da margem direita, em relação àquelas da margem esquerda do rio (Figura 4C).

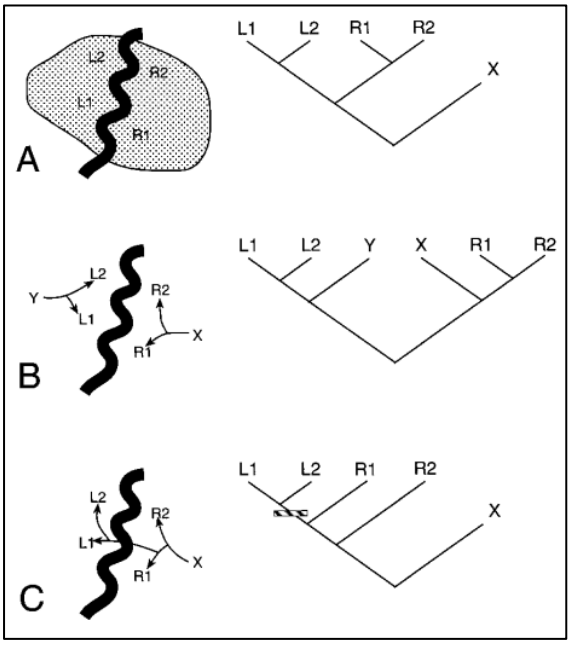


**Figura 4**: Três hipóteses filogeográficas alternativas. **A:** Diversificação Primária: monofilia recíproca e clados irmãos ligados por um rio que se impôs em meio à distribuição geográfica de uma espécie. **B:** Contato Secundário: monofilia recíproca, mas clados que não compartilham um ancestral em comum, ligados por um rio que atua como um ponto de encontro secundário destes clados que evoluíram em outro local. **C:** Dispersão: relações parafiléticas dos haplótipos da margem direita (R1 e R2) em relação aos haplótipos da margem esquerda (L1 e L2) devido a um episódio de cruzamento do rio. (Modificado da Figura 164 de Patton e cols., 2000)

Além da abordagem filogeográfica utilizando marcadores moleculares, os autores foram criteriosos com o planejamento amostral, buscando pontos estratégicos de coleta em ambas as margens ao longo de toda a extensão do rio Juruá, desde a nascente até a foz.

Das seis espécies para as quais a amostragem contemplou todos os pontos ao longo do rio, apenas uma apresentou um padrão de divergência de acordo com o esperado segundo a RBH. A diferenciação molecular encontrada entre populações do sagüi-de-cara-suja *Saguinus fuscicollis* foi atribuída à presença do rio, havendo compartilhamento de haplótipos apenas entre os pontos de coleta próximos à nascente (Peres e cols., 1996). As outras cinco espécies de roedores apresentaram várias situações de compartilhamento de haplótipos ao longo do rio, tanto entre sítios da mesma margem quanto de margens opostas do rio. O padrão filogeográfico do rato arbóreo *Mesomys hispidus* foi inclusive considerado "*nonriverine pattern*", ou seja, um padrão oposto ao esperado de acordo com a RBH. Isso porque os dois clados divergentes reportados para as amostras desta espécie separaram as localidades da parte alta do rio (nascente e região central alta) das localidades da parte baixa (região central baixa e foz), e não as localidades das margens opostas do rio Juruá (Patton e cols., 2000).

Os autores discutem a respeito de transições fitogeográficas e eventos geológicos históricos que poderiam justificar o padrão de divergência filogeográfica verificado ao longo do rio – e não em lados opostos do rio – encontrado para a maioria dos mamíferos analisados. O histórico de retração e expansão da floresta, causadas pelas alterações climáticas, bem como os efeitos causados pela elevação dos Andes, tornam extremamente dinâmica a história do bioma Amazônico. Os rios, em seus percursos e vazão contemporâneos, podem estar atuando como barreiras efetivas ao fluxo gênico entre determinadas linhagens, mas a estruturação verificada com os marcadores moleculares reflete um contexto muito mais antigo na história evolutiva destas espécies.

Seja para avaliar a força de um rio como barreira, ou para distinguir qual das três hipóteses sugeridas por Patton e cols. (2000) melhor explica o comportamento de um rio frente às linhagens de uma espécie habitando seu entorno; a utilização de marcadores moleculares em uma perspectiva filogeográfica e com planejamento amostral adequado, pode trazer importantes contribuições a respeito do efeito dos rios como barreira.

O tuco-tuco de colar – *Ctenomys torquatus*, protagonista desta tese, contempla vários atributos que o tornam um modelo convidativo ao estudo da RBH, entre eles: sua ampla distribuição

geográfica, abrangendo rios de grande porte, um bioma carente de estudos filogeográficos em grande escala, além das variações cariotípicas periféricas associados ao Canal São Gonçalo e o rio Ibicuí.

**OBJETIVOS**

● Descrever a distribuição, a estruturação espacial e relação histórica dos tuco-tucos com o ambiente no bioma Pampa;

● Inferir os padrões filogeográficos de *C. torquatus*;

● Caracterizar o efeito dos rios que separam populações de *C. torquatus* com variações cariotípicas periféricas;

● Avaliar a força do rio como barreira, utilizando como referência as localidades de ocorrência de *C. torquatus* ao longo do rio Jacuí, dada a sua extensão e a uniformidade cariotípica das populações;

● Avaliar o status de espécie plena dos espécimes com cariótipo e morfologia diferenciados encontrados no município de Manoel Viana;

● Inferir os padrões filogeográficos da espécie nova, determinando os limites de sua distribuição geográfica e seu status de conservação.

**CAPÍTULO I**

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# TETRANUCLEOTIDE MICROSATELLITE MARKERS IN *CTENOMYS TORQUATUS* (RODENTIA)

**Paula A. Roratto, Marlise L. Bartholomei-Santos e Thales Renato Ochotorena de Freitas**

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TECHNICAL NOTE

# Tetranucleotide microsatellite markers in *Ctenomys torquatus* (Rodentia)

**Paula A. Roratto · Marlise L. Bartholomei-Santos · Thales R. O. de Freitas**

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**Abstract** Eleven microsatellite markers were isolated from an enriched library developed specifically for the tuco-tuco *Ctenomys torquatus*, using tri and tetranucleotide probes. Ten of these were successfully amplified, and only one was monomorphic for the populations that have so far been analyzed. Analysis of two different populations yielded a mean of 2.6 (Cachoeira do Sul—CAC) and 4.3 (Butiá—BUT) alleles per locus, with a range from 1 to 6. The means for observed and expected heterozygosity were also lower in the CAC population (Ho = 0.37 and He = 0.39), but in BUT they were 0.63 and 0.61 respectively. These are the first microsatellite markers reported for a Brazilian species of tuco-tuco, and will be applied to investigate the genetic structure of their populations in the widely impacted Pampa Biome.

**Keywords** Fossorial rodent · Tetranucleotide · Selective hybridization · *Campos sulinos* biome

*Ctenomys torquatus* Lichtenstein 1830 is a subterranean rodent known as the collared tuco-tuco, which inhabits the grasslands of southern Brazil (Fernandes et al. 2007). They preferentially build their burrow systems in sandy soil. The patchy distribution, together with low vagility, generally leads to the isolation of populations of tuco-tucos in small demes (Reig et al. 1990).

This species occurs from the east coast of the state of Rio Grande do Sul (RS) to the western Argentina-Brazil border and northern Uruguay. Despite the broad distribution of the collared tuco-tuco, its habitat in the Pampa biome has been impacted over wide areas. Natural grasslands in southern Brazil have decreased due to the expansion of agricultural activities and silvicultural practices, making them unsuitable for the fossorial rodents. Despite its high floristic diversity, the southern Brazilian biome has not received great attention or concern about its conservation (Overbeck et al. 2007).

Moreover, the geographical distribution of this rodent in RS coincides almost exactly with the distribution of coal reserves. Studies using alkaline single-cell gel electrophoresis (SCG) and micronucleus assays indicated DNA damage induced by coal and coal byproducts in *C. torquatus* specimens exposed to mining activities (Silva et al. 2000a; Silva et al. 2000b). The authors suggested that this species would be a good sentinel organism for coal-mining hazards.

Understanding the effects of habitat change on the collared tuco-tuco in southern Brazilian grasslands requires a detailed knowledge of how species biology and landscape characteristics can structure populations. In order to investigate gene flow and genetic variation in *C. torquatus*, species-specific microsatellite loci were developed.

A sample of collared tuco-tuco (ear tip) was used for DNA extraction following Medrano et al. (1990) and for construction of a genomic library according to Refseth et al. (1997), with modifications to avoid the generation of chimeric sequences, as suggested by Roratto et al. (2008).

The DNA sample was digested with the restriction enzyme *Taq*I and ligated to adapters formed by the

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**Table 1** Characteristics of the 10 microsatellite loci isolated from *Ctenomys torquatus,* genotyped in two populations: (CAC) Cachoeira do Sul; (BUT) Butiá

| *Loci* | Primer 5′-3′ | Repeat motif | AT (°C) | Size range (bp) | CAC | | | | BUT | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Na | Ho | He | PHW | Na | Ho | He | PHW |
| *Tor1* | F: CGATGTCTTCTTATCTAGATATC<br>R: TGGTATTGTGTCTTTTCTGT | $(GATA)_{10}(GACA)_5(GATA)_4$ | 50 | 364–372 | 1 | – | – | – | 3 | 0.82 | 0.57 | 0.08 |
| *Tor2* | F: GCTAAACTACACAGCCTTATTTAATGT<br>R: GGAACAGACAATCAATGACAAG | $(GATA)_{15}AT(GATA)_3$ | 60 | 185–205 | 4 | 0.35 | 0.42 | 0.08 | 6 | 0.70 | 0.78 | 0.66 |
| *Tor3* | F: AGCCCTCCTGAAACTGGTCT<br>R: TGTGAAAGGAAGGATTGTATTGG | $(GATA)_{11}$ | 59 | 163–171 | 1 | – | – | – | 2 | 0.12 | 0.12 | 1.0 |
| *Tor4* | F: CAACCTTGACTAATCAGATACATAAA<br>R: AAAGCTGACATTCCTCCTGT | $(GATA)_{12}(GACA)_7$ | 62 | 206–242 | 2 | 0.55 | 0.48 | 0.65 | 4 | 0.70 | 0.76 | 0.55 |
| *Tor5* | F: TTTCCAAAGCAATGATAGGT<br>R: GGCTAGATAGCTAGATAAAAAGATAG | $(GATA)_{12}$ | 55 | 249–273 | 4 | 0.50 | 0.59 | 0.51 | 6 | 0.70 | 0.69 | 0.19 |
| *Tor6* | F: CTGCAAGTCAAAGCAACTCT<br>R: AGACCCTGTTTCAGGAAGAT | $(ATAG)_{11}$ | 55 | 184–200 | 3 | 0.45 | 0.41 | 0.77 | 4 | 0.70 | 0.70 | 0.81 |
| *Tor7* | F: TTACCGGTCAGTAACACTAAATATAA<br>R: TGTCCTAATAATCGGTTTTGAA | $(GATA)_6GATTA(GATA)_6$ | 57 | 190–210 | 1 | – | – | – | 5 | 0.82 | 0.73 | 0.37 |
| *Tor8* | F: CCCTAGCCCCTGCTGTTAG<br>R: CACTTGGGAGACTGAGGCA | $(GATA)_{11}GATG(GATA)_4$ | 58 | 238–250 | 4 | 0.15 | 0.23 | 0.25 | 3 | 0.47 | 0.39 | 1.0 |
| *Tor9* | F: GGGACTGGGATTTGAGGAAT<br>R: CAGGAGGAGCAGGAATTTGA | $(GT)_{16}$ | 60 | 192–206 | 4 | 0.25 | 0.23 | 1.0 | 6 | 0.64 | 0.81 | 0.16 |
| *Tor10* | F: TTGAAAACCATGACCACACAA<br>R: CGTGCTGAGATTACGGCATA | $(GT)_{11}$ | 61 | 162 | 1 | – | – | – | 1 | – | – | – |

*F* forward primer sequence, *R* reverse primer sequence, *AT* Annealing temperature, *Na* number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *PHW* probability of Hardy–Weinberg disequilibrium

annealing of two oligonucleotides (TaqI 20 Mer: 5′-ATGAAGCCTTGGTACTGGAT-3′ and TaqI 22 Mer: 5′ CGATCCAGTACCAAGGCTTCAT 3′). DNA fragments were hybridized with the biotinylated probes $(AAG)_8$ and $(GATA)_8$ and captured using streptavidin-coated magnetic beads (DynaBeads® M-280). Selected DNA was eluted from the beads as single strands and amplified in a polymerase chain reaction (PCR) using the oligonucleotide TaqI 20 Mer as primer. PCR was carried out in a volume of 25 µl containing 1.25 U Taq DNA polymerase, 20 mM pH 8.4 Tris–HCl, 50 mM KCl, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 5 µM of primer. The amplification conditions were as follows: 95°C for 5 min, 27 cycles of 95°C for 1 min, 61°C for 30 s, 72°C for 2 min and a final extension step of 8 min at 72°C. The PCR products were checked on 0.8% agarose gel, cloned into a pCR®2.1 vector (TA Cloning kit, Invitrogen) and transferred into competent *Escherichia coli* cells by the $CaCl_2$ method (Sambrook and Russel 2002).

Recombinant clones were sequenced with ABI 3700 (Applied Biosystems). Sequences with more than 6 repetition units and enough flanking region were selected for primer design using the software PRIMER 3 (Rozen and Skaletsky 2000). The only eight clones from the (AAG) library did not carry a repetitive sequence. A total of 99 clones from the (GATA) library were sequenced. Seventy-six clones were not redundant and 11 sequences were adequate for primer design (GenBank accession numbers JF274995 to JF275005). Except for the *Tor*11 primer pair, all the loci were successfully amplified. To assess the polymorphism of the 10 microsatellite loci, primer pairs were ordered with a fluorescent dye HEX and assayed in two populations of *C. torquatus*: Butia ($N = 17$) and Cachoeira do Sul ($N = 20$).

Alleles were identified through the software Peak Scanner 1.0 (Applied Biosystems). The genotyping results were analyzed using the software Micro-Checker (van Oosterhout et al. 2004) for the presence of null alleles, large allele drop out and stuttering. Arlequin 3.1 (Excoffier and Schneider 2005) was used to perform Hardy–Weinberg equilibrium and linkage disequilibrium tests, as well as to calculate the observed and expected heterozygosities.

The Micro-Checker results showed no evidence for scoring error due to stuttering, large allele dropout or null alleles. Table 1 shows the summary statistics for the markers.

These microsatellite loci are being applied to populations of the collared tuco-tuco throughout its distribution, in order to evaluate the genetic structure and the impact of habitat loss on this species. Although locus *Tor*10 was monomorphic for the Butia and Cachoeira do Sul samples, it showed two alleles in other populations (data not shown).

**Acknowledgments** Funding was provided by The National Council for Scientific and Technological Development (CNPq) and The National Council for the Improvement of Higher Education (CAPES).

## References

Excoffier L, Schneider S (2005) Arlequin version 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Fernandes FA, Fernández-Stoltz GP, Lopes CM et al (2007) The conservation status of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae), in southern Brazil. Braz J Biol 67(4 Suppl): 839–847

Lichtenstein H (1830) Darstellung neuer oder wenig bekannter Säugethiere in Abbildungen und Beschreibungen von fünf und sechzig Arten auf fünzig colorirten Steindrucktafeln nach den Orginalen des Zoologischen Museums der Universität zu Berlin. C. G. Lüderitz, Berlin

Medrano JF, Aesen E, Sharrow L (1990) DNA extraction from nucleated red blood cells. Biotechniques 8:43

Overbeck GE, Muller SC, Fidelis A et al (2007) Brazil's neglected biome: the South Brazilian campos. Perspect Plant Ecol 9: 101–116

Refseth UH, Fangan BM, Jakobsen KS (1997) Hybridization capture of microsatellites directly from genomic DNA. Electrophoresis 18:1519–1523

Reig OA, Busch C, Contreras MO et al (1990) An overview of evolution, systematics, population and speciation in *Ctenomys*. In: Nevo E, Reig OA (eds) Evolution of subterranean mammals at the organismal and molecular levels. Wiley-Liss, New York, pp 71–96

Roratto PA, Buchmann D, Santos S, Bartholomei-Santos ML (2008) PCR-mediated recombination in development of microsatellite markers: mechanism and implications. Genet Mol Biol 31(1): 58–63

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Sambrook J, Russel DW (2002) Molecular cloning: A laboratory manual, 3rd edition. (trans: Huang Pei-Tang et al.). Science Press, Beijing

Silva J, Freitas TRO, Heuser VD et al (2000a) Chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated with multiple methods and tissues. Mut Res 470(1):39–51

Silva J, Freitas TRO, Heuser VD et al (2000b) Genotoxicity biomonitoring in coal regions using wild rodent *Ctenomys torquatus* by comet assay and micronucleus test. Environ Mol Mutagen 35(4):270–278

Van Oosterhout C, Hutchinson WF, Wills DPM et al (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

**CAPÍTULO II**

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# RIVERS, PERIPHERIES AND KARYOTYPIC POLYMORPHISMS: THE PHYLOGEOGRAPHY OF *CTENOMYS TORQUATUS* (RODENTIA).

**Paula Angélica Roratto, Fabiano Araújo Fernandes e Thales Renato Ochotorena de Freitas**

Manuscrito a ser submetido à revista Journal of Biogeography.

ORIGINAL ARTICLE

**Rivers, peripheries and karyotypic polymorphisms: the phylogeography of *Ctenomys torquatus* (Rodentia).**

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Short running head: Rivers' effect on *Ctenomys torquatus* phylogeography.

**ABSTRACT**

**Aim** Rivers generally are appealing habitat discontinuities thought to promote diversification on terrestrial species. In South America, subterranean rodents of the genus *Ctenomys* are widespread throughtout open habitats, and the collared tuco-tuco *C. torquatus* has one of the widest ranges among these species, which is dissected by great water courses. The aims of the present study are describing the phylogeography of the collared tuco-tuco and evaluating the effect of main rivers structuring their distribution and karyotypically distinct populations, on the basis of the riverine barrier hypothesis.

**Location** Grasslands of the southern Brazil and northern Uruguay, known as Pampa biome.

**Methods** A broad sampling was performed, throughout the geographic range of *C. torquatus*, especially around main rivers. Phylogeographic patterns were approached through mitochondrial DNA hyper variable sequence (d-loop) and citochrome oxidase I gene. Additionally, 22 microsatellite loci were surveyed in order to estimate the effect of rivers as geographic barrier to gene flow. Index of genetic differentiation was calculated, beside approaches that take into account the spatial location of populations through genetic landscape shape interpolation and clustering analysis that incorporate the geographic coordinate. The effect of the geographic distance was evaluated with spatial autocorrelation analysis. Demographic changes were also estimated in a timescale framework.

**Results** This species showed a pattern of recent demographic expansion from the central region of the Rio Grande do Sul State. No geographic neither karyotypic structuration was observed, despite the wide range. Nevertheless, populations of the collared tuco-tuco were highly differentiated; except too near localities or those where the river is very narrow (headwater). Great rivers structure populations, but their effect was not proportionally higher from headwater to the mouth, as it is expected following the riverine barrier hypothesis.

**Main Conclusions** Geographic distance and the organization of tuco-tucos in small demes seem to promote the high population differentiation observed. The riverine hypothesis barrier was not fully demonstrated, but it could be a result of the recent history of occupation of the *C. torquatus* in that region. Our results showed that interpretation of demographic changes in a timescale, associated with geological knowledge of the region, brings wealthy inferences on the role of rivers as barriers.

**Keywords**

Hystricognathy, Pampa biome, riverine barrier hypothesis, underground habitat, Pleistocene

## INTRODUCTION

Species diversification is a broadly broached theme among biologists which seek to understand the evolutionary process that generate and maintain the diversity of life. Among the biogeographical processes underlying speciation, the river systems as barrier furthering diversification are appealing, mainly because great rivers as boundaries of closely related species or subspecies is a commonly observed pattern for several taxonomic groups. Based on his observations of primate species composition of the Amazon, Wallace (1852) brought up the 'Riverine Barrier Hypothesis' (RBH). He demonstrated that major Amazonian rivers limit different community of species from both sides. Moreover, Wallace noted that the sharing of species in opposite banks raises next origins of the river, and then he stated that the strength of a river as barrier decrease toward the headwaters where the channel is narrower and, consequently, it should be easier to cross it. Wallace's assumption was quantified later by Ayres & Clutton-Brock (1992) for Amazonian primate communities around several rivers, and they showed that opposite-banks similarity exhibited a significant decline as a function of increasing river width. Nevertheless, such pattern was not observed for mammals and frogs species diversity along both banks of the Juruá River, also in the Amazon Basin (Gascon *et al.,* 2000; but see the Colwell, 2000 commentary).

The riverine hypothesis considering species community, as proposed by Wallace, can be applied in an intraspecific approach where phylogeographic patterns of differentiation are assayed for populations of a widely spread species through a river system, in order to assess the strength of the river as barrier. Patton *et al.* (2000) analyzed divergence patterns in mitochondrial cytochrome *b* sequences for several wide range mammals of the Amazonia in the 1990s (Patton *et al.,* 2000 and references therein). Except the saddle-back tamarins *Saguinus fuscicollis* (Peres *et al.,* 1996), for all other taxa that exhibited a certain divergence pattern, the phylogeographic discontinuities were along the river, not on opposite banks as suggest the RBH. Although riverine effect was not frequently determinant on diversification, Patton *et al.* (2000) underlined the usefulness of phylogeographic analysis to distinguish three different hypotheses for riverine divergence: (i) Primary diversification, when a river is imposed on an existing taxon range (vicariant effect); (ii) Secondary contact, range expansion of different lineages where the river forms only a common meeting place; (iii) Dispersal, over water transfer from established populations on the opposite banks of the river (see Figure 164 in Patton *et al.,* 2000).

An excellent mammal evolutionary model to verify these hypotheses is the South American tuco-tucos (genus *Ctenomys*). These subterranean rodents have shown themselves an interesting model to study evolutionary divergence of lineages because of their extraordinary chromosomic variability and rate of speciation. Populations of tuco-tucos generally settle theirs burrow systems as small demes that associated with limited dispersal and territoriality, results in low local genetic variation and high population differentiation (Reig *et al.,* 1990; Lacey *et al.,* 2000). Speciation in ctenomyids is considered a recent and burst event among mammalian lineages, as the record fossil point about 3.5 mya for the origin of the genus that has approximately 60 recognized species nowadays (Lessa & Cook, 1998; Lacey *et al.,* 2000; Verzi *et al.*, 2010; Parada *et al.,* 2011).

Phylogeographic studies with species of *Ctenomys*, regardless not explicitly broached the RBH, had found that sometimes great rivers limit the range of species as well as karyomorphs or phylogeographic groups on an intraspecific scale (Mora *et al.,* 2007; Tomasco & Lessa, 2007; Mirol *et al.,* 2010; Lopes, 2011; Fernández-Stolz, 2006). As a subterranean rodent, it is suggestive that not only rivers but also wetlands take action on evolutionary process of diversification for tuco-tucos, as it was demonstrated for *C. minutus* and *C. lami* (Gava & Freitas, 2003; Lopes, 2011). Mirol *et al.,* (2010) fascinatingly demonstrated the effect of rivers and dynamic water bodies of the Iberá wetland

(Corrientes, Argentina) driving divergence of the called *perrensi* group (*C. dorbignyi*, *C. roigi*, *C. perrensi* and several forms of uncertain taxonomic status). Besides the Parana and Uruguay Rivers surround these Corrientes species range, the Santa Lucía River and Iberá Basin play an important role on the genetic structure, whether they are interspecific or intraspecific.

Phylogenetic approaches demonstrated that Corrientes species are a sister clade of the collared tuco-tucos *C. torquatus* Lichtenstein 1830, which are just on the Brazilian bank of the Uruguay river (Fernandes *et al.,* 2009a; Parada *et al.,* 2011). The Argentinean species show a diploid number range of 40-70 (Ortells *et al.,* 1990; Ortells, 1995; García *et al.,* 2000a; Giménez *et al.,* 2002) among which an undefined *Ctenomys* sp. has karyotype morphology similar to *C. torquatus* according to Ortells *et al.* (1990). The study of Fernandes *et al.,* (2009a) confirm such chromosomic similarity; nevertheless they showed through cytochrome *b* gene analysis that the Corrientes *Ctenomys* sp. and the Brazilian *C. torquatus* are clearly phylogenetically different and the Uruguay River is an effective barrier detaching these sister monophyletic groups (see Fig. 1).

Well delimited on west by the Uruguay River, *C. torquatus* has one of the widest geographical ranges among tuco-tucos, from the northern half of Uruguay to the southern half of the Rio Grande do Sul (RS) State in Brazil (Freitas, 1995; Fernandes *et al.,* 2009a). Despite its widespread distribution (Fig. 1), karyotypic variations of *C. torquatus* are punctual and peripheral (Fernandes *et al.,* 2009a; Gonçalves & Freitas, 2009), in a different way of the other specie belonging to the *torquatus* phylogenetic group (Parada *et al.,* 2011) as *C. pearsoni* (Tomasco & Lessa 2007), *C. lami, C. minutus* (Freitas, 2006) and the *perrensi* complex of species (as cited above), which have wide intraspecific chromosomal variation.

Karyotype 2n=44 is the commoner and widespread throughout the geographic distribution of the collared tuco-tuco, then considered plesiomorphic (Freitas & Lessa, 1984; Fernandes *et al.,* 2009a). In the south of the RS State, Freitas & Lessa (1984) described the restrict karyomorph 2n=46 surrounded by the Atlantic Ocean and wet lands, and isolated from the 2n=44 populations by the São Gonçalo Channel (Fig. 1), a water channel connecting the Patos and the Mirim Lagoons that arose 2,600 years ago (Jost *et al.,* 1975). Likewise, on the western end of its geographical range, distinct karyotypic populations of *C. torquatus* are apart by Ibicuí River (Fig. 1). On its south bank, populations have the common 2n=44 karyotype and there is no collared tuco-tucos on the north bank of Ibicuí River elsewhere, except the Itaqui population with 2n=40 (Fernandes *et al.,* 2009a).

Either on western end of the RS State, Gonçalves & Freitas (2009) described the 2n=40 and the 2n=42 chromosomal variations in allopatry with 2n=44 populations, on the Alegrete municipality, which was characterized as a high karyotypic polymorphism in a narrow range for *C. torquatus.* Despite this study had demonstrated the occurrence of chromosomic distinct forms without apparent geographic barrier, the peripheral karyotypic variations associated with great rivers for *C. torquatus* uncover an inviting phylogeographic scenery to test the RBH.

Interestingly, the common 2n=44 karyotype was observed in four populations along both sides of Vacacaí and Jacuí Rivers (Fig. 1), two of the greatest and oldest rivers crossing the range of the collared tuco-tuco (Fernandes *et al.,* 2009a). Widely distributed along Vacacaí-Jacuí extension (from headwater to mouth) and without karyotypic variation, *C. torquatus* meet the requirements to test the strength of these rivers as barrier.

Given the broad knowledge about geographic distribution and cytogenetic status of *C. torquatus*, it is important the assessment of its phylogeography with special concerning on the role of those rivers supra-cited. Therefore, the objectives of the present work are: (1) to describe the phylogeographic patterns of the collared tuco-tuco *C. torquatus*; (2) evaluate the strength of the river as barrier (headwater-outfall) using the Vacacaí-Jacuí Rivers as model, as there is no karyotypic polymorphism; and (3) attempt to identify which of the three hypothesis for the riverine divergence could explain the events of chromosomic divergence around the Ibicuí River and São Gonçalo Channel.

## MATERIAL AND METHODS

### Study area

The southern half of RS State and adjacent areas of Uruguay and Argentina make up the Pampean province, one of the biggest warm grassland areas in the world. Within its vast and diversified landscape, elements from different origins influence each ecoregion (Cabrera & Willink, 1980). The Uruguayan portion contains a mosaic of savanas, gallery forests, palm savanas and outcropings of submontane forests which differs physionomically form the Argentinean pampas and show close affinities with either the Chaco and Paranaense provinces as well as punctual elements from the Cerrado in Brazil (Grela, 2004).

All collared tuco-tuco specimens in Brazil were collected in the lowlands of the southernmost region of RS State characterized by subtropical grassland, named Pampa biome (IBGE, 2004). Specimens in Uruguay inhabited the Uruguayan savanna ecoregion that extends from the extreme southern part of the RS State to the entire country of Uruguay (Dinerstein *et al.,* 1995). This grassland biome of southernmost Brazil and Uruguay is strongly influenced by human activity. Transformations of the landscape by agriculture and pasture have markedly changed the original vegetation (Behling, 2002).

**Sample collection and cytogenetics**

The specimens of *C. torquatus* were collected in 33 localities, all around southern, central and western of the Rio Grande do Sul State, Brazil, besides collection sites sampled for previous studies (Appendix S1 in Supporting Information). DNA samples of two Uruguayan sites were kindly provided by Enrique Lessa and the revision of the collection from the Museo Nacional de Historia Natural (MNHN), Montevideo, Uruguay, was also relevant to improve our knowledge. Henceforth we will refer to abbreviations of the sample localities, see Appendix S1 for full names.

Sampling was intensified around Ibicuí River and São Gonçalo Channel. The Jacuí was chosen to test the strength of the river as barrier due the karyotypic monomorphism of populations around it, counteracting a likely chromosomic effect. For this purpose, we looked for additional populations along the river, from the headwater to the outfall. As the Jacuí River headwater, on the Brazilian Meridional Plateau, is out of the *C. torquatus* range (Fig. 1), its main tributary, the Vacacaí River, was included in the model. We collected two headwaters sites at São Gabriel municipality (SG1 and SG2), where the stream is less than ten meters large (Fig. 1). On the basis of the two middle course sites of the Vacacaí (SM1) and Jacuí (CS1) Rivers previously known (Fernandes *et al.,* 2009a), we look for another two middle course points on the opposite bank for each one of them. At SM1 locality, the Vacacaí River is roughly 80 meters large and we called it the upper-middle site. After soundly look for another upper-middle site on the opposite bank of the SM1, we were not able to find it. We noted that the right bank of the Vacacaí river has not a sandy soil suitable for tuco-tucos burrowing, and we just found another site at the left bank (SM2, see Fig. 1). SM2 is about 20 km apart from the SM1 locality and, although both are at the left bank of the Vacacaí River, they are on opposite sides of a tributary of the Vacacaí and then they were also analyzed. At CS1 locality, the Jacuí River is roughly 180 meters

large, so we call it the low-middle site of the Vacacaí-Jacuí system. We sampled the localities CS2 and CS3 on the south bank of the Jacuí River, completing the low-middle site. The GC, ML1 and BU localities previously described (see Appendix S1 for references) make up the outfall site of the Jacuí River, where the stream has a width about 300 meters and we also added the ML2 locality to this outfall site (Fig. 1).

The rodents were caught alive through an Oneida Victor nº 0 traps with a rubber cover, anaesthetized, measured and a little piece of theirs ear were cut off and stored in absolute ethanol alcohol. At least three individuals of each new locality were killed to obtain karyotypes and specimen vouchers. The femur marrows were obtained for cytogenetic analysis, following Ford & Hamerton (1956). The tissues, skulls and skins were deposited in the Mammalian Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil. All work with the animals was done with the permission of Sisbio-IBAMA (the official Brazilian environment protection agency) and following guidelines approved by the American Society of Mammalogists (Sikes *et al.,* 2011).

**Molecular methods**

The total DNA was extracted following Medrano *et al.,* (1990). For the phylogeographic analysis, two fragments of the mitochondrial DNA (mtDNA) were amplified through polymerase chain reaction (PCR) for 294 specimens of *C. torquatus*: the hyper variable sequence 1 (HVS) from control region (primers TucoPro, Tomasco & Lessa, 2007; and TDKD, Kocher *et al.,* 1989) and the cytochrome *c* oxidase I gene (COI) (primers LCO-1490 and HCO-2198, Folmer *et al.,* 1994).

PCR amplifications were carried out in a reaction volume of 20 µl containing 20-80 ng of DNA, 0.2 µM of each primer, 0.2 mM dNTP, 1x PCR buffer, 4 mM $MgCl_2$ for HVS1 and 2.5 mM for COI and 1.0 unit of *Taq* DNA polymerase (Invitrogen). Cycling consist of 94ºC for 1 min, followed by 35 cycles of 30s at 94ºC, annealing (30s at 48°C for HVS and 1 min at 50°C for COI), 1 min at 72°C and a final extension at 72ºC for 5 min. Sample size per population ranges from two to 15 individuals (see Appendix S1).

In order to infer substitution rates for estimate times of divergence and historical demography (see below), the complete cytochrome *b* gene (cyt *b*) sequence was obtained for 16 samples of *C. torquatus* and others tuco-tuco species included on these analyses as well as sequences got from

Genbank (Appendix S2 and S3). Two partially overlapping fragments of the cyt *b* were amplified using pairs primers MVZ05 – Tuco06 and Tuco07 – Tuco14 following Lessa and Cook (1998). PCR products were checked on agarose gel stained with ethidium bromide, purified using Exonuclease I and Shrimp Alkaline Phosphatase (GE Healthcare) performed following the guidelines of the suppliers and sent for sequencing at Macrogen Inc. (Korea).

To improve evaluation of the RBH, we carried out fine spatial scale analyses using microsatellite loci for samples of populations around the Jacuí River, the Ibicuí River and the São Gonçalo Channel (n=254, see populations on Appendix S1). We surveyed 22 microsatellite loci isolated from the Argentinean species *C. haigi* (*Hai*2, *Hai* 3, *Hai*4, *Hai*5, *Hai*6, *Hai*9, *Hai*10 and *Hai*12 – Lacey *et al.,* 1999), *C. sociabilis* (*Soc*1, *Soc*2, *Soc*3, *Soc*4, *Soc*5 and *Soc*6 – Lacey, 2001) and from the own *C. torquatus* (*Tor*1, *Tor*2, *Tor*4, *Tor*5, *Tor*6, *Tor*7, *Tor*8 and *Tor*9 – Roratto *et al.,* 2011). The polymerase chain reaction amplifications were carried out in a volume of 20µl following procedures of Wlasiuk *et al.,* (2003) for *Hai* and *Soc* loci and Roratto *et al.,* (2011) for *Tor* loci. All forward primers were stained with HEX or FAM fluorescence. PCR products were sent for genotyping at Macrogen Inc. (Korea) with the GeneScan 400HD ROX size standard (Applied Biosystems).

**Mitochondrial DNA data analysis**

Sequence electropherograms of the mitochondrial DNA (mtDNA) were visually inspected using Chromas 2.33 (Technelysium Pty Ltd), aligned and edited by eye using the Clustal W algorithm implemented in Mega 5.0 (Tamura *et al.,* 2011). All analyses described bellow were performed for HVS and COI sequence data separately and for both concatenated (COI+HVS).

The software DNASP 5.0 (Librado & Rozas, 2009) was used to define haplotypes (H) as well as to estimate nucleotide ($\pi$) and haplotype diversity (Hh) following Nei (1987). A median-joining network (Bandelt *et al.,* 1999) was computed in the Network 4.6.0.0 program (http://www.fluxux-engineering.com). The program ARLEQUIN 3.11 (Excoffier & Schneider, 2005) was used to infer departures from neutrality through Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics. The software DNASP 5.0 was also used to detect occurrence of past events of population expansion or decline, employing mismatch distribution analysis (Rogers and Harpending, 1992).

Spatial autocorrelation analysis was performed using the program Alleles in Space (Miller, 2005). Geographic distances are calculated between pair of individuals on the basis of its geographic

coordinate. The program uses an uncorrected proportional distance (p-distance) for DNA sequences and, for codominant markers as microsatellites, a genetic distance measure applied to pairs of individuals, identical to that used by Nei *et al.,* (1983) for population frequency data. The measure of autocorrelation used for analysis is quantified as the average genetic distance between pairs of individuals (Ay) that fell into distance class (y). Five to ten distance classes were applied and 5000 random replicates were used to identify distance classes where average genetic distances were significantly larger or smaller than random expectations.

An analysis of molecular variance (AMOVA) was implemented to estimates the partitioning of genetic variation and to test whether geographic distances or distinct karyotypes provides a good explanation for the genetic divergence observed in *C. torquatus*. By this reason, two analysis were performed: (1) five geographical groups (South, East, Central, Frontier and West – see Fig. 1) – taking into account the geographic distribution of the localities; and (2) four karyotypic groups (2n=40, 42, 44 and 46 – see Fig. 1) – considering localities according to theirs karyotype. Besides, separated AMOVAs were also performed for data sets of localities grouped by banks of each river, which is the same as groups of distinct karyotypes for Ibicuí River and São Gonçalo Channel (AMOVA I; see Appendix S4). The effect of different geological barriers of the coastal plain formation was also tested with populations of the São Gonçalo Channel (AMOVA II; Appendix S4) considering that localities RG2, TA1 and TA2 inhabit the IV barrier formed by the last Holocenic marine transgression (Villwock & Tomazelli, 1995). Localities along the Vacacaí-Jacuí Rivers were grouped in several ways to test the influence of the river on differentiation: (I) groups along the river; (II) localities on both banks of the Jacuí River; (III) localities on both banks of the low-middle site; (IV): localities on both banks of the outfall site (Appendix S4). AMOVA and $F_{ST}$ statistics for pairwise genetic differentiation between localities were computed using ARLEQUIN with 10000 random permutations to test the values for significance.

**Molecular clock analysis and Phylogeny**

Time estimates and calibration of molecular clock is often a hard task due the scarcity and uncertainty of the fossil records for some taxa. As an approximation, we firstly estimate a substitution rate for the cyt *b* gene of ctenomyids due large number of sequences for *Ctenomys* and other Caviomorpha species available on Genbank that comprise the usable calibration points from data

fossil. We use a total of 69 sequences, including representatives of the Caviomorpha families and one sample of the infraorder Phiomorpha (*Thryonomys swinderianus*) that was used as outgroup for Caviomorpha (Appendix S2). Three different calibration points were defined: the Caviomorpha radiation (Wyss et al, 1993), the Ctenomyidae-Octodontidae divergence (Quintana, 1994; Vucetich et al., 1999; Verzi, 2002) and the most recent common ancestor for *Ctenomys* at 3.5 mya (Verzi, 2008; Verzi et al, 2010). Detailed information of priors is given in Table 1. The Bayesian Inference used through program BEAST 1.6.1 (Drummond & Rambaut 2007) co-estimate divergence time and phylogenetic relationships. We used the Yule process, SRD06 substitution model (Shapiro et al., 2006) and the relaxed molecular clock (uncorrelated lognormal). Afterward, the same phylogeny was constructed adding 18 cyt *b* haplotypes of *C. torquatus* (Appendix S2), in order to estimate the time to most recent common ancestor (tMRCA) of this species.

Then the substitution rate for HVS and COI segments were estimated relative to the previously calculated rate for cyt *b*, which was employed as a normal prior, in a partitioned analysis using 38 specimens of *Ctenomys* that have sequences for the three DNA fragments, as discriminated on Appendix S3. We ran the analyses using the Yule prior, a relaxed molecular clock model, with uncorrelated rates, the Hasegawa-Kishino-Yano model (HKY) with a proportion of invariable sites (I) and the correction gamma value (G) for the HVS and the Tamura-Nei model (TrN) + (I) for the COI sequences, both selected by the Akaike Information Criterion (AIC) implemented in Modeltest version 3.7 (Posada and Candrall 1998).

Finally, we characterized past changes in the effective population size of *C. torquatus* testing the Bayesian skyline (Drummond et al., 2005) and skyride plots (Minin et al., 2008), both implemented in the software BEAST. These methods use a MCMC sampling procedure to obtain estimates of the posterior distribution of the effective population size through time. The skyline method employ a piece-wise constant model that allow abrupt changes between successive intervals, making it possible to recover sudden population demographic changes. On the other hand, the skyride plot uses a smoothing prior (the Gaussian Markov random field) that penalize effective population size changes between consecutive coalescent intervals, taking into account the length of the intervals (Ho and Shapiro, 2011). We used concatenate HVS and COI sequences for the whole 294 specimens, as well as for the South haplotype groups (with karyotype 2n = 46) separately. We ran these analyses with a

strict molecular clock, using the rates for COI and HVS estimated by us, drawn from a normal distribution for the mean-rate prior.

All BEAST analyses were performed in two independent runs of 30 million iterations sampling each 1,000 chains and the first 10% iterations were discarded as burn-in. We use the computational resources of Bioportal from University of Oslo (https://www.bioportal.uio.no). The program TRACER 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used to analyze the parameter distributions estimated from BEAST and check for convergence of the chains, taking into account that all ESS values be greater than 200. TRACER was also used for the skyline and skyride reconstructions. Runs were summarized in TreeAnnotator 1.5.4 (Drummond & Rambaut 2007) and the inferred tree visualized in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

**Microsatellite data analysis**

To determine the allele's size, electropherograms were imaged using the Peak scanner software (Applied Biosystems). The data set generated was submitted to software CONVERT (Glaubitz, 2004) to obtain input files for other analyses. The program MICROCHECKER 2.2.0 (Van Oosterhout *et al.,* 2004) was used to detect null alleles, any genotyping errors (including typing mistakes) and large allele dropout. Genetic diversity measures, as well as deviations from Hardy–Weinberg equilibrium (HWE) and inbreeding coefficient ($F_{IS}$) for each sampling site and tests for linkage disequilibrium (LD) across all pairs of loci were conducted using ARLEQUIN, with a strict Bonferroni correction applied for multiple comparisons (Rice, 1989).

Population differentiation was assessed with pair-wise $F_{ST}$ (Weir & Cockerham, 1984) between sampling sites. We also spatial autocorrelation analysis as described for mtDNA. Analyses of molecular variance (AMOVA) were first computed for all microsatellite data set without a priori groups and including the individual level ($F_{IT}$), and then separately for sets of localities around the Ibicui River, the São Gonçalo Channel and the Jacui River as described for the mtDNA data (Appendix S4).

**Population structure**

An interpolation-based graphical method was implemented to represent the genetic distance patterns across the landscape using the program Alleles in Space. This analysis provides a visual perspective of the spatial distribution of genetic structure over landscapes, with peaks in areas where

genetic distances between individuals are high, and valleys where genetic distances between individuals are low (Miller *et al.,* 2006). Georeferenced coordinates (Universal Transverse Mercator system) were provided for each individual and the genetic distances were the same used in spatial autocorrelation analysis described previously for microsatellite data and COI+HVS sequences. We tested different grid size and distance weighting parameters which did not affect landscape shape, and then we use a 50 x 100 grid and a distance weighting parameter of 0.25.

We also perform cluster analysis of microsatellite genotypes using the programs STRUCTURE 2.3.2 (Pritchard *et al.,* 2000, http://pritch.bsd.uchicago.edu/structure.html) and GENELAND 3.3 (Guillot *et al.,* 2005). STRUCTURE uses an iterative approach to cluster microsatellite genotypes into K populations without using the geographic locations of individuals, in a way that maximizes the Hardy–Weinberg equilibrium and the linkage equilibrium within the resulting clusters. We ran ten independent runs for each K, using $1 \times 10^6$ MCMC iterations, a burn-in of $5 \times 10^5$, the independent allele frequencies model and assuming admixture. Considering the data set of 21 localities (see Appendix S1), analysis were performed with values for K range from 1 – 30. The optimal K was determined based on the Pritchard *et al.,* (2000) methodology, considering the highest mean value of estimated logarithm of probability of the data [LnPr(X/K)], and the lowest standard deviation (SD) among the independent runs for each K. The graphical display of the STRUCTURE results was generated using DISTRUCT software (Rosenberg, 2004). GENELAND has the same assumptions of STRUCTURE, but it incorporates the spatial coordinates of specimens as it takes into account the fact that differentiated populations tend to be structured in spatially distinct areas. We first infer K (ranging from 1-30) performing five independent runs of $1 \times 10^6$ iterations saved at every 100, uncorrelated allele frequencies and the uncertainty attached to spatial coordinates fixed to 500 m (taking into account the low vagility of tuco-tucos and the spatial scale of the burrow systems). We then infer the assignment of individuals for the k fixed previously in a new round of five runs and the same parameters above. The posterior probability of population membership for each pixel of the spatial domain was computed for each run using a burn-in of 1000 iterations and following the manual recommendations (http://www2.imm.dtu.dk/~gigu/Geneland/Geneland-Doc.pdf).

