UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ESTUDOS EVOLUTIVOS, FILOGEOGRÁFICOS E DE CONSERVAÇÃO EM UMA ESPÉCIE ENDÊMICA DO ECOSSISTEMA DE DUNAS COSTEIRAS DO SUL DO BRASIL, *Ctenomys flamarioni* (RODENTIA - CTENOMYIDAE), ATRAVÉS DE MARCADORES MOLECULARES MICROSSATÉLITES E DNA MITOCONDRIAL

Gabriela Paula Fernández-Stolz

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Professor Orientador: Thales Renato Ochotorena de Freitas

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RESUMO

Ctenomys flamarioni, comumente chamado tuco-tuco-das-dunas, é um roedor subterrâneo que habita a primeira linha de dunas costeiras do Estado do Rio Grande do Sul. Devido à pouca informação sobre este roedor e ao fato de ser uma espécie endêmica e ameaçada de extinção (principalmente pela redução e modificações antrópicas a que é submetido o ecossistema costeiro) o principal esforço desta tese foi centralizado na caracterização genética da variabilidade (e em como esta se encontra distribuída) em populações ao longo de toda a área de distribuição da espécie. Para isto foram utilizados dois tipos de marcadores moleculares, *loci* nucleares (nove *loci* de microssatélites) e seqüências de DNA mitocondrial (389 pares de bases da região controladora e 665 pares de bases do citocromo-*b*). Para três das dez populações amostradas foram incorporadas análises demográficas tanto a partir de dados de campo quanto a partir de dados genéticos através da utilização de *loci* de microssatélites. Para estas populações foram observadas diferenças significativas na proporção de machos e fêmeas (1:2, para os indivíduos adultos) e nas medidas utilizadas como estimativa de dimorfismo sexual (machos com maior peso e comprimento do corpo). Estas evidências sugerem um padrão de poliginia para *C. flamarioni*.

As análises de estrutura genética indicaram forte diferenciação entre as populações, mas não a nível intrapopulacional. As estimativas de dispersão a partir dos dados genéticos utilizando índices de *assignement* (F_{ST}, F_{IS}, *mAIc*, *vAIc*) e teste de AMOVA, mostraram diferenças não significativas entre machos e fêmeas, sugerindo dispersão de ambos os sexos. Todavia, a partir de estimativas de F_{ST} entre as populações mais próximas, foi inferida uma maior mobilidade dos machos. Quando a dispersão foi comparada entre as

populações, esta foi significativamente maior para fêmeas, na população que habita a área mais preservada, e com menor densidade de indivíduos, em relação às outras duas.

Para o total de populações, ambos os marcadores mostraram baixa variabilidade genética intrapopulacional, embora esta fosse mais crítica para o mtDNA. Para o conjunto de seqüências da região controladora mitocondrial (n = 89) analisadas foram obtidos sete haplótipos, enquanto que para as do citocromo-*b* (n = 45), cinco. Para os *loci* de microssatélites foi observado um número baixo de alelos por *loci* (entre três e oito). A variabilidade genética obtida foi divergente entre as nove populações estudadas, com valores de diversidade alélica que variaram de 1,1 a 3,6 e valores de heterozigosidade média de 0,21 a 0,70.

As populações do sul da distribuição apresentaram os menores valores de variabilidade: a maioria dos *loci* nucleares em estado monomórfico e baixo número de haplotipos para os *loci* mitocondriais. O baixo polimorfismo obtido para os microssatélites não tem permitido o uso de testes estatísticos para detectar gargalos de garrafa. Todavia, para três das seis populações analisadas foi verificada a existência de reduções recentes do tamanho populacional.

Os níveis de diferenciação genética entre populações foram maiores para os *loci* de microssatélites que para os de mtDNA, sugerindo assim baixo fluxo gênico atual e maior conectividade histórica entre as populações.

Através dos testes de AMOVA, para os dois tipos de marcadores genéticos utilizados, foi observada correlação positiva entre a estruturação da variabilidade e a presença de duas das três possíveis barreiras geográficas ao fluxo gênico testadas. Os testes de Mantel evidenciaram um padrão de isolamento pela distância quando todas as populações foram consideradas na análise, mas não a nível regional.

As árvores filogenéticas obtidas a partir dos diferentes métodos empregados evidenciaram relações pouco profundas entre os haplótipos, também sugeridas através das análises de Network. Estas últimas indicaram os haplótipos do norte da distribuição como os ancestrais, a partir dos quais teriam se originado os demais.

A partir das diferentes abordagens utilizadas para testar a expansão demográfica (*mismatch distribution*, teste de Tajima, Fu, e modelo de crescimento exponencial), foi evidenciado sinal positivo, embora fraco, de expansão. O baixo poder dos testes empregados pode estar evidenciando, para a espécie, um padrão mais complexo de ocupação de sua atual área de distribuição, no qual flutuações do tamanho populacional, tanto históricas como contemporâneas, teriam um papel fundamental na redução da variabilidade genética.

A partir de métodos semi e não-paramétricos foi estimado o tempo de divergência das principais linhagens filogenéticas de *C. flamarioni* (aproximadamente 90.000 anos) assim como uma taxa de mutação para o citocromo-*b* (μ = 0,019⁄ sítio ⁄ milhão de anos). Baseado nesta taxa de mutação, o tempo de ocorrência da principal expansão demográfica foi estimado em 60.000 anos.

A partir dos resultados obtidos para os marcadores moleculares utilizados, e com o aporte de informação dos polimorfismos cariotípicos e protéicos reportados para a espécie, foi proposto: (1) duas Unidades Evolutivamente Significativas (ESUs) para *C. flamarioni*, a primeira formada pelas populações das regiões I, II e III, e a segunda incluindo as populações pertencentes à região IV; e (2) que cada uma das populações estudadas se constitua em uma unidade de manejo independente.

ABSTRACT

The tuco-tuco-das-dunas, *Ctenomys flamarioni*, is a subterranean rodent that inhabits the first line of the coastal dunes in State of Rio Grande do Sul. Due to the lack of information about this endemic rodent, and the threatened situation of the species (mainly as result of the anthropogenic changes over native coastal ecosystem), the effort of this thesis was focalized on the genetic variability characterization, and its distribution, over the total range of the species. With this aim two kinds of molecular markers were used: nine nuclear microsatellites loci and mitochondrial DNA sequences (389 base pairs of the control region and 665 base pairs of the cytochrome-*b*). In three of the ten populations sampled demographical analyses were done both from field and genetical data. For adult individuals significant differences were observed toward a female-biased sex-ratio (1 male:2 female) and sexual dimorphism (males heavier and larger than females). These evidences support a hypothesis of polygyny in *C. flamarioni*.

Analysis of genetic structure revealed strong differentiation among populations, but no significant structure at the intrapopulation level. Non-significant values were obtained for the tested indexes, from assignment tests (F_{ST}, F_{IS}, *mAIc*, *vAIc*) as well as from AMOVA, showing no evidence of sex-biased dispersal over populations. Nevertheless, for the nearest populations, non significant pairwise F_{ST} for males suggested a slightly male-biased dispersal pattern. In regard to inter-population differences, significant differences were clearer in the dispersal patterns among females with more dispersal for the population at the most preserved habitat than the two others.

For all populations both microsatellites and mtDNA datasets showed low within-population genetic variation but, it was more critical for mtDNA. For the mitochondrial

control-region set of sequences (n = 89) a total of seven haplotypes were obtained, and for the cytochrome-*b* dataset (n = 45) five haplotypes. The nine microsatellite loci surveyed were polymorphic with variable number of alleles by locus, ranging from three to seven. The same was observed with the genetic variability: the population mean diversity for varied between 1.1 to 3.6 alleles, and the mean observed heterozygosity across all loci ranged from 0.21 to 0.70.

The three populations from the southern region of the range showed the lowest values of diversity: most of nuclear loci in monomorphic state and low number of haplotypes for the mtDNA datasets. The low number of polymorphic loci in these populations precluded the use of statistical test for bottleneck detection. However, for three of the six populations examined, a genetic signature of recent population size reduction was observed.

Levels of genetic differentiation among populations were higher for microsatellites than mitochondrial loci, suggesting lower gene flow at the present than in the past.

For both kinds of markers, the AMOVA analyses showed a substantial relationship between the genetic variation partitioning and two of the three hypothesized historical barriers to gene flow. Positive correlation between the genetic and geographic distances (isolation-by-distance pattern) was found when the total sampled range was considered but not at regional level.

Phylogenetic tree analyses showed a shallow relationship between haplotypes, also suggested by the network approach. The northern haplotypes were suggested as the most ancient.

Weak evidences of recent population expansion were obtained from the mismatch distribution, Tajima and Fu's tests and using an exponential grow model. The lack of

power of the applied tests may indicate by a complex pattern of the species occupation in its distribution range, in which historical and current reductions in population size possibly had a primary role in the reduction of genetic variability.

Further analyses from nonparametric and semiparametric methods estimated a divergence time of haplotypic lineages of 90,000 years ago, in the Late Pleistocene, and a rate of cytochrome-*b* evolution $\mu = 1.9\%$ per million years. Using this rate and assuming a stepwise scenario, the major increase in effective population size was estimated as occurring 60,000 years ago.

Despite the lack of phylogeographic information to define Evolutionarily Significant Units (ESUs), based on the patterns of variation and divergence from the two kinds of markers and the previous allozyme and karyotype studies we propose, (1) two ESU, the first including north (I) and central (II and III) regions, and the second including all populations at the south of the distribution (IV); and (2) that each of the populations sampled should constitute an independent management unit.

