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E BIOLOGIA MOLECULAR

História evolutiva de *Ctenomys minutus* e *Ctenomys lami* na planície costeira do Sul do Brasil (Rodentia, Ctenomyidae)

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Orbital Consequences

The sun and the earth describe orbital changes
which drive climate cycles and modify ranges.
The shape of the land forms a number of places
that allow the survival of different races.
When enclaves advance with the ice in retreat
some form hybrid zones where two ranges meet.
Such regions are common and not very wide
so the mixing of genes affects neither side.
They divide up the range in a patchwork of pieces
with echoes and glimpses on the nature of species.
A brief rendez-vous and the ice comes again.

When the glaciers melt so that ranges expand
some plants will spread quickly where there's suitable land.
Those insects which eat them will follow this lead
some flying, some walking to establish their breed.
Those that try later meet a resident band,
they must somehow be better to make their own stand.
But the mixture will change as more types arrive
and warming conditions allow new species to thrive.
Some will move on to fresh places ahead,
those that remain must adapt, or are dead.
And then the tide turns and the ice comes again.

Each refuge could foster a deviant form,
new neighbours, chance changes and drift from the norm.
When the warm breakout comes, those few in the van
disperse from the edge and breed where they can.
Pioneer pockets grow to large populations,
a very good place to strike new variations.
Some may not work well with their parental kind
so stopping the spread of those from behind.
Continental theatres provide plenty of chances
to establish new morphs in both retreats and advances.
New species may form when the ice comes again.

So what will you do when the ice comes again?
It could be quite quick, if the ice cores speak plain.
The great ocean currents that warm our green spring
may stop in a season should the salt balance swing.
Great civilizations in north temperate lands
must migrate south to the sun and the sands.
But past pollen and dust tell us these will be drier,
wet forests will shrink and population grow higher.
Our forebears hung on near a sea or a cave.
They fished and they painted, they dreamed, they were brave.
So like Noah and Eric, we must adapt and survive.

G. M. Hewitt (2001)

À minha família

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SUMÁRIO

RESUMO

Ctenomys lami e *C. minutus* são espécies irmãs de roedores subterrâneos, pertencentes ao grupo filogenético *torquatus* do gênero *Ctenomys*. Estas espécies são indistinguíveis através da morfologia externa, no entanto *C. lami* foi recentemente descrita como uma nova espécie distinta de *C. minutus* devido às diferenças em seus cariótipos, principalmente com relação às formas dos cromossomos e números diplóides, os habitats distintos que ocupam e também pelas diferenças encontradas através de análises morfométricas de seus crânios e mandíbulas. Ambas as espécies são endêmicas da planície costeira do Sul do Brasil e apresentam um notável polimorfismo cromossômico. *Ctenomys minutus* se distribui em uma estreita faixa de dunas ou campos arenosos próximos da linha da costa, nos estados do Rio Grande do Sul (RS) e sul de Santa Catarina (SC). Apresenta números diplóides variando de 2n = 42 até 50 e números autossômicos (NA) de 68 a 80, compreendendo um total de 45 cariótipos descritos até o momento. *Ctenomys lami* é geograficamente restrito a uma área de 78 x 12 km, chamada Coxilha das Lombas, correspondente aos campos arenosos mais internos da planície costeira do RS. Apresenta 5 números diplóides variando de 2n = 54 a 58 e NA variando de 74 a 84, os quais combinados formam 26 cariótipos. Quatro blocos cariotípicas, denominados blocos A, B, C e D, foram descritos para esta espécie considerando os rearranjos Robertsonianos encontrados. Além disso, duas zonas híbridas intraespecíficas foram descritas, uma entre os blocos cariotípicos A x B e outra localizada entre os os blocos C x D. Ainda, nas proximidades da Lagoa dos Barros existem evidências da ocorrência de uma zona de hibridação interespecífica. Com os objetivos de acessar os padrões filogeográficos e populacionais dessas duas espécies, confirmar e caracterizar a zona híbrida interespecífica e providenciar informações para ajudar a traçar estratégias conservacionistas, foram analisados 2 fragmentos do DNA mitocondrial e 14 *loci* de microssatélites em 172 espécimes de *C. lami*, 340 espécimes de *C. minutus* e 19 possíveis híbridos, associados aos cariótipos de cada um dos espécimes e informações geomorfológicas a respeito do ambiente que ocupam. Para as duas espécies as descontinuidades no ambiente agem como um dos principais fatores estruturantes da variabilidade genética, representadas principalmente por cursos de água mais volumosos. Além disso, o isolamento pela distância também desempenha um papel importante no padrão de diferenciação

populacional para as duas espécies, sendo mais evidente em alguns trechos da distribuição geográfica do que em outros. Não foram observadas associações diretas entre mudanças significativas na estrutura genética com os diferentes rearranjos cromossômicos encontrados tanto em *C. lami* quanto em *C. minutus*, demonstrando que os diferentes cariótipos presentes nestas espécies desempenharam, até o momento, um papel secundário na estrutura genética das populações. Apesar da ausência de clados reciprocamente monofiléticos para o DNAmt, *C. lami* e *C. minutus* são consideradas como duas espécies distintas com base nos resultados das análises citogenéticas, dos *loci* de microssatélites, pelas diferenças na morfologia do crânio e mandíbula e a distinção dos habitats que ocupam. A presença de uma zona de hibridação interespecífica foi confirmada, os 19 híbridos apresentaram cariótipos intermediários entre as espécies analisadas, genoma mitocondrial proveniente de *C. minutus*, e a composição dos *loci* de microssatélites provenientes de *C. lami*, levando a conclusão de que houve um cruzamento preferencial, ou até mesmo exclusivo, ao menos nos primeiros estágios da formação dessa zona híbrida, entre fêmeas de *C. minutus* com machos de *C. lami*. Os dados demonstraram também uma introgressão substancial do genoma nuclear de *C. lami* nos espécimes de *C. minutus* coletados nas proximidades da zona híbrida. Mediante o exposto, medidas conservacionistas devem ser tomadas focando conter o avanço da hibridação e introgressão entre essas espécies, além de preservar a integridade de populações puras que não sofrem a influência desses eventos. Além disso, sugerimos que *C. minutus* seja incluído em listas vermelhas de espécies ameaçadas como quase ameaçado, e *C. lami* como vulnerável.

ABSTRACT

Ctenomys minutus and *C. lami* are burrowing rodents that have been considered sister species of the *torquatus* group in the *Ctenomys* genus. These species are undistinguishable trough the external morphology, however *C. lami* were recently described as a separated species from *C. minutus* due to differences in their karyotypes, mainly regards the chromosomal forms and diploid numbers, the distinct habitats that these species occupy, and even by differences in the morphology of their skulls and mandibles. Both species are endemic to the southern Brazilian coastal plain and have notable chromosomal polymorphisms. *Ctenomys minutus* have a narrow distribution along the first dunes or sand fields near to the coast, in Rio Grande do Sul (RS) and Santa Catarina (SC) Brazilian states. This species presents diploid numbers ranging from 2n = 42 to 50, and the autosomal arm numbers (AN) ranging from 68 to 80, comprising a total of 45 karyotypes described until now. *Ctenomys lami* are geographically restricted to an area of 78 x 12 km, named as Coxilha das Lombas, corresponding to more internalized sandy fields of the RS coastal plain. This species have five diploid numbers from 2n = 54 to 58 and ten AN from 74 to 84, which combined formed 26 karyotypes. Four karyotypic blocks, named as Blocks A, B, C and D, were described for this species considering the Robertsonian rearrangements. Also, two intra-specific hybrid zones were reported for this species, one between blocks A x B, and another between blocks C x D. Near to the Barros Lake there are evidences of an interspecific hybrid zone between these species. The aims of this study are assess the phylogeographical and populational patterns for both species, to confirm and characterize the interspecific hybrid zone and provide informations to help future conservation decisions and management strategies for both species. For this, 2 fragments of mtDNA and 14 microsatellite loci were analyzed in 172 specimens of *C. lami*, 340 specimens of *C. minutus*, and 19 possible hybrids, associated to the karyotypes of each specimen sampled and geomorphological informations about the habitat occupied. In both species the environmental discontinuities act as effective geographical barriers to the gene flow, mainly major water streams. Moreover the isolation-by-distance also plays a key role in the pattern of genetic differentiation of populations, being more evident is some areas of the geographical distributions. There were no direct associations between distinct chromosomal rearrangements and genetic structures in both *C. lami* and *C. minutus*,

evidencing that karyotypes do not play a causative role in the genetic structure of populations. The absence of monophyletic clades separating both species was not considered by us as enough evidence to detract *C. minutus* and *C. lami* to a single taxa, since all other analyses regarding the pattern retrieved in microsatellite data, cytogenetic analyzes, and morphological and habitat differences confirm their status as two separated sister species, only recently differentiated. The interspecific hybrid zone was strongly evidenced by 19 individuals with intermediate karyotypic forms between the species analyzed. All hybrids showed mtDNA content exclusively from *C. minutus*, and nuclear microsatellite composition from *C. lami*, suggesting that the hybrid zone was formed by a pattern of crossings between females of *C. minutus* with males of *C. lami*. Also, a substantial clinal introgression of the genomic content of *C. lami* into *C. minutus* individuals sampled around the hybrid zone was reported. Conservation efforts should focus in hold the progress of introgression, to preserve the integrity of pure populations. Moreover, *C. minutus* have to be included in red lists of endangered fauna as near threatened and *C. lami* have to be included as vulnerable.

CAPÍTULO I

INTRODUÇÃO GERAL

A delimitação de espécies e todos os fatores que permeiam o processo de especiação são questões centrais no âmbito da biologia evolutiva e têm sido amplamente debatidas há aproximadamente 200 anos (Schilthuizen, 2000; Hewitt, 2001). Apesar do grande esforço aplicado por inúmeros pesquisadores no intuito de solucionar o que é uma espécie, esta ainda continua sendo uma questão não resolvida (King, 1993). Existem aproximadamente 22 conceitos diferentes de espécies na literatura moderna, muitos deles sendo incompatíveis na descrição da diversidade biológica (Mayden, 1997). A falta de um consenso, com a possibilidade de inúmeras respostas para esta pergunta, leva à convicção de que a especiação é um processo multifatorial, dependente do contexto em que é analisada, e que não se adequa a generalizações (Schilthuizen, 2000).

Considerando que espécies não são entidades fixas, e que estas evoluem continuamente no tempo e no espaço, muitas vezes é mais proveitoso representar o quadro completo revelado por uma pesquisa, por mais complexo que possa parecer, do que simplificar artificialmente os resultados para adequá-los a conceitos pré-estabelecidos (Hey *et al*., 2003). Neste sentido, identificar o papel de fatores direta ou indiretamente envolvidos na diferenciação de populações ou grupos de populações é de grande importância na compreensão do processo evolutivo. Isto posto, nos próximos tópicos serão abordados alguns dos principais fatores para a compreensão e desenvolvimento deste estudo.

Rearranjos cromossômicos

Diferentes características cariotípicas, como números diplóides e rearranjos cromossômicos, são normalmente variáveis entre as diferentes espécies, porém, costumam ser estáveis no nível intraespecífico (White, 1978; Lukhtanova *et al*., 2011). O surgimento e a fixação de novos rearranjos cromossômicos têm sido considerados como responsáveis por diminuir o fluxo gênico entre formas cromossômicas distintas, agindo como mecanismos de isolamento pós-zigóticos, e desempenhando, em alguns casos, papel chave

no processo de especiação (King, 1993). Alguns modelos de especiação baseados em rearranjos cromossômicos propõem que apenas os rearranjos com efeitos negativamente heteróticos, ou potencialmente negativamente heteróticos, que afetem o *fitness* de indivíduos heterozigotos, originando produtos meióticos deletérios ou até mesmo letais, têm a possibilidade de desempenhar um papel no processo de cladogênese (King, 1987). No entanto, modelos mais recentes sugerem que as mudanças cromossômicas reduzem o fluxo gênico, agindo como barreiras genéticas, pela redução da recombinação em heterocariótipos e pelos efeitos de aproximar ou afastar genes anteriormente isolados, em lugar do efeito da sub-dominância (Rieseberg, 2001; Navarro & Barton, 2003; Jackson, 2011).

Em contrapartida, é questionável se todos os tipos de rearranjos cromossômicos realmente podem exercer algum papel no processo de especiação. De acordo com King (1987), fusões em *tandem*, translocações recíprocas, e fusões ou fissões cêntricas têm grande capacidade de serem negativamente heteróticas. No entanto, se esses rearranjos forem capazes de produzir sistemas meióticos balanceados, nos quais houver segregação normal dos produtos da meiose, eles não atuarão como mecanismos de isolamento pós-zigótico eficientes, geralmente resultando em polimorfismos cromossômicos. Do mesmo modo, as inversões pericêntricas e paracêntricas podem formar produtos meióticos letais ou deletérios, porém, elas não apresentarão nenhum efeito na meiose se o quiasma for localizado fora da alça de inversão, se o *crossing-over* for inibido nos heterocariótipos, ou ainda se não houver pareamento de cromossomos homólogos heterozigotos para a inversão.

Existem numerosos casos na natureza de variações cariotípicas entre espécies proximamente relacionadas, especialmente em plantas, devido à poliploidia (Coghlan *et al*., 2005). Entre os mamíferos alguns exemplos de variabilidade cariotípica são encontrados no gênero *Muntiacus* que inclui espécies de veados nos quais os números diplóides variam de 2n=6 até 2n=46 (Huang *et al.*, 2006); o gênero de roedores *Proechmys* com 2n variando entre 14-62 (Reig & Useche, 1976; Barros, 1978); e os gêneros de roedores subterrâneos (que geralmente apresentam as taxas mais altas de evolução cromossômica entre os mamíferos): *Ctenomys*, que apresenta uma enorme variação interespecífica, com 2n=10 até 2n=70 (Reig *et al*., 1990); *Thomomys* (2n=40 – 2n=82;

Thaeler, 1980); e *Spalax* com mais de 30 cariótipos (2n=38-62) representados por 8 espécies clássicas (Savic & Nevo, 1980).

No nível intraespecífico o polimorfismo cromossômico em mamíferos geralmente se apresenta de forma mais discreta, com variações nas proporções de heterocromatina constitutiva, ou alguns poucos rearranjos cromossômicos (incluindo fissões, fusões e translocações). Porém, algumas espécies representam exceções. É o caso do musaranho *Sorex araneus* que possui aproximadamente 70 raças cromossômicas (Wójcik *et al*., 2003), o roedor *Mus musculus domesticus* com 2n variando entre 22 até 40 (Piálek *et al*., 2005), e o roedor subterrâneo *Thomomys talpoides* com 38 diferentes cariótipos (Thaeler, 1985).

A fixação de rearranjos cromossômicos neutros está muitas vezes relacionada a pequenos tamanhos populacionais, ou à periferia das populações, uma vez que novos rearranjos que surgem no interior de grandes populações estão em minoria e costumam ser selecionados negativamente. Porém, quando os rearranjos surgem em populações isoladas de menor densidade, sua fixação é favorecida pelo efeito da deriva genética (Lande, 1979). Características como baixas taxas de dispersão dos adultos e distribuição de pequenas populações relativamente fragmentadas são comuns à maioria dos roedores subterrâneos, e têm sido associadas ao favorecimento da fixação de novos rearranjos cromossômicos tanto no nível intra como interespecífico (Lacey *et al*., 2000). Considerando que os roedores apresentam convergências e homoplasias morfológicas que geram inúmeras controvérsias taxonômicas, associadas a uma grande diversidade cromossômica intraespecífica, o que tem sugerido a existência de espécies crípticas, a citogenética se apresenta como uma ferramenta particularmente útil no estudo desse grupo (Bonvicino *et al*., 2005).

Fatores geográficos

As distribuições geográficas das espécies são determinadas por uma série de fatores e podem variar substancialmente em períodos curtos de tempo, devido a mudanças climáticas, colonização de novas áreas, extinção de competidores, pela presença de barreiras geográficas, dentre outros fatores (Slatkin, 1987; Losos & Glor, 2003). Evidências que indicam essas mudanças são baseadas em registros fósseis, estudos realizados ao longo de vários anos e através de análises genéticas das populações que podem demonstrar eventos de expansão populacional e/ou gargalos de garrafa (Losos &

Glor, 2003). Como resultado, a distribuição geográfica atual de uma espécie pode diferir grandemente de sua distribuição inicial, logo que se diferenciou no ambiente (Barracough & Vogler, 2000; Losos & Glor, 2003). Dada essa instabilidade apresentada pelas espécies, a diferenciação em alopatria é um dos cenários mais relevantes dentro da biologia evolutiva. Mesmo nos casos em que espécies irmãs apresentem atualmente distribuições simpátricas, não se pode refutar completamente a ideia de que estas se diferenciaram inicialmente em alopatria, e posteriormente expandiram seus territórios até se tornarem simpátricas (Barracough & Vogler, 2000).

A geografia é amplamente reconhecida como um fator chave nos processos de diversificação biológica (Barracough & Vogler, 2000). Os modelos de diferenciação alopátrica supõem que populações passem por um período de isolamento geográfico, com pouco ou nenhum fluxo gênico entre elas, suficiente para acumular diferenças genéticas a ponto de distinguí-las (White, 1978). Esse isolamento geográfico pode ser representado pela colonização de novos ambientes, como por exemplo, ilhas, lagoas, ou um habitat distinto; por barreiras geográficas, como cadeias montanhosas, rios, desertos, oceanos e até mesmo a fragmentação do habitat; ou ainda pelo efeito da distância geográfica entre as populações. As regiões pelas quais uma espécie vai se distribuir depende, entre outros fatores, de onde ela se originou e quais barreiras para a sua dispersão foram encontradas ao longo do caminho (Slatkin, 1987). Essas barreiras geográficas por vezes são largas e conspícuas o suficiente para conter a maior parte das espécies que chegam até elas, e por vezes são tão diminutas que é difícil compreender porque uma determinada espécie não a atravessou (Slatkin, 1987).

As oscilações climáticas do Quaternário, por exemplo, desempenharam um papel importante na diversidade genética e distribuição geográfica atual de inúmeros táxons (Hewitt, 2000). Espécies foram extintas de grande parte de suas distribuições, algumas dispersaram para novos locais, outras sobreviveram em refúgios expandindo posteriormente, enquanto outras formaram zonas de hibridação após contato secundário, e esses eventos se seguiram repetidas vezes (Hewitt, 2000). Diversos exemplos bem documentados de espécies que foram influenciadas pelos eventos glaciais e interglaciais se encontram principalmente na Europa e América do Norte, dentre as quais, além das formas vegetais, são listados ursos, musaranhos, salamandras, ouriços, roedores e gafanhotos (revisões em Taberlet *et al*., 1998; Hewitt, 2000).

Existem na literatura inúmeros exemplos sobre o papel das mais diferentes barreiras geográficas na estruturação genética de populações. Crawford *et al*. (2007) analisando 3 espécies de rãs de serrapilheira de florestas úmidas da América Central do gênero *Craugastor*, observaram que barreiras montanhosas e florestas secas tropicais reduziram fortemente o fluxo gênico nas espécies estritamente de florestas úmidas. Eriksson *et al*. (2004) analisaram sequências da região controladora do DNA mitocondrial (mtDNA) em 5 populações de bonobos (*Pan paniscus*) separadas por rios. Apesar dos bonobos serem primatas altamente móveis, todas as localidades amostradas apresentaram diferenças significativas de acordo com estimativas de distância genética (F_{ST}), e apesar da ausência de um padrão geográfico óbvio na estruturação das populações, com haplótipos sendo compartilhados por indivíduos de diferentes localidades, os autores sugerem que os rios são de fato um obstáculo para o fluxo gênico nesta espécie. Estudos conduzidos com pumas (*Puma concolor*) amostrados no sudoeste dos Estados Unidos demonstraram que o fluxo gênico entre as diferentes populações é fortemente limitado pela distância geográfica, particularmente na presença de barreiras no habitat, neste caso, representadas por áreas de deserto e pradarias (Mcrae *et al*., 2005). O gênero *Ctenomys* também possui exemplos de espécies nas quais as populações são fortemente estruturadas por barreiras geográficas. Ctenomídeos são conhecidos por sua baixa capacidade natatória, neste sentido, cursos de água mais volumosos podem representar barreiras geográficas efetivas impedindo a livre dispersão dos indivíduos no ambiente (Reig *et al.,* 1990). Como sugerido em *C. talarum* (Mora *et al.,* 2007) e *C. flamarioni* (Fernández-Stolz, 2007), algumas de suas populações se apresentam fortemente estruturadas pela presença de rios que interrompem suas distribuições geográficas.

Steinberg & Patton (2000) afirmam que o contexto geográfico é essencial no processo de especiação em roedores subterrâneos. Os autores sugerem que a especiação simpátrica é incomum neste grupo, uma vez que a maior parte das espécies desses roedores não compartilha o mesmo habitat. Além disso, o modelo de especiação parapátrica é também considerado improvável, uma vez que as estreitas zonas de contato comumente encontradas entre roedores subterrâneos são principalmente de origem secundária, contrariando os princípios do modelo. Desta forma, Steinberg & Patton (2000) sugerem que o contexto de especiação nos roedores subterrâneos é alopátrico, devendo seguir um processo de vicariância ou o isolamento periférico.

Dispersão e isolamento pela distância

Entre os animais há normalmente alguma diferença entre os sexos relativa à capacidade de dispersar distâncias e às taxas de dispersão (Handley & Perrin, 2007). Segundo a lei de Haldane, o sexo heterogamético é comumente o dispersor, como por exemplo, no caso das aves representadas pelas fêmeas (ZW), e em mamíferos representados pelos machos (XY). O sistema de cruzamento também tem sido considerado como um dos vieses da dispersão, nos mamíferos sendo comumente baseado no sistema poligínico de defesa das fêmeas, onde a dispersão das fêmeas é menor e se dá pela procura de recursos, e nos machos a dispersão é maior a fim de evitar o endocruzamento ou a competição por reprodução ou recursos com indivíduos aparentados (Greenwood, 1980; Waser, 1985). Se os espécimes de um dos sexos dispersar para evitar o endocruzamento, em teoria não há risco para o outro sexo, sendo que este permaneceria filopátrico (Gandon, 1999; Perrin & Mazalov, 1999). Pequenas distâncias de dispersão são muitas vezes suficientes para evitar o endocruzamento ou a competição com indivíduos aparentados, enquanto que as longas distâncias, dentre outros fatores, costumam ser percorridas para colonizar um novo território (Clobert *et al.*, 2001). Além disso, em muitas espécies os indivíduos jovens são mais propensos a dispersar do que os adultos (Greenwood, 1980).

Apesar do sexo dispersante entre os pequenos mamíferos ser normalmente representado pelos machos, entre os roedores subterrâneos é relativamente comum encontrar espécies onde os dois sexos dispersem, principalmente os indivíduos jovens, porém, ainda assim, as taxas são consideradas baixas (Busch *et al*., 2000). Em geral, a dispersão das fêmeas é mediada pela distribuição de recursos, enquanto a dispersão nos machos é mediada pela distribuição das fêmeas e pelas interações agressivas com os outros espécimes (Busch *et al*., 2000).

A dispersão diferencial entre os sexos apresenta importantes consequências na estrutura genética das populações (Clobert *et al.*, 2001). A direção e extensão do fluxo gênico para marcadores moleculares sexo-específicos, como o DNA mitocondrial (de herança materna), e o cromossomo Y (de herança paterna), por exemplo, são fortemente influenciados pela dispersão diferencial entre sexos (Handley & Perrin, 2007), podendo acarretar em introgressões diferenciais dependendo do marcador analisado. O fenômeno de disjunção genômica cito-nuclear em mamíferos e aves foi recentemente revisado por Petit

& Excoffier (2009), em um estudo sobre o papel do fluxo gênico na delimitação das espécies. Os autores demonstraram que os componentes do genoma, com padrões distintos de herança, comumente apresentam diferenças nos padrões filogeográficos e nos níveis de introgressão entre zonas de hibridação interespecíficas, e este padrão é fortemente associado à dispersão diferencial entre os sexos.

A capacidade de dispersão também apresenta reflexos na distância genética entre populações mediada pela distância geográfica. Quanto menor for a capacidade de dispersão de uma espécie, maiores são as chances de o fluxo gênico entre as populações ser reduzido com o aumento da distância geográfica entre elas. O modelo de isolamento pela distância é um padrão geralmente encontrado entre as populações de roedores subterrâneos, incluindo os tuco-tucos (Busch *et al*., 2000), principalmente em espécies que habitam faixas estreitas ao longo da linha da costa, como nos casos de *C. pearsoni*, *C. flamarioni* e *C. australis* (Mora *et al.,* 2006; Tomasco & Lessa, 2007; Fernández-Stolz, 2007). A distribuição estreita de uma espécie limita sua dispersão e fluxo gênico para uma direção espacial predominante quando comparado com uma espécie de ampla distribuição geográfica, influenciando no aumento da diferenciação genética entre populações quanto mais distantes estas forem umas das outras (Slatkin & Barton, 1989).

Zonas de hibridação

O papel das zonas de hibridação é altamente discutido entre os biólogos, sendo geralmente citadas como laboratórios naturais para estudos evolutivos, permitindo evidências sobre processos de variações genético/geográficos entre diferentes táxons, a manutenção de suas identidades, a origem e natureza das espécies e como importantes fontes de novas variantes e novas espécies principalmente em plantas (Barton & Hewitt, 1985; Dowling & Secor, 1997; Hewitt, 2001). Os eventos de hibridação são muito mais comuns entre animais do que era suposto por zoólogos, estimando-se que em alguns grupos a taxa de hibridação entre espécies pode passar de 25% (Mallet, 2005). No entanto, poucas zonas híbridas animais são descritas em detalhes, sendo que algumas questões relativas às causas e significados evolutivos desse processo para as espécies envolvidas permanece pouco compreendido (Trigo *et al*., 2008).

A hibridação ocorre quando grupos de indivíduos geneticamente distintos, se encontram, cruzam e resultam em uma prole de ancestralidade misturada (Barton & Hewitt, 1989; Harrison, 1993). São eventos encontrados tanto entre diferentes populações de uma mesma espécie, como entre espécies distintas, podendo aparecer como descontinuidades abruptas, ou como clinas suaves entre grupos de populações. Podem ocorrer de forma esporádica, principalmente entre espécies simpátricas, formando zonas híbridas estreitas e transitórias, ou até mesmo se caracterizando como eventos comuns através de um intenso processo de miscigenação (Harrison, 1993). As zonas de hibridação são normalmente estreitas, principalmente quando comparadas com a distribuição geográfica dos tipos parentais, e sua extensão é fortemente relacionada com a capacidade de dispersão dos espécimes envolvidos e com a pressão de seleção (Harrison, 1993). Normalmente são mais frequentes em espécies de origem recente, pois estas têm barreiras reprodutivas menos efetivas do que espécies mais antigas, assim espécies que não apresentam atualmente zonas de hibridação podem ter apresentado em algum momento no passado (Mallet, 2005). Como produto de uma zona de hibridação a progênie pode ser formada apenas por indivíduos decorrentes da F1, muitas vezes sendo inférteis ou com a fertilidade reduzida, ou por uma população numerosa formada por inúmeras gerações de cruzamentos e retrocruzamentos, dando origem a uma ampla variação de tipos recombinantes e até mesmo indivíduos híbridos tão semelhantes aos parentais que se torna impossível distinguí-los, fenômeno conhecido como "*hybrid swarm*" (Anderson & Hubricht, 1938; Allendorf *et al*., 2001).

Uma das principais questões com relação às zonas de hibridação é a respeito da dificuldade de distinguir zonas de contato primário ou secundário sem evidências históricas, pois ambas podem produzir padrões idênticos de variação (Barton & Hewitt, 1985; Harrison, 1993). As zonas híbridas de contato primário surgem localmente em resposta a pressões de seleção do gradiente ambiental, e no segundo caso, a hibridação é resultado do contato secundário entre populações que se diferenciaram em alopatria e entraram em contato posteriormente (Harrison, 1993). Muitas das zonas híbridas descritas são atribuídas ao contato secundário, tanto aquelas relacionadas com causas naturais, como no caso da interferência das flutuações climáticas no padrão de distribuição e colonização de muitas espécies da América do Norte e Europa, quanto aquelas que têm suas origens relacionadas a ações antrópicas, como modificações e fragmentação do habitat ou

introdução de espécies exóticas (Barton & Hewitt, 1985; Allendorf *et al*., 2001). Se a zona híbrida é formada naturalmente, esta é considerada como parte do processo evolutivo dos táxons envolvidos, no entanto, se a hibridação for consequência de ações humanas esta pode se tornar um problema e requer intervenções conservacionistas (Rhymer & Simberloff, 1996; Allendorf *et al*., 2001). Considerando que o impacto antrópico tem aumentado progressivamente, principalmente com relação às atividades que contribuem para a formação de zonas de híbridas (introdução de espécies exóticas, fragmentação e alteração do habitat), acredita-se que as zonas de hibridação vão se tornar cada vez mais recorrentes e problemáticas na natureza (Allendorf *et al*., 2001)

A introgressão pode ser uma das consequências dos eventos de hibridação, muitas vezes decorrendo de retrocruzamentos entre os híbridos e as formas parentais, resultando na incorporação de alelos de um dos táxons envolvidos no outro (Harrison, 1993; Rieseberg & Wendel, 1993). Mesmo poucos híbridos podem funcionar com uma ponte para a troca de alelos entre parentais (Mallet, 2005). A extensão da introgressão depende dos marcadores analisados que podem apresentar um padrão de direção assimétrico. Como consequências extremas desse processo é possível a fusão completa das formas parentais, o reforço das barreiras reprodutivas através da seleção por cruzamento preferencial entre indivíduos da mesma espécie, a extinção de um dos táxons envolvidos (especialmente no caso de espécies raras em contato com espécies mais abundantes) ou ainda os híbridos podem dar origem a uma nova espécie (Arnold, 1992; Rieseberg & Wendel, 1993; Allendorf *et al*., 2001). Desta forma, identificar e caracterizar eventos de introgressão é primordial em estudos de zonas de hibridação (Allendorf *et al*., 2001).

Reconhecer a ocorrência de indivíduos híbridos entre populações ou espécies, e compreender os padrões e processos envolvidos na formação de uma zona híbrida não consiste em uma tarefa simples, e normalmente se dá por meio de análises envolvendo múltiplos aspectos, como a morfologia, diferenças cromossômicas, etológicas, ecológicas e moleculares (Hewitt, 1988; Allendorf *et al*., 2001). Características morfológicas, por exemplo, podem facilitar a detecção de indivíduos híbridos nos casos em que as formas parentais sejam distintas e os híbridos apresentem características intermediárias. Porém, se os parentais não apresentarem distinções claras em sua morfologia a detecção dos híbridos será comprometida. Além disso, características morfológicas não permitem identificar se um indivíduo híbrido é proveniente da primeira geração de cruzamentos (F1), de um

retrocruzamento, ou de gerações posteriores (Allendorf *et al*., 2001). Nesses casos, marcadores moleculares são de grande utilidade para identificação e caracterização de zonas de hibridação, podendo contribuir com a identificação dos cruzamentos e de eventos de introgressão existentes nas populações (Allendorf *et al*., 2001).

Filogeografia e marcadores moleculares

O caminho percorrido por uma determinada variante genética no tempo e espaço é influenciada pela biologia do organismo e por circunstâncias pelas quais este passará, incluindo seu sucesso reprodutivo, tamanho populacional, seleção natural, deriva e eventos históricos. Os modelos de genética de populações investigam as relações entre esses fatores demográficos e a distribuição das variantes genéticas, através do uso de marcadores moleculares com características apropriadas para acessar informações sobre o processo evolutivo no qual aquele indivíduo está inserido (Sunnucks, 2000). Neste sentido, selecionar marcadores moleculares e análises genéticas apropriadas para as perguntas que se pretende responder é essencial para o sucesso de um estudo (Anne, 2006).

A disciplina de filogeografia surge como reflexo de debates recorrentes a respeito do papel dos processos microevolutivos (mutação, deriva genética, seleção, rearranjos cromossômicos) que operam dentro das espécies, extrapolados de forma a explicar as diferenças macroevolutivas entre espécies e táxons mais elevados (Avise *et al*., 1987). A filogeografia é definida como o estudo dos princípios e processos responsáveis pela distribuição geográfica de linhagens genéticas intraespecíficas ou entre espécies proximamente relacionadas (Avise, 2000). Visando analisar e interpretar a forma como as diferentes linhagens se distribuem no ambiente, estudos filogeográficos requerem a integração entre diversas disciplinas micro e macroevolutivas, através da utilização de dados de genética de populações, etologia, demografia, paleontologia, filogenia, morfologia, geomorfologia, entre outras (Avise *et al*., 1987; Avise, 2000).

Marcadores moleculares constituem a base de investigações filogeográficas e segundo Avise *et al*. (1987), um sistema molecular ideal deve: ser distintivo e presente nos mais diferentes organismos; ser facilmente isolado e analisado; ter uma estrutura genética simples; exibir um modo de transmissão genética preferencialmente direto, sem recombinações ou outros rearranjos; apresentar um série de estados de caracteres

qualitativos, através dos quais as inter-relações possam ser inferidas; e evoluir rapidamente. Dentro deste contexto os marcadores de DNA mitocondrial (DNAmt) são principalmente utilizados nas análises dos padrões filogeográficos de espécies animais, e os marcadores de *loci* de microssatélites são amplamente aplicados em estudos populacionais.

A utilização de sequências do DNAmt apresenta certas vantagens em relação aos marcadores moleculares do DNA nuclear (Avise, 2000). Características como sua herança matrilinear, a ausência de recombinação, altas taxas evolutivas e tamanho efetivo populacional quatro vezes menor do que segmentos equivalentes em autossomos fazem com que o DNAmt seja uma importante ferramenta para os estudos filogenéticos e de eventos evolutivos relativamente recentes, permitindo revelar grupos geográficos de indivíduos relacionados, analisar as relações entre as matrilinhagens dentro de uma população, auxiliar na compreensão do modo de dispersão de muitos organismos e de acasalamentos preferenciais, analisar zonas híbridas e também pode ser utilizado para traçar eventos históricos como gargalos-de-garrafa (Avise, 1994; Sunnucks 2000; Arias & Infante-Malachias, 2001; Hare, 2001). Estudos filogeográficos baseados em sequências da região controladora e do gene citocromo b do DNAmt, utilizando espécies do gênero *Ctenomys*, têm sido realizados com sucesso devido à possibilidade de melhor descrição dos padrões de distribuições geográficas, de relações filogenéticas e de distâncias genéticas e fluxo gênico entre as diferentes linhagens de cada uma das espécies, aumentando o conhecimento relativo à biogeografia e às áreas de endemismo em: *C. rionegrensis* (Wlasiuk *et al*., 2003); grupo *perrensi* (Giménez *et al*., 2002); *C. australis* (Mora *et al*., 2006); *C. talarum* (Mora *et al*., 2007); *C. pearsoni* (Tomasco & Lessa, 2007); *C. flamarioni* (Fernández-Stolz, 2007); e *C. torquatus* (Fernandes, 2008).

A vantagem do uso de *loci* de microssatélites em estudos populacionais consiste no fato de serem marcadores de herança biparental, altamente polimórficos, com alelos codominantes e seletivamente neutros (Schlötterer, 1998), sendo bastante variáveis para permitir a identificação dos indivíduos amostrados e a realização de inúmeras análises estatísticas de associação dos indivíduos às suas populações de origem (Hansen *et al*., 2000). *Loci* de microssatélites tem sido amplamente aplicados em estudos populacionais, com diferentes enfoques, em espécies do gênero *Ctenomys*, com o objetivo de estimar níveis de fluxo gênico entre populações, fazer inferências sobre deriva genética e

migração, estrutura populacional, comparações da variabilidade genética entre espécies e populações e em estudos de paternidade e parentesco (Lacey, 2001; Wlasiuk *et al.*, 2003; El Jundi & Freitas, 2004; Gava & Freitas, 2004; Cutrera *et al.*, 2005; Fernández-Stolz *et al.*, 2007; Gonçalves & Freitas, 2009; Mirol *et al*., 2010).

Planície costeira do Sul do Brasil

O litoral brasileiro se estende por aproximadamente 9200 km e apresenta um conjunto muito diverso de ambientes costeiros que se desenvolveram durante o Quaternário, em resposta às mudanças climáticas e flutuações no nível do mar, interagindo com uma fonte de sedimentos variados e uma herança geológica que remonta a separação do continente Africano e a América do Sul, durante o Mesozóico (Dominguez, 2009).

Planícies costeiras são habitats efêmeros, bastante instáveis e fortemente submetidos às influências dos oceanos (Dillenburg & Hesp, 2009). A fauna e flora presentes nessas regiões são adaptadas às mudanças ambientais as quais agem como forças evolutivas, moldando a estrutura genética dessas espécies. A costa dos estados do Rio Grande do Sul e de Santa Catarina faz parte de uma das mais extensas e amplas planícies costeiras do mundo, sendo limitada ao norte pelo Cabo de Santa Marta em SC-Brasil e ao sul pelo Cabo Polônio no Uruguai, com largura variando de 20 a 100 km e se estendendo por aproximadamente 800 km, no sentido Sudoeste-Nordeste (Tomazelli & Villwock, 2000; Weschenfelder, 2005; Dillenburg *et al*., 2009; Hesp *et al*., 2009).

A planície costeira do Sul do Brasil começou a ser formada no início do Cratáceo com a abertura do Oceano Atlântico (Tomazelli *et al*., 2000). O acúmulo de sedimentos erodidos de terras mais altas adjacentes e os depósitos aluviais deram origem ao sistema de leques aluviais ao longo da porção proximal (oeste) da planície costeira (Figura 1; Tomazelli *et al.*, 2000; Tomazelli & Villwock, 2005). Durante o Quaternário, flutuações glacio-eustáticas do nível do Oceano Atlântico produziram grandes deslocamentos laterais da linha da costa, que retrabalharam os depósitos aluviais por no mínimo quatro ciclos transgressivo-regressivos, moldando a costa do Sul do Brasil nos formatos que a encontramos atualmente (Villwock *et al.*, 1986; Tomazelli & Villwock, 2000).O primeiro, e mais antigo ciclo transgressivo-regressivo ocorreu no início do Pleistoceno, há aproximadamente 400 mil anos, e teria dado origem à região conhecida como Coxilha das

Lombas, com cerca de 250 km de extensão, uma largura de 5 a 10 km e orientada no sentido Nordeste-Sudoeste. A região ocupada pelo sistema lagunar associado a esta barreira abrange boa parte das bacias do Rio Gravataí e do complexo fluvial do lago Guaíba e sofreu a influência dos eventos transgressivo-regressivos que se sucederam (Villwock *et al.*, 1986; Tomazelli & Villwock, 2000). O segundo e terceiro ciclos (aproximadamente há 325 e 125 mil anos, respectivamente) deram origem à denominada "Barreira Múltipla Complexa", correspondente à segunda linha de dunas, e foram responsáveis pela separação das Lagoas dos Patos e Mirim. O máximo da fase regressiva que caracterizou o terceiro ciclo teria dado origem a uma ampla planície que atualmente forma parte da plataforma submarina, retraindo a linha de costa aproximadamente 120m abaixo do seu nível atual (Villwock *et al.*, 1986; Tomazelli & Villwock, 2000). O quarto e último evento ocorreu por volta do início do Holoceno e teria atingido seu máximo transgressivo há cerca de 5 mil anos, elevando o nível do mar de 2 a 4 m acima do atual (Tomazelli *et al*., 2000; Tomazelli & Villwock, 2005). A estabilização temporária no final deste evento foi a principal responsável pela implantação de uma barreira de dimensões reduzidas que foi aumentando com o acúmulo de areia e sedimentos fornecidos pela ante-praia inferior e pela plataforma continental interna (Tomazelli & Villwock, 2000). Esta barreira, de natureza dinâmica, constitui hoje o sistema de dunas costeiras, primeira linha de dunas, que se estende ao longo de toda a planície costeira (Villwock *et al.*, 1986; Tomazelli & Villwock, 2000).

Atualmente a planície costeira do Sul do Brasil apresenta um terreno bastante irregular, formado por um complexo sistema de lagos, lagoas, rios e dunas que, em alguns casos, podem representar barreiras geográficas naturais ao fluxo gênico entre diferentes populações das espécies do gênero *Ctenomys* que habitam a região (Moreira *et al*., 1991; Freitas, 2007; Fernández-Stolz, 2007; Lopes, 2007).