## RESULTS

### Geographic distribution and karyotypes

The distributional limits of *C. torquatus* enclose localities in the eastern, central, western and southern of Rio Grande do Sul State, in southern Brazil, and in western and northern Uruguay. Most of the Brazilian populations of *C. torquatus* occur in open areas along the *Depressão Central* region (low lands between the Brazilian Meridional Plateau and the *Escudo Sul-Riograndense*, Fig. 1) and *Planalto da Campanha*, southwest of the RS State. The northernmost registered locality of occurrence for *C. torquatus* is at Candelária Municipality, in the *Depressão Central* region border, where a skull was collected in 1969 by A. Ximenez, and deposited at MUNHINA, in Uruguay. We did not find records of tuco-tuco in the Brazilian Meridional and the *Escudo Sul-Riograndense* Plateaus (Fig. 1).

The revision of the MUNHINA mammals' collection in Uruguay corroborated the occurrence of *C. torquatus* in areas of savanna in northern Uruguay (Langguth & Abella, 1970; Kiblisky *et al.,* 1977; Freitas & Lessa, 1984; Novello *et al.,* 1990; Novello *et al.,* 1996; Villar, *et al.,* 2005; Villar & Novello, 2006). Populations of *C. torquatus* occur in almost all the Departments from the northern and western of Uruguay: Cerro Largo, Rivera, Tacuarembó, Salto, Paysandú and Rio Negro (Fig. 1).

New populations found beyond south bank of São Gonçalo Channel and north bank of Ibicui River showed 2n=46 and 40, respectively (Fig. 1, Appendix S1). All other populations have the most common Karyotype 2n=44.

### Phylogeographic structure and historical biogeography

We obtained 636 bp for the COI and 376 bp for HVS sequences that concatenated (1012 bp) draw up 40 haplotypes defined by 48 variable positions (GenBank Accession in Appendix S5) in a sample of 294 individuals. Haplotype diversity values were moderate to high, whereas nucleotide diversity values were low (Table 2), indicating that this species is comprised of a relatively high number of closely related haplotypes (diversity index for all localities are given on Appendix S1). Despite COI and HVS separately show less haplotypes than the concatenate data COI+HVS, the general patterns were concordant. Then we will present and comment only the COI+HVS haplotype network (Fig. 2 and Appendix S5).

Few haplotypes were shared between several populations. There was a high number of unique haplotypes that differed in one or very few nucleotide changes from the central and widespread H12 haplotype. This haplotype was represented since ML1 and BU localities on the east, SM1 on the central region, RO on the west of the Rio Grande do Sul state, till Río Negro on west of Uruguay (Figs. 1 & 2). Likewise, the haplotype (H10) of eastern samples (GC, BU, ML2) is also present in the southern population (CA) and diverge in one mutation from the H29 of QU, on the west. These central and widespread haplotypes H10 and H12 were found mainly in the *Depressão Central* region of the RS State. Two haplotype groups are slightly detached: the southern populations (H1-H9 and H11) and the western populations (H33-H37). The widespread haplotypes H10 and H12 were absent in those regions.

The Tajima's D neutrality test was significant only for COI sequences, but the more sensitive Fu's FS test resulted in negative and significant values for the three dataset (Table 2). Additionally, evidence of a recent history of population expansion comes from the analysis of mismatch distribution that showed a typical unimodal distribution under the exponential population expansion model (Table 2; Appendix S6).

The spatial autocorrelation analysis demonstrated a spatial phylogeographic structure in *C. torquatus* occurring until the order of approximately 200 km for all distance classes tested (Fig. 3), for both microsatellite loci and mtDNA concatenate data. Despite the concordance of the geographic distance threshold for both molecular markers, the mitochondrial data set yielded higher Ay value (0.92) than microsatellite loci (0.65).

In the two AMOVA tests performed, the highest percentages of genetic variation were found among localities within groups (around 50%), but there were also a great percentage of variation among the proposed groups, specially the geographic groups (Table 3). The low variation was always found within populations as a result of the high number of localities with one or few exclusive haplotypes. The low haplotype sharing among localities is also evident by the high and significant $F_{ST}$ values. The $F_{ST}$ pairwise estimates ranging from -0.3 to 1.00, but the lowest values were between next localities and those ones that were not statistically significant refer mainly for comparisons of localities with $N < 6$ individuals (Appendix S7).

The Bayesian tree of cyt *b* demonstrated a pattern among tuco-tucos species in accordance with the recent phylogeny proposed by Parada *et al.,* (2011) and shows the *torquatus* group species as a

monophyletic clade (Appendix S8). Results of molecular clock analysis revealed a mean rate of 0.0208 substitutions/site/Myr (standard deviation 0.000067) for the cyt *b* gene. The Effective Sample Size (ESS) values were >200 for all parameter estimates. A second bayesian phylogenetic analysis was performed, including the cyt *b* haplotypes obtained for *C. torquatus* (Appendix S2), in order to estimate the tMRCA of this species (see detail in Appendix S8), that was around 515000 yr before the present (BP) (95%IC 275000 - 800000).

On the basis of the substitution rate estimated for cyt *b*, we co-estimated the substitution rates of 0.0198 (standard deviation 0.000033) and 0.0331 (standard deviation 0.000086) substitutions/site/Myr for the COI gene and HVS1 control region, respectively.

For the whole *C. torquatus* sample (N = 294) concatenate COI+HVS data, we did not obtain reliable Bayesian skyline plot results. Using the default number of intervals (parameter m = 10), the MCMC chains did not stabilize and several parameters showed ESS values below 200 and even 100. Tests with higher numbers of intervals (m = 12, 15 and 17) were worst and as the m values were lowering (m = 7, 5, 3 and 2), less parameters had bad ESS values, but the MCMC chains did not stabilize even after very long runs. Although unreliable results, the Bayesian skyline reconstruction showed a demographic population jump about 75000 yr BP and the estimated tMRCA for *C. torquatus* dating from around 150000 yr BP (95%IC 66600 - 255400). These time estimatives (treeModel.rooHeight statistic) showed good ESS values, convergent chains and recurrent values over distinct runs.

The Bayesian skyride plot shows an ongoing population expansion for *C. torquatus* during the last 25000 yr BP (Fig. 4a). For this analysis, MCMC chains stabilized and the ESS values were higher than 200 for all parameters, except the treelikelihood. The tMRCA of the collared tuco-tuco was oddly recent, dating from 24700 yr BP (95%IC 14700 - 36600) in comparison with the cyt *b* phylogeny and skyline plot estimatives approximately in the Late Pleistocene (Fig. 4, Appendix S8).

As there were no detached haplotype groups, we performed the demographic analysis for the whole data set, and for the south localities (RG1, RG2, TA1 and TA2) that has the 2n=46 karyotype and occupy a geologically recent formed region. Results quality for skyline plot was better than those for the whole data set. For these 2n=46 population's data set, again both analysis estimated very different tMRCA. Whilst the tMRCA was around 15000 yr BP (95%IC 3300 - 29000) for the Bayesian skyride plot (Fig. 4b), it was about 40000 yr BP (95%IC 5000 - 95000) for the skyline reconstruction

(Fig. 4c). Nevertheless, both plots showed a stressed demographic growth in the last 5000 yr for the southernmost localities with 2n=46 karyotype of the collared tuco-tuco.

**Microsatellite data analysis**

Number of alleles per sampling site (and clusters), observed heterozygosity, deviations from Hardy-Weinberg equilibrium, linkage disequilibrium and $F_{IS}$ values are presented in Appendix S1. None locus was presumed to have null alleles or genotyping errors. After Bonferroni correction, four loci showed isolated situations of departures from Hardy-Weinberg equilibrium for different localities. All these deviations were caused by deficiency of heterozygotes, except locus *Hai*3 for TA1. Only four sample collections present between one and three different pairwise loci with linkage disequilibrium, even after the correction for multiple tests. The $F_{IS}$ index was positive and significant for localities RG1, CS2 and GC, suggesting a deficiency of heterozygotes (Appendix S1).

Pairwise $F_{ST}$ comparisons showed high and significant differentiation among all pairs of localities, except for comparisons involving ML1, which has few individuals (N = 4). The lowest comparison ($F_{ST}$ = 0.10) was for UR1 and UR2 localities and the greater was $F_{ST}$ = 0.74 between CS1 and TA2, distant one another over 280 km (Appendix S7). High population differentiation was also achieved using AMOVA. Considering all localities without groups, 47.09% of variation was a result of among-localities differences ($F_{ST}$ = 0.47, P<0.0001). Only 3.67% of the variation was among individuals within localities ($F_{IS}$ = 0.07, P < 0.0001) and the higher percentage of variation (49.24%, $F_{IT}$ = 0.50, P<0.0001) was at individual level.

The estimated number of clusters for STRUCTURE and GENELAND programs was k=14 (Fig. 5). For STRUCTURE, the mean logarithm of posterior probability increased until k=14 and reached a plateau (Fig. 5b). In the first run, GENELAND results strongly supported the existence of 14 clusters (Fig. 5c). Further analyses using a fixed K of 14 were checked for consistency of results. The 14 inferred clusters corresponded to the cluster assignment obtained with STRUCTURE (Fig. 5a).

**São Gonçalo Channel**

The six local of sampling around São Gonçalo Channel formed four separated clusters for both STRUCTURE and GENELAND approaches. Localities PE1 and PE2, as well as TA1 and TA2 become clustered and were denominated cPE and cTA, respectively (Fig. 5a).

The $F_{ST}$ values for microsatellite data were highly significant among all comparisons and the lowest value was $F_{ST}$ = 0.15 for PE1-PE2. The $F_{ST}$ values for COI+HVS data were even higher and significant among all comparisons, except pairs PE1-PE2 and TA1-TA2, both localities pairs that shared haplotypes (Table 4 and Fig. 2).

AMOVA considering groups according to river banks (PE1,PE2 x RG1,RG2,TA1,TA2) that is the same as different Karyotypes (2n = 44 x 2n = 46), showed low percentage and non significant $F_{CT}$ value for variation among the suggested group, for both mitochondrial and microsatellite data (Appendix S4).

The genetic landscape shape (GLS) interpolation analyses were roughly congruent for the spatial distribution of COI+HVS haplotypes (Fig. 6a) and microsatellite genotypes (Fig. 6b). The lowest genetic distances were observed between TA1-TA2 and PE1-PE2 localities pairs, and the highest divergence peaks were for pairwise comparisons involving locality RG2. The detached peaks among (PE1, PE2), RG1, RG2 and (TA1, TA2) agree with cluster analysis. The divergence peak between PE2 and RG1, which correspond to the São Gonçalo Channel, was less pronounced for mtDNA than microsatellite GLS.

**Ibicuí River**

The Bayesian analysis using STRUCTURE and GENELAND identified the presence of three clusters for sampled sites around the Ibicuí River. The three clusters represented localities IT1, IT2 and (UR1 + UR2), so called cUR (Fig. 5a). The haplotype H33 was shared among localities UR1, UR2 and IT1. The northernmost locality IT2 show an exclusive and divergent haplotype H37 (Fig. 2).

The $F_{ST}$ values for microsatellite data were highly significant among all comparisons and the lowest value was $F_{ST}$ = 0.10 for UR1-UR2. The $F_{ST}$ values for COI+HVS data were high and significant for all comparisons involving locality IT2 (Table 4). We also performed $F_{ST}$ pairwise considering UR1 and UR2 as one locality (cUR) and the result were congruent for both molecular markers (see grey cells on Table 4).

AMOVA considering groups according to river banks and karyotypes (IT1, IT2 x UR1, UR2), that is the same as different Karyotypes (2n = 40 x 2n = 44), showed low percentage and non significant $F_{CT}$ value for variation among the suggested group, for both mitochondrial and microsatellite data (Appendix S4).

The genetic landscape results show that the lowest pairwise genetic distances for Ibicuí localities were found between UR1-UR2 for the microsatellite GLS, which also showed the higher pairwise genetic distances between IT1 and UR1 localities that coincide with the presence of the Ibicuí River (Fig. 6d). The GLS for mtDNA showed high genetic divergence peaks between all site pairs, mainly for IT1-IT2 (Fig. 6c), which should be due the divergent H37 on IT2.

**Jacuí River**

STRUCTURE and GENELAND programs inferred the presence of 7 clusters for the 11 localities along the Vacacaí and Jacuí Rivers (Fig. 5a). These clusters correspond to four sampled sites SM1, SM2, CS1 and GC, and three localities groups: cSG (localities SG1 and SG2), cCS (CS2 and CS3) and cBU (BU, ML1 and ML2). These clustered localities represent the headwater points (cSG) and sampled sites on the same bank of the low-middle (cCS) and outfall points (cBU; Fig. 1).

The $F_{ST}$ values for microsatellite data were highly significant (Table 5), except for comparisons involving the locality ML1, which has few individuals (N = 4). The lowest values were $F_{ST}$ = 0.13 for SG1-SG2 (the headwater sites) and $F_{ST}$ = 0.18 for CS2-CS3 localities. The differentiation between localities of opposite river banks on the Jacuí outfall, CG and cBU ($F_{ST}$ = 0.28), was moderate in comparison with localities of the low-middle river CS1 and cCS ($F_{ST}$ = 0.47). The same was noted for the mtDNA data $F_{ST}$ (Table 5).

There was sharing of the haplotypes between the localities of the same bank river CS2 and CS3 (H17) and BU, ML1 and ML2 (H10, H12 and H14). However, there were also haplotype sharing between localities of opposite river banks: the H26 on headwater sites (SG1 and SG2) and the H10 on the outfall sites GC, BU and ML2 (Fig. 2). The $F_{ST}$ values for COI+HVS data were high and significant for all comparisons, except for locality ML1 and pairs of sites that shared haplotypes (Table 5, Fig. 2). Headwater sites shared the haplotype H26, but some individuals of SG2 had the differentiated H27. The complete list of individuals from each site and their respective haplotypes is given in Appendix S5.

The only AMOVA grouping that showed significant F-statistic, for both microsatellite and COI+HVS data, was for localities along the river (AMOVA group I, Appendix S4), where the presence of the river was not considered as the factor of segregation, but the distances among the sampled sites on headwater (SG1, SG2), upper-middle (SM1, SM2), low middle (CS1, CS2, CS3) and outfall (GC, BU, ML1, ML2). Despite non significant $F_{CT}$ value, the 100% percentage of variation among

groups (CS1) and (CS2, CS3) for mtDNA is evident by the fact that each group has fixed a different haplotype of COI+HVS (Fig. 2). Although non significant, microsatellite data AMOVA results for groups III and IV showed higher percentage of variation between banks of the low-middle (III) than the outfall river site (IV, Appendix S4) in agreement with $F_{ST}$ values (Table 5).

The mtDNA and microsatellite GLS graphs detached a high differentiation of the outfall localities GC, BU, ML1 and ML2 from the others in general. There are peaks of divergence between all pairs of localities on opposite banks of the river, except for headwater (SG1, SG2) localities (Fig. 7). For microsatellite data, the divergence between CS1 and (CS2, CS3) localities on the low-middle Jacuí site was higher than the divergence between GC and (BU, ML1, ML2) sites on the outfall (Fig. 7b).

## DISCUSSION

### Geographic boundaries and interespecific relationships

Several factors affect a species' geographical distribution. The suitable conditions may exist in many regions, but other biological and historical realities typically prevent a species from existing throughout its full potential geographical distribution (Anderson *et al.,* 2002). Geographic factors, vegetation structure and soils type has often acted to restrict a species' distribution and also play an important role on the populations' ongoing and fragmentation, particularly in relation to tuco-tucos (Busch *et al.,* 2000; Lizarralde *et al.,* 2001; Mirol *et al.,* 2010; Mora *et al.,* 2010).

The wide geographic distribution of *C. torquatus* has been reported previously (Freitas 1995, 2006; Fernandes *et al.,* 2007; Fernandes *et al.,* 2009a). The Brazilian Meridional Plateau, located at the north and northeast of RS State, has *Campos de altitude* (high elevation grasslands), at elevations above 1600 meters (Behling, 2002), but its hard soils must be a geographic barrier for the occurrence of collared tuco-tuco. Though tuco-tucos can occur at altitudes up to 5000 meters (Contreras & Bidau, 1999), Barlow (1969) suggested that *C. torquatus* prefers sandy, rock-free soils in very dry areas, and occurs at low (0–200 m) elevations. This information was corroborated in this study since the exclusive habitat of *C. torquatus* populations along their distribution was composed of sand fields with less than 200 meters of altitude. The lowlands on northwest of the Rio Grande do Sul State were also searched out but any record of tuco-tucos were found (Fig. 1), except a new species on the Manoel Viana municipality (Freitas *et al.,* in prep).

The most important barrier on eastern is the *Escudo Sul-Riograndense*, where none *C. torquatus* was found (Fig. 1). The lack of records could be due the higher altitudes and also the geomorphologic source in this region, where 91% (40.000/44.000 Km2) are covered by granite soil (Rambo, 2000). Moreover, the coastal system of lagoons partially may have limited the areas of occurrence of *C. torquatus*, except for RGs and TAs localities beyond the São Gonçalo Channel, as discussed below.

The western distribution is limited by the Uruguay River, one of the most important and abundant rivers in South America, that probably arose during the Pliocene (Souza *et al.,* 2005) and depart the collared tuco-tuco from the Corrientes species of the Argentinean side. Despite karyotypic similarities (Ortells *et al.,* 1990) between *C.* sp. from Corrientes and *C. torquatus*, Fernandes *et al.,* (2009a) showed that they are clearly phylogenetically different. The same was demonstrated in the cyt *b* phylogeny of the present work (Appendix S8) in relation to *C.* sp. from Contreras Cué and Tacuarita (2n = 42, Mirol *et al.,* 2010), which are not phylogenetically related with the 2n = 42 from Catimbau (***C. tor* CAT** in the Appendix S8, and see Gonçalves & Freitas, 2009).

The Uruguayan species *C. pearsoni* occurs at the southern boundary of *C. torquatus* distribution and have been phylogenetically related to them (D'Elia *et al.,* 1999; Castillo *et al.,* 2005). Remarkable differences between skulls' size and shape of *C. torquatus* and *C. pearsoni* were showed in a morphometric geometric study (Fernandes *et al.,* 2009b) and two putative barriers must be pointed between both species in Uruguay: Cuchilla Grande and Río Negro River (Fig. 1). Considering the recent expansion evidences found for the collared tuco-tuco in the present study, with probable origin on the central region of the RS State (as discussed below), those geographic barriers should consist of secondary contact between *C. torquatus* and *C. pearsoni*, but only more studies and a detailed sampling could provide the exact species delimitation, and the evolutionary and phylogeographic process involved in that scenario. Furthermore, García *et al.,* (2000b) pointed out the cytogenetic similarity between *C. pearsoni* and *C. dorbignyi* from Corrientes – Argentina; *C. pearsoni* was more related with Corrientes species in the Parada *et al.,* (2011) work, and it is closely related with *C. dorbignyi* from Sarandicito in the present study (Appendix S8), which is the southernmost locality of the Corrientes' tuco-tucos (see Fig. 1 in Mirol *et al.,* 2010).

For molecular clock analysis, the cyt *b* phylogenetic tree showed species assigned to *torquatus* group as monophyletic including the new specie *C. ibicuiensis* (Appendix S8), and our estimatives for the tMRCA of this group and the genus *Ctenomys* (Table 1) were more recent than those of Parada et

al., (2011). It must be because we use the origin of *Ctenomys* as calibration point, besides the Ctenomyidae-Octodontidae divergence. The prior tMRCA for *Ctenomys* had median age at 5 my (3.3 - 7.5) because fossil records of tuco-tucos supports the minimum age of 3.5 myr for the genus, but do not allow estimate its origin (Verzi et al., 2010). Fernandéz (2006) also used the origin of *Ctenomys*, among others, as calibration point and found the molecular rate 0.019 substitution/site/my very similar of that we obtained, but theirs estimative for the divergence time of *Ctenomys* was more recent (3.3 my ago). It is important to keep in mind that these estimatives of divergence time are approximations, given that the rate was estimated only for the cyt *b* gene (COI and HVS rates were co-estimated from cyt *b*), the calibration points were external to the genus *Ctenomys* and age estimates yield large confidence intervals.

**Phylogeography**

The geomorphological history of the Pampa biome domains was very intense. It was covered by the ocean during the Paleozoic era, bedspread by a desert along the Triassic, suffered by the Terciario's sea ingression, and finally originated similarly as it seems nowadays after the sea regression in the Quaternary beginning (Rambo, 2000). From the early Quaternary to now this region seems to be more stable and the regressive and transgressive sea levels had affected only the geographic variations of populations, particularly tuco-tucos species, living on the coastal plain as *C. australis* (Mora *et al.,* 2006), *C. pearsoni* (Tomasco & Lessa, 2007), *C. rionegrensis* (Kittlein & Gaggiotti, 2008), *C. flamarioni* (Fernández-Stolz, 2006) and *C. minutus* (Lopes, 2011). Palynological researches indicate that grasslands dominated in the Pampa region from the late Pleistocene, about 42,000–10,000 yr BP, including the end of the last maximum glacial (Overbeck *et al.,* 2007 and references therein).

The historical demographic reconstruction for *C. torquatus* (Fig. 4a) suggests that this species would have recently expanded in its area of distribution. Expansion inference is also supported by the significant negative values of the Fu's FS neutrality test and by the unimodal mismatch distribution graphs (Table 2 and Appendix S6). Despite the population growth emphasized on the skyride plot, the time estimated for the common ancestor of the *C. torquatus* lineages are about a hundred thousand years younger than the results of cyt *b* phylogeny and the Bayesian skyline plot. The Bayesian skyride method shrinks genealogies sometimes. It is not a case of bug, but it is possible that such shrinkage

occurs when the alignments do not have enough mutations, so the genealogical reconstruction falls back a little too much on the coalescent prior (Vladimir Minin, personal communication). Then, the skyride's historical demography of expansion is trustworthy, but the tMRCA is not.

The cyt *b* phylogenetic analysis suggested a tMRCA for *C. torquatus* dating from the middle to late Pleistocene. Although the Bayesian skyline plot did not result convergent chains, its estimative of tMRCA was reliable and fit the 95% IC of the cyt *b* phylogeny. Such estimatives suggest an occupation – and expansion – of the collared tuco-tuco in southern RS State and northern Uruguay from the middle Pleistocene, which may be associated with a cold and dry climate during the last glacial maximum, when grasslands dominated in these regions (Behling *et al.,* 2005). Mapelli *et al.* (2012) assigned the high population stability inferred for *C. porteousi* to these severe climatic conditions for the middle and late Pleistocene, when Argentinean Pampean region was covered by sand dunes and sparse vegetation, the ideal habitat demonstrated previously for this species (Mapelli & Kittlein, 2009). Such kind of habitat would be so crucial for *C. porteousi* survival that the population decrease during the Holocene could be related with reduction in the suitable habitat, mainly by higher vegetal cover, due changes to a warm and humid climate (Mapelli *et al.*, 2012).

*Ctenomys torquatus* lacks a study detailing its habitat specificities as that of *C. porteousi* (Mapelli & Kittlein, 2009), but some evidences allow to imply that they are distinct. First, all sites where *C. torquatus* were collected are thickly covered by grass, whereas the porteou's tuco-tuco inhabits preferentially less vegetated areas (Mapelli & Kittlein, 2009). Second, it seems that climatic conditions during the last maximum glacial brought on a more intense reduction of the vegetation cover in central Argentina (see references in Mapelli *et al.*, 2012) than in the south Brazil and north Uruguay (Behling *et al.,* 2005). These grasslands where the collared tuco-tuco inhabits are still predominant nowadays; the main change regards Holocenic moister and warmer conditions was expansion of gallery forests (Behling *et al.,* 2005) and *C. torquatus* did not show evidence of population reduction related with it, differently of *C. porteousi* (Mapelli *et al.*, 2012). Then, the climate during the last glacial maximum in the Pampean region could be related to the population stability of *C. porteousi* in central-west Buenos Aires province and to the expansion of *C. torquatus* throughout grasslands of the Brazil-Uruguay frontier.

Recent population expansion has been documented for *C. australis* (Mora *et al.,* 2006) and *C. flamarioni* (Fernández-Stolz, 2006) that occur in Quaternary coastal dune systems, i.e. young habitat

available for occupation since last sea level fluctuations in the middle Holocene. On the other hand, tuco-tuco species habiting inland more stable regions like *C. pearsoni*, *C. talarum*, *C. minutus* and *C. lami* had showed demographic inferences of long time population stability (Mora *et al.,* 2007; Tomasco & Lessa, 2007; Lopes, 2011). *Ctenomys torquatus* exhibits a different situation because its populations had recently expanded in a relatively stable area since early Pleistocene. Such grasslands were not flooded during the Holocene marine transgressions, but may be likewise affected by the dry climatic conditions during the last glacial maximum.

The spatial autocorrelation results showed that the genetic differentiation increase is related to the geographic distance up to 135km for mtDNA and 200km for microsatellite data. Values of Ay did not change linearly with geographic distances above it, which means that beyond this threshold further differentiation is unrelated to physical distance for *C. torquatus*. The lower geographic threshold value for mtDNA, corresponding to a higher genetic differentiation (Ay = 0.92) in comparison with microsatellites (Ay = 0.65), should be due the matrilinear inheritance of this marker and the female phylopatry common for tuco-tucos (Lacey, 2001; Cutrera *et al.,* 2005; Fernández-Stolz *et al.,* 2007; Lopes, 2011). These results are in agreement with spatial autocorrelation analysis using allozyme loci in *Thomomys* (Patton and Smith, 1990) and mtDNA sequences in *C. minutus* and *C. lami* (Lopes, 2011).

Neigel *et al.,* (1991) suggested that the most geographically widespread haplotypes should be the ancestral, under a limited gene flow model. On the other hand, haplotypes restricted to single locations should be of more recent origin. Following this line of reasoning, all peripheral areas of the *C. torquatus* distribution that presented unique haplotypes should be recently colonized, and the area that comprised the central-northeast distribution of *C. torquatus* at the *Depressão Central* could be ancestral, given that it harbors the most frequent mitochondrial haplotypes H10 and H12. The H12 is present, but not exclusively, in the eastern localities BU and ML1, besides CS1, SM1 and in the western localities RO and RN. The southern locality of CA provided another evidence of the central-northeast origin of the species because, besides its unique H9, this site shares the H10 with easternmost localities (Figs 1 & 2).

Uruguayan populations analyzed had different haplotype for the concatenate COI+HVS (Fig. 2). The H38 haplotype from TC is more related with southern haplotypes (H1 to H11) whilst RN locality shared the most common H12 haplotype with Brazilian localities besides its exclusive haplotypes H39

and H40. These differences are also noticeable on the cyt *b* phylogeny (Appendix S8). The morphometric geometric study of Fernandes *et al.,* (2009b) detected intraspecific differences in skull shape between *C. torquatus* 2n=44 populations from Brazil and Uruguay, even thought a clear effective geographic barrier, or ecological differences, are absent between these countries. Detailed studies in skulls differentiation must be employed to test if Cuchilla Haeda (between 200 and 500 meters of altitude, see Fig. 1) can be a putative barrier for the skulls' differentiation between samples from Brazil and Uruguay and also between eastern and western Uruguayan populations.

**Rivers, peripheries and karyotypic polymorphisms**

Since the expansion, subpopulations have differentiated essentially in isolation, under the influence of genetic drift and mutation. Considering the central and northeast region as older and the most common karyotype 2n = 44 as ancestral, peripheral karyotypic variations should be recent. Hybrid zone was not found between distinct chromosomic forms of the collared tuco-tuco, even for the Alegrete municipality where Gonçalves & Freitas (2009) described three allopatric forms (2n = 40, 42 and 44) without any apparent geographic barrier. In spite of the habitat restriction (sandy soils), *C. torquatus* occupy a geographic range nearly 600km from northeast to southwest. The recent process of occupation on such broad area may not have allowed a contact between localities with different karyotypes yet and it probably will be harder on the presence of a river detaching them (see below).

Genetic drift has been related as the main cause of chromosomic polymorphism fixation on several tuco-tuco species, besides pelage color polymorphism for *C. rionegrensis* (Wlasiuk *et al.,* 2003) and *C. torquatus* (Gonçalves & Freitas, 2009). For all AMOVA tests with microsatellite data, "within populations" level contained major percentage of variation (Appendix S4). The same was observed for *C. talarum* (Cutrera *et al.,* 2005) and *C. lami* (Lopes, 2011). It is interesting highlighted that when AMOVA is performed considering the individual level, the minor level of variation "within individuals" ($F_{IT}$) takes into account the differences between genes found within individuals (Excoffier *et al.,* 2006). Our data demonstrated that when including individual level, variation become far more "within individuals" (49.21%; $F_{IT}$ = 0.50; P < 0.001) than "among individuals within population" (3.68%; $F_{IS}$ = 0.07; P < 0.001). Therefore, the high percentage of variation that we observe within population level using microsatellite loci is due the multiloci nature of the molecular marker. In fact, there is low genetic variability among individuals within populations, as we observed for AMOVA performed with

mtDNA data (Appendix S4). Both data sets bring forward the probable effect of the drift over genetic variability, as a result of the territoriality, small demes, spatial isolation and restricted mobility of these subterranean rodents.

As pointed out by D'Annato & D'Elía (2011) for *C. pearsoni*, great watercourses are suggestive to promoting isolation and differentiation of karyomorphs, but there are evenly great rivers dissecting the distributional range of populations that share the same karyotype. As the distinct karyotypic forms of *C. torquatus* are due centric fusions/fissions (Freitas & Lessa, 1984; Fernandes *et al.,* 2009a), they probably were fixed with little or no negative heterosis by drift. Furthermore, the significant percentage of variation among karyotypic groups found to mtDNA AMOVA (Table 3), which considered all *C. torquatus* localities, should be reflecting the geographical structure, as AMOVAs performed for Ibicuí River and São Gonçalo Channel localities in a smaller scale did not show significant genetic structure explained by karyotypes (Appendix S4).

Major rivers appear to be associated with strong levels of genetic differentiation in *C. talarum* (Mora *et al.,* 2007) and *C. minutus* (Lopes, 2011), tuco-tuco species showing a long-time population stability pattern. On the other hand, the effect of rivers as main promoters of genetic divergence was not observed for species with a suggestive recent history of population expansion, like *C. australis* (Mora *et al.,* 2006). AMOVA performed on localitie' sets separated by rivers did not bear significant apportionment of the genetic diversity for this species and the authors argued that the lack of genetic structure reflects the imprinting of historical population process rather than current gene flow.

Considering the RBH, dispersal (over water transfer) must be the main event among those suggested by Patton *et al.,* (2000), especially for a young genus as *Ctenomys*. Probably main rivers already existed when tuco-tucos species colonized theirs actual geographic range and there was Pleistocenic and Holocenic periods under arid climate conditions, in which the discharge of the most important rivers could have been reached several times less than the present (Latrubesse *et al.,* 2005; Oliveira *et al.,* 2005), making it possible these subterranean rodents "to cross" the river. Additionally, the Secondary Contact hypothesis for riverine divergence (Patton *et al.,* 2000) may be the case, for example, for tuco-tuco species habiting coastal regions. When partially affected by sea level transgressive-regressive events, coastal populations could had took refuge in inland areas and then re-colonized shoreline, as it was shown for *C. rionegrensis* (Wlasiuk *et al.,* 2003; Kittlein & Gaggiotti,

2008). If the re-colonized area is dissected by rivers, they became an imposing barrier, as must be the scenery for *C. talarum* (Mora *et al.,* 2007) and *C. minutus* (Lopes, 2011).

An interesting example of Primary Diversification caused by river (vicariant effect) could be the São Gonçalo Channel, given its recent and known formation at about 2,600 years (Jost *et al.,* 1975). Southern populations of the collared tuco-tuco, mainly RG2, TA1 and TA2, should be established during the last 5,000 yr on this region, when the most recent barrier-lagoon system of RS coastal plain (Barrier IV) was formed, at the final stages of the Post-Glacial Marine transgression (Tomazelli *et al.,* 2000). The Bayesian skyline and skyride plot results for populations of south bank of the São Gonçalo Channel corroborate this estimative of occupation (Figs 4b & 4c) by showing an event of demographic expansion for the last 5,000 yr. Then, the São Gonçalo Channel formation probably was posterior to the collared tuco-tuco colonization of that area. Nevertheless, in spite of the fixed 2n = 46 karyotypic form, it has not passed enough time since its formation, to the São Gonçalo Channel assigns a genetic structure pattern between populations on north and south banks (AMOVA São Gonçalo (I) Appendix S4; GLS plots Fig. 6). Instead, the AMOVA São Gonçalo II (Appendix S4) for mtDNA and GLS plots for both molecular markers point out a more deep divergence between sites RG1 and RG2, faraway 20 km each other; whilst RG2 is over 55 km distant from TA1 and TA2 but genetic differentiation is not too high among them, mainly for mtDNA data (Fig. 6a). Landscape characteristics as soil and wetlands may play an important role on such differentiation and should be further investigated, but it seems to be an effect of the recent occupation of the RG2, TA1 and TA2 populations on the last sandy dunes system formed in the southern Brazil coast. Distinct geological formation of this region has played an important role on the phylogeographical structure for *C. minutus* (Lopes, 2011).

According to Behling *et al.,* (2005), southern Brazil grasslands were exposed to dry and too cold climate during the full and late glacial periods (about 20 to 10 thousand years BP), a condition of shallow waters and no formations of gallery forests along rivers, detected by the pollen core analysis. Such permissive characteristic of the streams easily would allow tuco-tucos to crossed great rivers as Jacuí and especially the Ibicuí River on the neighborhood of the sediment core studied by Behling *et al.,* (2005). Subtropical gallery forest only had developed during the last 5 thousand years BP, reflecting a change to wetter conditions, and expanded after 1500 years BP.

The *C. torquatus* establishment at westernmost end of the RS state suggests an event of crossing Ibicuí River, as indicated by sharing of haplotype (H33) among sites of both banks of the river. Although site IT2 has the differentiated haplotype H37, which causes the high divergence peak in the GLS plot (Fig. 6c), this haplotype has a common origin with H33 from UR1, and specimens from IT2 shared the chromosomic mutation 2n = 40 with IT1, both sites in the Ibicuí north bank (Fig. 1). Therefore, these information support the scenery of an ancestral lineage coming from the central region of the RS State (the probable origin of the collared tuco-tuco expansion), colonizing the south bank of the Ibicuí River and fixing the new karyomorph 2n = 40 after an overwater transference to north bank. Microsatellite data, which account for a more recent population relationship, showed the higher genetic divergence between sites IT1 and UR1 (Fig. 6d), indicating the going action of the Ibicuí River as barrier.

Despite the presence of sandy soil around all extension of the Ibicuí River, we only found *C. torquatus* populations on its south bank (DA, CQ, CAT, CAV, UR1 and UR2 sites), but IT1 and IT2 next its outfall on Uruguay River (Fig. 1). Nevertheless, our field work allowed the discovery of a new species on the Manoel Viana municipality on the north bank of the Ibicuí River (Freitas *et al.,* in prep), in allopatry with *C. torquatus* populations of Alegrete municipality (CAV, CAT and ALE). Phylogenetic and phylogeographical relationships between these two species are been investigated (Freitas *et al.,* in prep; Roratto *et al.,* in prep).

**The strength of river as barrier**

Based on the Wallace's assumption regards the strength of a river as barrier detaching distinct species community, we adapt this model to the intraspecific level, surveying the strength of a river as barrier through phylogeography of the collared tuco-tuco. The wide range of this specie along the banks of Vacacaí and Jacuí Rivers would allow such approach; despite we did not find localities on both banks of the upper-middle point (Santa Maria localities).

In general, we observed higher genetic similarity between localities on headwater point (SG1 and SG2) in comparison with all the other sampled points where the river is broader, as it is expected following the RBH. Sites SG1 and SG2 appeared as a unique cluster in the assignment analyses, they had low genetic differentiation ($F_{ST}$, GLS plots) and shared the haplotype H26.

The higher genetic differentiation expected, nevertheless, was not found for the outfall points GC – cBU. Instead, high $F_{ST}$ values, diverging peaks on GLS plots and no haplotype sharing were observed for the middle point. For the outfall sites, where the Jacuí river is wider, there was sharing of the haplotype H10 among GC, BU and ML2 localities and the $F_{ST}$ pairwise GC – cBU for microsatellite and mtDNA data were lower than the those between CS1 – cCS and SM1 – SM2 (see Table 5).

Additionally, none AMOVA results for mtDNA data considering the Jacuí River banks provided significant genetic partition explained by groups (groups II, III and IV, Appendix S4). However, the suggested group I, considering all sites along the Vacacaí-Jacuí Rivers, held on high and significant percentage of variation (group I, Appendix S4). It demonstrates that the phylogeographic structure is geographically perpendicular to the river, not on opposite banks of them. The same was observed for the Echimyid rodents of the Amazon Juruá River. Silva and Patton (1993) demonstrated that related haplotypes are patterned in a linear replacement from the headwaters to mouth, not along opposite banks as would be expected if the river itself served as a primary isolation barrier.

According to the recent history of population expansion that our results suggested for *C. torquatus*, we would expect that the colonization from the *Depressão Central* of the RS State would result in a migration along the Vacacaí and Jacuí Rivers. The Brazilian Meridional and *Escudo Rio Grandense* Plateaus would not allow another course (Fig. 1). We are not able to distinguish whether the CS1 and cCS populations independently colonized the opposite banks of the Jacuí River (Secondary Contact) or there were an event of crossing the river on the low-middle point. However, considering the lower genetic differentiation and the H10 haplotype sharing among sites of the outfall, dispersion over the Jacuí River outfall (cBU and GC) seems more probable. It could also be explained by the occurrence of a shifting of the mouth, allowing isolation of populations on opposite banks. Changes of the Jacuí course on its outfall could have happened due the marine transgression-regression during the Quaternary, as this River flows into the Guaíba and Patos Lagoons, both belonging to the coastal system of lagoons widely smitten by this sea level changes (Villwock & Tomazelli, 1995).

Therefore, except by comparison of the headwater site (narrow wide river associated with low genetic differentiation between SG1 and SG2), with all other sites where the river are wider, we were not able to demonstrated the strength of the river as barrier rising the genetic differentiation between opposite banks from the headwater to the outfall, for *C. torquatus*. Instead, we showed that rivers may

had been permeable to the collared tuco-tucos crossed them along their recent colonization process, although nowadays the Jacuí River acts as an important agent restrain gene flow.

**Conclusion remarks**

The riverine barrier hypothesis was born on the Amazon through Wallace's work. Despite recognized as harbouring the greatest biodiversity on Earth, phylogeographic information is unsatisfactorily assayed for this and other biomes of the Southern Hemisphere, as highlighted by Beheregaray (2008). The influence of global climate fluctuations on range shifts, extinctions, and speciation are well known for Northern Hemisphere biotas and such knowledge is increasing for some regions of the South Hemisphere. Despite the high biodiversity found in South American dry and grasslands habitats and the increasing knowledge of geological and climatic events, they have been much less studied than rainforests regions (Almeida *et al.,* 2007; Speranza *et al.,* 2007, but see Poljak *et al.,* 2010 and Chan & Hadly, 2011 as Argentinean Pampa examples). This study represents the first wide phylogeographical investigation of a mammal species endemic to Pampa biome.

A perspective about the timescale of interest of phylogeographic studies allow make inferences not only regards the polymorphism distributions, which is open to multiple interpretation. As shown in the present work, account for the timescale and merge in geological knowledge greatly make wealthy inferences on the past and current role of rivers as barriers, especially because it is well known that drainage patterns are dynamic and change over evolutionary time. *Ctenomys torquatus* is one of the most widely distributed among the genus *Ctenomys*, with a geographical range permeated by rivers. Our phylogeographic data suggest that this species expanded into its current geographic range at a relatively recent time in the past from the center of the Rio Grande do Sul State, the *Depressão Central*, throw the west and the southern Brazil and northern Uruguay, with the genetic signature of expansion still evident.

In general, the present work dismissed the riverine hypothesis barrier (at species level) as the primary cause of diversification for *C. torquatus* lineages and also do not completely noticed the strength of the river as barrier. However, we demonstrated the importance of telling the history about the riverine role in a timescale framework and, as argued by Colwell (2000) on Amazon Rivers, we let our contribution to refine the question or, in the case of the poorly assayed Pampa Biome, we open the discussion.

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**REFERENCES**

Almeida, F.C., Bonvicino, C.R. & Cordeiro-Estrela, P. (2007) Phylogeny and temporal diversification of the genus *Calomys* (Rodentia: Sigmodontinae): implications for the biogeography of open/dry biomes of South America. *Molecular Phylogenetics and Evolution*, **42**, 449-466.

Anderson, R.P., Gómez-Laverde, M. & Peterson, A.T. (2002) Geographical distributions of spiny pocket mice in South America: insights from predictive models. *Global Ecology and Biogeography*, **11**, 131–141.

Ayres, J.M. & Clutton-Brock, T.H. (1992) River boundaries and species range size in Amazonian primates. *American Naturalist*, **140**, 531-537.

Bandelt, H.J., Forster, P. & Rohl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**,37-48.

Barlow, J. C. (1969) Observations on the biology of rodents in Uruguay. *Life science contributions of Royal Ontario Museum*, **75**, 1-60.

Beheregaray, L. (2008) Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology,* **17**, 3754-3774.

Behling, H. (2002) South and southeast Brazilian grasslands during Late Quaternary times: a synthesis. *Palaeogeography, Palaeoclimatology and Palaeoecology,* **177**, 19-27

Behling, H., Pillar, V.D.P. & Bauermann, S.G. (2005) Late Quaternary grassland (Campos), gallery forest, fire and climate dynamics, studied by pollen, charcoal and multivariate analysis of the São Francisco de Assis core in western Rio Grande do Sul (southern Brazil). Review *of Palaeobotany and Palynology*, **133**, 235-248.

Busch, C., Antinuchi, C.D., del Valle, J.C., Kittlein, M.J., Malizia, A.I., Vassallo, A.I. & Zenuto, R.R. (2000) Population ecology of subterranean rodents. *Life Underground, the biology of subterranean*

*rodents* (ed. by E.A. Lacey, J.L. Patton and G.N. Cameron), pp. 181-226. The University of Chicago Press, Chicago.

Cabrera, A.L. & Willink, A. (1980) *Biogeografia de America Latina*, 2nd edn. OEA, Washington.

Chan, Y.L. & Hadly, E.A. (2011) Genetic variation over 10 000 years in *Ctenomys*: comparative phylochronology provides a temporal perspective on rarity, environmental change and demography. *Molecular Ecology*, doi, 10.1111/j.1365-294X.2011.05295.x

Castillo, A.H., Cortinas, M.N. & Lessa, E.P. (2005) Rapid diversification of South American tuco-tucos (*Ctenomys*; Rodentia; Ctenomyidae): contrasting mitochondrial and nuclear intron sequences. *Journal of Mammalogy*, **86**, 170-179.

Colwell, R.K. (2000) A barrier runs through it… or maybe just a river. *Proceedings of the National Academy of Science USA*, **97**, 13470-13472.

Contreras, J.R. & Bidau, C.J. (1999) Líneas generales del panorama evolutivo de los roedores excavadores sudamericanos del gênero *Ctenomys* (Mammalia, Rodentia, Caviomorpha, Ctenomyidae). *Ciencia Siglo XXI*, **1**, 1–22.

Cutrera, A.P., Lacey, E.A. & Bush, C. (2005) Genetic structure in a solitary rodent (*Ctenomys talarum*): implications for kinship and dispersal. *Molecular Ecology,* **14**, 2511-2523.

D'Annato, A. & D'Elia, G. (2011) Incongruent patterns of morphological, molecular, and karyotypic variation among populations of *Ctenomys pearsoni* Lessa & Langguth, 1983 (Rodentia, Ctenomyidae). *Mammalian Biology*, **76**, 36–40

D'Elia, G., Lessa, E.P. & Cook, J.A. (1999) Molecular phylogeny of tuco-tucos, genus *Ctenomys* (Rodentia, Octodontidae): Evaluation of the mendocinus species group and the evolution of asymmetric sperm. *Journal of Mammalian Evolution*, **6**, 19–38.

Dinerstein, E., Olson, D.M., Graham, D.J., Webster, A.L., Primm, S.A., Bookbinder, M.P. & Ledec, G. (1995) *A conservation assessment of the terrestrial ecoregions of Latin America and the Caribbean*. The World Bank, Washington.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Drummond, A.J., Rambaut, A., Shapiro, B. & Pybus, O.G . (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular and Biological Evolution*, **22**, 1185-1192

Excoffier, L. & Schneider, S. (2005) Arlequin version 3.0: an integrated software package for Population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50

Excoffier, L., Laval, G. & Schneider, S. (2006) Arlequin 3.1 Manual: an integrated software package for Population genetics data analysis. http://cmpg.unibe.ch/software/arlequin3.