I. INTRODUÇÃO

I.1 O gênero *Ctenomys*

Os tuco-tucos, nome comum dado aos roedores subterrâneos inclusos dentro do gênero *Ctenomys,* pertencem à família Ctenomyidae e subordem Hystricognathi (Woods, 1982). A especiação no gênero *Ctenomys* é considerada uma das mais explosivas entre os gêneros de mamíferos (Cook & Lessa, 1998; Lessa & Cook, 1998), gerando 56 espécies distribuídas na chamada Região Neotropical, Sub-região Patagônica (Reig e cols., 1990), desde a Terra do Fogo, na Argentina, até o sul da Bolívia e Peru, e desde o nível do mar até mais de 4.000 metros de altitude nos Andes peruanos (Pearson, 1959; Novak, 1999). O surgimento do gênero *Ctenomys* foi atribuído ao centro da Argentina, durante o Plioceno Tardio - Pleistoceno (Contreras e cols., 1987; Reig e cols*.,* 1990; Lessa & Cook, 1998; Verzi e cols., 1999). Em estudos mais recentes, Verzi (2002) reporta *Ctenomys* fósseis em formações do Terciário (Formação Chapadmalalal, Plioceno Superior) na província de Buenos Aires (Argentina), implicando uma antigüidade para o gênero maior do que 3 milhões de anos (Ma). Subseqüentemente o gênero sofreu uma explosiva cladogênese, produzindo o grande número de espécies atuais, e se tornou dominante na exploração do nicho subterrâneo na Região Neotropical (Reig e cols*.,* 1990; Cook & Lessa, 1998; Lessa & Cook, 1998; Maschereti e cols., 2000).

Estes roedores passam a maior parte de suas vidas abaixo da superfície da terra, em sistemas de túneis (Nowak, 1999) que podem ser construídos por um ou por vários indivíduos (Lacey e cols., 1998; Lacey, 2000). A estrutura destes sistemas consiste de uma galeria principal e várias ramificações partindo desta, terminando em aberturas ou em um fundo cego. Ao longo das galerias são encontradas câmaras especializadas para depósito de

comida ou defecação (Nevo, 1979; Gallardo & Anrique, 1991; Altuna e cols., 1999; Busch e cols., 2000). Os túneis são mantidos fechados, o que proporciona não só proteção contra os predadores, mas também condições mais estáveis do que as do meio externo: menores flutuações de temperatura, alto grau de umidade relativa, concentrações de O_2 de 15 a 21% e de CO_2 de 0,5 a 2% (McNab, 1966).

A versatilidade de hábitats em que ocorrem estes roedores é, de fato, reflexo da estabilidade conferida pelo tipo de vida subterrâneo. Contudo, estudos mais detalhados de sua distribuição, mostram que os tuco-tucos apresentam uma tendência a viver em solos arenosos ou, no mínimo, bem arejados (Contreras, 1973). Isto não é só devido às restrições impostas pela dependência da atividade escavatória que realizam os indivíduos, mas também por restrições relacionadas com a manutenção do calor e o intercâmbio de gases através do solo (McNab, 1966; 1979; Contreras & McNab, 1990).

Os tuco-tucos apresentam adaptações morfológicas relacionadas ao seu hábito subterrâneo, como o corpo robusto e cilíndrico, a cabeça grande, a cauda curta, a abertura bucal atrás dos incisivos e unhas fortes. O pescoço e os membros são curtos e musculosos (Nevo, 1979; Reig e cols., 1990; Nowak, 1999).

A constatação da presença destes animais no campo pode ser feita pela observação de amontoados de areia que correspondem aos "tampões" que os indivíduos usam para fechar suas tocas (Pearson e cols., 1968).

Alimentam-se especialmente de gramíneas, são generalistas na maioria dos casos e têm grande influência sobre as comunidades de plantas das regiões que habitam (Nevo, 1979; Gallardo & Anrique, 1991; Zenuto & Busch, 1995; Borrel e cols., 1998; Altuna e cols., 1999; Busch e cols., 2000).

Outras características do gênero são a marcada territorialidade e baixa dispersão dos indivíduos (Busch e cols., 2000), assim como estar constituído principalmente por espécies solitárias, embora tenham sido descritas algumas espécies sociais e semi-sociais (Lacey e cols.,1997; Lacey 2000).

O gênero *Ctenomys* apresenta uma grande diversidade cariotípica, variando desde 2n = 10 em *C. steinbachi* a 2n = 70 em *C. pearsoni* (Reig & Kiblisky, 1969; Kiblisky e cols., 1977; Gallardo, 1979; Lessa & Langguth 1983; Freitas & Lessa, 1984; Ortells e cols., 1990; Ortells, 1995; Massarini e cols., 1991b; Freitas, 1990; 1994; 1997; Giménez e cols., 1997; 1999; Mascheretti e cols., 2000; Garcia e cols., 2000; Slamovits e cols., 2001). Esta importante amplitude cariotípica tem sido motivo de especulações a respeito dos mecanismos promotores de tal diversificação (Nevo 1999). Reig & Kiblisky (1969) foram os primeiros a propor o modelo de especiação cromossômica para este clado de mamíferos. A diversificação do grupo estaria facilitada pela formação de pequenos demes isolados, característica da estrutura populacional da maioria das espécies de tuco-tucos (Reig e cols., 1990; Busch e cols., 2000), e a ação da deriva gênica que permitiria a fixação rápida de rearranjos cromossômicos. Numerosos autores explicam a grande variabilidade cromossômica observada (tanto no gênero quanto dentro de muitas das espécies pertencentes ao gênero), assim como a alta diversificação na origem dos ctenomídeos através da estrutura populacional que caracteriza estes roedores (e.g., Reig & Kiblisky, 1969; Nevo, 1979). Todavia, e como contrapartida ao modelo clássico de diversificação cromossômica, Tomasco e cols. (*in press*), através da análise de um fragmento da região controladora do ácido desoxirribonucléico (DNA) mitocondrial para um conjunto de populações de *C. pearsoni* com diferente cariótipo, rejeitam a hipótese de fixação dos cariomorfos em populações de tamanho reduzido.

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

I.2 O grupo mendocinus

Este grupo de espécies de Ctenomídeos, denominado mendocinus por Massarini e cols. (1991a), foi originalmente formado por quatro espécies morfologicamente iguais (Massarini e cols., 1986) distribuídas na região central da Argentina: *C. mendocinus*, *C. azarae*, *C. australis* e *C. porteousi*, (Massarini e cols., 1991b; D'Elia e cols., 1998; 1999; Slamovits e cols., 2001; Castillo e cols., 2005). Estudos posteriores permitiram a introdução de duas espécies mais a esse grupo: *C. flamarioni*, do Brasil e *C. rionegrensis*, do Uruguai (Freitas, 1994; D'Elia e cols., 1999).

Além de ter morfologia similar, estas espécies compartilham o mesmo número cariotípico 2n = 47-48, padrões similares de bandas G e C e exibem heterocromatina constitutiva em blocos pericentroméricos nos braços curtos dos cromossomos (Massarini e cols., 1991a; 1991b; 1998). A forma dos espermatozóides é do tipo simples assimétrico (Feito & Gallardo, 1982; Altuna e cols., 1986; Vitullo e cols., 1988; Freitas, 1995b). Todavia, estudos filogenéticos baseados no seqüenciamento completo do citocromo-b sugerem que o tipo de espermatozóide assimétrico não define o clado mendocinus, por ter uma origem difilética (D´Elia e cols., 1999).

Rossi e cols. (1990) caracterizam o maior DNA satélite conhecido do gênero *Ctenomys*, chamado de RPCS (*Repetitive Pvu II Ctenomys Sequences*), e posteriormente o descrevem em espécies argentinas do grupo mendocinus (Rossi e cols., 1993).

Estudos citogenéticos, morfométricos e de morfologia de espermatozóides nos tuco-tucos deste grupo, sugerem a ocorrência de eventos de especiação em alopatria, nos quais rearranjos cromossômicos não têm um papel central (Massarini e cols., 1998). Isto contrasta com o modo predominante de especiação dentro do gênero: rápida cladogênese com rearranjos cromossômicos (Reig & Kiblisky, 1969; Ortells, 1995; Slamovits e cols., 2001).

Trabalhos populacionais na espécie *C. rionegrensis*, uma espécie uruguaia de distribuição restrita, a partir de três tipos de marcadores moleculares independentes alozimas, citocromo-b mitocondrial (D'Elia e cols., 1996) e *loci* de microssatélites (Wlasiuk e cols., 2003) têm sugerido um processo de colonização e de expansão demográfica recente a partir de uma população de tamanho reduzido. Da mesma forma, mas através da utilização de um fragmento da região controladora mitocondrial, Mora e cols. (2006) descrevem para *C. australis* um padrão de expansão populacional indicando a ocupação recente desta espécie na sua atual distribuição (dunas costeiras da região central da Argentina).

I.3 *Ctenomys flamarioni*

Ctenomys flamarioni, Travi 1981, comumente chamado de tuco-tuco-das-dunas (Figura 1), é uma espécie endêmica do Estado do Rio Grande do Sul (RS) que habita a primeira linha de dunas dos mais recentes depósitos eólicos e marinhos da Planície Costeira do Rio Grande do Sul (PCRS; Freitas, 1995a). Sua distribuição atual estende-se desde Arroio Teixeira, ao norte, até a desembocadura do Arroio Chuí na divisa com Uruguai, ao sul (Figura 2).

Figura 1. Exemplar adulto de tuco-tuco-das-dunas no seu habitat natural, a primeira linha de dunas da Planície costeira do Rio Grande do Sul.