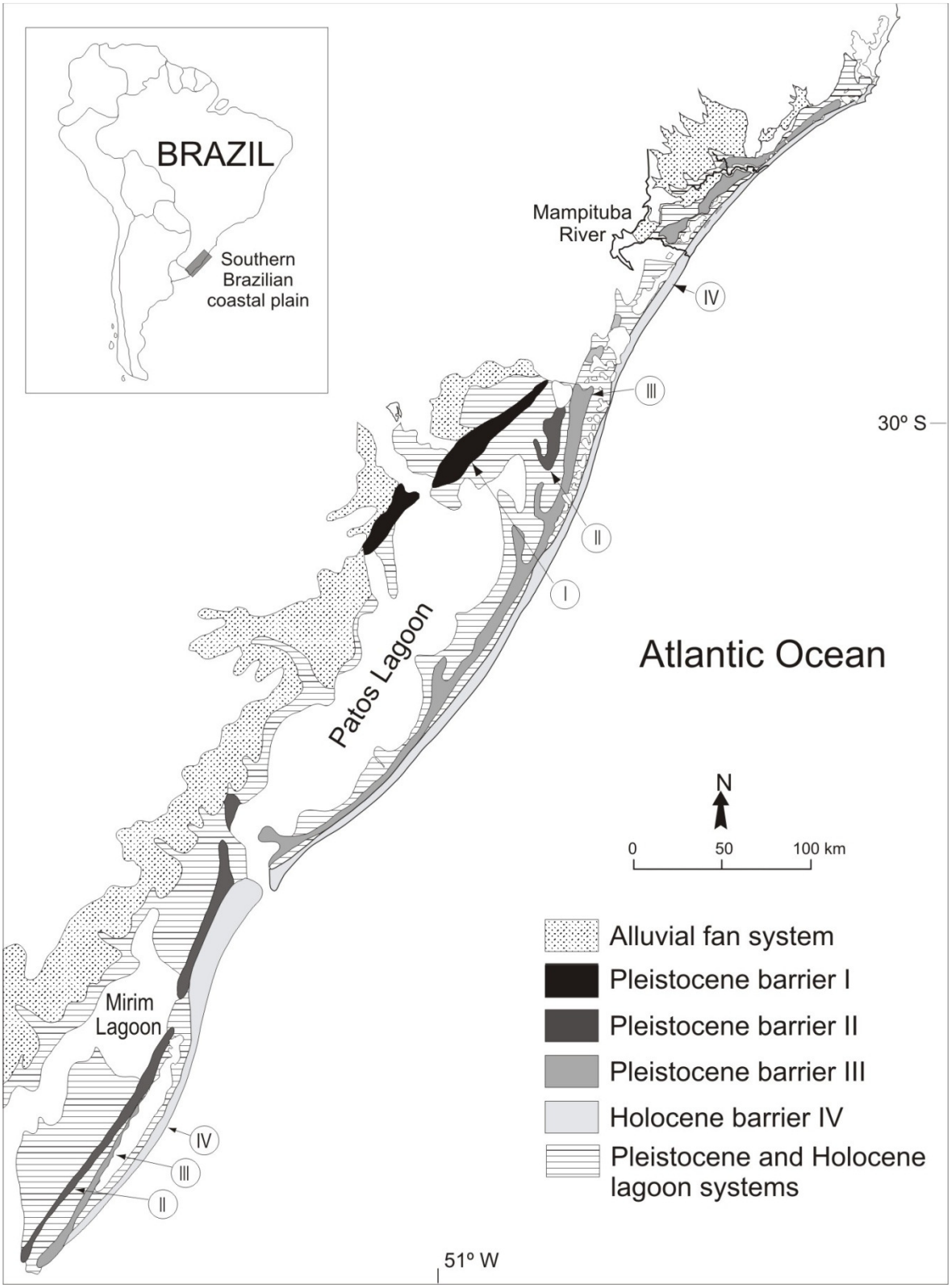

Figura 1. Mapa geológico da planície costeira do Sul do Brasil. Modificado de Tomazelli *et al.*, 1996 e Martin *et al*., 1988.

Roedores subterrâneos: Família Ctenomyidae

Considerado o grupo mais diverso entre os mamíferos, a ordem Rodentia possui aproximadamente 2.227 espécies distribuídas em 34 famílias e 481 gêneros (Honeycutt 2009). Ocorrem em praticamente todos os continentes, ocupando uma grande variedade de habitats. Podem ter hábitos arborícolas, terrestres, adaptações à vida semi-aquática e algumas espécies são especializadas à vida subterrânea (Nowak, 1999).

Os roedores subterrâneos são definidos por espécies escavadoras que vivem em sistemas de túneis abaixo do solo, onde passam a maior parte de seu tempo e realizam grande parte de suas atividades biológicas (Lacey *et al*., 2000). Ocorrem em quase todos os continentes com exceção da Austrália e Antártica, habitando geralmente áreas abertas de solos bem drenados como savanas, pradarias, estepes e raramente são encontrados em áreas florestais de vegetação densa (Nevo, 1979; Lacey *et al*., 2000). Por estas características suas populações costumam ser distribuídas no ambiente de forma fragmentada, ocupando regiões que apresentem condições favoráveis. Esse padrão de distribuição espacial possui consequências em vários aspectos da biologia desses animais, como em relação à variabilidade genética, dispersão e fluxo gênico (Lacey *et al*., 2000). O nicho subterrâneo é geralmente pouco variável, sendo que o interior dos túneis é normalmente caracterizado pela ausência de luz, excesso de umidade no ar, baixa amplitude térmica e altos níveis de dióxido de carbono em relação aos baixos níveis de oxigênio. Essas condições parecem impor uma série de adaptações comportamentais, fisiológicas e morfológicas à sua sobrevivência abaixo da superfície do solo (Nevo, 1979; Lacey *et al*., 2000). Os roedores subterrâneos são representados pelas famílias: Bathyergidae (África); Geomyidae (América do Norte); Muridae, dividida nas subfamílias Arvicolinae, Myospalacinae, Rhizomyinae e Spalacinae (Ásia, leste europeu e África) Octodontidae e Ctenomyidae (América do Sul; Figura 2; Wilson & Reeder 2005; Lacey *et al*., 2000).

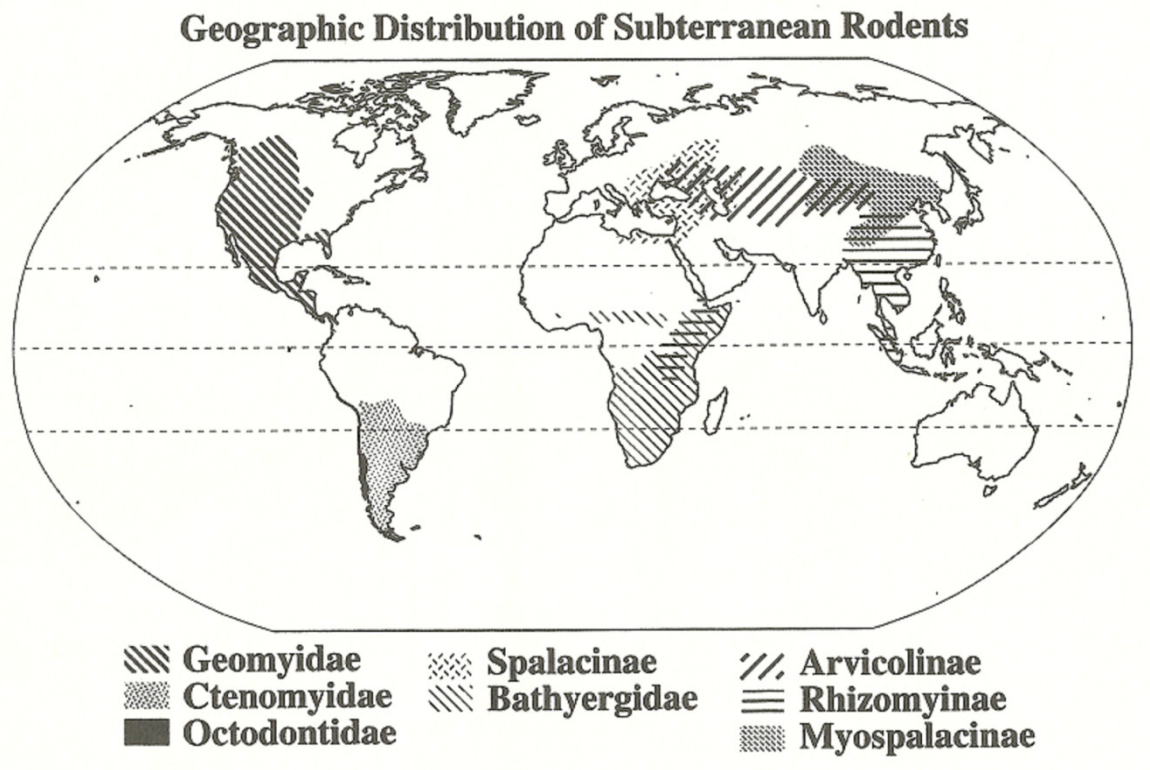

Figura 2. Distribuição geográfica dos roedores subterrâneos por família ou subfamília (Lacey *et al*., 2000).

A família Ctenomyidae Lesson, 1842 é formada apenas pelo gênero *Ctenomys* Blainville, 1826 (ordem Rodentia, subordem Hystricognathi), e seu surgimento tem sido atribuído ao Plioceno tardio, com o registro fóssil mais antigo datado de aproximadamente 3.5 milhões de anos, localizado no noroeste da Argentina (Verzi *et al*., 2010). O gênero é constituído por aproximadamente 60 espécies, amplamente distribuídas desde elevadas altitudes do Altiplano Andino no sul do Peru e Bolívia até o nível do mar no Chile, e através da Argentina até o leste da região sul do Brasil, sendo que sua especiação é considerada como uma das mais explosivas dentre os gêneros de mamíferos atuais (Reig *et al*., 1990; Cook *et al*., 2000; Wilson & Reeder, 2005). A alta velocidade de diversificação no gênero tem sido sugerida não só pelo grande número de espécies relativo ao intervalo de tempo de seu surgimento, mas através de estudos filogenéticos, pela persistência de politomias basais em filogenias obtidas tanto a partir de sequências do DNA mitocondrial (Cook & Lessa, 1998; Lessa & Cook, 1998, Mascheretti *et al*., 2000; Slamovits *et al.*,

2001; Parada *et al*., 2011) como a partir de dados provenientes do DNA nuclear (fragmentos dos genes da rodopsina e vimentina; Castillo *et al*., 2005).

Esses roedores, popularmente conhecidos como tuco-tucos, ocupam variados tipos de habitats, desde campos mais secos até áreas de florestas, ocorrendo preferencialmente em regiões abertas de solo pouco compacto (Lacey *et al*., 2000). A maioria das espécies possui hábitos solitários e territorialistas, com apenas um indivíduo ocupando cada toca, ou no caso das poucas espécies sociais e semi-sociais (*C. sociabilis*, *C. peruanus* e *C.porteousi*) e durante o período de cuidado parental, há o compartilhamento dos túneis por mais de um indivíduo. Esses hábitos, em geral, determinam uma baixa densidade populacional e assim como já mencionado anteriormente para outros roedores subterrâneos, as populações de ctenomídeos costumam apresentar tamanho reduzido, com distribuição de forma fragmentada, semi-isoladas umas das outras, podendo ser determinadas por condições ecológicas e/ou por barreiras geográficas (Nevo, 1979; Cook *et al*., 1990; Reig *et al*., 1990; Lacey *et al*., 1998, 2000; Busch *et al*., 2000; Lacey, 2000). A maioria das espécies apresenta distribuição alopátrica, embora existam casos de parapatria e simpatria (Reig *et al*., 1990).

As taxas de dispersão são geralmente referidas como baixas entre as espécies do gênero, sendo que os jovens, principalmente os machos, dispersam mais que os adultos. Malizia *et al*. (1995) sugerem que a procura sexual seja o primeiro determinante na dispersão dos machos e que outros fatores, como a disponibilidade da área, influenciem a dispersão das fêmeas. Essa dispersão reduzida parece reforçar o isolamento entre os demes, o que restringe o fluxo gênico entre os grupos (Nevo, 1979; Reig *et al*., 1990; Malizia *et al*.,1995; Freitas, 1995). Porém, dados obtidos sobre taxas de dispersão para espécies de *Ctenomys* são escassos e o uso de métodos diretos para determinar tais taxas é bastante limitado pela dificuldade de acesso ao ambiente subterrâneo (Smith, 1993; Busch *et al*., 2000; Lacey, 2000).

Aspectos como taxas baixas de dispersão dos adultos e populações de tamanho reduzido com distribuição fragmentada, associadas à ação da deriva genética, promovem o estabelecimento de pequenas unidades genéticas (demes) onde a variação intrapopulacional é baixa e a divergência interpopulacional é alta (Lessa & Cook, 1998; Lacey *et al*., 2000; Wlasiuk *et al*., 2003). Somado a isto, essas características também favorecem a fixação de novos rearranjos cromossômicos tanto entre espécies quanto dentro

das espécies (Reig *et al.,* 1990; Lessa & Cook, 1998). Entre as espécies os números cromossômicos variam de 2n = 10 em *C. steinbachi* até 2n = 70 em *C. pearsoni* e *C. dorbignyi* (Reig *et al.*, 1990), e no nível intraespecífico os exemplos mais conhecidos são em *C. pearsoni* (2n = 56-70; Tomasco & Lessa, 2007); C. *lami* (2n = 54-58; Freitas, 2007; El Jundi, 2003); *C. minutus* (2n = 42-50; Freitas, 1997; Freygang *et al.*, 2004); e *C. torquatus* (2n = 40-46; Fernandes, 2008).

Tamanha diversidade desperta o interesse de evolucionistas para a escolha de tuco-tucos como um modelo para o estudo de processos evolutivos, particularmente com relação à sua grande diversificação cromossômica, especiação e sociabilidade (Lessa & Cook, 1998).

Oito espécies de tuco-tucos foram descritas no Brasil. A espécie tipo do gênero, *C. brasiliensis* Blainville, 1826, permanece como uma grande questão para futuras investigações, pois após a sua descrição, no estado de Minas Gerais, a espécie não foi novamente registrada em nenhuma ocasião. No Norte e Centro-Oeste do Brasil os registros de ctenomídeos são escassos. As poucas amostras de *C. bicolor* Ribeiro, 1914, *C. naterreri* Wagner, 1848, e *C. rondoni* Ribeiro, 1914 foram coletadas nos estados de Rondônia e Mato Grosso, e permanecem como dúvidas taxonômicas, em parte devido à falta de precisão nas suas localidades tipo (Bidau & Avila-Pires, 2009). As quatro espécies de tuco-tucos restantes (*Ctenomys torquatus* Lichtenstein, 1830; *Ctenomys flamarioni* Travi, 1981; *Ctenomys minutus* Nehring, 1887 e *Ctenomys lami* Freitas, 2001) ocorrem no Sul do Brasil, particularmente nos estados do Rio Grande do Sul e Santa Catarina, e vem sendo amplamente investigadas, sendo as duas últimas alvos de estudo deste trabalho (Freitas & Lessa, 1984; Freitas, 1995, 1997; Silva *et al*., 2000a, b; Freitas, 2001; Gava & Freitas, 2002; Heuser *et al*., 2002; Gava & Freitas, 2003, 2004; El Jundi & Freitas, 2004; Freygang *et al*., 2004; Freitas, 2006; Freitas, 2007; Fernandes *et al*., 2007; Fernandez-Stolz *et al*., 2007; Fernandes *et al*., 2009a, b; Gonçalves & Freitas, 2009; Fornel *et al*., 2010).

Ctenomys minutus e *Ctenomys lami*

Ctenomys minutus e *C. lami* são endêmicos da planície costeira do Sul do Brasil, e são consideradas espécies irmãs pertencentes ao grupo *torquatus* no gênero *Ctenomys* (Freitas, 2001; Parada *et al*., 2011).

Ctenomys minutus (Figura 3) apresenta um distribuição estreita que vai desde a praia de Jaguaruna no estado de Santa Catarina (SC), até o município de São José do Norte no estado do Rio Grande do Sul (RS; Figura 4; Freygang *et al.*, 2004). Essa espécie ocupa no norte de sua distribuição preferencialmente o quarto sistema de barreiras-lagunas, correspondente à primeira linha de dunas da beira da praia, até as proximidades de Tramandaí (RS), e a partir de Tramandaí em direção ao sul a espécie passa a ocupar o segundo e terceiro sistemas de barreiras-lagunas, correspondente aos campos arenosos da segunda linha de dunas, interiorizando-se cerca de 2Km da costa (Freitas, 1995).

As populações de *C. minutus* possuem notável variação cariotípica (Gava & Freitas, 2004). Estudos realizados por Freitas (1997), Gava & Freitas (2003), Castilho 2004 e Freygang *et al*. (2004) demonstraram a existência de sete cariótipos parentais distribuídos parapatricamente (2n = 50a, 48a, 46a, 42, 46b, 48b e 50b), entre os quais se tem a formação de quatro zonas híbridas intra-específicas que dão origem a cariótipos intermediários entre os parentais: i) 2n = 46a x 2n = 48a → 2n = 47a; ii) 2n = 42 x 2n = 48a → 2n = 43, 44, 45, 46, 47 (foram encontrados 5 números diplóides, porém 25 combinações cariotípicas diferentes); iii) 2n = 46b x 2n = 48b → 2n = 47b; iv) 2n = 50b x 2n = 48b → 2n = 49b; e ainda o cariótipo 2n = 49a que possivelmente é híbrido entre 2n = 50a e outro cariótipo desconhecido até o presente momento (Figura 4; Freitas, 1997; Gava & Freitas, 2002; Freygang *et al*., 2004; Freitas, 2006). Cada cariótipo pode ser designado por uma área geográfica particular, com distribuição contígua ou separada por barreiras geográficas, sendo que nos dois extremos da distribuição tem-se 2n = 50, enquanto que em direção ao centro os cariótipos variam seguindo uma clina, e o número diplóide é progressivamente reduzido até 2n = 42, através de rearranjos Robertsonianos, fusões/fissões *in tandem*, inversões paracêntricas e pericêntricas, e no caso de um mesmo número diplóide, porém com cariótipos descritos como "a" ou "b", o que os diferencia são rearranjos em cromossomos distintos (Freitas, 1997; Freygang *et al*., 2004).

Além disso, ao longo da distribuição geográfica de *C. minutus* duas descontinuidades geográficas cruzam a planície costeira no sentido oeste-leste: o rio Araranguá em SC e o rio Mampituba na divisa dos estados de SC e RS (Figura 4). Estudos recentes também revelaram a existência de dois paleocanais que conectavam a Lagoa dos Patos ao Oceano Atlântico no passado. Porém, durante o último ciclo transgressivo-regressivo do nível do mar, esses canais foram progressivamente cobertos por sedimentos

até desaparecerem completamente e essas regiões da planície costeira se tornaram contínuas (Weschenfelder *et al*., 2008a, b).

Análises de morfometria geométrica do crânio e mandíbula de espécimes de *C. minutus* coletados ao longo de toda a sua distribuição geográfica demonstraram uma correlação fraca, porém significativa, entre as distâncias morfológicas e geográficas, demonstrando que as populações desta espécie se diferenciam seguindo um modelo de isolamento pela distância (Fornel *et al*., 2010). Os autores sugerem que a forma do crânio dos espécimes que habitam dunas ou campos arenosos difere significativamente. Além disso, inversões cromossômicas, rios e paleocanais parecem atuar como barreiras ao fluxo gênico entre as populações, ao contrário de fusões e fissões robertsonianas.

Figura 3. Exemplar de *C. minutus* adulto.

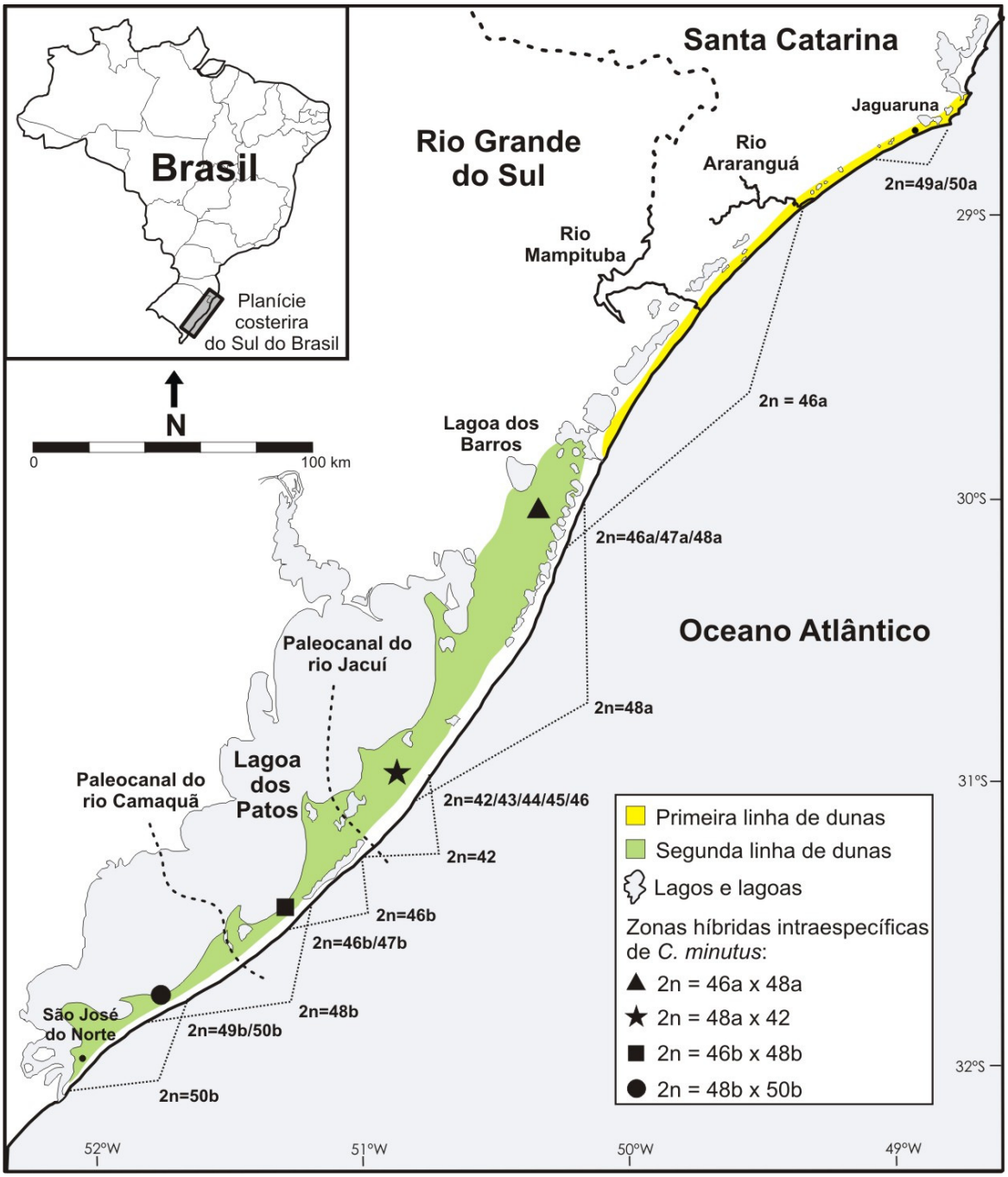

Figura 4. Distribuição geográfica de *Ctenomys minutus* na planície costeira do Rio Grande do Sul e Santa Catarina. As regiões de ocorrência dos diferentes cariótipos de *C. minutus* encontram-se destacadas no mapa. Os diferentes símbolos representam as zonas híbridas intra-específicas.

Ctenomys lami (Figura 5) é geograficamente restrito a uma área de ocorrência de 78 km x 12 km, em uma região de campos arenosos, conhecida como Coxilha das Lombas, correspondente ao primeiro e mais antigo sistema de barreiras-lagunas, estendendo-se do norte do Lago Guaíba até o noroeste da Lagoa dos Barros. Essa região é cercada a noroeste pelo banhado dos Pachecos e a sudeste pelo banhado dos Touros, os quais são conectados no centro da Coxilha das Lombas. (Figura 6; Freitas, 2001, 2007).

Essa espécie apresenta uma das maiores variabilidades cariotípicas conhecidas até então para o gênero *Ctenomys* proporcionalmente à sua distribuição geográfica, com cinco diferentes números diplóides (2n = 54, 55, 56, 57 e 58), dez números autossômicos (NA; de 74 até 82 e 84), que combinados formam 26 cariótipos descritos em uma área de 936 km^2 (Freitas, 2001, 2007). As variações nos números diplóides são devidas a rearranjos Robertsonianos, fusões/fissões cêntricas e inversões pericêntricas, e como observado nos espécimes de *C. minutus*, números diplóides descritos como "a" ou "b" apresentam rearranjos em cromossomos distintos (Freitas, 2007). Considerando os rearranjos cromossômicos encontrados em cada uma das localidades amostradas, Freitas (2007) descreveu quatro blocos cariotípicos, denominados bloco A (2n = 54, 55a e 56a), bloco B (2n = 57 e 58), bloco C (2n = 54 e 55a) e bloco D (2n = 55b e 56b). Entre as populações cromossômicas de *C. lami* há indicação de duas zonas híbridas estreitas. A primeira caracterizada pelo cruzamento entre os cariótipos 2n = 56a ou 2n = 55a (bloco A) com 2n = 58 (bloco B), e a segunda formada pela hibridação entre os cariótipos 2n = 54 (bloco C) com 2n = 56b (bloco D; Freitas, 2007).

Análises comparativas entre sexos, cariótipos e blocos populacionais utilizando dados de morfometria geométrica revelaram que a forma do crânio em *C. lami* varia significativamente para todos os fatores citados acima, porém, não há correlação entre as distâncias morfológicas e geográficas (Fornel, 2010).

Figura 5. Exemplar de *C. lami* adulto.

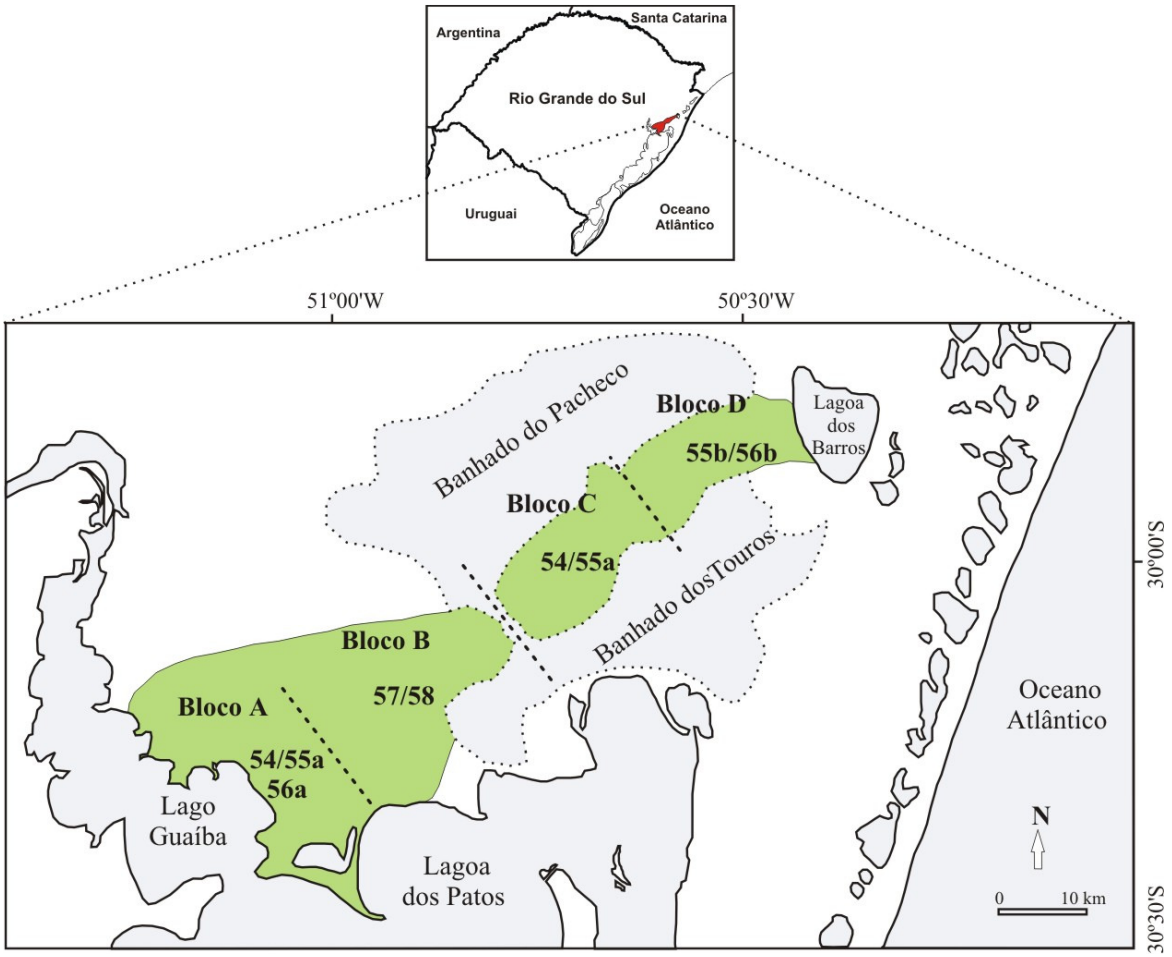

Figura 6. Distribuição geográfica de *Ctenomys lami* na planície costeira do Rio Grande do Sul, destacada em vermelho no mapa. As regiões de ocorrência das zonas híbridas intra-específicas foram descritas no texto.

Ctenomys minutus e *C. lami* são indistinguíveis através da morfologia externa, no entanto *C. lami* foi descrito recentemente como uma espécie distinta de *C. minutus* devido aos habitats distintos que essas espécies ocupam, às diferenças em seus cariótipos e também por diferenças na morfologia de seus crânios (Freitas, 2001). Os cariótipos presentes nas duas espécies são diferentes não só devido aos números diplóides distintos, mas também com relação aos rearranjos que deram origem a todo o polimorfismo cromossômico intraespecífico e principalmente com relação às formas dos cromossomos que podem ser exclusivas para cada uma das espécies, como por exemplo, o par cromossômico número 1 em todos os espécimes de *C. minutus* é apresentado como um

grande cromossomo submetacêntrico, enquanto que todos os espécimes de *C. lami* apresentam um grande par de cromossomos metacêntricos (Freitas, 2001).

A hipótese assumida atualmente sobre o processo de especiação entre essas duas espécies é baseado no papel dos rearranjos cromossômicos e barreias geográficas, resultando em um modelo alopátrico de especiação seguido pela diferenciação de vários rearranjos cromossômicos (Freitas, 2006). *Ctenomys lami* tem sido considerado como mais semelhante ao ancestral comum entre as duas espécies e *C. minutus* como sendo a espécie que apresenta características mais derivadas, uma vez que possui números diplóides menores formados por um maior número de rearranjos cromossômicos do que *C. lami*. Além disso, a região da planície costeira do sul do Brasil ocupada por *C. minutus* tem formação geológica mais recente do que a Coxilha das Lombas ocupada por *C. lami* (Freitas, 2001, 2006).

No entanto, o processo evolutivo no qual essas duas espécies estão envolvidas vem sofrendo gradativamente com as ações humanas. Dados citogenéticos de 6 indivíduos amostrados na margem oeste da Lagoa dos Barros, no estado do RS, demonstraram números diplóides e rearranjos cromossômicos intermediários entre indivíduos de *C. lami* (2n = 56) e *C. minutus* (2n = 48a) que habitam as proximidades dessa região. Esses espécimes foram considerados formas híbridas interespecíficas entre *C. lami* e *C. minutus* por Gava & Freitas (2003). Os autores sugerem que esta zona híbrida seria produto de contato secundário, uma vez que no passado, uma extensa área úmida, a oeste da Lagoa dos Barros, isolava geograficamente populações dessas duas espécies. No entanto, durante a década de 1950 a introdução de culturas de arroz na região drenou completamente o banhado, expondo uma região arenosa que permitiu a aproximação, reprodução e consequentemente a formação de indivíduos híbridos. As consequências dessa zona híbrida para ambas as espécies são incertas e requerem investigações.

OBJETIVOS

Apesar do grande conhecimento a respeito dos padrões de variação cariotípica encontrados em *C. minutus* e *C. lami*, pouco se sabe a respeito de suas estruturas genéticas. A análise de dados moleculares e cariotípicos associados aos conhecimentos a respeito da geografia e geologia da região que habitam possibilitariam esclarecer a história evolutiva destas duas espécies na planície costeira do Sul do Brasil. Tendo isto em vista os objetivos deste estudo são:

- Analisar os padrões filogeográficos e populacionais de *C. minutus* e *C. lami* ao longo de suas distribuições geográficas, utilizando como marcadores moleculares quatorze *loci* de microssatélites e dois fragmentos do DNA mitocondrial;
- Relacionar os resultados obtidos pelos dois marcadores com a existência de barreiras geográficas e os diferentes cariótipos dos espécimes de *C. minutus* e *C. lami*;
- Confirmar, quantificar e caracterizar a existência de eventos de hibridação e introgressão nas áreas de contato entre as duas espécies;
- Providenciar informações para o desenvolvimento de estratégias conservacionistas apropriadas, principalmente para *C. lami*;
- Propor um panorama sobre a ocupação e estabelecimento dessas duas espécies na planície costeira do Sul do Brasil;
- Contribuir com a compreensão taxonômica e sistemática do gênero *Ctenomys*, através da caracterização molecular das diferentes linhagens de *C. minutus* e *C. lami*.

CAPÍTULO II

Manuscrito submetido ao periódico *Journal of Heredity*

Human impact in naturally patched small populations: genetic structure and conservation of tuco-tuco (*Ctenomys lami*)

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Running title: Phylogeography of *Ctenomys lami*

ABSTRACT

Isolated or semi-isolated small populations are commonly found among species, due to a naturally patchy occupancy of suitable habitats, or also as a result of habitat alterations. These populations are subject to an increased risk of local extinction since they are more vulnerable to demographic, genetic and environmental stochasticity. Considering that natural areas have been becoming progressively more fragmented and smaller, understanding the genetic structure and evolutionary dynamics of small populations is critical. *Ctenomys lami* has 26 karyotypes distributed in a small area (936 km^2) continually modified by human actions. We assessed the genetic geographical structure of this species on a fine scale, using information from chromosomal variability, mitochondrial DNA, and microsatellite loci. The observed isolation-by-distance pattern and a clinal genetic variation suggest a stepping-stone population model. The results did not indicate genetic structuring associated with distinct karyotypes. However, both molecular markers demonstrated the existence of two demes, which are not completely isolated but are probably reinforced by a geographical barrier. The vulnerability of *C. lami* is greater than previously supposed, and our data support the designation of one Evolutionary Significant Unit and one Management Unit, and also the inclusion of this species' conservation status as vulnerable.
Keywords: phylogeography, population genetics, southern Brazilian coastal plain, metapopulation, chromosomal polymorphism

INTRODUCTION

Understanding how genetic variation is partitioned among populations in the environment is of prime importance in conservation biology, with important implications for making decisions to properly manage the genetic diversity of threatened species. Therefore it is necessary to identify boundaries of species and populations, assess evolutionary patterns and processes such as gene flow, genetic drift, and selection, and also the interaction between these micro-evolutionary processes and landscape features (Manel et al. 2003).

Small, declining, and isolated populations are more vulnerable to demographic, environmental and genetic stochasticity than larger and more stable ones, and therefore face a higher risk of local extinction (Ellstrand and Elam 1993; Shaffer 1981). Either naturally stable small populations or populations that have recently suffered or recurrently suffer reductions in their sizes are susceptible to genetic consequences of inbreeding and genetic drift, which can lead to reductions in genetic diversity and consequently the reduction of evolutionary potential, in addition to increasing differentiation among populations (Ellstrand and Elam 1993; Frankham 2008). Considering that natural areas have been becoming progressively more fragmented and smaller, understanding the ecological and evolutionary dynamics of small populations is increasingly important (Lande 1988).

The genus *Ctenomys*, popularly known as tuco-tucos, is composed of about 60 species of subterranean rodents that share some common characteristics including their solitary and territorial habits, small patchily distributed populations, and small effective population sizes, which associated with low rates of adult dispersal leads to a pattern of low genetic variation within populations, and high genetic divergence among populations. Furthermore, the species commonly show high levels of karyotypic variation (Lacey et al. 2000; Nowak 1999; Reig et al. 1990).

Ctenomys lami Freitas, 2001 is geographically restricted to an area of 78 x 12 km, named Coxilha das Lombas (Freitas 2001). This area consists of sandy fields derived from the oldest depositional system of the southern Brazilian coastal plain, formed by fluctuations in the Atlantic Ocean sea level, approximately 400 000 years ago (Tomazelli et al. 2000). This region is bounded on the northwest by the Pachecos swamp and on the

southeast by the Touros swamp, which are connected in the middle of the Coxilha das Lombas, and also by lakes, lagoons, and a river (Figure 1; Fernandes et al. 2007; Freitas 2001). Nowadays this region has been progressively transformed by the introduction of pastures and human-modified vegetation (Fernandes et al. 2007; Freitas 2001). *Ctenomys lami* has one of the highest chromosomal variability in the smallest geographical distribution among ctenomyids. Freitas (2007) identified five different diploid numbers (2n = 54, 55, 56, 57 and 58), and ten autosomal arm numbers (AN; from 74 to 82, and 84), which combined formed the 26 karyotypes described until now for this species. Considering the Robertsonian rearrangements found in each locality sampled, Freitas (2007) described four karyotypic blocks, named A (2n=54, 55a, 56a), B (57 and 58), C (54 and 55a) and D (56b and 55b), and two intra-specific karyotypic hybrid zones, one between blocks AxB, and another between blocks CxD (Table 1). Also, studies analyzing karyotypic data suggested a region of contact and hybridization between *C. lami* and *Ctenomys minutus* on the western shores of Barros Lake (Gava and Freitas 2003). Despite the relative good knowledge of the pattern of karyotypic variation in this species, little is known about its genetic structure, and only one study has been carried out along its distributional range using enzyme polymorphisms (Moreira et al. 1991).

The lack of knowledge about ecological and genetic structure has contributed to difficulties in developing conservation strategies for *C. lami*. Also, the high chromosomal polymorphism of this species associated with the progressive human disturbance of its limited geographical territory could lead to a great loss of genetic diversity and the evolutionary process in which this species is involved. In order to provide information for the development of appropriate conservation strategies for *C. lami,* we analyzed their geographical genetic structure throughout the Coxilha das Lombas area, using the control region and the cytochrome c oxidase subunit I mitochondrial regions and 14 nuclear microsatellite loci.

METHODS

Sampling, genotyping, and sequencing

A total of 178 individuals were used in this study; 172 were analyzed for microsatellite data and 166 individuals were sequenced for mitochondrial DNA (mtDNA), from 28 sampling sites (Table 1), which were previously collected and karyotyped by Freitas (2001) and El Jundi (2003). The tissue samples were preserved in 95% ethanol and stored at -20 °C in the collection of the Laboratório de Citogenética e Evolução of the Departamento de Genética of the Universidade Federal do Rio Grande do Sul.

Total DNA was extracted following a modified phenol-chloroform protocol from Sambrook and Russel (2001). All samples were genotyped for 14 microsatellite loci, using fluorescently labeled primers, isolated for the species *C. haigi* (Hai2, Hai3, Hai4, Hai5, Hai 6, Hai9, Hai10, Hai12; Lacey et al. 1999) and *C. sociabilis* (Soc2, Soc3, Soc4, Soc5, Soc6; Lacey 2001). The PCR amplifications were carried out following the protocols described in Lacey et al. (1999) and Lacey (2001), and were conducted using one primer pair at a time. The genotypes were obtained using an ABI 3730 DNA sequencer, joining at most three PCR products, combined based on their sizes and labels. The allele sizes were defined using the program PeakScanner 1.0 (http://www.appliedbiosystems.com). In order to obtain reliable results of allele sizes, the PCR and genotyping reactions were carried out as many times as needed, and also all peaks of allele sizes were double-checked.

Two fragments of mtDNA were analyzed: part of the HVS1 control region (CR), and the cytochrome c oxidase subunit I (COI). The CR sequences were amplified using the primers TucoPro (5'-TTCTAATTAAACTATTTCTTG-3', Tomasco and Lessa 2007) and TDKD (5'-CCTGAAGTAGGAACCAGATG-3', Kocher et al. 1989), following the PCR amplification conditions described by Tomasco and Lessa (2007). The PCR amplifications for the COI sequences followed the protocols suggested in http://www.barcoding.si.edu/DNABarCoding.htm, using the primers LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer et al. 1994). The PCR products were visualized on 1% agarose gels and purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California), following the guidelines of the suppliers. Sanger sequencing was conducted in an ABI 3730 automated sequencer, using the forward primers TucoPro and LCO-1490, for CR and COI, respectively. The ambiguous sequences were reamplified and resequenced as many times as needed to obtain a clean and reliable electropherogram.

Data analyses

The evidence of null alleles and scoring errors were checked using Micro-Checker 2.2.3 (van Oosterhout et al. 2004). Genepop 4.0 was used (Rousset 2008) to test for deviations from linkage equilibrium (LE) among each pair of loci in each locality sampled, and for tests of Hardy-Weinberg equilibrium (HWE) among each locus in each locality, using 10 000 dememorization steps, 500 batches, and 10 000 iterations per batch. Significance levels ($\alpha = 0.05$) of HWE and LE were adjusted using sequential Bonferroni corrections considering the multiple simultaneous comparisons (Rice 1989). The program Arlequin 3.5.1.2 (Excoffier and Schneider 2005) was used to compute measures of microsatellite diversity, including the mean number of alleles per locus, and the observed and expected heterozygosities (Ho and He). This software was also used to calculate pairwise genetic differentiation between localities (F_{ST}), and the inbreeding coefficient (F_{IS}), according to Weir and Cockerham (1984). Localities with fewer than 4 individuals were excluded from F_{ST} comparisons, and sequential Bonferroni corrections were applied, with $\alpha = 0.05$, to adjust the statistical significance levels. To detect genetic signatures of bottlenecks, the program Bottleneck (Cornuet and Luikart 1996) was used, following the Wilcoxon signed rank test, with both stepwise (SMM) and two-phase (TPM) mutation models. Only localities with more than 10 individuals were tested.

All analyses were performed with the mtDNA data sets of CR and COI separately, and a concatenated data set of CR+COI (CC); however, the results of the concatenated data will be preferentially presented here. The electropherograms were visually inspected using Chromas 2.33 (http://www.technelysium.com.au/chromas.html), and aligned using the Clustal W algorithm with default options, implemented in Mega 4.02 (Tamura et al. 2007). Alignments were checked and edited by hand if necessary. Measures of mtDNA diversity, including the mean number of pairwise differences (π, Nei 1987), definitions of haplotypes (H), and haplotype diversity (*Hd*), were calculated in the program DNAsp 5.00.03 (Librado and Rozas 2009). The program Arlequin 3.5.1.2 was used to infer the occurrence of past events of population expansion or decline, employing mismatch distribution analysis (Rogers and Harpending 1992), and Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) neutrality tests. To check the topological relationship between the haplotypes, a concatenated median-joining haplotype network was constructed in Network 4.6.0.0

(http://www.fluxux-engineering.com), including sequences of species closely related to *C. lami* (GenBank acession numbers of *C. torquatus* HM443438 and HM443439, and *C. pearsoni* JQ341031 and JQ341042).

To test for positive correlations between genetic and geographical distances, a Mantel test (Mantel 1967), implemented in Arlequin 3.5.1.2, was used to examine patterns of isolation by distance along geographical distribution, for mitochondrial CC and microsatellite data independently. Moreover, two additional Mantel tests were performed considering that *C. lami* geographical distribution is interrupted by the connection between Pachecos and Touros swamps: i) Considering only localities corresponding to karyotypic blocks A and B; and ii) Considering sampling sites corresponding to karyotypic blocks C and D. Statistical significance was tested using 1 000 random permutations.