Fernandes, F.A. (2008) *Filogeografia de* Ctenomys torquatus *(Rodentia: Ctenomyidae)*. Ph.D Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Fernandes, F.A., Gonçalves, G.L., Ximenes, S.S.F. & Freitas, T.R.O. (2009a) Karyotypic and molecular polymorphisms in the *Ctenomys torquatus* (Rodentia: Ctenomyidae): taxonomic considerations. *Genetica*, **136**, 449-459.

Fernandes, F.A., Fornel, F., Cordeiro-Estrela, P. & Freitas, T.R.O. (2009b) Intra- and interespecific skull variation in two sister species of the subterranean rodent genus *Ctenomys* (Rodentia, Ctenomyidae): coupling geometric morphometrics and chromosomal polymorphism. *Zoological Journal of the Linnean Society*, **155**, 220-237.

Fernández-Stolz, G. (2006) *Estudos evolutivos, filogeográficos e de conservação em uma espécie endêmica do ecossistema de dunas costeiras do sul do Brasil,* Ctenomys flamarioni *(Rodentia - Ctenomyidae), através de marcadores moleculares microssatélites e DNA mitocondrial.* Ph.D Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Fernández-Stolz, G., Stolz, J.F.B. & Freitas, T.R.O. (2007) Bottlenecks and Dispersal in the Tuco-tuco das Dunas, *Ctenomys flamarioni* (RODENTIA: CTENOMYIDAE), in Southern Brazil. *Journal of Mammalogy*, **88**,935-945.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294-297.

Ford, C.E. & Hamerton, J.L. (1956) A colchicines, hypotonic citrate, squash sequence for mammalian chromosomes. *Stain Technology*, **31**, 247-251.

Freitas, T.R.O. (1995) Geographic distribution and conservation of four species of the genus *Ctenomys* in southern Brazil. *Studies on Neotropical Fauna and Environment*, **30**, 53-59.

Freitas, T.R.O. (2006) Cytogenetics status of four *Ctenomys* species in the south of Brazil. *Genetica*, **126**,227-235.

Freitas, T.R.O. & Lessa, E.P. (1984) Cytogenetics and morphology of *Ctenomys torquatus* (Rodentia - Octodontidae). *Journal of Mammalogy*, **65**, 637-642.

Fu, Y.X. (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**,915–925.

García, L., Ponsà, M., Egozcue, J., García, M. (2000a) Cytogenetic variation in *Ctenomys perrensi* (Rodentia, Octodontidae). *Biological Journal of the Linnean Society,* **71**, 615–624.

García, L., Ponsà, M., Egozcue, J. & García, M. (2000b) Comparative chromosomal analysis and phylogeny in four *Ctenomys* species (Rodentia,Ocotodontidae). *Biological Journal of the Linnean Society*, **69**, 103–120.

Gascon, C., Malcolm, J., Patton, J.L., Silva, M.N.F., Bogart, J.P., Lougheed, S.C., Peres, C.A., Neckel, S. and Boag, P.T. (2000) Riverine barriers and the geographic distribution of Amazonian species. *Proceedings of the National Academy of Science USA*, **97**, 13672-13677.

Gava, A. & Freitas, T. R. O. (2003) Inter and intra-specific hybridization in tuco-tucos (*Ctenomys*) from Brazilian coastal plains (Rodentia: Ctenomyidae). *Genetica*, **119**, 11–17.

Giménez, M.D., Mirol, P.M., Bidau, C.J., Searle, J.B. (2002) Molecular analysis of populations of *Ctenomys* (Caviomorpha, Rodentia) with high karyotypic variability. *Cytogenetic and Genome Research,* **96**, 130–136.

Glaubitz, J.C. (2004) CONVERT: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. *Molecular Ecology Notes*, **4**, 309-310.

Gonçalves, G.L. & Freitas, T.R.O. (2009) Intraspecific variation and genetic differentiation of the collared tuco-tuco (*Ctenomys torquatus*) in sourthern Brazil. *Journal of Mammalogy,* **90**, 1020-1031.

Grela, I. (2004) *Geografía florística de especies arbóreas de Uruguai: propuesta para la delimitación de dendroflora*. M.Sc. Thesis, Universidad de la República, Montevideo.

Guillot, G., Mortier, F. & Estoup, A. (2005) GENELAND: a computer package for landscape genetics. *Molecular Ecology Notes*, **5**, 712–715.

Ho, S.Y.W. & Shapiro, B. (2011) Skyline-plot methods for estimating demographic history from nucleotide sequences. *Molecular Ecology Resources*, **11**, 423-434.

Instituto Brasileiro de Geografia e Estatıstica – IBGE (2004). Brazilian maps of vegetation and biomes. http://www.ibge.gov.br. Accessed 18 November 2011

Jost, H., Soliani, E. & Godalphin, M.F. (1975) Evolução Paleogeográfica da região da Lagoa Mirim, RS, SE do Brasil. *Actas del Congresso Argentino de Paleontología y Bioestratigrafía, Tucumán*, **2**, 575-587.

Kittlein, M.J. & Gaggiotti, O. (2008) Interactions between environmental factors can preclude the detection of isolation by distance patterns: A case study of *Ctenomys rionegrensis* in Uruguay. *Proceedings of the Royal Society of London B*, **275**, 2633-2638.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F. & Wilson, A.C. (1989) Dynamics of the mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science USA*, **89**, 6196-6200.

Lacey, E. A. (2001) Microsatellite variation in solitary and social tuco-tucos: molecular properties and population dynamics. *Heredity*, **86**, 628–637.

Lacey, E. A., Maldonado, J.E. & Clabaugh, J. P. (1999) Interspecific variation in microsatellites isolated from tuco-tucos (Rodentia: Ctenomyidae). *Molecular Ecology*, **8**, 1753–1768.

Lacey, E.A., Patton, J.L. & Cameron, G.N. (2000) *Life underground: the biology of subterranean rodents*. The University of Chicago Press, Chicago.

Latrubesse, E.M., Stevaux, J.C., Santos, M.L. & Assine, M.L. (2005) Grandes sistemas fluviais: geologia, geomorfológica e paleoidrologia. *Quaternário do Brasil* (ed. by C.R.G. Souza, K. Suguio, A.M.S. Oliveira and P.E. Oliveira), pp. 276-297. Holos Editora, Ribeirão Preto.

Lessa, E.P. & Cook, J.A. (1998) The molecular phylogenetics of tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae) suggest an early burst of speciation. *Molecular Phylogenetics and Evolution*, **9**, 88-99.

Librado, P. & Rozas, J. (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451-1452.

Lichtenstein, H. (1830) *Darstellung neuer order wenig bekannter Saügethiere in Abbildung und Beschreibungen*. Luderitz Edit, Berlin.

Lizarralde, M., Deferrari, G., Alvarez, S. & Escobar, J. (2001) Evolutionary differentation in *Ctenomys magellanius*: allozymic, morphological variation and biogeographic considerations of 2 chromosomal forms. *Interciencia*, **26**, 13-17.

Lopes, C.M. (2011) *História evolutiva de* Ctenomys minutus *e* Ctenomys lami *na planície costeira do Sul do Brasil*. Ph.D Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Lopes, C.M. & Freitas, T.R.O. (2012) Naturally patched small populations in human-impacted areas: genetic structure and conservation of tuco-tuco (*Ctenomys lami*). *Journal of Heredity*, in press.

Mapelli, F.J. & Kittlein, M.J. (2009) Influence of patch and landscape characteristics on the distribution of the subterranean rodent *Ctenomys porteousi*. *Landscape Ecology*, **24***,* 723-733.

Mapelli, J.F., Mora, M.S., Mirol, P.M. & Kittlein, M.J. (2012) Effects of Quaternary climatic changes on the phylogeography and historical demography of the subterranean rodent *Ctenomys porteousi*. *Journal of Zoology*, **286**, 48-57.

Medrano, J.F., Aesen, E. & Sharrow, L. (1990) DNA extraction from nucleated red blood cells. *Biotechniques*, **8**, 43.

Miller, M.P. (2005) Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. *Journal of Heredity*, **96**, 722–724.

Miller, M.P., Bellinger, M.R., Forsman, E.D. & Haig, S.M. (2006) Effects of historical climate change, habitat connectivity, and vicariance on genetic structure and diversity across the range of the red tree vole (*Phenacomys longicaudus*) in the Pacific Northwestern United States. *Molecular Ecology*, **15**, 145–159.

Minin, V.N., Bloomquist, E.W. & Suchard, M.A. (2008) Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. *Molecular Biology and Evolution*, **25**, 1459-1471.

Mirol, P., Giménez, M.D., Searle, J.B., Bidau, C.J. & Faulkes, C.G. (2010) Population and species bounderies in the South America subterranean rodent *Ctenomys* in a dynamic environment. *Biological Journal of the Linnean Society*, **100**, 368-383.

Mora, M., Lessa, E.P., Kittlein, M.J. & Vassalo, A.I. (2006) Phylogeography of the subterranean rodent *Ctenomys australis* (Rodentia: Ctenomyidae) in sand-dune habitats: evidence of recent population expansion. *Journal of Mammalogy*, **87**,1192–1203.

Mora, M., Lessa, E.P., Cutrera, A.P., Kittlein, M.J. & Vassalo, A.I. (2007) Phylogeographical structure in the subterranean tuco-tuco *Ctenomys talarum* (Rodentia: Ctenomyidae): contrasting the demographic consequences of regional and habitat-specific histories. *Molecular Ecology*, **16**, 3453-3465.

Mora, M.S., Mapelli, F.J., Gaggiotti, O.E., Kittlein, M.J. & Lessa, E.P. (2010) Dispersal and population structure at different spatial scales in the subterranean rodent *Ctenomys australis*. *BMC Genetics*, **11**, 9.

Nei, M. (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei, M., Tajima, F. & Tateno, Y. (1983) Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. *Journal of Molecular Evolution*, **19**, 153-170.

Neigel, J.E., Ball Jr, R.M. & Avise, J.C. (1991) Estimation of single generation dispersal migration distances from geographic variation in animal mitochondrial DMA. *Evolution*, **45**, 423-432.

Oliveira, P.E., Behling, H., Ledru, M. P., Barberi, M., Bush, M., Salgado-Labouriau, M. L., Garcia, M. J., Medeanic, S., Barth, O. M., Barros, M. A. & Scheel-Ybert, R. (2005) Paleovegetação e paleoclimas do Quaternário do Brasil. *Quaternário do Brasil* (Ed. by C.R.G. Souza, K. Suguio, A.M.S. Oliveira and P.E. Oliveira), pp. 52-74. Holos Editora, Ribeirão Preto.

Ortells, M.O. (1995) Phylogenetic analysis of G-banded karyotypes among the South American subterranean rodents of the genus *Ctenomys* (Caviomorpha: Octodontidae), with special reference to chromosomal evolution and speciation. *Biological Journal of the Linnean Society*, **54**, 43–70.

Ortells, M.O., Contreras, J.R. & Reig, O.A. (1990) New *Ctenomys* karyotypes (Rodentia, Octodontidae) from north-eastern Argentina and from Paraguay confirm the extreme chromosomal multiformity of the genus. *Genetica*, **82**, 189–201.

Overbeck, G.E., Müller, S.C., Fidelis, A., Pfadenhauer, J., Pillar, V.D., Blanco, C.C., Boldrini, I.I., Both, R. & Forneck, E.D. (2007). Brazil's neglected biome: The South Brazilian *Campos*. *Perspectives in Plant Ecology, Evolution and Systematics*, **9**, 101-116.

Parada, A., D'Elía, G., Bidau, C.J. & Lessa, E.P. (2011) Species groups and the evolutionary diversification of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). *Journal of Mammalogy*, **92**, 671-682.

Patton, J.L., Silva, M.N. & Malcolm, J.R. (2000) Mammals of the Rio Juruá and the evolutionary and ecological diversification of Amazonia. *Bulletin of the American Museum of the National History*, **244**, 1-306.

Patton, J.L. & Smith, M.F. (1990) Evolutionary dynamics of the pocket gopher *Thomomys bottae*, with anphasis on California populations. *University of California Publications in Zoology*, **123**, 1-161.

Peres, C.A., Patton, J.L. & da Silva, M.N.F. (1996) Riverine barriers and gene flow in Amazonian saddle-back tamarins. *Folia Primatologica*, **67**, 113–124.

Poljak, S., Confalonieri, V., Fasanella, M., Gabrielli, M. & Lizarralde, M.S. (2010) Phylogeography of the armadillo Chaetophractus villosus (Dasypodidae Xenarthra): Post-glacial range expansion from Pampas to Patagonia (Argentina). *Molecular Phylogenetics and Evolution*, **55**, 38–46

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Quintana, C.A. (1994) Ctenominos primitivos (Rodentia, Octodontidae) del Mioceno de la Provincia de Buenos Aires, Argentina. *Boletin de la Real Sociedad Espanola de Historia Natural - Seccion Geologica*, **89**, 19-23.

Rambo, B. (2000) A *fisionomia do Rio Grande do Sul: ensaio de monografia natural*. Editora Unisinos, São Leopoldo.

Reig, O.A., Busch, C., Ortells, M.O. & Contreras, J.R. (1990) An overview of evolution, systematic, population biology and speciation in *Ctenomys*. *Evolution of subterranean mammals at the organismal and molecular levels* (ed. by E. Nevo E and O.A. Reig), pp. 71-96. Wiley-Liss, New York.

Rice, W.R. (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Rogers, A.R., Harpending, H. (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**, 552-569.

Rosenberg, N.A. (2004) Distruct: a program for the graphical display of population structure. *Molecular Ecology Notes*, **4**, 137-138.

Roratto, P.A., Bartholomei-Santos, M.L. & Freitas, T.R.O. (2011) Tetranucleotide microsatellite markers in *Ctenomys torquatus* (Rodentia). *Conservation Genetics Resource*, **3**, 725-727.

Shapiro, B., Rambaut, A. & Drummond, A.J. (2006) Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. *Molecular Biology and Evolution*, **23**, 7-9.

Sikes, R.S., Gannon, W.L. & the Animal Care and Use Committee of the American Society of Mammalogists (2011) Guidelines of the American Society of Mammalogists for the use of wild mammals in research. *Journal of Mammalogy,* **92**, 235–253.

Silva, J., Freitas, T.R.O., Heuser, V.D., Marinho, J.R., Bittencourt, F., Cerski, C.T.S., Klassmann, L.S. & Erdtmann, B. (2000a) Chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated with multiple methods and tissues. *Mutation Research*, **470**, 39-51.

Silva, J., Freitas, T.R.O., Heuser, V.D., Marinho, J.R. & Erdtmann, B. (2000b) Genotoxicity biomonitoring in coal regions using wild rodent *Ctenomys torquatus* by comet assay and micronucleus test. *Environmental and Molecular Mutagenesis*, **35**, 270-278.

Silva, N.F. & Patton, J.L. (1993) Amazonian Phylogeography: mtDNA sequences variation in arboreal echimyid rodents (Caviomorpha). *Molecular Phylogenetics and Evolution*, **2**, 243-255.

Speranza, P.R., Seijo, J.G., Grela, I.A. & Solís-Neffa, V.G. (2007) Chloroplast DNA variation in the *Turnera sidoides* L. complex (Turneraceae): biogeographical implications. *Journal of Biogeography*, **34**, 427–436.

Souza, C.R.G., Suguio, K., Oliveira, A.M.S. & Oliveira, P.E. (2005) *Quaternário do Brasil*. Holos Editora, Ribeirão Preto.

Tajima, F. (1989) Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. *Genetics*, **123**,585-595

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, **28**, 2731-2739

Tomasco, I.H. & Lessa, E.P. (2007) Phylogeography of the Tuco-tuco *Ctenomys pearsoni*: mtDNA variation and its implication for chromosomal differentiation. *The quintessential naturalist. Honoring life and legacy of Oliver P. Pearson* (ed. by D.A. Kelt, E.P. Lessa, J. Salazar-Bravo and J.L. Patton), pp. 859-882. University of California Publications, Berkeley.

Tomazelli, L.J., Dillenburg, S.R. & Villwock, J. A. (2000) Late quaternary geological history of Rio Grande do Sul coastal plain, southern Brazil. *Revista Brasileira de Geocienciências*, **30**, 474-476.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Verzi, D.H. (2002) Patrones de evolución morfológica em Ctenomyinae (Rodentia, Octodontidae). *Mastozoología Neotropical*, **9**, 309-328

Verzi, D.H. (2008) Phylogeny and adaptive diversity of rodents of the family Ctenomyidae (Caviomorpha): delimiting lineages and genera in the fossil record. *Journal of Zoology*, **274**, 386-394

Verzi, D.H., Olivares, A.I. & Morgan, C.C. (2010) The oldest South American tuco-tuco (late Pliocene, northwestern Argentina) and the boundaries of genus *Ctenomys* (Rodentia, Ctenomyidae). *Mammalian Biology*, **75**, 243-252.

Villwock, J.A. & Tomazelli, L.J. (1995) Geologia costeira do Rio Grande do Sul. *Notas Técnicas do Centro de Estudos de Geologia Costeira e Oceânica*, **8**, 1-45.

Vucetich, M.G., Verzi, D.H. & Hartenberger, J.L. (1999) Review and analysis of the radiation of the South American Hystricognathi (Mammaha, Rodentia). *Paleontology*, **329**, 763-769

Wallace, A.R. (1852) On the monkeys of the Amazon. *Proceedings of the Zoological Society of London*, **20**, 107-10.

Weir, B.S. & Cockerhan, C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358-1370

Wyss, A.R., Flynn, J.J., Norell, M.A., Swisher, C.C., Charrier, R., Novacek, M.J. & McKenna, M.C. (1993) South America's earliest rodent and recognition of a new interval of mammalian evolution. *Nature*, **365**, 434-437

Wlasiuk, G., Garza, J.C. & Lessa, E.P. (2003) Genetic and geographic differentiation in the Rio Negro tuco-tuco (*Ctenomys rionegrensis*): inferring the roles of migration and drift from multiple genetic markers. *Evolution*, **57**, 913-926.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Collections sites, karyotype number, geographic coordinate and summary of genetic variability of the mitochondrial and microsatellite markers for *Ctenomys torquatus*.

**Appendix S2** Species and Genbank accession numbers of cytochrome *b* sequences used in the phylogenetic and molecular clock analysis.

**Appendix S3** Specimens and Genbank accession numbers of cytochrome *b*, cytochrome *c* oxidase I and hiper variable sequence 1 (d-loop) sequences used in molecular clock analysis.

**Appendix S4** AMOVAs results.

**Appendix S5** Distribution of each haplotype by localities and the GenBank accession numbers for COI and HVS of mitochondrial DNA of *Ctenomys torquatus*.

**Appendix S6** Mismatch distribution graphs for cytochrome *c* oxidase I and hiper variable sequence 1 (d-loop) data sets.

**Appendix S7** Pairwise $F_{ST}$ results for concatenate COI+HVS and microsatellite data for all localities.

**Appendix S8** Bayesian time-calibrated tree of the cytochrome *b* gene from ctenomyids and other caviomorph species.

**TABLES**

**Table 1** Priors used as calibration points for the cytochrome *b* phylogeny and the estimated time to most recent common ancestor (tMRCA) for some nodes. M is Mean for normal distribution and Median for lognormal distribution; 95% HPD (high probability density). All values are expressed in millions of years (my) ago.

| Node | Priors for calibration points | | tMRCA |
|---|---|---|---|
| | Distribution | M (95% HPD) my | Mean (95% HPD) my |
| Caviomorpha origin | Normal | 34 (31 – 37) | 30.90 (27.26 – 34.48) |
| Octodontidae/Ctenomyidae | Normal | 10.65 (9.8 – 11.5) | 11.17 (10.20 – 12.13) |
| Ctenomyidae origin | Lognormal | 5 (3.3 – 7.5) | 5.64 (4.38 – 6.98) |
| *Ctenomys torquatus* | | | 0.51 (0.27 – 0.80) |

**Table 2** Genetic diversity estimatives for cytochrome *c* oxidase I (COI), hyper variable sequence 1 (HVS) markers and for the concatenated data set (COI+HVS). Number of segregating sites (S), nucleotide diversity (π), number of haplotypes (H), haplotype diversity (*Hd*), Tajima's *D* and Fu's $F_S$ neutrality tests and graphic distribution of the mismatch analysis.

| Data set | S | π | H | Hd | Tajima's D* | Fu's FS** | Mismatch distribution |
|---|---|---|---|---|---|---|---|
| HVS | 18 | 0.0062 | 25 | 0.91 | NS | -9.26 | Unimodal |
| COI | 30 | 0.0033 | 24 | 0.82 | -1.51 | -9.43 | Unimodal |
| COI+HVS | 48 | 0.0044 | 40 | 0.95 | NS | -13.7 | Unimodal |

*Significant values for P < 0.05; ** Significant values for P < 0.02; NS non significant value

**Table 3** Analysis of molecular variance for concatenated mtDNA (COI+HVS) data considering the geographic regions of the *Ctenomys torquatus* range and the four distinct karyomorphs (see Appendix S1). All F's statistic values were significant (P < 0.0001)

| Group | S x C x E x W x F | Karyotypes |
|---|---|---|
| Among Groups | 41.99 | 35.20 |
| ($F_{CT}$) | (0.42) | (0.35) |
| Among localities | 49.44 | 57.20 |
| within groups ($F_{SC}$) | (0.85) | (0.88) |
| Within localities | 8.57 | 7.61 |
| ($F_{ST}$) | (0.91) | (0.92) |

Letters refer to the geographic region of the grouped localities, as detached in Figure 1: (S) South, (C) Central, (W) West, (E) East, (F) Frontier.

**Table 4** Genetic differentiation of localities and clusters of Ibicuí River and São Gonçalo Channel. $F_{ST}$ values of the COI+HVS mtDNA data are above diagonal and for microsatellite data are below diagonal. Locality names follow Appendix S1.

| River | | $F_{ST}$ values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ibicui | | IT1 | IT2 | UR1 | UR2 | cUR | | | |
| | IT1 | | **0.96** | 0.40 | 0.73 | 0.39 | | | |
| | IT2 | **0.52** | | **0.97** | **0.98** | **0.93** | | | |
| | UR1 | **0.43** | **0.36** | | **0.73** | - | | | |
| | UR2 | **0.52** | **0.43** | **0.10** | | - | | | |
| | cUR | **0.43** | **0.34** | - | - | | | | |
| S. Gonçalo | | PE1 | PE2 | cPE | RG1 | RG2 | TA1 | TA2 | cTA |
| Channel | PE1 | | 0.21 | **-** | **0.91** | **0.95** | **1.00** | **1.00** | - |
| | PE2 | **0.15** | | **-** | **0.78** | **0.88** | **0.81** | **0.80** | - |
| | cPE | **-** | **-** | | **0.85** | **0.91** | **-** | **-** | **0.87** |
| | RG1 | **0.31** | **0.24** | **0.23** | | **0.89** | **0.84** | **0.83** | **0.89** |
| | RG2 | **0.44** | **0.35** | **0.35** | **0.43** | | **0.92** | **0.92** | **0.95** |
| | TA1 | **0.33** | **0.27** | **-** | **0.33** | **0.44** | | 0.00 | - |
| | TA2 | **0.43** | **0.37** | **-** | **0.38** | **0.56** | **0.33** | | - |
| | cTA | **-** | **-** | **0.26** | **0.29** | **0.43** | - | - | |

$F_{ST}$ bold values were significant after Bonferroni adjustments (α = 0.00023 for microsatellites, α = 0.00009 for COI+HVS). Grey cells refer to clustered localities: cUR (UR1+UR2), cPE (PE1+PE2) and cTA (TA1+TA2).

**Table 5** Pairwise comparisons between localities and clusters of the Jacuí River. $F_{ST}$ values of mtDNA data COI+HVS are above diagonal and for microsatellite data are below diagonal. Localities names follow Appendix S1.

| | SM1 | SM2 | SG1 | SG2 | cSG | BU | ML1 | ML2 | cBU | GC | CS1 | CS2 | CS3 | cCS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SM1 | | **0.94** | **1.00** | **0.74** | **0.82** | 0.19 | 0.80 | **0.67** | 0.23 | **1.00** | 0.00 | **1.00** | **1.00** | **1.00** |
| SM2 | **0.54** | | **0.98** | **0.83** | **0.88** | **0.81** | 0.91 | **0.88** | **0.77** | **0.96** | **0.95** | **0.97** | **0.97** | **0.97** |
| SG1 | **0.51** | **0.40** | | 0.07 | **-** | **0.90** | 0.98 | **0.94** | **-** | **1.00** | **1.00** | **1.00** | **1.00** | **-** |
| SG2 | **0.47** | **0.39** | **0.13** | | **-** | **0.71** | 0.68 | **0.74** | **-** | **0.79** | **0.77** | **0.82** | **0.82** | **-** |
| cSG | **0.45** | **0.35** | **-** | **-** | | **-** | - | - | **0.79** | **0.85** | **0.84** | **-** | **-** | **0.90** |
| BU | **0.45** | **0.37** | **0.37** | **0.33** | **-** | | 0.26 | 0.23 | - | 0.37 | 0.24 | **0.78** | **0.78** | **-** |
| ML1 | 0.46 | 0.42 | 0.36 | 0.32 | **-** | **0.19** | | 0.66 | - | 0.92 | 0.85 | 0.95 | 0.95 | - |
| ML2 | **0.46** | **0.39** | **0.38** | **0.35** | **-** | **0.17** | 0.26 | | - | 0.01 | **0.71** | **0.88** | **0.88** | **-** |
| cBU | **0.39** | **0.32** | **-** | **-** | **0.28** | **-** | - | **-** | | 0.19 | 0.26 | **-** | **-** | **0.77** |
| GC | **0.54** | **0.49** | **0.45** | **0.41** | **0.39** | **0.30** | 0.47 | **0.36** | **0.28** | | **1.00** | **1.00** | **1.00** | **1.00** |
| CS1 | **0.68** | **0.63** | **0.57** | **0.57** | **0.50** | **0.55** | **0.65** | **0.56** | **0.46** | **0.57** | | **1.00** | **1.00** | **1.00** |
| CS2 | **0.53** | **0.43** | **0.37** | **0.37** | **-** | **0.30** | 0.41 | **0.35** | **-** | **0.38** | **0.57** | | 0.00 | - |
| CS3 | **0.47** | **0.43** | **0.38** | **0.35** | **-** | **0.28** | 0.36 | **0.31** | **-** | **0.36** | **0.54** | **0.18** | | **-** |
| cCS | **0.45** | **0.38** | **-** | **-** | **0.30** | **-** | - | **-** | **0.23** | **0.32** | **0.47** | **-** | **-** | |

Bold values were significant after Bonferroni adjustments (α = 0.00023 for microsatellites, α = 0.00009 for COI+HVS); Grey cells refer to clustered localities: cSG (SG1+SG1), cBU (BU+ML1+ML2) and cCS (CS2+CS3).

**FIGURE LEGENDS**

**Figure 1** Map of the Rio Grande do Sul State (Brazil) and Uruguay, indicating the geographical placement of sampling sites for *Ctenomys torquatus*. Abbreviations used are as in Appendix S1. Colors of points refer to karyotipe: pink for 2n = 40, purple for 2n = 42, black for 2n = 44 and green for 2n = 46. Gray areas represent elevations above 200 meters, named: (A) Brazilian Meridional Plateau, (B) *Escudo Sul-Riograndense*, (C) Cuchilla de Haedo and (D) Cuchilla Grande. * Departments of Uruguay where there were registers of the *C. torquatus* occurrence. Sites for AMOVA's geographic groups are surrounded by dotted lines. ✦ Candelária Municipality.

**Figure 2** Topological relationship between the COI+HVS haplotypes of collared tuco-tuco *Ctenomys torquatus.* Haplotypes are depicted according to sampled sites (localities names for code on Appendix S1 and Figure 1) and areas are proportional to frequencies, cross hatches represent mutation steps and red points represent medium vectors. Karyotypic variations are represented by vertical stripes (2n = 46), horizontal stripes (2n = 42) and dots (2n = 40).

**Figure 3** Results of spatial autocorrelation analysis of *Ctenomys torquatus* for microsatellite loci and the concatenate HVS control region and cytochrome *c* oxidase I mitochondrial sequences (mtDNA). Analyses performed using five to ten distance classes showed similar results, and then the eight distance classes' results are presented. $A_y$ quantifies the average pairwise genetic distances that fall within the boundaries specified for each distance class *y*. The horizontal line indicates the average value of $A_y$ for a data set.

**Figure 4** Bayesian demographic histories derived from concatenated COI+HVS mtDNA sequences. **(a)** Bayesian skyride plot for the total *Ctenomys torquatus* sample (*n* = 294); **(b)** Bayesian skyline and **(c)** skyride plots for the southernmost localities with karyotype 2n=46 (*n* = 41). The thin and the thick dotted lines are the lower and the median estimated tMRCA, projected on the time line, and the blue area overlay show the 95% highest posterior density (HPD) limits for the effective population size.

**Figure 5** Results of clustering analysis. (a) Posterior estimates of cluster membership for STRUCTURE. Labels above the graph are the sampling sites (see names for codes on Appendix S1). Labels below graph are clusters that grouped more than one locality. (b) Mean value of estimated logarithm of probability of the data (open circles) and the standard deviation (vertical bars) for ten STRUCTURE runs with k ranging from 1 to 30. (c) Index of MCMC iterations along the whole chain (left) and density of each number of clusters along the chain after a burning $10^5$ iterations (right), from GENELAND.

**Figure 6** Graphical interpolation-based representation of the spatial patterns of the COI+HVS mtDNA (a and c) and microsatellite (b and d) diversity for samples of the São Gonçalo Channel and Ibicuí River. X- and Y-axes represent geographic coordinates, while surface heights are proportionate to the genetic distance of the population (Z axis). Peaks (dark blue) and valleys (yellow) are indicative of areas with high or low pairwise genetic distance between individuals, respectively. The site codes on the landscape plots are given in Appendix S1 and they represent approximately the geographic localization of the sites.

**Figure 7** Graphical interpolation-based representation of the spatial patterns of the COI+HVS mtDNA (a) and microsatellite (b) diversity for samples of theVacacaí- Jacuí Rivers. X- and Y-axes represent geographic coordinates, while surface heights are proportionate to the genetic distance of the population (Z axis). Peaks (dark blue) and valleys (yellow) are indicative of areas with high or low pairwise genetic distance between individuals, respectively. The site codes on the landscape plots are given in Appendix S1 and they represent approximately the geographic localization of the sites.

**FIGURES**

Figure 1:

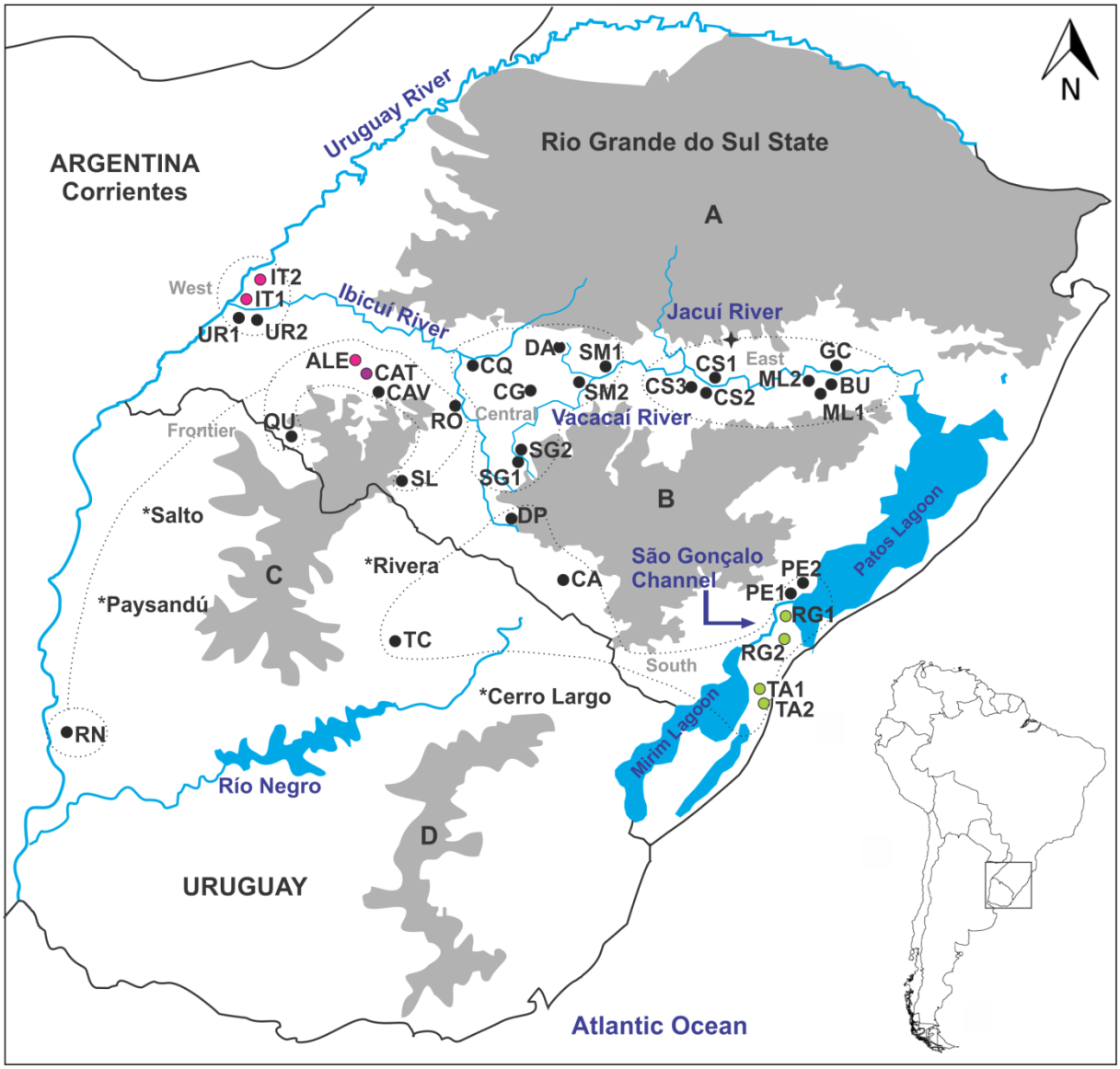


Figure 2:

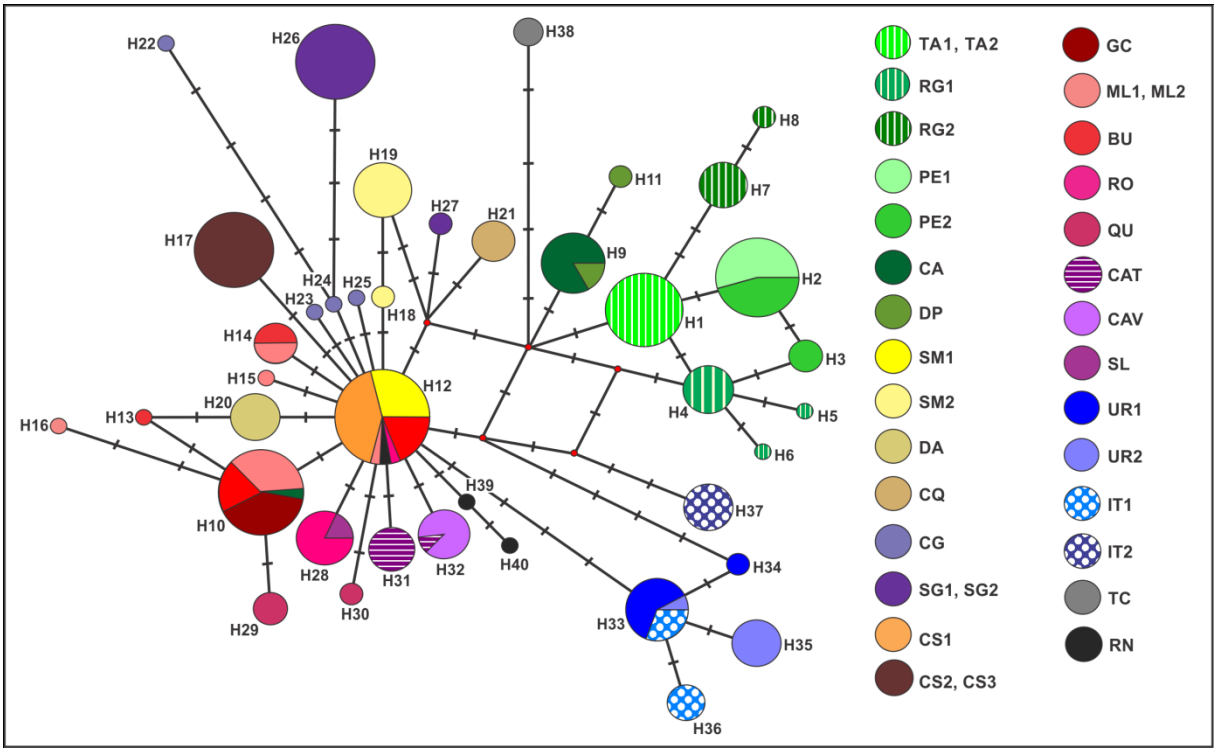



Figure 3:

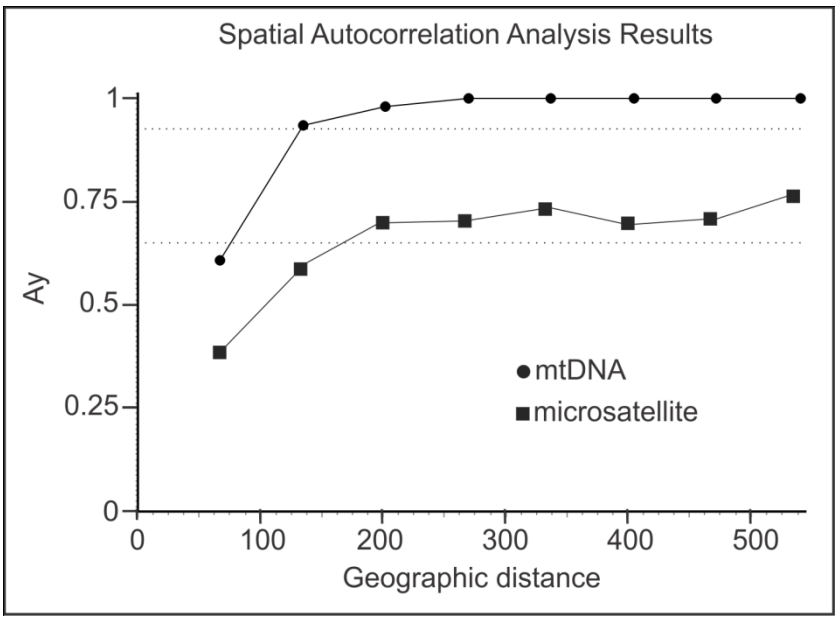


Figure 4:

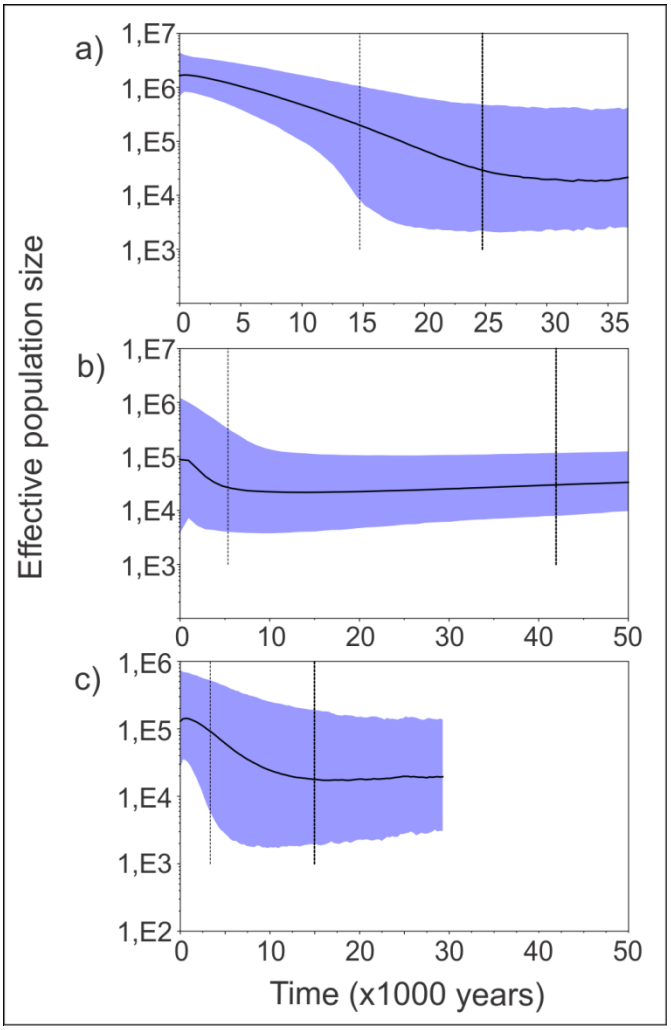

a)
b)
c)
Effective population size
1,E7
1,E6
1,E5
1,E4
1,E3
1,E2
0
5
10
15
20
25
30
35
40
50
Time (x1000 years)

Figure 5:

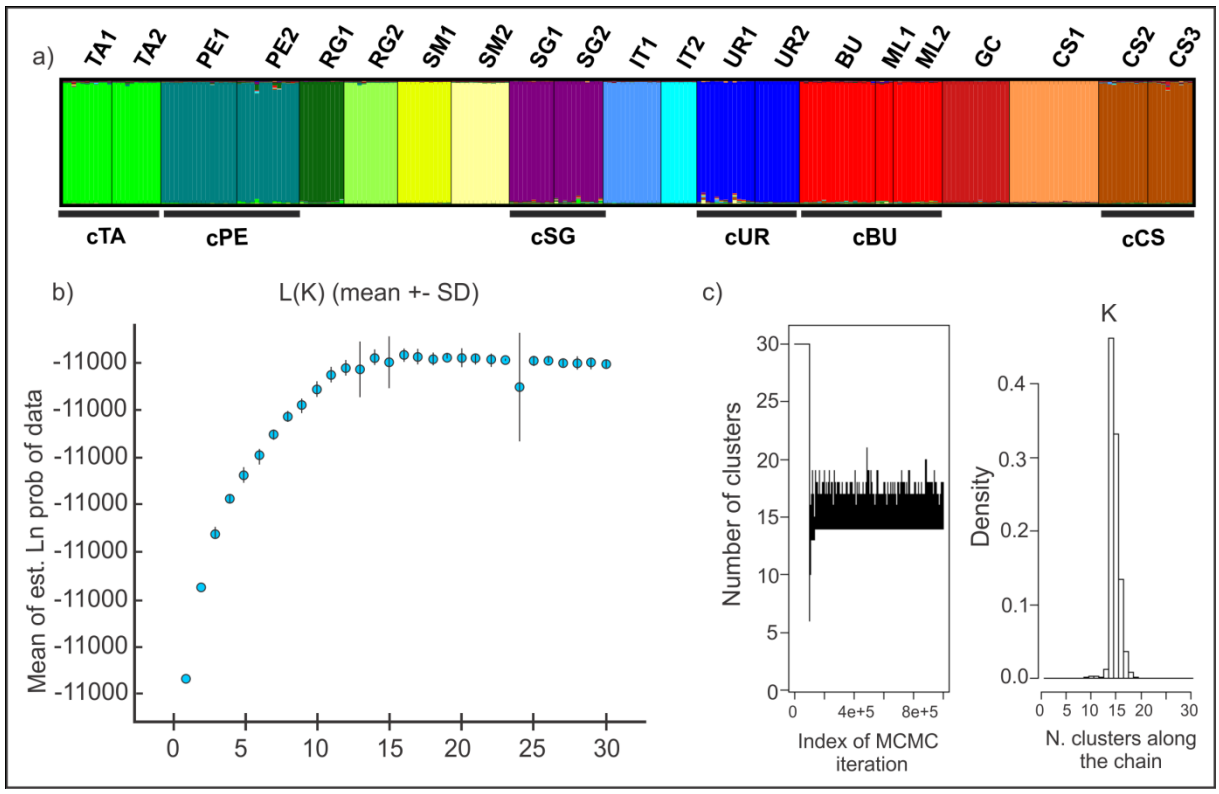

a)
TA1
TA2
PE1
PE2
RG1
RG2
SM1
SM2
SG1
SG2
IT1
IT2
UR1
UR2
BU
ML1
ML2
GC
CS1
CS2
CS3
cTA
cPE
cSG
cUR
cBU
cCS
b)
L(K) (mean +- SD)
Mean of est. Ln prob of data
-11000
-11000
-11000
-11000
-11000
-11000
-11000
-11000
0
5
10
15
20
25
30
c)
Number of clusters
30
25
20
15
10
5
0
0
4e+5
8e+5
Index of MCMC iteration
K
Density
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
30
N. clusters along the chain