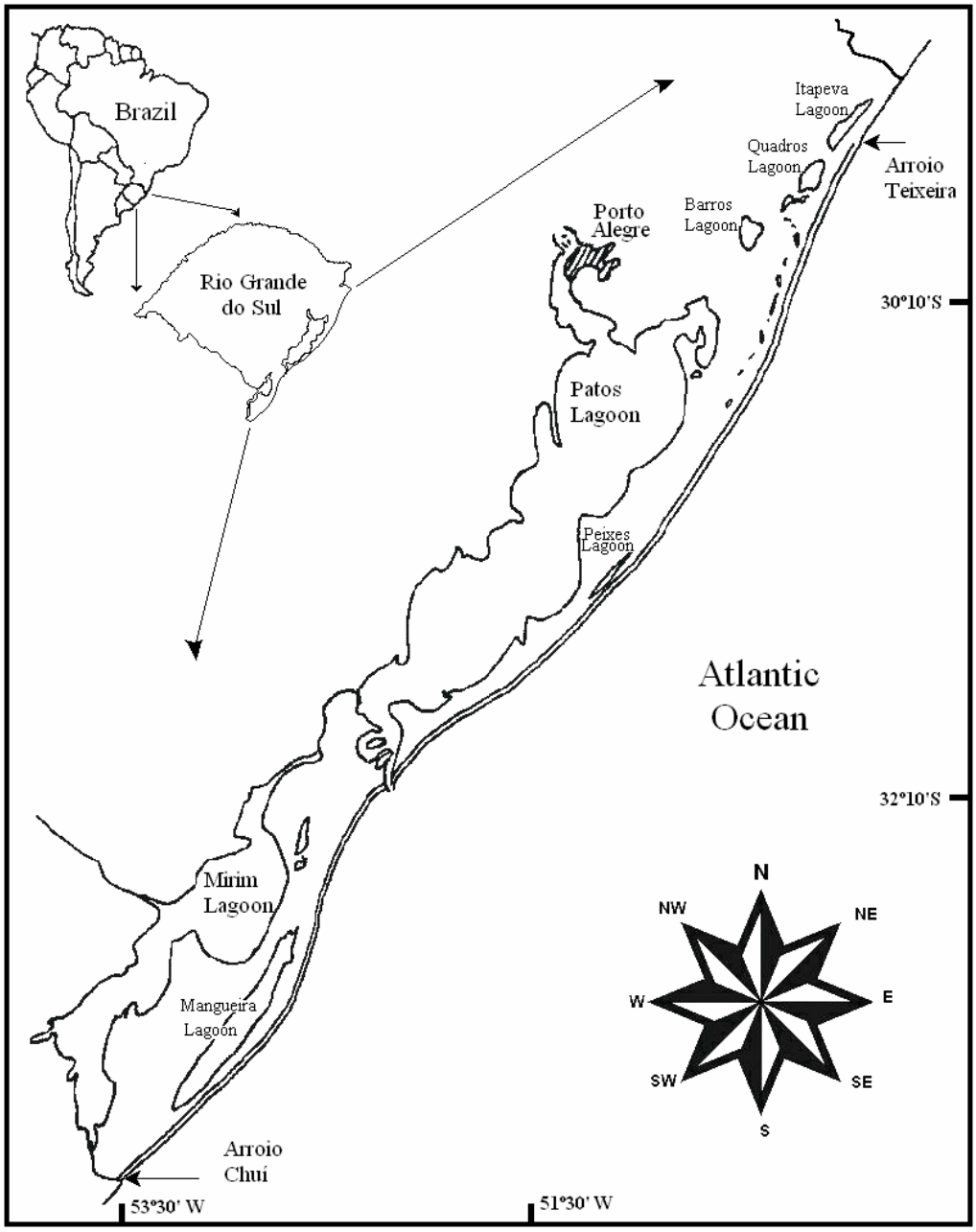

Figura 2. Mapa da distribuição geográfica de *Ctenomys flamarioni* ao longo da região litorânea da Planície Costeira do Rio Grande do Sul, indicando os limites de distribuição norte (Arroio Teixeira) e sul (Arroio Chuí).

Uma associação entre características cariotípicas e morfologia dos espermatozóides tem posicionado *C. flamarioni* dentro do grupo de espécies denominado mendocinus. A origem desta espécie foi sugerida por Freitas (1994) e Massarini & Freitas (2005), a partir de uma forma ancestral proveniente da Argentina, por migração, isolamento, e diferenciação posterior de *C. australis*. Esta migração teria acontecido durante o Pleistoceno, quando a Planície Costeira era aproximadamente 100 km mais ampla do que no presente e se encontrava sob condições de aridez nas quais o Rio da Prata não representava uma barreira geográfica expressiva (Corrêa e cols., 1992).

Além das características cromossômicas compartilhadas entre *C. flamarioni* e as outras espécies do grupo mendocinus, existem outras que reforçam a hipótese de que *C. australis* e *C. flamarioni* teriam um ancestral comum mais recente baseado nos padrões de distribuição geográfica de ambas as espécies (restritas às dunas costeiras): possuem a mesma coloração clara (Busch, 1989; Malizia e cols., 1991; Freitas, 1994; 1995a; Mora e cols., 2006; Fernández-Stolz e cols., submetido), e compartilham um único par cromossômico com marcação na banda NOR e 67% de suas bandas-G (Freitas, 1994). Todavia, estudos comparando o crânio destas duas espécies através do Método de Diagrama de Simpson (Travi & Freitas, 1984) e por análise multivariada (Massarini & Freitas, 1995; 2005) revelam diferenças morfológicas significativas entre elas.

Os indivíduos de *C. flamarioni* são morfologicamente mais robustos que os outros tuco-tucos do Rio Grande do Sul. O tamanho maior foi também observado na espécie argentina *C. australis,* e determinado como fator causal da restrição dos locais de ocorrência às dunas de areia da região costeira da Província de Buenos Aires onde o solo é mais frouxo e arejado (Contreras & McNab, 1990).

Ctenomys flamarioni se caracteriza por possuir um cariótipo único (2n = 48) em todos os indivíduos estudados, diferente das outras espécies brasileiras do gênero, as quais apresentam alta variabilidade cromossômica (Freitas, 1994). Embora todos os indivíduos exibam o mesmo número diplóide, variam quanto ao número de braços autossômicos através de um gradiente norte - sul, sendo o número máximo de braços encontrados 78, e o mínimo 50. Isto determina diferenças significativas na quantidade de heterocromatina constitutiva na distribuição geográfica da espécie, também decrescendo na direção sul. Todavia, o maior decréscimo em heterocromatina constitutiva é devido à perda do braço curto do par autossômico 1 nas populações ao sul da Barra de Rio Grande (saída da Laguna dos Patos; Freitas, 1994).

Moreira e cols. (1991), através de um estudo de eletroforese de proteínas reportam para duas populações da espécie um índice de similaridade baixo para populações co-específicas (S = 0.773), na ordem de magnitude ao obtido para estimativas interespecíficas entre *C. flamarioni* e outras espécies do gênero (*C. torquatus*, *C. minutus* e *C. sp*; S = 0.789).

Quanto à estrutura de idades, *C. flamarioni* descreve o padrão exibido na maior parte dos taxa de roedores subterrâneos, onde a classe etária mais representada é a dos indivíduos adultos (Busch e cols., 1989; Gallardo & Anrique, 1991; Rosi e cols., 1992; Zenuto & Busch, 1998; Busch e cols., 2000; Fernández, 2002).

Estimativas feitas sobre as áreas de vida dos indivíduos sugerem que os machos fazem uso de uma área maior que as fêmeas (Bretschneider, 1987; Fernández, 2002). Esta diferença pode ser atribuída às maiores necessidades energéticas decorrentes do maior tamanho dos machos, ou explicada como resposta a um padrão de poliginia, na qual os

machos se mostram ativos através de áreas maiores do que as fêmeas, presumivelmente para entrar em contato com um maior número delas (Fernández, 2002).

Assim como na maioria das espécies do gênero *Ctenomys*, nas populações de *C. flamarioni* estudadas não houve registros de compartilhamento de sistemas de túneis entre os adultos, tanto entre indivíduos do mesmo sexo quanto de sexos opostos. Túneis com mais de um indivíduo foram limitados a fêmeas com filhotes (Bretschneider, 1987; Fernández, 2002).

A sazonalidade dos registros de fêmeas amamentando, restritas à Primavera e ao Verão, permitiu estimar um único período de acasalamento para as populações estudadas (Fernández, 2002). Do mesmo modo, Bretschneider (1987) sugere um único período aproximado de acasalamento de seis meses, entre maio e setembro, com os nascimentos entre setembro e fevereiro.

Atualmente a espécie *C. flamarioni* tem sido citada na categoria de vulnerável à extinção tanto na Lista Nacional de Espécies da Fauna Brasileira Ameaçadas de Extinção (Ibama, 2004) e Lista da Fauna Ameaçada de Extinção no Rio Grande do Sul (Fontana e cols., 2003) principalmente devido à expressiva descaracterização do ambiente na qual ocorre.

I.4 Contexto Geológico: Planície Costeira do Rio Grande do Sul

I.4.1 Características gerais

A Planície Costeira (PC), pertencente ao pacote sedimentar Cenozóico no RS representado pela Bacia de Pelotas, estende-se através de uma área de aproximadamente 33.000 km^2 e alcança em alguns setores uma largura de mais de 100 km. A atual linha da costa, praticamente retilínea, possui uma orientação NE-SW e se estende por uma distância

de aproximadamente 620 km, desde Torres, no extremo norte (29° S), até a desembocadura do Arroio Chuí, no extremo Sul (34° S). Ao longo desta distância, a costa, de característica baixa arenosa, é interrompida de forma permanente em dois locais: as desembocaduras da Laguna de Tramandaí e da Laguna dos Patos (ver Figura 1). O clima da região é definido como temperado, úmido, e com uma distribuição de chuvas homogênea ao longo do ano (aproximadamente 1.300 mm anuais) (Tomazelli e cols., 2000).