To determine the geographical genetic structure of *C. lami* based on microsatellite data and to assign individuals to specific cluster, we used the Bayesian clustering approach with and without spatial data, implemented in the programs Geneland 3.2.4 (Guillot et al. 2008) and Structure version 2.2.3 (Pritchard et al. 2000), helping us to identify distinct evolutionary units that could be used to define conservation strategies. To determine the most appropriate number of clusters (Ks) in Structure 2.2.3, ten independent runs of K=1-15 were tested, assuming the admixture model, independent allele frequencies, no a priori population information, 500 000 iterations for the burn-in period and 2 000 000 for the Markov chain Monte Carlo (MCMC). The optimal K was determined using the highest mean value of the estimated logarithm of probability of the data [LnPr(X/K)] and the lowest standard deviation (SD) among the independent runs for each K, before reaching instability or a plateau in the means of LnPr(X/K). Mixed ancestry of specimens was considered when $q \leq 0.7$. For the analysis procedure in Geneland 3.2.4 we used the geographical coordinates of each sampling site. First, we inferred the appropriate number of clusters (K), following ten independent runs, each one varying K between 1 to 10, 1 500 000 MCMC iterations of which every 100 iteration was saved, allele frequencies uncorrelated, and the uncertainty of spatial coordinates set at 0.5 km. After that, ten additional independent runs were processed, with the same parameters described above, but with k fixed at the value inferred in the first step. The posterior probability of population membership was computed with a burn-in of 2 000 iterations, and the pixels

were set to 400 on both x and y axes. The best run was chosen by the highest value of posterior probability of the data.

To investigate patterns of hierarchical partitioning of genetic structure, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was implemented in Arlequin 3.5.1.2, using both mitochondrial CC and microsatellite data, under four different scenarios of hypothesized subdivision: i) considering the four karyotypic blocks independently (A x B x C x D); and ii) considering two major groups separated by the link between the Pachecos and Touros swamps, blocks A+B x C+D; iii) considering 6 groups subdivided according to the clustering of localities retrieved in the Structure analysis; and iv) also 6 groups according to the subdivisions found in the Geneland results.

RESULTS

Genetic diversity

Departures from HWE were found in 5 of a total of 378 comparisons carried out (BC: Hai4; CO: Hai10; FV: Hai3; PI: Hai5; and SR: Soc4; the abbreviations of locality names correspond to those in Table 1). These deviations could be explained by evidence of null alleles detected with Microchecker in eight localities (BC: Hai4 and Hai6; CO: Hai10; FF: Hai10; FP: Hai4; FV: Hai3 and Soc5; PI: Hai5; PL: Hai4 and Soc2; and SR: Soc4), or even by deviations from random mating evidenced in six localities (PI, BC, PL, CO, FV, LB2) through the significance values of the inbreeding coefficient (F_{IS}; Table 1). None of the localities showed heterozygote excess, and also the localities tested for evidence of a recent bottleneck did not show significances for any of the models of mutation analyzed. No deviations from LE were detected among loci. The overall microsatellite genetic diversity was moderate compared with other tuco-tucos (see Table S1). Overall, 120 alleles were found, the number of alleles per locus ranged from 2 to 13 with a mean of 8.57, the number of alleles per locus per locality ranged from 1 to 8, and the means are given in Table 1.

For mitochondrial data, 418 and 623 base pairs (bp) were analyzed for CR and COI, respectively. CR data retrieved 11 polymorphic sites, resulting in 14 haplotypes (GenBank accession numbers JQ322885 to JQ322898), with an overall haplotype diversity (*Hd*) of

0.871, and a global nucleotide diversity (π) of 0.00591. COI sequences resulted in 10 variable sites, 9 haplotypes (GenBank accession numbers JQ322899 to JQ322907), *Hd* = 0.648, and π = 0.00349. Considering these two mitochondrial regions together, the 1041bp resulted in 21 polymorphic sites, giving rise to 18 haplotypes (*Hd* = 0.908, π = 0.00446). These moderate values of *Hd*, together with the low levels of π indicate that the few haplotypes found are closely related. The karyotypic blocks A and C showed the highest values of π; however, the same pattern was not followed by the haplotype diversity measures, which were higher in blocks B and D (Table 2). Differences among diversity measures could indicate that blocks A and C have more admixtures than blocks B and D, resulting in higher π values, while common haplotypes shared by most specimens reduces the *Hd*.

Demographic history, geographical genetic variation, and gene flow

There was no evidence of a recent expansion of population sizes according to the global mismatch distribution tests, which showed multimodal graphs for the three mtDNA data sets (data not shown), and according to the neutrality tests (**CR**: D = 0.67, P = 0.80; F_S = -0.98, P = 0.42; **COI**: D = 0.55, P = 0.75; F_S = 0.83, P = 0.68; **CC**: D = 0.70, P = 0.79; F_S = 0.14, P = 0.57). Also, the neutrality tests did not show evidence of recent population expansion when analyzed for each karyotypic block (Table 2).

There was a significant pattern of isolation by distance (IBD) for all three tests performed with CC mtDNA (all range: r = 0.51, P = 0.00; blocks A+B: r = 0.30, P = 0.03; blocks C+D: r = 0.60, P = 0.00), and also for microsatellite data considering the entire range (r = 0.53, P = 0.00), and the localities from blocks C+D (r = 0.34, P = 0.03). The exception was found in localities from blocks A+B, with microsatellite data, where no significant evidence of IBD was found, although the correlation coefficient showed moderate values (r = 0.30, P = 0.07).

In general, the microsatellite estimates of F_{ST} values showed moderate to high levels of genetic differentiation in pairwise comparisons between localities, ranging from 0.01 to 0.67 (Table S2), the overall F_{ST} was 0.28, and among the 253 pairwise comparisons, 102 showed significant values. For mtDNA data, the F_{ST} pairwise estimates ranged from -0.33 to 1.00 (overall 0.75), and although most of them were much higher

than those found for the microsatellite, only 63 pairwise comparisons were significant. Most of the non-significant estimates were found in comparisons involving localities with fewer than 6 individuals, suggesting that localities with small sample sizes may led to erroneous interpretations by overestimating the gene-flow levels. Also, sex-biased dispersion may occur in tuco-tucos, being more pronounced in males than in females, accounting for the higher values of F_{ST} found in mtDNA than from the microsatellite data.

Relantionship among haplotypes and clustering of localities

The median-joining network obtained with the CC data (Figure 2) revealed a topology with various haplotypes directly connected by few mutational steps to the central haplotype 5, which is shared by 12 localities from three karyotypic blocks (A, B, and C). It is suggested, under a limited-gene-flow model, that the most geographically widespread haplotypes should be the ancestral ones, which could be the case for haplotype 5. Among the 18 haplotypes recovered, most are shared among different localities (Table 1), even within or between different karyotypic blocks, providing evidence that ancestral polymorphisms are shared by specimens with different karyotypes or even that distinct karyotypes do not act as reproductive barriers to this species, allowing gene flow (Figure 2). All specimens from block D and some from block C formed a distinct group, separated in the network by a large number of mutational steps from the other haplotypes. The outgroup sequences of *C. torquatus* and *C. pearsoni* included in the median-joining network are separated from our ingroup by many mutational steps, as represented in Figure 2.

Bayesian clustering inferred from the Structure program recovered a multimodal distribution of the mean values of LnPr(X/K) through different Ks. However, the K with the highest mean of the logarithm of the probability of the data, with the lowest standard deviations between the independent runs, before reaching instability in the graph of mean LnPr(X/K), was K = 6, which was considered the real number of clusters (Figure 3A and Figure 3B). Geneland analysis recovered K=7 as the most likely number of clusters for all the ten runs; nevertheless, when the runs were performed with K fixed at 7, the 28 sampling sites were distributed in 6 clusters, and the remaining one was a “ghost cluster”. The “ghost clusters” occurs when the number of real populations is smaller than the

number of inferred populations, due to departures of the data from modelling assumptions or by a lack of MCMC mixing (Guillot et al. 2005). This is a rare, but possible event, and according to Guillot et al. (2005) these “ghost" populations may be interpreted as spurious, and the focus can be restricted to modal populations. Comparing the 6 clusters retrieved for Structure and Geneland analysis, four of them grouped localities in the same way as depicted in Figures 3A and 3C and Table 1. The discrepancies were found in the clustering of the localities from the entire karyotypic block B (IT, CO, PV2, FV, LB2, FP, BM, and AC) plus the localities VJ, PA, and GV from block A. For Structure there is one large cluster formed by all localities cited above (cluster III) except VJ, which is isolated in an exclusive cluster (IV). In Geneland these localities are also subdivided in two clusters, but VJ is grouped with PA and FP (cluster 4), and the remaining localities are grouped in another cluster (III). The karyotypic block A showed more admixtures than the others, with their localities distributed in four of the six clusters found (clusters I, II, III, and IV); also the karyotypic block D is the most homogeneous, with all of its localities sampled in cluster VI. As in the results retrieved with mtDNA, the genetic structuring of microsatellite data was not directly associated either with the four karyotypic blocks or with the chromosomal rearrangements.

Genetic clustering was also tested using microsatellite and CC mtDNA data through an AMOVA approach. The four analyses performed, testing the proportion of genetic variation considering the four karyotypic blocks (A x B x C x D), two clusters (A+B x C+D), or 6 groups according to the clusters retrieved in the Structure or Geneland analyses, indicates that both molecular markers were highly structured in *C. lami*, at all levels (Table S3). For mtDNA in the four tests performed, the highest percentages of genetic variation were found among groups (more than 40%), but were slightly higher in the test considering the 6 cluster retrieved for Structure (54.90%). For microsatellite data, the lowest levels of genetic variation were found among groups; the highest percentages of genetic variation were attributed within localities (more than 68%). It is commonly to find a lower proportion of genetic variance among groups of populations in microsatellites than in mtDNA, which can be due to high levels of variation and potential homoplasy in microsatellites, and the lower *Ne* of mtDNA.

DISCUSSION

Phylogeographical pattern and population structure

The importance of identifying the spatial structure of populations and their dynamics in the environment is widely recognized in conservation biology. The species that shows a pattern of patch occupancy, with small populations, requires more attention because of a higher probability of extinction risk, than species with large and continuously distributed populations (Hanski 1998; Manel 2003).

The species of the genus *Ctenomys* commonly show a pattern of metapopulation distribution, consisting of small populations patchily distributed in favorable habitats (Mirol et al. 2010). The patchy distribution can be observed in the field, with groups of several burrows close together, and separated from other groups by long distances. This behavior, together with the low levels of adult dispersal, reported for subterranean rodents (Lacey et al. 2000), could explain the low levels of gene flow found in *C. lami*, denoted by the high significant values of pairwise F_{ST} estimates (Table S2), and the occurrence of deviations from random mating in six of the 28 localities sampled (Table 1). Also, although this species occurs in a small geographical area, the Mantel tests demonstrated for both molecular markers a positive and significant association between genetic and geographical distance. This pattern of isolation by distance could result both from low levels of dispersal, and limited dispersal among neighbor demes due to the narrow distribution of *C. lami*, engendering a stepping-stone model of genetic variation. The pattern of isolation by distance has been widely reported in species of ctenomyids that are nearly linearly distributed (*Ctenomys talarum*, by Mora et al. 2007; *Ctenomys flamarioni*, by Fernández-Stolz 2007; *Ctenomys pearsoni*, by Tomasco and Lessa 2007; *Ctenomys minutus*, by Lopes et al. in prep.). The narrow distribution of a species limits its dispersal and gene flow to a dominant spatial direction as compared to a wide distributional range, more easily allowing an increasing genetic differentiation between populations the farther they are from each other (Slatkin and Barton 1989). With these considerations, we were able to explain a pattern of isolation by distance that was more pronounced in the localities from karyotypic blocks C and D, which have a narrower distribution than the specimens from blocks A and B (Figure 1), in which the correlation between genetic and geographical distance was low for both markers, and also not significant for microsatellites.

A clinal pattern of genetic variation could be observed in *C. lami*, since the haplotypes and alleles are commonly shared between geographically close localities, mainly among localities within karyotypic blocks A and B, and among localities within blocks C and D (Figures 2, 3A and 3C). Inspection of the haplotype network topology revealed that three haplotypes (2, 4, and 7) are shared between localities from blocks A and B, haplotype 5 is shared between blocks A, B, and C, and block D shares only one haplotype (15) only with localities from block C. Also, this pattern of differentiation between blocks A and B versus C and D was observed in the clusters retrieved from microsatellite data. Cluster III, in both methodologies analyzed (Structure and Geneland), grouped localities from blocks A and B, while cluster VI grouped localities from blocks C and D. The existence of these two demes is possibly reinforced by the connection between the Pachecos and Touros swamps. However, the demes do not seem to be completely isolated, since specimens from blocks A, B and C share haplotype 5, which could be an effect of sharing of an ancestral polymorphism, or even evidence of some degree of gene flow. Moreover, some migrants and hybrids between the karyotypic blocks A, B and C were detected in the Structure analysis (Figure 3A). Moreira et al. (1991) analyzed the genetic variation of 20 structural loci encoding 13 enzymes, for the four southern Brazilian ctenomyid species (*C. minutus*, *C. torquatus*, *C. lami*, and *C. flamarioni*). For C. *lami*, 87 specimens from 10 sampling sites along its distributional range were analyzed. Moreira and colleagues found that 47.5% of the loci were shared by specimens from all localities, but in two loci they detected a clinal pattern of geographical variation. Also, similar to our suggestion, they observed the existence of two demes, one formed by the specimens from karyotypic blocks A and B, and the other formed by the specimens from karyotypic blocks C and D, however showing some degree of gene flow between them. *Ctenomys lami* has a notable chromosomal polymorphism, showing 26 karyotypes.distributed in four karyotypic blocks. The dynamics of chromosomal rearrangements along the geographical distribution were analyzed trough the G-banding pattern by Freitas (2007). The referred study showed that diploid numbers primarily varies due to centric fusions/fissions in chromosome pairs 1 and 2, changing between a metacentric or acrocentric chromosome forms depending on the karyotype observed. Moreover, pericentric inversions are mainly related with variations in autosomic arm numbers. Diploid number 54 presents both chromosome pairs 1 and 2 as metacentric forms. Specimens with 2n = 55 present one chromosome pair as metacentric

and a heterozygote form of the chromosome pair 1 (55b) or pair 2 (55a), with one metacentric homologue replaced by two acrocentrics. In forms with 2n = 56 both homologues of pair 1 (56a) or pair 2 (56b) are replaced by 4 acrocentrics. In 2n = 57 the chromosome pair 1 is heterozygote and chromosome pair 2 are replaced by 4 acrocentrics. Finally, specimens with 2n = 58 have all elements of pairs 1 and 2 fissioned originating 8 acrocentrics. Freitas (2007) also proposed four karyotypic blocks considering the pattern of fusion/fission in chromosome pairs 1 and 2. Karyotipic blocks A and C present just populations with chromosome pair 1 metacentric. Block B is composed by specimens with both chromosome pairs 1 and 2 fissioned, and populations from Block D present specimens with chromosome pair 2 metacentric.

Our results did not indicate genetic structuring associated with the four different karyotypic blocks separately, or even with the chromosomal rearrangements found in this species, suggesting that the karyotypic blocks described by Freitas (2007) have proven to be inconsistent for mtDNA and microsatellite data, and also that the chromosomal rearrangements probably do not prevent reproduction among individuals carrying distinct karyotypes, i.e., not act as reproductive barriers. Fixation of new chromosomal rearrangements seems to be frequent in species of the genus *Ctenomys*, and it is commonly not followed by sterility, reductions in fitness, or negative heterosis of heterozygote carriers (Tomasco and Lessa 2007). There are some examples of chromosomal polymorphic ctenomyids that fail to show genetic differentiation of karyotypic populations. For instance, the Corrientes group includes species that are genetically extremely similar to each other despite their high degree of karyotypic differentiation (Giménez et al. 2002; Mirol et al. 2010). For *C. pearsoni,* all populations and karyomorphs studied were polyphyletic in their mtDNA (Tomasco and Lessa 2007). The Brazilian species *C. torquatus* and *C. minutus*, the latter being the sister species of *C. lami*, also fail to show a common pattern of genetic and karyotypic variation (Fernandes et al. 2009; Lopes et al. in prep).

In spite of the absence of evidence of a recent bottleneck pattern in populations of *C. lami*, and although the levels of microsatellite variability were moderate to high compared with other tuco-tucos (Table S1), the mtDNA markers showed moderate to low levels of genetic diversity. Comparing the CR data available among tuco-tucos species, *C. torquatus*, *C. australis*, *C. flamarioni*, and *C. lami* showed the lower levels of genetic

diversity (Table S1), given that *C. flamarioni* have the lowest levels of mitochondrial genetic diversity among all tuco-tucos analyzed until now, and is included as "vulnerable" in the National List of Endangered Species of the Brazilian Fauna (Machado et al. 2008) and in the Regional List of Endangered Fauna in Rio Grande do Sul (Fontana et al. 2003), and as “endangered” in the IUCN red list of threatened species. The results retrieved by Moreira et al. (1991) also showed low levels of genetic variation for *C. lami* in enzymatic loci, which were not lower than the levels of variation found in *C. flamarioni*.

Implications for conservation and conclusions

The demographic and genetic characteristics of *C. lami*, together with the progressive urbanization, human occupation, development of cattle ranching and agriculture, mainly rice and soybean cultivation, in the region of Coxilha das Lombas, impact the limited geographical distribution of this species and place at risk the process of differentiation and evolution in which the specimens are involved, deserving of special attention (Fernandes et al. 2007).

In order to aid in the identification of priority areas for conservation, the concept of evolutionarily significant units (ESU) was created and has been widely discussed since 1986 (Fraser and Bernatchez 2001). One of the most widespread concepts of ESUs is that described by Moritz (1994), in which he considered an ESU as reciprocally monophyletic for mtDNA alleles and showing significant divergence of allele frequencies at nuclear loci. One problem regarding this concept is that recently founded populations or rapidly radiated species could not demonstrate reciprocal monophyly. To circumvent this, Moritz proposed the management unit (MU), which considers statistically significant divergence in allele frequencies (nuclear or mitochondrial), no matter the phylogenetic differentiation of the alleles. Our data support the designation of one ESU for *C. lami*, including the sampling localities of FE, FR, LB4, LB3, CL1, LB5, FF, CL2, CL, CL3, CL4 and MG, based on their genetic differentiation for both molecular markers used here; and also all the other localities could be treated as a MU.

Despite all the threats, *C. lami* was only recently given “vulnerable” status in the IUCN Red List of threatened species. Considering the situation described, the vulnerability of *C. lami* is greater than previously supposed. Its extinction could lead to a great loss of

genetic diversity, since it shows one of the highest chromosomal variabilities among *Ctenomys* species (Fernandes et al. 2007; Freitas 2007). Therefore some measures must be taken in order to protect their biological diversity and evolutionary process, mainly with regard to the designation of this species as vulnerable in national and regional Brazilian Red Lists of endangered fauna, and also with regard to conservation units.

Most of the distributional area of *C. lami* is not covered by conservation units. There is one state park, called "Parque Itapuã" (approximately 5 500 hectares in area), located entirely within the area of occurrence of this species, where the specimens from our PI sampling site were collected. Also there is a wildlife refuge, called "Banhado dos Pachecos" (approximately 2 500 hectares in area), near our sampling site of AC; however, only part of its area overlaps the distribution of *C. lami* (Figure 1). We suggest that conservation units of "Parque Itapuã" and "Banhado dos Pachecos" are expanded to overlap a greater area of *C. lami* geographical distribution. Moreover, based on our results, another conservation unit must to be implemented around the region corresponding to the sampling sites of CL1, LB5, FF, CL2 and CL, aiming to protect a combination of a unique genetic diversity found in this area.

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FIGURES

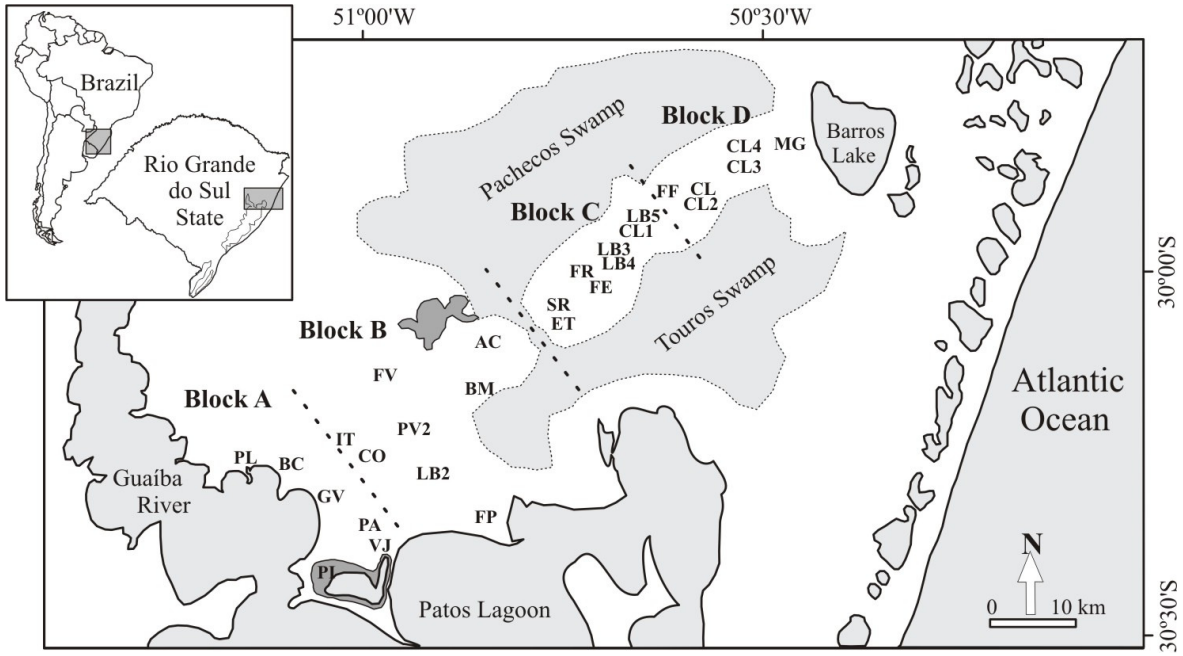


Figure 1. Localities sampled for *C. lami*, showing the four karyotypic blocks. Locality abbreviations correspond to those in Table 1. The two areas highlighted in dark gray are conservation units.

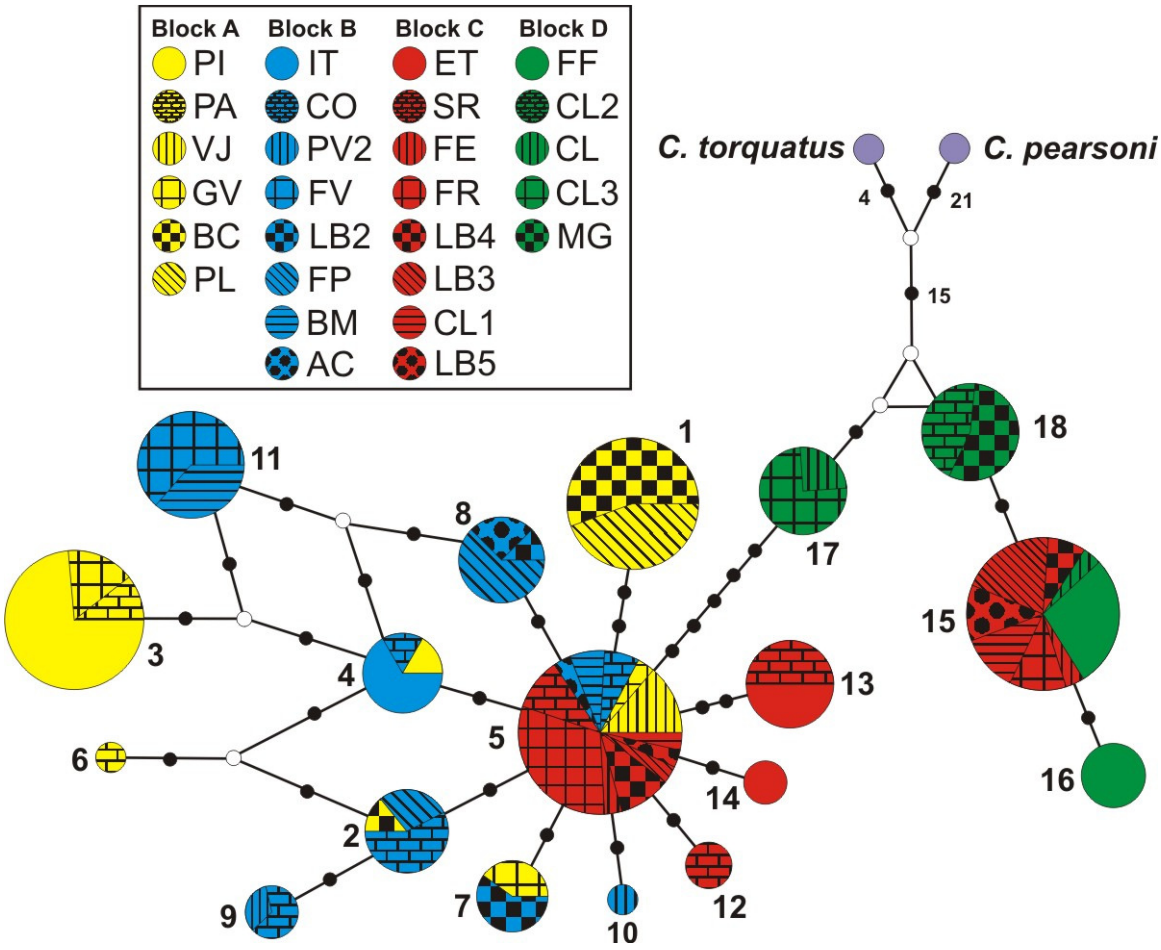
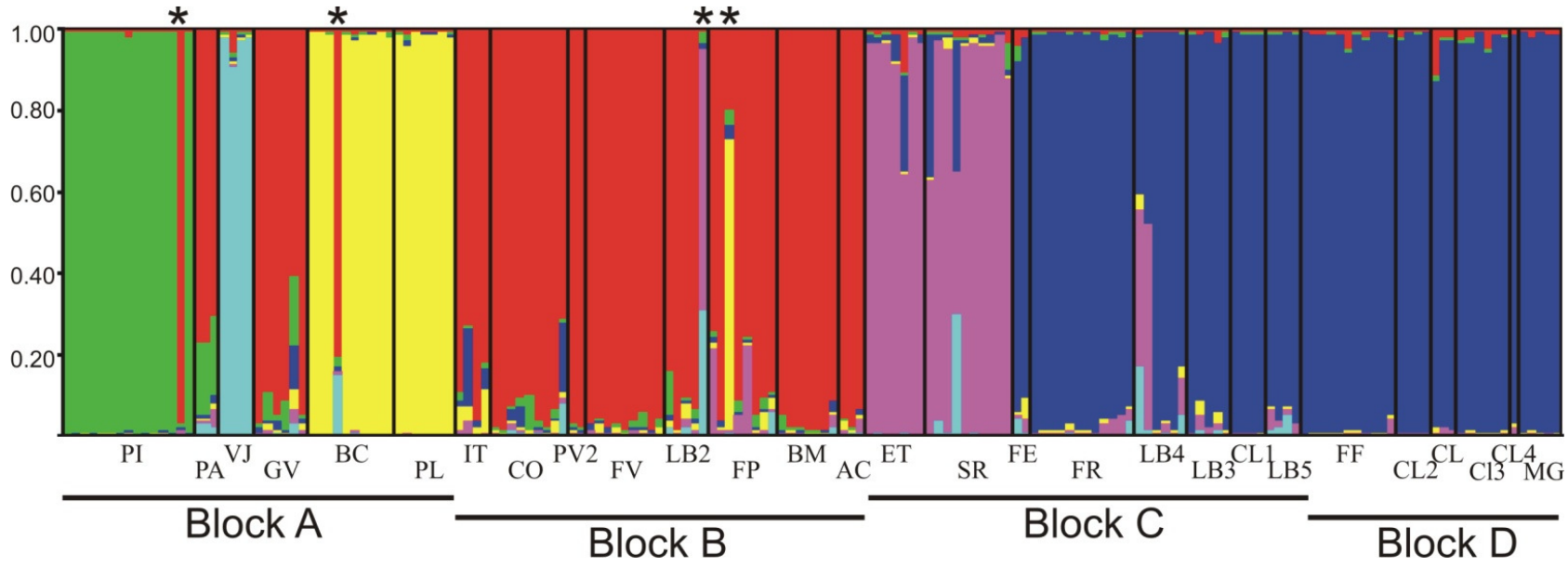


Figure 2. Median-joining haplotype network topology obtained with 1024bp of concatenated CR and COI mtDNA data, from 166 *C. lami* specimens, 1 *C. torquatus* and 1 *C. pearsoni*. Shading indicates localities, and each color represents the four karyotypic blocks following the legend. Small black circles represents nucleotide differences, and small white circles represent extinct or unsampled haplotypes. Abbreviations of the localities are given in Table 1.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CL
Cl3
CL4
MG
Block A
Block B
Block C
Block D

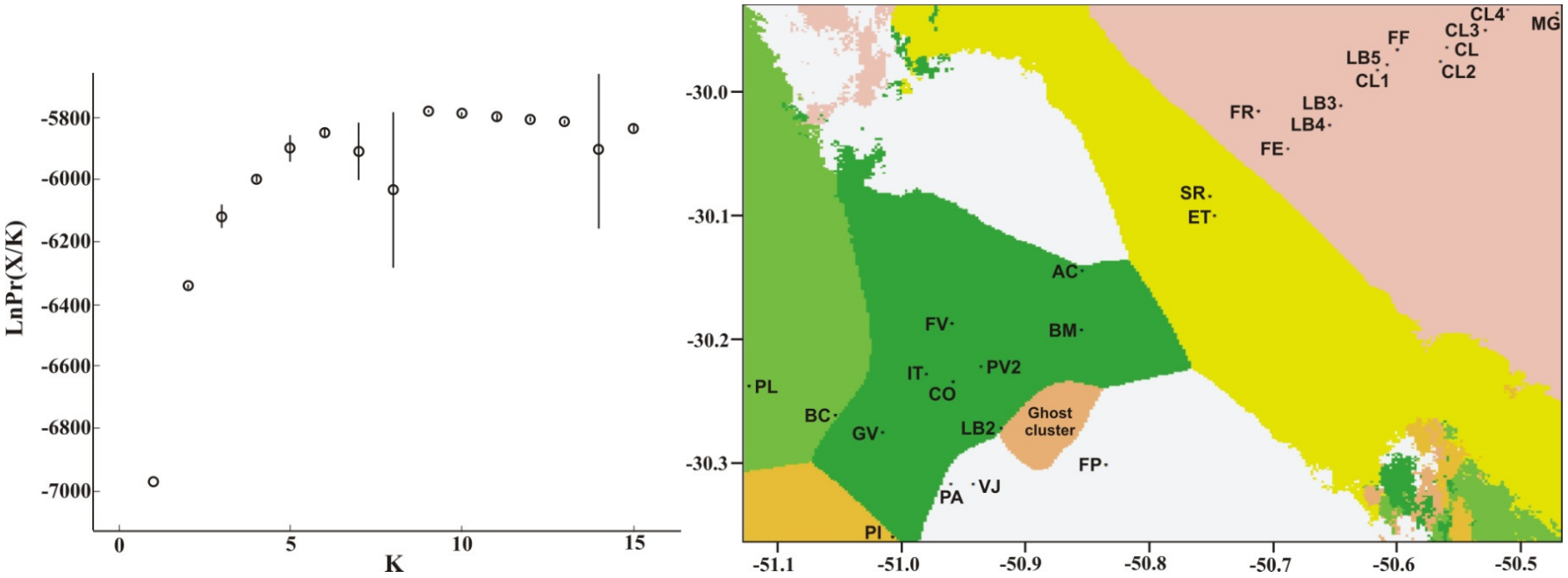

Figure 3. A) six clusters identified by the Structure program. Each specimen is identified by a single vertical bar, and each cluster by a color, below the plot is the correspondence of localities and karyotypic blocks. The asterisk indicates migrants from other clusters; B) estimation of the *C. lami* Structure clusters. The vertical lines across the dots are proportional to the standard deviations among the ten runs for each k; C) Spatial Bayesian cluster in the program Geneland. The sampling sites are shown. Each color represents one cluster.

TABLES

Table 1. Description of samples and genetic diversity of *C. lami*. Locality names and their abbreviations in parentheses, diploid and autosomal arm numbers [2n(AN)], number of individuals genotyped (nG) and sequenced (nS) per locality, overall number of alleles per locality (nA), mean number of alleles per locus per locality (*A*), inbreeding estimates (F_{IS}), and the number of concatenated haplotypes retrieved in each locality. *Statistical significance at $P \leq 0.05$.

Locality name	2n(AN)	Karyotypic block	Structure clusters	Geneland clusters	nG	nS	nA	*A*	F_{IS}	CC haplotypes
Parque Itapuã (PI)	54(76,78) 55a(78,80)	A	I	I	15	15	38	2.71	0.19*	CC3=14/CC4=1
Passo da Areia (PA)	54(76,78) 55a(76)	A	III	IV	3	3	39	2.79	-0.29	CC3=2/CC6=1
Varzinha do Jacaré (VJ)	54	A	IV	IV	4	4	25	1.79	-0.14	CC5=4
Gravatá (GV)	56a	A	III	III	6	6	38	2.71	0.01	CC3=3/CC5=1/CC7=2
Beco do Cego (BC)	54(76,82) 55a(76,78,80) 56a(76,78,80)	A	II	II	10	11	39	2.79	0.21*	CC1=10/CC2=1
Praia do Lami (PL)	-	A	II	II	7	8	30	2.14	0.31*	CC1=8
Itapuã (IT)	58(78,80,83)	B	III	III	4	4	29	2.07	0.17	CC4=4
Costa do Oveiro (CO)	58(78/79)	B	III	III	9	9	54	3.86	0.21*	CC2=4/CC4=1/CC5=2/CC9=2
Passo do Vigário 2 (PV2)	58(80,82)	B	III	III	2	2	28	2.00	-0.36	CC9=1/CC10=1
Fervura (FV)	58/54	B	III	III	9	7	37	2.64	0.30*	CC11=7
Lombas 2 (LB2)	57(77) 58(78,80,86)	B	III	III	5	4	55	3.93	0.33*	CC7=2/CC8=1
Fazenda Pimenta (FP)	58(78)	B	III	IV	8	7	54	3.86	0.08	CC2=2/CC8=5

Beco da Macega (BM)	58	B	III	III	6	6	37	2.64	-0.16	CC5=2/CC11=4
Águas Claras (AC)	58(78,80)	B	III	III	4	3	42	3.00	-0.02	CC5=1/CC8=2
Estiva (ET)	54(74,76,77,78,79) 55a(76)	C	V	V	7	6	47	3.36	0.03	CC13=4/CC14=2
Sanga da Rapadura (SR)	54	C	V	V	10	9	56	4.00	0.07	CC5=3/CC12=2/CC13=4
Fazenda do Estácio (FE)	54	C	VI	VI	2	2	33	2.36	-0.10	CC5=1/CC15=1
Fazenda Rita Maria (FR)	54	C	VI	VI	12	12	54	3.86	-0.02	CC5=9CC15=3
Lombas 4 (LB4)	54(75,76,77)	C	VI	VI	6	5	50	3.57	0.01	CC5=3/CC15=2
Lombas 3 (LB3)	54(75,76,78)	C	VI	VI	5	6	44	3.14	-0.01	CC5=1/CC15=5
Chico Lomã 1 (CL1)	54	C	VI	VI	4	4	31	2.21	0.01	CC5=1/CC15=3
Lombas 5 (LB5)	54(75,76,78)	C	VI	VI	4	4	44	3.14	0.04	CC5=1/CC15=3
Fazenda dos Freitas (FF)	56b(78,80,82)	D	VI	VI	11	11	40	2.86	0.10	CC15=7/CC16=4
Chico Lomã 2 (CL2)	-	D	VI	VI	4	4	38	2.71	-0.10	CC18=4
Chico Lomã (CL)	56b(80)	D	VI	VI	3	3	30	2.14	0.31	CC15=1/CC17=2
Chico Lomã 3 (CL3)	56b	D	VI	VI	6	6	36	2.57	0.10	CC17=6
Chico Lomã 4 (CL4)	56b	D	VI	VI	1	-	19	1.36	0.00	-
Morro Grande (MG)	54	D	VI	VI	5	5	25	1.79	0.12	CC18=5

Table 2. Genetic estimates in each karyotypic block. Number of samples genotyped (nG) and sequenced (nS) per karyotypic block, nucleotide diversity (π), haplotype diversity (*Hd*), Tajima's *D* (*D*), Fu's F_S (F_S), total number of alleles (nA), and the mean number of alleles per locus per block (*A*). None of the neutrality tests were significant ($P > 0.05$).

Karyotypic Blocks	**nG**	**nS**	**π**	***Hd***	***D***	***F_S***	**nA**	***A***
A	45	47	0.00209	0.690	0.99	1.27	90	5.29
B	47	42	0.00197	0.856	0.71	-0.61	94	6.50
C	50	48	0.00403	0.701	2.02	6.44	100	6.21
D	30	29	0.00152	0.759	1.42	1.61	60	4.50

SUPPLEMENTARY DATA

Supplementary Table S1. Comparative table of indexes of genetic diversity for ctenomyid species, for microsatellite (nL: number of polymorphic loci analyzed; nAL: range of number of alleles per locus; *A*: mean number of alleles per locus) and mtDNA control region [nH (Hd): overall number of haplotypes, and haplotype diversity; and π: nucleotide diversity].

Species	**Reference**	**nL**	**nAL**	***A***	**nH (Hd)**	**π**
C. sociabilis	Lacey, 2001	7	2-3	2.29	-	-
C. rionegrensis	Wlasiuk et al. 2003	11	6-14	8.3	-	-
C. haigi	Lacey, 2001	15	3-13	7.47	-	-
perrensi group	Mirol et al., 2010	16	5-19	13	-	-
C. roigi	Mirol et al., 2010	16	1-7	3.31	-	-
C. perrensi	Mirol et al., 2010	16	2-12	6.75	-	-
C. dorbignyi	Mirol et al., 2010	16	5-11	7.13	-	-
C. porteousi	Mapelli et al., 2011a, b	8	7-17	12.12	12 (0.89)	0.01
C. flamarioni	Fernández-Stolz, 2007	9	3-8	5.3	7 (0.790)	0.003
C. torquatus	Gislene et al., 2009; Fernandes, 2008	9	2-12	4.2	13 (0.819)	0.0068
C. talarum	Cutrera et al., 2006; Mora et al., 2007	12	2-9	3.67-6.17	32 (0.93)	0.022
C. minutus	Lopes et al., personal communication	14	7-16	13	40 (0.961)	0.0236
C. australis	Mora et al., 2006; Mora et al., 2010	8	3-6	4.75	24 (0.83)	0.0055
C. pearsoni	Tomasco and Lessa, 2007	-	-	-	21 (-)	0.20 – 5.76
C. lami	**This study**	**14**	**2-13**	**8.57**	**14 (0.871)**	**0.00591**

Supplementary Table S2. Pairwise F_{ST} values between sampling sites with more than four individuals, estimated with concatenated mtDNA data (above diagonal), and for 14 microsatellite loci (below diagonal). Values in bold are significant after Bonferroni correction.