Figure 6:

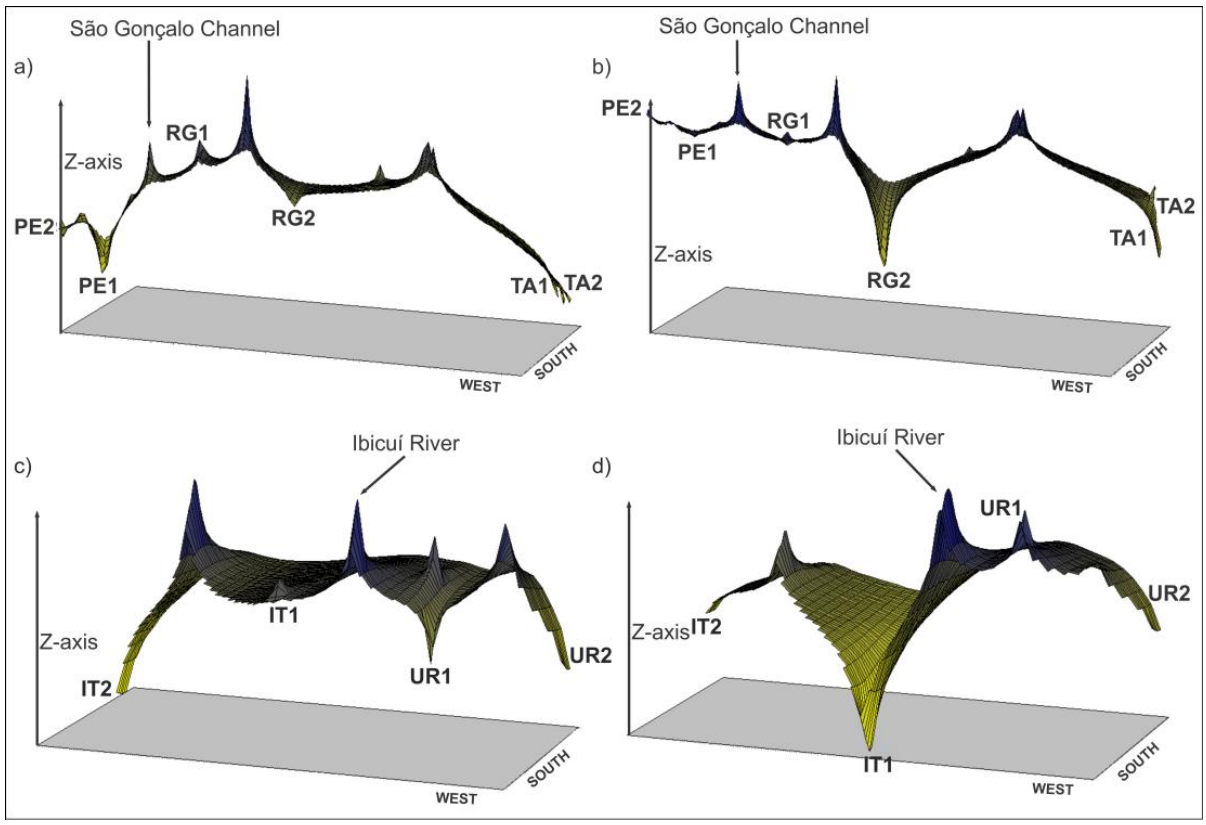
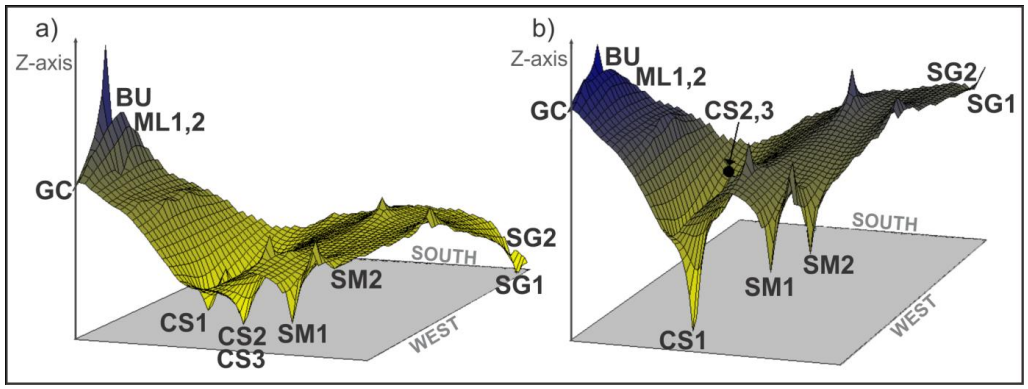



Figure 7:



**APPENDIX**

**Appendix S1** Collection sites of *Ctenomys torquatus*, their geographic coordinate, diploid number (2n), estimatives of genetic diversity for the concatenated mitochondrial data set (COI and HVS) and for the 21 localities assayed with microsatellite loci. N number of individuals for each assay, H(Hd) number of haplotypes (haplotypic diversity), π nucleotide diversity, A mean number of alleles per locus, LP number of polymorphic loci, Ho mean observed heterozygosity, LHW loci that showed evidence for departure from Hardy-Weinberg equilibrium, DL number of pairwise loci that showed linkage disequilibrium, $F_{IS}$ inbreeding coefficient values.

| | Locality | Geographic coordinate | Karyotype | | mtDNA | | | microsatellite | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2n | N | N | H (Hd) | π | N | A | LP | Ho | LHW* | DL* | $F_{IS}$** |
| Brazil | ALE-Alegrete [a] | 29°53'41,7"S 55°43'24,6"W | 40 | 5 | - | | | - | | | | | | |
| | BU-Butiá [b] | 30°03'19,7"S 51°56'12,3"W | 44 | 5 | 15 | 4(0.73) | 0.0202 | 17 | 3.54 | 19 | 0.53 | | | |
| | CA-Candiota [b] | 31°32’59,9”S 53°43’10,18”W | 44 | 5 | 12 | 2(0.16) | 0.0136 | - | | | | | | |
| | CAT-Catimbau [a] | 29°57'46,8"S 55°39'9,2"W | 42 | 11 | 8 | 2(0.25) | 0.0102 | - | | | | | | |
| | CAV-Caverá [a] | 30°06'47,2"S | 44 | 5 | 8 | 1(0) | 0 | - | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 55°33'14,6"W | | | | | | | | | | | | |
| | CG-Caboaté Grande | 30°6'37,9"S | 44 | 4 | 4 | 4(1.0) | 0.0884 | - | | | | | | |
| | | 54°20'7,4"W | | | | | | | | | | | | |
| | CQ-Cacequi | 29°53'48"S | 44 | 3 | 6 | 1(0) | 0 | - | | | | | | |
| | | 54°48'25,9"W | | | | | | | | | | | | |
| | CS1-Cachoeira do Sul 1[c] | 30°02'5"S | 44 | 4 | 13 | 1(0) | 0 | 20 | 1.50 | 11 | 0.33 | | | |
| | | 52°51'27,5"W | | | | | | | | | | | | |
| | CS2-Cachoeira do Sul 2 | 30°06'4,4"S | 44 | 4 | 10 | 1(0) | 0 | 11 | 2.91 | 21 | 0.36 | | | 0.25 |
| | | 52°55'30,9"W | | | | | | | | | | | | |
| | CS3-Cachoeira do Sul 3 | 30°04'29,6"S | 44 | 3 | 10 | 1(0) | 0 | 10 | 2.81 | 20 | 0.51 | | | |
| | | 53°3'28,1"W | | | | | | | | | | | | |
| | DA-Dilermando de | 29°45'5"S | 44 | 3 | 8 | 1(0) | 0 | - | | | | | | |
| | Aguiar | 54°6'57,7"W | | | | | | | | | | | | |
| | DP-Dom Pedrito[c] | 31°08'10,3"S | 44 | 3 | 4 | 2(0.66) | 0.0136 | - | | | | | | |
| | | 54°29'32"W | | | | | | | | | | | | |
| | GC-General Câmara[c] | 29°55'7,5"S | 44 | 4 | 10 | 1(0) | 0 | 15 | 2.27 | 17 | 0.36 | *Soc*4, | 2 | 0.26 |
| | | 51°53'28,2"W | | | | | | | | | | *Tor*6 | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IT1-Itaqui 1 [c] | 29°24'26,2"S 56°38'30,8"W | 40 | 9 | 9 | 2(0.55) | 0.0113 | 13 | 1.95 | 15 | 0.48 | | | |
| IT2-Itaqui 2 | 29°15'50,7"S 56°37'03,2"W | 40 | 3 | 8 | 1(0) | 0 | 8 | 1.22 | 12 | 0.32 | | 1 | |
| ML1-Minas do Leão 1 [b] | 30°07’56”S 52°01’30,1”W | 44 | 4 | 4 | 2(0.5) | 0.0102 | 4 | 1.91 | 16 | 0.48 | | | |
| ML2-Minas do Leão2 | 30°2'2,8"S 52°6'42,4"W | 44 | 3 | 11 | 3(0.34) | 0.0148 | 11 | 2.63 | 18 | 0.48 | | | |
| PE1-Pelotas 1 [b] | 31°44'27,4"S 52°13'22,3"W | 44 | 10 | 12 | 1(0) | 0 | 17 | 2.77 | 17 | 0.43 | | | |
| PE2-Pelotas 2 | 31°40'1,7"S 52°11'35,3"W | 44 | 3 | 14 | 2(0.44) | 0.0089 | 14 | 3.31 | 18 | 0.57 | | | |
| QU-Quarai[a] | 30°28'14,7"S 56°15'36,4"W | 44 | 3 | 6 | 2(0.53) | 0.0435 | - | | | | | | |
| RG1-Rio Grande 1 [c] | 31°54'23,2"S 52°17'45,7"W | 46 | 6 | 10 | 3(0.38) | 0.0081 | 10 | 2.18 | 15 | 0.43 | | 3 | 0.26 |
| RG2-Rio Grande 2 | 32°5'18,0"S | 46 | 3 | 10 | 2(0.2) | 0.0072 | 12 | 1.91 | 15 | 0.45 | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 52°18'57,3"W | | | | | | | | | | | |
| RO-Rosário[a] | 30°13'29,3"S | 44 | 6 | 10 | 2(0.2) | 0.0040 | - | | | | | |
| | 54°54'51,8"W | | | | | | | | | | | |
| SG1-São Gabriel 1 | 30°36'29,8"S | 44 | 3 | 10 | 1(0) | 0 | 11 | 2.95 | 20 | 0.46 | | |
| | 54°25'33,3"W | | | | | | | | | | | |
| SG2-São Gabriel 2 | 30°38'54,9"S | 44 | 3 | 12 | 2(0.3) | 0.0433 | 11 | 3.18 | 21 | 0.51 | | |
| | 54°25'57,7"W | | | | | | | | | | | |
| SL-Santana do | 30°49'56,7"S | 44 | 2 | 2 | 1(0) | 0 | - | | | | | |
| Livramento[c] | 55°22'35,8"W | | | | | | | | | | | |
| SM1-Santa Maria 1[c] | 29°55'35,3"S | 44 | 5 | 9 | 1(0) | 0 | 12 | 2.18 | 19 | 0.39 | | 1 |
| | 53°44'28,7"W | | | | | | | | | | | |
| SM2-Santa Maria 2 | 29°59'45,8"S | 44 | 3 | 13 | 2(0.28) | 0.0057 | 13 | 2.09 | 19 | 0.47 | | |
| | 53°56'23,2"W | | | | | | | | | | | |
| TA1-Taim 1[d] | 32°32'15,9"S | 46 | 8 | 11 | 1(0) | 0 | 11 | 2.09 | 16 | 0.47 | *Hai*3 | |
| | 52°32'21,8"W | | | | | | | | | | | |
| TA2-Taim 2 | 32°35'16,8"S | 46 | 3 | 10 | 1(0) | 0 | 11 | 1.54 | 13 | 0.31 | | |
| | 52°29'48,2"W | | | | | | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UR1-Uruguaiana 1[a] | 29°28'57,4"S 56°38'15,6"W | 44 | 3 | 10 | 2(0.2) | 0.0072 | 13 | 3.81 | 22 | 0.58 | |
| | UR2-Uruguaiana 2 | 29°32'14,4"S 56°36'51,1"W | 44 | 3 | 9 | 2(0.22) | 0.0045 | 10 | 2.59 | 19 | 0.48 | *Tor*7 |
| Uruguay | RN- Río Negro | 32º40'S 57º57'W | 44 | - | 3 | 3(1.0) | 0.0272 | - | | | | |
| | TC-Tacuarembó | 31º42'S 55º58'W | 44 | - | 3 | 1(0) | 0 | - | | | | |
| | Total | | | | 294 | 40(0.95) | 0.0044 | 254 | | | | |

Collection sites sampled for previous studies [a](Gonçalves & Freitas, 2009), [b](Silva et al, 2000a;b), [c](Fernandes et al, 2009a), [d](Freitas & Lessa, 1984)

* After applying the Bonferroni correction for multiple tests; **Only significant values (P<0.001)

**Appendix S2** List of species and the Genbank accession number for the cytochrome *b* sequence applied to phylogenetic and molecular clock analysis. *Ctenomys* species groups are detached.

| Family | Species | Genbank number |
|---|---|---|
| Thryonomyidae | *Thryonomys swinderianus* | NC002658 |
| Dasyproctidae | *Myoprocta acouchy* | AF437781 |
| | *Dasyprocta leporina* | AF437808 |
| Caviidae | *Galea musteloides* | GU082485 |
| | *Cavia aperea* | GU136759 |
| Hydrochaeridae | *Hydrochaerus hydrachaeris* | GU136721 |
| Erethizontidae | *Erethizon dorsatum* | FJ357428 |
| Capromyidae | *Capromys pilorides* | AF422915 |
| Echimyidae | *Dactylomys dactylinus* | L23335 |
| | *Echimys didelphoides* | EU302705 |
| Octodontidae | *Spalacopus cyanus* | AF007061 |
| | *Octodontomys gliroides* | AF370706 |
| | *Octodon degus* | AF007059 |
| | *Tympanoctomys barrerae* | AF007060 |
| Ctenomyidae | *C. sociabilis* | HM777495 |
| | *C. leucodon* | AF007056 |
| | *C. tuconax* | AF370693 |
| | *C.* sp. *ITA* | AF007047 |
| | *C.* sp. *MINUT* | AF007052 |
| | *C.* sp. *MONTE* | AF007053 |
| | *C. maulinus* | AF370703 |
| *frater* group | *C. frater* | AF007046 |
| | *C. conoveri* | AF007055 |
| | *C.* sp. *LLATHU* | AF007048 |
| | *C. lewisi* | AF007049 |
| *boliviensis* group | *C. boliviensis* | AF007040 |
| | *C. goodfellowi* | AF007051 |

| | | |
|---|---|---|
| | *C. nattereri* | HM777484 |
| | *C.* sp. *ROBO* | AF007039 |
| | *C. steinbachi* | AF007044 |
| *tucumanus* group | *C. tucumanus* | AF370694 |
| | *C. occultus* | HM777485 |
| | *C. latro* | AF370705 |
| | *C. argentinus* | AF370680 |
| *magellanicus* group | *C. magellanicus* | AF370690 |
| | *C. colburni* | HM777474 |
| | *C. haigi* | AF007063 |
| | *C. coyhaiquensis* | AF119112 |
| | *C. fodax* | HM777475 |
| | *C. sericeus* | HM777496 |
| | *C. sociabilis** | U34853 |
| | *C.* sp. 1 | HM777500 |
| | *C.* sp. 2 | HM777501 |
| | *C.* sp. 3 | HM777502 |
| | *C.* sp. 4 | HM777503 |
| | *C.* sp. 5 | HM777504 |
| | *C.* sp. 6 | HM777505 |
| | *C.* sp. 7 | HM777506 |
| *talarum* group | *C. talarum* | AF370699 |
| | *C. pundti* | HM777490 |
| *mendocinus* group | *C. mendocinus* | AF007062 |
| | *C. rionegrensis* | AF119114 |
| | *C. flamarioni* | AF119107 |
| | *C. australis* | AF370697 |
| | *C. porteousi* | AF370682 |
| *opimus* group | *C. opimus* | AF007042 |
| | *C. fulvus* | AF370688 |

| | | |
|---|---|---|
| | *C. saltarius* | HM777493 |
| | *C. scagliai* | HM777494 |
| *torquatus* group | *C. lami* | HM777477 |
| | *C. minutus* | HM777481 |
| | *C. ibicuiensis* | JQ389020 |
| | *C. pearsoni* | AF119108 |
| | *C. perrensi* | HM777487 |
| | *C. roigi* | M777492 |
| | *C.* sp. Contreras Cue, Tacuaritas | JQ389032 |
| | *C. dorbignyi* Sarandicito | JQ389030 |
| | *C. dorbignyi* Mbarigui | JQ389031 |
| | *C. torquatus* (SM1) | JQ389033 |
| Additional sequences | *C. torquatus* (CAV) | EF372280 |
| | *C. torquatus* (ALE) | EF372282 |
| | *C. torquatus* (ML2 - TR1382) | JQ389042 |
| | *C. torquatus* (CS2) | JQ389036 |
| | *C. torquatus* (ML2 - TR1387) | JQ389040 |
| | *C. torquatus* (GC) | JQ389035 |
| | *C. torquatus* (SG1) | JQ389038 |
| | *C. torquatus* (RO, QU) | EF372283 |
| | *C. torquatus* (CAT) | EF372286 |
| | *C. torquatus* (RN - CA655) | JQ389043 |
| | *C. torquatus* (RN - CA654) | AF119109 |
| | *C. torquatus* (UR1, IT1) | JQ389034 |
| | *C. torquatus* (TC - CA743) | AF119111 |
| | *C. torquatus* (TC - CA744) | JQ389044 |
| | *C. torquatus* (DP) | JQ389045 |
| | *C. torquatus* (PE2) | JQ389041 |
| | *C. torquatus* (TA2) | JQ389039 |
| | *C. torquatus* (IT2) | JQ389037 |

*A probable *C. haigi* sequence, according to Parada et al. (2011).

**Appendix S3** List of *Ctenomys* specimens, vouchers and the Genbank accession number for the cytochrome *b* (cyt *b*), cytochrome *c* oxidase I (COI) genes and hyper variable sequence 1 (HVS) sequences used for the substitution rate estimatives.

| | | Genbank accession numbers | | |
|---|---|---|---|---|
| Species | voucher | cyt *b* | COI | HVS |
| *C. australis* | UNMDP3* (C1) | AF370697 | JQ341043 | JQ341032 |
| *C. azarae* | UNMDP7 | JN791406 | JQ341046 | JQ341035 |
| *C. boliviensis* | NK15367 | AF155869 | JQ341048 | JQ341037 |
| *C. flamarioni* | G118 * (T29) | AF119107 | JQ341052 | JQ341041 |
| *C. nattereri* | TR1110 | AF144298 | JQ389082 | JQ389117 |
| *C. pearsoni* | CA369 | AF500067 | JQ341042 | JQ341031 |
| *C. porteousi* | UNMDP10 | AF370681 | JQ341045 | JQ341034 |
| *C. rionegrensis* | EV1043 | AF119114 | JQ341044 | JQ341033 |
| *C. sociabilis* | MVZ166425 | EU035177 | JQ341051 | JQ341040 |
| *C. steinbachi* | NK12133 | JN791407 | JQ341047 | JQ341036 |
| *C. talarum* | IF02 | HM777498 | JQ341049 | JQ341038 |
| *C. torquatus* | CA743 | AF119111 | JQ389072 | JQ389106 |
| | TR913 | EF372287 | HM443439 | JQ389101 |
| | TR914 | EF372285 | JQ389066 | JQ389088 |
| | TR917 | EF372286 | HM443439 | JQ389102 |
| | TR1236 | JQ389034 | JQ389068 | JQ389103 |
| | TR1255 | JQ389035 | JQ389062 | JQ389095 |
| | TR1258 | JQ389036 | HM443439 | JQ389090 |
| | TR1324 | JQ389037 | JQ389071 | JQ389105 |
| | TR1332 | JQ389038 | JQ389064 | JQ389099 |
| | TR1355 | JQ389039 | JQ389051 | JQ389083 |
| | TR1387 | JQ389040 | HM443439 | JQ389087 |
| | TR1422 | JQ389041 | JQ389051 | JQ389084 |
| *C. minutus* | Cf02 | JQ389046 | HM237009 | HM236969 |

| | | | | |
|---|---|---|---|---|
| | Cf08 | JQ389047 | HM237011 | HM236972 |
| | L05 | JQ389048 | HM237032 | HM236997 |
| | TR1122 | JQ389049 | HM237039 | HM237003 |
| | TR1215 | JQ389050 | HM237038 | HM237002 |
| | TR431 | HM777481 | HM237028 | HM236993 |
| | TR40 | HM777482 | HM237030 | HM236995 |
| | TR02 | HM777483 | HM237040 | HM237005 |
| *C. lami* | TJ186 | HM777477 | JQ322899 | JQ322885 |
| | TJ115* (TR431) | HM777481 | JQ322900 | JQ322889 |
| *C. ibicuiensis* | TR1066 | JQ389020 | JQ389075 | JQ389108 |
| | TR1319 | JQ389024 | JQ389078 | JQ389111 |
| | TR1372 | JQ389026 | JQ389079 | JQ389113 |
| | TR1510 | JQ389028 | JQ389079 | JQ389112 |
| | TR1515 | JQ389029 | JQ389079 | JQ389112 |

**Appendix S4** AMOVA considering groups as localities from different banks of the rivers or different karyotypes for Ibicuí River and São Gonçalo Channel. Localities along the Vacacaí-Jacuí River were tested for AMOVA in four ways (see text). Locality names follow Appendix S1.

| Group/marker | | Partition | | |
|---|---|---|---|---|
| | | AG ($F_{CT}$) | APWG ($F_{SC}$) | WP ($F_{ST}$) |
| Ibicuí | (IT1, IT2) (UR1, UR2) | | | |
| | MS | 19.3 (0.19) | 24.9 (**0.32**) | 55.8 (**0.44**) |
| | mtDNA | -5.46 (-0.05) | 98.75 (**0.93**) | 6.72 (**0.93**) |
| São Gonçalo | (I): (PE1, PE2) (RG1, RG2, TA1, TA2) | | | |
| | MS | 5.8 (0.05) | 31.4 (**0.33**) | 62.7 (**0.37**) |
| | mtDNA | 36.6 (0.36) | 53.4 (**0.83**) | 20.3 (**0.89**) |
| | (II): (PE1, PE2, RG1) (RG2, TA1, TA2) | | | |
| | MS | 6.2 (0.06) | 31.1 (**0.33**) | 62.6 (**0.37**) |
| | mtDNA | 25.5 (**0.25**) | 63.5 (**0.89**) | 10.9 (**0.85**) |
| Jacuí | (I): (SG1, SG2) (SM1, SM2) (CS1, CS2, CS3) (GC, BU, ML1, ML2) | | | |
| | MS | 12.2 (**0.12**) | 32.8 (**0.37**) | 55.0 (**0.45**) |
| | mtDNA | 62.0 (**0.62**) | 24.0 (**0.63**) | 14.0 (**0.86**) |
| | (II): (CS1, GC) (CS1, CS2, BU, ML1, ML2) | | | |
| | MS | 10.1 (0.10) | 33.44 (**0.37**) | 56.44 (**0.43**) |
| | mtDNA | -11.9 (-0.11) | 83.8 (**0.75**) | 28.1 (**0.72**) |
| | (III): (CS1) (CS2, CS3) | | | |
| | MS | 34.8 (0.34) | 16.7 (**0.25**) | 48.5 (**0.51**) |
| | mtDNA | 100.0 (1.0) | 0 (0) | 0 (**1.0**) |
| | (IV): (GC) (BU, ML1, ML2) | | | |
| | MS | 17.0 (0.17) | 16.4 (**0.19**) | 66.6 (**0.33**) |
| | mtDNA | 17.3 (0.17) | 3.1 (**0.04**) | 79.6 (**0.20**) |

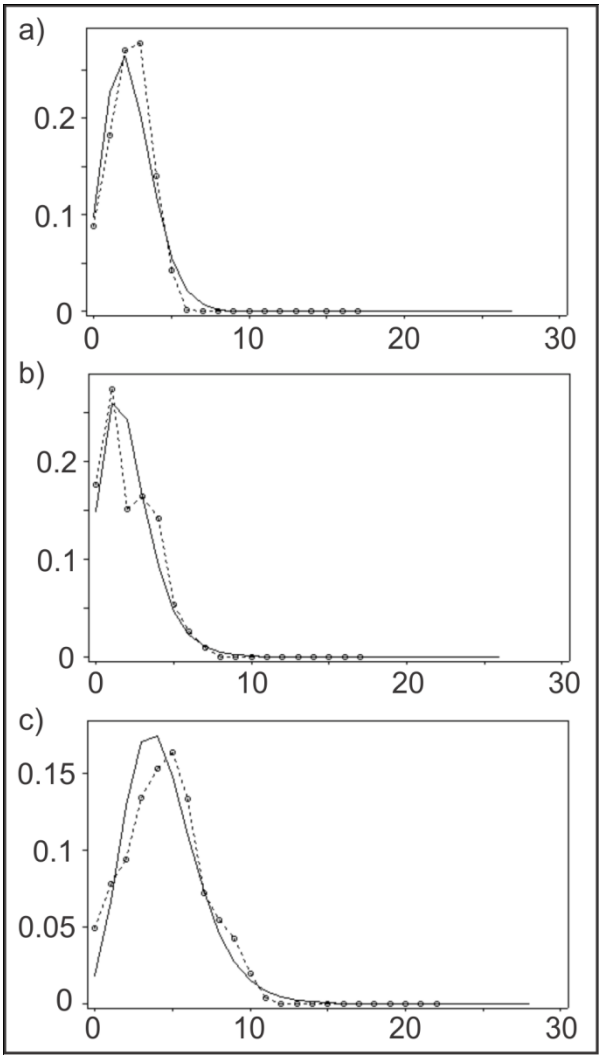
Significant values ($P < 0.05$) for F-statistic from AMOVA are in bold. AG, Among groups; APWG, Among populations within groups; WP, Within populations; MS microsatellite data; mtDNA mitochondrial DNA concatenate COI+HVS data.

**Appendix S5** Haplotype number for the concatenate data, distribution of each haplotype by localities and the GenBank accession numbers of the respective COI and HVS of mitochondrial DNA of *Ctenomys torquatus*.

| Concatenate Haplotype | Concatenate haplotype per locality | Haplotype per marker | |
|---|---|---|---|
| | | COI | HVS |
| H1 | TA1 (11), TA2 (10) | H1 (JQ389051) | H1 (JQ389083) |
| H2 | PE1 (10), PE2 (10) | H1 | H2 (JQ389084) |
| H3 | PE2 (4) | H2 (JQ389052) | H2 |
| H4 | RG1 (8) | H2 | H1 |
| H5 | RG1 (1) | H3 (JQ389053) | H1 |
| H6 | RG1 (1) | H2 | H3 (JQ389085) |
| H7 | RG2 (8) | H4 (JQ389054) | H4 (JQ389086) |
| H8 | RG2 (2) | H5 (JQ389055) | H4 |
| H9 | CA (11), DP (2) | H6 (JQ389056) | H1 |
| H10 | CA (1), GC (10), BU (5), ML2 (9) | H7 (HM443439) | H5 (JQ389087) |
| H11 | DP (2) | H6 | H2 |
| H12 | BU (6), ML1 (1), CS1 (13), SM1 (9), RO (1), RN (1) | H7 | H6 (JQ389088) |
| H13 | BU (1) | H7 | H7 (JQ389089) |
| H14 | BU (3), ML1 (3) | H8 (JQ389057) | H6 |
| H15 | ML2 (1) | H9 (JQ389058) | H6 |
| H16 | ML2 (1) | H10 (JQ389059) | H5 |
| H17 | CS2 (10), CS3 (10) | H7 | H8 (JQ389090) |
| H18 | SM2 (2) | H11 (JQ389060) | H9 (JQ389091) |
| H19 | SM2 (11) | H11 | H10 (JQ389092) |
| H20 | DA (8) | H7 | H11 (JQ389093) |
| H21 | CQ (6) | H12 (JQ389061) | H12 (JQ389094) |
| H22 | CG (1) | H13 (JQ389062) | H13 (JQ389095) |
| H23 | CG (1) | H7 | H14 (JQ389096) |
| H24 | CG (1) | H14 (JQ389063) | H15 (JQ389097) |

| | | | |
|---|---|---|---|
| H25 | CG (1) | H7 | H16 (JQ389098) |
| H26 | SG1 (10), SG2 (9) | H15 (JQ389064) | H17 (JQ389099) |
| H27 | SG2 (2) | H16 (JQ389065) | H12 |
| H28 | RO (9), SL (2) | H7 | H18 (JQ389100) |
| H29 | QU (4) | H7 | H19 (JQ389101) |
| H30 | QU (2) | H17 (JQ389066) | H6 |
| H31 | CAT (7) | H7 | H20 (JQ389102) |
| H32 | CAT (1), CAV (8) | H18 (JQ389067) | H6 |
| H33 | UR1 (8), UR2 (1), IT1 (4) | H19 (JQ389068) | H21 (JQ389103) |
| H34 | UR1 (2) | H19 | H22 (JQ389104) |
| H35 | UR2 (8) | H20 (JQ389069) | H21 |
| H36 | IT1 (5) | H21 (JQ389070) | H21 |
| H37 | IT2 (8) | H22 (JQ389071) | H23 (JQ389105) |
| H38 | TC (3) | H23 (JQ389072) | H24 (JQ389106) |
| H39 | RN (1) | H24 (JQ389073) | H25 (JQ389107) |
| H40 | RN (1) | H7 | H25 |

**Appendix S6** The observed (dashed line) and expected (solid line) mismatch distributions in an expanding population model for HVS1 control region **(a)**, for COI gene **(b)** and for the concatenate HVS+COI data **(c)**.



**Appendix S7** Genetic differentiation results for populations of collared tuco-tuco *Ctenomys torquatus*. Values above the diagonal represent pairwise $F_{ST}$ for concatenate COI+HVS data, values below the diagonal represent pairwise $F_{ST}$ for microsatellite data.

| | TA1 | TA2 | PE1 | PE2 | RG1 | RG2 | SM1 | SM2 | SG1 | SG2 | BU | ML1 | ML2 | GC | CS1 | CS2 | CS3 | IT1 | IT2 | UR1 | UR2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TA1 | | 0.00 | **1.00** | **0.81** | **0.84** | **0.92** | **1.00** | **0.96** | **1.00** | **0.85** | **0.85** | 0.97 | **0.91** | **1.00** | **1.00** | **1.00** | **1.00** | **0.97** | 1.00 | **0.98** | **0.99** |
| TA2 | **0.34** | | **1.00** | **0.80** | **0.83** | **0.92** | **1.00** | **0.96** | **1.00** | **0.84** | **0.84** | 0.97 | **0.91** | **1.00** | **1.00** | **1.00** | **1.00** | **0.97** | **1.00** | **0.97** | **0.99** |
| PE1 | **0.34** | **0.44** | | 0.21 | **0.92** | **0.95** | **1.00** | **0.97** | **1.00** | **0.87** | **0.86** | **0.98** | **0.90** | **1.00** | **1.00** | **1.00** | **1.00** | **0.97** | **1.00** | **0.98** | **0.99** |
| PE2 | **0.27** | **0.37** | **0.15** | | **0.78** | **0.88** | **0.94** | **0.93** | **0.97** | **0.86** | **0.83** | 0.91 | **0.84** | **0.92** | **0.95** | **0.96** | **0.96** | **0.95** | **0.95** | **0.95** | **0.96** |
| RG1 | **0.34** | **0.38** | **0.31** | **0.24** | | **0.89** | **0.95** | **0.94** | **0.98** | **0.84** | **0.85** | 0.91 | **0.89** | **0.96** | **0.96** | **0.97** | **0.97** | **0.95** | **0.95** | **0.95** | **0.97** |
| RG2 | **0.44** | **0.56** | **0.45** | **0.35** | **0.43** | | **0.96** | **0.95** | **0.98** | **0.86** | **0.87** | 0.93 | **0.91** | **0.97** | **0.97** | **0.98** | **0.98** | **0.95** | **0.97** | **0.96** | **0.97** |
| SM1 | **0.56** | **0.68** | **0.57** | **0.53** | **0.56** | **0.63** | | **0.94** | **1.00** | **0.74** | 0.19 | 0.80 | **0.67** | **1.00** | 0.00 | **1.00** | **1.00** | **0.94** | **1.00** | **0.96** | **0.98** |
| SM2 | **0.54** | **0.64** | **0.56** | **0.50** | **0.50** | **0.53** | **0.54** | | **0.98** | **0.83** | **0.81** | 0.91 | **0.88** | **0.96** | **0.95** | **0.97** | **0.97** | **0.95** | **0.97** | **0.96** | **0.97** |
| SG1 | **0.45** | **0.59** | **0.47** | **0.41** | **0.42** | **0.49** | **0.51** | **0.40** | | 0.07 | **0.90** | 0.98 | **0.94** | **1.00** | **1.00** | **1.00** | **1.00** | **0.97** | **1.00** | **0.98** | **0.99** |
| SG2 | **0.37** | **0.51** | **0.41** | **0.36** | **0.35** | **0.45** | **0.47** | **0.39** | **0.13** | | **0.71** | 0.68 | **0.74** | **0.79** | **0.77** | **0.82** | **0.82** | **0.84** | **0.85** | **0.85** | **0.86** |
| BU | **0.41** | **0.52** | **0.44** | **0.39** | **0.38** | **0.45** | **0.45** | **0.37** | **0.37** | **0.33** | | 0.26 | 0.23 | 0.37 | 0.24 | **0.78** | **0.78** | **0.84** | **0.86** | **0.85** | **0.87** |
| ML1 | 0.46 | 0.64 | 0.51 | 0.42 | 0.43 | 0.56 | 0.46 | 0.42 | 0.36 | 0.32 | **0.19** | | 0.66 | 0.92 | 0.85 | 0.95 | 0.95 | 0.90 | 0.97 | 0.92 | 0.95 |
| ML2 | **0.45** | **0.57** | **0.49** | **0.44** | **0.44** | **0.52** | **0.46** | **0.39** | **0.38** | **0.35** | **0.17** | 0.26 | | 0.01 | **0.71** | **0.88** | **0.88** | **0.89** | **0.92** | **0.90** | **0.92** |
| GC | **0.50** | **0.62** | **0.51** | **0.46** | **0.51** | **0.57** | **0.54** | **0.49** | **0.45** | **0.41** | **0.30** | 0.47 | **0.36** | | **1.00** | **1.00** | **1.00** | **0.95** | **1.00** | **0.97** | **0.98** |
| CS1 | **0.66** | **0.74** | **0.65** | **0.59** | **0.65** | **0.68** | **0.68** | **0.63** | **0.57** | **0.57** | **0.55** | **0.65** | **0.56** | **0.57** | | **1.00** | **1.00** | **0.95** | **1.00** | **0.96** | **0.98** |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CS2 | **0.42** | **0.54** | **0.45** | **0.38** | **0.43** | **0.47** | **0.53** | **0.43** | **0.37** | **0.37** | **0.30** | 0.41 | **0.35** | **0.38** | **0.57** | | 0.00 | **0.96** | **1.00** | **0.97** | **0.98** |
| CS3 | **0.44** | **0.58** | **0.47** | **0.40** | **0.41** | **0.49** | **0.47** | **0.43** | **0.38** | **0.35** | **0.28** | 0.36 | **0.31** | **0.36** | **0.54** | **0.18** | | **0.96** | **1.00** | **0.97** | **0.98** |
| IT1 | **0.56** | **0.65** | **0.53** | **0.47** | **0.51** | **0.59** | **0.59** | **0.54** | **0.49** | **0.43** | **0.47** | 0.53 | **0.52** | **0.53** | **0.68** | **0.48** | **0.52** | | **0.97** | 0.40 | 0.73 |
| IT2 | **0.53** | **0.67** | **0.54** | **0.46** | **0.53** | **0.64** | **0.60** | **0.57** | **0.49** | **0.40** | **0.46** | 0.55 | **0.50** | **0.54** | **0.69** | **0.50** | **0.52** | **0.52** | | **0.97** | **0.99** |
| UR1 | **0.41** | **0.52** | **0.44** | **0.39** | **0.39** | **0.49** | **0.39** | **0.37** | **0.31** | **0.27** | **0.34** | 0.32 | **0.34** | **0.39** | **0.52** | **0.34** | **0.36** | **0.43** | **0.36** | | **0.73** |
| UR2 | **0.51** | **0.63** | **0.52** | **0.45** | **0.48** | **0.57** | **0.51** | **0.48** | **0.41** | **0.36** | **0.42** | 0.45 | **0.44** | **0.49** | **0.63** | **0.44** | **0.47** | **0.52** | **0.44** | **0.10** | |

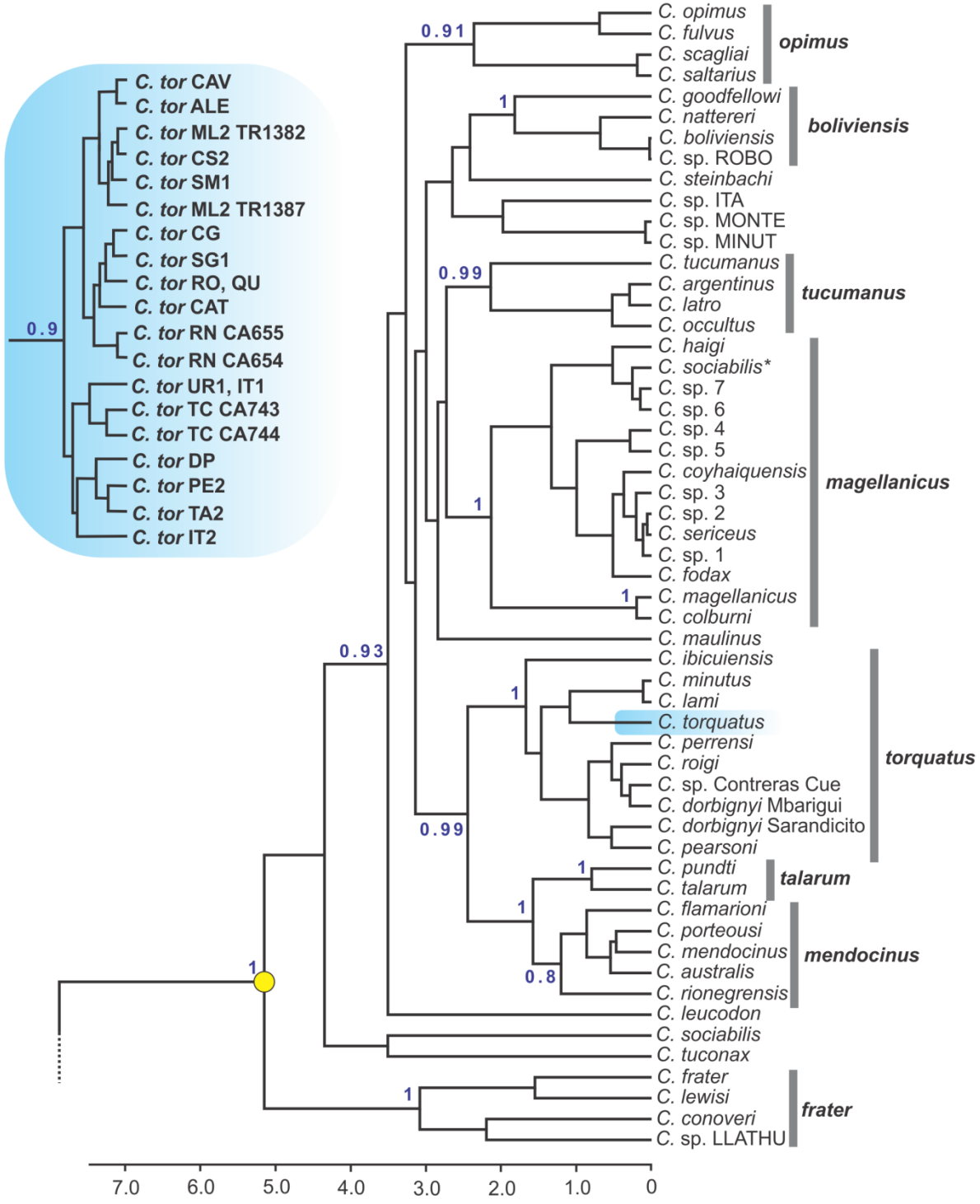
All significant estimates for multiple comparisons between populations are in bold, after Bonferroni adjustments (α = 0.00023 for microsatellites, α = 0.00009 for COI+HVS ); - Localities not analyzed with microsatellite data. Refer to Appendix S1 for population code definitions.

Continuation Appendix S7:.