I.4.2 Fatores físicos na evolução geomorfológica da Planície Costeira

Dois tipos de fatores governam os processos sedimentares que ocorrem em uma determinada região: estáticos e dinâmicos (Nimer, 1977). Quanto aos primeiros os mais importantes são a posição geográfica e o relevo. A Planície Costeira está incluída dentro da zona subtropical sul entre os paralelos 29° e 43° de latitude (Hasenack & Ferraro, 1989). O relevo influencia principalmente a região norte da Planície Costeira do Rio Grande do Sul (PCRS) devido à presença do Planalto da Serra Geral, que, com máximos de quase 1.000 m de altura, atua no controle de alguns parâmetros climáticos, tal como as precipitações.

Quanto aos fatores dinâmicos moduladores do clima, o mais importante está determinado pela ação conjunta de dois centros anticiclônicos: o Anticiclone Semipermanente do Atlântico Sul (Anticiclone Santa Helena) e o Anticiclone Móvel Polar. O comportamento dinâmico das massas de ar provenientes destes dois anticiclones modifica-se conforme as estações do ano. Assim, durante os meses de primavera-verão, em função de uma maior insolação, o Anticiclone do Atlântico se fortalece e se desloca para posições mais meridionais. Em conseqüência, o Anticiclone Móvel Polar se retrai, determinando o clima quente com ventos provenientes de NE-E que caracteriza estes meses. Já durante o outono inverno, devido a menor insolação, o Anticiclone do Atlântico

enfraquece e o Anticiclone Móvel Polar, caracterizado por frentes frias, aumenta o seu poder de penetração no continente, deslocando-se na direção SW-NE (Hasenack & Ferraro, 1989).

O regime de ventos tem um papel fundamental na morfogênese da PCRS já que, além de determinar a dinâmica de formação e erosão de dunas, é o fator básico que modula a hidrodinâmica tanto oceânica quanto dos corpos lagunares da região. De acordo com Tomazelli (1993) a PC encontra-se submetida a um regime bimodal de ventos de alta energia, sendo predominante o originado no Anticiclone do Atlântico Sul e, como conseqüência de sua incidência NE, as dunas eólicas migram no sentido SW.

Quanto ao regime hidrodinâmico, o transporte e deposição de sedimentos na Planície Costeira estão determinados principalmente pela ação de ondas de longo período proveniente do SE e por vagas locais provenientes principalmente do E-NE (Tomazelli & Villwock, 1992). Vários indicadores geomorfológicos registram um movimento de sedimentos na direção NE, também chamado de "deriva litorânea de sedimentos", sendo o mais evidente o sentido de deslocamento das desembocaduras não estabilizadas dos rios, arroios e lagunas que deságuam no mar. Este fenômeno pode ser exemplificado pela barra da Laguna de Tramandaí que, antes de ser fixada em 1961, migrava em média 200 m por ano, assim como a desembocadura da Lagoa do Peixe, próxima a Mostardas, migra constantemente em direção NE (Tomazelli & Villwock, 2000).

O regime normal de ondas é ocasionalmente perturbado pela ocorrência de ondas de tempestade associadas à passagem de frentes frias que acontecem durante outono e inverno, responsáveis pelos impactos erosivos mais marcantes na costa do RS (Tomazelli, 1990; Tomazelli & Villwock, 2000).

1.4.3 Evolução Paleogeográfica da PCRS

O modelo proposto para retratar os principais eventos que caracterizam a evolução paleogeográfica da PC prediz que, a partir do Terciário, se originou um sistema de leques aluviais coalescentes ao longo da parte oeste da Planície, devido à acumulação de um pacote de sedimentos clásticos terrígenos alimentados por fluxos de água provenientes de terras altas submetidos a um clima semi-árido. A porção superior do sistema de leques aluviais teria o apogeu do seu desenvolvimento no período que se estende do Plioceno ao Pleistoceno Inferior. Estes depósitos teriam sido subseqüentemente retrabalhados por no mínimo quatro ciclos transgressivo-regressivos ocorridos durante o Quaternário, sendo os três primeiros associados aos eventos glaciais do Pleistoceno, e o último ao Holoceno, originando quatro sistemas deposicionais complexos chamados de "laguna-barreira" (I – IV; Figura 3).

O primeiro ciclo transgressivo-regressivo pleistocênico teria dado origem, há aproximadamente 400 mil anos (ka), à Barreira das Lombas, com cerca de 250 km de extensão, uma largura de 5 a 10 km e orientada na direção NE. A região ocupada pelo sistema lagunar associado a esta barreira abrange boa parte das bacias do Rio Gravataí e do complexo fluvial do Guaíba e sofreu a influência dos eventos transgressivo-regressivos que se sucederam posteriormente.

O segundo e terceiro ciclos (aproximadamente há 325 ka e 120 ka respectivamente) deram origem à barreira arenosa denominada "Barreira Múltipla Complexa" que finalmente isolou as lagunas dos Patos e Mirim. A fase regressiva que caracterizou o terceiro ciclo teria atingido o seu máximo há 17 ka, dando origem a uma ampla planície que atualmente forma parte da plataforma submarina, retraindo a linha de costa aproximadamente 120 m abaixo do seu nível atual.

O quarto e último evento interglacial, iniciado no final do Pleistoceno, teria atingido seu máximo há cerca de 5 ka, elevando o nível do mar de 2 a 4 m acima do atual (Tomazelli e cols., 2000). A estabilização temporária no final deste evento transgressivo-regressivo foi a principal responsável pela implantação de uma barreira transgressiva de, provavelmente, 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 que se estende ao longo da PC.

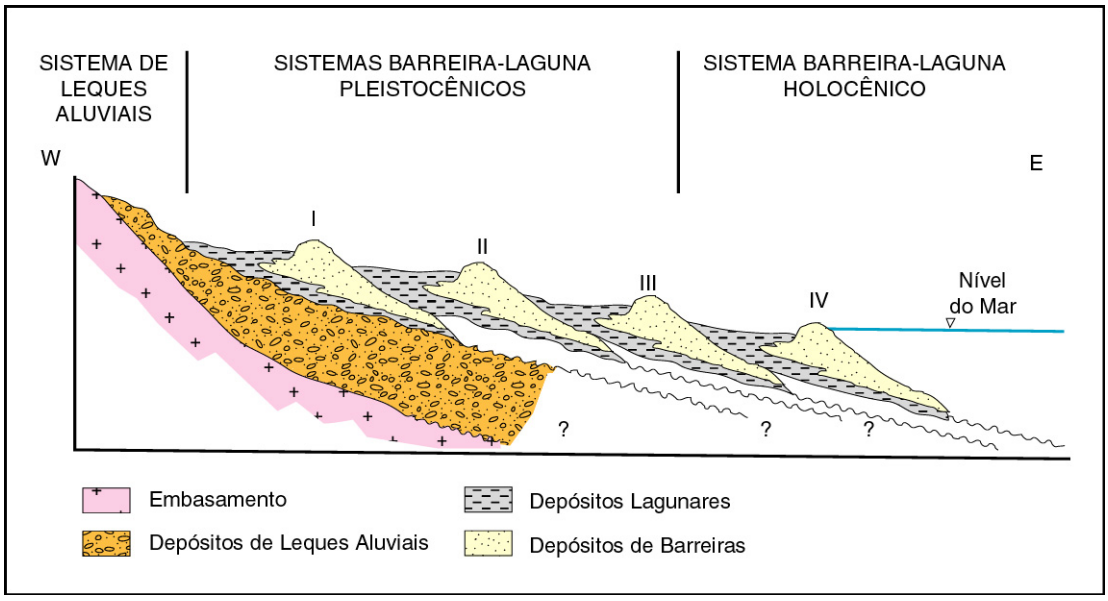

Figura 3. Perfil geológico esquemático, transversal aos sistemas laguna-barreira existentes no Rio Grande do Sul. Extraído e modificado de Tomazelli & Villwock (2000; *apud* Clerot, 2004).

I.4.4 Evidências de erosão no litoral do Rio Grande do Sul

Vários indicadores geológicos mostram que a maior parte da linha de costa está sofrendo erosão e retração na atualidade (Tomazelli, 1990; Tomazelli & Villwock, 1992;

Tomazelli e cols., 1997; 1998a; 1998b; Tomazelli & Dillenburg, 1998; Esteves e cols., 2002). Alguns destes indicadores são os afloramentos de turfa e outros sedimentos de idade holocênica (argilas orgânicas e camadas de conchas e moluscos estuarino-lagunares, etc.) na praia, e a existência de uma escarpa praticamente contínua ao longo das dunas frontais.

Além dos processos erosivos provocados por fatores climáticos e geomorforfológicos, existe um outro componente que, embora difícil de quantificar, tem mudado em níveis variáveis a paisagem das dunas costeiras do RS. Trata-se da atividade humana manifestada através de suas mais diversas formas, tais como a urbanização, o aumento de balneários com a conseqüente afluência de turistas, a destruição de dunas para a utilização de areia com diferentes fins (e.g., construção e aterros), a plantação de árvores exóticas e a presença de gado sobre a linha de dunas costeiras.

A partir do final da década de 80, vários estudos têm focalizado a atenção nestes processos, tentando determinar a importância relativa dos fatores naturais e humanos nos processos de longo e curto prazo.