	PI	**VJ**	**GV**	**BC**	**PL**	**IT**	**CO**	**FV**	**LB2**	**FP**	**BM**	**AC**	**ET**	**SR**	**FR**	**LB4**	**LB3**	**CL1**	**LB5**	**FF**	**CL2**	**CL3**	**MG**
PI		**0.92**	0.44	**0.92**	**0.96**	**0.89**	**0.84**	**0.91**	**0.89**	**0.87**	**0.70**	**-**	**0.80**	**0.76**	**0.66**	**0.75**	**0.90**	**0.88**	**0.88**	**0.97**	**0.98**	**0.98**	**0.98**
VJ	**0.50**		0.28	**0.79**	1.00	1.00	0.31	1.00	**0.50**	0.42	0.53	-	0.44	0.18	0.03	0.19	0.76	0.67	0.67	**0.95**	1.00	1.00	1.00
GV	**0.26**	0.37		0.68	**0.74**	0.28	0.40	0.62	0.25	0.43	0.21	-	0.31	0.25	0.25	0.31	0.71	0.62	0.62	**0.89**	0.84	0.83	0.85
BC	**0.41**	**0.45**	**0.31**		-0.03	0.86	**0.71**	**0.93**	0.77	**0.75**	0.76	-	**0.74**	**0.67**	**0.50**	0.60	**0.85**	0.82	0.82	**0.95**	**0.95**	**0.95**	0.96
PL	**0.47**	0.55	**0.36**	**0.15**		1.00	**0.80**	1.00	0.90	**0.85**	**0.83**	-	**0.81**	**0.75**	**0.53**	0.63	0.87	0.84	0.84	**0.97**	1.00	**1.00**	1.00
IT	**0.40**	0.54	0.26	**0.34**	0.38		0.58	**1.00**	0.75	0.70	0.44	-	0.64	0.53	0.29	0.38	0.79	0.71	0.71	0.96	1.00	1.00	1.00
CO	**0.27**	0.31	0.13	0.25	**0.29**	0.16		**0.83**	0.46	0.36	0.54	-	0.51	0.38	0.25	0.38	**0.77**	0.71	0.71	**0.92**	0.90	**0.89**	**0.91**
FV	**0.26**	0.39	0.11	**0.28**	**0.36**	0.23	0.09		0.90	0.81	0.23	-	0.84	**0.80**	**0.59**	0.65	**0.86**	0.83	0.83	**0.97**	1.00	**1.00**	1.00
LB2	0.26	0.29	0.11	0.22	0.25	0.17	0.05	0.10		0.41	0.49	-	0.49	0.38	0.18	0.26	0.73	0.63	0.63	0.93	0.94	0.94	0.94
FP	**0.29**	0.37	0.17	**0.25**	**0.29**	0.19	**0.11**	**0.16**	0.08		0.38	-	0.53	0.41	0.21	0.32	0.75	0.68	0.68	**0.92**	**0.91**	**0.91**	**0.92**
BM	**0.29**	0.40	0.15	0.35	**0.40**	0.28	0.08	**0.11**	0.08	**0.12**		-	0.56	0.50	0.32	0.37	0.74	0.66	0.66	**0.91**	0.88	0.89	0.89
AC	**0.38**	0.44	0.17	0.32	0.40	0.27	0.14	**0.16**	0.05	0.05	0.12		-	-	-	-	-	-	-	-	-	-	-
ET	**0.42**	0.42	0.26	**0.35**	**0.41**	0.30	**0.22**	**0.30**	0.19	**0.21**	0.29	0.24		0.03	0.30	0.36	0.74	0.67	0.67	0.91	0.89	0.88	0.90
SR	**0.36**	0.36	**0.23**	**0.28**	0.33	0.22	**0.19**	**0.22**	0.13	**0.18**	**0.26**	0.19	0.06		0.22	0.34	0.75	0.68	0.68	**0.90**	0.87	**0.85**	0.88
FR	**0.40**	**0.45**	**0.28**	**0.39**	**0.41**	**0.28**	**0.23**	**0.32**	**0.20**	**0.27**	**0.30**	0.32	**0.15**	**0.15**		-0.10	0.43	0.29	0.29	0.70	0.58	0.55	0.60
LB4	**0.46**	0.44	0.31	**0.40**	0.43	0.31	0.23	**0.31**	0.19	**0.25**	0.29	0.26	**0.15**	**0.12**	**0.13**		0.19	-0.01	-0.01	0.61	0.43	0.47	0.48
LB3	**0.39**	0.40	0.17	**0.33**	0.37	0.24	**0.18**	0.26	0.14	0.17	0.22	0.21	0.12	0.11	0.08	0.09		-0.24	-0.24	0.14	0.25	0.60	0.29
CL1	**0.47**	0.56	0.27	**0.42**	**0.46**	0.34	0.25	0.36	0.25	**0.26**	0.35	0.35	0.22	**0.25**	0.17	0.29	0.11		-0.33	0.24	0.20	0.53	0.26
LB5	**0.43**	0.39	0.23	**0.36**	0.41	0.26	0.18	0.27	0.17	0.22	0.28	0.28	0.12	0.11	0.08	0.09	0.01	0.18		0.24	0.20	0.53	0.26
FF	**0.49**	**0.51**	**0.32**	**0.46**	**0.50**	0.30	**0.27**	**0.35**	**0.26**	**0.33**	**0.34**	**0.35**	0.27	**0.29**	**0.22**	**0.28**	**0.17**	0.17	**0.25**		0.73	**0.90**	**0.75**
CL2	0.44	0.54	0.26	0.43	0.46	0.26	0.21	**0.30**	0.21	0.25	0.30	0.26	0.16	0.16	**0.12**	0.18	0.11	0.18	0.17	**0.21**		1.00	0.00
CL3	**0.42**	0.51	0.20	**0.39**	**0.44**	0.25	**0.20**	**0.26**	0.19	0.26	0.26	0.24	**0.23**	**0.22**	**0.16**	0.23	0.12	0.24	0.15	**0.21**	0.09		1.00
MG	**0.50**	0.67	0.38	0.50	0.52	0.42	**0.29**	0.38	0.31	**0.33**	0.36	0.39	0.32	**0.28**	0.22	0.30	0.24	0.41	0.26	**0.37**	0.20	0.24	

Supplementary Table S3. Analysis of molecular variance for concatenated mtDNA and microsatellite data considering: each karyotypic block separately (A x B x C x D); the link between the Pachecos and Touros swamps (A+B x C+D); the six clusters retrieved by the Structure program; and the six clusters retrieved by the Geneland program. Asterisks indicates significance at $P \leq 0.05$.

	A x B x C x D		**A+B x C+D**		**6 clusters Structure**		**6 clusters Geneland**	
CC mtDNA	%	Fixation indices	%	Fixation indices	%	Fixation indices	%	Fixation indices
Among groups	43.94	$F_{CT} = 0.44$*	40.31	$F_{CT} = 0.40$*	54.90	$F_{CT} = 0.55$*	53.58	$F_{CT} = 0.54$*
Among localities within groups	32.23	$F_{SC} = 0.57$*	38.38	$F_{SC} = 0.64$*	22.00	$F_{SC} = 0.49$*	22.83	$F_{SC} = 0.49$*
Within localities	23.83	$F_{ST} = 0.76$*	21.32	$F_{ST} = 0.79$*	23.11	$F_{ST} = 0.77$*	23.58	$F_{ST} = 0.76$*
Microsatellites								
Among groups	11.34	$F_{CT} = 0.11$*	11.48	$F_{CT} = 0.11$*	16.98	$F_{CT} = 0.17$*	15.09	$F_{CT} = 0.15$*
Among localities within groups	18.12	$F_{SC} = 0.20$*	20.13	$F_{SC} = 0.23$*	13.51	$F_{SC} = 0.16$*	14.74	$F_{SC} = 0.17$*
Within localities	70.54	$F_{ST} = 0.29$*	68.39	$F_{ST} = 0.32$*	69.51	$F_{ST} = 0.30$*	70.18	$F_{ST} = 0.30$*

CAPÍTULO III

Manuscrito em preparação

The role of karyotypes and geographical barriers in the divergence of lineages in a South American subterranean rodent (Rodentia: Ctenomyidae: *Ctenomys minutus*)

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Running title: Phylogeography of *Ctenomys minutus*

ABSTRACT

Ctenomys minutus is a highly karyotype-polymorphic species of subterranean rodent that inhabits the southern Brazilian coastal plain. This region has a complex geological history, with several potential geographical barriers acting on different time scales. The results recovered with mitochondrial DNA (mtDNA) and microsatellite loci data, over the entire distributional range of *C. minutus*, suggest that the main structuring factor of the genetic diversity of populations of this species was closely associated with geographical barriers, including rivers and the transition between different habitats. The isolation by distance pattern also plays an important role in fine-scale genetic differentiation among populations, which is strengthened by the narrowness of the coastal plain and by common features of subterranean rodents (small fragmented populations and low dispersal rates), which limit gene flow among populations. Chromosomal rearrangements seem to play only a minor role in the genetic structuring of *C. minutus* populations.

Keywords: allopatry - chromosomal polymorphism - coastal plain - isolation by distance - phylogeography

INTRODUCTION

The role of new chromosomal rearrangements in the speciation process has been widely discussed over the last 60 years. Some models of chromosomal speciation propose that the fitness of heterozygous carriers is diminished, leading to reductions in gene flow between chromosomally divergent populations and thus facilitating reproductive isolation (reviewed by King, 1993 and Rieseberg, 2001). However, recent models suggest that chromosomal changes reduce gene flow, acting as genetic barriers, by the reduction of recombination in heterokaryotypes and by the effects of linking isolated genes, and not because of underdominance (Rieseberg, 2001). Moreover, it is questionable whether all kinds of rearrangements could be involved in the speciation process (King, 1987).

While some authors have stressed the importance of chromosomal rearrangements in species formation (King, 1973; White, 1978), others have evaluated the contribution of factors such as geographical barriers and environmental adaptations that can lead to reproductive isolation and initiate the speciation process. Steinberg and Patton (2000), analyzing speciation in subterranean rodents, suggested that the geographical influence on species formation within this group is trivial. Their review suggested that sympatric speciation is uncommon in subterranean rodents, since most species do not share the same habitat; and that the parapatric model is also unlikely, since the narrow contact zones commonly found between subterranean rodents are mainly secondary in their origins, a situation incompatible with the requirements of the model. Thus, Steinberg and Patton (2000) suggested that the context of speciation in subterranean rodents is allopatric, and must follow processes of vicariance or peripheral isolation.

The burrowing rodents of the genus *Ctenomys*, popularly known as tuco-tucos, comprise a useful model for research on evolution. Some studies have employed tuco-tucos to test hypotheses about the evolutionary role of chromosomal rearrangements and discontinuities in the environment, leading to chromosomally or allopatrically mediated divergence of lineages (Tomasco and Lessa, 2007; Mora *et al.,* 2006, 2007; Fernández-Stolz, 2007).

The genus *Ctenomys* comprises approximately 56 living species, and is thought to result from an explosive speciation, showing impressive intra- and interspecific karyotypic variation (Reig *et al*., 1990). Some of their characteristics, such as low rates of adult

dispersal and their distribution in relatively small and fragmented populations, promote the establishment of small genetic units where genetic variation is low and interpopulation divergence is high. These characteristics favor the fixation of new chromosomal rearrangements, which have been considered to play a key role in the diversification and speciation of the genus (Reig *et al.,* 1990; Lessa and Cook, 1998; Wlasiuk *et al*., 2003), and also make the species more vulnerable to stochastic demographic factors and changes in their habitats (Patton *et al*., 1996).

Ctenomys minutus Nehring, 1887 is endemic to the southern Brazilian coastal plain, with a narrow distribution from Jaguaruna Beach in Santa Catarina state (SC) to São José do Norte Municipality in Rio Grande do Sul state (RS; Figure 1; Freygang *et al.*, 2004). It occupies the second and third barrier-lagoon systems (sand fields) from São José do Norte to Tramandaí; and from near Barco Beach to Jaguaruna, the species preferentially occupies the fourth barrier-lagoon system (first dune line; Figure 1).

This species has notable chromosomal variation, comprising seven parapatrically distributed parental karyotypes (2n = 42, 46a, 46b, 48a, 48b, 50a, and 50b) and four intra-specific hybrid zones, among them: i) 46a x 48a = 47a; ii) 46b x 48b = 47b; iii) 42 x 48a = 43, 44, 45, 46 (25 different karyotypic combinations were found among these five chromosomal numbers); and iv) 50b x 48b = 49b (Freitas, 1997; Gava and Freitas, 2002, 2003; Castilho, 2004; Freygang *et al*., 2004). The distribution of these karyotypes shows 2n = 50 at both ends of the geographical range, whereas toward the middle of the distribution, it is progressively reduced to 2n = 42 (see Figure 1) through Robertsonian rearrangements, tandem fusions/fissions, and a pericentric inversion which originated the distinct karyotypic systems “a” and “b” (Freitas, 1997; Freygang *et al.,* 2004).

Also, along the species’ range, two permanent discontinuities cross its distribution in the west-east direction: the Araranguá River in SC, and the Mampituba River along the border between SC and RS (Figure 1). Recent studies have also revealed the existence of two former inlets that connected the Patos Lagoon to the Atlantic Ocean and caused discontinuities in the former range of *C. minutus* (Figure 1). However, during the last trangressive-regressive sea-level event, these inlets were progressively filled with sediments until they completely disappeared, and these parts of the coastal plain became continuous (Weschenfelder *et al*., 2008a, b).

Coastal plains are ephemeral habitats, highly subject to ocean influences (Dillenburg and Hesp, 2009). Their endemic faunas and floras are adapted to the environmental changes, which could act as evolutionary forces, shaping the genetic structure of these species.

The coastline of the Brazilian states of RS and southern SC is part of one of the widest and most extensive coastal plains in the world, bounded on the north by the Cabo de Santa Marta in SC, and on the south by Cabo Polônio in Uruguay (Dillenburg and Hesp, 2009). During the Quaternary, glacio-eustatic fluctuations in the Atlantic Ocean sea level produced great lateral displacements of the shoreline, during at least four transgressive-regressive cycles, originating four barrier-lagoon systems that shaped the present southern Brazilian coastline (Villwock *et al*., 1986; Tomazelli *et al*., 2000). The first three barrier-lagoon systems originated during the Pleistocene (hereafter called Barriers I, II, and III), and the fourth system was formed in the Holocene (Barrier IV; Tomazelli *et al.,* 2000). Barrier I is the westernmost and smallest, behind the northern Patos Lagoon region; Barriers II and III are contiguous and extend over a wide area of the central coastal plain; and Barrier IV is the easternmost, corresponding to the first dune line, and extends along the entire coastline (Figure S1; Tomazelli *et al*., 2000).

At present, the landscape of the region is a mosaic of many lakes and lagoons, rivers, sand fields, and dunes. The roles of this complex geological formation and of the presence of some potential geographical barriers in shaping the distribution and genetic structure of the species of the coastal fauna remain poorly understood.

In this contribution, we address phylogeographical questions regarding the coastal-plain dynamics, using *C. minutus* as a model of genetic variation. Considering the narrow and patchy distribution of *C. minutus*, together with its limited dispersal rate, it is expected that genetic differentiation among localities will increase with increased geographical distance. Therefore, the question arises, as to whether the populations of *C. minutus* follow a pattern of isolation-by-distance.

A second question is, which scenario is more likely to cause the divergence of *C. minutus* populations: allopatric or chromosomal? Along the distribution of *C. minutus* we find several present and former environmental discontinuities, represented mainly by rivers and paleochannels. Since tuco-tucos are very poor swimmers (Reig *et al*., 1990), perennial streams could act as geographical barriers, reducing or preventing gene flow between

populations on opposite sides, leading to the differentiation of clusters of *C. minutus* populations in allopatry. On the other hand, *C. minutus* is a highly karyotypically polymorphic species. If the chromosomal rearrangements found in *C. minutus* are underdominant, or suppress recombinations, they could reduce gene flow and act as genetic barriers between chromosomally divergent populations, leading to the differentiation of clusters of *C. minutus* populations by means of chromosomal rearrangements.

To date, studies of the phylogeographical patterns and population genetics of *Ctenomys* species have been based mainly on information regarding geographical features and/or chromosomal rearrangements when the species is polymorphic, together with mtDNA information (Giménez *et al*., 2002; Wlasiuk *et al*., 2003; Mora *et al*., 2006, 2007; Fernández-Stolz, 2007; Tomasco and Lessa, 2007; Fernandes *et al.*, 2009), or with microsatellite data (Lacey, 2001; Wlasiuk *et al*., 2003; Gava and Freitas, 2004; El Jundi and Freitas, 2004; Cutrera *et al.*, 2005; Fernández-Stolz, 2007; Gonçalves and Freitas, 2009; Mirol *et al.*., 2010). Often, these studies have sampled only a few populations within the species' range. This study provides one of the best-documented reports about a ctenomyid, with sites sampled on a fine scale over the species' entire geographical range, analyzing sequences from the mitochondrial DNA (mtDNA) control region and cytochrome c oxidase subunit I, 14 microsatellite nuclear loci, and chromosomal rearrangements for each specimen analyzed, correlating these results with current knowledge of the local geomorphology. Together, this information provided a broad overview of the genetic picture of *C. minutus*.

MATERIAL AND METHODS

Sample collection and cytogenetics

The samples analyzed included 3 to 25 specimens of *C. minutus* from each of 30 localities, totaling 276 individuals analyzed for mtDNA, and 340 individuals analyzed for microsatellite loci, across the species' entire distributional range (see Table 1 and Figure 1). Most of the specimens were collected and karyotyped previously in studies by Freitas

(1997), Gava and Freitas (2002, 2003), Castilho (2004), and Freygang *et al*. (2004). The tissue samples were preserved in 95% ethanol and stored at -20 °C in the collection of the Laboratório de Citogenética e Evolução of the Departamento de Genética of the Universidade Federal do Rio Grande do Sul. Additional specimens were collected for this study: 15 specimens from Ilhas (SC), 5 specimens from Jaguaruna (SC), 15 specimens from Farol de Santa Marta (SC), 10 specimens from 17 Km south of Mostardas (RS), and 11 specimens from 26 Km south of Mostardas (RS; Table 1). For these specimens, the mitotic preparations were obtained from bone marrow, according to Ford and Hamerton (1956). The diploid and autosomal numbers were determined by analyses of at least 20 metaphase spread cells stained with Giemsa.

DNA amplification, sequencing and genotyping

Genomic DNA was extracted using a standard phenol:chloroform protocol (Sambrook and Russel, 2001). Two fragments of mtDNA were amplified by polymerase chain reaction (PCR): Part of HVS1 from the control region (CR) was amplified using the primers TucoPro (5'-TTCTAATTAAACTATTTCTTG-3', Tomasco and Lessa, 2007) and TDKD (5'-CCTGAAGTAGGAACCAGATG-3', Kocher *et al*., 1989), following a protocol modified from Tomasco and Lessa (2007). The cytochrome c oxidase subunit I (COI) followed the protocols suggested in http://www.barcoding.si.edu/DNABarCoding.htm, using the primers LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer *et al*., 1994).

The PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California, USA), following the guidelines of the suppliers, and sequenced in an ABI 3730 (Applied Biosystems, Foster City, California, USA) automated sequencer, using the forward primers TucoPro and LCO-1490. The ambiguous sequences were reamplified and resequenced as many times as needed to obtain clean and reliable electropherograms.

In addition to mtDNA sequences, 14 microsatellite markes, isolated from the Argentinean species *C. haigi* and *C. sociabilis* (Lacey *et al.*., 1999; Lacey, 2001), were selected for use in this study (13 dinucleotides Hai2, Hai3, Hai4, Hai5, Hai 6, Hai9, Hai10,

Hai12, Soc2, Soc3, Soc4, Soc5, Soc6; and 1 trinucleotide Hai 12). PCR amplifications were carried out following the protocols described by Lacey *et al*. (1999) and Lacey (2001), using one fluorescent-labeled primer pair at a time. The genotypes were obtained in the sequencer ABI 3730, mixing two primers with distinct labels, per reaction. To define the allele sizes, we used the program PeakScanner 1.0 (http://www.appliedbiosystems.com). In order to obtain reliable peaks, the PCR and genotyping reactions were repeated as many times as needed, and also the genotyping results were analyzed three times.

Data analysis

Mitochondrial DNA. Sequence electropherograms were visually inspected using Chromas 2.33 (http://www.technelysium.com.au/chromas.html), and aligned using the CLUSTAL W algorithm with default options, implemented in Mega 4.0.2 (Tamura *et al*., 2007). Alignments were checked and edited by hand if necessary. All analyses were performed with the three data sets: CR and COI separately, and a concatenated data set of CR+ COI (CC); however, mainly the CC results will be presented here. Measures of mtDNA diversity, such as polymorphic sites, average number of nucleotide differences (k), average number of nucleotide differences per site between all pairs of sequences (π), definitions of haplotypes (H), and haplotype diversity (*Hd*), were calculated with DnaSP 5.00.03 (Librado and Rozas, 2009).

The program Arlequin 3.5.1.2 (Excoffier and Schneider, 2005) was used to assess the demographic history of populations, employing mismatch distribution analysis, and Tajima's *D* and Fu's F_S neutrality tests. In addition, to investigate patterns of hierarchical levels of population structure, analyses of molecular variance (AMOVA) were performed under two different scenarios of hypothesized population subdivisions. First, the samples were subdivided into eight groups according to parental karyotypes. Specimens collected in intra-specific karyotypic hybrid zones were excluded from this analysis: i) only specimens with 2n = 50a, from FSM (the complete names of the localities and the corresponding abbreviations are listed in Table 1); ii) Specimens with 2n = 48c, from ILH; iii) 2n = 46a, all specimens from MC to OSO; iv) 2n = 48a, from PAS to 96; v) 2n = 42, from 115 to MOS; vi) 2n = 46b, from TV1; vii) 2n = 48b, from BJ1; and viii) Specimens

with 2n = 50b, from SJN. The second test separated all the samples into 6 groups, considering present and possible former geographical barriers: i) Specimens from FSM to ILH, isolated to the south by the Araranguá River; ii) From MC to PST, isolated to the south by the Mampituba River; iii) Specimens collected in GUA and BAR, delimited to the south by the transition between sand fields and dunes; iv) All localities from TRA to MOS, isolated in the past to the south by the paleochannel of the Jacuí River; v) From 17S to TV2, isolated in the past to the south by the paleochannel of the Camaquã River; and vi) From BJ1 to SJN, isolated in the southernmost part of the *C. minutus* distribution by the paleochannel of the Camaquã River.

To test for positive correlations between genetic and geographical distances, a Mantel test implemented in Arlequin 3.5.1.2 was used to examine patterns of isolation by distance, over the entire geographical range. Since the geographical distribution of *C. minutus* is interrupted by several potential geographical barriers, additional Mantel tests were performed, following the same subdivision assumed in the second AMOVA test. Statistical significance was assessed using 1 000 random permutations. Also, a spatial autocorrelation analysis (SAC) was performed in the program Alleles In Space 1.0 (Miller, 2005), with 1 000 random permutations.

To determine the appropriate model of nucleotide sequence evolution, we used the Akaike Information Criterion (AIC) as implemented in MrModeltest 2.2 (Nylander, 2004). The HKY+I+G model provided the best fit to the CR data set, and for COI the best model obtained was HKY+G. A phylogenetic analysis of the concatenated data using Bayesian inference was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The CR and COI sequences were also concatenated into a single data set, with the data partitioned by fragment, for which the best evolutionary model was used. Two independent replicates of the Markov chain Monte Carlo search were performed with 1 000 000 simulations, each with four chains, sampling trees every 100 generations. After the convergence test, the first 500 trees (burn-in) were discarded. Homologous sequences of *Ctenomys torquatus* Lichtenstein, 1830 (GenBank accession numbers CR: HM443438; and COI: HM443439) and *Ctenomys flamarioni* Travi, 1981 (GenBank accession numbers CR: JQ341041; and COI: JQ341052) were used as the outgroup. Also, the topological relationship between the haplotypes was estimated using the program Network 4.5.1.0 (http://www.fluxus-engineering.com) with the median-joining approach, for the three data sets.

Microsatellite data. We used Arlequin 3.5.1.2 and Genepop 4.0 (Rousset, 2008) to test deviations from the Hardy-Weinberg equilibrium (HWE), to calculate the observed and expected heterozygosis (*Ho* and *He*), and to perform an analysis of linkage equilibrium (LE). The significance levels ($\alpha = 0.05$) of HWE and LE were adjusted using sequential Bonferroni corrections, including the multiple simultaneous comparisons. Genotyping errors were tested in Micro-Checker (van Oosterhout *et al*., 2004), under a 95% confidence interval. In order to assess the measurements of microsatellite diversities, we used Arlequin 3.5.1.2.

Wright's *F*-statistics were used to analyze the within (F_{IS}) and between (F_{ST}) population structures. Localities with fewer than 5 individuals were excluded from F_{ST} comparisions, and sequential Bonferroni corrections were applied, with $\alpha = 0.05$, to adjust the statistical significance levels. Tests of AMOVA were designed for the same two groups coused in the mtDNA data. All calculations were performed with Arlequin 3.5.1.2.

To test positive correlations between genetic and geographical distances, Mantel tests were implemented in Arlequin 3.5.1.2, and a SAC analysis was implemented in Alleles In Space 1.0, both in the same manner as in the mtDNA analyses.

To determine the clustering of populations and their possible associations with geographical barriers and/or chromosomal rearragements, two different approaches were used. An analysis was performed through the Bayesian Markov Chain Monte Carlo (MCMC) approach, implemented in Structure 2.3.3 (Pritchard *et al*., 2000), following ten independent runs of K = 1-30, with1 500 000 iterations, and a burn-in period of 500 000 steps, assuming an admixture model, independent allele frequencies, and no a priori population information. The most likely number of clusters was based on the highest mean of the estimated logarithm of probability of the data [ln Pr (X/K)] and the lowest standard deviations (SD), among ten runs, before reaching instability or plateaus in the graph of mean ln Pr (X/K) among runs. This is a powerful tool for identifying genetic structure; however, it assumes that HWE and LE were violated in some localities sampled for *C. minutus*. Therefore, a principal components analysis (PCA), based on a multivariate ordination method, which does not account for HWE and LE, was implemented in PCA-GEN 1.2.1 (http://www2.unil.ch/popgen/softwares/pcagen.htm), using 10 000 randomizations.

RESULTS

Cytogenetics

All specimens recovered in FSM had the karyotype 2n = 50a, previously described by Freitas (1997); the specimens from ILH had a previously unknown karyotype, 2n = 48c (Table S1, Figure S2E). At locality JG, between ILH and FSM, the specimens had 2n = 48c, 50a and hybrid forms between them, 2n = 49a. These results demonstrate that the parapatric karyotypes 2n = 50a and 48c have a contact region around the JG sampling site, leading to the formation of a previously undescribed hybrid zone.

Of the 21 specimens collected at 17S and 26S, 12 individuals were successfully karyotyped, revealing 3 specimens with a previously known karyotype (2n = 42, NA = 74), described by Freitas (1997), and 9 specimens with four new karyotypes, intermediate between the forms 2n = 42 and 46b, described in Table S1 and Figure S2A-D. We did not find any specimen carrying the expected karyotype for the F1 of a cross between 2n = 42 and 46b. However, we found several other intermediate karyotypic forms, providing evidence of another, previously undescribed hybrid zone in the contact region between these parental karyotypes.

Considering all cytogenetic data available for *C. minutus*, there is evidence that this species has nine diploid numbers (2n = 42, 43, 44, 45, 46, 47, 48, 49, and 50), eight parental karyotypes (2n = 42, 46a, 46b, 48a, 48b, 48c, 50a, and 50b), and six hybrid zones (50a x 48c, 46a x 48a, 48a x 42, 42 x 46b, 46b x 48b, and 48b x 50b), comprising a total of 45 karyotypes known so far.

Genetic diversity

From 398 bp of the CR, 43 polymorphic sites were obtained, resulting in a total of 40 haplotypes (GenBank accession numbers HM236969 to HM237008). The COI sequences were 620 bp in length, resulting in 44 variable sites and 35 haplotypes (GenBank accession numbers HM237009 to HM237043), and the concatenated data comprised 1018 bp, generating a total of 52 haplotypes. The levels of diversity in the three

data sets were mostly moderate to high (Table 2), compared with indices of mtDNA diversity for other ctenomyids.

The total number of alleles per locus among the 14 microsatellite loci analyzed ranged from 7 to 16, and from 1 to 12 per locus per locality. The localities FSM, JG, ILH, BAR, EBL, 35, and 96 showed one monomorphic locus; TV2, BJ1, and BJ2 showed two monomorphic loci; and SJN had six monomorphic loci. The monomorphic loci differed among the localities. The mean numbers of alleles per locality are presented in Table 1.

Six sampling sites showed departures from HWE: OSO (Hai12 and Soc3); EBL (Hai4); SBL (Soc5); 96 (Soc3); MOS (Hai6); and BJ2 (Hai4 and Soc6). With the exception of BJ2 (Soc6), all deviations were detected by Micro-Checker as evidence of null alleles. Pairwise comparisons of LE within localities revealed significant results among five pairs of loci: Hai3xSoc5 (OSO); Hai10xSoc2 (JG, OSO, 108); Hai12xSoc1 (GAI); Soc3xSoc6 (MOS); and Soc4xSoc5 (GAI). However, these loci were not excluded from the subsequent analysis because there are no significant deviations of LE, for the same loci, in pairwise comparisions between other localities, and also among the same loci analysed here for other ctenomyidae species.

Population expansion and isolation by distance

The overall results of Tajima's *D* showed positive values for CR and CC data and a negative *D* for COI, although none of the values were significant (Table 2). For the global Fu's F_S test, all values were negative but not significant (Table 2). With respect to the neutrality test results for the 7 haplogroups of mtDNA, explained below, only Barros Lake and Tavares showed negative and significant Fu's F_S values, for the CR data set. All other Tajima's *D* and Fu's F_S values, for the three data sets, were not significant (Table 2). The rejection of a recent history of population expansion for *C. minutus* was also reinforced by the high average number of nucleotide differences (ranging from k = 6.20 to 15.59; Table 2), recovered for all mtDNA data sets, and by multimodal plots of mismatch distributions (Figure S3).

The Mantel's test detected a positive and significant ($P < 0.05$) correlation between genetic and geographical distances for the overall geographical range in both molecular markers (CC mtDNA: $r = 38.44\%$; microsatellite data: $r = 53.48\%$; Table 3). This

correlation was also verified through the SAC (Alleles In Space) analysis. The mean genetic distances estimated were 0.015 and 0.77, for the CC mtDNA and microsatellite data, respectively, and a significant increase of genetic distance from 105 kilometers of distance among sampling sites was observed (Figure S4). However, for the tests considering geographical barriers, only the sampling sites located between the paleochannel of the Jacuí River and the transition between sand fields and dunes (from MOS to TRA) showed significant positive correlations between genetic and geographical distances (r = 54.054%) for the CC mtDNA data. For the microsatellite data, the sampling sites located between the Jacuí River paleochannel and the transition between sand fields and dunes (from MOS to TRA), and the sampling sites between the paleochannels of the Jacuí and Camaquã rivers (from 17S to TV2) showed significant positive correlations between genetic and geographical distances (r = 30.13%, and r = 80.29%, respectively; Table 3).

Phylogeographic pattern of haplotype distributions

The Bayesian phylogenetic tree generated from concatenated data (Figure 2) showed a basal polytomy between the *C. minutus* branches and the outgroup. Polytomies are commonly found among and within species of *Ctenomys,* and the persistence of these polytomies has been suggested to be the result of an early simultaneous cladogenesis event.

Seven main clades were highlighted in the phylogenetic tree, all of them showing a strong relationship between specimen clustering withthe geographical structure. The northern localities were subdivided into two separate clades: **North 1** was formed by all specimens from MC and 14 specimens from JG (6 specimens with 2n = 50a, 3 with 2n = 49a, and 5 with 2n = 48c); and **North 2** included the specimens from FSM and ILH, and 3 specimens from JG (2 with 2n = 49a and 1 with 2n = 50a). Despite this subdivision, specimens from the sampling site JG, which shares the same karyotypes (2n = 48c, 49a and 50a), without subdivisions of its microsatellite genetic variation, or any apparent morphological difference, were found in both the North1 and North 2 clades, suggesting that this subdivision is probably an effect of a retention of ancestral polymorphisms in one of the two clades. The southern sampling sites were represented by the **South** clade, comprising specimens from BJ1, BJ2, and SJN (2n = 48b, 49b, and 50b). It is followed by

the **Tavares** clade, represented by specimens from 17S, 26S, TV1, and TV2 (2n = 42, 43, 44, 45, 46b, and 47b). The middle of the *C. minutus* distribution was subdivided into three other clades, among them: **Coast**, formed by specimens inhabiting only the shoreline (GAI, PST, GUA, and BAR; 2n = 46a); **Barros Lake**, represented by the specimens from TRA, OSO, EBL, SBL, PAS, FOR, PIT, and PAL, and 1 specimen from 35 (2n = 46a, 47a, and 48a); and **Mostardas**, referring to the specimens that live in the Mostardas region, comprising the sampling sites 35, 53, 64, 96, 108, 115, and MOS (2n = 42, 43, 44, 45, 46, 48a).

The relationship among the CC mtDNA haplotypes, retrieved by the median-joining network, revealed seven haplogroups similar to the main clades highlighted in the phylogenetic tree, strongly correlated with the geographical distributions of the specimens (Figure 3). In general, most haplotypes are separated by several mutational steps, and are limited to single localities, i.e., private alleles. The shared haplotypes are mainly among neighboring sampling sites, following an approximately clinal model, with a predominance of haplotypes with restricted distributions and not closely related (Table 1 and Figure 3). With respect to karyotypes, only the parapatric neighboring ones and their hybrid forms shared haplotypes in the three mtDNA data sets, mainly among specimens collected within or around the hybridization regions (Table S2). These results may indicate sharing of ancestral polymorphisms by neighboring karyotypes, and/or may suggest that different chromosomal rearrangements do not prevent gene flow between neighboring karyotypic forms, i.e., do not act as reproductive barriers.

Population structure

Local estimates of F_{IS} recovered five significant values (Table 1). In addition, the F_{ST} analysis revealed highly significant differences among most pairwise locality comparisions, evidencing high levels of genetic differentiation and therefore low levels of gene flow among most sampling sites (Table S3).

The Structure analysis recovered a multimodal distribution of the mean values of Ln Pr (X/K) through different Ks, although, considering the highest mean of Ln Pr (X/K) with the lowest SD, before reaching a plateau and the instability along Ks, the most likely number of clustering was K = 12 (Figure S7). All runs at K = 12 showed a very similar

pattern of clustering, which is presented in Figure 4 by the plot of membership assignment for the run with the highest ln Pr (X/K). Most localities had specimens with mixed ancestry ($q < 0.8$), usually among neighboring clusters, with the exception of clusters I (FSM), III (MC), and XII (BJ1, BJ2, and SJN), which contained no individuals with mixed ancestry. The pattern of clustering of localities did not follow the karyotypic variation: in some cases a karyotype was present in more than one cluster (e.g., 2n = 46a and 48a), or different karyotypes and hybrid zones were clustered together (e.g., clusters XI and XII). Instead, the clusters were mainly related to the geographical proximity of sampling sites, grouping together neighboring localities; and in some cases the subdivisions were mediated by geographical barriers, as in the case of the Araranguá River between clusters II and III, and the paleochannels of the Camaquã and Jacuí Rivers between clusters X and XI, and clusters XI and XII, respectively. For the Mampituba River and the transition between sand fields and the dune line, there was no clear association with cluster separation. Also, regarding the CC mtDNA haplogroups associated with the Structure clusters (Figure 4), we can suggest that among the specimens corresponding to the neighboring haplogroups South x Tavares, Tavares x Mostardas, and Coast x North (1 and 2) there is little microsatellite gene flow, since few specimens showed mixed ancestry between each other. The specimens corresponding to the haplogroups Coast x Barros Lake showed more evident levels of microsatellite gene flow, with some individuals of mixed ancestry. Finally, the haplogroups Barros Lake x Mostardas showed the largest number of specimens with microsatellite mixed ancestry among each other, or even sharing the same Structure cluster, evidencing a greater gene flow.

The PCA analysis showed that 25.87% of the genetic variation was explained by the first two principal components (15.20% and 10.67%, respectively). The distribution of clusters was not the same as that found with the Structure program, although some patterns were recurrent. As in Structure, the pattern of clustering of localities in PCA followed the geographical structure rather than the karyotypic variation. The FSM sampling site was isolated from the other northern localities, in which JG and ILH were clearly grouped (cluster A); however, MC appeared to be transitional between clusters A and B (from GAI to BAR). The sampling sites from cluster B were distributed in the middle of the plot, together with PAL and 35. Cluster C was recovered at the bottom of the y axis, formed by localities from TRA to PIT, in the middle of the *C. minutus* distribution. Cluster D grouped

from 53 to TV2, and was isolated in the upper right of the plot. Cluster E was most differentiated from the others, formed by localities BJ1, BJ2, and SJN (Figure S8).

With respect to the AMOVA results, the tests using eight groups of parental karyotypes showed most of the genetic variation distributed among populations within karyotypes, for the three mtDNA data sets. On the other hand, the second test, in which the populations were subdivided into six groups considering present and past geographical barriers, revealed that the percentages of genetic variation among groups were higher than in other levels (ranging from 50.73% to 51.12%, Table 4). Nevertheless, although all fixation indices for all tests showed highly significant *P* values, the subdivision of populations into six groups considering geographical barriers seems to be more reasonable to explain the partition of the mtDNA genetic variation for *C. minutus,* than does the karyotypic variation. For microsatellite data, the two AMOVA tests (karyotypic, geographic) showed more than 67% genetic variation within populations (Table 4).

DISCUSSION

Chromosomal rearrangements

Different karyotypic features, including diploid numbers and chromosomal rearrangements, are often variable among species, but are usually stable within species (White, 1978; Lukhtanova *et al*., 2011). Unlike gene mutations, each chromosomal rearrangement is unique, has a single point of origin in space and time in a specimen, and sometimes has deleterious effects (Chiarelli and Capanna, 1973). However, some taxa can maintain their adaptability and fertility in chromosomally heterozygous carriers by means of mechanisms that partially or totally suppress recombinations with harmful effects during meiosis (White, 1978; Rieseberg, 2001). King (1987) separated classes of chromosomal rearrangements which could be involved in speciation. According to King, tandem fusions, reciprocal translocations, centric fusion/fission, multiple centric fusions/fissions, and pericentric and paracentric inversions have the capacity to be powerfully negatively heterotic, and may produce deleterious or lethal meiotic products. However, if any of these rearrangements produce a balanced meiotic system, in which normal segregation of the meiotic products occurs, they will no longer be able to form a postmating isolating

mechanism and will generally result in chromosomal polymorphisms. In cases of inversion, the rearrangements may not have any effect on meiosis if the chiasma is located outside the inversion loop, if crossing-over is inhibited in heterokaryotypes, or even by non-homologous pairing in inversion heterozygotes.

Subterranean rodents show some of the highest rates of chromosomal evolution known in mammals (Reig *et al*., 1990), with many highly karyotypic polymorphic species (for examples see Thaeler, 1985; Savic and Nevo, 1990). All cytogenetic data available for *C. minutus* showed that this species has a total of 45 known karyotypes (Freitas, 1997; Gava and Freitas, 2002; 2003; Castilho, 2004; Freygang *et al*., 2004; this study), suggesting that it is one of the most karyotype-polymorphic species among all subterranean rodents, and perhaps among all mammals.

In 6 of 7 transition regions between neighboring karyotypes, we found specimens carrying chromosomal rearrangements of both karyotypes, forming well-established intra-specific hybrid zones, often represented by forms beyond the F1 generation. The hybrid zones are present even between karyotypes differing by one Robertsonian rearrangement (2n = 50a x 48c; 46a x 48a; 46b x 48b; and 48b x 50b), or more (2n = 48a x 42), and between karyotypes differing by Robertsonian rearrangements and a pericentric inversion (2n = 42 x 46b). The pericentric inversion in *C. minutus* is responsible for the distinction between the karyotypic systems "a" (2n = 46a, 47a, 48a, 49a, 50a, plus 2n = 42 and 48c) and "b" (2n = 46b, 47b, 48b, 49b, and 50b; Freygang *et al*., 2004; Freitas, 2006). This might act as a postmating isolation mechanism between specimens from divergent systems, through the formation of aneuploid gametes.

The cytogenetic results presented here showed that chromosomal rearrangements in *C. minutus* (Robertsonian rearrangements, tandem fusions/fissions, and a pericentric inversion) are possibly neutral, occurring as transient or balanced polymorphisms, and do not prevent reproduction among specimens of neighboring parapatric karyotypes, i.e., do not act as reproductive barriers. In addition, studies by Gava and Freitas (2002, 2003) using cytogenetic data for the *C. minutus* hybrid zones between 2n = 46a x 48a, and 48a x 42, showed no evidence of underdominance, and demonstrated that chromosomal polymorphism fails to cause sterility in heterozygous carriers.

The genetic patterns of the mtDNA and microsatellite markers were not primarily structured following variations in karyotypes. The mtDNA results of the AMOVA tests,

considering eight karyotypic groups, showed that the genetic variation was mainly distributed among populations within karyotypes. For the microsatellite data, the lowest apportionment of genetic variation was found among karyotypic groups (PK test in Table 4). The seven haplogroups retrieved with mtDNA were mainly structured by geographical barriers, explained below, followed by the clinal pattern of sharing of haplotypes among neighboring sampling sites, which is another important structuring factor for mtDNA. Ultimately, the chromosomal rearrangements could have only a smal effect on *C. minutus* mtDNA genetic variation, since only parapatric karyotypes and their hybrid forms share haplotypes (Figures 2, 3, S5 and S6). Considering microsatellite data, the patterns of clustering retrieved with Structure and PCA analyses were not genetically structured by chromosomal rearrangements, but by the geographical proximity of localities and eventually by geographical barriers (Figures 4 and S8). Moreover, the skull morphometric data for *C. minutus* indicate that there was no direct relationship between karyotype and skull morphology, and these characters are independent, although both vary with the geographical distribution (Freitas, 2005; Fornel *et al.*, 2010).

Fixation of new chromosomal rearrangements seems to be frequent in species of the genus *Ctenomys*, and is commonly not followed by sterility, reductions in fitness, or negative heterosis of heterozygote carriers. There are some examples of chromosomal polymorphic ctenomyids that fail to show genetic differentiation of karyotypic populations. For instance, the Corrientes group includes species that are genetically extremely similar to each other despite the high degree of karyotypic differentiation (Giménez *et al*., 2002; Mirol *et al*., 2010). For *Ctenomys pearsoni,* all populations and karyomorphs studied were polyphyletic in their mtDNA (Tomasco and Lessa, 2007). *Ctenomys torquatus* also fails to show a common pattern of genetic and karyotypic variation (Fernandes *et al*., 2009).

Isolation by distance

Ctenomys minutus has a nearly linear geographical distribution along the southern Brazilian coastal plain. When species have a linear distribution, dispersal and gene flow will be geographically limited. This feature, together with their low ability to disperse over significant distances relative to the species' range, which is a common feature in subterranean rodents (Busch *et al*., 2000), including tuco-tucos, can cause a pattern of

isolation by distance. Species of tuco-tucos that inhabit narrow stretches of coastal areas, such as *C. pearsoni*, *C. flamarioni,* and *C. talarum,* have low levels of gene flow among populations, and a pattern of isolation by distance associated with their nearly linear geographical distributions, based on phylogeographical studies with control region and/or cytochrome b mtDNA data (Mora *et al*., 2007; Tomasco and Lessa, 2007; Fernández-Stolz, 2007).

In the case of *C. minutus,* the Mantel tests and the spatial autocorrelation analysis indicated positive and significant correlations between the overall distribution of genetic and geographical distances, for the CC mtDNA and microsatellite data sets. Also, genetic distances increased significantly from 105 kilometers of distance between sampling sites (Figure S4). However, although most Mantel analyses of subgroups with respect to geographical barriers did not show significant values, the percentages of correlation between genetic and geographical distances were relatively high, several of them above 50% (Table 3).

Several other lines of evidence indicate that isolation by distance plays an important role in the genetic structure of this species. The distribution of karyotypes is parapatric (Figure 1), and no specimen was found outside the limits of its source karyotypic population. Similarly, it was possible to observe a geographical limitation on the distribution of haplotypes, since most haplotypes were limited to single sampling sites, and shared ones were mainly among neighboring localities (Table 1 and Figure 2). The most widely separated localities that shared haplotypes, for CR, COI, and CC data, respectively, were FOR and 35 (41.5 km apart, haplotype CR20), SBL and MOS (140 km apart, haplotype COI8), and 96 and MOS (32 km apart, CC13). Low levels or absence of gene flow, mainly among distant localities, were observed in the high and significant values of most pairwise microsatellite F_{ST} comparisons, and also, a process of inbreeding was indicated in some localities by the significance of the F_{IS} estimates (Tables S2 and 1).