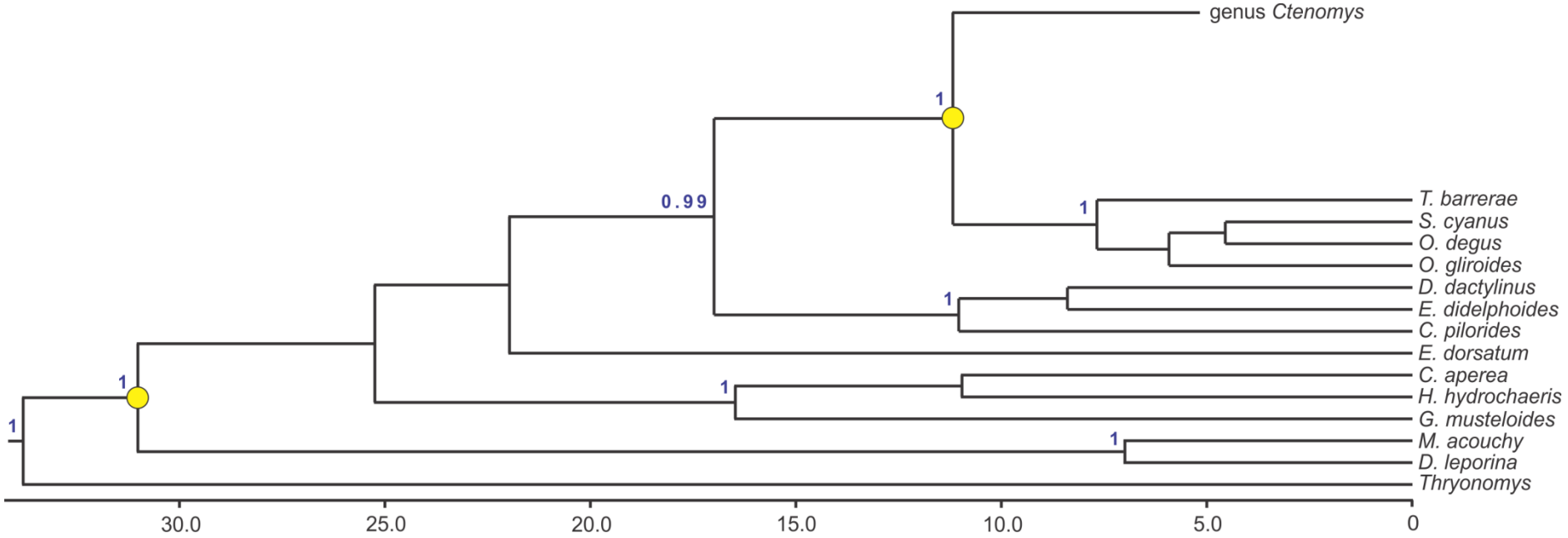
| | CA | DP | DA | CQ | CG | RO | SL | QU | CAT | CAV | TC | RN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TA1 | **0.84** | 0.94 | **1.00** | **1.00** | 0.79 | **0.98** | 1.00 | **0.85** | **0.95** | **1.00** | 1.00 | 0.94 |
| TA2 | **0.83** | 0.93 | **1.00** | **1.00** | 0.77 | **0.97** | 1.00 | 0.84 | **0.94** | **1.00** | 1.00 | 0.94 |
| PE1 | **0.89** | 0.94 | **1.00** | **1.00** | 0.82 | **0.98** | 1.00 | **0.85** | **0.96** | 1.00 | 1.00 | 0.96 |
| PE2 | **0.83** | 0.82 | **0.95** | **0.93** | **0.78** | **0.93** | 0.92 | **0.80** | **0.91** | **0.95** | 0.94 | 0.89 |
| RG1 | **0.84** | 0.87 | **0.96** | 0.94 | 0.77 | **0.94** | 0.93 | 0.83 | **0.91** | **0.96** | 0.95 | 0.89 |
| RG2 | **0.88** | 0.91 | **0.97** | **0.96** | 0.81 | **0.95** | 0.95 | **0.86** | **0.93** | **0.97** | 0.96 | 0.91 |
| SM1 | **0.87** | 0.95 | **1.00** | 1.00 | 0.36 | **0.88** | 1.00 | **0.55** | **0.76** | **1.00** | 1.00 | 0.65 |
| SM2 | **0.89** | 0.92 | **0.95** | **0.94** | 0.76 | **0.93** | 0.93 | **0.82** | **0.91** | **0.95** | 0.97 | 0.88 |
| SG1 | **0.95** | 0.98 | **1.00** | 1.00 | 0.77 | **0.98** | 1.00 | 0.89 | **0.96** | **1.00** | 1.00 | 0.96 |
| SG2 | **0.80** | 0.77 | **0.77** | **0.77** | 0.47 | **0.77** | 0.67 | **0.67** | **0.74** | **0.77** | 0.83 | 0.65 |
| BU | **0.75** | 0.76 | **0.60** | **0.74** | 0.28 | **0.58** | 0.50 | 0.33 | **0.52** | **0.63** | 0.87 | 0.35 |
| ML1 | 0.83 | 0.86 | 0.91 | 0.93 | 0.26 | 0.83 | 0.79 | 0.47 | 0.71 | 0.91 | 0.96 | 0.51 |
| ML2 | **0.82** | 0.81 | **0.81** | **0.85** | 0.39 | **0.77** | 0.71 | **0.33** | **0.71** | **0.81** | 0.92 | 0.60 |
| GC | **0.90** | 0.95 | **1.00** | **1.00** | 0.52 | **0.95** | 1.00 | **0.47** | **0.89** | **1.00** | 1.00 | 0.86 |
| CS1 | **0.89** | 0.96 | **1.00** | **1.00** | 0.46 | **0.90** | 1.00 | **0.62** | **0.81** | **1.00** | 1.00 | 0.73 |
| CS2 | **0.92** | 0.97 | **1.00** | **1.00** | 0.71 | **0.97** | 1.00 | **0.80** | **0.93** | **1.00** | 1.00 | 0.91 |
| CS3 | **0.92** | 0.97 | **1.00** | **1.00** | 0.71 | **0.97** | 1.00 | **0.80** | **0.93** | 1.00 | 1.00 | 0.91 |
| IT1 | **0.92** | 0.93 | **0.95** | **0.95** | 0.76 | **0.93** | 0.91 | 0.82 | 0.90 | 0.95 | 0.96 | 0.87 |
| IT2 | **0.92** | 0.96 | 1.00 | 1.00 | 0.78 | **0.98** | 1.00 | **0.85** | 0.95 | 1.00 | 1.00 | 0.94 |
| UR1 | **0.92** | 0.94 | **0.96** | 0.96 | 0.78 | **0.95** | 0.94 | **0.84** | **0.92** | **0.96** | 0.97 | 0.89 |
| UR2 | **0.94** | 0.96 | **0.98** | 0.98 | 0.80 | **0.96** | 0.97 | 0.86 | **0.94** | **0.98** | 0.98 | 0.92 |
| CAN | | 0.11 | **0.90** | **0.85** | 0.68 | **0.88** | 0.85 | **0.75** | **0.84** | **0.90** | 0.89 | 0.79 |
| DP | **-** | | 0.95 | 0.93 | 0.57 | 0.93 | 0.89 | 0.68 | 0.88 | 0.95 | 0.93 | 0.79 |
| DA | **-** | **-** | | 1.00 | **0.56** | **0.94** | 1.00 | 0.69 | 0.88 | **1.00** | 1.00 | 0.83 |
| CQ | **-** | **-** | **-** | | 0.61 | 0.96 | 1.00 | 0.73 | **0.90** | 1.00 | 1.00 | 0.87 |
| CG | **-** | **-** | **-** | **-** | | **0.45** | 0.05 | 0.26 | 0.45 | 0.56 | 0.70 | 0.14 |
| RO | **-** | **-** | **-** | **-** | **-** | | -0.3 | **0.67** | **0.82** | **0.94** | 0.98 | 0.76 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SL | - | - | - | - | - | - | | 0.46 | 0.79 | 1.00 | 1.00 | 0.57 |
| QU | - | - | - | - | - | - | - | | 0.59 | **0.69** | 0.82 | 0.38 |
| CAT | - | - | - | - | - | - | - | - | | 0.86 | 0.95 | 0.63 |
| CAV | - | - | - | - | - | - | - | - | - | | 1.00 | 0.83 |
| UYT | - | - | - | - | - | - | - | - | - | - | | 0.90 |

**Appendix S8** Bayesian time-calibrated tree of the cytochrome *b* gene from ctenomyids and other caviomorph species. Calibration points (Table 1) are indicated by yellow circles. The Bayesian posterior probabilities were given for nodes of phylogenetic groups with values above 0.8. Sub-tree with haplotypes of *Ctenomys torquatus* is also shown. Localities names for codes on Appendix S1 and Figure 1. Bottom rule is the divergence dates (in million years before the present) from the relaxed uncorrelated lognormal clock analysis. Group names are the same proposed by Parada et al. (2011).



Continuation Appendix 8:



**CAPÍTULO III**

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# NOVELTY AND RARITY IN THE SOUTH BRAZIL PAMPA'S BIOME: THE SANDY LAND TUCO-TUCO *CTENOMYS IBICUIENSIS* (RODENTIA) WAS DESCRIBED ALREADY ENDANGERED.

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Manuscrito a ser submetido à revista Conservation Genetics.

**Novelty and rarity in the south Brazil Pampa's biome: the sandy land tuco-tuco Ctenomys ibicuiensis (Rodentia) was described already endangered.**

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**Abstract** Very small population or very restricted geographic distribution is one of the five IUCN criteria used to determine whether a taxon is threatened or not. Rarity can be a natural aspect of species biology or caused by habitat reduction and loss, especially due anthropogenic actions. The subterranean rodents of the genus *Ctenomys* are characterized by small effective population size, habitat specialization due the underground life style and the majority of species have restricted geographic distribution, which are the three main factors accounting for rarity. The recently described tuco-tuco, *C. ibicuiensis,* is one of the most geographically restrict species of tuco-tucos, been endemic to patches of sandy soil in an area around 540km$^2$ in southern Brazilian grassland. Habitat loss and fragmentation due to silviculture, pastures and desertification are the most pervasive threats to this region. Endemic species are commonly hypothesized to have little genetic variation because of inbreeding, bottlenecks, besides the proneness to lose variability when submitted to stochastic events. We investigated genetic variation in *C. ibicuiensis* through 14 microsatellite loci and two mitochondrial DNA sequences in 34 individuals distributed along its geographic range. Overall, low nucleotide diversity ($\pi$ = 0.0047) and mean number of microsatellite alleles per locus (3.26) were found, besides high and significant values of population differentiation. There were no evidence of genetic bottlenecks

and inbreeding. Nevertheless, the endemism and low dispersal ability of these rodents, associated with the highly impacted grasslands of that region, suggest concerns about the conservation status and the evolutionary destine of *C. ibicuiensis*.

**Keywords** subterranean rodent, endemism, Pampa biome, habitat specialization.

**Introduction**

Fragmentation and isolation due habitat reduction and modification has decreased the population size of several species of flora and fauna throughout the world, especially as a consequence of human civilization development and over exploration of the natural resources. In the remaining habitat patches, gene flow can be hampered among isolated populations, which generally became highly differentiated. However, the most worrying effects of habitat loss are those of stochastic nature, such genetic drift and inbreeding, which can result in low genetic variability and reduce the evolutionary potential of populations (Allendorf and Luikart 2007).

To the subterranean rodents of the genus *Ctenomys* (tuco-tucos), patchy distribution is a typical trait. The burrow system habitat, associated to territoriality and the solitary behavior for most species, lead to limited dispersal abilities, low genetic variation within populations and high inter-population divergence (Lacey 2000). Under such circumstances, genetic drift is an important process shaping populations and it has been particularly associated to the fixation of chromosomal rearrangements for tuco-tucos, one of the most karyotypically diverse clades of mammals known, with diploid number ranging from 2n = 10 in *C. steinbachi* to 2n = 70 in *C. pearsoni*, besides several cases of intraspecific variation (Cook et al. 1990; Lopes 2011, Mirol et al 2010; Ortells 1995; Reig et al. 1990).

The particular fragmented distribution in small demes of these rodents seems not to be a constraint to genus evolution as a whole. Instead, it is behind the mechanisms of speciation (Steinberg and Patton 2000). The genus *Ctenomys* has been considered an example of explosive speciation due the high species richness (about 60 species, Parada et al. 2011; Reig et al. 1990) achieved since its origins and diversification in the late Pliocene (Verzi 2010); and it is widely distributed throughout the southern half of the South America, from the coastal of the Atlantic Ocean (sea level) to the Andes, where elevation is above 4000m (Lacey et al 2000; Reig et al. 1990).

Nowadays, nevertheless, these demographic attributes associated to the increasingly environmental destruction by human impact may subject tuco-tuco species to population decline, mostly taking into account endemic species with small geographic distributions as *C. flamarioni* (Fernandéz-Stolz et al. 2007), *C. australis* (Mora et al. 2006), *C. sociabilis* (Chan and Hardy 2011) and *C. lami* (Freitas 2001). The sand-dune habitat occupied by *C. flamarioni* and *C. australis* constrains theirs distribution to a narrow linear range along the Brazilian and Argentinean coasts, respectively, and have been affected by urbanization (Fernandéz-Stolz et al. 2007; Mora et al. 2010). Both species are assigned as "endangered" in the IUCN red list of threatened species and *C. flamarioni* have been included as "vulnerable" in the National List of Endangered Species of the Brazilian Fauna (Machado et al. 2008) and in the List of Endangered Fauna in Rio Grande do Sul State (Fontana et al. 2003).

Populations of *C. lami* are geographically restricted to an area of 78 x 12 km in the most inland region of the southern Brazilian coastal plain, known as Coxilha das Lombas, which has been intensively modified by agriculture, pastures and human occupation (Fernandes et al. 2007; Freitas 2001; Lopes 2011). This species was recently included as "vulnerable" in the IUCN red list of threatened species, and Lopes and Freitas (in press) highlighted the vulnerability of *C. lami*, suggesting the designation of an evolutionarily significant unit (ESU) for conservation purpose. Likewise, *C. sociabilis* is listed as Critically Endangered in the IUCN Red list because actually all individuals are in one restrict area (about 1400 $km^2$) within the Reserva Nacional del Parque Nacional Nahuel Huap, Argentina, and it has an historic of population declining (Bidau et al. 2008; Chan and Hadly 2011).

A new tuco-tuco species was recently described for the southern Brazil grasslands, the so called Pampa biome. *Ctenomys ibicuiensis* has an impressive narrow geographic range, been restrict to 6 registered sites (Figure 1) on west of the Rio Grande do Sul (RS) State (Freitas et al. in press). Just like *Ctenomys* species cited above, this tuco-tuco has its limited distribution threatened by human impact, mainly associated with land use for cattle, agriculture and the replacement of the native grassland landscape for exotic trees like *Pinus* sp. and *Eucalyptus* sp. (Overbeck et al. 2007; Roesch et al. 2009). Moreover, there is an increase in the number of areas undergoing sandy patch process nowadays in southwest of the RS State (Suertegaray et al. 2011), on the vicinity of the *C. ibicuiensis* occurrence.

Endemic species are commonly hypothesized to have little genetic variation because of inbreeding, bottlenecks and other factors that, associated with habitat fragmentation, could impair theirs

evolutionary potential (Frankham et al. 1999; Lande 1995). In order to assess the variability of the endemic *C. ibicuiensis*, the main goal of the present study was to estimate its genetic diversity and structure through microsatellite loci and mitochondrial sequences analysis, in a comparative framework with the allopatric and widespread species *C. torquatus.* We also argued about the ecological and historical factors accounting for its rarity and how to ensure the maintenance of this species regarding threats of environmental changes.

**Material and Methods**

Sample collection

Samples used here were the same applied for the *C. ibicuiensis* description work (Freitas et al. in press). The sampling period was between 2007 and 2011, when the westernmost region of the Pampa was searched out in order to delimit the geographic range of the new specie (Figure 1). Individuals were caught with Oneida Victor Nº 0 snap trap protected with rubber strips to avoid injury. Samples were obtained as freshly preserved (96% ethanol) amounts of tissue, being liver from sacrificed individuals and ear-skin from those released. All procedures with animals followed the American Society of Mammalogists guidelines (Sikes et al. 2011). The geographic coordinate of each individual burrow was taken.

Laboratory procedures

Total DNA was extracted from each sample following the protocol of Medrano et al. (1990). On the basis of the microsatellite assay for *C. torquatus* (Roratto et al. in prep), the 14 most polymorphic microsatellite loci were applied for *C. ibicuiensis* populations. They were previously described for species *C. haigi* (*Hai* 3 and *Hai*12 – Lacey et al. 1999), *C. sociabilis* (*Soc*1, *Soc*2, *Soc*4 and *Soc*5 – Lacey 2001) and *C. torquatus* (*Tor*1, *Tor*2, *Tor*4, *Tor*5, *Tor*6, *Tor*7, *Tor*8 and *Tor*9 – Roratto et al. 2011). The PCRs were carried out in a volume of 20µl following procedures of Wlasiuk et al. (2003) for *Hai* and *Soc* loci and Roratto et al. (2011) for *Tor* loci. All forward primers were stained with HEX or FAM fluorescence. PCR products were sent for genotyping at Macrogen Inc. (Korea) with the GeneScan 400HD ROX size standard (Applied Biosystems).

Phylogeographic analysis were based on the hyper variable sequence 1 (HVS) from control region using primers TucoPro (Tomasco and Lessa 2007) and TDKD (Kocher et al. 1989) and the cytochrome *c* oxidase I (COI) gene of the mitochondrial DNA (mtDNA) using LCO-1490 and HCO-2198 primers (Folmer et al. 1994). Polymerase Chain Reactions (PCR) for the whole C. ibicuiensis sample (N = 34) were performed in a reaction volume of 20 µl containing 20-80 ng of DNA, 0.2 µM of each primer, 0.2 mM dNTP, 1x PCR buffer, 4 mM MgCl2 for HVS1 and 2.5 mM for COI and 1.0 unit of Taq DNA polymerase (Invitrogen). Cycling consist of 94ºC for 1 min, followed by 35 cycles of 30 s at 94ºC, annealing (30 s at 48°C for HVS and 1 min at 50°C for COI), 1 min at 72°C and a final extension at 72ºC for 5 min. PCR products were checked on agarose gel stained with ethidium bromide, purified using Exonuclease I and Shrimp Alkaline Phosphatase (GE Healthcare) performed following the guidelines of the suppliers and sent for sequencing at Macrogen Inc. (Korea), using the forward primer.

Microsatellite loci statistical analysis

Electropherograms were analyzed with Peak scanner software (Applied Biosystems) to define the allele's size. The program MICROCHECKER version 2.2.0 (Van Oosterhout et al. 2004) was used to detect null alleles, any genotyping errors (including typing mistakes) and large allele dropout.

Two Bayesian MCMC approaches were used in order to find the distinctiveness of populations through clustering of individual genotypes. For the program Structure 2.3.2 (Pritchard et al. 2000), ten independent runs without the locality information were performed for each K, which ranges from 1 – 8, considering the 5 sampled localities. Runs were performed with $1 \times 10^6$ MCMC iterations, a burn-in of $5 \times 10^5$, the independent allele frequencies model and assuming admixture. The optimal K was determined based on the Pritchard et al. (2000) methodology, considering the highest mean value of estimated logarithm of probability of the data [LnPr(X/K)], and the lowest standard deviation (SD) among the independent runs for each K. The graphical display of the STRUCTURE results was generated using DISTRUCT software (Rosenberg 2004). The GENELAND 3.3 (Guillot et al. 2005) software incorporates the spatial coordinates of specimens, besides the genotypic data, as it takes into account the fact that differentiated populations tend to be structured in spatially distinct areas. We first infer K (ranging from 1 – 8) performing five independent runs of $1 \times 10^6$ iterations saved at every 100, uncorrelated allele frequencies and the uncertainty attached to spatial coordinates fixed to 500 m

(taking into account the low vagility of tuco-tucos and the spatial scale of the burrow systems). We then infer the assignment of individuals for the k fixed previously in a new round of five runs and the same parameters above. The posterior probability of population membership for each pixel of the spatial domain was computed for each run using a burnin of 1000 iterations and following the manual recommendations (http://www2.imm.dtu.dk/~gigu/Geneland/Geneland-Doc.pdf).

On the basis of the genetic clusters results, diversity measures as well as deviations from Hardy–Weinberg equilibrium (HWE) and inbreeding coefficient ($F_{IS}$) for each population and tests for linkage disequilibrium (LD) across all pairs of loci were conducted using ARLEQUIN 3.1 (Excoffier and Schneider 2005), with a strict Bonferroni correction applied for multiple comparisons (Rice 1989). Population differentiation was assessed with pair-wise $F_{ST}$ (Weir and Cockerham 1984) and the effective numbers of migrants per generation according to Slatkin (1995). Analyses of molecular variance (AMOVA) were first computed for all microsatellite data set without a priori groups and including the individual level ($F_{IT}$), and then separately for sets of localities on both banks of the Itu River, being (L1, L2 and L3) from south bank and (L5 and L6) from north bank (see Figure 1), considering that this River seems the only great watercourse crossing the *C. ibicuiensis* range. The gene flow, $F_{ST}$ estimates and AMOVA were performed in ARLEQUIN 3.1 (Excoffier and Schneider 2005) with 10000 random permutations to test the values for significance.

The program Alleles in Space (Miller 2005) was used to perform spatial autocorrelation analyses. Geographic distances are calculated between pair of individuals on the basis of its geographic coordinate. The measure of autocorrelation used for analysis (Ay) was quantified as the average genetic distance between pairs of individuals that fell into distance class y. Five and ten distance classes were applied and 5000 random replicates were used to identify distance classes where average genetic distances were significantly larger or smaller than random expectations.

In order to compare the indexes of genetic diversity for *C. ibicuiensis* and the allopatric species *C. torquatus*, the original genotypic data set of this species, containing 22 microsatellite loci from Roratto et al. (in prep), was re analyzed using the same14 microsatellite loci surveyed for *C. ibicuiensis*.

mtDNA statistical analysis

Sequence electropherogram were visually inspected using CHROMAS 2.33 (Technelysium Pty Ltd), aligned and edited using the CLUSTALW algorithm implemented in MEGA 5.0 (Tamura et al. 2011).

The genetic diversity at the mtDNA level was calculated as the number of polymorphic sites, the number of haplotypes, haplotype diversity and the nucleotide diversity (π) as the average number of nucleotide differences per site between two sequences (Nei 1987), which were calculated with DNASP 5.0 (Librado and Rozas 2009). We also calculated $F_{ST}$, gene flow and performed the AMOVA as described for microsatellite loci, considering groups of populations separated by the Itu River. Three haplotype networks were constructed (HVS, COI and the both data set concatenated) to examine the evolutionary relationships between mtDNA haplotypes, and theirs geographic localization. The phylogenetic relationships between the mtDNA haplotypes were calculated by the median-joining method using the program NETWORK 4.6.0.0 (http://www.fluxux-engineering.com). Spatial autocorrelation analysis was performed for the concatenate mtDNA as described for the microsatellite data.

Demographic history inferences

Bottlenecks leave a genetic signature in populations noticed by the loss of low-frequency alleles and an increase in relative abundance of intermediate and high-frequency alleles. In order to infer the occurrence of a recent bottleneck event, the microsatellite data set was submitted to the program BOTTLENECK (Cornuet and Luikart 1996), using only samples of localities L1 and L5+L6 that have more than 10 individuals. The two-phase model (TPM) (DiRienzo et al. 1994) and the stepwise mutation model (SMM) (Kimura and Ohta 1978) of microsatellite evolution were tested. Parameters for the TPM were firstly tested with the default set at 70% single step mutations and then at 90%, as suggested for microsatellite data (Cornuet and Luikart 1996). Only the Wilcoxon sign-rank test of heterozygote excess provides sufficient power for our number of loci and individuals.

To test for deviation of the mtDNA sequence variation from evolutionary neutrality, the Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) statistics were performed using ARLEQUIN 3.11 (Excoffier and Schneider 2005). Negative values of these statistics can be attributed to positive selective sweeps, recent population growth, or background selection. Besides, the software DNASP 5.0 (Librado and Rozas 2009) was used to detect occurrence of past events of population expansion, employing mismatch distribution analysis (Rogers and Harpending 1992).

Two Bayesian coalescence frameworks were also used to simulate the demographic history of the *C. ibicuiensis* populations. The Bayesian skyline (Drummond et al., 2005) and skyride plots (Minin et al.

2008), as implemented in the software BEAST 1.6.1 (Drummond & Rambaut 2007), recover the effective population size dynamics over time in a coalescence-based estimative. Analyzes were performed employing a strict molecular clock with the substitution rates for COI and HVS sequences estimated by Roratto et al. (in prep) as normal priors. Models of molecular evolution were the Hasegawa-Kishino-Yano model (HKY) with a proportion of invariable sites (I) and the correction gamma value (G) for the HVS and the Tamura-Nei model (TrN) + (I) for the COI sequences, all selected by the Akaike Information Criterion (AIC) implemented in Modeltest version 3.7 (Posada and Candrall 1998). Chain length for MCMC sampling was 30 million generations, sampling every 1,000 generations and the first 10% was discarded as burning. We use the computational resources of Bioportal from University of Oslo (https://www.bioportal.uio.no). The program TRACER 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used for the skyline and skyride reconstructions, to analyze the parameter distributions estimated from BEAST and check for convergence of the chains, taking into account that the Effective Sample Size (ESS) values for all parameters be greater than 200. Runs were summarized in TreeAnnotator 1.5.4 (Drummond & Rambaut 2007).

The time to most recent common ancestor (tMRCA) of *C. ibicuiensis* was estimated through Bayesian phylogenetic analysis that co-estimate divergence time. The phylogeny using cytochrome *b* (cyt *b*) sequences of *C. ibicuiensis* and other ctenomyids in Freitas et al. (in press) was reconstructed incorporating the molecular rate for this gene, estimated by Roratto et al. (in prep). The same sequences data set, evolutive model and parameters of Freitas et al. (in press) were used with program BEAST. Molecular rate 0.0208 substitutions/site/my (standard deviation 0.000067) for the cyt *b* gene (Roratto et al., in prep) was used as normal prior.

**Results**

No evidence for scoring error due to stuttering, large allele dropout or null alleles was reported for the 14 microsatellite loci surveyed. All of them were polymorphic (Table 1) and the total number of alleles per locus range from 4 (loci *Tor*6 and *Soc* 1) to 13 (locus *Tor*8). Exact tests of genotypic linkage disequilibrium yielded no significant values ($P > 0.0005$, after Bonferroni correction), suggesting that loci are independent. Significant positive departures from Hardy–Weinberg equilibrium was found only to locus *Tor*8 for the cluster L5+L6 ($P = 0.015$), but it was not significant after Bonferroni Correction (corrected P-value = 0.0035).

The Bayesian analysis using Structure and Geneland clearly identified the presence of four clusters (Figure 2) that correspond to sample collections L1, L2, L3 and the northernmost localities L5 and L6 clustered together. Individual assignment did not vary among 10 independent simulations of Structure and the results were also concordant for the 5 independent Geneland runs. The proportion of individual genotypes (*q*) belonging to each of the respective inferred cluster was always high (*q* > 0.91). On the basis of the genotypic assign results, the L5 and L6 sites were considered as one population for all the other analysis, represented as L5+L6.

The concatenate mtDNA sequences for *C. ibicuiensis* presented a total of 21 variable sites, which define 12 haplotypes (see Appendix S1 in Supporting Information; GenBank accession numbers JQ389074-JQ389081 for COI and JQ389108-JQ389116 for HVS). Values of haplotype diversity were moderate to high, but the nucleotide diversity was low for the two mitochondrial markers and the concatenate data set (Table 2). Genealogical relationships among haplotypes were concordant for the distinct markers and the concatenate data and showed spatial structure (Figure 3 and Appendix S2). In general, all haplotypes were limited to single populations, except $H5_{HVS}$ and $H6_{COI}$ which were shared between localities L3 and L5+L6. The four individuals of site L2 had always the same haplotype whilst the five individuals of site L3 were polymorphic for HVS (Appendix S1 and S2). Distinct haplotypes from the same locality diverged by low mutational steps, except the H4 of the concatenate data, from L1 (Figure 3). This haplotype was found for individual TR1075 that showed the divergent $H3_{HVS}$ and the $H4_{COI}$. However, this sample had the H4 for cyt *b* (Freitas et al. in press) that was not so divergent from the others of the same locality (Appendix S2). The Appendix S1 in the Supporting Information shows the complete list of HVS and COI for each locality.

The differentiation analysis among populations pair by pair indicates high and significant $F_{ST}$ values for microsatellite data, ranging from 0.24 to 0.42, and over 0.39 for the concatenate mtDNA data (Table 3). Pairwise estimates of gene flow based on sequence data showed lower values than those of microsatellite data (Table 3). A hierarchical analysis of variance (AMOVA) showed substantial subdivision among *C. ibicuiensis* populations, mostly for the mtDNA concatenate data. Regions separated by the Itu River did not provide significant source of variation for both molecular markers (Table 4). Spatial autocorrelation analysis confirm the high population differentiation, suggesting that the extent of spatial phylogeographic structure in *C. ibicuiensis* occurs in the order of approximately 15 km considering five distance classes (Figure 4a) and 10 km for ten distance classes (Figure 4b), for

both microsatellite loci and mtDNA concatenate data. Despite the concordance of the geographic distance threshold for both molecular markers, the mitochondrial data set yielded higher Ay value (0.94) than microsatellite loci (0.64).

Genetic signatures of bottlenecks for L1 and L5+L6 localities were not observed for any of the models of mutation analyzed. The distribution of allele frequencies for *C. ibicuiensis* was clearly L-shaped, with the largest proportion of alleles at low frequencies (0.01–0.20) for both localities tested. Estimates of intrapopulation structure calculated using the inbreeding coefficient ($F_{IS}$) indicated low and no significant values for all localities (Table 1).

The Tajima's D and the Fu'Fs tests performed did not show departures from strict neutrality (Table 2), as well as the multimodal mismatch distribution graphs for the two mtDNA markers (Figure 5), which supports a population equilibrium scenario. Likewise, the Bayesian skyline and skyride plots showed little change in effective population size for *C. ibicuiensis* over time (Figure 6) since its origins. The estimated time to most recent common ancestor (tMRCA, in million years) revealed divergence times of 0.15 mya (95%CI: 0.08 – 0.22) and 0.11 mya (95%CI: 0.06 – 0.16) for the modern *C. ibicuiensis* mtDNA lineages, from the skyline and the skyride analysis, respectively. The ESS values were over 700 for all parameters estimated and convergence chains were reached for both approaches.

The cyt *b* phylogenetic tree showed the same relationships among species showed in Freitas et al., in press (data not shown). The estimated tMRCA of *C. ibicuiensis* was 0.31 mya (95%CI: 0.14 – 0.53) and the divergence between *C. ibicuiensis* and *C. torquatus* was 1.6 mya (95%CI: 1.08 – 2.33).

## Discussion

### Range restriction

Rabinowitz (1981) proposed a model to explain rarity of species based on three characteristics: the local population density, the area of the species range and the number of different kinds of habitats that species occupy, known as Rabinowitz's '7 forms of rarity'.

Yu et al. (2000) adapted such model to examine rarity and commonness of a large sample of species from the mammalian fauna of the world and found that this group exhibits a strong bimodal pattern of many relatively common and rare species. In spite of the macroecological framework (considering that "kinds of habitats" where generalized to forest, woodland, scrub, savanna, steppe, desert, and

aquatic), authors considered the 'niche-based' hypothesis (Brown 1984; 1995) to explain such bimodal patterns. The Brown's hypothesis suggest that generalist species should be widespread and abundant because of the environmental flexibility, whilst the specialists ones should be geographically restricts because their narrow conditions of suitable habitat generally are thinly spread in nature.

When thinking about the subterranean niche, crucial characteristics such as the soil porosity, drainage and vegetation impose selective pressures on herbivorous mammalian like tuco-tucos (Busch et al. 2000). Underground rodents hardly subdivide their subterranean niche. Except few situations of marginally overlapping range, it is found one species of subterranean rodent in a given area of habitable soil (Lessa 2000; Pearson 1959).

Brown et al. (1996) invoked two classes of processes, not mutually exclusive, shaping the geographic range of species. Besides the niche limitations by environmental variables, they suggest dynamic processes of colonization and extinction (and sometimes speciation) accounting for patterns of range size. Such processes are common to subterranean rodents due their metapopulation model of geographic structure (Steinberg and Patton 2000).

Thus, underground life style naturally provides demographic and ecological characteristics that constrain geographic range of species. Variation of these factors or combination of them can explain rarity of tuco-tuco species as those previously cited and the new endemic *C. ibicuiensis*, that has a range around 540 $km^2$ restrict to sandy soils in the southwestern grasslands of the RS State.

Genetic variability and demography

As it was expected due small geographic range and, consequently, the reduced sampling of *C. ibicuiensis*, levels of microsatellite and mtDNA variability found in the present work were low. Twelve haplotypes, with a diversity of 0.88, was found for the concatenated mtDNA data, but the nucleotide diversity was low (0.0047). When we compare the nucleotide and haplotype diversity index only for the control region, which has been data published for other ctenomyids in the literature, values for *C. ibicuiensis* are as low as those of tuco-tuco species with restrict geographic range, like *C. lami*, *C. sociabilis* and *C. australis* (Table 5). *Ctenomys flamarioni*, besides restrict and linear range, has the lowest values due habitat instability and bottleneck effects (Fernández-Stolz et al. 2007). On the other hand, despite its wide geographic range, large sampling and high haplotype diversity, the low

nucleotide diversity of *C. torquatus* probably concerns the recent population expansion described for this species (Roratto et al. in prep).

The mean numbers of microsatellite alleles per locus (3.26) of *C. ibicuiensis* was inferior to most other ctenomyid species, been comparable with values for *C. sociabilis*, *C. roigi* and *C. torquatus* (Table 5). The low variability found in *C. sociabilis* may be due social behavior and historical population reduction whereas *C. roigi* sample constitute a single population (see references in Table 5); but these should not be the cases for *C. torquatus.* Nevertheless, even with low mean number of alleles per locus, the microsatellite data set was enough to discriminate 14 clusters for this widespread tuco-tuco (Roratto et al. in prep). Likewise, four clusters where found for *C. ibicuiensis* that closely fit the geographical distribution of the localities (Figure 2). Only individuals of localities L5 and L6 clustered together, which was expected considering their geographical closeness (Figure 1). Moreover, a significant proportion of the variability was explained by the among-population comparison in AMOVA (35.12%, $P < 0.001$) performed with microsatellite data. The higher source of variation was at the individual level and the Itu River did not significantly structure the molecular variation for both microsatellite loci and mtDNA markers (Table 4).

The inter population differentiation was even further glaring with the mtDNA concatenate AMOVA (Table 4), low haplotype sharing (Figure 3), high genetic distances measured by pairwise $F_{ST}$ values and consequently low estimative of gene flow (Table 3). Although both haplotype and allele frequencies data results showed strong genetic structure among *C. ibicuiensis* populations, the higher differentiation provided by mtDNA sequences can be attributed to the matrilineal heritance of this marker associated with evidences of female phylopatry in ctenomyids (Cutrera et al. 2005; Fernández et al. 2007; Lacey 2001; Lopes 2011). The spatial autocorrelation analysis clearly indicated the presence of strong phylogeographic structure between sampled sites, which reflects the influence of relatively short geographic distances (between 10 and 15 km) structuring populations of these subterranean rodents (Figure 4).

Low genetic variability within populations and high genetic differentiation among tuco-tuco populations is recurrently observed and it is expected due the propensity of these species to be distributed into many, relatively small and no continuously distributed demes (Lacey et al. 2000; see references in Table 5). In addition, historical demographic events such as recent colonization, population expansion or reduction (bottleneck), behavior (solitary versus social) and habitat instability has been reported to

shape spatial and temporal variability for tuco-tuco species (Chan and Hadly 2011; Fernández-Stolz et al. 2007; Lacey 2001; Lopes 2011; Mirol et al. 2010; Mora et al. 2006).

Results of the present study did not find a signature of population bottleneck for *C. ibicuiensis*, on the basis of changes in microsatellite allele frequencies, neither indication of recent expansion according to the multimodal mismatch distribution analysis (Figure 5) and non significant neutrality tests (Table 2). The tMRCA of the present lineages of *C. ibicuiensis* was relatively recent, dating from the mid to late Pleistocene, but a pattern of population stability was recovered from the Bayesian skyline and skyride plots, showing little changes in the effective population size over time, despite large confidence intervals (Figure 6). Therefore, *C. ibicuiensis* is a geographically restrict tuco-tuco species and its rarity apparently could not be attributed to any historical demographic event.

That was not the situation for widespread and neighboring collared tuco-tuco, *C. torquatus*. The tMRCA for this species was dated around 0.51 mya (95%IC 0.27 – 0.80 mya) and it exhibits an historic of recent population expansion throughout the half south of the RS State and north Uruguay (Roratto et al. in prep), surrounding the *C. ibicuiensis* range. Both species belongs to the same phylogenetic group (the *torquatus* group, following Parada et al. 2011) but they diverged early, approximately 1.6 mya (see Figure S2 in Roratto et al., in prep).

Considering the ancient divergence between these species and the probable phylogeographic origin of the *C. torquatus* mtDNA lineages from the central region of the RS State (Roratto et al. in prep), their neighboring around the Ibicuí River seems rather a secondary contact zone or proximity than a primary resource for allopatric vicariance or peripheral isolation in a process of speciation.

The contrasting patterns of range and demography lead us to discuss about which intrinsic characteristics and evolutionary factors would be shaping the trajectory of these species up to now, as well as how it can influence the existence and survival of the rare *C. ibicuiensis* facing the present and future environmental changes and threat.

Phylogeographic history of tuco-tucos in Pampa's biome

Before try to explain such differences, it is important to keep in mind that a single measurement of the present genetic diversity would not accurately reflect the variability over longer time periods, as it was demonstrated for *C. sociabilis* using fossil records haplotypes (Chan and Hadly 2011). Unfortunately, we do not have knowledge about any archaeological site harboring *Ctenomys* samples in south Brazil

up to now. Anyway, the Chan and Hadly (2011) study provide a comparative framework about differences in rarity and behavior of two parapatric species – *C. sociabilis* and *C. haigi*, and the way these specific characteristics result in contrasting demographic histories.

Both, *C. torquatus* and *C. ibicuiensis* are solitary species. Then, sociality is not a determining factor explain their differences as it was for *C. sociabilis* and *C. haigi* (Chan and Hardy 2011; Lacey 2001). In spite of *C. torquatus* has a bigger body size than *C. ibicuiensis* (Freitas et al. in press) the hypothesis of competition between both species seems not probable, mainly because without having fossil evidences we are not able to infer whether both species had contact. If so, it probably would be at the moment that *C. torquatus* expansion reached surrounds of the *C. ibicuiensis* range, but still there is the Ibicuí River detaching the occurrence of these species nowadays. Moreover, we did not find evidence of population decline in *C. ibicuiensis*.

Past environmental changes in the Pampean region could not have influenced demography and genetic variability of *C. torquatus* and *C. ibicuiensis*. Both species inhabit the grasslands of the southern half of the RS State in Brazil, the so-called Pampa Biome in the Brazilian biome classification (IBGE 2004). After the extensive marine transgressions of the Tertiary (Rambo 2005), this region got through cold and dry climatic conditions during the ice age, which allowed the grassland predominance since the Pleistocene (Behling 2002). As suggested for the Argentinean armadillo *Chaetophractus villosus* in southern Patagonia (Poljak et al. 2010), the fossorial habit, that enable thermal regulation, would allow survive and extend of individuals in areas with adverse climatic conditions, mainly a grassland recovered region ideal for tuco-tucos such as these southern Brazilian open areas inhabit by *C. ibicuiensis* and *C. torquatus*. Pollen analysis performed through peat deposit core in the city of São Francisco de Assis (see Figure 1), 5 km north from the Ibicuí River, showed high abundance of grass pollen (over 90%) since the full-glacial age in this region, with subtropical gallery forests developed along rivers only since mid Holocene times and expanded during the last 1500 ya, reflecting a change to wetter conditions (Behling et al. 2005).

Thus, the predominant grassland and probably suitable habitat for the underground life style, even during the cold and dry periods, may have been the ideal conditions for the expansion of the collared tuco-tuco throughout its wide geographic range in the Pampean region. The contrasting scenery of demographic stability for the endemic and highly differentiated *C. ibicuiensis* populations should be related with other evolutionary factors.

An important ecological aspect of subterranean rodents is the habitat specialization, mainly regards kind of soil (Busch et al. 2000). The grater preference of *C. sociabilis* for the wet meadows habitats was suggested to cause the high genetic structuring of its populations and restrict geographic range, as a consequence of its reduced ability to deal with environmental changes through time such as volcanic eruptions and shifting of steppe habitat during the last glacial maximun (Chan and Hardy 2011). A broader field knowledge is needed in order to infer whether the high structuring and rarity of *C. ibicuiensis* is related with habitat specialization, particularly considering that the few sampling sites recorded for this sandy soil tuco-tuco were exactly sandy land sites (Freitas et al. in press), while the widespread *C. torquatus* was sampled in a great variety of sandy-like soils, with different degrees of hardness (personal communication).

A demographic pattern of long time stability was recovered for populations of *C. porteousi*, occuping a restrict area in central west Buenos Aires province – Argentina, during the cold and dry climatic conditions of the last glacial maximum (Mapelli et al. 2012b). The highly specific habitat requirements of this species, consisting of reduced vegetation cover on sand dunes (Mapelli and Kittlein 2009), would have been larger and less fragmented during this period. Porteous tuco-tuco's habitat specialization is so crucial that shrinkin of this typical habitat during the moister and warmer Holocenic climate would be responsible for its population decrease (Mapelli et al. 2012b). *Ctenomys ibicuiensis* seems likewise highly associated with sand soils, but widely covered by grass. Considering that vegetal coverage did not change substantially in this region since *C. ibicuiensis* origins in the middle Pleistocene (Behling et al. 2005), we could suppose the restrict habitat requirements associated with the grassed sandy lands as justification for the rarity and stability of *C. ibicuiensis*.

Unlike historic perturbation events suffered by *C. sociabilis* (Chan and Hardy 2011), there is no register of drastic geological events for the lowlands inhabit by *C. ibicuiensis* and *C. torquatus* since the Brazilian Plateau formation in the Mesozoic (Rambo 2005). In order to evaluate whether the recent lineages of both species show distinct geographic and demographic patterns due niche specialization, a refinement of the Pampa's habitat changes, in response to Quaternary oscillating climate, is needed to be merged in ecological niche modeling approach. Such approach has wisely shedding light on environmental changes effects over range and niche of species (e.g. Jezkova et al. 2011).

Implications for conservation

The south Brazilian Pampa biome and adjacent areas of Uruguay and Argentina are a mosaic vegetation of shrubland and different forest types, dominated by a high diversity of grass species, underestimated and neglected under current conservation policies as pointed out by Overbeck et al. 2007. According to Bolzon et al. (2002), the higher floristic diversity found in open areas than in sparse forests is due the relatively recent gallery forests expansion associated with the Holocenic warmer and wetter climatic conditions. The grasslands established earlier in dry and cold climate and had larger evolution time to differentiate and speciate. It is reflected also in the higher number of endemic, rare, and actually threat, species (Deble 2011). The dwarf palm *Butia lallemantii* is an interesting example of endemism for the sandy soil grasslands of the southwest RS State. It is a palm species lacking stem and its distribution almost coincides with the *C. ibicuiensis* occurrence (Deble and Marchiori, 2006). As well this endemic tuco-tuco has the related widespread species *C. torquatus* surrounding its range; the dwarf palm has the related and widespread *B. paraguayensis* species (Deble 2011), native to Brazil, Argentina, Uruguay and Paraguay. The critically endangered fish *Austrolebias ibicuiensis* is another example of a threat species endemic of the Ibicuí River basin (Machado et al. 2008). Independently of the environmental and ecological factors that lead to *C. ibicuiensis* rarity, the path distribution in small demes, low vagility and effective population size – characteristic of subterranean rodents – seems not to be a concern for the maintenance of its restrict geographic range, considering that the southwest grasslands of the RS State appeared stable since Pliocene (Behling et al. 2005). However, these fragile conditions, intrinsic to tuco-tuco populations, certainly will be endangered as human activities fragment landscape and reduce the suitability of the habitat, as it has been demonstrated for *C. flamarioni* (Fernández-Stolz et al. 2007). Despite not shown evidences of population reduction and inbreeding, *C. ibicuiensis* meet the Endangered threatened category following the IUCN criteria (IUCN Standards and Petitions Subcommittee 2011) due the small extent of occurrence and low number of locations (B1a criteria). Thus, we recommend the inclusion of *C. ibicuiensis* as threatened in the IUCN red list, the national and regional Brazilian red lists of endangered fauna.

This mammal species, added to others fauna and flora species evenly endemic and threat (Deble 2011; Pillar et al. 2009), are appealing evidences of the critical need of conservation actions of the Pampa biome in order to preserve its peculiar biodiversity and safeguard its evolution fate.

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**References**

Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell, London.

Behling H (2002) South and southeast Brazilian grasslands during Late Quaternary times: a synthesis. Palaeogeogr Palaeoclimateol Palaeoecol 177: 19-27

Behling H, Pillar VD, Bauermann SG (2005) Late Quaternary grassland (Campos), gallery forest, fire and climate dynamics, studied by pollen, charcoal and multivariate analysis of the São Francisco de Assis core in western Rio Grande do Sul (southern Brazil). Rev Paleo-bot Palynol 133: 235-248

Bidau C, Lessa E, Ojeda R (2008) *Ctenomys sociabilis*. IUCN Red List of Threatened Species Version 2011.1. http://www.iucnredlist.org. Accessed 09 December 2011

Bolzon RT, Marchiori JNC (2002) A vegetação no sul da América, perspectiva paleoflorística. Ciência e Ambiente 24: 5-24

Brown JH (1984) On the relationship between abundance and distribution of species. American Naturalist 124: 255-279.

Brown J H (1995) Macroecology. University of Chicago Press, Chicago.