Como causas de erosão de longo prazo, têm sido citadas: a elevação atual do nível do mar (Tomazelli, 1990; Tomazelli e cols., 1997; 1998; Villwock & Tomazelli, 1998), a concentração de energia de ondas controlada por feições morfológicas em grande escala (Tomazelli & Dillenburg, 1998; Dillenburg e cols., 2000) e o déficit de areia (Tomazelli e cols., 1998). Por outra parte, dentro dos fatores de curto prazo podem ser incluídos: a ressaca por tempestades (Calliari e cols., 1998b; Esteves e cols., 2000; Tozzi & Calliari, 2000), a concentração de energia de ondas devida a características topográficas da costa em pequena escala (Calliari e cols., 1998a; 2000), a interferência no transporte de sedimentos ao longo da linha da costa (Almeida e cols., 2001) e a atividade humana (Dillenburg e cols., 2000; Esteves e cols., 2002). Tomazelli & Villwock (2000) também

indicam o aquecimento global como provável determinante tanto da elevação do nível do mar quanto do aumento da freqüência e⁄ou magnitude das tempestades.

Esteves e cols. (2002), através de uma análise utilizando todos estes fatores, concluem que, com o propósito de manejo costeiro, os eventos de curto prazo são mais críticos e importantes de serem controlados que os de longo prazo. Neste sentido o efeito da atividade humana ocupa um lugar de destaque com quase um terço da linha de costa impactada por algum tipo de expressão de tal atividade, podendo afetar o balance natural de areia nas dunas e agravar processos erosivos naturais. Exemplo disto é a fixação permanente da saída ao mar da Laguna dos Patos através da construção de dois molhes de 4 km cada um, resultando no aumento acelerado de acúmulo areia nas praias ao sul destas construções devido à obstrução no transporte natural de sedimentos (na direção S-N) ao longo da linha da costa (Dillenburg e cols., 2000).

I.5 Contexto genético

I.5.1 Ferramentas moleculares no estudo de padrões populacionais

Duas metas fundamentais da genética da conservação são: a prevenção de problemas associados ao endocruzamento e à deriva genética em pequenas populações, e a descrição da estrutura populacional com a finalidade de identificar unidades de conservação. A genética molecular tem proporcionado numerosas técnicas de acesso à variabilidade genética dentro e entre as populações, tanto através de polimorfismos de proteínas quanto de DNA (Avise, 1994). Por serem altamente polimórficos e possuírem altas taxas de mutação (dentre outras características), os marcadores moleculares mais utilizados em estudos populacionais e filogeográficos são o DNA mitocondrial e os *loci* de microssatélites.

I.5.1.i DNA mitocondrial

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

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

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

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

dentro das linhagens celulares; (3) o fato de estar livre de histonas, que são evolutivamente conservadas e poderiam limitar a taxa evolutiva do DNA nuclear (Avise, 2000).

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

De especial utilidade para análises filogenéticas sob escalas de tempo microevolutivas, é a região controladora (CR). As taxas de substituição de bases encontram-se acentuadas em duas regiões da CR chamadas de segmentos hipervariáveis (*Hiper-Variable Segment*, HVS) HVS1 e HVS2, com extensões aproximadas de 350 pb e separadas por uma região mais conservada com cerca de 200 pb (Vigilant e cols., 1989).

Análises de seqüências desta região têm gerado resultados com boa resolução em estudos populacionais e evolutivos, em diferentes grupos taxonômicos de mamíferos, como primatas (e.g., humanos: Bonatto & Salzano, 1997; *Ateles*: Collins & Dubach, 2000), ursos (Talbot & Shields, 1996; Waits, 2000), baleias (Rooney e cols., 2001), manatis (Cantanhede e cols., 2005), lêmures (Wyner e cols., 1999), marsupiais (Moritz e cols., 1997; Firestone e cols., 1999) e roedores (Faulkes e cols., 1997; Méndez-Harclerode e cols., 2005; Whorley e cols., 2004; Zheng e cols., 2003).

I.5.1.ii *Loci* de microssatélites

O uso de microssatélites de DNA permite superar algumas das limitações das outras técnicas (Jarne & Lagoda, 1996), por apresentarem altos níveis de variabilidade em estado diplóide e possuírem um caráter altamente polimórfico (Tautz, 1993). Outra vantagem no

uso destes marcadores é que são seletivamente neutros (o que possibilita a manutenção de sua alta variabilidade) e acumulam mutações a uma taxa relativamente constante.

Os microssatélites são pequenas seqüências repetidas *in tandem*, formadas por unidades de repetição de 1 a 6 pb de comprimento, altamente abundantes no genoma de eucariontes. Em mamíferos, tem-se estimado que os motivos mais comuns (GT/AC) ocorrem em média cada 30 mil bases (kb). A maioria dos microssatélites encontra-se em simples cópia, facilitando a identificação dos alelos sem ambigüidade (Schlötterer, 1998).

As mutações em regiões de microssatélites são mudanças no número de unidades de repetição causadas por um mecanismo de mutação intramolecular chamado de *DNA slippage*, baseado no fato de que as repetições dentro de um determinado arranjo são idênticas e então as duas cadeias de DNA homólogas podem ser facilmente alinhadas fora de fase durante a replicação. A resolução de produtos de *slippage* por reparo de DNA ou outros mecanismos podem permitir o ganho ou perda de unidades de repetição, mudando o tamanho final do arranjo (Amos & Hoeltzel, 1992; Tautz, 1993; Comings, 1998). Os alelos dos *loci* de microssatélites podem ser "acessados" pela análise de Reação em Cadeia da Polimerase (*Polymerase Chain Reaction*, PCR), requerendo menores quantidades de DNA do que através de outros métodos moleculares (Bennett e cols., 1998).

Sua utilização tem aumentado significativamente em estudos sobre comparações da variabilidade genética entre espécies e populações, história evolutiva e estrutura populacional (Blundell e cols., 2002; Broders e cols., 1999; Ciofi & Bruford, 1999; Cutrera e cols., 2005; Waits e cols., 2000; D'Elia e cols., 1998; 1999; Lacey, 2001; Wlasiuk e cols., 2003), relações filogeográficas (Wyner e cols., 1999; Johnson e cols., 1999), paternidade e parentesco (Blouin e cols., 1996; Nesje e cols., 2000; Cerchio e cols., 2005).

I.5.2 Reduções do tamanho efetivo populacional (gargalos de garrafa)

Numerosas causas, tanto naturais quanto provocadas pela presença humana (direta ou indiretamente), têm sido descritas como responsáveis pelas reduções no tamanho efetivo populacional de variadas espécies (Pechmann e cols., 1991; Packer e cols., 1991; Gaines e cols., 1997; Leijs e cols., 1999; Frankham e cols., 2002).

Populações que têm experimentado reduções severas no tamanho efetivo populacional são mais suscetíveis a apresentar: 1. aumento da identidade por descendência; 2. perda da variabilidade genética; 3. efeito da deriva genética sobre o tamanho e a composição populacional (Lande, 1988). Estas mudanças podem levar ao aumento da homozigosidade, expressão de alelos recessivos deletérios, depressão por endocruzamento e, em casos mais extremos, extinção local (Shields, 1993; Packer e cols., 1993; Gaines e cols., 1997; Saccheri e cols., 1998; Leijs e cols., 1999; Whitehouse & Harley, 2001). Do mesmo modo, a perda da variabilidade genética pode reduzir o potencial evolucionário das populações (Lacy, 1997; Frankham e cols.,1999; 2002), assim como a habilidade de responderem frente à introdução de patógenos e parasitas (Allendorf & Leary, 1986; O´Brien & Evermann, 1989).

Apesar de que a discussão a respeito do real impacto da depressão por endocruzamento em populações naturais ainda exista (Hoelzel e cols., 1993; Craig, 1994), a maioria dos planos de conservação atuais monitoram tanto o tamanho quanto a estrutura e a variabilidade genética das populações (Lande, 1995; Gaines e cols., 1997; Frankham e cols., 2002; Primack & Rodrigues, 2002).

Como conseqüência disto, o interesse em detectar reduções no tamanho efetivo populacional tem aumentado nos últimos anos, assim como os métodos para abordar estas questões a partir de conjuntos de dados genéticos (Cornuet & Luikart, 1996; Luikart e

cols., 1998; Beaumont, 1999; Spencer e cols., 2000; Garza & Williamson, 2001; Leberg, 2002).

A maior parte destes métodos baseia-se no fato de que, na medida em que se reduz o tamanho efetivo populacional, aumenta a ação da deriva genética, levando a mudanças nas freqüências alélicas e a perda ou fixação de alelos ao acaso.

I.5.3 Filogeografia: estrutura genética a escala macro-geográfica

A filogeografia, como definida por Avise (2000), é o campo de estudo envolvido com os princípios e processos que determinam as distribuições geográficas das linhagens genealógicas. A análise e interpretação da distribuição destas linhagens, usualmente requerem o aporte de dados da genética molecular, genética de populações, etologia e demografia. Desta forma, a filogeografia constitui-se em um esforço integrativo, entre diversas disciplinas micro e macroevolutivas. Em particular, a filogeografia proporciona uma ponte empírica e conceitual entre as tradicionalmente desacopladas disciplinas de biologia filogenética e genética de populações (Avise e cols., 1987).

A ferramenta molecular mais usada neste tipo de abordagens é a análise de DNA mitocondrial (mtDNA), que permite o acompanhamento de rastros genealógicos além dos limites genéticos entre as populações, espécies e grupos taxonômicos mais elevados. O sucesso da filogeografia baseada no DNA mitocondrial, deve-se principalmente à descrição aperfeiçoada da distribuição geográfica, relações filogenéticas, distâncias genéticas e tempos de divergência entre linhagens evolutivas de animais (Bermingham & Moritz, 1998; da Silva & Patton, 1998).