Analyses of the variation in the skull shape of *C. minutus* also found a weak but significant association between morphometric and geographical distances, and the authors suggested that differences among populations follow a subtle pattern of isolation by distance (Fornel *et al*., 2010). According to Hoelzer *et al.* (2008), the isolation-by-distance mechanism can lead to breaks in the distribution of genetic variation across a species' range, giving rise to discrete and spatially segregated lineages, which are still

reproductively compatible and can precede a speciation event. This mechanism may account for the reinforcement of cluster subdivisions along the *C. minutus* distribution, in areas where specimens share haplotypes and/or karyotypes without any geographical barrier, but there is evidence of genetic structuring (between the Mostardas and Barros Lake clusters).

Geographical barriers and genetic structure

Considering that members of the genus *Ctenomys* seem to be very poor swimmers, streams may in some cases constitute strong geographical barriers to dispersal (Reig *et al*., 1990). As suggested for *C. talarum* (Mora *et al*., 2007) and *C. flamarioni* (Fernández-Stolz, 2007), major rivers within their ranges appear to be associated with genetic structuring of populations.

The range of *C. minutus* includes many water bodies, but only the Araranguá and Mampituba rivers form discontinuities that currently cross its distribution in the west-east direction. In addition, two ancient inlets, the paleochannels of the Camaquã and Jacuí rivers, caused discontinuities in its former range.

The results of the mtDNA and microsatellite data regarding the role of the Araranguá River as a geographical barrier are conflicting. For the mtDNA data, the three networks and the phylogenetic tree retrieved two haplogroups, called North 1 and North 2, whichgrouped all specimens found on the north bank of the Araranguá River, and also specimens from MC, located on the south side. These results suggest that, at least in their mtDNA content, the specimens from MC are genetically closer to individuals from northern localities than to individuals from the same side of the Araranguá, such as GAI. However, the Structure analysis showed MC isolated in an exclusive cluster (III), and in PCA it appeared to be transitional between cluster A (specimens from the north bank of the Araranguá River) and cluster B (specimens from the south bank). Commonly, mtDNA has lower effective population sizes (*N*e) than microsatellite markers, which have higher levels of variation and are potentially homoplasic. Therefore, mtDNA can reflect historical events, while microsatellites are more accurate to explain recent events. The past transient status of the Araranguá River mouth (Tomazelli and Villwock, 2000; S. Dillenburg, personal communication) may have allowed contact between populations from opposite

banks. Taking this possibility into consideration, our results suggest that in the past, the Araranguá River mouth may have left MC isolated for a longer period on its north side, and only recently has this population come to be located on the south side.

For the Mampituba River, the median-joining networks and the phylogenetic tree demonstrated that localities sampled on the north (PST and Gai) and south banks (Gua and Bar) were grouped together in the Coastal haplogroup. However, Structure analyses resulted in two clusters for this region around the Mampituba River: cluster IV, primarily specimens from GAI (north bank); and cluster V, formed by specimens from GUA and BAR (south bank). Specimens from PST (north of Mampituba) showed mixed ancestry. For PCA, only one cluster (B) was retrieved among the localities sampled on both sides of this river. The reason for the absence of mtDNA genetic structuring caused by the Mampituba River may lie in its low discharge volume, resulting in a past transient status of the river mouth (Tomazelli and Villwock, 2000; S. Dillenburg, personal communication), which may sometimes have allowed contact between populations from opposite banks. After 1970, the banks of Mampituba River were stabilized by human activity, and the period of time since this was done is apparently not sufficient to allow complete genetic differentiation of lineages between opposite banks.

The two former inlets that connected the Patos Lagoon to the Atlantic Ocean probably acted as effective barriers to free dispersal of *C. minutus*, leaving signs of genetic differentiation between populations that were on opposite sides up to now. The paleochannel corresponding to the mouth of the Jacuí River on the Atlantic Ocean, described by Weschenfelder *et al*. (2008a), crossed the coastal plain near the city of Mostardas. This same region showed a genetic subdivision among sampling sites which were located on opposite sides of this former stream, for mtDNA haplogroups (Mostardas x Tavares) and Structure analysis (clusters X x XI). The paleochannel known as Barra Falsa, described by Weschenfelder *et al*. (2008a, b), which corresponded to the mouth of the Camaquã River, crossed the region immediately north of BJ1. All analyses showed a recurrent clustering of specimens from BJ1, BJ2, and SJN, named the South haplogroup (mtDNA), cluster E (PCA), and cluster XII (Structure).

Both of these channels have caused discontinuities in the past range of *C. minutus*. However, during the last sea-level trangressive-regressive event, approximately 5 thousand years ago, the channels were progressively filled with sediments until they completely

disappeared, converting these portions of the coastal plain into continuous regions (Weschenfelder *et al*., 2008a, b). Although the two former inlets do not now exist, the period of time during which these channels acted as barriers to free access between their sides was long enough to leave signs of genetic structuring. Probably the genetic structures still exist today due to the metapopulation structure, common to ctenomyids, allied with low levels of gene flow.

Fornel *et al*. (2010), using geometric analyses of cranium morphometry, found a phenotypic structuring of *C. minutus* into three morphological groups, which suggested the existence of populations at the extreme north of the distribution, isolated from the other localities by the Araranguá River; populations at the extreme south, isolated by the Jacuí paleochannel; and all other populations associated with lakes and lagoons, in the middle of the distribution.

The transition between the sand fields and dune line, another environmental discontinuity along the range of *C. minutus*, seems to have acted, at some time, as an impediment to free dispersal among specimens from different habitats. The coastal specimens, which inhabit the stretch from GAI to BAR, live only in the first dune line, corresponding to the fourth barrier-lagoon system. South of TRA, specimens live only in the second dune line (second and third barrier-lagoon systems). For mtDNA, we found two separate genetic groups in these habitats, the Coastal and Barros Lake haplogroups. For microsatellite data the genetic structuring was retrieved but was not so evident, with specimens with mixed ancestry between both environments. Environmental characteristics such as soil hardness leads to morphological adaptations in tuco-tucos, principally in the anterior and posterior limbs and in the cranium, since they are burrowing rodents and use their teeth, paws, and legs to dig tunnels. Freitas (2005) and Fornel *et al.* (2010) found significant differences in skull morphology among specimens of *C. minutus* from this transition region between sand fields and dunes. Therefore, the discontinuity between the first and second dune lines may also have led to the genetic discontinuities apparent from our data.

CONCLUSIONS

Our results demonstrated that the main structuring factor of the genetic diversity in *C. minutus* was geographical features and the geological evolution of the southern Brazilian coastal plain, which were associated with most of the mtDNA haplogroups identified, and some clusters retrieved by microsatellite loci variation. Among geographical structuring factors, we can highlight the major rivers (Araranguá), the paleochannels (Camaquã and Jacuí), and the discontinuity between habitats (sand fields x dune line). It is also important to separate the effect of geographical distances associated with low rates of dispersal on the increasing of genetic distances. This plays a very important role in fine-scale genetic differentiation among populations of our species.

On the other hand, chromosomal rearrangements did not show a primary role in the genetic structure of *C. minutus* populations. Some highly chromosomal polymorphic species, such as *Mus musculus domesticus* and *Sorex araneus,* also show a step-by-step mechanism of karyotype evolution, as in *C. minutus*. However, although the presence of karyotypic populations is well defined, molecular markers do not match this subdivision, and the karyotypic populations are still considered as part of a single species (Wójcik *et al.*, 2002, 2003; Piálek *et al.*, 2005). The step-by-step chromosomal subdivision was suggested by Lukhtanova *et al*. (2011) as reflecting the intial phase of the chromosomal speciation process. For *C. minutus* there is no chromosomal polymorphism within populations (outside hybrid zones), and differences among neighboring parental karyotypes, although minor, are fixed. As suggested by Lukhtanova *et al*. (2011) "we cannot exclude that geographically distant and chromosomally divergent populations would display reduced fertility if crossed, although they are connected by a chain of compatible populations that should allow gene flow".

Two different scenarios of chromosomal evolution in *C. minutus* are suggested. The first is a step-by-step mechanism of chromosomal evolution, in which chromosomal rearrangements arose randomly on the periphery of populations, became fixed by genetic drift and colonization of new habitats, and remained fixed in the new populations. Another hypothesis is that initially the entire coastal plain was colonized by only one karyotypic form. New chromosomal rearrangements arose independently along the species' range, were fixed in allopatry, and mediated by geographical barriers (mainly rivers). Subsequently, these rivers disappeared from the coastal plain, allowing contact between neighboring karyotypes and giving rise to secondary-contact hybrid zones.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURES

Figure 1. Localities sampled along the entire distributional range of *Ctenomys minutus*, and the distribution of the parental karyotypes. Localities with overlapping parapatric karyotypes correspond to the intra-specific hybrid zones. Locality abbreviations correspond to those in Table 1.

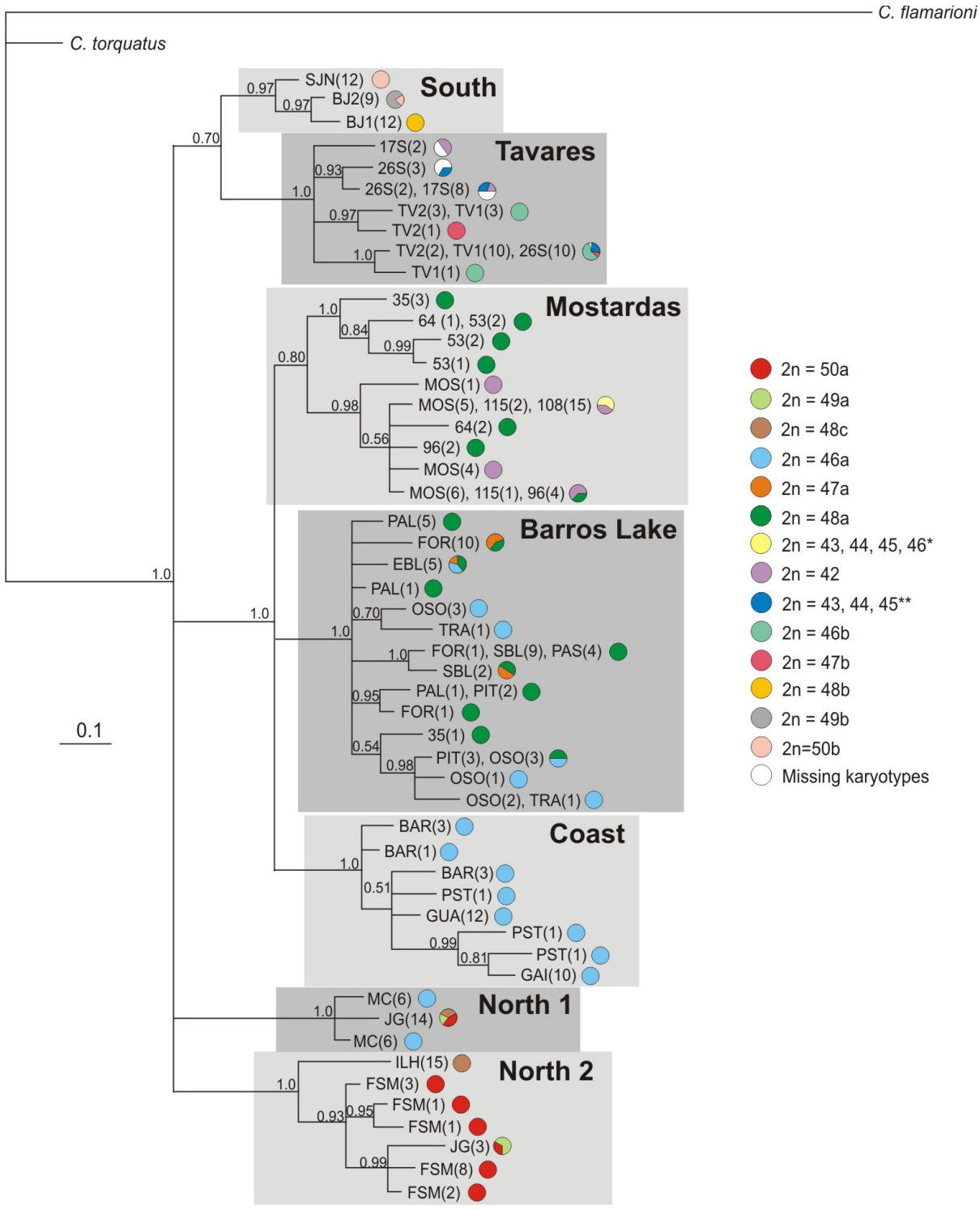

Figure 2. Bayesian phylogenetic tree of the concatenated mtDNA data for *Ctenomys minutus*. The seven main genetic clades are highlighted by gray squares. The abbreviations of the localities, the number of specimens (in parentheses), and the proportion of karyotypes are shown for each branch. Locality abbreviations correspond to those in Table

1. The node posterior probabilities are given on the branches. *Ctenomys flamarioni* and *Ctenomys torquatus* were included as outgroup. * diploid numbers of hybrids from crossing between 2n = 42 x 48a. ** diploid numbers of hybrids from crossing between 2n = 42 x 46b.

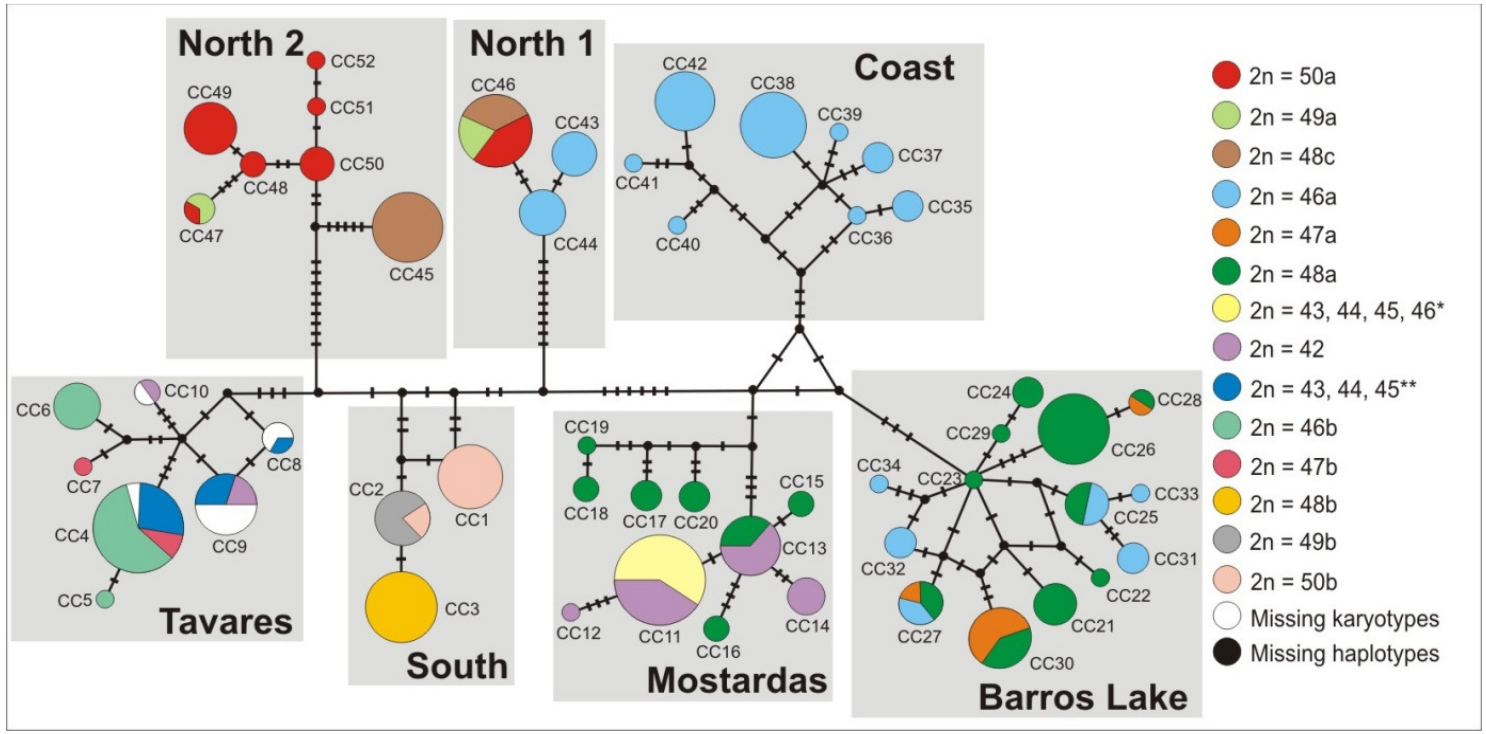

Figure 3. Median-joining haplotype network topology obtained for concatenated mtDNA data. The colors represent diploid numbers of parental karyotypes and intra-specific hybrids, as indicated in the legend, the specimens unsuccessfully karyotyped are presented in white. The seven main genetic haplogroups identified for *C. minutus* are indicated by gray squares. Circle areas are proportional to the haplotype frequencies. Correspondence of haplotypes numbers and diploid numbers of sampling sites are in Table 1. * diploid numbers of hybrids from crossing between 2n = 42 x 48a. ** diploid numbers of hybrids from crossing between 2n = 42 x 46b.

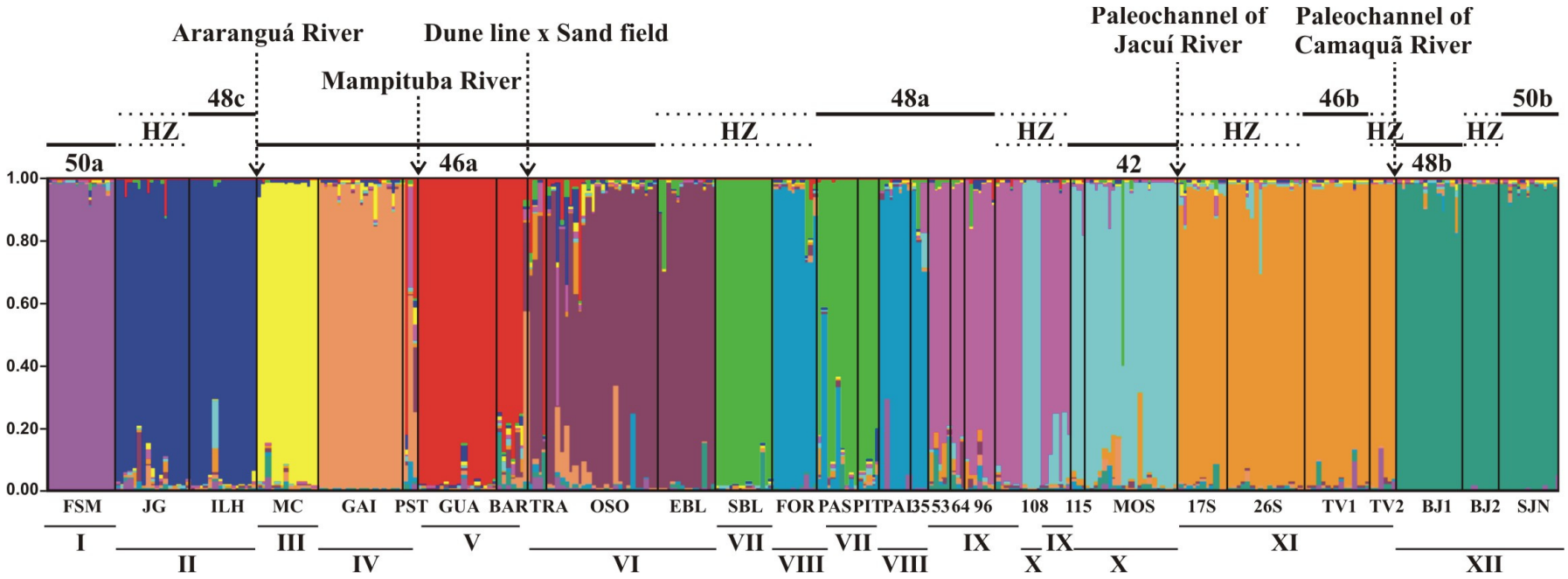

Figure 4. Bayesian clustering and individual assignment for the twelve clusters identified by Structure program. Each specimen is represented by a single bar, and each cluster by a color. Sampling sites are plotted geographically in the North-South direction, from left to right. Below the plot is identified the locality names, and the twelve clusters. Above the plot are indicated the parental parapatrical karyotypes and hybrid zones among them, and main possible geographical barriers along *C. minutus* distribution.

TABLES

Table 1. Localities sampled for *Ctenomys minutus* (abbreviations in parentheses), diploid and autosomal numbers found within the localities, in this and other studies [2n(AN)], the seven main genetic haplogroups of CC mtDNA data, number of samples sequenced (No. S), number of specimens per haplotype of CC mtDNA (CC), the 12 clusters retrieved with Structure analysis, number of samples genotyped (No.G), mean number of alleles per locality (*A*), and F_{IS} estimates. [1]statistical significance ($P < 0.05$).

Localities	2n(AN)	mtDNA haplogroup	No. S	CC	Structure clusters	No.G	*A*	F_{IS}
Farol de Santa Marta (FSM)	50a(76)	North 2	15	CC48=2CC49=8/CC50=3/ CC51=1/CC52=1	I	15	2.57	0.038
Jaguaruna (JG)	48c(76), 49a(76), 50a(76)	North 1, North 2	17	CC46=14/CC47=3	II	17	4.14	0.087
Ilhas (ILH)	48c(76)	North 2	15	CC45=15	II	15	2.93	0.200[1]
Morro dos Conventos (MC)	46a(76)	North 1	12	CC43=6/CC44=6	III	14	4.36	0.467
Gaivota Beach (GAI)	46a(76)	Coast	10	CC42=10	IV	19	5.21	0.218
Passo de Torres (PST)	46a(76)	Coast	3	CC39=1/CC40=1/CC41=1	IVand V	3	3.57	0.404
Guarita Beach (GUA)	46a(76)	Coast	12	CC38=12	V	18	3.71	0.443
Barco Beach (BAR)	46a(76)	Coast	7	CC35=3/CC36=1/CC37=3	V	7	3.71	0.0712
Tramandaí (TRA)	46a(76)	Barros Lake	2	CC31=1/CC34=1	V and VI	4	3.29	0.464
Osório (OSO)	46a(76)	Barros Lake	9	CC25=3/CC31=2/CC32=3/ CC33=1	VI	25	6.36	0.198[1]
East Barros Lake (EBL)	46a(76), 47a(76), 48a(76)	Barros Lake	5	CC27=5	VI	13	3.64	0.295[1]
South Barros Lake (SBL)	47a(76), 48a(76)	Barros Lake	11	CC26=9/CC28=2	VII	13	3.50	0.447
Passinhos (PAS)	48a(76)	Barros Lake	4	CC26=4	VII	9	3.71	0.224

Pitangueira (PIT)	48a(76)	Barros Lake	5	CC24=2/CC25=3	VII	5	2.79	-0.041
Fortaleza Lake (FOR)	47a(76), 48a(76)	Barros Lake	12	CC26=1/CC29=1/ CC30=10	VIII	10	4.00	0.130
Palmares do Sul (PAL)	48a(76)	Barros Lake	7	CC21=5/CC23=1/CC24=1	VIII	7	3.64	0.029
Road km 35 (35)	48a(76)	Barros Lake Mostardas	4	CC20=3/CC22=1	VIII	4	3.07	-0.137
Road km 53 (53)	48a(76)	Mostardas	5	CC17=2/CC18=2/CC19=1	IX	5	3.21	-0.131
Road km 64 (64)	48a(76)	Mostardas	3	CC16=2/CC17=1	IX	3	2.86	0.424
Road km 96 (96)	48a(76)	Mostardas	6	CC13=4/CC15=2	IX	7	4.71	0.287[1]
Road km 108 (108)	42(68,69,70,71,72,73,74), 43(70,72,73,74,75), 44(72,73,74,75,76), 45(74,75,76,78,80), 46(71,74,76,77,78)	Mostardas	15	CC11=15	IX and X	17	5.07	0.149
Road km 115 (115)	42(74)	Mostardas	3	CC11=2/CC13=1	X	3	3.21	0.244
Mostardas (MOS)	42(74)	Mostardas	16	CC11=5/CC12=1/CC13=6/ CC14=4	X	21	6.21	0.177[1]
17 Km south of Mostardas (17S)	42(74), 43(74)	Tavares	10	CC9=8/CC10=2	XI	11	5.43	0.087
26 Km south of Mostardas (26S)	42(74), 43(70,72,74), 44(74), 45(76), 46b(76)	Tavares	15	CC4=10/CC8=3/CC9=2	XI	18	5.64	0.023
Tavares 1 (TV1)	46b(76)	Tavares	14	CC4=10/CC5=1/CC6=3	XI	14	4.93	0.645
Tavares 2 (TV2)	46b(76), 47b(76)	Tavares	6	CC4=2/CC6=3/CC7=1	XI	6	3.07	-0.031
Bujuru 1 (BJ1)	48b(76,78)	South	12	CC3=12	XII	15	3.29	0.001
Bujuru 2 (BJ2)	49b(76,77), 50b(76)	South	9	CC2=9	XII	9	2.50	0.174
São José do Norte (SJN)	50b(76,77)	South	12	CC1=12	XII	13	2.07	0.134
30 localities	45 karyotypes	7 haplogroups	276	52 haplotypes	12 clusters	340	3.88	

Table 2. Number of specimens (N), nucleotide diversity (π), average nucleotide differences (k), haplotype diversity (*Hd*), Tajima's *D* neutrality test (*D*), and Fu's F_S neutrality test (F_S) estimates for the seven main genetic haplogroups identified for *Ctenomys minutus.* Control region (CR), cytochrome c oxidase subunit I (COI), concatenated data (CC).

	North 1	**North 2**	**Coast**	**Barros Lake**	**Mostardas**	**Tavares**	**South**	**Total**
N	26	33	32	55	52	45	33	276
π (CR)	0.0026	0.0069	0.0066	0.0049	0.0059	0.0035	0.0024	0.0236
π (COI)	0.0006	0.0047	0.0029	0.0040	0.0019	0.0029	0.0007	0.0100
π (CC)	0.0014	0.0055	0.0044	0.0043	0.0035	0.0031	0.0014	0.0153
k (CR)	1.03	2.75	2.64	1.96	2.35	1.41	0.95	9.39
k (COI)	0.37	2.88	1.81	2.47	1.19	1.80	0.48	6.20
k (CC)	1.40	5.63	4.46	4.43	3.55	3.20	1.43	15.59
***Hd* (CR)**	0.517	0.699	0.758	0.846	0.740	0.702	0.682	0.961
***Hd* (COI)**	0.369	0.722	0.758	0.859	0.483	0.643	0.477	0.947
***Hd* (CC)**	0.628	0.735	0.764	0.878	0.773	0.702	0.682	0.965
***D* (CR)**	2.07	0.44	0.20	0.32	-1.16	-0.64	1.87	1.01
***D* (COI)**	0.67	1.37	0.13	0.07	-1.30	1.42	1.42	-0.36
***D* (CC)**	1.93	1.73	0.19	0.21	-1.33	0.24	2.15	0.33
F_S (CR)	3.21	2.84	0.56	-9.58[1]	-5.22	-4.96[1]	1.58	-2.46
F_S (COI)	1.00	2.85	-0.65	-0.22	-1.32	2.66	1.60	-4.67
F_S (CC)	2.43	3.89	1.68	-4.52	-4.35	-0.94	2.80	-1.59

[1]P < 0.02 Fu's F_S significance, according to Arlequin manual.

Table 3. Mantel analyses for CC mtDNA data, and microsatellite loci considering overall geographical distribution and the isolated geographical subgroups.

	CC mtDNA	**Microsatellite**
FSM to ILH	r = 99.79% (P = 0.18)	r = 33.22% (P = 0.49)
MC to PST	r = 58.88% (P = 0.33)	r = 51.52% (P = 0.34)
GUA to BAR	*	*
TRA to MOS	r = 54.04% (P = 0.00)	r = 30.13% (P = 0.01)
17S to TV2	r = 22.21% (P = 0.41)	r = 80.29% (P = 0.04)
BJ1 to SJN	*	r = 86.24% (P = 0.33)
Overall range	r = 38.44% (P = 0.00)	r = 53.48% (P = 0.00)

* the program was unable to proceed with the calculations.

Table 4. Results of analyses of molecular variance for mtDNA data sets and microsatellites. The percentage of variation for each hierarchical level and the fixation indices (in parentheses) are shown. PK: 8 groups, considering parental karyotypes; GB: 6 groups, considering potential present and former geographical barriers. mtDNA = CR (control region); COI (cytochrome c oxidase subunit I); CC (concatenated data between CR + COI). Values in bold are significant (P = 0.00)

	CR		**COI**		**CC**		**Microsatellite**	
	PK	**GB**	**PK**	**GB**	**PK**	**GB**	**PK**	**GB**
Among groups (F_{CT})	30.30% **(0.30)**	51.12% **(0.51)**	41.81% **(0.42)**	50.73% **(0.51)**	35.00% **(0.35)**	50.99% **(0.51)**	10.49% **(0.10)**	9.28% **(0.09)**
Among populations within groups (F_{SC})	62.16% **(0.89)**	40.56% **(0.83)**	49.75% **(0.85)**	38.04% **(0.77)**	57.09% **(0.88)**	39.57% **(0.81)**	22.09% **(0.25)**	22.70% **(0.25)**
Within populations (F_{ST})	7.54% **(0.92)**	8.32% **(0.92)**	8.44% **(0.92)**	11.23% **(0.89)**	7.91% **(0.92)**	9.44% **(0.90)**	67.43% **(0.33)**	68.03% **(0.32)**

SUPPLEMENTARY DATA

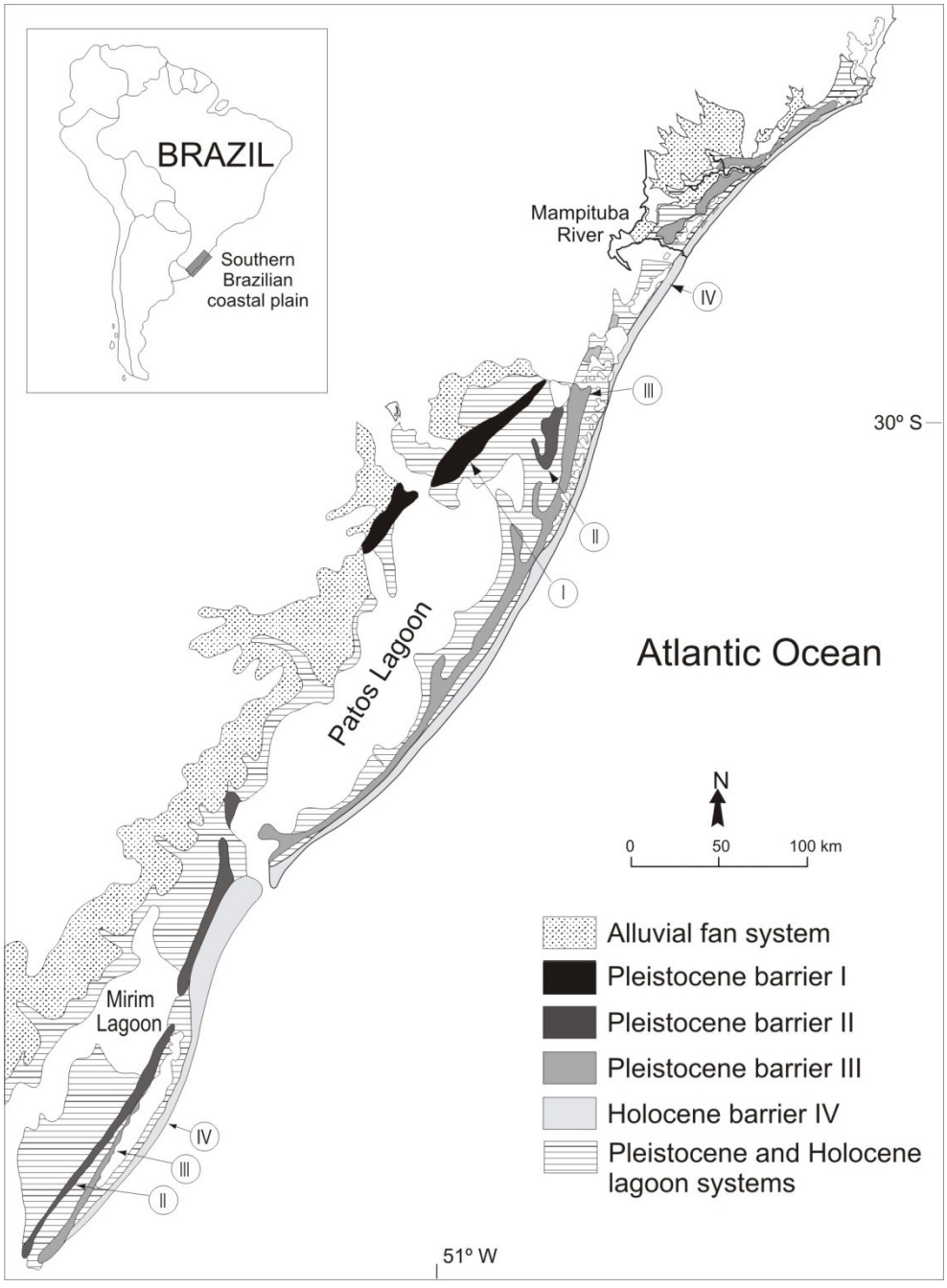

Figure S1. Geological map of the southern Brazilian coastal plain (Modified from Tomazelli *et al.*, 1996; Martin *et al.*, 1988).

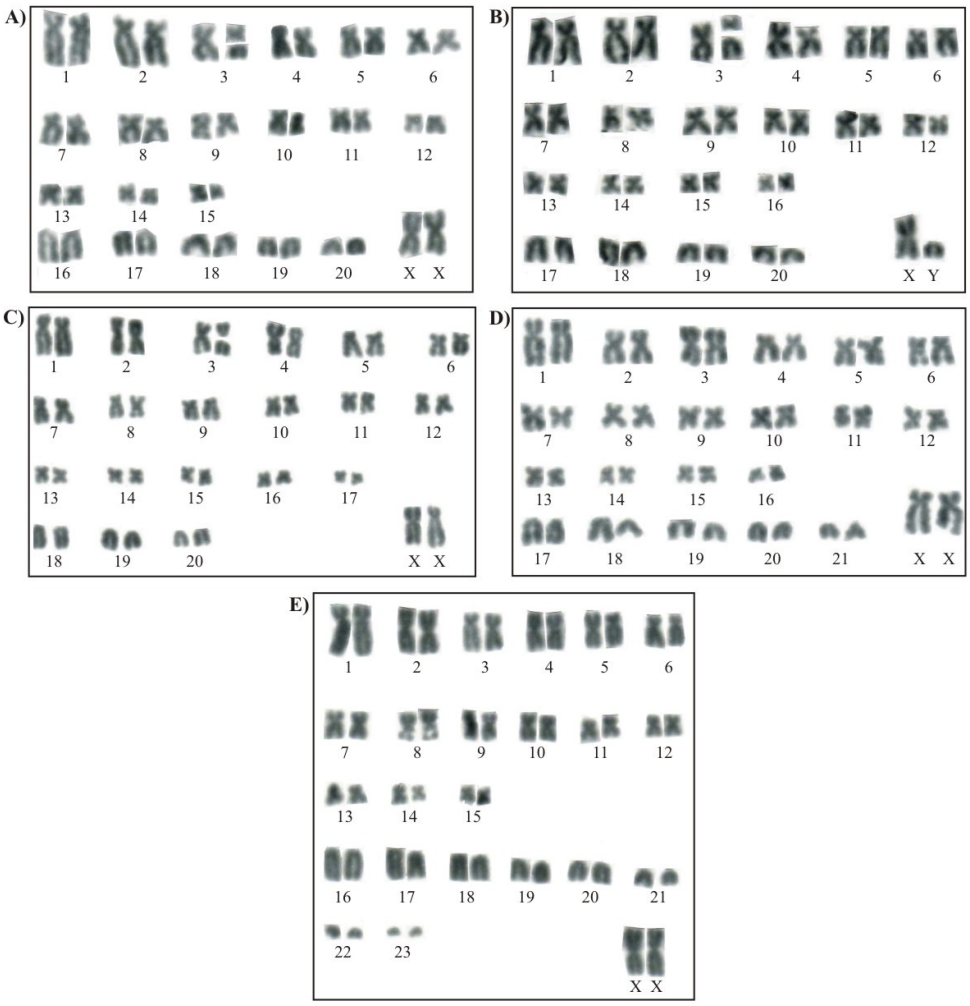

Figure S2**.** New karyotypes obtained in this study from *Ctenomys minutus* bone marrow. Karyotypes found in the hybrid zone between 2n = 42 and 2n = 46b: A) 2n = 43, AN = 70; B) 2n = 43, AN= 72; C) 2n = 43, AN= 74; D) 2n = 44, AN= 74. Karyotype found in specimens from Esteves Lake, Ilhas, and Jaguaruna: E) 2n = 48, AN = 76.

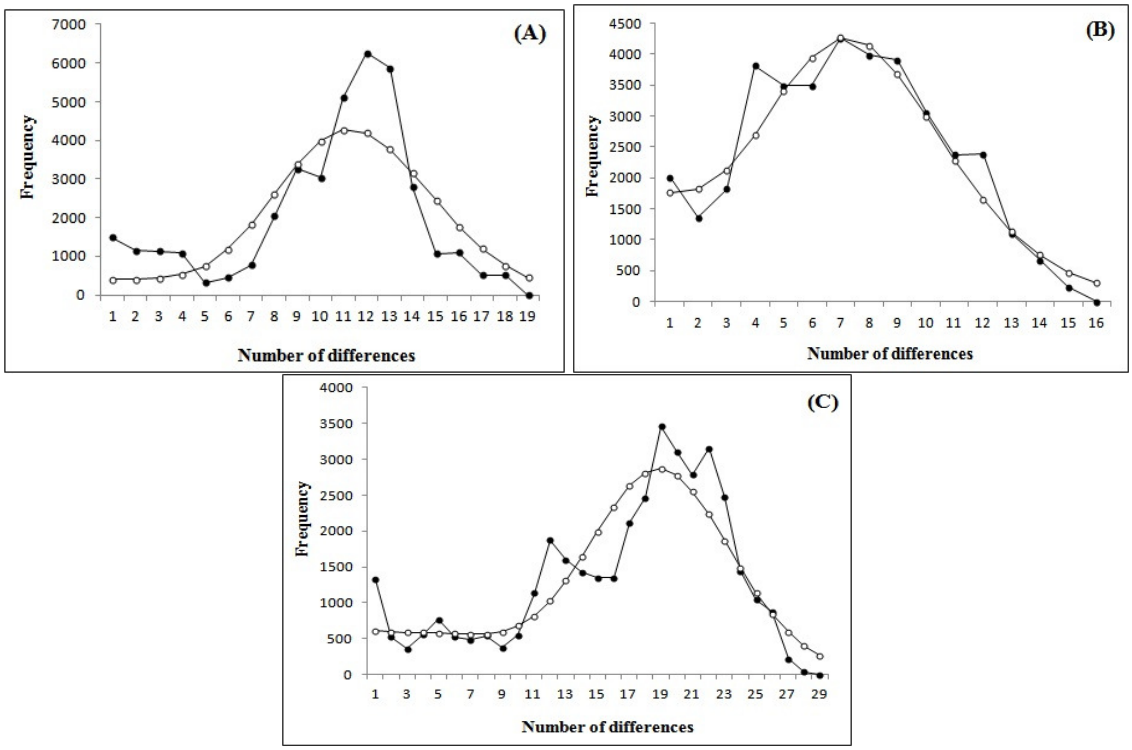

Figure S3. Observed and expected mismatch distributions for *C. minutus*, for (A) control region, (B) cytochrome c oxidase subunit I, and (C) concatenated data. Open circles: simulated distributions; solid circles: observed distributions.

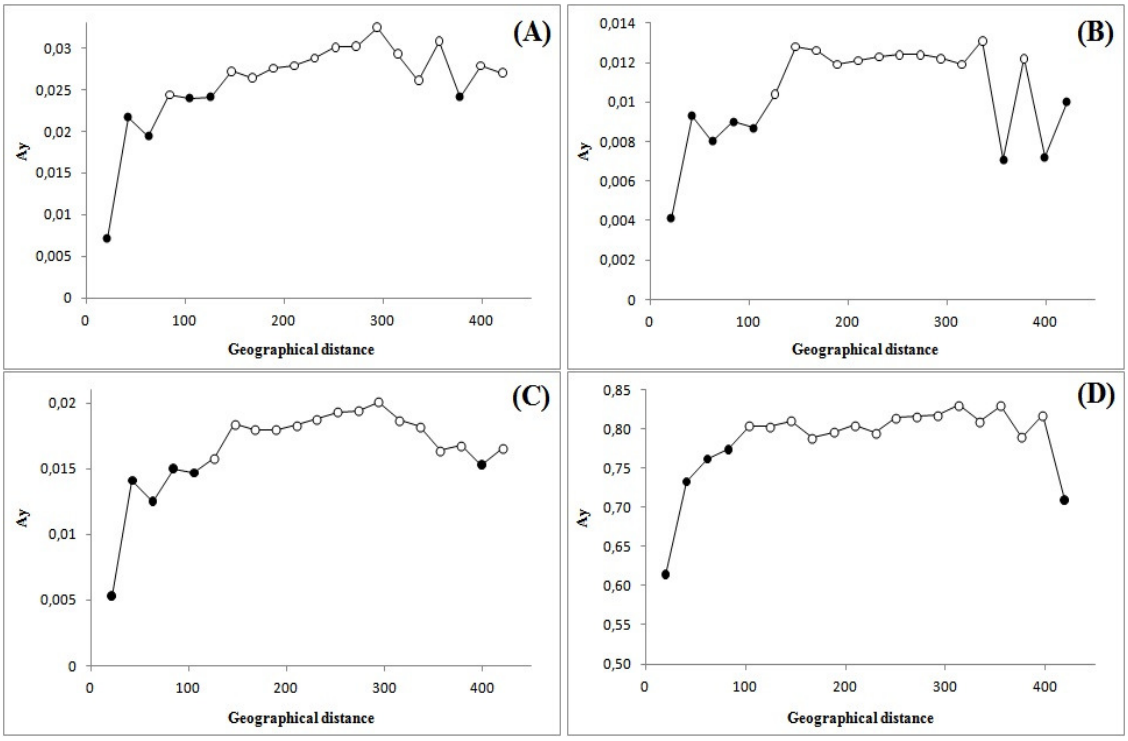

Figure S4. Plots of spatial autocorrelation analysis for (A) control region, (B) cytochrome c oxidase I, (C) concatenated data, and (D) microsatellite data. *Ay* is the mean pairwise genetic distance among specimens. White circles correspond to statistical significance ($P < 0.05$).