Brown JH, Stevens GC, Kaufman DM (1996) THE GEOGRAPHIC RANGE: Size, Shape, Boundaries, and Internal Structure. Annu Rev Ecol Syst 27: 597-623

Busch C, Antinuchi CD, del Valle JC, Kittlein MJ, Malizia AI, Vassallo AI, Zenuto RR (2000) Population ecology of subterranean rodents. In: Lacey EA, Patton JL, Cameron GN (eds) Life Underground, the biology of subterranean rodents. The University of Chicago Press, Chicago and London, pp 181-226

Chan YL, Hadly EA (2011) Genetic variation over 10 000 years in *Ctenomys*: comparative phylochronology provides a temporal perspective on rarity, environmental change and demography. Mol Ecols. doi: 10.1111/j.1365-294X.2011.05295.x

Cook JA, Anderson S, Yates TL (1990) Notes on Bolivian mammals. 6: The genus *Ctenomys* (Rodentia: Octodontidae) in the highlands. Amer Mus Novit 2980: 1-27

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001-2014

Cutrera AP, Lacey EA, Busch C (2005) Genetic Structure in a solitary rodent (*Ctenomys talarum*): implications for kinship and dispersal. Mol Ecol 14: 2511-2523

Deble LP (2011) A Vegetação Campestre no Bioma Pampa. In: Deble LP, Deble ASO, Leão ALSO (eds) Bioma Pampa, Contribuições Científicas. Ediurcamp, Bagé, pp. 84-143

Deble LP, Marchiori JNC (2006) *Butia lallemantii*, uma nova arecacea do Brasil. Balduinia 9: 1-3

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple sequence repeat loci in human populations. Proc Natl Acad Sci USA 91: 3166-3170

Drummond AJ, and Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214

Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol 22, 1185-1192

Excoffier L, Schneider S (2005) Arlequin version 3.0: an integrated software package for Population genetics data analysis. Evol Bioinform Online 1: 47-50

Fernandes FA, Fernandez-Stolz GP, Lopes CM, Freitas TRO (2007). The conservation status of the tuco tucos, genus *Ctenomys* (Rodentia, Ctenomyidae) in Southern Brazil. Braz J Biol 67: 839-847

Fernández-Stolz G, Stolz JFB, Freitas TRO (2007) Bottlenecks and Dispersal in the Tuco-tuco das Dunas, *Ctenomys flamarioni* (RODENTIA: CTENOMYIDAE), in Southern Brazil. J Mammal 88: 935-945

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294-297

Fontana CS, Bencke GA, Reis RE (2003) Livro Vermelho da Fauna Ameaçada de Extinção no Rio Grande do Sul. EDIPUCRS, Porto Alegre

Frankham R, Lees K, Montgomery ME, England PR, Lowe EH, Briscoe DA (1999) Do population size bottlenecks reduce evolutionary potential? Anim Conserv 2: 255-260

Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. Mol Ecol 10: 2741-2752

Freitas TRO (2001) Tuco-tucos (Rodentia, Octodontidae) in Southern Brazil: *Ctenomys lami* spec. nov. separated from *C. minutus* Nehring 1887. Stud Neotrop Fauna Environ 36: 1-8

Freitas TRO, Fernandes FA, Fornel R, Roratto PA (2012) An endemic new species of tuco-tuco, genus *Ctenomys* (Rodentia: Ctenomyidae), with a restrict geographic distribution in southern Brazil. J Mammal, in press.

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915-925

Guillot G, Mortier F, Estoup A (2005) GENELAND: a computer package for landscape genetics. Mol Ecol Notes 5: 712-715

Instituto Brasileiro de Geografia e Estatıstica – IBGE (2004). Mapa da vegetacao do Brasil e Mapa de Biomas do Brasil. http://www.ibge.gov.br. Accessed 18 November 2011

IUCN Standards and Petitions Subcommittee (2011) Guidelines for Using the IUCN Red List Categories and Criteria, Version 9.0. Prepared by the Standards and Petitions Subcommittee. http://www.iucnredlist.org/documents/RedListGuidelines.pdf. Downloaded 21 November 2011

Jezkova T, Olah-Hemmings V, Riddle BR (2011) Niche shifting in response to warming climate after the last glacial maximum: inference from genetic data and niche assessments in the chisel-toothed kangaroo rat (*Dipodomys microps*). Glob Chan Biol 17: 3486-3502

Kimura M, Ohta T (1978) Stepwise mutation model and distribution of allelic frequencies in a finite population. Proc Natl Acad Sci USA 75: 2868-2872

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca F, Wilson AC (1989) Dynamics of the mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Scie USA 89: 6196-6200

Lacey EA (2000) Spatial and social systems of subterranean rodents. In: Lacey EA, Patton JL, Cameron GN (eds) Life Underground: The Biology of Subterranean Rodents University of Chicago Press, Chicago and London, pp 257-293

Lacey EA (2001) Microsatellite variation in solitary and social tuco-tucos: molecular properties and population dynamics. Heredity 86:628-637

Lacey EA, Maldonado JE, Clabaugh JP (1999) Interspecific variation in microsatellites isolated from tuco-tucos (Rodentia: Ctenomyidae). Mol Ecol 8:1753-1768

Lacey EA, Patton JL, Cameron GN (2000) Life Underground, the biology of subterranean rodents. The University of Chicago Press, Chicago and London

Lande R (1995) Breeding plans for small populations based on the dynamics of quantitative genetic variance. In: Ballou JD, Gilpin M, Foose TJ (eds) Population management for survival and recovery. Columbia University Press, New York, pp. 318-340

Lessa EP (2000) The evolution of subterranean rodents. In: Lacey EA, Patton JL, Cameron GN (eds) Life Underground, the biology of subterranean rodents. The University of Chicago Press, Chicago and London. pp. 389-420

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452

Lopes CM (2011) História evolutiva de *Ctenomys minutus* e *Ctenomys lami* na planície costeira do Sul do Brasil. Ph.D Thesis, Universidade Federal do Rio Grande do Sul.

Lopes CM, Freitas TRO (2012) Naturally patched small populations in human-impacted areas: genetic structure and conservation of tuco-tuco (*Ctenomys lami*). Journal of Heredity, in press.

Machado ABM, Drummond GM, Paglia AP (2008) Livro vermelho da fauna brasileira ameaçada de extinção 1ª Ed. MMA, Brasília

Mapelli FJ, Kittlein MJ (2009) Influence of patch and landscape characteristics on the distribution of the subterranean rodent *Ctenomys porteousi*. Landscape Ecol 24: 723-733.

Mapelli JF, Mora MS, Mirol PM, Kittlein MJ (2012a) Population structure and landscape genetics in the endangered subterranean rodent *Ctenomys porteousi*. Conserv Genet 13: 165-181.

Mapelli JF, Mora MS, Mirol PM, Kittlein MJ (2012b) Effects of Quaternary climatic changes on the phylogeography and historical demography of the subterranean rodent *Ctenomys porteousi*. J Zool 286: 48-57.

Medrano JF, Aesen E, Sharrow L (1990) DNA extraction from nucleated red blood cells. Biotechniques 8: 43

Miller MP (2005) Alleles In Space: computer software for the joint analysis of interindividual spatial and genetic information. J Hered 96: 722-724

Minin VN, Bloomquist EW, Suchard MA (2008) Smooth Skyride through a Rough Skyline: Bayesian Coalescent-Based Inference of Population Dynamics. Mol Bio Evol 25: 1459-1471

Mirol P, Giménez MD, Searle JB, Bidau CJ, Faulkes CG (2010) Population and species boundaries in the South American subterranean rodent *Ctenomys* in a dynamic environment. Biol J Linn Soc 100: 368-383

Mora MS, Lessa EP, Kittlein MJ, Vassalo AI (2006) Phylogeography of the subterranean rodent *Ctenomys australis* (Rodentia: Ctenomyidae) in sand-dune habitats: evidence of recent population expansion. J Mammal 87: 1192-1203

Mora MS, Mapelli FJ, Gaggiotti OE, Kittlein MJ, Lessa EP (2010) Dispersal and population structure at different spatial scales in the subterranean rodent *Ctenomys australis*. BMC Genetics 11: 9

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York

Ortells MO (1995) Phylogenetic analysis of G-banded Karyotypes among the South American subterranean rodents of the genus *Ctenomys* (Caviomorpha: Octodontidae), with special reference to chromosomal evolution and speciation. Biol J Linn Soc 54: 43-70

Ortells MO, Barrantes GE (1994) A study of genetic distances and variability in several species of the genus *Ctenomys* (Rodentia: Octodontidae) with special reference to a probable causal role of chromosomes in speciation. Biol J Linn Soc 53: 189-208

Overbeck GE, Müller SC, Fidelis A, Pfadenhauer J, Pillar VD, Blanco CC, Boldrini II, Both R, Forneck ED (2007) Brazil's neglected biome: The South Brazilian Campos. Persp Plant Ecol Evol Syst 9: 101-116

Parada A, D'Elía G, Bidau CJ, Lessa EP (2011) Species groups and the evolutionary diversification of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). J Mammal 92: 671-682

Pearson OP (1959) Biology of subterranean rodents, *Ctenomys,* in Peru. Mem. Mus. Hist. Nat. "Javier Prado" 9: 1-56

Pillar VP, Müller SC, Castilhos ZMS, Jacques AVA (2009) Campos sulinos: conservação e uso sustentável da biodiversidade. Instituto do Meio Ambiente e dos Recursos Naturais Renováveis, Brazil

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945-959

Poljak S, Confalonieri V, Fasanella M, Gabrielli M, Lizarralde MS (2010) Phylogeography of the armadillo *Chaetophractus villosus* (Dasypodidae Xenarthra): Post-glacial range expansion from Pampas to Patagonia (Argentina). Mol Phylo Evol 55: 38-46

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818

Rabinowitz D (1981) Seven forms of rarity. In: Synge H (ed) The Biological Aspects of Rare Plant Conservation, John Wiley & Sons, Chichester, pp. 205-217

Rambo B (2005) A fisionomia do Rio Grande do Sul: ensaio de monografia natural. Editora Unisinos, São Leopoldo.

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43: 223-225

Reig OA, Busch C, Ortells MO, Contreras JR (1990) An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. In: Nevo E, Reig OA (eds) Evolution of subterranean mammals at the organismal and molecular levels Alan R. Liss Inc., New York, pp. 71-96

Roesch LFW, Vieira FCB, Pereira VA, Schünemann AL, Teixeira IF, Senna AJT, Stefenon VM (2009) The Brazilian Pampa: A Fragile Biome. Diversity 1: 182-198

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9: 552-569

Roratto PA, Bartholomei-Santos ML, Freitas TRO (2011) Tetranucleotide microsatellite markers in *Ctenomys torquatus* (Rodentia). Conservation Genet Resour 3: 725-727

Rosenberg NA (2004) Distruct: a program for the graphical display of population structure. Mol Ecol Notes 4: 137-138

Sikes RS, Gannon WL, the Animal Care and Use Committee of the American Society of Mammalogists (2011) Guidelines of the American Society of Mammalogists for the use of wild mammals in research. J Mammal 92: 235-253

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457-462

Steinberg EK, Patton JL (2000) Genetic structure and the geography of speciation in subterranean rodents: opportunities and constraints for evolutionary diversification. In: Lacey EA, Patton JL, Cameron GN (eds) Life Underground, the biology of subterranean rodents. University of Chicago Press, Chicago and London, pp. 301-331

Suertegaray DMA, Guasseli LA, Verdum R (2001) Atlas de arenização: Sudoeste do Rio Grande do Sul. Secretaria da Coordenaçnao e Planejamento, Porto Alegre

Tajima F (1989) Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics 123: 585-595

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28: 2731-2739

Tomasco IH, EP Lessa (2007) Phylogeography of the Tuco-tuco *Ctenomys pearsoni*: mtDNA variation and its implication for chromosomal differentiation. In: Kelt DA, Lessa EP, Salazar-Bravo J, Patton JL (eds) The quintessential naturalist. Honoring life and legacy of Oliver P. Pearson. University of California Publications, Berkeley, pp 859-882.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4: 535-538

Verzi DH, Olivares AI, Morgan CC (2010) The oldest South American tuco-tuco (late Pliocene, northwestern Argentina) and the boundaries of the genus *Ctenomys* (Rodentia, Ctenomyidae). Mammal Biol 75: 243-252

Weir BS, Cockerhan CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38: 1358-1370

Wlasiuk G, Garza JC, Lessa, EP (2003) Genetic and geographic differentiation in the Rio Negro tuco-tuco (*Ctenomys rionegrensis*): inferring the roles of migration and drift from multiple genetic markers. Evolution 57: 913-926

Yu J, Dobson FS (2000) Seven forms of rarity in mammals. J Biogeo 27: 131-139

**TABLES**

**Table 1** Summary of the microsatellite variation in *Ctenomys ibicuiensis* sampled localities. Site L5 and L6 were clustered. All $F_{IS}$ values were no significant.

| Locality/Cluster | N | A | NLP | Ho | $F_{IS}$ |
|---|---|---|---|---|---|
| L1 | 12 | 3.83 | 12 | 0.56 | 0.05 |
| L2 | 4 | 2.45 | 11 | 0.45 | 0.06 |
| L3 | 5 | 3.00 | 14 | 0.65 | -0.09 |
| L5+L6 | 13 | 4.85 | 14 | 0.64 | 0.01 |
| Total/mean | 34 | 3.26 | 14 | - | |

N, number of specimens; A, mean number of alleles per locus; NLP, number of polymorphic loci; Ho, mean observed heterozygosity; $F_{IS}$, inbreeding coefficient.

**Table 2** Summary of the diversity and neutrality tests for the control region (HVS), cytochrome *c* oxidase I (COI), and the concatenate mitochondrial data set in *Ctenomys ibicuiensis*.

| | Ns | L(bp) | S | H(Hd) | π | Tajima's D | Fu's FS |
|---|---|---|---|---|---|---|---|
| HVS | 34 | 425 | 9 | 9 (0.83) | 0.0056 | 0.28 | -1.19 |
| COI | 30 | 650 | 12 | 8 (0.72) | 0.0039 | -0.53 | -0.49 |
| Concatenate | 30 | 1075 | 21 | 12 (0.88) | 0.0047 | -0.14 | -0.89 |

Ns number of samples sequenced; L(pb) length of the sequence in base pairs; S number of polymorphic sites; H(Hd) number of haplotypes and haplotype diversity in parenthesis; π nucleotide diversity. All values of neutrality tests were no significant, $p>0.05$ for Tajima's D and $p>0.02$ for Fu's FS.

**Table 3** Genetic differentiation of *Ctenomys ibicuiensis* populations. $F_{ST}$ values for pairwise comparisons of microsatellite loci are above diagonal and for concatenate mtDNA are below diagonal. The effective numbers of migrants per generation are also reported in parentheses.

| Locality | L1 | L2 | L3 | L5+L6 |
|---|---|---|---|---|
| L1 | - | 0.42 (0.34) | 0.30 (0.58) | 0.27 (0.65) |
| L2 | 0.62 (0.15) | - | **0.38** (0.40) | 0.36 (0.43) |
| L3 | 0.67 (0.12) | 0.88 (0.03) | - | 0.24 (0.79) |
| L5+L6 | 0.71 (0.10) | 0.91 (0.02) | 0.39 (0.38) | - |

All values were significant after Bonferroni Correction (corrected P-value < 0.008), except the bold value.

**Table 4** Hierarchical analysis of molecular variance (AMOVA), considering groups of populations from both sides of the Itu River, using microsatellite and mitochondrial concatenate data sets. The fixation indices (F-statistics) are shown in parentheses. Significant values are in bold.

| Hierarchical level | Microsatellite | Concatenate mtDNA |
|---|---|---|
| Among Groups ($F_{CT}$) | -5.02 (-0.05) | -22.97 (-0.22) |
| Among Populations Within Groups ($F_{SC}$) | 35.12 **(0.33)** | 94.11 **(0.76)** |
| Within Population ($F_{ST}$) | 69.90 **(0.30)** | 28.87 **(0.71)** |

**Table 5** Comparative table of indexes of genetic diversity for ctenomyid species, for microsatellite or mtDNA control region. N: number of specimens analyzed; nL: number of loci analyzed; nAL: range number of alleles per locus; *A*: mean number of alleles per locus; nH(Hd): overall number of haplotypes, and haplotype diversity; and π: nucleotide diversity.

| **Marker** | **Microsatellite** | | | | **Control region** | | | **Reference** |
|---|---|---|---|---|---|---|---|---|
| **Specie** | **N** | **nL** | **nAL** | **A** | **N** | **nH (Hd)** | **π** | |
| *C. sociabilis* | 35 | 15 | 1-3 | 2.29 | | - | - | Lacey 2001 |
| *C. rionegrensis* | 142 | 11 | 6-14 | 8.3 | | - | - | Wlasiuk et al. 2003 |
| *C. haigi* | 35 | 15 | 3-13 | 7.5 | | - | - | Lacey 2001 |
| *perrensi* group | 169 | 16 | 5-19 | 13 | | - | - | Mirol et al. 2010 |
| *C. roigi* | 12 | 16 | 1-7 | 3.31 | | - | - | Mirol et al. 2010 |
| *C. perrensi* | 21 | 16 | 2-12 | 6.75 | | - | - | Mirol et al. 2010 |
| *C. dorbignyi* | 26 | 16 | 5-11 | 7.13 | | - | - | Mirol et al. 2010 |
| *C. flamarioni* | 85 | 9 | 3-8 | 5.3 | 89 | 7 (0.790) | 0.0030 | Fernández-Stolz et al. 2007 |
| *C. talarum* | 134 | 12 | 2-9 | 6.17 | 71 | 32 (0.93) | 0.0220 | Cutrera et al. 2006; Mora et al. 2007 |
| *C. minutus* | 340 | 14 | 7-16 | 13 | 276 | 40 (0.961) | 0.0236 | Lopes 2011. |
| *C. lami* | 178 | 14 | 2-13 | 8.57 | 178 | 14 (0.871) | 0.0059 | Lopes et al. in press. |
| *C. australis* | | - | - | - | 70 | 24 (0.83) | 0.0055 | Mora et al. 2006 |
| *C. pearsoni* | | - | - | - | 98 | 21 | 0.0186** | Tomasco and Lessa. 2007 |
| *C. torquatus* | 254 | 14* | 6-15 | 3.08 | 294 | 25 (0.91) | 0.0062 | Roratto et al. in prep |
| ***C. ibicuiensis*** | **34** | **14** | **4-13** | **3.26** | **34** | **9 (0.83)** | **0.0056** | **This study** |

***** Data modified from Roratto et al. (in prep), with a reduced number of loci. **Overall nucleotide diversity estimated by us from the control region sequences downloaded from genbank.

**LEGEND TO FIGURES**

**Fig. 1** Detail of western Rio Grande do Sul State (Brazil), indicating the geographical placement of sampling sites for *Ctenomys ibicuiensis* (black points) and neighboring *C. torquatus* localities (black squares). Gray areas represent elevations above 200 meters of Brazilian Meridional Plateau, emphasizing the Serra do Iguariaçá mountain range.

**Fig. 2** Results of clustering analysis. (a) Index of MCMC iterations along the whole chain (left) and density of each number of clusters along the chain after a burning of $10^5$ iterations (right), from GENELAND. (b) Mean value of estimated logarithm of probability of the data (open circles) and the standard deviation (vertical bars) for ten STRUCTURE runs with k ranging from 1 to 8. (c) Posterior estimates of cluster membership for STRUCTURE. Labels below the graph are the sampling sites (see Fig. 1).

**Fig. 3** Median-joining network of concatenate (cytochrome *c* oxidase I and hyper variable sequence 1) mitochondrial DNA sequences of *Ctenomys ibicuiensis*. Circle size reflects the number of individuals exhibiting each haplotype (smallest = 1, largest = 7). Cross hatches represent mutation steps and red points represent medium vectors.

**Fig. 4** Results of spatial autocorrelation analysis of *Ctenomys ibicuiensis* using fourteen microsatellite loci (squares) and the concatenate HVS control region and cytochrome *c* oxidase I mitochondrial sequences (circles). Analyses were performed using five (a) and teen (b) distance classes. $A_y$ quantifies the average pairwise genetic distances that fall within the boundaries specified for each distance class *y*. The horizontal line indicates the average value of $A_y$ for a data set.

**Fig. 5** The observed and expected mismatch distributions in an expanding population model for HVS control region (a) and COI gene (b). The expected and observed mismatch distributions are indicated by solid and dashed lines, respectively.

**Fig. 6** Bayesian skyline (a) and skyride (b) plots derived from concatenate mitochondrial sequences of *Ctenomys ibicuiensis*, respectively. The thin and the thick dotted lines are the lower and the median estimated tMRCA, projected on the time line, and the blue area overlay show the 95% highest posterior density (HPD) limits for the effective population size, represented on Y-axis.

**FIGURES**

Figure 1:

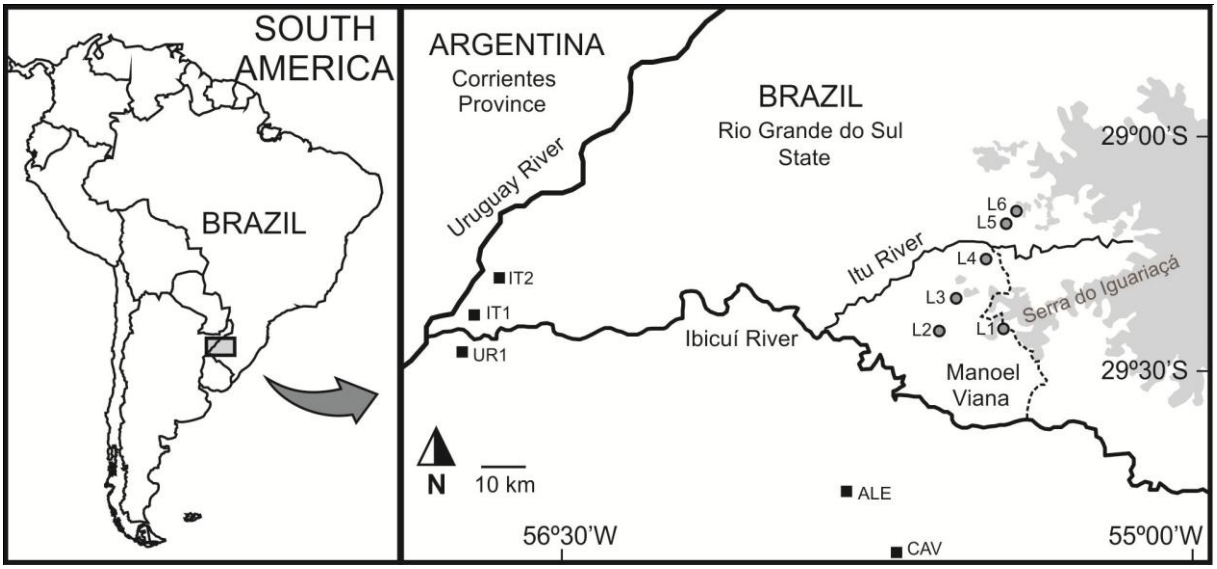


Figure 2:

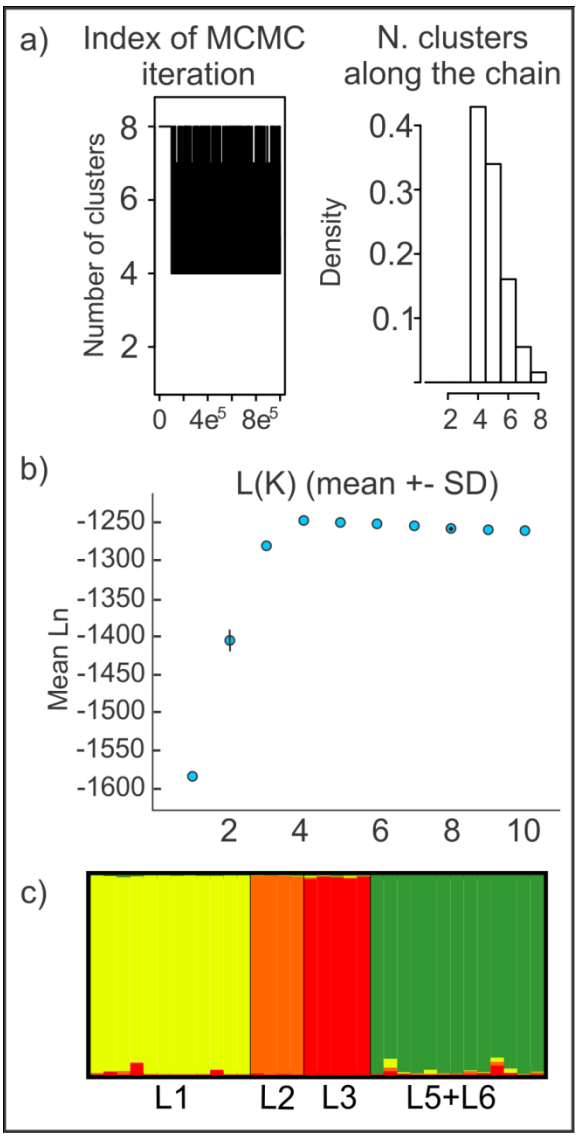

a)
Index of MCMC iteration
N. clusters along the chain
Number of clusters
8
6
4
2
0
4e5
8e5
Density
0.4
0.3
0.2
0.1
2 4 6 8
b)
L(K) (mean +- SD)
Mean Ln
-1250
-1300
-1350
-1400
-1450
-1500
-1550
-1600
2 4 6 8 10
c)
L1
L2
L3
L5+L6

Figure 3:

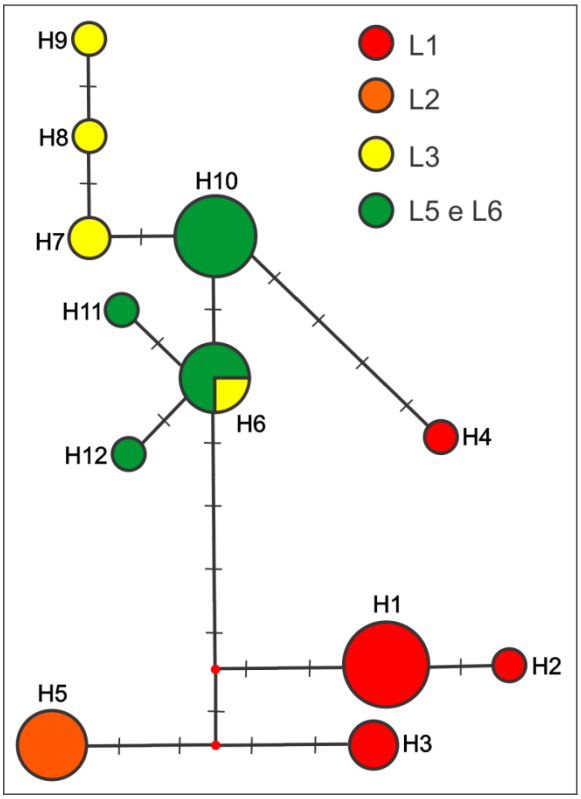



Figure 4:

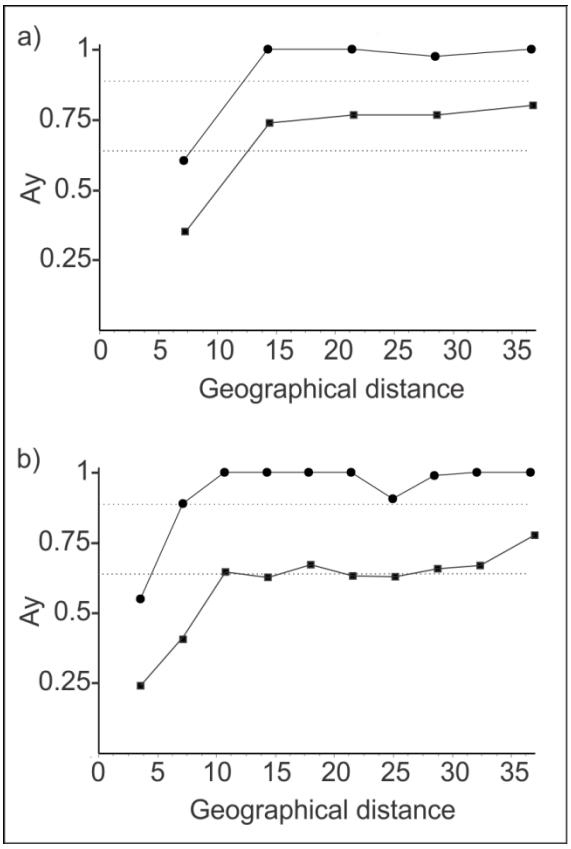


Figure 5:

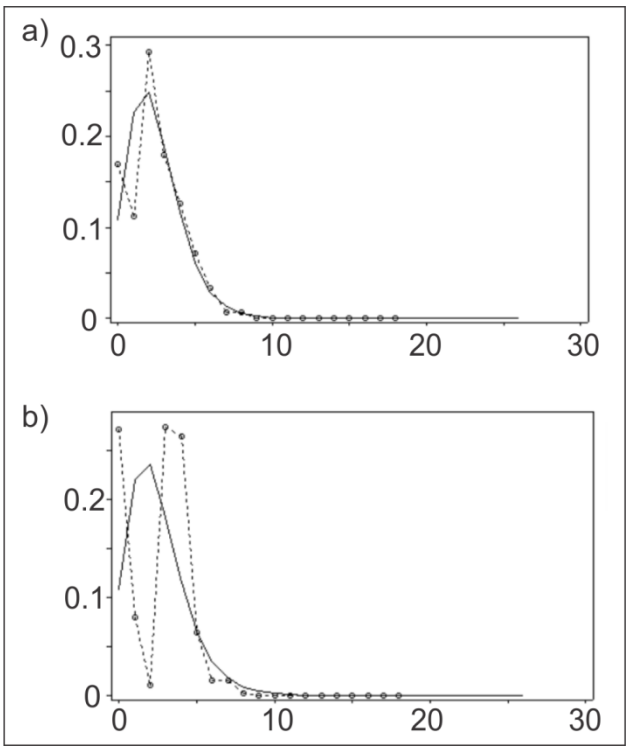
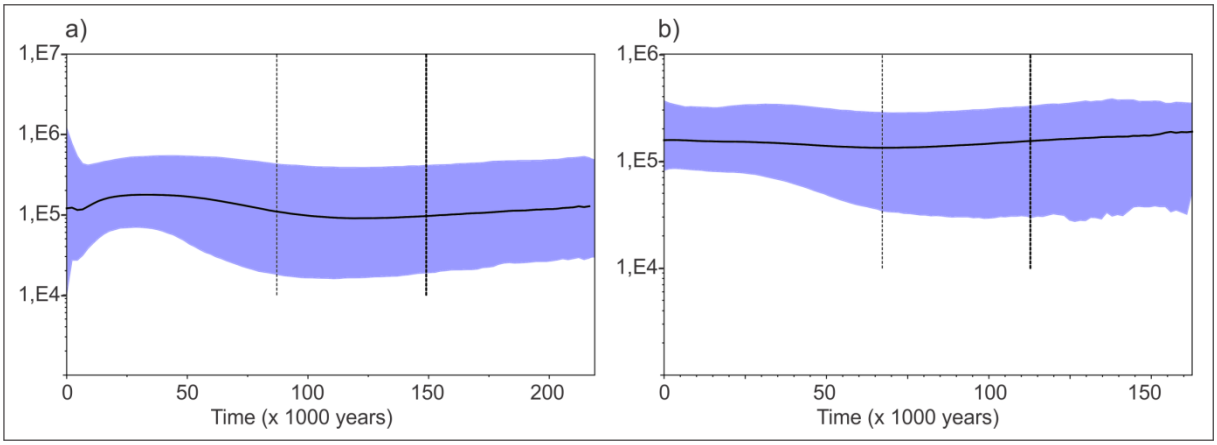



Figure 6:



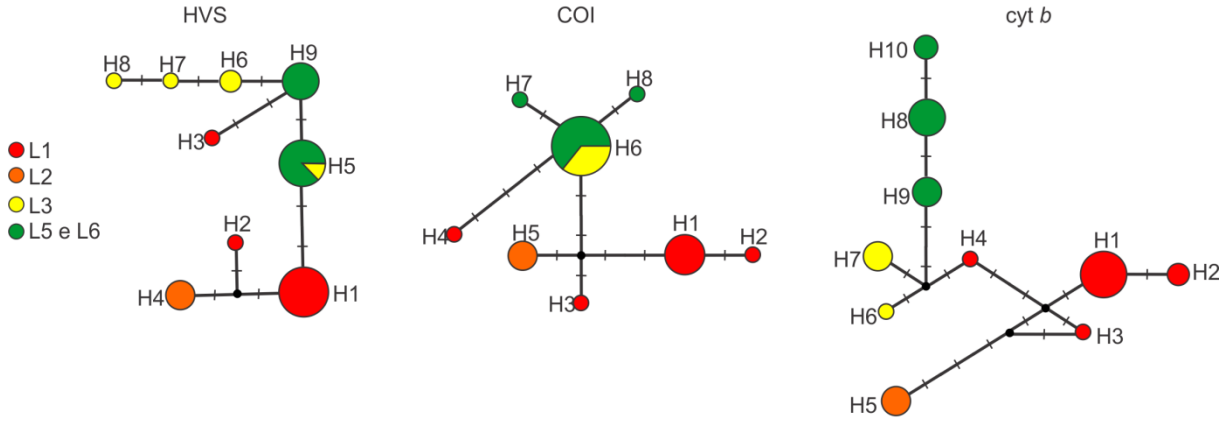
**SUPPORTING INFORMATION**

**Appendix S1** Number of *Ctenomys ibicuiensis* specimens (N), per locality, that compound each cytochrome *c* oxidase I (COI) and hyper variable sequence 1 (HVS) haplotype and the respective concatenate haplotype for both mitochondrial sequences.

| Locality (N) | COI | HVS | concatenate |
|---|---|---|---|
| L1 (7) | H1 | H1 | H1 |
| L1 (1) | H2 | | H2 |
| L1 (2) | - | | - |
| L1 (1) | H3 | H2 | H3 |
| L1 (1) | H4 | H3 | H4 |
| L2 (4) | H5 | H4 | H5 |
| L3 (1), L5 (2), L6 (1) | H6 | H5 | H6 |
| L3 (2) | | H6 | H7 |
| L3 (1) | | H7 | H8 |
| L3 (1) | | H8 | H9 |
| L5 (6) | | H9 | H10 |
| L6 (2) | - | H5 | - |
| L5 (1) | H7 | H5 | H11 |
| L6 (1) | H8 | H5 | H12 |

- Samples that did not amplified by PCR for COI.

**Appendix S2** Median-joining network of mitochondrial hyper variable sequence 1 from control region (HVS), cytochrome *c* oxidase I (COI) and cytochrome *b* (cyt *b*, modified from Freitas et al. in prep) genes for *Ctenomys ibicuiensis* samples. Circle areas are proportional to frequencies, cross hatches represent mutation steps and black points represent medium vectors.



## DISCUSSÃO GERAL

Reconhecida como uma das regiões de maior biodiversidade da Terra, a America do Sul ocupa a última posição num ranking continental de produtividade em estudos filogeográficos (Beheregaray, 2008). O Brasil, com seu imenso território e grandiosa biodiversidade, ocupou a 15º posição neste estudo (que considerou as publicações entre 1987 – 2006), ficando atrás de diversos países do hemisfério norte, inclusive países europeus com territórios infimamente menores.

Apesar deste viés de trabalhos filogeográficos, concentrados principalmente no hemisfério norte, as publicações nesta área vem aumentando de forma extraordinária (Beheregaray, 2008). Entretanto, se um estudo como este fosse realizado com foco nos biomas brasileiros, certamente outro viés seria verificado, com um número significativamente maior de publicações de âmbito filogeográfico relacionadas a biomas florestais como a Amazônia e a Mata Atlântica.

Antes mesmo de estudos filogeográficos, conhecimentos básicos sobre os biomas não florestais brasileiros são defasados. Considerada apenas pela produtividade agropecuária, não houve uma preocupação histórica como área prioritária para conservação do bioma Pampa (Overbeck e cols., 2007), que somente foi reconhecido como um bioma independente, em nível nacional, a partir da classificação do IBGE (2004).

Além de resguardar uma das maiores diversidades de gramíneas (Boldrini, 2009), a região dos pampas também abriga várias espécies de vertebrados emblemáticas de ambientes abertos que ocorrem em toda a Província Pampeana, e pelo menos 21 espécies endêmicas (Bencke, 2009). A única espécie de mamífero desta lista é *Ctenomys lami*, cuja distribuição é restrita a região conhecida como Coxilha das Lombas, próxima a capital do estado do Rio Grande do sul (RS), Porto Alegre.

Os artigos apresentados nesta tese resultam em uma enriquecedora contribuição à fauna de mamíferos endêmicos do bioma Pampa. Assim como *C. lami*, a nova espécie descrita para o oeste do estado, *C. ibicuiensis*, é endêmica e extremamente restrita. Já o tuco-tuco de colar, *C. torquatus*, passa a ser considerada a espécie endêmica de mamífero mais amplamente distribuída nos campos do sul do Brasil. A ausência de barreiras biogeográficas entre Brasil e Uruguai propicia a extensão de sua distribuição aos campos do norte do país vizinho. Esta espécie seria, portanto, mais adequadamente tratada como subendêmica dos campos sul brasileiros, de acordo com Bencke (2009).

A história geomorfológica do bioma Pampa remete a um longo período de estabilidade desde o final das grandes transgressões marinhas do Terciário, com o predomínio de gramíneas recobrindo os campos e clima frio e seco durante o período glacial (Rambo, 2005; Behling e cols., 2005). De acordo com os resultados de datação molecular apresentados nesta tese, a origem estimada para *C. ibicuiensis* e *C. torquatus* (da metade para o final do Pleistoceno) sugere que ambas teriam se estabelecido na região neste cenário de estabilidade geológica, o que torna ainda mais intrigante os padrões demográficos e de distribuição diferenciados entre as duas espécies.

Apesar da proximidade geográfica, separadas apenas pelo rio Ibicuí, *C. torquatus* e *C. ibicuiensis* não compartilham um ancestral comum direto. Ambas pertencem ao grupo filogenético *torquatus*, mas a divergência entre suas linhagens foi datada há cerca de 1,6 milhões de anos, no início do Pleistoceno, sendo que *C. torquatus* é mais intimamente relacionado às espécies de tuco-tuco habitantes da planície costeira do RS, *C. minutus* e *C. lami*. Portanto, um processo de diferenciação e especiação recente entre as espécies do Pampa, como o observado entre *C. minutus* e *C. lami* (Freitas, 2001; Lopes, 2011), não foi verificado.

À distribuição extremamente restrita de *C. ibicuiensis* era esperada uma associação a eventos de redução demográfica como consequência da degradação ambiental que a região vem sofrendo nas últimas décadas (Roesch e cols., 2009; Suertegaray e cols., 2011). Apesar baixa variabilidade genética encontrada, tanto para o DNA mitocondrial quanto para microssatélites, *C. ibicuiensis* não apresentou evidências de redução populacional nem depressão endogâmica. Um padrão de estabilidade foi recuperado nas análises demográficas para as populações de *C. ibicuiensis*, desde a sua origem no final do Pleistoceno.

Diferentemente da estabilidade demográfica descrita para *C. ibicuiensis*, *C. torquatus* apresentou um padrão demográfico de expansão recente. A origem destas linhagens remete à região central do estado, denominada Depressão Central, localizada entre o Planalto Sul-Brasileiro e a Serra do Sudeste. Como não ocorre em altitudes acima de 200 m, a colonização provavelmente ocorreu em direção ao oeste do estado, norte no Uruguai e, contornando a Serra do Sudeste, o tuco-tuco de colar teria se expandido até o litoral sul do estado, na região do Taim.

A ocorrência de *C. ibicuiensis* na borda da distribuição do tuco-tuco de colar provavelmente se caracteriza como um ponto de encontro entre linhagens que divergiram em outras regiões, e que atualmente habitam as margens opostas do rio Ibicuí. Em seu processo de expansão, *C. torquatus*

não ultrapassou o limite ao norte do rio Ibicuí em toda sua extensão, exceto na localidade de Itaqui, na foz deste rio.

Em contrapartida, o igualmente volumoso rio Jacuí foi colonizado em ambas as margens por populações de *C. torquatus* e provavelmente foi atravessado em algumas situações. Assim como o rio Ibicuí não serviu como barreira justamente em sua desembocadura, onde é mais caudaloso, o rio Jacuí não se enquadrou perfeitamente no padrão esperado de acordo com a hipótese de rios atuando como barreiras, na qual se espera uma diminuição mais efetiva do fluxo gênico entre margens opostas à medida que as populações se aproximam da foz de um rio (Wallace, 1852; Patton e cols., 2000). Apesar de não caracterizarem-se como causadores primários da divergência no passado, os grandes cursos d'água que dissecam a distribuição de *C. torquatus* podem estar atuando como promotores de diferenciação na forma como se apresentam atualmente, e participando como coadjuvantes de um processo de especiação futura, principalmente nas situações periféricas associadas à fixação de variações cromossômicas.

A filogeografia e o comportamento demográfico de *C. ibicuiensis* e *C. torquatus* apresentados neste estudo revelam um panorama de evolução diferenciada entre duas espécies que vivem na mesma região, sob as mesmas condições, com o mesmo estilo de vida subterrâneo associado a todas as restrições que essa condição impõe. Independente do perfil de estabilidade ou expansão exibido por estas espécies, ou da presença de barreiras apelativas como grandes rios, as análises de variância molecular (AMOVA) frequentemente atribuem a maior parte da variação genética às diferenças entre populações de tuco-tucos. A organização destes roedores em pequenos demes isolados, associada à baixa dispersão, territorialidade e filopatria das fêmeas (especialmente para o DNA mitocondrial) deixam um sinal profundo de estruturação em nível de populações, e isso é recorrente em estudos com outras espécies do gênero *Ctenomys* (Cutrera e cols*.,* 2005; Mapelli e cols., 2012a e b; Lopes, 2011).

Essa configuração espacial e demográfica peculiar das populações de tuco-tucos, além de resultar em estruturação a nivel intraespecífico, pode ter desempenhado um papel de extrema importância como propulsor da especiação explosiva documentada para o gênero *Ctenomys*, (Lacey e cols., 2000; Reig e cols., 1990). Entretanto, esse sistema é bastante sucetível a alterações climáticas e perturbações geológicas passadas, resultando em reflexos demográficos negativos como verificado

para *C. sociabilis* (Chan e Hadly, 2011) e *C. porteousi* (Mapelli e cols., 2012a); especialmente em se tratando de espécies consideravelmente especialistas em relação ao habitat.

Atualmente, as modificações no ambiente causadas por ação antrópica, como o desenvolvimento urbano, tem causado impacto na variabilidade genética e tamanho populacional em *C. flamarioni* (Fernández-Stolz e cols., 2007). A fragmentação dos campos no sul do Brasil, causada pela prática crescente da silvicultura, produção agrícola e pecuária intensiva, pode acarretar em efeitos ainda mais agravantes que os eventos naturais históricos, afetando a conectividade entre as populações de tuco-tuco dos Pampas.

Assim como os padrões filogeográficos de tuco-tucos (Fernandéz-Stolz, 2006; Lopes, 2011), petúnias (*Petunia integrifolia* – AMC Ramos-Fregonezi, comunicação pessoal), anfíbios (*Melanophryniscus* spp. – JB Silva, comunicação pessoal) e lagartos (*Liolaemus* spp. – CM Silva, comunicação pessoal) têm agregado conhecimento sobre a dinâmica evolutiva da planície costeira do sul do Brasil, bem como os impactos da ação antrópica; os resultados apresentados neste estudo para *C. ibicuiensis* e *C. torquatus* caracterizam-se como uma contribuição primordial para o entendimento dos processos evolutivos do Pampa, como os rios e as condições geografias podem interferir nos padrões filogeográficos, e como a interferência humana pode influenciar na preservação de espécies com características demográficas tão peculiares como os tuco-tucos.

## CONCLUSÕES

● *Ctenomys torquatus* apresenta um padrão de expansão populacional recente em sua ampla distribuição, tendo como origem provável a região da Depressão Central do estado do Rio Grande do Sul;

● As populações de *C. torquatus* com cariótipo 2n = 40 em Itaqui colonizaram a margem ao norte do rio Ibicuí somente neste local, caracterizando um evento de Dispersão através do rio;

● As populações de *C. torquatus* com cariótipo 2n = 46 em Rio Grande e Taim colonizaram a planície costeira no sul do Rio Grande do Sul e, provavelmente, foram posteriormente isoladas pelo surgimento do Canal São Gonçalo, caracterizando um evento de Diferenciação Primária, quando há imposição de um curso d'água (vicariância). Por se tratar de um evento recente, o efeito da estruturação não foi verificado.

● Exceto pela comparação dos pontos na nascente em relação aos demais ao longo do rio Jacuí, não foi verificado um padrão de aumento da diferenciação genética à medida que aumenta a largura do rio, de acordo com o esperado pela hipótese de rios como barreira;

● *Ctenomys ibicuiensis* foi descrita como uma nova espécie de tuco-tuco endêmica do bioma Pampa e com distribuição geográfica extremamente restrita aos campos arenosos de Manoel Viana e Maçambará, no oeste do Rio Grande do Sul;

● *Ctenomys ibicuiensis* e *C. torquatus* são próximas geograficamente, separadas pelo rio Ibicuí, pertencem ao mesmo grupo filogenético *torquatus*, mas não compartilham um ancestral comum direto e recente;

● A espécie nova apresentou baixos índices de diversidade genética, estabilidade demográfica e a maior parte da variação corresponte a diferenças entre populações, que apesar de próximas geograficamente mostraram-se altamente diferenciadas;

● Apesar de não haver evidências de redução populacional e depressão endogâmica; a raridade e o baixo número de localidades registradas para *C. ibicuiensis*, associados à intensa modificação e desgaste ambiental na região, preenchem os requisitos para sua inclusão na lista de espécies ameaçadas da IUCN.