O principal avanço da aplicação da filogeografia tem-se dado em três áreas, cada uma delas vinculada a diferentes aspectos testáveis de "concordância genealógica" (Avise 1996; 1998): (1) concordância na partilha genealógica ao longo de vários *loci* não ligados dentro dos limites da espécie; (2) concordância na posição geográfica das partições das árvores de genes ao longo de múltiplas espécies co-distribuídas, ajudando na determinação dos principais fatores biogeográficos históricos que modularam as filogenias intra-específicas; e (3) concordância entre partições nas árvores de genes e os limites geográficos entre regiões biogeográficas conhecidas, ou separadas por barreiras históricas ao fluxo gênico.

Isto levou a melhor compreensão de biogeografias regionais e áreas de endemismo, auxiliando na elaboração de propostas prioritárias para a conservação das biodiversidades taxonômicas e locais (Rojas, 1995; Bermingham & Moritz, 1998; da Silva & Patton, 1998; Firestone e cols., 1999).

I.6 Tópicos de conservação: a contribuição dos dados genéticos

I.6.1 Unidades Evolutivamente Significativas: conceitos e aplicações

A maioria das espécies distribui-se em populações geograficamente estruturadas, muitas das quais podem experimentar pouco ou nenhum contato por longos períodos de tempo. Entretanto, a dinâmica populacional de outras espécies pode estar caracterizada por expansões recentes, o que determina estreitas conexões genealógicas entre as populações resultantes (Ibrahim e cols., 1996).

Características demográficas históricas e contemporâneas podem afetar a estrutura espacial de populações co-específicas e com ela influenciar as genealogias matrilineares em vários sentidos. Um importante desafio dos estudos filogeográficos é decifrar os fatores

demográficos passados e presentes que geram um determinado padrão de relações genealógicas (Avise, 2000).

No contexto da conservação, tanto as divergências genealógicas superficiais quanto as profundas, podem ser informativas. Existem várias tentativas de formalizar as relações entre o *status* de conservação e o *status* taxonômico dentro das espécies (e.g., Vane-Wright e cols., 1991; Dizon e cols., 1992; Vogler & DeSalle, 1994). Dentro deste contexto, o conceito mais amplamente utilizado é o de Unidade Evolutivamente Significativa (*Evolutionary Significant Unit*, ESU). As ESUs foram desenvolvidas para prover uma abordagem objetiva na escolha de unidades prioritárias para conservação abaixo do nível de espécie (Ryder, 1986), uma vez que a taxonomia existente pode não refletir exatamente a diversidade genética subjacente. Desde as origens da definição proposta por Ryder, o conceito vem mudando de acordo com necessidades práticas e as limitações dos conceitos vigentes (ver Anexo I; Waples, 1991, Dizon e cols., 1992; Avise, 1994; Moritz, 1994; Vogler & DeSalle, 1994; Crandall e cols., 2000; Fraser & Bernatchez, 2001). A principal divergência entre as diferentes definições de ESUs está no papel dos marcadores genéticos neutros em relação a outros critérios utilizados na tomada de decisões relativas à conservação.

Uma das definições mais utilizadas na literatura é a proposta por Moritz (1994) sob uma abordagem filogeográfica, combinando idéias de Dizon e cols. (1992) e particularmente de Avise (1994). Moritz propôs um critério genético para a identificação de ESU, a qual define-se pela população (ou conjunto de populações) que apresentam recíproca monofilia para 'alelos' (linhagens) de mtDNA e divergência significativa nas freqüências alélicas para *loci* nucleares. Como tal, uma ESU reflete a separação filogenética histórica de diferentes populações, constituindo-se numa fonte de diversidade

genética dentro das espécies. O objetivo de designar estas unidades é permitir o manejo destas de forma independente, e de manter a diversidade evolutiva histórica (Avise, 2000).

A consideração de casos nos quais a monofilia recíproca não era atingida entre as linhagens filogenéticas levou à formalização do conceito de Unidade de Manejo (*Management Unit*, MU; Moritz, 1994) definido como populações (ou grupos destas) identificadas pela divergência significativa nas freqüências alélicas de *loci* neutros (nucleares ou mitocondriais), independentemente das relações filogenéticas entre os alelos. Estas unidades são mais apropriadas para objetivos de conservação em curto prazo, tal como monitoramento populacional.

Assim, existem desvantagens e/ou limitações no uso de marcadores moleculares na identificação das unidades de conservação. Uma delas, relacionada à determinação de filogenias que reflitam com precisão a história filogeográfica das espécies, é intrínseca da própria escolha dos marcadores, já que uma determinada árvore de genes pode não ser congruente com a árvore das unidades taxonômicas que estão sendo consideradas. Dado que as árvores de genes podem ou não representar as relações hierárquicas entre os organismos, os padrões de divergência devem ser acessados a partir de diversos marcadores moleculares, a fim de minimizar erros na determinação da história filogenética (Avise, 2000).

O segundo problema está relacionado com a transferência horizontal de genes através de eventos de introgressão. Esta transferência horizontal interferirá na filogenia em maior ou menor grau, dependendo do marcador utilizado: mais com marcadores de mtDNA do que com marcadores nucleares, devido aos diferentes tempos de coalescência e às taxas de introgressão (Steinberg & Patton, 2000).

Um novo critério mais amplo e 'adaptativo' tem sido proposto baseado na idéia central de que, em diferentes situações, a identificação de unidades de conservação irá requerer o uso de um ou mais critérios, adequados às necessidades de cada caso (Fraser & Bernatchez, 2001). Esta abordagem chamada de *Adaptative Evolutionary Conservation* (AEC), constitui um consenso dos diferentes critérios vigentes, que visa contribuir com os esforços de conservação de uma forma mais integrada através do estudo de cada caso.

Independente do critério usado na definição das unidades de conservação, a utilização de dados genéticos é amplamente difundida, assim como é sugerida a investigação de *loci,* tanto de DNA nuclear quanto de mtDNA. Estes *loci* são marcadores genéticos extremamente efetivos no estudo da variabilidade, diferenciação e estrutura ao nível populacional (Pope e cols., 1996; Moritz e cols., 1997; da Silva & Patton, 1998; Firestone e cols., 1999; Johnson e cols., 1999; Wyner e cols., 1999; Roach e cols., 2001; Maudet e cols., 2002; Blundell e cols., 2002).

II. OBJETIVOS

1. Caracterizar a espécie *Ctenomys flamarioni* no que se refere à variabilidade genética e diferenciação geográfica através da utilização de marcadores moleculares nucleares (*loci* de microssatélites) e mitocondriais (fragmentos da região controladora e do citocromo b).

2. Comparar os padrões gerados a partir de cada um dos marcadores.

3. Inferir os padrões filogeográficos de *C. flamarioni* ao longo da área de distribuição da espécie e determinar os limites de sua distribuição geográfica.

4. Fazer estimativas do tempo de divergência das principais linhagens encontradas.

5. Examinar a concordância entre os padrões filogenéticos achados a partir dos dados moleculares e as barreiras geográficas existentes, isto é, analisar a efetividade das barreiras geográficas na diferenciação filogenética.

6. Definir Unidades Evolutivamente Significativas (ESUs) e Unidades de Manejo (MUs) para a espécie.

7. Gerar dados para a elaboração de planos de monitoramento, manejo e preservação da espécie e de seu entorno.

III. CAPÍTULO I

BOTTLENECKS AND DISPERSAL IN THE TUCO-TUCO-DAS-DUNAS, *Ctenomys flamarioni* (RODENTIA: CTENOMYIDAE) IN SOUTHERN BRAZIL

GABRIELA P. FERNÁNDEZ-STOLZ, J. F. B. STOLZ AND THALES R. O. DE FREITAS

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Gabriela Paula Fernández-Stolz

Departamento de Genética, Avenida Bento Gonçalves 9500, Prédio 43323

Caixa postal 15053 - CEP 91501-970, Bairro Agronomia, Porto Alegre,

Rio Grande do Sul, Brazil.

gabriela.fernandez@ufrgs.br

BOTTLENECKS AND DISPERSAL IN THE TUCO-TUCO-DAS-DUNAS, *CTENOMYS FLAMARIONI* (RODENTIA: CTENOMYIDAE) IN SOUTHERN BRAZIL

GABRIELA P. FERNÁNDEZ-STOLZ*, J. F. B. STOLZ, AND THALES R. O. DE FREITAS

Programa de Pós-Graduação em Genética e Biologia Molecular, Prédio 43323, P. O. BOX 15053, Universidade Federal do Rio Grande do Sul, Porto Alegre, 91501-970, Rio Grande do Sul, Brazil (GPFS and TROF)

Programa de Pós-Graduação em Ecologia, Prédio 43422, P. O. Box 15007, Universidade Federal do Rio Grande do Sul, Porto Alegre, 91540-000, Rio Grande do Sul, Brazil (JFBS and TROF)

Departamento de Genética, Prédio 43323, P. O. BOX 15053, Universidade Federal do Rio Grande do Sul, Porto Alegre, 91501-970, Rio Grande do Sul, Brazil (TROF)

** Correspondent: gabriela.fernandez@ufrgs.br*

The tuco-tuco-das-dunas, *Ctenomys flamarioni*, is a subterranean rodent endemic to sand-dune ecosystems along the southern coast of Brazil. We studied three populations that differ in the degree of human impact, and used direct and indirect methods to assess demographic and genetic information. Field studies revealed a tendency toward a female-biased sex-ratio, and sexual dimorphism in both weight and length in all three populations. This evidence supports a hypothesis of polygyny in *C. flamarioni*. Using 9 microsatellite loci, we explored patterns of variation and genetic structure among the populations. Our findings suggested that the Xangri-lá and Remanso populations, living in more-disturbed locations, could have experienced demographic reductions in population size, but not the Pinhal population. However, other factors such as a polygynous breeding system and the environmental instability that characterizes the coastal dunes may have influenced the observed pattern. Analysis of genetic structure revealed strong differentiation among populations, but no significant structure at the intrapopulation level. Non-significant values for the tested indexes from assignment tests (F_{ST}, F_{IS}, *mAIc*, *vAIc*) over population showed no evidence of sex-biased dispersal. The same was observed from AMOVA tests. Nevertheless, lower pairwise F_{ST} and higher *Nm* values between Xangri-lá and Remanso males indicated greater gene flow among males, suggesting a slightly male-biased dispersal pattern. Significant differences in interpopulation dispersal patterns were clearer among females: more dispersal for Pinhal females than those at Remanso and Xangri-lá.