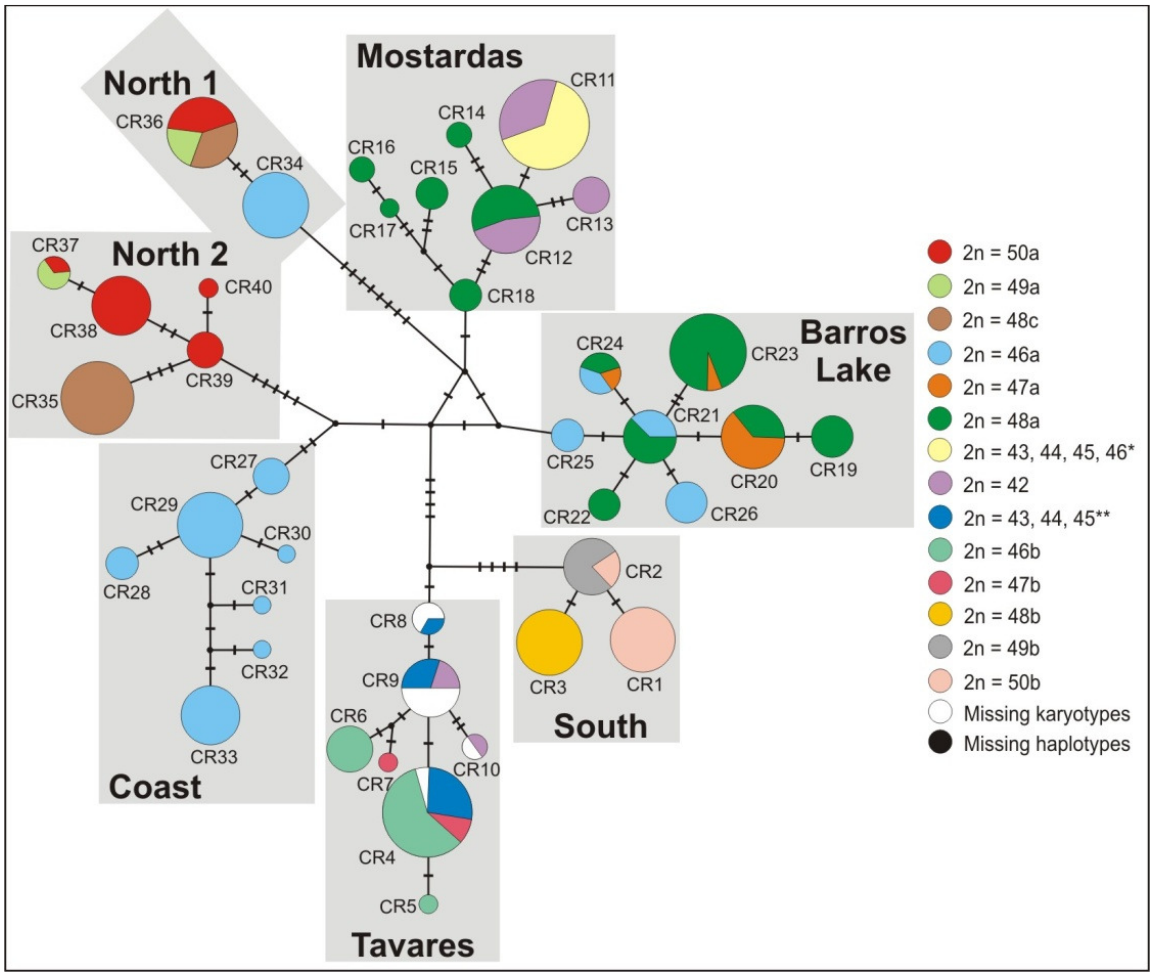

Figure S5. Median-joining haplotype network topology obtained for mitochondrial DNA control region data. The colors represent the diploid numbers of parental karyotypes and the intra-specific hybrids, as indicated in the legend. The seven main haplogroups of *C. minutus* are indicated by gray squares. Circle areas are proportional to the haplotype frequencies. * diploid numbers of hybrids from crossing between 2n = 42 x 48a. ** diploid numbers of hybrids from crossing between 2n = 42 x 46b.

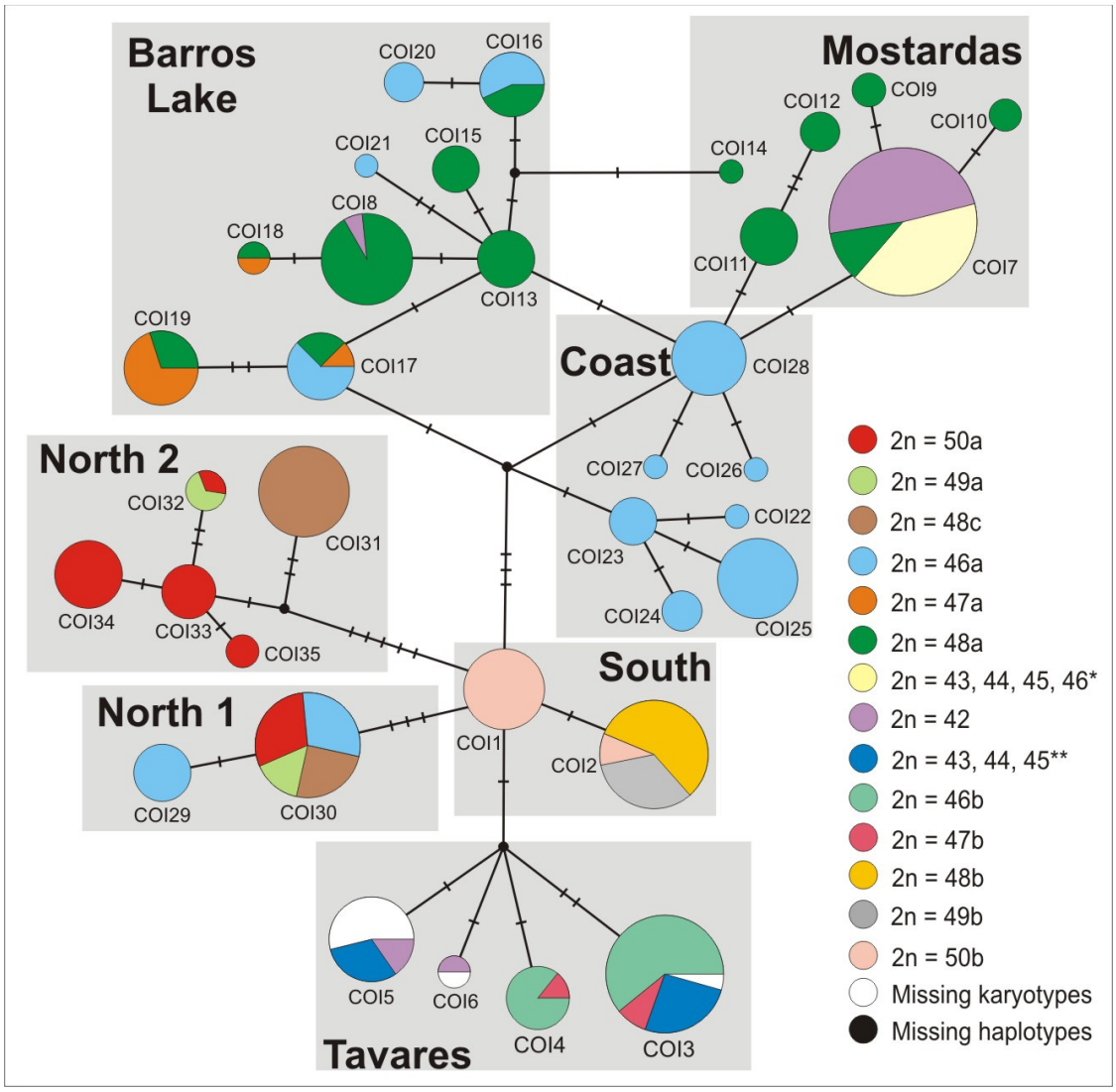

Figure S6**.** Median-joining haplotype network topology obtained with mtDNA cytochrome c oxidase subunit I. The colors represent the diploid numbers of parental and the intra-specific hybrids, as indicated in the legend. The seven main genetic haplogroups of *C. minutus* are indicated by gray squares. Circle areas are proportional to the haplotype frequencies. * diploid numbers of hybrids from crossing between 2n = 42 x 48a. ** diploid numbers of hybrids from crossing between 2n = 42 x 46b.

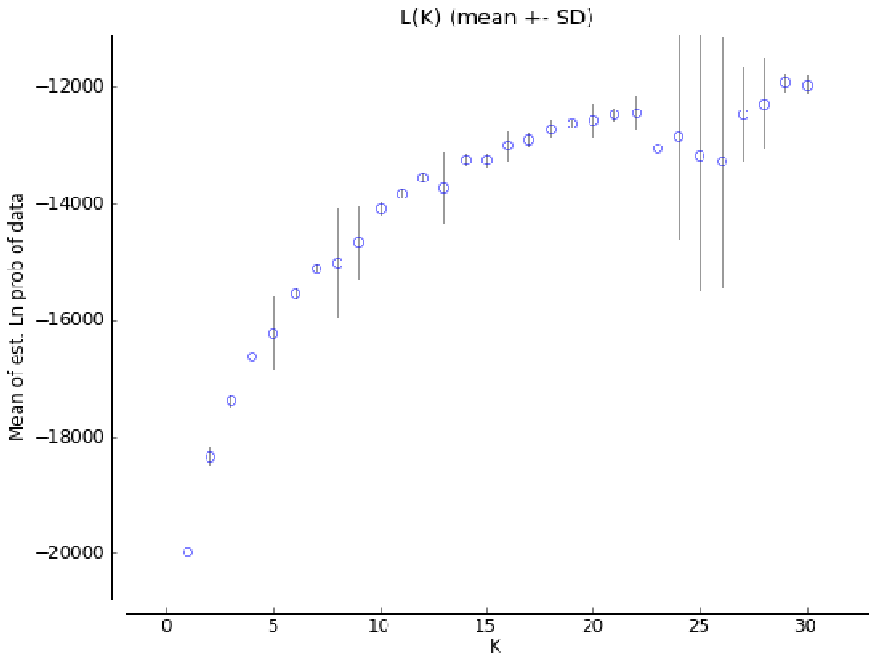

Figure S7. Plot of the mean logarithm probability of the data Ln (X/K) and Standart deviations (SD) of Structure analyses.

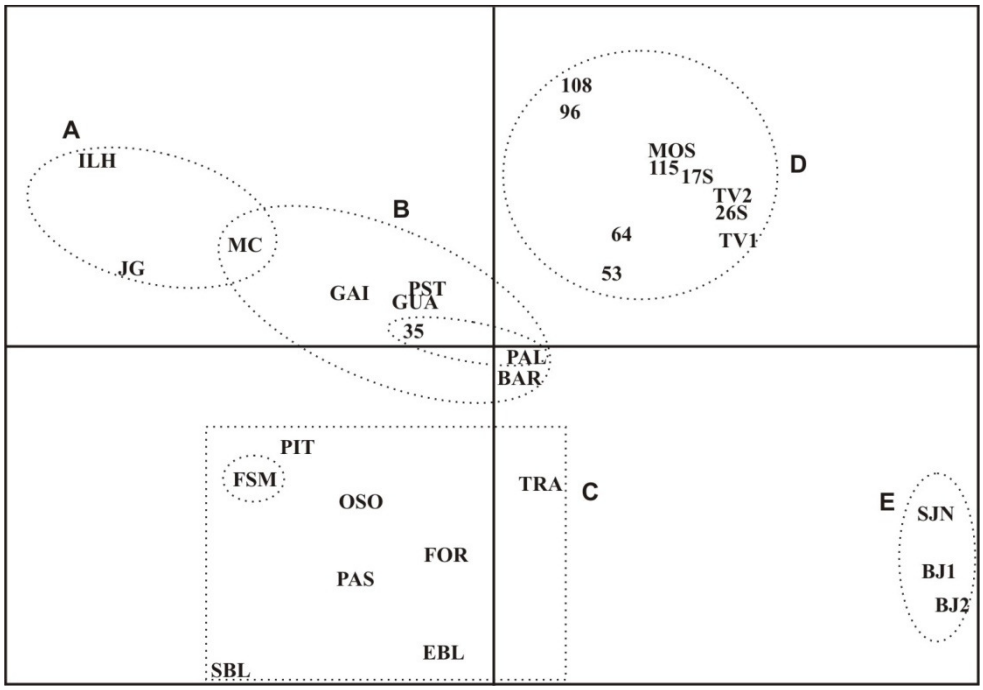

Figure S8. Sampling sites clustered according to the two first principal components. Sampling site names correspond to those in Table 1.

Table S1. New karyotypes for *Ctenomys minutus* obtained in this study. Collecting sites (Localities), number of specimens per karyotype (No. S), diploid numbers (2n), chromosomal arm numbers (AN), number of chromosomal biarmed pairs (Bi), number of chromosomal acrocentric pairs (Ac), and the heteromorphic chromosomal pair (HP).

Localities	**No. S**	**2n**	**AN**	**Bi**	**Ac**	**HP**
Ilhas and Jaguaruna	20	48c	76	15	8	-
17 and 26 Km south of Mostardas	2	43	70	14	6	pair 3
	2	43	72	15	5	pair 3
	2	43	74	16	4	pair 3
	3	44	74	16	5	-

Table S2. Mitochondrial DNA haplotypes found for each diploid number. Diploid numbers from hybrid zones are italicized.

	control region	**citochrome c oxidase I**	**concatenated data**
2n = 50b	CR1	COI1	CC1, CC2
2n = 49b	CR2	COI2	CC2
2n = 48b	CR3	COI2	CC3
2n = 47b	CR4, CR7	COI3, COI4	CC4, CC7
2n = 46b	CR4, CR5, CR6	COI3, COI4	CC4, CC5, CC6
2n = 45	CR4	COI3	CC4
2n = 44	CR4, CR9	COI3, COI5	CC4, CC9
2n = 43	CR4, CR8, CR9	COI3, COI5	CC4, CC8, CC9
2n = 42	CR9, CR10, CR11, CR12, CR13	COI5, COI6, COI7, COI8	CC9, CC10, CC11, CC12, CC13, CC14
2n = 43	CR11	COI7	CC11
2n = 44	CR11	COI7	CC11
2n = 45	CR11	COI7	CC11
2n = 46	CR11	COI7	CC11
2n = 48a	CR12, CR14, CR15, CR16, CR17, CR18, CR19, CR20, CR21, CR22, CR23, CR24	COI7, COI8, COI9, COI10, COI11, COI12, COI13, COI14, COI15, COI16, COI17, COI18, COI19	CC13, CC15, CC16, CC17, CC18, CC19, CC20, CC21, CC22, CC23, CC24, CC25, CC26, CC27, CC28, CC29, CC30
2n = 47a	CR20, CR23, CR24	COI17, COI18, COI19	CC27, CC28, CC30
2n = 46a	CR21, CR24, CR25, CR26, CR27, CR28, CR29, CR30, CR31, CR32, CR33, CR34	COI16, COI17, COI20, COI21, COI22, COI23, COI24, COI25, COI26, COI27, COI28, COI29, COI30	CC25, CC27, CC31, CC32, CC33, CC34, CC35, CC36, CC37, CC38, CC39, CC40, CC41, CC42, CC43, CC44
2n = 48c	CR35	COI31	CC45
2n = 49a	CR36, CR37	COI30, COI32	CC46, CC47
2n = 50a	CR36, CR37, CR38, CR39, CR40	COI30, COI32, COI33, COI34, COI35	CC46, CC47, CC48, CC49, CC50, CC51, CC52

Table S3. F_{ST} pairwise comparisions among localities for microsatellite data. Bold numbers were non-significant (P > 0.05).

	FSM	**JG**	**ILH**	**MC**	**GAI**	**GUA**	**BAR**	**OSO**	**EBL**	**SBL**	**FOR**	**PAS**	**PIT**	**PAL**	**53**	**96**	**108**	**Mos**	**17S**	**26S**	**TV1**	**TV2**	**BJ1**	**BJ2**	**SJN**
FSM	0.00																								
JG	0.38	0.00																							
ILH	0.43	0.24	0.00																						
MC	0.36	0.26	0.27	0.00																					
GAI	0.35	0.22	0.28	0.20	0.00																				
GUA	0.44	0.36	0.39	0.33	0.28	0.00																			
BAR	0.46	0.33	0.39	0.28	0.25	0.25	0.00																		
OSO	0.32	0.22	0.32	0.21	0.17	0.23	0.20	0.00																	
EBL	0.44	0.36	0.43	0.32	0.30	0.38	0.31	0.19	0.00																
SBL	0.42	0.33	0.42	0.31	0.27	0.35	0.34	0.20	0.31	0.00															
FOR	0.42	0.35	0.42	0.30	0.26	0.33	0.36	0.22	0.33	0.28	0.00														
PAS	0.43	0.33	0.42	0.31	0.24	0.34	0.31	0.19	0.32	0.15	0.25	0.00													
PIT	0.46	0.33	0.39	0.29	0.23	0.38	0.37	0.21	0.33	0.21	0.21	**0.17**	0.00												
PAL	0.45	0.35	0.46	0.32	0.28	0.31	0.32	0.22	0.37	0.37	0.29	0.21	0.30	0.00											
53	0.43	0.35	0.41	0.29	0.26	0.31	0.33	0.23	0.32	**0.34**	0.29	**0.29**	**0.27**	**0.31**	0.00										
96	0.44	0.34	0.38	0.28	0.23	0.29	**0.29**	0.24	0.37	0.37	**0.31**	0.32	0.31	0.30	**0.19**	0.00									
108	0.39	0.29	0.32	0.23	0.21	0.26	0.24	0.20	0.33	0.33	0.30	0.29	0.27	0.28	0.20	0.13	0.00								
MOS	0.38	0.28	0.35	0.25	0.20	0.32	0.26	0.21	0.30	0.32	0.28	0.27	0.25	0.27	0.21	0.21	0.12	0.00							
17S	0.39	0.29	0.35	0.23	0.21	0.30	0.27	0.21	0.31	0.32	0.28	0.27	0.26	0.25	0.19	0.21	0.17	0.15	0.00						
26S	0.39	0.27	0.35	0.25	0.20	0.28	0.25	0.19	0.29	0.30	0.27	0.25	0.24	0.23	0.20	0.21	0.18	0.12	0.09	0.00					
TV1	0.38	0.31	0.37	0.27	0.24	0.29	0.27	0.21	0.31	0.34	0.29	0.28	0.29	0.23	0.20	0.23	0.22	0.19	0.11	0.10	0.00				
TV2	0.42	0.36	0.42	0.31	0.27	0.34	0.30	0.24	0.37	0.38	0.35	0.32	**0.35**	0.29	**0.26**	0.25	0.22	0.21	0.17	0.16	0.09	0.00			
BJ1	0.49	0.43	0.48	0.38	0.32	0.43	0.37	0.31	0.40	0.41	0.37	0.40	0.43	0.42	0.38	0.37	0.33	0.30	0.31	0.28	0.30	0.35	0.00		
BJ2	0.51	0.43	0.53	0.42	0.35	0.45	0.40	0.32	0.42	0.43	0.38	0.40	0.45	0.43	0.39	0.43	0.35	0.30	0.32	0.27	0.32	0.40	0.15	0.00	
SJN	0.56	0.53	0.57	0.49	0.42	0.48	0.50	0.38	0.48	0.55	0.51	0.54	0.61	0.56	0.50	0.50	0.40	0.40	0.40	0.38	0.38	0.49	0.43	0.48	0.00

CAPÍTULO IV

Manuscrito em preparação

Introgressive secondary contact hybridization mediated by habitat alterations in burrowing rodents of southern Brazil

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Running title: Interspecific introgressive hybrid zone

ABSTRACT

Hybridization between animal species has been increasingly reported; however, its underlying causes and evolutionary significance are not yet fully understood. Natural hybrid zones are considered to be part of the evolutionary history of the taxa involved, although such zones become a problem and require conservation interventions when they occur as a result of human actions. *Ctenomys minutus* and *Ctenomys lami* are sister species of burrowing rodents, endemic to the southern Brazilian coastal plain. An interspecific hybrid zone between them, mediated by habitat alterations, has been identified through cytogenetic data. In order to evaluate the evolutionary history of these two species, and to confirm and characterize the hybrid zone between them, mitochondrial DNA (mtDNA) sequences and nuclear microsatellite markers were used, together with cytogenetic data for each individual sampled. Despite the absence of reciprocal monophyletic mtDNA clades, *C. minutus* and *C. lami* were considered as separate sister species by means of microsatellite, cytogenetic and skull morphometric data, and habitat occupancy. The interspecific hybrid zone was strongly evidenced by 19 individuals with intermediate karyotypic forms between the two species. All hybrids showed mtDNA content exclusively from *C. minutus*, and nuclear microsatellite composition from *C. lami*, suggesting that the hybrid zone was formed by crossings between females of *C. minutus* and males of *C. lami*. Also, a substantial clinal introgression of the genomic content of *C. lami* into *C. minutus* individuals collected around the hybrid zone was reported. Conservation efforts should focus on halting the process of introgression, in order to preserve the integrity of the pure populations. Moreover, *C. minutus* and *C. lami* must be treated as two distinct species, and therefore, conservation strategies should be individually designed to take account of their particularities.

INTRODUCTION

The concept of species, species boundaries, and the process of speciation are among the central questions in biology, and in this context the role of hybrid zones in the evolutionary process is also actively debated (Dowling & Secor 1997; Hewitt 2001). Hybrid zones are often cited as laboratories for evolutionary studies, providing insights into the processes of geographical genetic variation among taxa and the maintenance of their identity (Dowling & Secor 1997; Barton & Hewitt 1985). The event of hybridization is much more common among animals than previously supposed; however, few animal hybrid zones have been described in detail, and certain questions about the underlying causes and evolutionary significance of this process for the species involved remain to be elucidated (Trigo *et al.* 2008).

One of the most troublesome issues regarding hybrid zones is the difficulty of distinguishing primary from secondary contact without historical evidence, since both can produce identical patterns of variation (Barton & Hewitt 1985; Harrison 1993). Primary contact may arise in situ in response to selection pressures from the environmental gradient, and secondary contact may occur between populations that had previously differentiated in allopatry (Harrison 1993). Most of the know hybrid zones can be explained by secondary contact. This contact may have natural causes, such as the climate fluctuations that commonly account for hybrid zones found in most species from Europe and North America; or may originate from human disturbances, caused by habitat modifications or the introduction of exotic species (Barton & Hewitt 1985; Allendorf *et al.* 2001). If the process of hybridization occurs naturally, it is considered as part of the evolutionary history of the taxa involved; however, it becomes a problem and requires conservation interventions when it is driven by human actions (Rhymer & Simberloff 1996; Allendorf *et al*. 2001).

Hybrid zones in which hybrids survive beyond the F1 generation, interbreeding with each other and backcrossing with their parental types, may lead to the formation of a population consisting of a wide variety of recombinant types, sometimes blurring the boundaries between the parental taxa (Harrison 1993; Allendorf *et al.* 2001). Such populations are known as “hybrid swarms” and can account for the introgression phenomenon, in which alleles are incorporated from one taxon into the other (Harrison

1993; Rieseberg & Wendel 1993). The estimated extent of the introgression depends on the markers analyzed, and sometimes its direction can be asymmetrical; moreover, the introgression may have as extreme consequences the total fusion of the parental species, the extinction of one of the taxa involved, or even the formation of a distinct species from the hybrids (Arnold 1992; Rieseberg & Wendel 1993; Allendorf *et al.* 2001). Thus, identifying and characterizing introgression patterns is a fundamental step in the study of hybrid zones (Allendorf *et al.* 2001).

The tuco-tucos *Ctenomys minutus* Nehring, 1887 and *Ctenomys lami* Freitas, 2001 are burrowing rodents that have been considered sister species within the *torquatus* group in the genus *Ctenomys* (Freitas 2001; Parada *et al.* 2011). Both species are endemic to the southern Brazilian coastal plain. *Ctenomys minutus* has a narrow distribution along the first dune line and sand fields near the coast, in the states of Santa Catarina (SC) and Rio Grande do Sul (RS; Figure 1; Lopes *et al.* b in prep.), and *C. lami* is restricted to an area of 78 x 12 km named as Coxilha das Lombas, of sand fields farther inland on the RS coastal plain (Figure 1; Freitas 2001). These species have notable chromosomal polymorphisms. *Ctenomys minutus* shows diploid numbers ranging from 2n = 42 to 50, and autosomal arm numbers (AN) ranging from AN = 68 to 80, comprising a total of 45 karyotypes described until the present (Freitas 1997; Gava & Freitas 2002, 2003; Castilho 2004; Freygang *et al.* 2004; Lopes *et al* a in prep.). The main karyotypes (2n = 42, 46a, 46b, 48a, 48b, 48c, 50a, and 50b) are distributed parapatrically, and between each pair of parapatric karyotypes, except one, is an intra-specific hybrid zone (Lopes *et al* in prep. a). *Ctenomys lami* has five different diploid numbers (2n = 54, 55, 56, 57 and 58), and ten AN (from 74 to 82, and 84), which combined form 26 karyotypes. Four karyotypic blocks (A, B, C, and D) were described for this species based on the Robertsonian rearrangements. Also, two intra-specific hybrid zones were reported for this species, one between blocks A x B, and another between blocks C x D (Freitas 2007).

These species are indistinguishable by their external morphology. However, *C. lami* was recently described as a separate species from *C. minutus* based on differences in their karyotypes (mainly regarding the chromosomal forms and diploid numbers), the distinct habitats that the species occupy, and also on differences in skull morphology (Freitas 2001). The present hypothesis regarding the process of speciation between them is based on the role of chromosomal rearrangements and geographical barriers, resulting in an

allopatric model of speciation followed by chromosomal rearrangements (Freitas 2006). *Ctenomys lami* has been considered to be closer to their common ancestor, and *C. minutus* presenting more derived characteristics, since the latter has lower diploid numbers, showing more chromosomal rearrangements than *C. lami*, and also the region of the southern Brazilian coastal plain occupied by *C. minutus* is geologically newer than the Coxilha das Lombas occupied by *C. lami* (Freitas 2001, 2006).

However, the evolutionary process in which these species are involved and their status as two distinct species may be affected by human actions. Cytogenetic data from six individuals collected from the western shore of Barros Lake (RS) revealed diploid numbers and chromosomal rearrangements intermediate between the individuals of *C. lami* (2n = 56) and *C. minutus* (2n = 48a) that inhabit the areas surrounding this region. These specimens were considered inter-specific hybrid forms between *C. lami* and *C. minutus* by Gava & Freitas (2003). The authors suggested that this hybrid zone is the product of a secondary contact, because in the past, a wide wetland west of Barros Lake formed a geographical barrier separating *C. minutus* from *C. lami*. However, in the 1950s the introduction of rice farming in the region resulted in the swamp being completely drained, exposing a sandy area that allowed the meeting and mating of the two species, and consequently the production of hybrids. The consequences of this hybrid zone for both species are uncertain, requiring further investigation.

The goals of the present study were to (i) describe the evolutionary history of *C. lami* and *C. minutus* on the southern Brazilian coastal plain; (ii) confirm the existence of the inter-specific hybrid zone west of Barros Lake, genetically characterizing the tuco-tucos living there, and assessing the magnitude of mixture and introgression; and (iii) provide information to help future conservation decisions and management strategies for both species. To evaluate these issues, we used mitochondrial DNA sequences and nuclear microsatellite markers, together with cytogenetic data for each individual sampled.

MATERIAL AND METHODS

Samples and laboratory procedure

The samples covered the entire currently known area of occurrence of these species on the southern Brazilian coastal plain. A total of 531 individuals were analyzed, comprising 172 specimens of *C. lami* from 28 sampling sites, 340 specimens of *C. minutus* from 30 sites, and 19 possible hybrids from 3 different sites (Table 1, Figure 1). The tissue samples and cytogenetic data were provided by Freitas (2001), and El Jundi (2003), for *C. lami*; and by Freitas (1997), Gava & Freitas (2002, 2003), Castilho (2004), Freygang *et al.* (2004), and Lopes *et al*. a (in prep.), for *C. minutus*. The possible hybrids were collected using Oneida-Victor no. 0 snap-traps in the beginning of May (site 1), late June (site 2), and October (site 3), in 2009. The diploid and autosomal numbers were determined by analyses of at least 20 metaphase spread cells, stained with Giemsa, following the protocol described by Ford & Hamerton (1956). To evaluate the behavior of chromosomes and Robertsonian rearrangements during meiosis, meiotic analyses were provided for males, using the technique of Ford & Evans (1969). All tissue samples were preserved in 95% ethanol and stored at -20 °C in the collection of the Laboratório de Citogenética e Evolução of the Departamento de Genética of the Universidade Federal do Rio Grande do Sul.

All mitochondrial and microsatellite data for *C. lami* and *C. minutus* were provided by Lopes et al. a (in prep.), and Lopes et al. b (in prep.), respectively. Total DNAs from the possible hybrids were extracted following a modified phenol-chloroform protocol from Sambrook & Russel (2001). Two fragments of mitochondrial DNA (mtDNA) were analyzed. A segment of the HVS1 control region (CR) was amplified using the primers TucoPro (5'-TTCTAATTAAACTATTTCTTG-3', Tomasco & Lessa, 2007) and TDKD (5'-CCTGAAGTAGGAACCAGATG-3', Kocher *et al*., 1989), following the PCR amplification conditions described by Tomasco & Lessa (2007). The amplification of cytochrome c oxidase subunit I (COI) followed the protocols suggested in http://www.barcoding.si.edu/DNABarCoding.htm, using the primers LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer *et al.* 1994). PCR products were visualized on 1% agarose gels and purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California), following the guidelines of the suppliers. Sanger sequencings were conducted in an ABI3730 automated sequencer, using the forward primers TucoPro and LCO-1490, for CR and COI,

respectively. The ambiguous sequences were reamplified and resequenced as many times as needed, and the electropherograms were visually inspected using Chromas 2.33 (http://www.technelysium.com.au/chromas.html), and aligned using the Clustal W algorithm with default options, implemented in Mega 4.02 (Tamura *et al.* 2007). Alignments were checked and edited by hand if necessary.

In addition to the mtDNA data, the samples were genotyped for 14 microsatellite loci, using fluorescently labeled primers (thirteen dinucleotides Hai2, Hai3, Hai4, Hai5, Hai 6, Hai9, Hai10, Hai12, Soc2, Soc3, Soc4, Soc5, Soc6; and one trinucleotide Hai 12; Lacey et al., 1999; Lacey, 2001). The PCR amplification conditions were carried out following the protocols described by Lacey *et al.* (1999) and Lacey (2001), and were conducted using one primer pair at a time. The genotypes were obtained using an ABI3730 DNA sequencer, joining at most three PCR products, combined considering their sizes and labels. The allele sizes were defined using the program PeakScanner 1.0 (http://www.appliedbiosystems.com). In order to obtain reliable results for allele sizes, the PCR and genotyping reactions were carried out as many times as needed, and also all peaks of allele sizes were double-checked.

Data analyses

mtDNA data

Some analyses were performed with the mtDNA data sets of CR and COI separately, but most results shown here were obtained using a concatenated data set of CR+COI (CC). Measures of mtDNA diversity, including the number of polymorphic sites, mean number of pairwise differences (π, Nei 1987), average number of nucleotide differences (k), definitions of haplotypes (H), and haplotype diversity (*Hd*), were calculated in the program DNAsp 5.00.03 (Librado & Rozas 2009).

In order to estimate pairwise genetic differentiation between localities sampled near the western shore of Barros Lake, F_{ST} statistics were computed using the software Arlequin 3.5.1.2 (Excoffier & Schneider 2005). Localities with fewer than 4 individuals were excluded from comparisons, and sequential Bonferroni corrections were applied, for α = 0.05, to adjust the statistical significance levels. In addition, the same program was used to

assess the level of genetic differentiation between species, excluding the hybrids from analyses, employing an Analysis of Molecular Variance (AMOVA; Excoffier *et al*. 1992), using 10000 permutations to test statistical significance.

Phylogenetic analyses were performed using Maximum Parsimony (MP) and a distance-based (Neighbor Joining - NJ) method implemented in Paup 4.0b10 (Swofford 1998), Maximum Likelihood (ML) performed in PhyML 3.0 (Guindon *et al.* 2010). A Bayesian Inference (BI) was employed in Beast 1.6.1 (Drummond & Rambaut 2007), using only the CC data. However, to determine the appropriate model of nucleotide sequence evolution, we analyzed each mtDNA data set separately and also concatenated, both using the Akaike Information Criterion (AIC) estimated in MrModelTest 2.2 (Nylander 2004). The Hasegawa-Kishino-Yano model with a proportion of invariable sites and a gamma distribution of rate heterogeneity across sites (HKY+I+G) provided the best fit to all data sets and was applied in the subsequent model-based analyses (ML, NJ, and BI).

The MP phylogeny was 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. For the NJ search, the ML distance was used. In all cases the nodal supports were evaluated using 1000 bootstrap replicates. ML analyses started with an NJ tree, following the nearest-neighbor interchange (NNI) branch-swapping, and 1000 replicates of randomly added taxa. The BI analysis is explained below. Trees were rooted with homologous sequences of *C. torquatus*, *C. pearsoni*, *C. australis*, *C. rionegrensis*, *C. porteousi*, *C. azarae*, *C. steinbachi*, *C. boliviensis*, *C. talarum*, *C. haigi*, *C. sociabilis*, and *C. flamarioni* as outgroups (GenBank accession numbers for CR: HM443438, JQ341031 to JQ341041 respectively; and COI: HM443439, JQ341042 to JQ341052, respectively). Also, the topological relationship between the haplotypes was estimated using the program Network 4.5.1.0 (http://www.fluxux-engineering.com) with the median-joining approach, for the CC data set.

The time of the most recent common ancestor (TMRCA) between *C. lami* and *C. minutus*, as well as the ages of their main mtDNA clades were estimated using the Bayesian approach implemented in the program Beast 1.6.1, employing strict molecular clock models with substitution rates estimated for ctenomyids by Roratto *et al.* (in prep.). For CR the rate used was 3.44×10^{-8} site/year (95% confidence interval: 1.83×10^{-8} -

$5.26x10^{-8}$), and for COI the substitution rate was $2.63x10^{-8}$ site/year (95% confidence interval: $1.64\ x10^{-8}$ - $3.76\ x10^{-8}$). Both the rates and models of substitution were used separately for each partition of the mtDNA data (CR and COI). The Bayesian skyline approach was used, starting with a UPGMA tree, following 100 million iterations of the Markov chain Monte Carlo (MCMC) procedure, sampling every 10000 steps, and discarding the first 20% iterations as burn-in. Results were visually inspected in the program Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/), summarized in TreeAnnotator 1.6.1, and the resulting BI tree was visualized in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Microsatellite data

Measures of microsatellite diversity were computed separately for *C. lami*, *C. minutus* and possible hybrids as the number of alleles per locus, private alleles, and observed and expected heterozygosities (H_O and H_E), using the programs Arlequin 3.5.1.2 and Genepop 4.0 (Rousset 2008). Deviations from linkage equilibrium (LE), and Hardy-Weinberg equilibrium (HWE) were checked using the exact test based on the Guo & Thompson (1992) method, for each locus, in the three localities sampled for possible hybrids, with the Genepop 4.0 program, following 10000 dememorization steps, 500 batches, and 10000 iterations per batch. Significance levels ($\alpha = 0.05$) of HWE and LE were adjusted using sequential Bonferroni corrections considering the multiple simultaneous comparisons (Rice 1989). Genotyping errors were tested in Micro-Checker (Van Oosterhout *et al*. 2004), with the 95% confidence interval. To estimate pairwise genetic differentiation between localities sampled near the western shore of Barros Lake, and to assess the level of genetic differentiation between the species, analyses of F_{ST} and AMOVA were carried out as for the mtDNA data.

Bayesian clustering approaches were implemented in the program Structure version 2.2.3 (Pritchard *et al.* 2000), to determine genetic clusters (Ks), to assign the genetic identity of individuals in each species, to identify hybrids, and to assess the extent and direction of possible introgression events from one species into the other. Two sets of analyses were performed. First, to identify the real number of clusters among all individuals sampled for *C. lami*, *C. minutus*, and possible hybrids, ten independent runs

from K = 1-25 were tested, assuming the admixture model, independent allele frequencies, no a priori population information, 500000 iterations for the burn-in period, and 2000000 for MCMC. From these runs, the optimal K was determined by the ΔK approach of Evanno *et al.* (2005). After that, we performed another set of analyses, using prior species information for individuals that we could identify with the confidence as *C. lami* or *C. minutus* (based on their sampling sites and karyotypes). For individuals that were possible hybrids, collected from the western shore of Barros Lake, we did not provide information, allowing the program to assign their identities alone. Twenty independent runs were applied, assuming K=2, 500000 steps of burn-in period, followed by 2000000 iterations of MCMC, using the admixture model, and inferring the lambda and gamma values separately for each species.

The program NewHybrids 1.1 (Anderson & Thompson 2002), which also uses the Bayesian approach, was used to compute the posterior distribution of individuals into different genotypic categories, as pure individuals of a species (P1 or P2), F1, F2, back-crossings between hybrids and pure parental, and so on. This analysis was performed using the genotypic classes and the assumptions for allele frequencies as described by Anderson & Thompson (2002) in a run of 1000000 steps after a burn-in period of 500000 sweeps. For both Structure and NewHybrids analyses, individuals were considered as "pure" when the q score was ≥ 0.9.

RESULTS

Cytogenetic data

Of the 19 individuals collected from western shore of Barros Lake, 18 were successful karyotyped. A total of 10 karyotypes were retrieved, comprising 6 diploid numbers (2n = 48, 50, 53, 54, 55, and 56), and 4 autosomal arm numbers (AN = 74, 76, 78, and 80). The karyotypes for each sampling site are described in Table S1 and in Figure S1. The karyotypic forms 2n = 56, AN = 80; 2n = 56, AN = 78; and 2n = 55, AN = 76 were previously recorded for *C. lami*, and the form 2n = 48, AN = 76 was previously described for *C. minutus*. The other karyomorphs retrieved, for 13 individuals, showed intermediate diploid numbers and chromosome rearrangements between individuals of *C. lami* (2n =

56b) and *C. minutus* (2n = 48a), which occur near this region and are probably the source of this variation. The karyotypes of the hybrids were geographically distributed on a clinal gradient through the landscape, ranging from 2n = 56 in individuals from sampling site 1, near the populations of *C. lami*, and were progressively reduced to 2n = 48 at sampling site 3, near the populations of *C. minutus*.

Males collected at sampling sites 2 and 3 showed normal meiosis and proper segregation of chromosomes. Of the 2 males analyzed from locality 2, one had 2n = 50, NA = 74, and gametic cells with n = 25; and the other had 2n = 53, AN = 80, and gametic cells with n = 26 or 27. At locality 3, only one male was collected, which had 2n = 48, AN = 76, and gametic cells with n = 24. However, the 3 males collected at sampling site 1 had small testicles, and we were not able to observe cells in spermatogenesis. The sex chromosome pair, with a sub-metacentric X and an acrocentric Y chromosome was the same for all karyotypes.

Genetic diversity and population structure

The global estimates of microsatellite genetic diversity were always higher in *C. minutus*, followed by *C. lami* and the hybrids. The total number of alleles in *C. minutus* was 182, ranging from 7 in Hai10 and Hai2 to 17 in Hai6, comprising a total of 62 private alleles, which were distributed in all loci ranging from 2 to 7. *Ctenomys lami* showed a total of 121 alleles, ranging from 2 in Hai10 to 13 in Soc2 and Soc5, and only 4 alleles were private, 1 in Hai4, 1 in Soc1, and 2 in Soc 2. Among the 19 individuals considered hybrids were, 48 alleles were found, and none of these was private. At the three sampling sites in the hybrid region, no genotyping errors were detected, nor deviations from LE and HWE. These results for each sampling site for *C. lami* and *C. minutus* were described by Lopes *et al.* a (in prep.), and Lopes *et al.* b (in prep.), respectively.

The mtDNA amplifications retrieved 398 bp in length for CR (GenBank accession numbers: *C. lami* – JQ322885 to JQ322898; *C. minutus* - HM236969 to HM237008; and hybrids - HM236991), 620 bp for COI (GenBank accession numbers: *C. lami* – JQ322899 to JQ322907; *C. minutus* - HM237009 to HM237043; and hybrids - HM237016), comprising a total of 1018 bp for CC data. The measures of genetic variability were always

higher in *C. minutus* than in *C. lami*, especially those related to the number of nucleotide differences (k and π). The estimated measures are presented in Table 2.

Microsatellite estimates of F_{ST} showed moderate to high levels of genetic differentiation in pairwise comparisons between localities sampled near the supposed hybrid zone region, most of them with values ≥ 0.25, ranging from 0.01 to 0.48 (Table 3). For the mtDNA CC data, the F_{ST} pairwise estimates ranged from -0.33 to 1.00, with various comparisons showing estimates much higher than those found with the microsatellite data, most of them ≥ 0.40. The non-significant estimates were mainly found in comparisons involving sampling sites with fewer than 6 individuals, suggesting that data from localities with small sample sizes may lead to erroneous interpretations by overestimating the gene-flow levels. Considering the two sampling sites of possible hybrids (named 1 and 2), the highest levels of gene flow for mtDNA CC data were found between them ($F_{ST} = 0.00$), and for *C. minutus* individuals from PSS and SBL ($F_{ST} = 0.00$, and 0.06, respectively). On the other hand, the sampling sites of hybrids showed the lowest levels of gene flow with *C. minutus* localities, for the microsatellite data, with most pairwise estimates of F_{ST} significant and higher than 0.30.