**REFERÊNCIAS BIBLIOGRÁFICA**

Atlas Ambiental de Buenos Aires (AABA), 2010. Pampa – Ecología: Caracterización de los agroecosistemas <http://www.atlasdebuenosaires.gov.ar>, acessado em outubro de 2011.

Ayres JM & Clutton-Brock TH (1992) River boundaries and species range size in Amazonian primates. American Naturalist 140: 531-537.

Beheregaray L (2008) Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. Mol Ecol 17: 3754-3774.

Behling H, Pillar VD, Orlóci L & Bauermann SG (2004) Late Quaternary Araucaria forest, grassland (Campos), fire and climate dynamics, studied by high resolution pollen, charcoal and multivariate analysis of the Cambará do Sul core in southern Brazil. Palaeogeogr Palaeoclimatol Palaeoecol 203: 277-297.

Behling H, Pillar VD & Bauermann SG (2005) Late Quaternary grassland (Campos), gallery forest, fire and climate dynamics, studied by pollen, charcoal and multivariate analysis of the São Francisco de Assis core in western Rio Grande do Sul (southern Brazil). Rev Paleo-bot Palynol 133: 235-248.

Bencke GA (2009) Diversidade e conservação da fauna dos campos do sul do Brasil. In: Pillar VP, Müller SC, Castilhos ZMS & Jaques AVA (eds) Campos sulinos, conservação e uso sustentável da biodiversidade. Brasília, MMA, pp 101-121.

Boldrini I I (2009) A flora dos campos do Rio Grande do Sul. In: Pillar VP, Müller SC, Castilhos ZMS e Jaques AVA (eds) Campos sulinos, conservação e uso sustentável da biodiversidade. Brasília, MMA, pp 63-77.

Busch C, Antinuchi CD, del Valle JC, Kittlein MJ, Malizia AI, Vassallo AI & Zenuto RR (2000) Population ecology of subterranean rodents. In: Lacey EA, Patton JL & Cameron GN (eds) Life Underground, the biology of subterranean rodents. The University of Chicago Press, Chicago and London, pp 181-226.

Cabrera AL & Willink A (1980) Biogeografia da America Latina. OEA, Washington.

Carvalho PCF & Batello C (2009) Access to land, livestock production and ecosystem conservation in the Brazilian Campos biome: The natural grasslands dilemma. Livest Sci 120: 158-162.

Castilho CS (2004) Análise cromossômica e de microssatélites em uma zona de hibridação intra-

específica de *Ctenomys minutus* (Rodentia: Ctenomyidae). Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Castillo AH, Cortinas MN & Lessa EP (2005) Rapid diversification of South American tuco-tucos (*Ctenomys*; Rodentia; Ctenomyidae): contrasting mitochondrial and nuclear intron sequences. J Mammal 86: 170-179.

Chan, Y.L. & Hadly, E.A. (2011) Genetic variation over 10 000 years in Ctenomys: comparative phylochronology provides a temporal perspective on rarity, environmental change and demography. Molecular Ecology, doi, 10.1111/j.1365-294X.2011.05295.x

Cutrera AP, Lacey EA & Bush C (2005) Genetic structure in a solitary rodent (*Ctenomys talarum*): implications for kinship and dispersal. Mol Ecol 14: 2511-2523.

Deble LP (2011) A Vegetação Campestre no Bioma Pampa. In: Deble LP, Deble ASO & Leão ALSO (eds) Bioma Pampa, Contribuições Científicas, Ediurcamp, Bagé, pp. 84-143.

D'Elia G, Lessa EP & Cook JA (1999) Molecular phylogeny of tuco-tucos, genus *Ctenomys* (Rodentia, Octodontidae): Evaluation of the mendocinus species group and the evolution of asymmetric sperm. J Mammal Evol 6: 19-38.

Fernandes FA (2008) Filogeografia de *Ctenomys torquatus* (Rodentia: Ctenomyidae). Tese de doutorado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Fernandes FA, Gonçalves GL, Ximenes SSF & Freitas TRO (2009) Karyotypic and molecular polymorphisms in the *Ctenomys torquatus* (Rodentia: Ctenomyidae): taxonomic considerations. Genetica 136: 449-459.

Fernández-Stolz GP (2006) Estudos evolutivos, filogeográficos e de conservação em uma espécie endêmica do ecossistema de dunas costeiras do sul do Brasil, *Ctenomys flamarioni* (Rodentia - Ctenomyidae), através de marcadores moleculares microssatélites e DNA mitocondrial. Tese de doutorado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Fernández-Stolz GP, Stolz JFB & Freitas TRO (2007) Bottlenecks and dispersal in the tuco-tuco-das-dunas, *Ctenomys flamarioni* (Rodentia: Ctenomyidae) in southern Brazil. J Mammal 88: 935-945.

Freitas TRO (1995) Geographic distribution and conservation of four species of the genus *Ctenomys* in southern Brazil. Stud Neot Fauna Environ 30: 53-59.

Freitas TRO (1997) Chromosome polymorphism in *Ctenomys minutus* (Rodentia: Octodontidae). Genet Mol Biol 20: 1-7.

Freitas TRO (2001) Tuco-tucos (Rodentia, Octodontidae) in Southern Brazil: C*tenomys lami* spec. nov. separated from *C. minutus* Nehring 1887. Stud Neot Fauna Environ 36: 1-8.

Freitas TRO (2006) Cytogenetics status of four *Ctenomys* species in the south of Brazil. Genetica 126: 227-235.

Freitas TRO & Lessa EP (1984) Cytogenetics and morphology of *Ctenomys torquatus* (Rodentia - Octodontidae). J Mammal 65: 637-642.

Freygang CC, Marinho JR & Freitas TRO (2004) New karyotypes and some considerations of *Ctenomys minutus* (Rodentia: Ctenomidae) on the coastal plain of the Brazilian State of Rio Grande do Sul. Genetica 121:125-132.

Gava A & Freitas TRO (2002) Characterization of a hybrid zone between chromosomally divergent populations of *Ctenomys minutus* (Rodentia; Ctenomyidae). J Mammal 83:843-851.

Gava A & Freitas TRO (2003) Inter and intra-especfic hybridization in tuco-tuco (*Ctenomys*) from Brazilian coastal plains (Rodentia: Ctenomidae). Genetica 119: 11-17.

Gonçalves GL & Freitas TRO (2009) Intraspecific variation and genetic differentiation of the collared tuco-tuco (*Ctenomys torquatus*) in sourthern Brazil. J Mammal 90: 1020-1031.

Instituto Brasileiro de Geografia e Estatística – IBGE (2004) Mapa da vegetacao do Brasil e Mapa de Biomas do Brasil. http://www.ibge.gov.br. Acessado dia 18 de Novembro de 2011.

Jost H, Soliani E & Godalphin MF (1975) Evolução Paleogeográfica da região da Lagoa Mirim, RS, SE do Brasil. Actas del Congresso Argentino de Paleontología y Bioestratigrafía, Tucumán 2: 575-587.

Kiblisky P, Brum-Zorrilla N, Perez G & Saez F A (1977) Variabilidad cromosómica entre diversas poblaciones uruguayas del roedor cavador del género *Ctenomys* (Rodentia-Octodontidae). Mendeliana 2: 85-93.

Lacey EA, Patton JL & Cameron GN (2000) Life underground: the biology of subterranean rodents. The University of Chicago Press, Chicago.

Lessa EP & Cook J A (1998) The molecular phylogenetics of tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae) suggest an early burst of speciation. Mol Phylogenet Evol 9: 88-99.

Lopes, C.M. (2011) História evolutiva de *Ctenomys minutus* e *Ctenomys lami* na planície costeira do Sul do Brasil. Tese de doutorado, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Mapelli FJ & Kittlein MJ (2009) Influence of patch and landscape characteristics on the distribution of the subterranean rodent *Ctenomys porteousi*. Landscape Ecol 24: 723-733.

Mapelli JF, Mora MS, Mirol PM & Kittlein MJ (2012a) Effects of Quaternary climatic changes on the phylogeography and historical demography of the subterranean rodent *Ctenomys porteousi*. J Zool 286: 48-57.

Mapelli JF, Mora MS, Mirol PM & Kittlein MJ (2012b) Population structure and landscape genetics in the endangered subterranean rodent *Ctenomys porteousi*. Conserv Genet 13: 165-181.

Mascheretti S, Mirol PM, Giménez MD, Bidau CJ, Contreras JR & Searle JB (2000) Phylogenetics of the speciose and chromosomally variable rodent genus *Ctenomys* (Ctenomyidae: Octodontoidea), based on mitochondrial cytochrome *b* sequences. Biol J Linnean Soc 70: 361-376.

Mirol P, Giménez MD, Searle JB, Bidau CJ & Faulkes CG (2010) Population and species boundaries in the South American subterranean rodent *Ctenomys* in a dynamic environment. Biol J Linneam Soc 100: 368-383

Moritz C, Patton JL, Schneider CL & Smith TB (2000) Diversification of rainforest faunas: an integrated molecular approach. Annu Rev Ecol Syst 31: 533-563.

Nevo E (1979) Adaptative convergence and divergence of subterranean mammals. Annu Rev Ecol Syst 10: 269-308.

Overbeck GE, Müller SC, Fidelis A, Pfadenhauer J, Pillar VD, Blanco CC, Boldrini II, Both R & Forneck ED (2007) Brazil's neglected biome: The South Brazilian Campos. Perspect Plant Ecol Evol Syst 9: 101-116.

Parada A, D'Elía G, Bidau CJ & Lessa EP (2011) Species groups and the evolutionary diversification of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). J Mammal 92: 671-682.

Patton JL, Silva MN & Malcolm JR (2000) Mammals of the Rio Juruá and the evolutionary and ecological diversification of Amazonia. Bull Am Mus Nat Hist 244: 1-306.

Peres CA, Patton JL & Silva MNF (1996) Riverine barriers and gene flow in Amazonian saddle-back tamarins. Folia Primatol 67: 113-124.

Rambo B (2005) A fisionomia do Rio Grande do Sul: ensaio de monografia natural. Editora Unisinos, São Leopoldo.

Reig OA, Busch C, Ortells MO & Contreras JR (1990) An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. In: Nevo E & Reig OA (eds) Evolution of subterranean mammals at the organismal and molecular levels. Alan R. Liss Inc., Nova York, pp. 71-96.

Roesch LFW, Vieira FCB, Pereira VA, Schünemann AL, Teixeira IF, Senna AJT & Stefenon VM (2009) The Brazilian Pampa: A Fragile Biome. Diversity 1: 182-198.

Silva J, Freitas TRO, Heuser VD, Marinho JR, Bittencourt F, Cerski CTS, Klassmann LS & Erdtmann B (2000a) Chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated with multiple methods and tissues. Mutat Res 470: 39-51.

Silva J, Freitas TRO, Heuser VD, Marinho JR & Erdtmann B (2000b) Genotoxicity biomonitoring in coal regions using wild rodent *Ctenomys torquatus* by comet assay and micronucleus test. Environ Mol Mutagen 35: 270-278.

Slamovits CH, Cook JA, Lessa EP & Rossi MS (2001) Recurrent amplification and deletions of satellite DNA accompanied chromosomal diversification in South American tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae): A phylogenetic approach. Mol Biol Evol 18: 1708-1719.

Suertegaray D.M.A., Guasseli L.A. & Verdum R (2001) Atlas de arenização: Sudoeste do Rio Grande do Sul. Secretaria da Coordenação e Planejamento, Porto Alegre

Tomasco IH & EP Lessa (2007) Phylogeography of the Tuco-tuco *Ctenomys pearsoni*: mtDNA variation and its implication for chromosomal differentiation. In: Kelt DA, Lessa EP, Salazar-Bravo J & Patton JL (eds) The quintessential naturalist. Honoring life and legacy of Oliver P. Pearson. University of California Publications, Berkeley, pp 859-882.

Tomazelli LJ, Dillenburg SR & Villwock JA (2000) Late quaternary geological history of Rio Grande do Sul coastal plain, southern Brazil. Rev Bras Geocienciên 30: 474-476.

Verzi DH, Olivares AI & Morgan CC (2010) The oldest South American tuco-tuco (late Pliocene, northwestern Argentina) and the boundaries of the genus *Ctenomys* (Rodentia, Ctenomyidae). Mammal Biol 75: 243-252.

Wallace AR (1852) On the monkeys of the Amazon. Proceedings of the Zoological Society of London, 20: 107-10.

**ANEXO**

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# AN ENDEMIC NEW SPECIES OF TUCO-TUCO, GENUS *CTENOMYS* (RODENTIA: CTENOMYIDAE) WITH A RESTRICT GEOGRAPHIC DISTRIBUTION IN SOUTHERN BRAZIL.

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Running heading:

New tuco-tuco from southern Brazil

**An endemic new species of tuco-tuco, genus *Ctenomys* (Rodentia: Ctenomyidae), with a restricted geographic distribution in southern Brazil**

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A new species of tuco-tuco, genus *Ctenomys*, is described from sandy soils on western slopes of the state of Rio Grande do Sul in southern Brazil. This species is distinguished from other named members of this South American endemic genus by several analyses. Diagnostic traits for this new propose species are the diploid number of 50 chromosomes and the autosomal fundamental number of 68 arms, with the 1st pair much longer than in other related species. Qualitative and quantitative (geometric morphometrics) analyses of the skull morphology and phylogenetics analysis of the mitochondrial cytochrome b gene support this

status, especially when comparing with phylogenetically related and geographically neighboring species. Populations of this species have a narrow geographic distribution in a small area (~500 $km^2$) that have been suffering from anthropogenic pressure from soybean, pine and eucalyptus plantations, as well as desertification. This scenario suggests that it could be characterized as an endangered species.

Keywords: endangered, endemism, fossorial, geometric morphometrics, Pampa biome.

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The Neotropical subterranean rodents of the genus *Ctenomys* (tuco-tucos) comprise more than 60 described species. They have been considered one of the most chromosomally variable mammals, with karyotypes ranging from 2n = 10 to 70, and a model for studies of speciation and evolution (Reig et al. 1990; Freitas 2006; Mirol et al. 2010; Lopes, 2011).

The relatively recent origin of the genus (Verzi et al. 2010) and its high species diversity suggest an explosive speciation event in tuco-tucos, which may be the reason for the unsolved 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; Parada et al. 2011; Slamovits et al. 2001) and even with nuclear loci (Castillo et al. 2005). The most recent phylogenetic analysis, with a wide taxonomic and geographic coverage, recovered 8 relatively well supported clades that comprise groups of tuco-tuco species, although some taxa remained without a defined phylogentic position and the relationships among them and the species groups were weakly supported (Parada et al. 2011).

The *torquatus* group is composed of geographically neighboring species, which generally show high karyotypic polymorphism. A complex of the species *C. roigi* (2n = 48), *C. perrensi* (2n = 50), *C. dorbignyi* (2n = 70), and several forms of uncertain taxonomic status (2n = 42 to 65) were found in Corrientes, Argentina, where the intricate pattern of genetic and karyotypic organization among these metapopulations was attributed to the unstable dynamics of the habitat (Mirol et al. 2010). *Ctenomys pearsoni* (2n = 56 to 70) is another chromosomally polytypic species of the *torquatus* group and inhabits the coastal plains of southern Uruguay by the Rio de la Plata and the Atlantic Ocean, with some populations in Argentina (Garcia et al. 2000). On the southern Brazilian coastal plain, two species show high levels of chromosomal variability. A total of 45 karyotypes were described for *C. minutus* (2n = 42 to 50; Freitas 1997; Lopes 2011) and 26

distinct karyotypes for *C. lami* (2n = 54 to 58), which has an extremely restricted geographic range (Freitas 2001). Finally, *C. torquatus*, the nominate species of the group, inhabits the grasslands of southern Rio Grande do Sul (RS), Brazil, and northern Uruguay. Despite its wide geographic range, there is a most common and widespread chromosomal form (2n = 44), and only some restricted karyotypic polymorphisms in the southern (2n = 46) and western (2n = 40 and 42) parts of its distribution (Fernandes et al. 2009a; Freitas and Lessa 1984; Gonçalves and Freitas 2009).

Between 2007 and 2011, while we were trying to cover the geographic distribution of *C. torquatus* in western RS, we found new localities where the specimens seemed to be slightly differentiated. Surveys of cytogenetic, morphometric, phylogenetic, and geographic information suggested that these specimens, collected in the Manoel Viana municipality and initially presumed to be *C. torquatus,* in fact represent a separate taxon. This report describes this new species of *Ctenomys*, and compares its populations with species of the *torquatus* group and other congeners.

## MATERIALS AND METHODS

*Samples and cytogenetic analysis* – A total of 34 individuals were collected from localities L1, L2, L3, L5 and L6 (Fig. 1, Table 1). In locality L4, burrows were observed in the field, but no individual was collected. The specimens were caught alive using Oneida Victor No. 0 traps with a rubber cover, anesthetized, measured, and a small piece of the ear was cut off and stored in absolute ethanol.

Some individuals from each new locality were killed, to obtain karyotypes and specimen vouchers: all 16 specimens from L1 and L2, 6 from L5, and 1 specimen from each of L3 and L6. One hour before the procedure, colchicine (0.01%) was administered at 1ml/100mg of body weight. Samples of femur marrow were incubated in hypotonic solution for 20 min and fixed in methanol : acetic acid (3:1). Cytogenetic analysis was performed following Ford and Hamerton (1956). The tissue samples, skulls and skins were deposited in the Mammal Collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil. All work with the animals was done with the permission of Sisbio-IBAMA (the official Brazilian environmental protection agency) and following guidelines approved by the American Society of Mammalogists (Sikes et al. 2011).

*Linear measurements* – Morphometric measurements (Langguth and Abella 1970) were taken to the nearest 0.05 mm with a caliper to describe the holotype skull. Furthermore, corporal measurements and the weight of holotype specimen were taken.

*Geometric morphometrics approach* – The sample consisted of 16 skulls of adult specimens (6 males, 10 females) collected at five localities in Manoel Viana and Maçambara municipalities, western RS (Fig. 1, Table 1). We compared these specimens with skulls from different species of the torquatus group: *C. lami* (N = 30), *C. minutus* (N = 30), *C. pearsoni* (N = 30), *C. perrensi* (N = 9), *C. roigi* (N = 7), and *C. torquatus* (N = 30). All specimens had been previously deposited in collections or museums (Appendix I).

Each skull was photographed in dorsal, ventral and lateral views with a digital camera at 3.1 megapixels resolution (2048 x 1536). Two-dimensional morphological landmarks were defined for dorsal (29), ventral (30) and lateral (21) views of the skull respectively (Fernandes et al. 2009b). The coordinates of each landmark were obtained using tpsDig 1.40 software (Rohlf 2004). Coordinates were superimposed using a generalized Procrustes analysis (GPA) algorithm (Dryden and Mardia 1998). The size of each skull was estimated using its centroid size, the square root of the sum of the squares of the distances of each landmark from the centroid (Bookstein 1991).

Size was compared between species with a one-way analysis of variance (ANOVA) of centroid size values and for multiple comparisons we used Tukey's Test. Principal components analysis (PCA) was carried out using the variance-covariance matrix of generalized least-squares superimposition residuals (Cordeiro-Estrela et al. 2006 ; Baylac et al. 2003). PCs of the covariance matrix of superimposition residuals were used as new shape variables, to reduce the dimensionality of the data set as well as to work on independent variables. The matrixes of PCA scores for each view of the cranium (dorsal, ventral and lateral) were joined into 1 total matrix. Shape differences between species were tested through multivariate analysis of variance (MANOVA) and Bonferroni correction for multiple comparisons. To choose the number of PCs to be included in the Linear Discriminant Analysis (LDA), we computed correct classification percentages with each combination of PCs (Baylac and Friess 2005). We selected the subset of PCs giving the highest overall good classification percentage. We used a leave-one-out cross-validation procedure that allows an unbiased estimate of classification percentages (Baylac and Friess 2005).

Cross-validation is used to evaluate the performance of classification by LDA. In the leave-one-out cross-validation, all the data except those for one individual are used to calculate the discriminant function, and the individual not used is then classified (Cordeiro-Estrela et al. 2006). The visualization of shape differences between species (the new species *C*. sp. x *C. torquatus*; *C*. sp. x *C. dorbignyi*, *C*. sp. x *C. perrensis*, and *C*. sp. x *C. roigi*), for the three skull views, was obtained through multivariate regression of discriminant axes of the shape variables. Mahalanobis distances were used to compute a neighbor-joining tree to visualize the

morphological relationships among species. For all statistical analyses and to generate graphs, we used the "R" language and environment for statistical computing version 2.2.1 for Linux (R Development Core Team, http://www.r-project.org) and the following libraries: MASS (Venables and Ripley 2002), APE version 1.8-2 (Paradis et al. 2004), GenKern 1.1-0 (Lucy and Aykroid 2004). Geometric morphometric procedures were carried out with the Rmorph package (Baylac 2006), a geometric and multivariate morphometrics library.

*Molecular Methods and Phylogenetic Analysis* – The tissue samples (preserved in absolute ethanol and refrigerated at -20°C) were obtained from the 34 specimens. The total DNA was extracted following Medrano et al. (1990).

The mitochondrial cytochrome b gene was amplified using the primers MVZ05-tuco06 and tuco07-tuco14a (Smith and Patton 1999; Wlasiuk et al. 2003). PCR amplifications were carried out in a reaction volume of 20 µl containing 20-80 ng of DNA, 0.2 µM of each primer, 0.2 µM dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase (Invitrogen). The thermocycling profile included an initial denaturing at 94ºC for 5 min, followed by 30-34 cycles of denaturing at 94ºC for 30 s, annealing at 45ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min. PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (GE Healthcare) following the guidelines of the suppliers, and sent for sequencing at Macrogen Inc. (Korea), using the forward primer.

Sequence electropherograms were visually inspected using Chromas 2.31 (http://www.technelysium.com.au/chromas_lite.html) and the two partially overlapping fragments obtained were aligned using Clustal W, implemented in MEGA 5.0 (Tamura et al. 2011). Alignments were checked and edited by hand.

Measurements of mtDNA diversity, including the mean number of pairwise differences ($\pi$ –Nei 1987), definitions of haplotypes (H), and haplotype diversity (*Hd*), were calculated in the program DNAsp 5.0 (Librado and Rozas 2009). A median-joining haplotype network was constructed in Network 4.6 (http://www.fluxux-engineering.com).

The interspecific analyses were performed using the cytochrome b haplotypes found for those samples, together with 54 different recognized species and sequences from tuco-tucos of undefined taxonomic status, available in GenBank (Accession numbers provided in Appendix II). We also included five haplotypes of *C. torquatus* from localities around the geographic range of the new species: H1, H2, and H3 from Alegrete municipality, and IT1-UR1 and IT2 from Itaqui and Uruguaiana municipalities (Fig. 1). The octodontid *Octodon degus* was incorporated as outgroup.

Uncorrected genetic distances (p-distance) were computed for all pairs of sequences using MEGA. Distance was also computed between species within the *torquatus* group, but for the new species and for *C. torquatus*, the p-distance was computed considering the mean distance for all haplotypes listed in Appendix II.

We performed a maximum parsimony (MP) analysis with PAUP* 4.0b10 (Swofford 1998), based on a heuristic search using 1000 replicates of random taxon addition, and tree bisection-reconnection (TBR) branch-swapping, storing a maximum of 10000 trees. Support for nodes was assessed using non-parametric bootstrapping with 1000 replications.

In addition, a maximum likelihood (ML) analysis was performed under the HKY + I + G model selected by Modeltest 3.06 (Posada and Crandall 1998) based on the Akaike Information Criterion (AIC). Besides the molecular evolution model, five substitution categories with an estimated gamma distribution parameter (1.18), proportion of invariant sites (0.49), and the transition/transversion ratio (8.79) were estimated by Modeltest. ML searches were carried out with PhyML (Guindon and Gascuel 2003), starting with an NJ tree, following the nearest-neighbor interchange (NNI) branch-swapping and empirical base frequencies. Clade support was assessed by bootstrapping (B) with 1000 replicates. The ML analysis was performed at the online South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml/).

Bayesian inference (BI) was carried out using the Beast 1.6.2 program (Drummond and Rambaut 2007). We used a Yule tree prior, and the search was performed with the model chosen using Modeltest (HKY+ I + G) and the SRD06 substitution model (Shapiro et al. 2006), which allow partitioning data considering the 1st and 2nd codon positions in a separate partition from the 3rd position. This model incorporates information from the genetic code, and this has been suggested to optimize the performance for coding sequences. Two independent Markov chain Monte Carlo runs were performed with 30 million generations, sampling trees every 1000 generations, using the computational resources of Bioportal from the University of Oslo (https://www.bioportal.uio.no). The effective sample sizes (ESSs) of parameters sampled from MCMC were verified to be higher than 200 using the software TRACER 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Independent runs were summarized in TreeAnnotator 1.6.2 (Drummond and Rambaut 2007); the first 10% of trees (3000) were discarded and the remaining trees were used to infer a maximum a posteriori tree that was visualized in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

## RESULTS

### DESCRIPTION. – *Ctenomys ibicuiensis,* new species

*Holotype*. – Adult female, 2n = 50 and FN = 68. Skin and skull number TR1065, collected by Thales R. O. Freitas and Fabiano A. Fernandes. Deposited in the Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul. Total length 234 mm; tail 75 mm; weight 200 g; hind foot (with claws) 37 mm; and hind foot (without claws) 33 mm. Total length of skull, 42.90 mm; nasal length, 16.25 mm; nasal width, 6.39 mm; zygomatic breadth, 26.58 mm; bimeatal breadth, 25.20 mm; mastoid breadth, 24.63 mm; rostral breadth, 10.65 mm; pre-orbital foramen length, 9.10 mm; diastema length 10.41 mm; braincase breadth, 18.14 mm; length of maxillary toothrow, 9.21 mm; palatal length, 79.45 mm; upper incisor breadth 3.30 mm; 4th premolar length, 3.88 mm; auditory bulla width, 7.53 mm; auditory bulla length, 14.02 mm; and mandibular width, 31.49 mm.

*Type locality*. – Manoel Viana (Fig. 1B), in central-western Rio Grande do Sul, southern Brazil, at 29º23'37''S, 55º25'43''W. Habitat: sand dunes and grasslands, disturbed by agricultural activities and desertification.

*Paratypes*. – 11 specimens (5 males and 6 females) were deposited in the Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil, from locality L1 (Table 1): TR 1066 to TR 1076.

*Etymology*. – The name 'ibicuiensis' refers to the Ibicuí River near the type locality. Moreover, the word "ibicuí" in the Tupi-Guarani language means "sand dunes" (or "terra de areia" in Portuguese), exactly the habitat where these tuco-tucos live.

*Geographic distribution*. – *Ctenomys ibicuiensis* is known only from six sites, most of them in Manoel Viana and two in Maçambará municipalities, in western Rio Grande do Sul, southern Brazil, and at an elevation of around 200 m (Fig. 1).

From the 1st locality sampled (L1), we searched through the Manoel Viana, São Francisco de Assis, Maçambará, Itaqui, São Borja, Santo Antônio das Missões and Santiago municipalities, for new collection sites of *C. ibicuiensis*. However, we found only the few localities shown in Fig. 1.

*Ctenomys torquatus* has been recorded along the entire south bank of the Ibicuí River, including Alegrete municipality just south of Manoel Viana. This species was only found on the north bank near the mouth of the Ibicuí River, in Itaqui municipality, over 100 km west of the known range of *C. ibicuiensis*. No tuco-tuco population, of either species, was found between these localities (see stars in Fig. 1).

In spite of the predominance of sandy soil, we did not find *C. ibicuiensis* populations west and northwest of the L1 to L6 localities, nor did we find mention of its occurrence from folk knowledge of local residents. The geographic distribution of *C. ibicuiensis* is bordered on the east by the Serra do Iguariaçá, a mountain range of basaltic geomorphology that is part of the Meridional Plateau in an Atlantic Forest biome. Probably due to these characteristics there is no record of tuco-tucos in this region.

*Karyotype analysis*. – These individuals showed a diploid number of 50 chromosomes and an autosomal fundamental number of 68 arms; they had the 1st submetacentric pair much longer and the 11th acrocentric pair relatively longer than observed in the other related species. This karyotype has 10 biarmed and 14 acrocentric pairs (Fig. 2). The sexual pair in males is formed by a medium-sized X-metacentric and small Y-acrocentric chromosomes.

*Geometric morphometric analysis*. – Skull Size: By ANOVA, we found significant differences in skull centroid size for dorsal ($F = 7.901$; d.f. = 6; $P < 0.001$), ventral ($F = 8.638$; d.f. = 6; $P < 0.001$), and lateral ($F = 11.88$; d.f. = 6; $P < 0.001$) views of the skull. For pairwise tests, *C. ibicuiensis* showed significant differences only from the larger *C. torquatus* (dorsal view: $P = 0.0004$; ventral view: $P = 0.0001$; and lateral view: $P = 0.006$; for all views of pairwise tests d.f. = 15); *C. torquatus* always had a larger skull than *C. ibicuiensis*.

Skull Shape: The LDA showed the highest percentages of correct classification for the dorsal and lateral views of the skull, and in all analyses, *C. ibicuiensis* was correctly classified more than 87% of the time (Table 2). For MANOVA results, we found significant differences in skull shape for dorsal ($\lambda_{Wilks} = 0.0001$, $F = 5.781$; d.f. = 6; $P < 0.001$), ventral ($\lambda_{Wilks} = 0.0001$, $F = 5.172$; d.f. = 6; $P < 0.001$), and lateral ($\lambda_{Wilks} = 0.0002$, $F = 7.583$; d.f. = 6; $P < 0.001$) views of the cranium. All pairwise comparisons of *C. ibicuiensis* with species from the *torquatus* group were significant (Table 3). The graph of the discriminant analysis showed the variation in skull shape for *C. ibicuiensis* and other species from the *torquatus* group (Fig. 4). The plots of discriminant analysis for three views of the skull showed *C. ibicuiensis* with intermediate scores on the 1st axis, among other species (Figs. 4A, 4E, and 4I). For the ventral view of the skull, the 2nd axis showed positive scores for *C. ibicuiensis* (Fig. 4E) and the main difference in skull shape was the rostrum length, which is proportionally shorter in *C. ibicuiensis* (Figs. 4C and 4G).

The unrooted neighbor-joining tree of Mahalanobis distances for three views of the skull pooled, as in the discriminant analysis, placed *C. ibicuienesis* between two subgroups. On one side there is the southern Brazilian species *C. lami* and *C. minutus*, and on the other side the species *C. torquatus*, *C. pearsoni* from

Uruguay, and *C. perrensi*, and *C. roigi* from Argentina (Fig. 5). In the phenogram (Fig.5), *C. ibicuiensis* takes an intermediary place among *Ctenomys* Brazilian species (*C. lami*, *C. minutus*, and *C. torquatus*).

Qualitative morphological skull traits: The skull of *C. ibicuiensis* is shorter than that of *C. torquatus*, and is more similar to the juveniles than to the larger skulls and robust zygomatic arches in *C. torquatus* adults. The jugal process is smaller in *C. ibicuiensis,* and in general the cranium is more compact than in *C. torquatus*.

*Phylogenetic analysis*. – The 1110 bp of the cytochrome b gene resulted in 14 polymorphic sites that provided 10 haplotypes for the 34 specimens of *C. ibicuiensis* examined (Table 1; GenBank accession number in Appendix II). The nucleotide and haplotype diversities were 0.00413 and 0.88, respectively. The network (Fig. 6A) represents the topological relationships among the haplotypes regarding their localities of occurrence. Despite the overall small number of samples, there was high haplotype diversity, mainly for L1, where a larger number of specimens were collected (see Table 1). All haplotypes were exclusively from one locality; only haplotype H9 was shared between L5 and L6, which are neighboring sites (Fig. 1).

The intraspecific p-distance among haplotypes of *C. ibicuiensis* varied from 0.1 to 0.8%. Within the *torquatus* group, the range of interspecific divergence was from 0.3% (*C. lami* and *C. minutus*) to 4.8% (*C. pearsoni* and *C. ibicuiensis*). Other comparisons are given in Table 4. There were no values of divergence lower than 3.7% between *C. ibicuiensis* and other species of the *torquatus* group (Table 4). Similarly, there were no values lower than 4.8% comparing *C. ibicuiensis* and any species of the genus *Ctenomys* outside of the *torquatus* group (data not shown).

Topologies obtained from MP, ML and BI approaches were congruent, except in relation to nodes with weak support on the basis of the phylogeny. Therefore, we present only the BI phylogeny (Fig. 6B). A monophyletic clade, with strong support, was recovered for all cytochrome b haplotypes of the new species. *Ctenomys ibicuiensis* belongs to the *torquatus* group, which had high support from the BI and ML analysis, and showed three major clades (Fig. 6B). One clade included the *C. ibicuiensis* haplotypes. The other clade was composed by *C. torquatus* haplotypes as a monophyletic group, and sister of the branch leading to *C. minutus* and *C. lami*. The third and major clade comprised the tuco-tuco species from Corrientes – Argentina, and *C. pearsoni* from Uruguay.

On the whole, phylogenetic analyses recovered the eight species groups of *Ctenomys* that were distinguished by Parada et al. (2011), except some cases for MP that showed a basal polytomy for the genus.

## DISCUSSION

There are seven species of tuco-tucos described in Brazil 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, *C. brasiliensis*, until recently was presumed to have been described from Brazil, specifically in the State of Minas Gerais. As it was never collected again in that state, it has been a matter for investigation (F. A. Fernandes, pers. comm.). The present study adds one more species to the mammal diversity of RS, on the basis of strong phylogenetic, chromosomal, and morphological evidences.

*Chromosomal evidence* – The numbers of chromosomes and arms (fundamental number) are different between *C. ibicuiensis* and other *Ctenomys* species in southern Brazil, but chromosomal morphology is the most important diagnostic characteristic that differentiates the karyotype of the new species. Peculiarities of this karyotype are the long submetacentric 1st pair and the relatively longer acrocentric 1st pair in comparison with others acrocentric pairs. The diploid number of 50 chromosomes is the same as in *C. minutus*, in which the diploid number ranges from 2n = 42 to 50 (Freitas 1997). However, *C. ibicuiensis* and *C. minutus* with 2n = 50 are morphologically very different, because this latter species, which occurs on the RS coastal plain, has 14 biarmed and 10 acrocentric pairs of chromosomes (Freitas 1997).

One of the most widely distributed species of *Ctenomys*, from central Uruguay to central RS in southern Brazil, is *C. torquatus.* Its karyotypes range from 2n = 40 to 46, with a metacentric 1st pair and Robertsonian rearrangements thought to be responsible for the variation in chromosome numbers (Fernandes et al. 2009a).

Other species in southern Brazil are morphologically and numerically different. *Ctenomys lami* has a diploid number ranging from 2n = 54 to 58; and *C. flamarioni* exhibits 2n = 48 (Freitas 1994). In Uruguay, *C. pearsoni* has 2n = 56, 64, 66 and 70 (Kiblisky et al. 1977; Novello and Lessa 1986; Villar et al. 2005) and *C. rionegrensis*, 2n = 52 and 56 (Ortells et al. 1990). On the other side of the Uruguay River, in Argentina, the Corrientes species group (*C. dorbignyi, C. perrensis* and *C. roigi*; Ortells and Barrantes 1994) shows a diploid number ranging from 42 to 70 (Ortells et al. 1990). Although the members of this species group from Corrientes are closely related to *C. torquatus* with respect to the karyotype morphology, they are phylogenetically distant (Giménez et al. 2002; Fernandes et al. 2009a).

*Morphometric comparisons* – The geometric morphometrics approach using data from coordinates of landmarks revealed significant differences in skull shape and size for *C. ibicuienis* compared with species from the *torquatus* group. Both geometric morphometric and qualitative analysis of the skull showed that *C. ibicuiensis* is more compact and smaller than that of *C. torquatus*.

*Phylogenetic relationships* – The phylogenetic trees (Fig. 6B) and the p-distances (Table 4) clearly detached the cytochrome b haplotypes of *C. ibicuiensis* as a monophyletic group.

This species belongs to the *torquatus* species group, which was composed of three well defined clades: that of *C. ibicuiensis* haplotypes; one including all tuco-tuco species of the *perrensi* complex (Mirol et al. 2010) together with the Uruguayan *C. pearsoni*; and another containing the southern Brazilian species *C. torquatus*, *C. minutus* and *C. lami* (Fig. 6B). In spite of the allopatry with *C. torquatus*, the new species did not share a direct common ancestor with it. Instead, *C. torquatus* has a common ancestor with species of the RS coastal plain, *C. lami* and *C. minutus*. These relationships were not well supported in the most recent and complete phylogenetic analysis for the genus *Ctenomys* (Parada et al. 2011), but that study did not include the new species described here. Adding *C. ibicuiensis* provided good phylogenetic support for the *torquatus* group and clarified relationships among taxa.

Phylogenetic relationships among tuco-tuco species always result in a basal polytomy, although some species groups can be detached. The recent and explosive speciation of the ctenomyids has been cited as the cause for such unresolved relationships (Parada et al. 2011 and references therein). Future studies would be more enlightening considering each phylogenetic group individually, especially those such as the *torquatus* group with geographically proximate species ranges.

Morphological similarities between karyotypes of the westernmost localities of *C. torquatus* (2n = 40 and 42) and *Ctenomys* sp. from Corrientes, Argentina (2n = 42, 44 and 46), encouraged Fernandes et al. (2009a) to assay the taxonomic relationships of these neighboring populations on opposite sides of the Uruguay River. The phylogenetic relationships using the cytochrome b gene showed that *C. torquatus* and species from Corrientes form two separate but sister clades, and there was no support for a subdivision attributed to karyotypes. In the present study, anew populations in southern Brazil showed a karyotypic number that resembled species from Corrientes (2n = 50), but phylogenetic analysis, in addition to karyotype morphology, provided evidences of ancient divergence between the Brazilian and Argentinean lineages of tuco-tucos along the Uruguay River.

Likewise, inferences regarding speciation mode and intraspecific variation were made for *C. pearsoni* and *C. torquatus* using geometric morphometrics and chromosomal polymorphism (Fernandes et al. 2009b), considering their phylogenetic closeness (Castillo et al. 2005). Nevertheless, current knowledge of the *torquatus* group species (Parada et al. 2011) and phylogenetic relationships shown in the present study point to a closer association of *C. pearsoni* with the Argentinean *C. dorbignyi*; whereas *C. torquatus* is related with the Brazilian coastal species *C. lami* and *C. minutus* (Fig. 6B). These results open new perspectives for the application of morphometric and karyotypic assays within the *torquatus* group.

*Conservation* – Tuco-tucos are at risk of predation by humans because they have been considered agricultural pests for decades, due their fossorial habits (Massoia 1970; Pearson et al. 1968). In Brazil, lack of knowledge about these small mammals' areas of occurrence and life history has contributed to difficulties in developing preservation initiatives (Fernandes et al. 2007).

The situation of *C. ibicuiensis* is an issue of the utmost concern because of its small geographic range and habitat restriction to sandy-soil grasslands. Such habitat specificity, combined with the low population density and patchy distribution typical of tuco-tucos, allows the assignment of this species within the highest degree of rarity for mammals (category H) suggested by Yu and Dobson (2000). Besides its narrow distribution, *C. ibicuiensis* populations are also endangered by the intensive agricultural and silvicultural activities, and the expanding areas undergoing desertification in southwest RS (Suertegaray et al. 2001).

Recent studies have highlighted the worry for conservation of the flora in that region, because of its peculiar floristic composition and the presence of endemic species such as *Froelichia tomentosa* and *Butia lallemantii*. Nevertheless, the south Brazilian grasslands are not adequately protected under current conservation policies (Freitas et al. 2009; Overbeck et al. 2007; Roesch et al. 2009). The present study adds on a new mammal species endemic to the southwest grasslands, which intensifies concerns regarding the conservation status of this region.

The findings reported here suggest that *C. ibicuiensis* occurs as an endemic species in a narrow region in western RS, which is one of the most endangered areas in southern Brazil (Fernandes et al. 2007). Karyotype morphology, with the large 1st submetacentric chromosome, is a diagnostic trait for this species. The geometric morphometric and phylogenetics analysis support the singularity of this species.

Further studies are needed in order to assess the factors, such as habitat specificity or demography, that may had influenced the rarity and persistence of *C. ibicuiensis* through ecological or evolutionary time.

Furthermore, investigation of its genetic diversity is recommended to determine the current status of its populations and their potential for continued survival.

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**RESUMO**

Uma nova espécie de tuco-tuco, gênero *Ctenomys*, é descrita para a região de solos arenosos no oeste do estado do Rio Grande do Sul, Brazil. Esta espécie distinguiu-se das demais espécies do gênero, endêmico da América do Sul, através de diversas abordagens. O número diplóide 2n = 50 (número de braços autossômicos ou número fundamental NF = 68) com o primeiro par consideravelmente maior que outras espécies relacionadas, são características diagnósticas para a nova espécie. Análises qualitativas e quantitativas de morfologia do crânio, utilizando morfometria geométrica, bem como análises filogenéticas do gene mitocondrial citocromo b, suportam este status, principalmente em comparação com outras espécies do gênero relacionadas filogeneticamente e próxima geograficamente. Com área de ocorrência restrita a aproximadamente 500 km$^2$, esta espécie encontra-se extremamente ameaçada devido à intensa produção de soja e silvicultura na região, além de processos de desertificação. Este cenário sugere que a nova espécie seja categorizada como ameaçada.

**LITERATURE CITED**

BAYLAC, M. 2006. Rmorph: a R geometric and multivariate morphometrics library. Available from the author.

BAYLAC, M., AND FRIESS, M. 2005. Fourier descriptors, procrustes superimposition and data dimensionality: an example of cranial shape analysis in modern human populations. Pp. 145 – 162 in Modern Morphometrics in Physical Anthropology (D. E. Slice, ed.). Springer-Verlag, New York.

BAYLAC, M., VILLEMANT, C., AND SIMBOLOTTI, G. 2003. Combining geometric morphometrics to pattern recognition for species complexes investigations. Biological Journal of the Linnean Society 80: 89–98.

BOOKSTEIN, F. L. 1991. Morphometric tools for landmark data. Cambridge University Press, New York.

CASTILLO, A. H., CORTINAS, M. N., AND LESSA, E. P. 2005. Rapid diversification of South American tuco-tucos (*Ctenomys*; Rodentia; Ctenomyidae): contrasting mitochondrial and nuclear intron sequences. Journal of Mammalogy 86: 170–179.

CORDEIRO-ESTRELA, P., BAYLAC, M., DENYS, C., AND MARINHO-FILHO, J. 2006. Interspecific patterns of skull variation between sympatric Brazilian vesper mice: geometric morphometrics assessment. Journal of Mammalogy 87: 1270 – 1279.

D'ELIA, G., LESSA, E. P., AND COOK, J. A. 1999. Molecular phylogeny of tuco-tucos, genus *Ctenomys* (Rodentia, Octodontidae): Evaluation of the mendocinus species group and the evolution of asymmetric sperm. Journal of Mammalian Evolution 6: 19–38.

DRUMMOND, A. J., AND RAMBAUT, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7: 214.

DRYDEN, I. L., AND MARDIA, K.V. 1998. Statistical shape analysis. John Wiley and Sons, Inc., New York.

FERNANDES, F. A., FERNÁNDEZ-STOLZ, G. P., LOPES, C. M., AND FREITAS, T. R. O. 2007. The conservation status of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae), in southern Brazil. Brazilian Journal of Biology 4: 839–847.

FERNANDES, F. A., GONÇALVES, G. L., XIMENES, S. S. F., AND FREITAS, T. R. O. 2009a. Karyotypic and molecular polymorphisms in the *Ctenomys torquatus* (Rodentia: Ctenomyidae): taxonomic considerations. Genetica 136: 449–459.

FERNANDES, F. A., FORNEL, F., CORDEIRO-ESTRELA, P., AND FREITAS, T. R. O. 2009b. Intra- and interspecific skull variation in two sister species of the subterranean rodent genus *Ctenomys* (Rodentia,

Ctenomyidae): coupling geometric morphometrics and chromosomal polymorphism. Zoological Journal of the Linnean Society 155: 220–237.