Key words: bottleneck, dispersal, *Ctenomys flamarioni*, demography, genetic structure, microsatellite, polygyny

Fossorial rodents of the genus *Ctenomys* inhabit the southern part of the Neotropical region, from 17°S to 54°S latitude and from the Andes to the Atlantic Ocean. These small subterranean herbivores are among the most geographically variable mammals, and are also the most numerous in species of all subterranean rodents (Reig et al. 1990).

The biological and ecological characteristics of members of this genus remain poorly known, because most of their activities occur inside the burrows. As a result, their demography, reproductive success, spatial distribution, population genetic structure, and other social behavior must be studied using different methods from those used to observe surface-living animals (Lacey 2000).

Ctenomys flamarioni Travi 1981, called the tuco-tuco-das-dunas, is one of the four species of the genus in southern Brazil, and is endemic to coastal sand-dune grasslands in the State of Rio Grande do Sul. Its range is bounded by Arroio Teixeira City on the north (Freitas 1995a) and by the Chuí River on the south.

An association between cytogenetic characteristics (the same karyotype with 2n = 48 and a large amount of heterochromatin) and the simple asymmetrical form of the spermatozoa (Freitas 1994 and 1995b) suggest that *C. flamarioni* belongs to the mendocinus-group of *Ctenomys*, which also includes four Argentinean species: *C. azarae, C. australis, C. porteousi*, and *C. mendocinus* (D'Elia et al. 1999; Massarini and Freitas 2005; Massarini et al. 1991; Slamovits et al. 2001), and an Uruguayan species, *C. rionegrensis* (Freitas 1994; D'Elia et al. 1999).

As a result of human activity, such as urbanization, native habitats have become increasingly fragmented or destroyed. Both habitat loss and isolation of patches resulting from fragmentation can reduce population sizes to levels that lead to genetic drift, inbreeding, and loss of genetic variation and evolutionary potential (Frankham et al. 1999;

Lande 1995). In extreme cases, increase in population homozygosity, expression of deleterious alleles, and the effect of stochastic changes can result in local extinction (Gaines et al. 1997; Leijs et al. 1999).

Mark-recapture studies provide a good means to assess temporal changes in spatial and social relationships (Busch et al. 1989; Lacey 2000). Molecular genetic studies provide the means to measure patterns of parentage and kinship within groups with different degrees of sociality, as well as patterns of dispersion of genetic traits in space and time. Combining genetic analysis with demographic attributes provides valuable insights into processes that maintain variation within local populations and distribute variation among them (Steinberg and Patton 2000). Highly variable markers such as microsatellite loci are well suited for such studies, and their use is increasing in comparative studies of genetic variation and evolutionary history in *Ctenomys* (El Jundi and Freitas 2004; Gava and Freitas 2004; Lacey 2001; Wlasiuk et al. 2003).

The focal conservation issue for the tuco-tuco-das-dunas is the rapid urban development in coastal environments and the progressive destruction of the natural landscape (Esteves et al. 2002; Tomazelli and Villwock 2000). The main goals of this study were to examine patterns of genetic variation and population size reductions (bottlenecks) in populations of *C. flamarioni* that are subject to different levels of disturbance in their habitat, and to describe the existence (or absence) of sex-biased dispersal patterns in the species in a population context. With these aims, we analyzed microsatellite variation in three populations of tuco-tucos subject to different degrees of human impact.

MATERIALS AND METHODS

Study sites and sample collection.–Our studies were conducted in coastal dunes in the state of Rio Grande do Sul, Brazil. The sampling period extended from March 1999 through March 2001, with eight trapping periods, one per season at each location. The procedures with animals followed American Society of Mammalogists guidelines (Animal Care and Use Committee 1998). Oneida Victor No. 0 snap traps protected with rubber strips were used to catch the animals without injury. Each trap was introduced into the entrance of the burrow and checked every ten minutes to avoid overstressing the animals caught.

Tuco-tucos were sampled at 3 locations: Xangri-lá (XA; 29°47'S, 50°01'W; n = 24 individuals), Remanso (RE; 29°49'S, 50°02'W; n = 30 individuals), and Pinhal (PI; 30°18'S, 50°15'W; n = 31 individuals; Fig. 1). The choice of these populations was based on their different degree of human impact. The Xangri-lá and Remanso populations are subject to a higher degree of disturbance than Pinhal, consisting mainly of the strong reduction of the first line of coastal dunes and adjacent inland foredune plains because of urbanization on active dunes. Anthropogenic changes are related to the high levels of human visitation, the existence of several types of recreational structures and food-service buildings on the seaward face of the dunes, and the presence of non-native vegetation (*Pinus* cf. *maritime* and *Eucalyptus*) and road construction on the landward side. However, the richness of endemic plant species is similar in the three locales, and they contain the same native dune species, such as *Blutaparon portulacoides*, *Senecio crassiflorum*, and *Panicum racemosum.*

Although the geographic distance between the XA and RE populations is short (2.5 km), they were considered two different populations because of the existence of a

geographic barrier to gene flow: the continuity of the dunes is interrupted along a nearly 500 m stretch, and no evidence of the tuco-tuco's burrow system was observed.

The areas of the study sites were 2.5 ha for XA, 3.0 ha for RE and 7.5 ha for Pinhal. All of them extended for 400 m along the coastline. Differences in the sizes of sites depended on the available area between the frontal dunes and the first line of housing construction (for RE and XA) or the limits of the sand dunes (for PI).

After each capture, the sex, body weight, and length (excluding the tail), and the reproductive condition of females were recorded. Samples for genetic studies were obtained as ear-skin biopsies and preserved in absolute ethanol. In order to avoid overestimating the number of individuals, each captured animal was tattooed with an identification number on the left rear leg. The tuco-tucos were then released at the same point where they were captured.

The individuals were classified following the age criteria taken from Wilks (1963): 1. young (less than the mean weight calculated between the heaviest individual found in the mother's tunnel system and the lightest one found in its own tunnel system); 2. subadults (Male: less than the mean between the heaviest and lightest individual in its own tunnel system; Female: less than the lightest sexually mature female found; and 3. adults (more than the upper limit of the subadults, and all considered mature). Sexual maturity of the females was identified from signs of copulation such as the vagina opening (recent copulation) or the presence of a scar in the vagina entrance (non-recent copulation).

The position of capture was recorded with a GPS. This allowed us to determine density (number of individuals/area) and the minimum area occupied by the individuals in each population. The Minimum Number Known to be Alive method (MNKA; Krebs 1966) was used to estimate the number of individuals and their density in each population.

Chi-square tests were applied to determine whether sex-ratios deviated from parity in *C. flamarioni* populations, for all individuals and for adult individuals only. The significance of differences in body weight and length between adult males and females was assessed using Student's t-tests.

DNA extraction and Microsatellite analysis.–Skin-tissue samples collected in the field were deposited in the collection of the Cytogenetics and Evolution Laboratory, Genetics Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were stored at -20°C. Genomic DNA was extracted following a protocol from Medrano et al. (1990).

Molecular genetic analysis was performed with 9 polymorphic microsatellite loci, isolated from the Argentinean species *C. haigi and C. sociabilis*, with two (Hai 3, Hai 4, Hai 5, Hai 6, Hai 7, Hai 9, Soc 2, and Soc 3) and three (Hai 12) base-pair motifs (Lacey 2001; Lacey et al. 1999).

PCR amplifications were carried out in a reaction volume of 20 µl containing 25-100 ng DNA, 0.2 µM of each primer, 0.2 µM dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase (GIBCO-BRL Life Sciences/ Invitrogen, Carlsbad, California). The thermocycling profile included an initial denaturing at 94ºC for 5 min, followed by 30-34 cycles of denaturing at 94ºC for 30 s, annealing at 50-64ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 1 min. The products were run in non-denaturing 6% polyacrylamide gels and 8% polyacrylamide-40% urea denaturing sequencing gels at 1000V for 2-4 hrs, as dictated by the size of the amplification products for each locus. The products were developed with silver-nitrate stain.

Microsatellite statistical analysis.–Genetic diversity within each study population was measured as the number of alleles per locus (N), per locus per population (N_i), the mean number of alleles per locus (allelic richness, A), the percentage of polymorphic loci (%P), observed heterozygosity (H_o), and the heterozygosity expected from Hardy-Weinberg proportions (H_e – Nei 1978). The mean number of alleles per locus was corrected for variation in sample size using bootstrapping ($A_{c[B]}$, sampling with replacement) and jackknifing ($A_{c[J]}$, sampling without replacement) implemented in the AGARst program (Harley 2001). Differences among populations in the number of alleles per locus and expected heterozygosity were assessed using Wilcoxon's signed ranks test. Private alleles, defined as those present in only one population, were identified for each population through pairwise population comparisons.