The differentiation between the two species was obscure in the AMOVA tests, since this difference was overwhelmed by the high intraspecific diversities. For both mtDNA and microsatellite data, the results indicated significant fixation indices in all levels of genetic structure ($P = 0.00$; mtDNA: $F_{CT} = 0.20$; $F_{SC} = 0.87$; $F_{ST} = 0.89$. Microsatellites: $F_{CT} = 0.09$; $F_{SC} = 0.30$; $F_{ST} = 0.36$). For mtDNA, most of the genetic variation was apportioned among populations within species (69.69%), followed by among species (19.52%), and, last, within populations (10.79%). For microsatellite data, the highest percentage of variation was found within populations (63.82%), followed by among populations within species (27.09%), and among species (9.09%).

Phylogenetic and genealogical relationships among haplotypes

All phylogenetic trees generated by the methods of MP, ML, NJ, and BI showed congruent topologies regarding the main features (Figure 2). For the MP, ML, and NJ trees their roots were represented by a polytomy between *C. sociabilis* and *C. haigi*, whereas for the BI analysis the root of the tree was represented only by *C. sociabilis*. In all analyses *C.*

torquatus was well supported as the sister group of *C. lami* and *C. minutus*; however, there were no reciprocal monophyletic clades supporting the delimitation of two species of our in-group. Instead, eight main clades were highlighted in the trees, showing clustering of individuals following a strong geographic structure. The first two clades to differentiate in the trees were those represented by the northern sampling sites of *C. minutus*, identified as North 1 and North 2, followed by the clades South and Tavares, represented by the southern localities of the *C. minutus* geographical distribution. The remaining clades corresponded to the middle of the *C. minutus* geographical distribution (Coast, Barros Lake, and Mostardas clades), all individuals identified as *C. lami* (Barros Lake, and Block C+D clades), and all of the possible hybrids (Barros Lake clade).

The relationship among mtDNA CC haplotypes, retrieved by the median-joining network, again revealed eight haplogroups, which were more highly correlated with the geographical distribution of the individuals than with the species level (Figure 3). Six of these haplogroups (North 1, North 2, Coast, Mostardas, Tavares, and South) were formed exclusively by individuals identified as *C. minutus*, one haplogroup (Blocks C+D) consisted only of *C. lami* individuals, and the Barros Lake haplogroup was common to both species and hybrids. In this haplogroup were all individuals of *C. lami* collected south of the connection between the Pachecos and Touros swamps (corresponding to karyotypic Blocks A and B) and some specimens collected north of this connection (from karyotypic Block C), plus all individuals of *C. minutus* surrounding the Barros Lake region, ranging from sampling sites TRA to 35, and also the 19 possible hybrids (Table 1, Figures 1 and 3). The Barros Lake haplogroup has a star-like shape, with most haplotypes connected by few mutational steps to a central one (H23) which is the only one shared between both species. Moreover, all 19 hybrids share the same haplotype (H26) among each other and with *C. minutus* collected from SBL and PAS, leading us to believe that *C. minutus* females are the main, if not the only, donors of mtDNA to the hybrids.

Divergence time estimates

The TMRCA among all ctenomyids analyzed here was estimated as around 3 529 242 years ago (ya; 95% confidence interval: 1 998 357 – 5 861 413 ya). The retrieved divergence time between our in-group and the sister-species *C. torquatus* was 650 676 ya

(95% confidence interval: 350 119 – 1 013 415 ya), and the TMRCA for the in-group was estimated as 478 034 ya (95% confidence interval: 276 022 – 757 046 ya). The divergence times and the 95% confidence interval among haplotypes of each of the eight main clades were: **North 1**: 83 177 ya (20 367 – 117 399 ya); **North 2**: 181 087 ya (60 805 – 284 736 ya); **Coast**: 154 606 ya (56 932 – 231 119 ya); **Barros Lake** 137 936 ya (60 490 – 199 255 ya); **Blocks C+D**: 102 824 ya (27 243 – 153 504 ya); **Mostardas**: 181 952 ya (75 543 – 275 965 ya); **Tavares**: 126 110 ya (44 453 – 187 975 ya); and **South**: 94 294 ya (21 945 – 144 943 ya).

Genetic assignment of individuals

The first set of analyses performed with Structure 2.2.3 was used to validate the real subdivision of groups of individuals in clusters, without providing any information about sampling sites or species source. According to the ΔK approach of Evanno et al. (2005), the most likely number of clusters was K = 2 (Figure S2). Each of the species was assigned predominantly to one of the two clusters, i.e. *C. lami* to cluster 1 (q = 0.9817), and most *C. minutus* individuals to cluster 2 (q = 0.9021), while the 19 possible hybrids were assigned to cluster 1 together with *C. lami* (q = 0.9905).

After that, we performed 20 runs, providing species information and considering K = 2, to assign the genetic composition of hybrids, and to assess the extent and direction of possible introgression events. All runs retrieved identical patterns of genetic assignment of individuals, and the pattern chosen to display the results was that with the highest mean value of the estimated logarithm of probability of the data [LnPr(X/K) = -27563.6]. The plot of the individual cluster assignments is given in Figure 4. Again, each species was assigned predominantly to one of the two clusters; 98% of the 172 individuals classified as *C. lami* were attributed to cluster 1 ($q \geq 0.9$), and the remaining individuals showed mixed ancestries. The mean probability of these 172 individuals being assigned to cluster 1 was 0.9769. Of the 340 individuals identified as *C. minutus*, approximately 70% were assigned to cluster 2, ~12% were assigned to cluster 1 (similar to *C. lami*), and the remaining showed mixed ancestry. The total mean probability of these individuals in cluster 2 was 0.7752. The 19 possible hybrids were attributed to cluster 1, with a mean probability of 0.9938.

Although the program NewHybrids showed some similar results to those obtained with Structure, it was less sensitive in assigning individuals to genetic categories (Figure 4). Almost 96% of the individuals identified as *C. lami* were assigned to the genetic category of pure parental P1 ($q \geq 0.9$), the remaining individuals showed mixed contents in various genetic categories, and only one was identified as pure *C. minutus* (P2); the total mean probability was 0.9737. Of 340 individuals identified as *C. minutus*, 91% were assigned to the genetic category of pure parental P2, and the remaining 9% showed mixed identification among some categories; the total mean probability in the P2 category was 0.9587. Fifteen hybrids (8 from sampling site 1 and 7 from sampling site 2) were identified as pure P1 (similar to *C. lami*), and the other 4 hybrids (1 from sampling site 2 and 3 from site 3) showed mixed genetic compositions distributed among the six categories analyzed.

Most individuals classified as *C. minutus* which were assigned to cluster 1, or to category P1, such as *C. lami*, or those that showed mixed ancestry ($q \leq 0.9$), are geographically distributed near the Barros Lake region, where the contact zone between the two species is located. These individuals, despite having diagnostic karyotypes for *C. minutus* such as 2n = 46a, 47a, and 48a, and although they occur in a region of the southern Brazilian coastal plain that is characteristic of this species, had at least a considerable fraction of their nuclear microsatellite loci genetic composition from *C. lami*. In addition, most hybrids were identified as pure *C. lami* and none of them were classified as F1, F2, or as backcrossing with the parental form. This pattern must be the consequence of a complex system of mating involving advanced introgression of some nuclear genes from *C. lami* into *C. minutus*.

DISCUSSION

Evolutionary history of *C. minutus* and *C. lami* on the southern Brazilian coastal plain

Ctenomys minutus and *C. lami* have only recently been considered as two separate taxonomic units, based on differences in their karyotypes, areas of occurrence, and analyses of their skull morphology (Freitas 2001). Although ample information about their cytogenetic differences was available, there was a lack of knowledge regarding the use of molecular markers to confirm or reject their status as two distinct species.

The mtDNA phylogenetic trees provided here do not support reciprocal monophyletic clades separating *C. minutus* and *C. lami*. Instead, the main clades retrieved highlighted the clustering of individuals following a strong pattern of geographical subdivision. This strong geographical association in both species was previously assessed in phylogeographical studies conducted by Lopes *et al.* a (in prep.) and Lopes *et al.* b (in prep.). For *C. lami*, both mtDNA and microsatellite data supported a subdivision of the populations in two demes, which were not completely isolated but were probably reinforced by the connection between the Pachecos and Touros swamps (Lopes *et al.* a, in prep.). For *C. minutus* the observed phylogeographical patterns were primarily associated with the presence of geographical discontinuities in the landscape, represented by rivers, paleochannels, and the transition between sand fields and dunes. Lopes *et al.* b (in prep) suggested that these geographical barriers could impede the free dispersal of *C. minutus* specimens along the environment, leading to partial or total isolation among populations, and giving rise to clusters of geographically isolated individuals.

Unlike the mtDNA results, the microsatellite data analyses provided in the Structure and New Hybrids software were congruent in demonstrating that each species has its own genetic makeup (see Figure 4), being assigned predominantly to one particular cluster. 98% of the individuals classified as *C. lami* were attributed to cluster 1 (q = 0.9769) in the Structure, and 96% to the genetic category of pure parental P1 (q = 0.9737) in the New Hybrids analyses. Approximately 70% of the individuals identified as *C. minutus* were assigned to cluster 2 (q = 0.7752) in Structure, and 91% were assigned to the genetic category of pure parental P2 (q = 0.9587) in New Hybrids.

The species of the genus *Ctenomys* commonly show low rates of adult dispersal, and occur in relatively small and fragmented populations, which promotes the establishment of small genetic units where genetic variation is low and interpopulation divergence is high (Reig *et al.* 1990; Lessa & Cook 1998; Nowak 1999; Wlasiuk *et al*. 2003). These life-history characteristics favor the action of genetic drift, which can maintain ancestral polymorphisms fixed in the populations longer than in larger, randomly mating ones. The effect of the maintenance of ancestral lineages in populations can be even more pronounced in molecular markers experiencing low levels of gene flow, both by means of the action of genetic drift, and by reduced selective sweeps within species, which can be important in establishing or maintaining differences between species (Petit &

Excoffier 2009). Considering that mtDNA has a slower molecular evolutionary rate compared to microsatellite markers, and also that the heritance of mtDNA is matrilineal, and that females of ctenomyids commonly have lower rates of dispersal than males (explained below), it is expected that the power of taxonomic resolution of mtDNA is lower than that observed in microsatellite markers when the process of speciation is a recent event.

The estimate of the TMRCA among all individuals of *C. minutus* and *C. lami* was around 478 thousand years ago (kya; black arrow in Figure 2), however this node age does not match the starting point of the process of speciation between these species, since the first clades to differentiate in the phylogenetic trees for the in-group were represented only by individuals from the northern and southern sampling sites of *C. minutus*. Thus, the splitting process between *C. minutus* and *C. lami* would have begun approximately 278 kya, which corresponds to the estimated age of the node that includes both species and hybrids, represented by the clades from Barros Lake, Coast, Blocks C+D, and Mostardas (gray arrow in Figure 2). Possibly, this divergence time (~278 kya) was too brief to accumulate sufficient new mutations to mask the signals of ancestral polymorphisms, and thus did not lead to a distinct pattern of reciprocal monophyletic clades of mtDNA for each species. However, given the evolutionary differences between molecular markers, this time of divergence seemed to be sufficient to establish a recognizable pattern of genetic disjunction for each species, as assessed by microsatellite data.

One of the well studied complexes of closely related species in the genus *Ctenomys* is the *perrensi* group, which, similarly to *C. minutus* and *C. lami*, does not show a congruent pattern of genetic structure among the different methods of analysis. Traditionally, based on geographical range, morphology, and chromosomes, the *perrensi* group is described as formed by three species (*C. roigi*, *C. perrensi,* and *C. dorbignyi*) and several forms of uncertain taxonomic status (*Ctenomys* sp.; Ortells 1995). Later studies based on chromosomes and cytochrome b sequences suggested the existence of two other species in addition to the first three, despite the absence of reciprocally monophyletic species (Giménez *et al.* 2002). Recently, Mirol *et al.* (2010), analyzing microsatellite data that indicated a more complex evolutionary scenario than previously described, subdivided this complex into eight major groups. The authors suggested that populations were not sufficiently isolated to complete allopatric speciation, and thus when they come into

contact the hybridization process takes place, leading to the lack of congruence among the various aspects analyzed.

Considering these issues we did not consider that the absence of monophyletic clades separating the two species is sufficient evidence to recombine *C. minutus* and *C. lami* into a single taxon, since all other analyses regarding the cytogenetic and morphological differences, and the pattern retrieved in the microsatellite data confirm their status as two separate sister species, only recently differentiated. This hypothesis is reinforced by the pattern of crossings retrieved in the hybrid zone, with only females of *C. minutus* breeding with males of *C. lami*, as explained above. This pattern of unilateral crossing may reflect some kind of reproductive isolation mechanism between females of *C. lami* and males of *C. minutus*.

Moreover, the results showed that the North 1 and North 2 clades were the first ones to split (basal branches), represented only by *C. minutus* individuals distributed in the northern range, around 478 and 443 kya, respectively. After that the earlier *C. lami* clades arose, about 278 kya. Also, *C. minutus* showed proportionally higher levels of polymorphisms in their microsatellite loci and in their mtDNA sequences than *C. lami*, which can suggest an ancestral condition (see Table 2 and results). Thus, in contrast to our first hypothesis, the probable earlier clades that had differentiated in the southern Brazilian coastal plain are nowadays represented by *C. minutus* individuals.

The hybrid zone and the cyto-nuclear genome disjunction

The mtDNA, microsatellite, and cytogenetic data presented here concord closelywith the hypothesis of a hybridization region between *C. minutus* and *C. lami* around the western shore of Barros Lake in Rio Grande do Sul (Figure 1), and also provide evidence of a substantial clinal introgression of the genomic content of *C. lami* into *C. minutus* individuals living around the hybrid zone.

The cytogenetic data for the 19 possible hybrids demonstrated that 13 individuals have chromosomal rearrangements and diploid numbers intermediate between the karyotypic forms of the parental forms (*C. lami* 2n = 56b, and *C. minutus* 2n=48a; see Table S1 and Figure S1), geographically distributed on a clinal gradient through the environment. The wide range of karyotypic forms found here demonstrates the presence of

hybrids derived from several crossings beyond F1 generations, and back-crossings with parental forms. The supposed low effectiveness of chromosomal differences as barriers to gene flow may be caused by a neutral or only weakly underdominant condition of the chromosomal rearrangements, and thus their effects in reducing hybrid fitness are less likely (Rieseberg 2001). The heterozygous carriers of chromosomal rearrangements can maintain their adaptability and fertility by means of mechanisms that partially or totally suppress recombinations with harmful effects during meiosis (Rieseberg 2001).

The hybrid males collected from sampling sites 2 and 3 (sampled in June and October, respectively) showed normal meiosis and proper segregation of chromosomes. However, the males from site 1 (sampled in May) had small testicles with a lack of cells in spermatogenesis. This seems more likely to be associated with reproductive seasonality than with infertility of the hybrid males. As in other mammals, the reproductive success and duration of the breeding season of subterranean rodents are closely associated with environmental factors (Bennet *et al.* 2000). For ctenomyids, studies of reproductive patterns have demonstrated clear seasonality in *C. talarum*, *C. mendocinus*, *C. pearsoni*, and *C. rionegrensis*. Depending on the tuco-tuco species, reproduction begins between the late austral autumn/midwinter (May-August) and extends until the austral late spring/summer (December-March), and during the interval between breeding seasons the females are reproductively inactive (Malizia & Busch1991; Rossi *et al.* 1992, 1996; Altuna *et al.* 1999; Tassino & Passos 2009). Considering that the hybrids from sampling site 1 were collected in May, during the interval between breeding seasons, the males were probably reproductively inactive at that time, explaining the absence of cells in spermatogenesis.

All 19 hybrids share the same haplotype (H26; see Figure 3) with each other and with *C. minutus* from the SBL and PAS sampling sites, located near the hybridization region. Since the mitochondrial genome is only transmitted maternally, the pattern of mtDNA recovered in the hybrids indicates that *C. minutus* females are the main, if not the only donors of mitochondria to the hybrids. In addition, the microsatellite content of most hybrids was identified as pure *C. lami*, and none of them was classified as F1, F2, or a backcross with a parental form. Unlike mtDNA, the nuclear genome is biparentally inherited. Therefore, considering the prevalence of genomic content from *C. lami* and of mtDNA from *C. minutus* in the hybrids, this pattern must have been formed in the hybrid

zone by mating of males of *C. lami* with females of *C. minutus*. This unilateral pattern of crossing, together with the different inheritance of cytoplasmic and nuclear genomes, and the sex-biased dispersal, led to a pattern of cyto-nuclear genome disjunction, resulting in different directions and levels of introgression depending on the molecular marker analyzed (Arnold 1993).

Sex-biased dispersal is common in animals, with differences in terms of distances travelled and/or dispersal rates (Handley & Perrin 2007). In the great majority of mammal species, males show higher rates and distances of dispersal, whereas females are philopatric or disperse over shorter distances (Greenwood 1980; Waser 1985). Although no data are available on the rates and distances of dispersal in *C. lami* and *C. minutus*, our pairwise estimates of F_{ST} among sites sampled near the hybrid zone demonstrated that mtDNA has lower levels of gene flow compared with microsatellite data (Table 3), suggesting that the females of these species disperse over shorter distances than the males. The sex-biased gene flow could account for the differential introgression between molecular markers observed here. The cyto-nuclear genome disjunction in mammal and bird species was recently reviewed by Petit & Excoffier (2009), in a study of the role of gene flow in delimiting species. The authors demonstrated that genome components, with different patterns of inheritance, commonly show differences in phylogeographic patterns and levels of introgression between hybridizing species, and are closely associated with sex-biased dispersal. The mtDNA of the females of *C. minutus* involved in the formation of the hybrid zone was spread only among individuals collected inside the hybridization region, apparently not moving into the boundaries of *C. lami*. However, microsatellite alleles of *C. lami* constitute most of the genomic content of hybrids, and are highly introgressed into *C. minutus* individuals collected around the hybridization region, in a clinal pattern (Figure 4). This pattern of differential introgression was well described by Ishida *et al*. (2011) for African elephants, in which females remain with their natal core social group and males disperse between herds. Thus, while the mtDNA gene flow between different herds is nonexistent, males can transmit nuclear alleles from their natal herd to other herds, and their offspring can transmit these alleles to even more distant herds, and so on, so that nuclear alleles have the potential to disperse over long distances through the landscape.

Implications for conservation

Hybridization and introgression are not usually listed among the major extinction threats. However, these phenomena must be considered with greater caution than is commonly realized (Rhymer & Sinberloff, 1996). Non-natural hybrid zones mediated by human interventions deserve special attention from conservationists, since the effects of such zones may compromise the genetic integrity and evolutionary process in which the species are involved. For example, habitat modifications and fragmentation can disrupt the reproductive isolation between previously isolated species, leading to gene exchanges, which could be especially problematic for rare species contacting more abundant ones, and could result, in extreme situations, in the extinction of populations or species (Rhymer & Sinberloff 1996; Allendorf *et al.* 2001).

The hybridization between *C. minutus* and *C. lami* had its origin approximately 60 years ago, by means of human-mediated habitat alteration, through the introduction of rice farming on the western shore of Barros Lake, which dried a swamp and allowed the two species to come into contact (Gava & Freitas 2003). Considering that the hybridization between these species is now quite advanced, with fertile hybrids mating both among themselves and with parental types, it is difficult to stop, since the existence of hybrid swarms hampers conservation strategies and the recovery of threatened taxa (Allendorf *et al.* 2001). However, this hybrid zone and the extent of introgression are geographically limited, and also there are several parental populations that have not experienced the effects of hybridization and introgression. Therefore, conservation efforts should focus mainly on halting the progress of introgression, to preserve the integrity of pure populations of both species. Moreover, *C. minutus* and *C. lami* must be treated as two distinct species, and conservation strategies must be developed separately, respecting the particularities of each.

Ctenomys lami was included in the 2010 IUCN Red List of threatened species only as “vulnerable”. The study conducted by Lopes *et al.* a (in prep.) demonstrated that the vulnerability of this species is greater than previously supposed and its extinction could lead to a great loss of genetic diversity, since it shows one of the highest chromosomal variabilities among the species of *Ctenomys*. The results of the study by Lopes and colleagues supported the designation of one ESU and one MU within the geographical

distribution of *C. lami*, which must be considered in the development of conservation strategies and the implementation of conservation units. Moreover, the authors suggested that *C. lami* must also be included as "vulnerable" in regional and national Brazilian red lists of endangered fauna.

Ctenomys minutus is presently cited in the IUCN Red List as "data deficient." The data provided in this study and by Lopes *et al.* b (in prep.) showed that despite having a larger geographical distribution and greater genetic variability than *C. lami*, *C. minutus* also deserves attention from conservation efforts. Beyond the consequences that the hybridization with *C. lami* and the introgression events may have for *C. minutus* populations, this species is subject to threats stemming from the vulnerability of the southern Brazilian coastal plain, such as global warming and sea-level rise, urbanization, shore armoring, sand mining, construction of jetties, the introduction of domestic animals, and exotic vegetation (Fernandes *et al.* 2007). Thus, in addition to conservation measures to contain the effects of introgression and human actions on populations of *C. minutus*, this species must be included in the regional and national Brazilian Red Lists as "near threatened."

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DATA ACCESSIBILITY

Mitochondrial sequence data are deposited at GenBank under the following accession numbers: *Ctenomys minutus* CR (HM236969 to HM237008), and COI (HM237009 to HM237043); *Ctenomys lami* CR (JQ322885 to JQ322898), and COI (JQ322899 to JQ322907); hybrids CR (HM236991), and COI (HM237016); and outgroups CR (HM443438, JQ341031 to JQ341041), and COI (HM443439, JQ341042 to JQ341052).

FIGURES

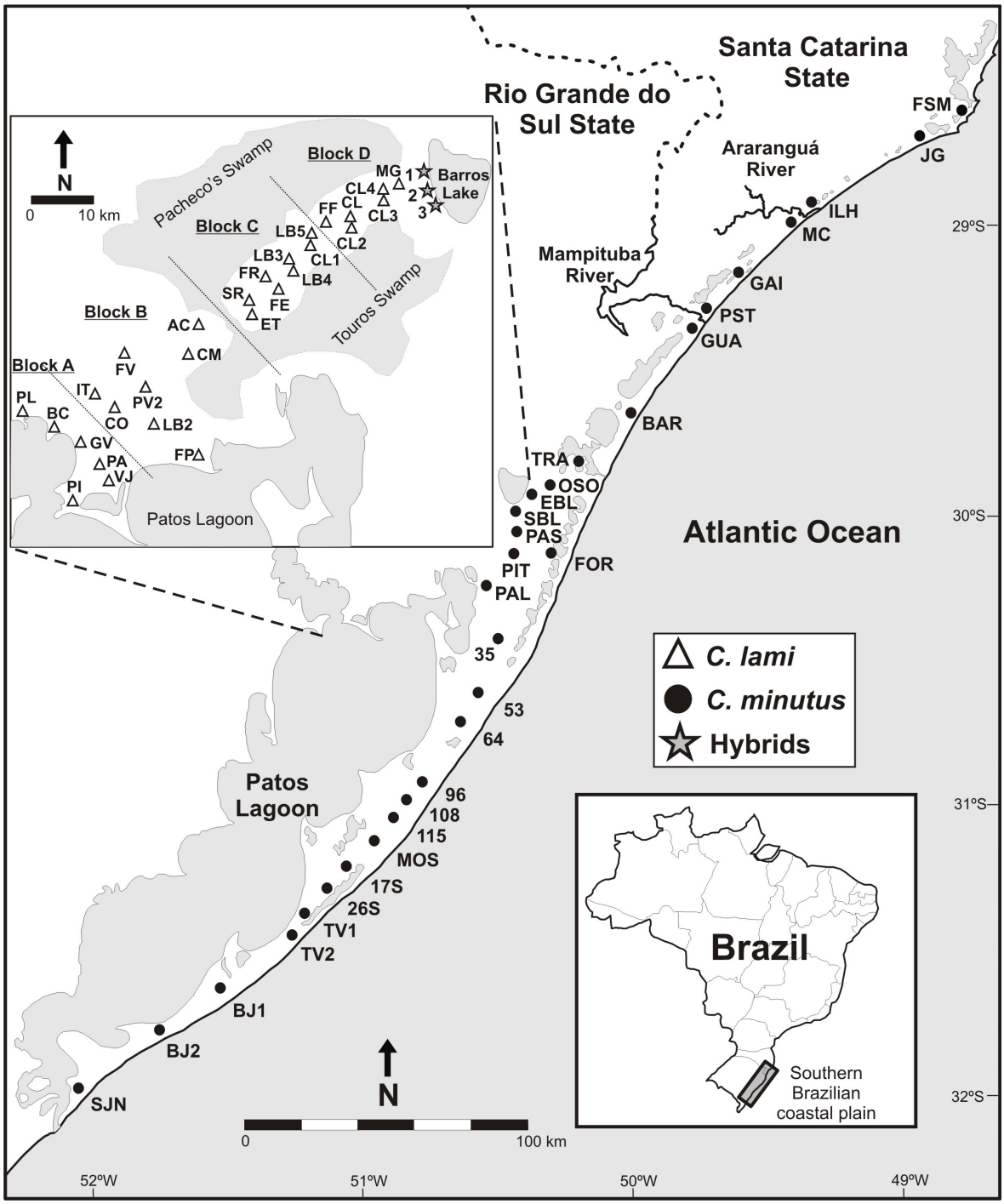


Figure 1. Sampling sites of *C. lami*, *C. minutus*, and possible hybrids between them on the southern Brazilian coastal plain. Locality abbreviations correspond to those in Table 1.

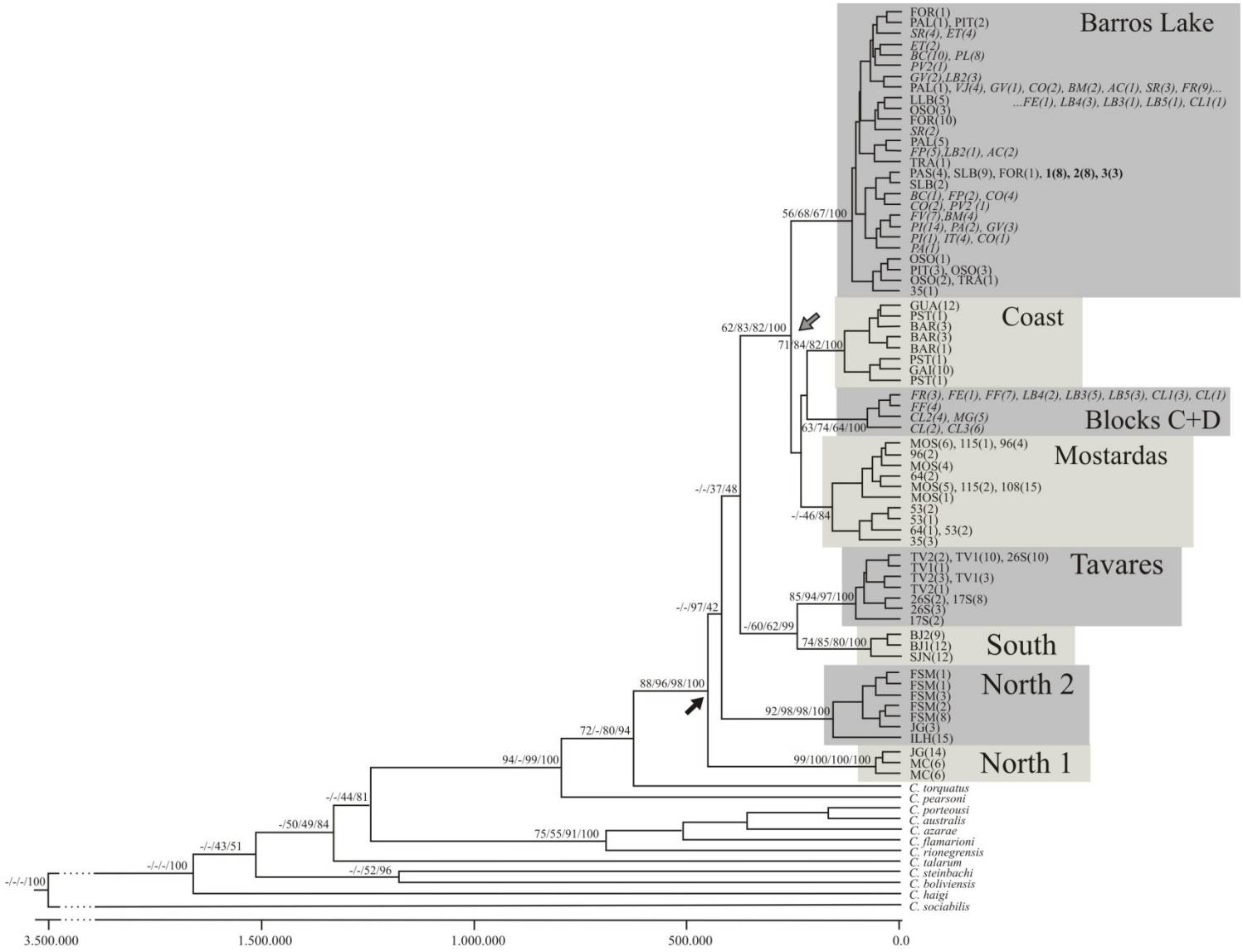

Barros Lake
Coast
Blocks C+D
Mostardas
Tavares
South
North 2
North 1
FOR(1)
PAL(1), PIT(2)
SR(4), ET(4)
ET(2)
BC(10), PL(8)
PV2(1)
GV(2),LB2(3)
PAL(1), VJ(4), GV(1), CO(2), BM(2), AC(1), SR(3), FR(9)...
LLB(5)
...FE(1), LB4(3), LB3(1), LB5(1), CL1(1)
OSO(3)
FOR(10)
SR(2)
PAL(5)
FP(5),LB2(1), AC(2)
TRA(1)
PAS(4), SLB(9), FOR(1), 1(8), 2(8), 3(3)
SLB(2)
BC(1), FP(2), CO(4)
CO(2), PV2 (1)
FV(7),BM(4)
PI(14), PA(2), GV(3)
PI(1), IT(4), CO(1)
PA(1)
OSO(1)
PIT(3), OSO(3)
OSO(2), TRA(1)
35(1)
GUA(12)
PST(1)
BAR(3)
BAR(3)
BAR(1)
PST(1)
GAI(10)
PST(1)
FR(3), FE(1), FF(7), LB4(2), LB3(5), LB5(3), CL1(3), CL(1)
FF(4)
CL2(4), MG(5)
CL(2), CL3(6)
MOS(6), 115(1), 96(4)
96(2)
MOS(4)
64(2)
MOS(5), 115(2), 108(15)
MOS(1)
53(2)
53(1)
64(1), 53(2)
35(3)
TV2(2), TV1(10), 26S(10)
TV1(1)
TV2(3), TV1(3)
TV2(1)
26S(2), 17S(8)
26S(3)
17S(2)
BJ2(9)
BJ1(12)
SJN(12)
FSM(1)
FSM(1)
FSM(3)
FSM(2)
FSM(8)
JG(3)
ILH(15)
JG(14)
MC(6)
MC(6)
C. torquatus
C. pearsoni
C. porteousi
C. australis
C. azarae
C. flamarioni
C. rionegrensis
C. talarium
C. steinbachi
C. boliviensis
C. haigi
C. sociabilis
56/68/67/100
62/83/82/100
71/84/82/100
63/74/64/100
-/-/37/48
-/-46/84
-/-/97/42
-/60/62/99
85/94/97/100
74/85/80/100
88/96/98/100
92/98/98/100
72/-/80/94
94/-/99/100
99/100/100/100
-/-/44/81
75/55/91/100
-/50/49/84
-/-/43/51
-/-/-/100
-/-/52/96
-/-/-/100
3.500.000
1.500.000
1.000.000
500.000
0.0

Figure 2. Bayesian phylogenetic tree of mitochondrial DNA concatenated data (control region and cytochrome c oxidase subunit I) for *C. lami* (italics), *C. minutus* (normal font), and possible hybrids (bold). Sampling sites are abbreviated according to Table 1, and are followed by the corresponding number of individuals, in parentheses. Support nodes are indicated by values above branches for MP/NJ/ML/BI, respectively, - indicates absence of the branch according to the methodology analyzed. The eight main mtDNA clades are highlighted by gray squares. Black arrow indicates the most recent common ancestor between all *C. minutus* and *C. lami* individuals, and gray arrow indicates the node representing the splintting process between the two species. Bottom rule shows the divergence time in years.

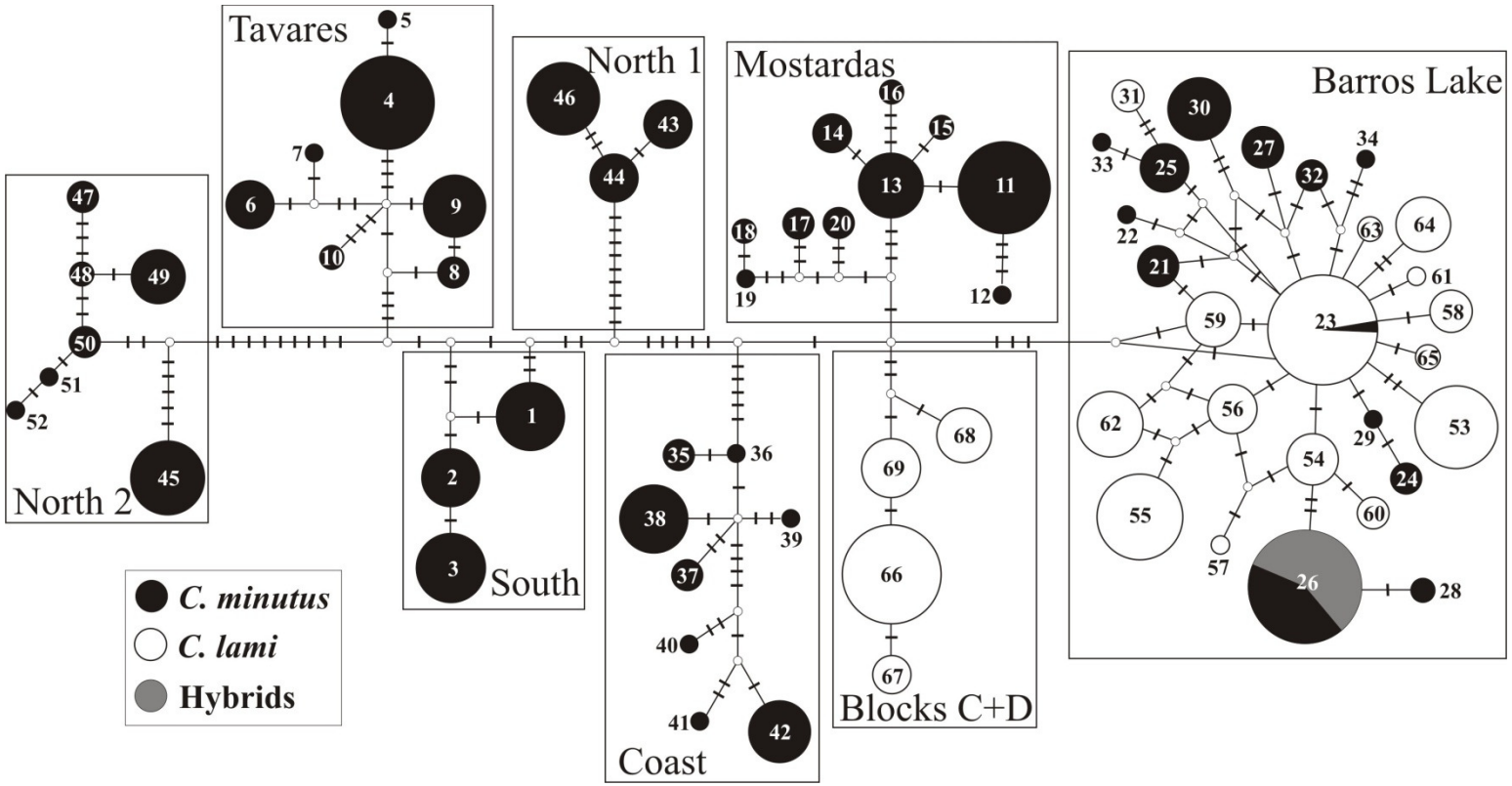


Figure 3. Median-joining haplotype network of mitochondrial DNA concatenated data. The colors represent the species and hybrids, as in the legend. The area of the circles is proportional to the haplotype frequency, each trait represents one mutational step, and small white dots correspond to unsampled haplotypes. Haplotype numbers correspond to those in Table 1. The eight main haplogroups are indicated by squares.

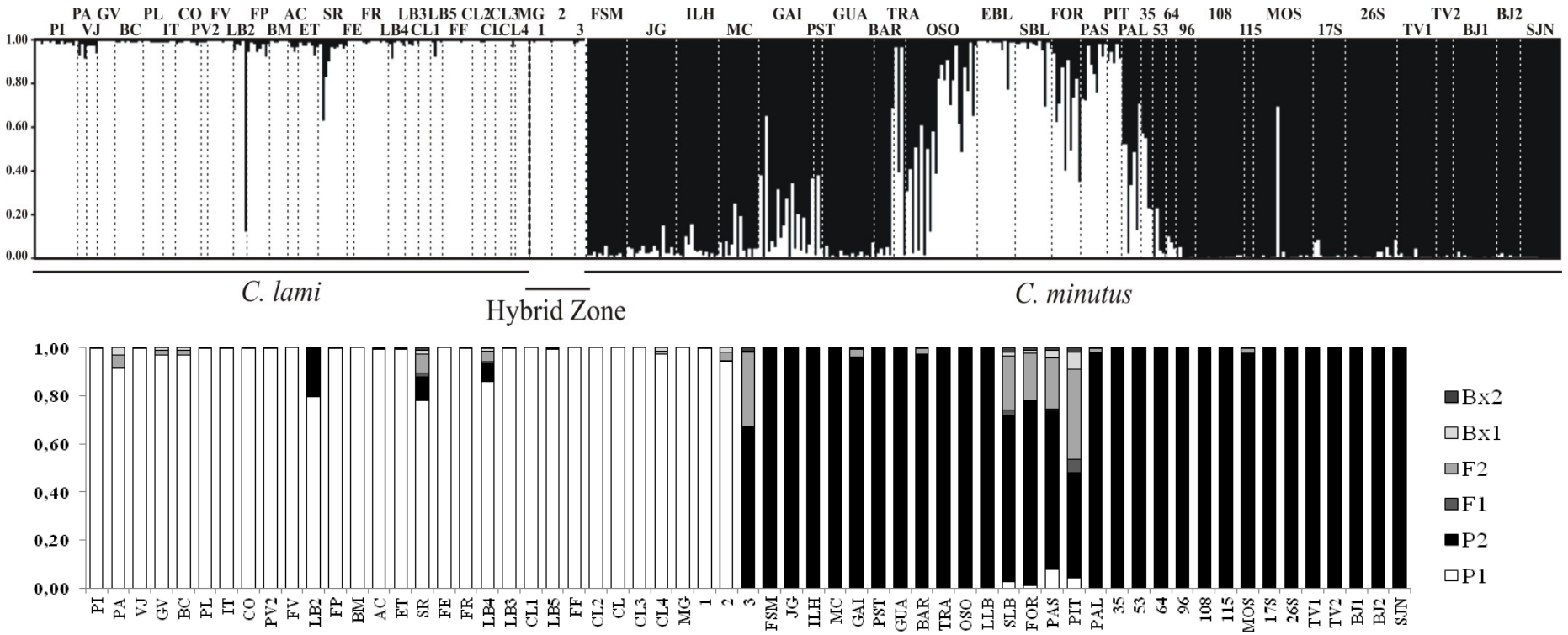


Figure 4. **Above:** Plot of Bayesian mixture analysis for individual assignment of species and hybrids performed with Structure. Each individual is identified by a single vertical bar, and each parental cluster by a color (white: *C. lami*; black: *C. minutus*). Above the plot are the abbreviations for the corresponding sampling sites, and below are the species and hybrids identifications. **Below:** Plot of Bayesian mixture analysis to identify the genetic category of individuals performed in NewHybrids: P1 – parental 1(*C. lami*); P2 – parental 2 (*C. minutus*); F1 – first generation of hybrid offspring; F2 - the offspring of F1; Bx1- backcrossing between F1 with P1; and Bx2 - backcrossing between F1 with P2. Each bar represents one sampling site, and each color represents one genetic category, as in the legend.

TABLES

Table 1. Locality names and abbreviations in parentheses, total number of individuals per locality (n), diploid and arm numbers [2n(AN)], and the number of concatenated mtDNA haplotypes retrieved in each locality. *Ctenomys lami* localities are in italics, those of putative hybrids in bold, and *C. minutus* localities in normal font. mtDNA clusters – BL: Barros Lake; BC+D: Blocks C+D; N2: North 2; N1: North 1; CO: Coastal; MO: Mostardas; TA: Tavares; SO: South.