FORD, C. E., AND HAMERTON, J. L. 1956. A colchicines, hypotonic citrate, squash sequence for mammalian chromosomes. Stain Technology 31: 247–251.

FREITAS, E. M., TREVISAN, R., SCHNEIDER, A. A., AND BOLDRINI, I. I. 2009. Floristic diversity in areas of sandy soil grasslands in Southwestern Rio Grande do Sul, Brazil. Brazilian Journal of Biosciences 8: 112 – 130.

FREITAS, T. R. O. 1994. Geographic variation of heterochromatin in *Ctenomys flamarion* (Rodentia-Octodontidae) and its cytogenetic relationships with other species of the genus. Cytogenetics and Cell Genetics, 67:193-198.

FREITAS, T. R. O. 1997. Chromosome polymorphism in *Ctenomys minutus* (Rodentia-Octodontidae). Genetics and Molecular Biology 20: 1-7.

FREITAS, T. R. O. 2001. Tuco-tucos (Rodentia, Octodontidae) in Southern Brazil: *Ctenomys lami* spec. nov. separated from *C. minutus* Nehring 1887. Studies on Neotropical Fauna and Environment 36: 1–8.

FREITAS, T. R. O. 2006. Cytogenetics status of four *Ctenomys* species in the south of Brazil. Genetica 126:227–235.

FREITAS, T. R. O., AND LESSA, E. P. 1984. Cytogenetics and morphology of *Ctenomys torquatus* (Rodentia - Octodontidae). Journal of Mammalogy 65: 637–642.

GARCÍA, L., PONSA, M. M., EGOZCUE, J., AND GARCIA, M. 2000. Comparative chromosomal analysis and phylogeny in four *Ctenomys* species (Rodentia, Ocotodontidae). Biological Journal of the Linnean Society 69: 103–120.

GIMÉNEZ, M. D., MIROL, P., BIDAU, C. J., AND SEARLE, J. B. 2002. Molecular analysis of populations of *Ctenomys* (Caviomorpha, Rodentia) with high karyotypic variability. Cytogenetics and Genome Research 96: 130–136.

GONÇALVES, G. L., AND FREITAS, T. R. O. 2009. Intraspecific variation and genetic differentiation of the collared tuco-tuco (*Ctenomys torquatus*) in southern Brazil. Journal of Mammalogy 90: 1020–1031.

GUINDON, S., AND GASCUEL, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52:696–704.

KIBLISKY, P., BRUM-ZORRILLA, N., PEREZ, G., AND SAEZ, F. A. 1977. Variabilidad cromosómica entre diversas poblaciones uruguayas del roedor cavador del género *Ctenomys* (Rodentia-Octodontidae). Mendeliana 2: 85–93.

LANGGUTH, A., AND ABELLA, A. 1970. Las especies uruguayas del género *Ctenomys* (Rodentia, Octodontidae). Comunicaciones Zoológicas del Museo de Historia Natural de Montevideo 10: 1–20.

LESSA, E. P., AND COOK, J. A. 1998. The molecular phylogenetics of tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae) suggest an early burst of speciation. Molecular Phylogenetics and Evolution 9: 88–99.

LIBRADO, P., AND ROZAS, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

LOPES, C. M. 2011. História evolutiva de *Ctenomys minutus* e *Ctenomys lami* na planície costeira do Sul do Brasil. Ph.D. Thesis, Universidade Federal do Rio Grande do sul, Porto Alegre, Brazil.

LUCY, D., AND AYKROYD, R. 2004. GenKern: Functions for generating and manipulating kernel density estimates, R package version 1.1.0.

MASCHERETTI, S., MIROL, P. M., GIMÉNEZ, M. D., BIDAU, C. J., CONTRERAS, J. R., AND SEARLE, J. B. 2000. Phylogenetics of the speciose and chromosomally variable rodent genus *Ctenomys* (Ctenomyidae: Octodontoidea), based on mitochondrial cytochrome b sequences. Biological Journal of the Linnean Society 70: 361–376.

MASSOIA, E. 1970. Mamíferos que contribuyen a deteriorar suelos y pasturas em la República Argetina. INTA - Instituto Nacional de Tecnología Agropecuaria, Buenos Aires – Argentina. Série IDIA: 14–17.

MEDRANO, J. F., AESEN, E., AND SHARROW, L. 1990. DNA extraction from nucleated red blood cells. Biotechniques 8: 43.

MIRANDA RIBEIRO, A. 1914. História Natural. Zoologia. Commissão de Linhas telegraphicas estratégicas de Matto-Grosso ao Amazonas.

MIROL, P., GIMÉNEZ, M. D., SEARLE, J. B., BIDAU, C. J., AND FAULKES, C.G. 2010. Population and species boundaries in the South American subterranean rodent *Ctenomys* in a dynamic environment. Biological Journal of the Linnean Society 100: 368–383.

NEI, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

NOVELLO, A., AND LESSA, E. P. 1986. G-Band homology in two karyomorphs of the *Ctenomys pearsoni* complex (Rodentia: Octodontidae) of Neotropical fossorial rodents. Zeitschrift für Säugetierkunde 51: 378–380.

ORTELLS, M. O., AND BARRANTES, G. E. 1994. A study of genetics distances and variability in several species of the genus *Ctenomys* (Rodentia, Octodontidae) with special reference to probable causal role of chromosome in speciation. Biological Journal of the Linnean Society 53: 189–208.

ORTELLS, M. O., CONTRERAS, J. R., AND REIG, O. A. 1990. New *Ctenomys* karyotypes (Rodentia, Octodontidae) from northeastern Argentina and from Paraguay confirm the extreme chromosomal multiformity of the genus. Genetica 82: 189–191.

OVERBECK, G.E., ET AL. 2007. Brazil's neglected biome: the South Brazilian Campos. Perspectives in Plant Ecology, Evolution and Systematics 9: 101–116.

PARADA, A., D'ELÍA, G., BIDAU, C. J., AND LESSA, E.P. 2011. Species groups and the evolutionary diversification of the tuco-tucos, genus *Ctenomys* (Rodentia: Ctenomyidae). Journal of Mammalogy 92: 671–682.

PARADIS, E., CLAUDE, J., AND STRIMMER, K. 2004. APE: Analyses of phylogenetic and Evolution R language. Bioinformatics 20: 289–290.

PEARSON, O., ET AL. 1968. Estructura social, distribución espacial y composición por edades de una población de tuco-tucos (*Ctenomys talarum*). Investigaciónes Zoológicas Chilenas: 47–79.

POSADA, D., AND CRANDALL, K. A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

REIG, O. A., BUSCH, C., ORTELLS, M. O., AND CONTRERAS, J. R. 1990. An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. Pp. 71–96 in Evolution of subterranean mammals at the organismal and molecular levels (E. Nevo and O. A. Reig, eds.). Wiley-Liss, New York.

ROESCH, L. F. W., ET AL. 2009. The Brazilian Pampa: A Fragile Biome. Diversity 1: 182–198.

ROHLF, F. J. 2004. TpsUtil, ver. 1.26 software; TpsDig, ver. 1.40 software and TpsREGR, ver. 1.29, F. James Rohlf. Dept. Ecology and Evolution, State University of New York, Stony Brook.

SHAPIRO, B., RAMBAUT, A., AND DRUMMOND, A. J. 2006. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Molecular Biology and Evolution 23: 7–9.

SIKES, R.S., GANNON, W.L., AND THE ANIMAL CARE AND USE COMMITTEE OF THE AMERICAN SOCIETY OF MAMMALOGISTS. 2011. Guidelines of the American Society of Mammalogists for the use of wild mammals in research. Journal of Mammalogy 92:235–253.

SLAMOVITS, C. H., COOK, J. A., LESSA, E. P., AND ROSSI, M. S. 2001. Recurrent amplification and deletions of satellite DNA accompanied chromosomal diversification in South American tuco-tucos (genus *Ctenomys*, Rodentia: Octodontidae): A phylogenetic approach. Molecular Biology and Evolution 18: 1708–1719.

SMITH, M. F., AND PATTON, J. L. 1999. Phylogenetic relationships and the radiation of sigmodontine rodents in South America: evidence from cytochrome b. Journal of Mammalian Evolution 6:89–128.

SUERTEGARAY, D. M. A., GUASSELLI, L. A., AND VERDUM, R. 2001. Atlas da Arenização: Sudoeste do Rio Grande do Sul. Porto Alegre: Secretaria da Coordenação e Planejamento.

SWOFFORD, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M., AND KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731–2739.

VENABLES, W. N., AND RIPLEY, B. D. 2002. MASS: Modern applied statistics with S. 4ed. Springer, New York.

VERZI, D. H., OLIVARES, A. I., AND MORGAN, C. C. 2010. The oldest South American tuco-tuco (late Pliocene, northwestern Argentina) and the boundaries of genus *Ctenomys* (Rodentia, Ctenomyidae). Mammalian Biology 75:243–252.

VILLAR, S., MARTÍNEZ-LÓPEZ, W., FOLLE, G., AND NOVELLO, A. 2005. Cytogenetic analysis of different *Ctenomys* (Rodentia, Octodontidae) species from Uruguay using G-banding. Mammalian Biology 70: 255–260.

WLASIUK, G., GARZA, J. C., AND LESSA, E. P. 2003. Genetic and geographic differentiation in the Rio Negro tuco-tuco (*Ctenomys rionegrensis*): inferring the roles of migration and drift from multiple genetic markers. Evolution 57: 913–926.

YU, J., AND DOBSON, F. S. 2000. Seven forms of rarity in mammals. Journal of Biogeography 27, 131–139.

## FIGURE LEGENDS

**Fig. 1.-** Locality records of *Ctenomys ibicuiensis* new species in Manoel Viana and Maçambara municipalities (state of Rio Grande do Sul) in southern Brazil. L1 to L6 correspond to localities listed in Table 1. Squares represent *C. torquatus* sites and stars are locations with no records of tuco-tuco occurrence. Dashed lines are municipality borders.

**Fig. 2.-** Conventional Giemsa staining of *Ctenomys ibicuiensis* (female) from Manoel Viana, state of Rio Grande do Sul, Brazil. Presenting 2n = 50 and FNa = 68 with 10 biarmed and 14 acrocentric pairs. Scale bar = 10µ.

**Fig. 3.-** Dorsal, ventral, and lateral views of the skull, and lateral view of the mandible of *Ctenomys ibicuiensis* new species holotype (TR 1065) from Manoel Viana municipality in Rio Grande do Sul in southern Brazil. Scale bar = 10 mm.

**Fig. 4.-** Skull shape variation of *Ctenomys ibicuiensis* for dorsal (A to C), ventral (E to G) and lateral views (I to K), compared with species from the *torquatus* group. Plot of discriminant analyses with the 1st 2 axes (A, E and I); frames of shape differences on the 1st axis (B, F and J) and 2nd axis (C, G and K); indication of landmarks in dorsal, ventral and lateral skull views of *C. minutus* (D, H and L, respectively). Frames represent extreme morphologies, the solid lines depict shape on the positive scores of discriminant axis, and the dotted lines on the negative ones. The percent of variance explained for each axis is given in parentheses.

**Fig. 5.-** Phenogram computed from the Mahalanobis distances among *Ctenomys ibicuiensis* and species from the *torquatus* group. The tree is made by the neighbor-joining method with branch lengths proportional to morphological distances for dorsal, ventral, and lateral views of the skull pooled.

**Fig. 6.-** Phylogenetic relationships of *Ctenomys ibicuiensis*. A) Intraspecific topological relationship between the cytochrome b haplotypes. Areas are proportional to frequencies, depictions refer to sampling sites (codes in Table 1 and Fig. 1), crosshatches represent mutation steps and black points represent medium vectors. B) Phylogenetic tree of the cytochrome b gene from ctenomyid species resulting from

Bayesian analysis in BEAST. The haplotypes of *C. ibicuiensis* are shown in **boldface**. Haplotype labels follow Appendix I. Numbers in nodes indicate support from posterior probability, likelihood bootstrap (%) and parsimony bootstrap (%), respectively. Hyphens (-) indicate nodes with posterior probability < 0.8 and bootstrap support < 60%. Vertical bars and letters A to H correspond to species groups and D indicates the *torquatus* group.

## APPENDIX

**APPENDIX I. –** List of *Ctenomys* skull specimens used in this study, with collection numbers.

| Species | Vouchers |
|---|---|
| *C. ibicuiensis* | TR-1065, 1066, 1067, 1068, 1070, 1072, 1073, 1074, 1505, 1506, 1507, 1508, 1509, 1510, 1513, 1517 |
| *C. roigi* | MMP-2410, 2411, 2442, 3417, 3461 ; MVZ-179153, 179154 |
| *C. perrensi* | MMP-2413, 2414, 2437, 2440, 2441, 2447, 3418; MVZ-179151, 179152 |
| *C. pearsoni* | MACN-19763, 19843, 19844, 19851, 20211; MLP-30XI93-3, 30XI93-4; MNHN-1806, 1807, 1835, 1836, 1837, 1838, 1841, 2249, 2300, 2306, 2324, 2795, 2796, 4167, 4168, 4170, 4177, 4179, 4183, 4188, 4189, 4194, 4197 |
| *C. torquatus* | TR-374, 900, 926, 927, 928, 929, 930, 933, 934, 935, 936, 939, 940, 941, 942, 944, 945, 946, 938, 949, 950, 951, 952, 954, 955, 956, 959, 960, 963, 964. |
| *C. lami* | TR-79, 86, 87, 89, 95, 97, 109, 110, 113, 114, 115, 136, 228, 340, 344, 503, 504, 507, 508, 511, 515, 517, 518, 520, 522, 524, 530, 540, 549, 621 |
| *C. minutus* | TR-49, 123, 124, 126, 127, 128, 130, 229, 230, 231, 234, 281, 288, 289, 290, 291, 293, 294, 299, 300, 302, 304, 305, 307, 308, 309, 311, 554, 567, 632 |

MACN - Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires, Argentina.

MMP - Museo de Ciencias Naturales “Lorenzo Scaglia”, Mar del Plata, Argentina.

MLP - Museo de La Plata, La Plata, Argentina.

MNHN - Museo Nacional de Historia Natural, Montevideo, Uruguay.

MVZ - Museum of Vertebrate Zoology, University of California, Berkeley, USA.

TR - Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil.

**APPENDIX II.** – List of species and their GenBank accession number for cytochrome b sequences of *Ctenomys* and outgroup employed in the phylogenetic analyses.

| Group | Species | GenBank number |
|---|---|---|
| Outgroup | *Octodon degus* | AF007059 |
| *Ctenomys* | *C. sociabilis* | HM777495 |
| | *C. leucodon* | AF007056 |
| | *C. tuconax* | AF370693 |
| | *C.* sp. ITA | AF007047 |
| | *C.* sp. MINUT | AF007052 |
| | *C.* sp. MONTE | AF007053 |
| | *C. maulinus* | AF370703 |
| *frater* group | *C. frater* | AF007046 |
| | *C. conoveri* | AF007055 |
| | *C.* sp. LLATHU | AF007048 |
| | *C. lewisi* | AF007049 |
| *boliviensis* group | *C. boliviensis* | AF007040 |
| | *C. goodfellowi* | AF007051 |
| | *C. nattereri* | HM777484 |
| | *C.* sp. ROBO | AF007039 |
| | *C. steinbachi* | AF007044 |
| *tucumanus* group | *C. tucumanus* | AF370694 |
| | *C. occultus* | HM777485 |
| | *C. latro* | AF370705 |
| | *C. argentinus* | AF370680 |
| *magellanicus* group | *C. magellanicus* | AF370690 |
| | *C. colburni* | HM777474 |
| | *C. haigi* | AF007063 |
| | *C. coyhaiquensis* | AF119112 |
| | *C. fodax* | HM777475 |
| | *C. sericeus* | HM777496 |

| | | |
|---|---|---|
| | *C. sociabilis** | U34853 |
| | *C.* sp. 1 | HM777500 |
| | *C.* sp. 2 | HM777501 |
| | *C.* sp. 3 | HM777502 |
| | *C.* sp. 4 | HM777503 |
| | *C.* sp. 5 | HM777504 |
| | *C.* sp. 6 | HM777505 |
| | *C.* sp. 7 | HM777506 |
| *talarum* group | *C. talarum* | AF370699 |
| | *C. pundti* | HM777490 |
| *mendocinus* group | *C. mendocinus* | AF007062 |
| | *C. rionegrensis* | AF119114 |
| | *C. flamarioni* | AF119107 |
| | *C. australis* | AF370697 |
| | *C. porteousi* | AF370682 |
| *opimus* group | *C. opimus* | AF007042 |
| | *C. fulvus* | AF370688 |
| | *C. saltarius* | HM777493 |
| | *C. scagliai* | HM777494 |
| *torquatus* group | *C. torquatus* H1 | EF372280 |
| | *C. torquatus* H2 | EF372282 |
| | *C. torquatus* H3 | EF372283 |
| | *C. torquatus* IT2 | JQ389037 |
| | *C. torquatus* IT1 | JQ389034 |
| | *C. lami* | HM777477 |
| | *C. minutus* | HM777481 |
| | *C. pearsoni* | AF119108 |
| | *C. perrensi* | HM777487 |
| | *C. roigi* | M777492 |
| | *C.* sp. Contreras Cue, Tacuaritas | JQ389032 |

| | |
|---|---|
| *C. dorbignyi* Sarandicito** | JQ389030 |
| *C. dorbignyi* Mbarigui** | JQ389031 |
| *C. ibicuiensis* H1 | JQ389020 |
| *C. ibicuiensis* H2 | JQ389021 |
| *C. ibicuiensis* H3 | JQ389022 |
| *C. ibicuiensis* H4 | JQ389023 |
| *C. ibicuiensis* H5 | JQ389024 |
| *C. ibicuiensis* H6 | JQ389025 |
| *C. ibicuiensis* H7 | JQ389026 |
| *C. ibicuiensis* H8 | JQ389027 |
| *C. ibicuiensis* H9 | JQ389028 |
| *C. ibicuiensis* H10 | JQ389029 |

ITA, MINUT, MONTE LLATHU and ROBO are ctenomyids with undefined taxonomic status. *A probable *C. haigi* sequence, according to Parada et al. (2011). **Samples of *C. dorbignyi* from Mbarigui and Sarandicito were considered separately because they came from different regions of the Corrientes Province, Argentina (see Mirol et al. 2010).

## TABLES

**TABLE 1.** – Sampling locations, geographic coordinates, number of individuals caught N (M – males; F – females), and the frequency of cytochrome b haplotypes described for each locality of *Ctenomys ibicuiensis*.

| Code | Locality | Geographic coordinates | N (M, F) | Haplotype frequency |
|---|---|---|---|---|
| L1 | BR176, Manoel Viana | 29°23'34.8"S 55°25'45.9"W | 12 (5, 7) | H1 (8), H2 (2), H3 (1), H4 (1) |
| L2 | Piraju, Manoel Viana | 29°24'11.9"S 55°34'49"W | 4 (2, 2) | H5 (4) |
| L3 | Lageado, Manoel Viana | 29°19'18.5"S 55°32'10.8"W | 5 (1, 4) | H6 (1), H7 (4) |
| L4 | Barragem do Itu, Manoel Viana | 29°13'50.6"S 55°28'1.8"W | - | - |
| L5 | Passo do Narciso, Maçambará | 29°9'34.6"S 55°25'15.6"W | 9 (3, 6) | H8 (6), H9 (3) |
| L6 | Passo do Narciso, Maçambará | 29°7'34.6"S 55°23'59.6"W | 4 (0, 4) | H9 (1), H10 (3) |

**TABLE 2.** – Percentage of correct classification from the linear discriminant analysis (LDA) for three views of the skull unified. Data for *Ctenomys ibicuiensis* and species of the *torquatus* group.

| Skull View | *C. ibicuiensis* | *C. lami* | *C. minutus* | *C. pearsoni* | *C. perrensi* | *C. torquatus* | *C. roigi* |
|---|---|---|---|---|---|---|---|
| Dorsal | 96.66 | 96.66 | 86.66 | 93.33 | 55.57 | 96.66 | 57.14 |
| Ventral | 86.95 | 86.66 | 90.00 | 86.67 | 66.67 | 96.66 | 85.71 |
| Lateral | 95.65 | 80.00 | 90.00 | 90.00 | 88.89 | 100.00 | 100.00 |

**TABLE 3.** – Pairwise MANOVA for comparisons of skull shape of *Ctenomys ibicuiensis* with species from the *torquatus* group (results for dorsal, ventral, and lateral skull views pooled).

| Comparison | $\lambda_{Wilks}$ | *F* | *d.f.* | *P* |
|---|---|---|---|---|
| *C. ibicuiensis* x *C. roigi* | 0.112 | 7.429 | 15 | 0.0043 * |
| *C. ibicuiensis* x *C. perresi* | 0.135 | 6.859 | 15 | 0.0035 * |
| *C. ibicuiensis* x C. *torquatus* | 0.187 | 10.678 | 15 | $5.43 \times 10^{-8}$ ** |
| *C. ibicuiensis* x *C. lami* | 0.06 | 38.939 | 15 | $4.62 \times 10^{-15}$ ** |
| *C. ibicuiensis* x *C. minutus* | 0.105 | 21.02 | 15 | $2.36 \times 10^{-12}$ ** |
| *C. ibicuiensis* x *C. pearsoni* | 0.167 | 12.251 | 15 | $8.23 \times 10^{-9}$ ** |

After Bonferroni correction: * $P < 0.01$; ** $P < 0.001$

**TABLE 4.** – Divergence among pairs of species within the *torquatus* group of genus *Ctenomys*, as uncorrected p-distance of the cytochrome b gene (shown as percentage). *dorbignyi* S and *dorbignyi* M refer to *C. dorbignyi* from Sarandicito and Mbarigui, respectively. sp. refers to *C.* sp. specimens from Contreras Cue and Tacuaritas. For *C. ibicuiensis* and *C. torquatus*, the p-distance was computed considering the mean distance for all haplotypes listed in Appendix II.

| *torquatus* group species | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *ibicuiensis* | | | | | | | | | |
| 3.7 | *torquatus* | | | | | | | | |
| 4.2 | 3.1 | *lami* | | | | | | | |
| 4.1 | 3.0 | 0.3 | *minutus* | | | | | | |
| 4.8 | 4.2 | 3.9 | 4.0 | *pearsoni* | | | | | |
| 4.1 | 4.0 | 3.7 | 3.8 | 1.7 | *dorbignyi* S | | | | |
| 3.8 | 3.7 | 3.7 | 3.6 | 1.9 | 2.0 | *dorbignyi* M | | | |
| 3.8 | 3.7 | 3.7 | 3.6 | 1.9 | 2.0 | 0.5 | sp. | | |
| 4.3 | 4.1 | 3.9 | 3.8 | 3.0 | 3.1 | 1.6 | 1.6 | *roigi* | |
| 4.4 | 4.0 | 3.8 | 3.9 | 2.4 | 2.3 | 1.4 | 1.4 | 2.3 | *perrensi* |

**FIGURES**

Figure 1:

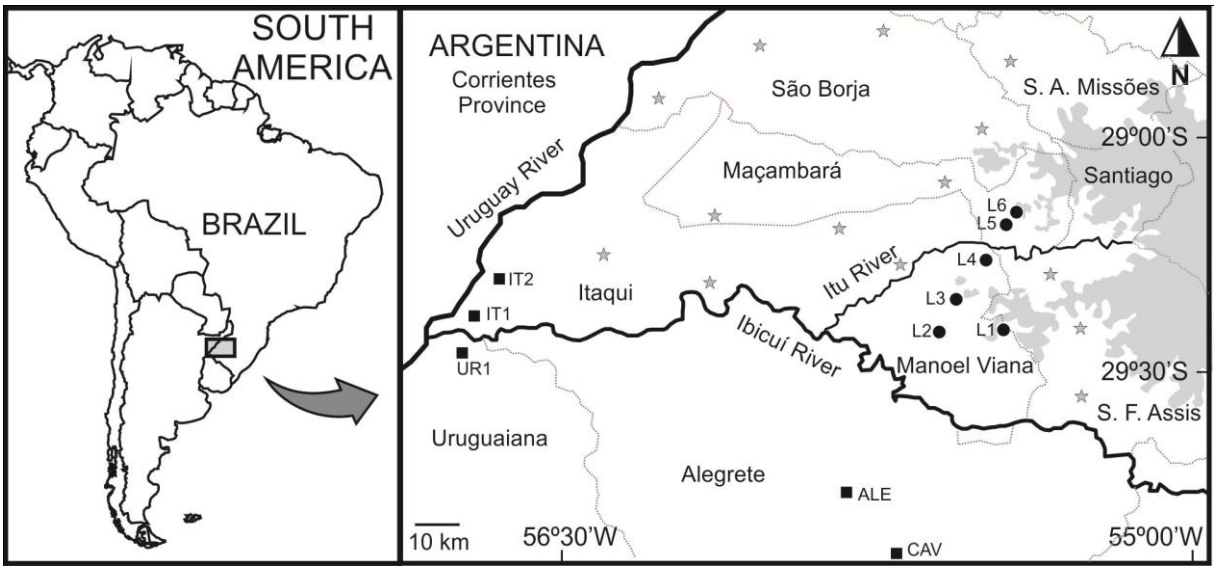


Figure 2:

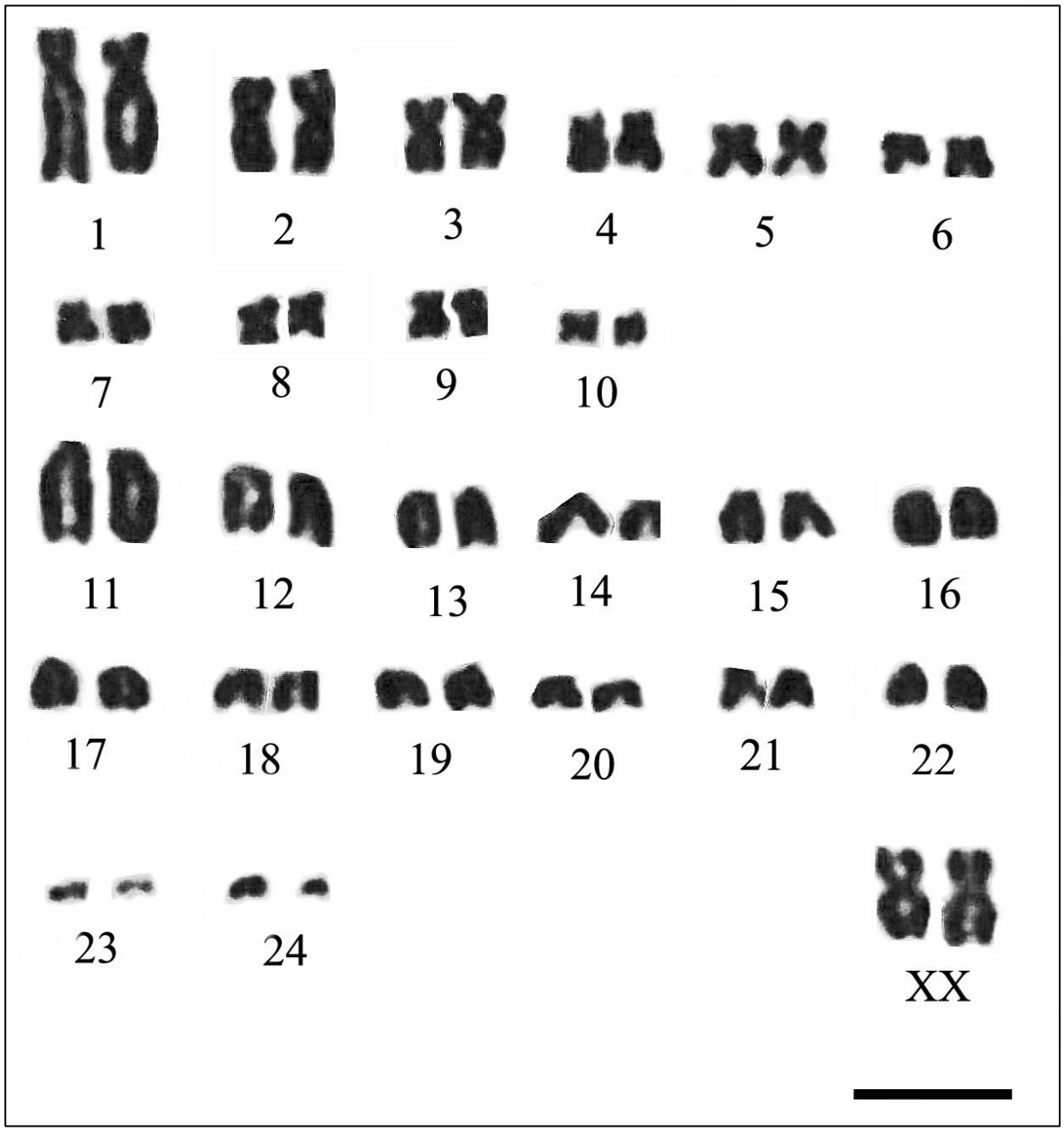


Figure 3:

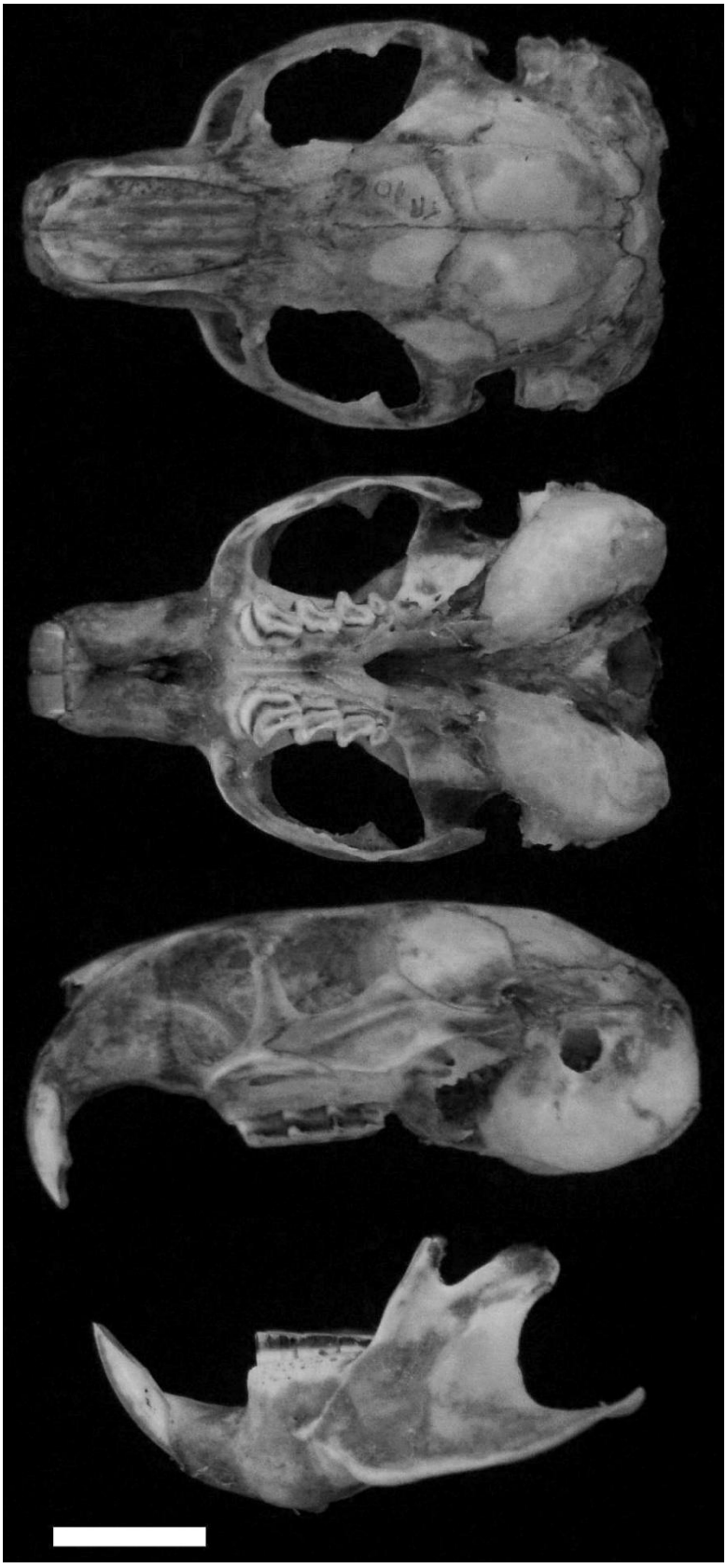


Figure 4:

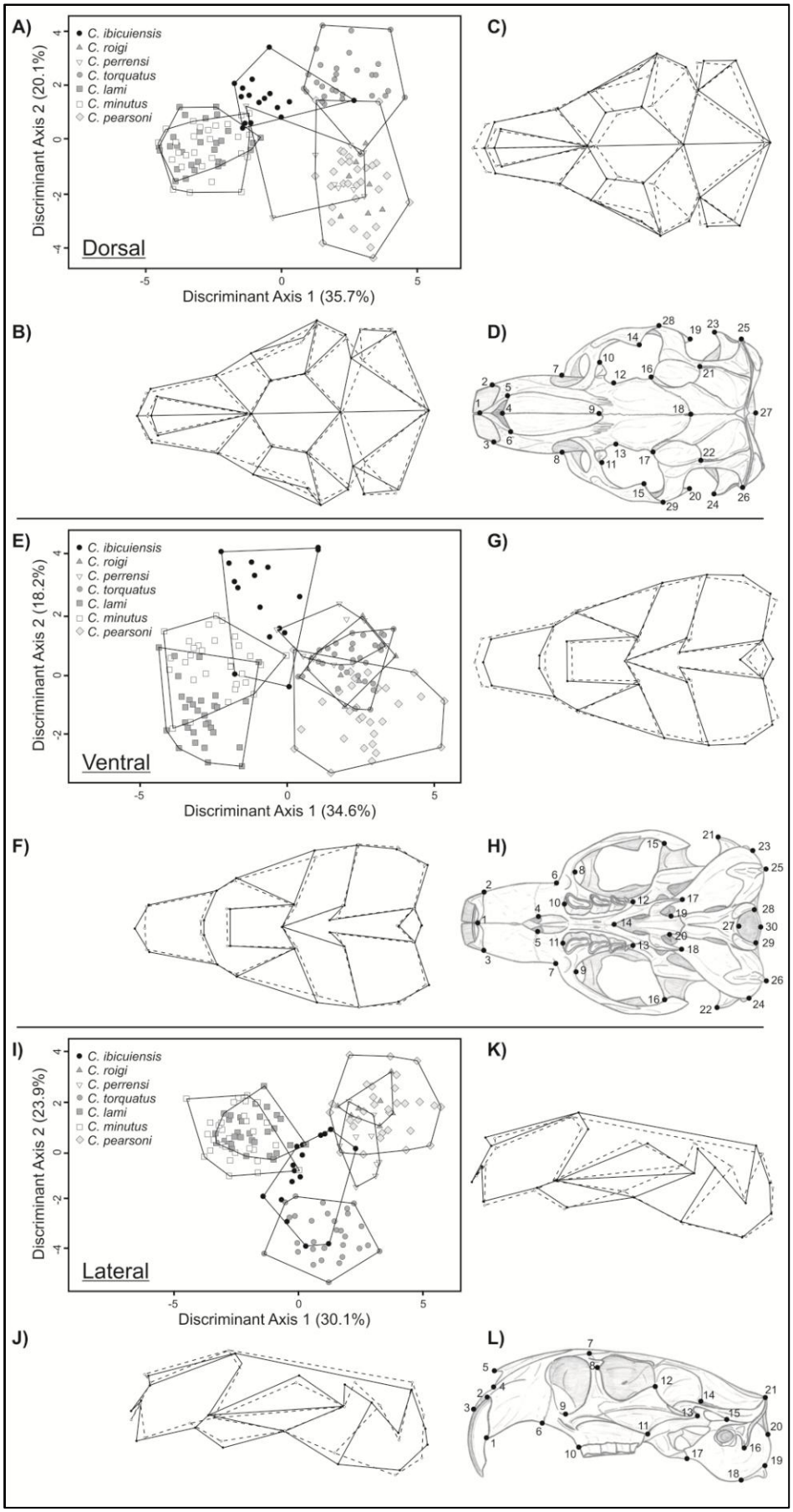

A)
C. ibicuiensis
C. roigi
C. perrensi
C. torquatus
C. lami
C. minutus
C. pearsoni
Discriminant Axis 2 (20.1%)
Dorsal
Discriminant Axis 1 (35.7%)
C)
B)
D)
E)
Discriminant Axis 2 (18.2%)
Ventral
Discriminant Axis 1 (34.6%)
G)
F)
H)
I)
Discriminant Axis 2 (23.9%)
Lateral
Discriminant Axis 1 (30.1%)
K)
J)
L)

Figure 5:

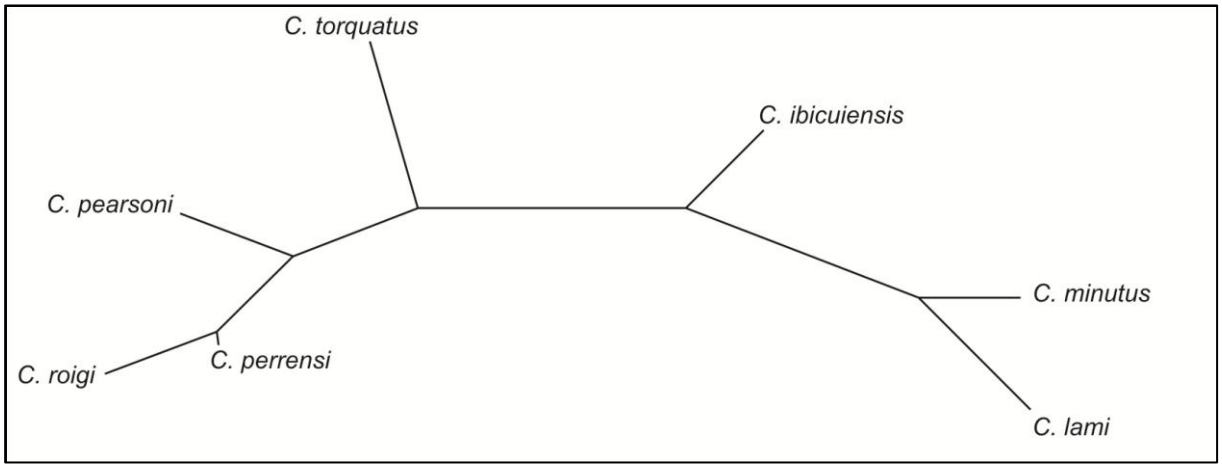
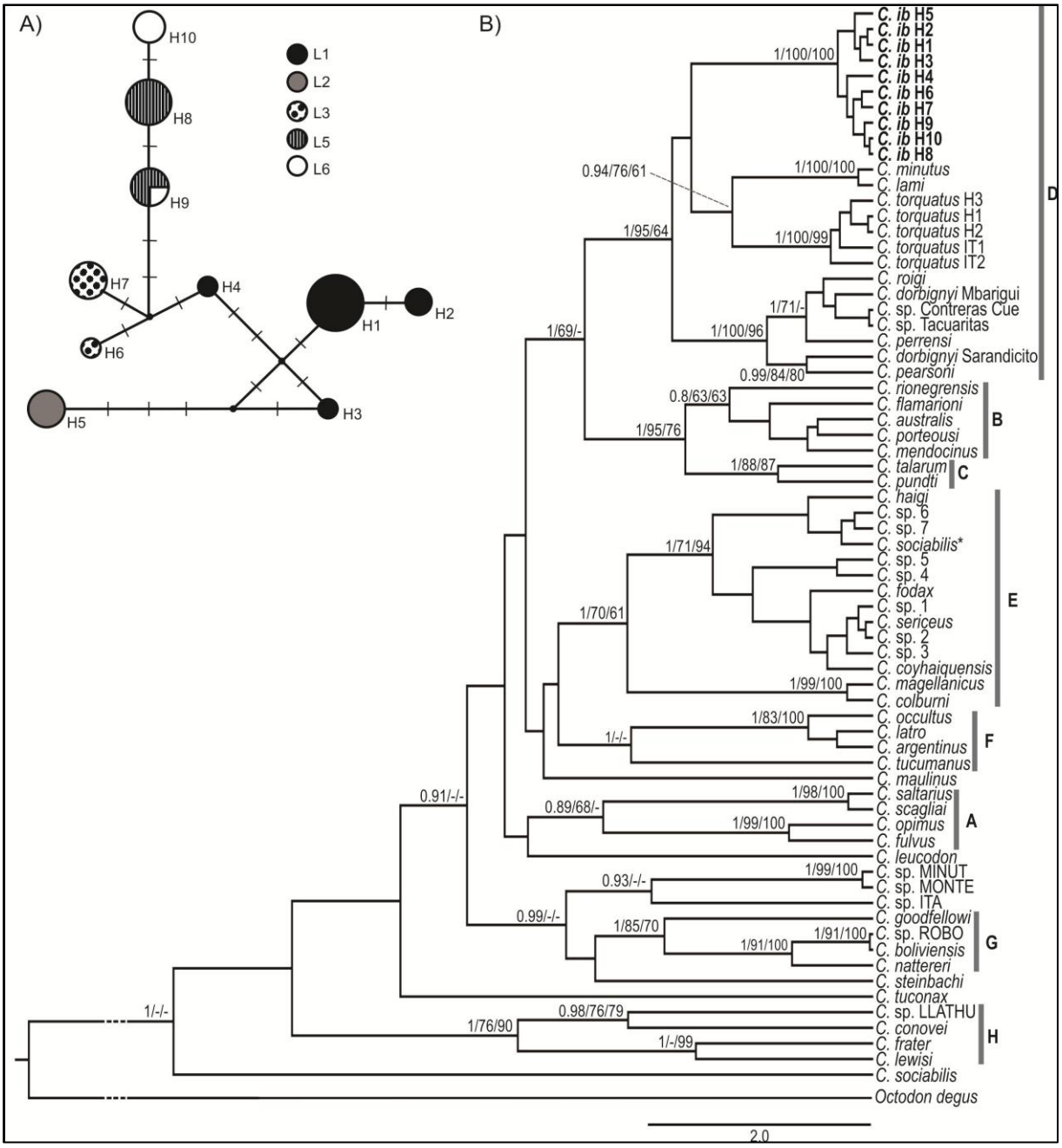

C. torquatus
C. ibicuiensis
C. pearsoni
C. minutus
C. roigi
C. perrensi
C. lami

Figure 6:


A)
H10
H8
H9
H7
H4
H2
H1
H6
H5
H3
L1
L2
L3
L5
L6
B)
C. ib H5
C. ib H2
C. ib H1
C. ib H3
C. ib H4
C. ib H6
C. ib H7
C. ib H9
C. ib H10
C. ib H8
C. minutus
C. lami
C. torquatus H3
C. torquatus H1
C. torquatus H2
C. torquatus IT1
C. torquatus IT2
C. roigi
C. dorbignyi Mbarigui
C. sp. Contreras Cue
C. sp. Tacuaritas
C. perrensi
C. dorbignyi Sarandicito
C. pearsoni
C. rionegrensis
C. flamarioni
C. australis
C. porteousi
C. mendocinus
C. talarum
C. pundti
C. haigi
C. sp. 6
C. sp. 7
C. sociabilis*
C. sp. 5
C. sp. 4
C. fodax
C. sp. 1
C. sericeus
C. sp. 2
C. sp. 3
C. coyhaiquensis
C. magellanicus
C. colburni
C. occultus
C. latro
C. argentinus
C. tucumanus
C. maulinus
C. saltarius
C. scagliai
C. opimus
C. fulvus
C. leucodon
C. sp. MINUT
C. sp. MONTE
C. sp. ITA
C. goodfellowi
C. sp. ROBO
C. boliviensis
C. nattereri
C. steinbachi
C. tuconax
C. sp. LLATHU
C. conovei
C. frater
C. lewisi
C. sociabilis
Octodon degus
1/100/100
0.94/76/61
1/100/100
1/95/64
1/100/99
1/71/-
1/69/-
1/100/96
0.99/84/80
0.8/63/63
1/95/76
1/88/87
1/71/94
1/70/61
1/99/100
1/83/100
1/-/-
0.91/-/-
1/98/100
0.89/68/-
1/99/100
1/99/100
0.93/-/-
0.99/-/-
1/85/70
1/91/100
1/91/100
1/-/-
0.98/76/79
1/76/90
1/-/99
D
B
C
E
F
A
G
H
2.0