Locality name	mtDNA clusters	n	2n(AN)	CC haplotypes
Parque Itapuã (PI)	BL	17	54(76,78)/55a(78,80)	CC55=14/CC56=1
Passo da Areia (PA)	BL	3	54(76,78)/55a(76)	CC55=2/CC57=1
Varzinha do Jacaré (VJ)	BL	4	54	CC23=4
Gravatá (GV)	BL	6	56a	CC55=3/CC23=1/CC58=2
Beco do Cego (BC)	BL	11	54(76,82)/55a(76,78,80)/56a (76,78,80)	CC53=10/CC54=1
Praia do Lami (PL)	BL	8	-	CC53=8
Itapuã (IT)	BL	4	58(78,80,83)	CC56=4
Costa do Oveiro (CO)	BL	9	58(78,79)	CC54=4/CC56=1/CC543= 2/CC60=2
Passo do Vigário 2 (PV2)	BL	2	58(80,82)	CC60=1/CC61=1
Fervura (FV)	BL	9	54/58	CC62=7
Lombas 2 (LB2)	BL	5	57(77)/58(78,80,86)	CC58=2/CC59=1
Fazenda Pimenta (FP)	BL	8	58(78)	CC54=2/CC59=5
Beco da Macega (BM)	BL	6	58	CC23=2/CC62=4
Águas Claras (AC)	BL	5	58(78,80)	CC23=1/CC59=2
Estiva (ET)	BL	7	54(74,76,77,78,79)/55a(76)	CC64=4/CC65=2
Sanga da Rapadura (SR)	BL	10	54	CC23=3/CC63=2/CC64=4
Fazenda do Estácio (FE)	BL/BC+D	2	54	CC23=1/CC66=1
Fazenda Rita Maria (FR)	BL/BC+D	12	54	CC23=9/CC66=3
Lombas 4 (LB4)	BL/BC+D	6	54(75,76,77)	CC23=3/CC66=2
Lombas 3 (LB3)	BL/BC+D	6	54(75,76,78)	CC23=1/CC66=5
Chico Lomã 1 (CL1)	BL/BC+D	4	54	CC23=1/CC66=3
Lombas 5 (LB5)	BL/BC+D	4	54(75,76,78)	CC23=1/CC66=3
Fazenda dos Freitas (FF)	BC+D	12	56b(78,80,82)	CC66=7/CC67=4
Chico Lomã 2 (CL2)	BC+D	4	-	CC69=4
Chico Lomã (CL)	BC+D	3	56b(80)	CC66=1/CC68=2

Chico Lomã 3 (CL3)	BC+D	6	56b	CC68=6
Chico Lomã 4 (CL4)	BC+D	1	56b	-
Morro Grande (MG)	BC+D	5	54	CC69=5
1	BL	8	53(74)/54(78)/55(76)/56(78, 80)	CC26=8
2	BL	8	50(74,76)/53(74,80)/54(78,80)	CC26=8
3	BL	3	48(76)/50(76)	CC26=3
Farol de Santa Marta (FSM)	N2	15	50a(76)	CC48=2/CC49=8/CC50=3/CC51=1/CC52=1
Jaguaruna (JG)	N1/N2	17	48c(76)/49a(76)/50a(76)	CC46=14/CC47=3
Ilhas (ILH)	N2	15	48c(76)	CC45=15
Morro dos Conventos (MC)	N1	14	46a(76)	CC43=6/CC44=6
Gaivota Beach (GAI)	CO	19	46a(76)	CC42=10
Passo de Torres (PST)	CO	3	46a(76)	CC39=1/CC40=1/CC41=1
Guarita Beach (GUA)	CO	18	46a(76)	CC38=12
Barco Beach (BAR)	CO	7	46a(76)	CC35=3/CC36=1/CC37=3
Tramandaí (TRA)	BL	4	46a(76)	CC31=1/CC34=1
Osório (OSO)	BL	25	46a(76)	CC25=3/CC31=2/CC32=3/CC33=1
East Barros Lake (EBL)	BL	13	46a(76)/47a(76)/48a(76)	CC27=5
South Barros Lake (SBL)	BL	13	47a(76)/48a(76)	CC26=9/CC28=2
Passinhos (PAS)	BL	9	48a(76)	CC26=4
Pitangueira (PIT)	BL	5	48a(76)	CC24=2/CC25=3
Fortaleza Lake (FOR)	BL	12	47a(76), 48a(76)	CC26=1/CC29=1/CC30=10
Palmares do Sul (PAL)	BL	7	48a(76)	CC21=5/CC23=1/CC24=1
Road km 35 (35)	BL/ MO	4	48a(76)	CC20=3/CC22=1
Road km 53 (53)	MO	5	48a(76)	CC17=2/CC18=2/CC19=1
Road km 64 (64)	MO	3	48a(76)	CC16=2/CC17=1
Road km 96 (96)	MO	7	48a(76)	CC13=4/CC15=2
Road km 108 (108)	MO	17	42(68,69,70,71,72,73,74) / 43(70,72,73,74,75) / 44(72,73,74,75,76) / 45(74,75,76,78,80) / 46(71,74,76,77,78)	CC11=15
Road km 115 (115)	MO	3	42(74)	CC11=2/CC13=1
Mostardas (Mos)	MO	21	42(74)	CC11=5/CC12=1/CC13=6/CC14=4
17 Km south of Mostardas (17S)	TA	11	42(74)/43(74)	CC9=8/CC10=2
26 Km south of Mostardas (26S)	TA	18	42(74)/43(70,72,74)/44(74)/46b(76)	CC4=10/CC8=3/CC9=2
Tavares 1 (TV1)	TA	14	46b(76)	CC4=10/CC5=1/CC6=3
Tavares 2 (TV2)	TA	6	46b(76)/47b(76)	CC4=2/CC6=3/CC7=1

Bujuru 1 (BJ1)	SO	15	48b(76,78)	CC3=12
Bujuru 2 (BJ2)	SO	9	49b(76,77)/50b(76)	CC2=9
São José do Norte (SJN)	SO	13	50b(76,77)	CC1=12

Table 2. Genetic diversity for *C. lami*, *C. minutus* and putative hybrids using the mtDNA concatenated data (1018bp between the control region and cytochrome c oxidase subunit I). Number of samples (N), number of haplotypes (N°H), polymorphic sites (Pol. Sites), average nucleotide differences (k), nucleotide diversity (π), and haplotype diversity (*Hd*).

Identification	N	N°H	Pol. Sites	k	π	*Hd*
C. lami	166	18	21	4.63	0.00456(±0.00021)	0.908(±0.009)
C. minutus	276	52	87	15.59	0.01533(±0.00025)	0.965(±0.003)
Hybrids	19	1	0	0.00	0.00000(±0.00000)	0.000(±0.000)

Table 3. Pairwise F_{ST} estimates among sampling sites located around the hybridization region, more than four individuals. Above diagonal: concatenated mtDNA data estimates; below diagonal: microsatellite loci estimates. Values in bold are significant after Bonferroni correction.

	FR	LB4	LB3	CL1	LB5	FF	CL2	CL3	MG	TRA	OSO	LLB	SLB	FOR	PSS	PIT	PAL	1	2
FR		-0.10	0.43	0.29	0.29	**0.70**	0.58	0.55	0.60	-	**0.31**	0.47	**0.64**	**0.54**	0.54	0.23	0.28	**0.62**	**0.62**
LB4	**0.13**		0.19	-0.01	-0.01	0.61	0.43	0.47	0.48	-	0.35	0.54	**0.72**	0.58	0.57	0.26	0.34	0.70	0.70
LB3	0.08	0.09		-0.24	-0.24	0.14	0.25	0.60	0.29	-	**0.69**	0.83	**0.88**	**0.79**	0.83	0.68	0.73	**0.88**	**0.88**
CL1	0.17	0.29	0.11		-0.33	0.24	0.20	0.53	0.26	-	0.61	0.78	0.86	0.74	0.78	0.58	0.65	0.86	0.86
LB5	0.08	0.09	0.01	0.18		0.24	0.20	0.53	0.26	-	0.61	0.78	0.86	0.74	0.78	0.58	0.65	0.86	0.86
FF	**0.22**	**0.28**	**0.17**	0.17	0.25		0.73	0.90	**0.75**	-	**0.85**	0.97	**0.96**	**0.90**	0.97	0.89	**0.90**	**0.97**	**0.97**
CL2	0.12	0.18	0.11	0.18	0.17	0.21		1.00	0.00	-	0.78	1.00	0.98	**0.86**	1.00	0.83	0.87	1.00	1.00
CL3	**0.16**	0.23	0.12	0.24	0.15	**0.21**	0.09		1.00	-	0.76	1.00	**0.97**	**0.86**	1.00	0.85	0.88	1.00	1.00
MG	**0.22**	0.30	0.24	0.41	0.26	**0.37**	0.20	0.24		-	0.80	1.00	**0.98**	0.87	1.00	0.85	0.88	1.00	1.00
TRA	0.31	0.22	0.25	0.40	0.26	0.42	0.33	0.39	0.49		-	-	-	-	-	-	-	-	-
OSO	**0.25**	**0.20**	**0.20**	**0.31**	**0.20**	**0.33**	**0.28**	**0.29**	**0.37**	0.12		0.55	**0.74**	**0.59**	0.66	0.11	**0.48**	**0.73**	**0.73**
LLB	**0.35**	**0.30**	**0.31**	0.44	0.31	**0.45**	**0.38**	**0.42**	**0.48**	0.14	**0.19**		**0.96**	**0.68**	1.00	0.70	0.76	1.00	1.00
SLB	**0.31**	**0.27**	**0.24**	0.36	0.22	**0.38**	0.31	**0.31**	**0.36**	0.28	**0.20**	**0.31**		0.82	-0.04	0.81	**0.84**	0.06	0.06
FOR	**0.28**	**0.25**	**0.21**	0.35	0.23	**0.35**	0.31	**0.29**	**0.37**	0.30	**0.22**	**0.33**	**0.28**		0.77	0.63	**0.58**	**0.82**	**0.82**
PSS	**0.29**	**0.21**	0.20	0.35	0.20	**0.37**	0.28	**0.28**	0.35	0.27	**0.19**	**0.32**	**0.15**	**0.25**		0.73	0.79	0.00	0.00
PIT	**0.19**	0.23	0.16	0.30	0.17	0.29	0.22	0.18	0.33	0.30	**0.21**	**0.34**	**0.21**	0.21	0.17		0.45	0.82	0.82
PAL	**0.38**	0.30	0.33	0.46	0.33	**0.47**	0.38	0.40	0.48	0.27	**0.22**	**0.37**	**0.37**	**0.29**	**0.21**	0.30		**0.85**	**0.85**
1	**0.25**	0.29	0.22	0.40	0.25	**0.36**	0.26	0.27	0.25	0.43	**0.31**	**0.41**	**0.30**	**0.38**	**0.26**	0.31	**0.43**		0.00
2	**0.28**	0.29	0.25	0.43	0.27	**0.38**	0.28	**0.28**	0.29	0.43	**0.29**	**0.41**	**0.24**	**0.39**	**0.23**	0.30	**0.44**	0.05	

SUPLEMENTARY DATA

Table S1. Karyotypes of individuals from the western shore of Barros Lake. Sampling sites, number of specimens per karyotype (No. S), sex, diploid numbers (2n), autosomal arm numbers (AN), number of biarmed chromosome pairs (Bi), number of acrocentric chromosome pairs (Ac), and the heteromorphic chromosome pair (HP) are shown.

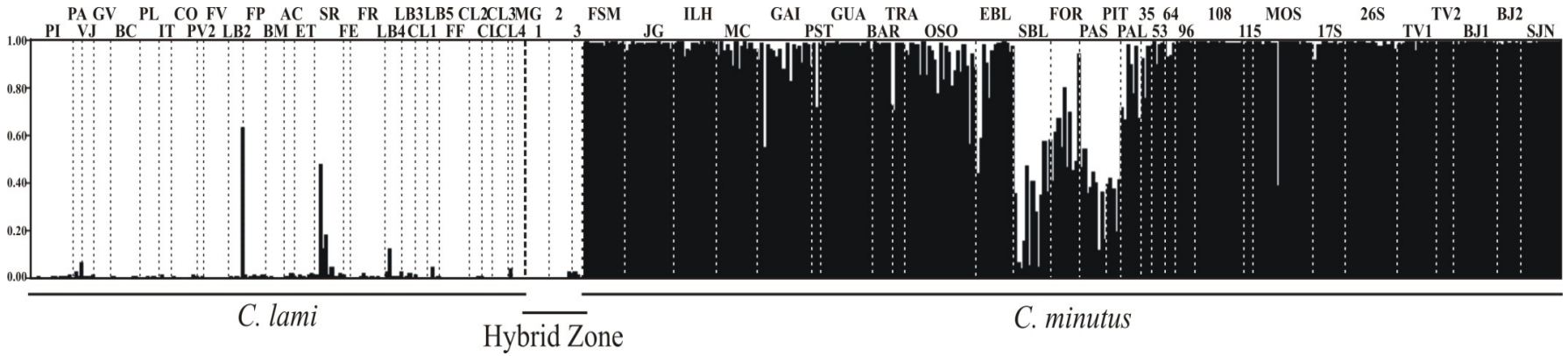
Sampling sites	No. S	Sex	2n	AN	Bi	Ac
1	2	1 M / 1 F	56*	80	26	28
	2	M	56*	78	24	30
	2	F	55*	76	23	30
	1	F	54	78	26	26
	1	F	53	74	23	28
2	1	F	54	80	28	24
	1	F	54	78	26	26
	2	1 M / 1 F	53	80	27	24
	1	F	53	74	23	28
	1	F	50	76	28	20
	1	M	50	74	26	22
3	1	F	50	76	28	20
	2	1 M / 1 F	48±	76	30	16

*Karytotypic forms previously described for *C. lami*

± Karytotypic form previously described for *C. minutus*

Figure S1. Karyotypes obtained in the interspecific hybrid zone between *C. lami* and *C. minutus*.

Figure S2. Plot of Bayesian mixture analysis of the most likely number of clusters [k = 2, based on ΔK approach of Evanno et al. (2005)], performed with Structure, testing ten independent runs from K=1-25, and no a priori population information. Each individual is identified by a single vertical bar, and each cluster by a color. Above the plot are the abbreviations of the corresponding sampling sites, and below are the species and hybrids identifications.



CAPÍTULO V

DISCUSSÃO GERAL

Neste estudo foram abordadas diversas questões relacionadas aos padrões filogeográficos e à estrutura populacional de *C. lami* e *C. minutus* na planície costeira do Sul do Brasil. Alguns tópicos receberam destaque nos capítulos II e III, como o papel de potenciais barreiras geográficas, eventos geológicos e rearranjos cromossômicos na estruturação da variabilidade genética de populações dessas espécies e a associação de fatores intrínsecos da biologia dos organismos, como dispersão, com os padrões observados de estrutura populacional e distribuição da diversidade genética. No capítulo IV foram resgatados e analisados em conjunto os dados utilizados nos capítulos II e III, com o intuito de propor um panorama a respeito da ocupação, estabelecimento e diferenciação dessas duas espécies na planície costeira do Sul do Brasil, bem como compreender melhor as questões relacionadas, com a agora confirmada, zona de hibridação interespecífica.

Tanto em *C. lami* quanto em *C. minutus* alguns padrões encontrados foram similares e se apresentam influenciados pelos mesmos fatores. Em ambas as espécies as descontinuidades no ambiente agem como um dos principais fatores estruturantes da variabilidade genética nas populações, representadas principalmente por cursos de água mais volumosos. No caso de *C. lami* os diferentes marcadores moleculares analisados (DNAmt e *loci* de microssatélites) demonstraram que as populações se encontram estruturadas em dois demes, que apesar de não serem completamente isolados, apresentam fluxo gênico reduzido e sua separação é reforçada pela presença da conexão entre o Banhado dos Pachecos e o Banhado dos Touros. Em *C. minutus* a subdivisão da maior parte dos haplogrupos obtidos através dos dados concatenados do DNAmt e alguns dos grupos obtidos pelas análises dos *loci* de microssatélites, foram associados à presença de barreiras geográficas, dentre as quais podemos destacar o rio Araranguá, a transição entre 1ª e 2ª linha de dunas e os paleocanais dos rios Jacuí e Camaquã.

Ctenomídeos são conhecidos por sua baixa capacidade natatória, neste sentido, cursos de água mais volumosos podem representar barreiras geográficas efetivas impedindo a livre dispersão dos indivíduos no ambiente (Reig *et al.,* 1990). Em *C.*

flamarioni a desembocadura das lagunas de Tramandaí e dos Patos foram indicadas como barreiras efetivas ao fluxo gênico, sendo responsáveis por explicar grande parte da divergência genética encontrada ao longo da distribuição da espécie. (Fernández-Stolz, 2007). Em *C. talarum* a subdivisão da variabilidade genética em grupos regionais foi atribuída à presença rios maiores que interrompem sua distribuição geográfica (Mora *et al.,* 2007).

Ctenomys lami e *C. minutus* também apresentaram um padrão de diferenciação populacional seguindo um modelo de isolamento pela distância, que apesar de mais evidente em alguns trechos da distribuição geográfica, foi confirmado através dos testes de Mantel, e da distribuição clinal da variação genética, com haplótipos ocorrendo normalmente em um único ponto de coleta (alelos privados) ou sendo compartilhados principalmente entre localidades vizinhas, um padrão muitas vezes observado entre os diferentes alelos de microssatélites. O modelo de isolamento pela distância é um padrão geralmente encontrado entre as populações de roedores subterrâneos, incluindo os tuco-tucos (Busch *et al*., 2000), principalmente em espécies que habitam faixas estreitas ao longo da linha da costa, como nos casos de *C. pearsoni*, *C. flamarioni* e *C. australis* (Mora *et al.,* 2006; Tomasco & Lessa, 2007; Fernández-Stolz, 2007). Esse padrão recorrente de isolamento pela distância em ctenomídeos tem sido atribuído à baixa capacidade de dispersão dos espécimes, principalmente nos adultos, associado à distribuição das populações que normalmente têm um tamanho reduzido e se distribuem no ambiente de forma fragmentada, ocupando habitats que apresentem condições favoráveis, o que geralmente promove o estabelecimento de pequenas unidades genéticas, com baixos níveis de variabilidade dentro das populações, porém grande divergência entre estas (Lacey, *et al*., 2000; Wlasiuk *et al*., 2003; Mora *et al.,* 2006; Fernández-Stolz, 2007; Tomasco & Lessa, 2007)

Todas estas características associadas à ação da deriva genética favorecem também a fixação de novos rearranjos cromossômicos que surgem nas populações, e estes têm sido considerados como responsáveis por desempenhar um papel chave na diversificação e especiação do gênero *Ctenomys* (Reig *et al.,* 1990; Lessa & Cook, 1998; Wlasiuk *et al*., 2003). No entanto, os resultados obtidos através deste estudo não evidenciaram associações diretas entre mudanças significativas na estrutura genética com os diferentes rearranjos cromossômicos encontrados tanto em *C. lami* quanto em *C. minutus*,

demonstrando que os diferentes cariótipos presentes nestas espécies desempenharam, até o momento, apenas um papel secundário na estrutura genética das populações.

Os resultados obtidos através das análises realizadas no capítulo IV revelaram a ausência de clados reciprocamente monofiléticos entre *C. lami* e *C. minutus*, porém, este dado não foi considerado como evidência suficiente para agrupar as duas espécies em uma unidade taxonômica comum, pois todas as outras análises com relação aos dados citogenéticos, os padrões obtidos através de análises dos *loci* de microssatélites e até mesmo as diferenças na morfologia do crânio e mandíbula (Freitas, 2001; Fornel, 2010) e a distinção dos habitats que ocupam, confirmam seus *status* como espécies irmãs distintas, pertencentes ao grupo *torquatus*, definido por Parada *et al*. (2011). As árvores filogenéticas e as estimativas de datação demonstraram que o ancestral comum mais recente entre as duas espécies data de aproximadamente 478 mil anos e que as duas espécies começaram a se diferenciar há ~ 278 mil anos. Além disso, as formas ancestrais que deram origem aos espécimes atuais de *C. minutus* foram provavelmente as primeiras a se diferenciarem e colonizarem a planície costeira do Sul do Brasil.

Ainda com relação ao capítulo IV, foram abordadas questões relativas à zona híbrida interespecífica. Os dados de DNAmt revelaram que todos os indivíduos considerados como híbridos apresentam o genoma mitocondrial proveniente de *C. minutus*, e em média 99% da composição dos *loci* de microssatélites analisados (segundo o programa Structure) são provenientes de *C. lami*. Esses dados nos permitem inferir que houve um cruzamento preferencial, se não exclusivo, ao menos nos primeiros estágios da formação dessa zona híbrida, entre fêmeas de *C. minutus* com machos de *C. lami*. Os dados demonstram também um introgressão substancial do genoma nuclear, no caso representado por *loci* de microssatélites, de *C. lami* nos espécimes de *C. minutus* amostrados nas proximidades da Lagoa dos Barros. Componentes genômicos com padrões distintos de herança (materno, paterno ou biparental) comumente apresentam diferenças nos padrões e níveis de introgressão em zonas de hibridação, sendo fortemente associado com a dispersão diferencial entre os sexos (Petit & Excoffier, 2009). Esse padrão de introgressão diferencial entre marcadores moleculares é conhecido como disjunção cito-nuclear do genoma e um dos exemplos melhor documentados na natureza foi descrito nos elefantes africanos de florestas e savanas por Ishida *et al*. (2011). Neste caso as fêmeas de elefantes se mantêm por toda a vida em seu grupo social natal enquanto os machos

dispersam entre diferentes bandos. Assim, enquanto o fluxo gênico do DNAmt entre os bandos é inexistente, os machos podem transmitir seus alelos nucleares do seu bando natal para outros bandos, seus descendentes podem transmitir esses mesmos alelos para bandos ainda mais distantes, e assim por diante, fazendo com que os alelos nucleares tenham o potencial de dispersar longas distâncias através do ambiente.

A zona de hibridação entre *C. lami* e *C. minutus* foi formada em consequência de alterações no habitat mediadas por intervenções humanas. Zonas híbridas não naturais requerem atenção de conservacionistas, pois podem comprometer a integridade genética e os processos evolutivos nos quais as espécies estão envolvidas (Rhymer & Sinberloff, 1996; Allendorf *et al*., 2001). Porém, uma vez que a hibridação entre essas espécies encontra-se em estágio avançado, com híbridos férteis cruzando entre si e com ambos parentais, torna-se difícil interromper este processo (Allendorf *et al*., 2001). Neste sentido, medidas conservacionistas devem ser tomadas focando conter o avanço da hibridação e introgressão e preservar a integridade de populações puras das duas espécies que não sofrem a influência desses eventos. Além disso, sugerimos que *C. minutus* seja incluído em listas vermelhas de espécies ameaçadas como quase ameaçado, e *C. lami* como vulnerável.

Aspectos importantes a respeito dos padrões filogeográficos, populacionais e de hibridação entre *C. minutus* e *C. lami* foram esclarecidos neste estudo, porém algumas questões permanecem em aberto e requerem o desenvolvimento de trabalhos paralelos a este, como por exemplo, o uso de diferentes técnicas para marcação de cromossomos a fim de investigar a fundo os rearranjos cromossômicos envolvidos nos polimorfismos encontrados nas duas espécies; o desenvolvimento de *primers* para amplificação do DNA de fragmentos dos cromossomos X e Y, no intuito de observar os padrões de herança e introgressão desses marcadores na zona híbrida interespecífica, a fim de comparar com os dados já obtidos; e juntamente com o depto de geologia da UFRGS propor locais ao longo da planície costeira para que sejam aprofundadas as investigações da existência de possíveis “paleobarreiras” geográficas.

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ANEXOS

Tabela 1. Lista de haplótipos e seus respectivos números de acesso no GenBank utilizados neste estudo para a região controladora, citocromo c oxidase subunidade I (COI) e dados concatenados do DNA mitocondrial. O número de coleção dos espécimes e a espécie correspondente a cada haplótipo são apresentados na tabela.

Espécie	Controladora	COI	Concatenado
C. minutus	**CR1 - HM236969** CF02, CF03, CF04, CF05, CF06, CF25, CF26, CF27, CF28, CF29, CF30, CF31	**COI1 - HM237009** CF02, CF03, CF04, CF05, CF06, CF25, CF26, CF27, CF28, CF29, CF30, CF31	**CC1** CF02, CF03, CF04, CF05, CF06, CF25, CF26, CF27, CF28, CF29, CF30, CF31
C. minutus	**CR2 - HM236970** CF49, CF50, CF51, CF52, CF53, CF54, CF55, CF56, CF57	**COI2 - HM237010** CF49, CF50, CF51, CF52, CF53, CF54, CF55, CF56, CF57, CF35, CF36, CF37, CF38, CF39, CF40, CF41, CF42, CF43, CF44, CF45, CF46	**CC2** CF49, CF50, CF51, CF52, CF53, CF54, CF55, CF56, CF57
C. minutus	**CR3 - HM236971** CF35, CF36, CF37, CF38, CF39, CF40, CF41, CF42, CF43, CF44, CF45, CF46	**COI3 - HM237011** CF08, CF09, CF10, CF11, CF12, CF13, CF14, CF15, CF18, CF20, CF21, CF58, CF61, TR461, TR462, TR463, TR464, TR1097, TR1099, TR1101, TR1102, TR1103, TR1106	**CC3** CF35, CF36, CF37, CF38, CF39, CF40, CF41, CF42, CF43, CF44, CF45, CF46
C. minutus	**CR4 - HM236972** CF08, CF09, CF10, CF11, CF12, CF14, CF15, CF18, CF20, CF21, CF58, CF61, TR461, TR462, TR463, TR464, TR1097, TR1099, TR1101, TR1102, TR1103, TR1106	**COI4 - HM237012** CF16, CF17, CF19, CF59, CF60, CF62, CF63	**CC4** CF08, CF09, CF10, CF11, CF12, CF14, CF15, CF18, CF20, CF21, CF58, CF61, TR461, TR462, TR463, TR464, TR1097, TR1099, TR1101, TR1102, TR1103, TR1106
C. minutus	**CR5 - HM236973** CF13	**COI5 - HM237013** TR1098, TR1100, TR1107, TR1104, TR1105, TR1086, TR1087, TR1088,	**CC5** CF13

		TR1089, TR1090, TR1091, TR1092, TR1094	
C. minutus	**CR6 - HM236974** CF16, CF17, CF19, CF60, CF62, CF63	**COI6 - HM237014** TR1095, TR1096	**CC6** CF16, CF17, CF19, CF60, CF62, CF63
C. minutus	**CR7 - HM236975** CF59	**COI7 - HM237015** TR867, TR868, TR869, TR870, TR878, TR871, TR872, TR874, TR876, TR877, TR879, TR873, TR880, TR855, TR856, TR853, TR852, TR854, TR857, TR858, TR863, TR865, TR866, C01, C02, C03, C04, C05, C06, C09, C11, C12, C13, TR848, TR849, TR850, TR851	**CC7** CF59
C. minutus	**CR8 - HM236976** TR1098, TR1100, TR1107	**COI8 - HM237016** TR875, 311, 314, 315, 316, TR408, TR410, TR411, TR412, TR416, TR419, TR414, TR417, TR418, 230	**CC8** TR1098, TR1100, TR1107
C. minutus	**CR9 - HM236977** TR1104, TR1105, TR1086, TR1087, TR1088, TR1089, TR1090, TR1091, TR1092, TR1094	**COI9 - HM237017** TR859, TR860	**CC9** TR1104, TR1105, TR1086, TR1087, TR1088, TR1089, TR1090, TR1091, TR1092, TR1094
C. minutus	**CR10 - HM236978** TR1095, TR1096	**COI10 - HM237018** TR846, TR847	**CC10** TR1095, TR1096
C. minutus	**CR11 - HM236979** TR867, TR868, TR869, TR870, TR875, TR878, TR852, TR854, TR857, TR858, TR863, TR865, TR866, C01, C02, C03, C04, C05, C06, C09, C11, C12, C13	**COI11 - HM237019** TR845, TR843, TR840, TR841, TR842, TR844	**CC11** TR867, TR868, TR869, TR870, TR878, TR852, TR854, TR857, TR858, TR863, TR865, TR866, C01, C02, C03, C04, C05, C06, C09, C11, C12, C13
C. minutus	**CR12 - HM236980** TR871, TR872, TR874, TR876, TR877, TR879, TR853, TR859, TR860, TR848, TR849, TR850, TR851	**COI12 - HM237020** TR837, TR838, TR839	**CC12** TR875
C. minutus	**CR13 - HM236981** TR873, TR880, TR855, TR856	**COI13 - HM237021** 325, 433, 321, 322, 323, 319	**CC13** TR871, TR872, TR874, TR876, TR877, TR879, TR853, TR848, TR849, TR850, TR851
C. minutus	**CR14 - HM236982** TR846, TR847	**COI14 - HM237022** TR836	**CC14** TR873, TR880, TR855 TR856

C. minutus	**CR15 - HM236983** TR845, TR843, TR840	**COI15 - HM237023** 318, TR444, TR447, TR448	**CC15** TR859, TR860
C. minutus	**CR16 - HM236984** TR841, TR842	**COI16 - HM237024** TR443, TR445, TR446, TR20, TR21, TR23, TR24	**CC16** TR846, TR847
C. minutus	**CR17 - HM236985** TR844	**COI17 - HM237025** 124, 435, 436, 442, TR209, 430, 438, 447	**CC17** TR845, TR843, TR840
C. minutus	**CR18 - HM236986** TR837, TR838, TR839	**COI18 - HM237026** 129, TR413	**CC18** TR841, TR842
C. minutus	**CR19 - HM236987** 325, 433, 321, 322, 323	**COI19 - HM237027** TR450, TR451, 293, 294, 326, 327, 448, 457, 460, 462	**CC19** TR844
C. minutus	**CR20 - HM236988** TR836, TR450, TR451, 293, 294, 326, 327, 448, 457, 460, 462	**COI20 - HM237028** 431, 444, TR35	**CC20** TR837, TR838, TR839
C. minutus	**CR21 - HM236989** 319, TR443, TR445, TR446, TR448, TR21, TR23, TR24	**COI21 - HM237029** TR33	**CC21** 325, 433, 321, 322, 323
C. minutus	**CR22 - HM236990** 318, TR444, TR447	**COI22 - HM237030** TR40, TR41, TR42	**CC22** TR836
C. minutus	**CR23 - HM236991** 311, 314, 315, 316, 129, TR408, TR410, TR411, TR412, TR413, TR416, TR419, TR414, TR417, TR418, 230	**COI23 - HM237031** TR1138, TR43, TR46, TR1137	**CC23** 319
C. minutus	**CR24 - HM236992** 124, 435, 436, 442, TR209	**COI24 - HM237032** L03, L04, L05, L06, L08, L07, L09, L11, L12, L13, L25, L26	**CC24** 318, TR444, TR447
C. minutus	**CR25 - HM236993** 431, 444, TR35	**COI25 - HM237033** TR212	**CC25** TR443, TR445, TR446, TR21, TR23, TR24
C. minutus	**CR26 - HM236994** 430, 438, 447, TR20, TR33	**COI26 - HM237034** 312	**CC26** 311, 314, 315, 316, TR408, TR410, TR411, TR412, TR416, TR419, TR414, TR417, TR418, 230
C. minutus	**CR27 - HM236995** TR40, TR41, TR42, TR1138	**COI27 - HM237035** 313	**CC27** 124, 435, 436, 442, TR209
C. minutus	**CR28 - HM236996** TR43, TR46, TR1137	**COI28 - HM237036** L14, L16, L17, L18, L19, L20, L21, L22, L24, L32	**CC28** 129, TR413
C. minutus	**CR29 - HM236997** L03, L04, L05, L06,	**COI29 - HM237037** 610, MC03, MC04,	**CC29** TR448

	L08, L07, L09, L11, L12, L13, L25, L26	MC08, MC09, TR1216	
C. minutus	**CR30 - HM236998** TR212	**COI30 - HM237038** MC02, MC05, MC10, TR1215, TR1217, TR1218, 281, 282, 291, TR01, TR07, TR03, TR08, TR04, TR06, TR1117, TR1118, TR1119, TR1120, TR1121	**CC30** TR450, TR451, 293, 294, 326, 327, 448, 457, 460, 462
C. minutus	**CR31 - HM236999** 312	**COI31 - HM237039** TR1122, TR1123, TR1124, TR1129, TR1130, TR1131, TR1132, TR1133, TR1134, TR1135, TR1136, TR1125, TR1126, TR1127, TR1128	**CC31** 431, 444, TR35
C. minutus	**CR32 - HM237000** 313	**COI32 - HM237040** 288, TR02, TR05	**CC32** 430, 438, 447
C. minutus	**CR33 - HM237001** L14, L16, L17, L18, L19, L20, L21, L22, L24, L32	**COI33 - HM237041** TR1200, TR1209, TR1210, TR1213, TR1214	**CC33** TR20
C. minutus	**CR34 - HM237002** 610, MC02, MC03, MC04, MC05, MC08, MC09, MC10, TR1215, TR1216, TR1217, TR1218	**COI34 - HM237042** TR1201, TR1202, TR1203, TR1204, TR1205, TR1206, TR1207, TR1208	**CC34** TR33
C. minutus	**CR35 - HM237003** TR1122, TR1123, TR1124, TR1129, TR1130, TR1131, TR1132, TR1133, TR1134, TR1135, TR1136, TR1125, TR1126, TR1127, TR1128	**COI34 - HM237043** TR1211, TR1212	**CC35** TR40, TR41, TR42
C. minutus	**CR36 - HM237004** 281, 282, 291, TR01, TR07, TR03, TR08, TR04, TR06, TR1117, TR1118, TR1119, TR1120, TR1121	-	**CC36** TR1138
C. minutus	**CR37 - HM237005** 288, TR02, TR05	-	**CC37** TR43, TR46, TR1137
C. minutus	**CR38 - HM237006** TR1200, TR1201, TR1202, TR1203, TR1204, TR1205, TR1206, TR1207, TR1208, TR1213	-	**CC38** L03, L04, L05, L06, L08, L07, L09, L11, L12, L13, L25, L26

C. minutus	**CR39 - HM237007** TR1209, TR1210, TR1211, TR1214	-	**CC39** TR212
C. minutus	**CR40 - HM237008** TR1212	-	**CC40** 312
C. minutus	-	-	**CC41** 313
C. minutus	-	-	**CC42** L14, L16, L17, L18, L19, L20, L21, L22, L24, L32
C. minutus	-	-	**CC43** 610, MC03 MC04 MC08, MC09, TR1216
C. minutus	-	-	**CC44** MC02, MC05, MC10, TR1215, TR1217, TR1218
C. minutus	-	-	**CC45** TR1122, TR1123, TR1124, TR1129, TR1130, TR1131, TR1132, TR1133, TR1134, TR1135, TR1136, TR1125, TR1126, TR1127, TR1128
C. minutus	-	-	**CC46** 281, 282, 291, TR01, TR07, TR03, TR08, TR04, TR06, TR1117, TR1118, TR1119, TR1120, TR1121
C. minutus	-	-	**CC47** 288, TR02, TR05
C. minutus	-	-	**CC48** TR1200, TR1213
C. minutus	-	-	**CC49** TR1201, TR1202, TR1203, TR1204, TR1205, TR1206, TR1207, TR1208
C. minutus	-	-	**CC50** TR1209, TR1210, TR1214
C. minutus	-	-	**CC51** TR1211
C. minutus	-	-	**CC52** TR1212
C. lami	**CR1 - JQ322885** BC01, BC02, BC04, BC07, BC08, BC09, BC10, BC12, M1127, M1129, TJ184, TJ185,	**COI1 - JQ322899** BC01, BC02, BC04, BC07, BC08, BC09, BC10, BC12, M1127, 1129, TJ184, TJ185,	**CC1** BC01, BC02, BC04, BC07, BC08, BC09, BC10, BC12, M1127, M1129, TJ184, TJ185,

	TJ186, TJ187, TJ188, TJ189, TJ190, TJ191	TJ186, TJ187, TJ188, TJ189, TJ190, TJ191	TJ186, TJ187, TJ188, TJ189, TJ190, TJ191
C. lami	**CR2 - JQ322886** BC05, TJ174, TJ176, TJ137, TJ138, TJ142, TJ144	**COI2 - JQ322900** BC05, TJ01, TJ07, TJ11, TJ25, TJ33, TJ41, TJ45, TJ50, TJ56, TJ60, TJ71, TJ82, NC130, 367, NC131, TJ152, TJ154, TJ155, TJ156, NC139, NC140, TJ145, TJ149, TJ151, TJ147, TJ172, TJ173, TJ175, TJ177, TJ178, TJ174, TJ176, NC124, NC125, NC126, NC133, NC134, NC136, TR202, TJ137, TJ138, TJ139, TJ140, TJ141, TJ142, TJ143, TJ144, TJ157, TJ158, TJ159, TJ180, TJ181, TJ182, TJ183, NC143, TJ160, TJ162, TJ163, TJ164, TJ165, TJ166, NC147, NC148, NC150, TJ128, TJ129, TJ130, TJ131, TJ132, TJ133, TJ135, ET01, NC171, NC172, NC175, TJ114, TJ115, TJ116, TJ117, TJ118, TJ119, TJ120, TJ122, TJ124, TJ126, NC108, NC112, 355, 340, NC180, TJ97	**CC2** BC05, TJ174, TJ176, TJ137, TJ138, TJ142, TJ144
C. lami	**CR3 - JQ322887** TJ01, TJ07, TJ11, TJ25, TJ33, TJ41, TJ45, TJ50, TJ56, TJ60, TJ71, TJ82, NC130, 367, NC139, NC140, TJ145, TJ149, TJ151	**COI3 - JQ322901** NC138	**CC3** TJ01, TJ07, TJ11, TJ25, TJ33, TJ41, TJ45, TJ50, TJ56, TJ60, TJ71, TJ82, NC130, 367, NC139, NC140, TJ145, TJ149, TJ151
C. lami	**CR4 - JQ322888** NC131, NC124, NC125, NC126, NC133, TJ140	**COI4 - JQ322902** TJ148, TJ150, NC141, NC142, NC144	**CC4** NC131, NC124, NC125, NC126, NC133, TJ140
C. lami	**CR5 - JQ322889** TJ152, TJ154, TJ155, TJ156, TJ147, TJ148, TJ150, TJ139, TJ141, NC141, NC142, NC144, TJ160, TJ163, NC148, TJ127, TJ128, TJ129, TJ130, TJ136, NC176, NC177, TJ114, TJ115, TJ116, TJ117, TJ118, TJ119, TJ120, TJ122, TJ124, TJ126, NC108, NC112, 355, 340, NC180, TJ97	**COI5 - JQ322903** TJ127, TJ136	**CC5** TJ152, TJ154, TJ155, TJ156, TJ147, TJ139, TJ141, TJ160, TJ163, NC148, TJ128, TJ129, TJ130, TJ114, TJ115, TJ116, TJ117, TJ118, TJ119, TJ120, TJ122, TJ124, TJ126, NC108, NC112, 355, 340, NC180, TJ97

C. lami	**CR6 - JQ322890** NC138	**COI6 - JQ322904** NC176, NC177	**CC6** NC138
C. lami	**CR7 - JQ322891** TJ172, TJ173, TJ175, TJ177, TJ178, NC143, NC147, NC150	**COI7 - JQ322905** TJ113, TJ121, TJ123, TJ125, NC101, NC102, NC103, NC104, NC105, NC106, NC107, 344, SA04, SA02, TR520, NC109, NC111, NC116, NC117, NC119, NC120, 372, NC173, NC178, NC179, TJ95, TJ98, TJ99, NC168	**CC7** TJ148, TJ150, NC141, NC142, NC144
C. lami	**CR8 - JQ322892** NC134, TR202, TJ143	**COI8 - JQ322906** NC167, NC169, TJ104, TJ105, TJ106, TJ107, TJ108, TJ109	**CC8** TJ172, TJ173, TJ175, TJ177, TJ178, NC143, NC147, NC150
C. lami	**CR9 - JQ322893** NC136	**COI9 - JQ322907** TJ100, TJ101, TJ102, TJ103, TJ167, TJ168, TJ169, TJ170, TJ171	**CC9** NC134, TR202, TJ143
C. lami	**CR10 - JQ322894** TJ157, TJ158, TJ159, TJ180, TJ181, TJ182, TJ183, TJ162, TJ164, TJ165, TJ166	**-**	**CC10** NC136
C. lami	**CR11 - JQ322895** TJ131, TJ132, TJ133, TJ135, ET01, NC171, NC172, NC175	**-**	**CC11** TJ157, TJ158, TJ159, TJ180, TJ181, TJ182, TJ183, TJ162, TJ164, TJ165, TJ166
C. lami	**CR12 - JQ322896** TJ113, TJ121, TJ123, TJ125, NC101, NC103, NC104, NC106, SA4, SA2, TR520, NC109, NC111, NC116, NC117, NC119, NC120, 372, NC173, NC178, NC179, TJ95, TJ98, TJ99, NC168, TJ100, TJ101, TJ102, TJ103, TJ167, TJ168, TJ169, TJ170, TJ171	**-**	**CC12** TJ127, TJ136
C. lami	**CR13 - JQ322897** NC102, NC105, NC107, 344	**-**	**CC13** TJ131, TJ132, TJ133, TJ135, ET01, NC171, NC172, NC175
C. lami	**CR14 - JQ322898** NC167, NC169, TJ104, TJ105, TJ106, TJ107, TJ108, TJ109	**-**	**CC14** NC176, NC177
C. lami	**-**	**-**	**CC15** TJ113, TJ121, TJ123, TJ125, NC101, NC103,

	-	-	NC104, NC106, SA04, SA02, TR520, NC109, NC111, NC116, NC117, NC119, NC120, 372, NC173, NC178, NC179, TJ95, TJ98, TJ99, NC168
C. lami	-	-	**CC16** NC102, NC105, NC107, 344
C. lami	-	-	**CC17** NC167, NC169, TJ104, TJ105, TJ106, TJ107, TJ108, TJ109
C. lami	-	-	**CC18** TJ100, TJ101, TJ102, TJ103, TJ167, TJ168, TJ169, TJ170, TJ171
Híbridos	**CR23 - HM236991** TR1219, TR1220, TR1221, TR1222, TR1223, TR1224, TR1225, TR1226, TR1227, TR1228, TR1229, TR1230, TR1231, TR1232, TR1233, TR1234, TR1273, TR1274, TR1275	**COI8 - HM237016** TR1219, TR1220, TR1221, TR1222, TR1223, TR1224, TR1225, TR1226, TR1227, TR1228, TR1229, TR1230, TR1231, TR1232, TR1233, TR1234, TR1273, TR1274, TR1275	**CC26** TR1219, TR1220, TR1221, TR1222, TR1223, TR1224, TR1225, TR1226, TR1227, TR1228, TR1229, TR1230, TR1231, TR1232, TR1233, TR1234, TR1273, TR1274, TR1275
C. torquatus	**HM443438** J209	**HM443439** J209	-
C. pearsoni	**JQ341031** CA369	**JQ341042** CA369	-
C. australis	**JQ341032** UNMDP3	**JQ341043** UNMDP3	-
C. rionegrensis	**JQ341033** EV1043	**JQ341044** EV1043	-
C. porteousi	**JQ341034** UNMDP10	**JQ341045** UNMDP10	-
C. azarae	**JQ341035** UNMDP7	**JQ341046** UNMDP7	-
C. steinbachi	**JQ341036** NK12133	**JQ341047** NK12133	-
C. boliviensis	**JQ341037** NK15367	**JQ341048** NK15367	-
C. talarum	**JQ341038** IF02	**JQ341049** IF02	-
C. haigi	**JQ341039** MVZ184888	**JQ341050** MVZ184888	-
C. sociabilis	**JQ341040** MVZ166425	**JQ341051** MVZ166425	-
C. flamarioni	**JQ341041** G83	**JQ341052** G83	-