Analysis of linkage disequilibrium and deviations from the Hardy-Weinberg equilibrium (Guo and Thompson 1992) were tested using ARLEQUIN 2.1 (Schneider et al. 2000). Sequential Bonferroni corrections were applied to correct for multiple simultaneous comparisons (Rice 1989), with $\alpha = 0.05$ to adjust the statistical significance levels.

In spite of the current geographical separation between the nearest populations, XA and RE, a Bayesian MCMC approach was used in order to examine the distinctiveness of populations and the clustering of individual genotypes using the program STRUCTURE (Pritchard et al. 2000).

To detect the genetic signatures of bottlenecks, three methods based on changes in allele frequencies were used. The first is a simple graphical method that tests whether a deficit in rare alleles exists in a sample of loci (Luikart et al. 1998a, 1998b), through the shape of the allele frequency distribution: L-shaped (as expected under mutation-drift distribution) or not (if recent bottlenecks caused a mode shift). Bottleneck cause a

characteristic change in the distribution of allele frequencies seen as loss of low-frequency alleles and an increase in relative abundance of intermediate and high-frequency alleles. The test was performed using the program BOTTLENECK (Cornuet and Luikart 1996) in which the alleles were grouped into ten frequency classes (0-0.10; 0.11-0.2 and so on until 0.91-1.0). For the graphical presentation of the results, the classes of allele frequency were 0.19 wide (i.e., 0.01-0.20) in order to show the pattern more clearly. The second method tests for excess of heterozygosity compared to the expected values from the observed number of alleles at each locus and population, assuming mutation-drift equilibrium. The probability of significant heterozygosity excess was calculated using a Wilcoxon signed rank test, and the computations were based on both stepwise mutation (SMM) and two-phase mutation (TPM) models (Di Rienzo et al. 1994) as performed in BOTTLENECK (Cornuet and Luikart 1996). The third method, described by Garza and Williamson (2001), uses the frequency of alleles, the total number of alleles (k), the difference between alleles in the number of repeats, and the overall range of allele size (r) to obtain the mean ratio of number of alleles to total range of allele size, $M = k/r$. This empirical method is based on the principle that, through the effect of genetic drift, the loss of any allele will contribute to a reduction in k but only a loss of the largest or smallest allele will contribute to a reduction in r. Thus, for bottlenecked populations, k is expected to be reduced more quickly than r, leading to reduced values of M. The M-values were calculated using the program AGARst (Harley 2001). The critical value used was that proposed by Garza and Williamson (2001), where values of M less than 0.68 generated by a limited number of alleles of different size, characterized a bottlenecked population. Additional Kolmogorov-Smirnov tests were performed in order to test for differences in the distribution of allele frequencies among populations.

Wright's *F*-statistics, based on the variance in the allele frequencies, were used to analyze within (F_{IS}) and between (F_{ST}) population structure, according to Weir and Cockerham (1984) and implemented in GENEPOP 3.4 (Raymond and Rousset 1995). As an alternative measure of population differentiation based on the variance in allele size under the stepwise mutation model, an R_{ST} estimator was used here noted as ρ_{ST} (Michalakis and Excoffier 1996; and see also Rousset 1996). Estimates of global levels of gene flow were calculated by the private allele method (Slatkin 1985), also using GENEPOP 3.4 (Raymond and Rousset 1995).

Measures of population structure (*F*-statistics; Wright 1951) were used to test for biases in dispersal tendencies of males, females, and populations as implemented by ARLEQUIN 2.1 (Schneider et al. 2000). A test of AMOVA considering two groups (males and females) with three samples of each (XA, RE, and PI) was performed to assess differences in the genetic variation apportioned among sex, among samples within sex, and within samples.

Other genetic estimates of sex-biased dispersal patterns were tested using four statistical indices calculated using FSTAT 2.9.3 (Goudet 1995). The first two are traditional global descriptors of population structure, F_{ST} and F_{IS}; and the others, based on a more recent approach relying on individual genotypes (assignment index, *AI*), are the mean of the corrected assignment index (*mAIc*), and the variance of *AIc* (*vAIc*; Goudet et al. 2002). Assignment indices determine the probability that an individual genotype may occur in the population from which it was sampled (Favre et al. 1997). In the absence of linkage disequilibrium, the probability of occurrence of a multi-locus genotype is calculated as the product of the probabilities of the individual loci (Goudet et al. 2002). Because differences between populations are not of interest, the assignment indices of individuals to their own

populations are standardized to remove the effect of population. In this way, the corrected assignment index (*AIc*) values are calculated by subtracting the mean assignment index for a given population from the assignment index of an individual, after log-transformation to avoid rounding errors with very small numbers. Thus, the distribution of *AIc* is centered on zero. A positive value indicates a genotype more likely than average to occur in its sample, whereas a negative value indicates a genotype less likely than average. Because immigrants tend to have lower *AIc* values than residents, and members of the dispersing sex will include both residents and immigrants, under sex-biased dispersal we expect that the sex which disperses most will have a lower mean *AIc* and larger variance than the more philopatric sex. These indexes were calculated for males, for females, and for all individuals within each population, and tested for significant differences between sex and between populations. The calculations were done excluding the loci from HWE.

RESULTS

Age Structure.–The limits established for each age class following Wilks's (1963) criterion were: 113 g for young individuals, 150 g for subadult females, and 265 g for subadult males. The mean weight for adult females was 222.8 g ± 29.8 SE (n = 42), and for adult males was 332.8 ± 41.6 g (n = 19). The age structure in all the populations of *C. flamarioni* was skewed toward adult (mature) individuals: mature/ (mature + immature) = 0.74.

Sexual Structure.–Non-significant deviations from the proportion 1:1 were observed for the total number of individuals (Chi-square test, n males = 36, n females = 42, χ^2 = 1.28, $P > 0.10$). The adult sex-ratio, expressed as R = male/ (female + male), was 0.31,

showing a significantly lower number of males than females (Chi-square test, *n* males = 19, *n* females = 42, $\chi^2 = 7.22$, $P < 0.05$).

Sexual size dimorphism.–Adult individuals belonging to all three populations showed significant sexual differences, both in body weight and body length, with males being heavier and longer than females (weight: 332.8 ± 41.6 g for males and 222.8 ± 29.8 g for females, $t = 10.70$, *d.f.* = 28, $P < 0.05$; body length: 212.0 ± 7.7 mm for males and 188.2 ± 9.5 mm for females, $t = 10.66$, *d.f.* = 45, $P < 0.05$).

Population size and density.– The population sizes estimated from captured and recaptured animals scored for two years were: *n* = 24 individuals at XA, *n* = 30 individuals at RE, and *n* = 31 at PI. The estimated densities for each population (based on the individuals scored in the field) were approximately 10 ind/ ha for XA and RE, and 4 ind/ ha for PI.

Microsatellite polymorphism and genetic variability.– All loci were polymorphic, except Hai 5 at XA and RE was monomorphic (Table 1). The fixed allele in these populations was also most frequent in the PI population. The total number of alleles was 24 for RE (*n* = 27) and XA (*n* = 24), and 32 for PI (*n* = 30). The number of alleles detected per locus ranged from three (Hai 4 and Hai 6) to six (Soc 2), with a mean of 4.2 alleles. A total of 12 private alleles were observed in the PI tuco-tuco population, and 2 for RE; no private alleles were obtained for XA. The allelic richness (*A*), calculated as the mean number of alleles across populations, and the values corrected for variation in sample size (bootstrapping and jackknifing A_c) showed lower values for XA and RE than PI (Table 1). Despite these observed differences among populations, the Wilcoxon signed ranks test shown non-significant interpopulation differences in the number of alleles ($T^+ = 5$, $n = 4$, P

= 0.50 for RE and XA population comparison; $T^+ = 5$, $n = 7$, $P = 0.20$ for XA and PI population comparison; and $T^+ = 6$, $n = 8$, $P = 0.13$ for RE and PI population comparison).

The expected values (H_e) for each locus ranged from 0 for Hai 5 (XA and RE) to 0.780 for Soc 2 at PI. Likewise, the observed values (H_o) ranged from 0 for Hai 5 at XA and RE to 0.767 for Soc 2 at PI. Values of H_o for each population over all the loci examined were $0 \leq H_o \leq 0.704$ for RE, $0 \leq H_o \leq 0.625$ for XA, and $0.100 \leq H_o \leq 0.767$ for PI. The Wilcoxon signed rank test to assess interpopulation differences in the expected heterozygosity produced non-significant *p*-values for all the pairwise comparisons ($T^+ = 12$, $n = 8$, $P = 0.50$ for RE and XA population comparison; $T^+ = 14.5$, $n = 9$, $P = 0.43$ for XA and PI population comparison; and $T^+ = 9$, $n = 8$, $P = 0.28$ for RE and PI population comparison).

Two loci (Hai 12 for XA; Hai 6 for PI) showed significant deviations from the genotype proportion expected according to the Hardy-Weinberg equilibrium (HWE) after the Bonferroni correction for multiple tests ($P < 0.003$). A global Test of HWE using all loci showed a deviation from HWE equilibrium only for XA, but it disappeared when Hai 12 was excluded from the analysis. Pairwise comparisons of allele frequencies revealed significant linkage disequilibrium between Hai 6 and Hai 9 in XA, after α-levels were corrected for multiple tests ($P < 0.0006$).

Using a Bayesian MCMC approach, the genetic distinctiveness of the three populations was inferred solely on the basis of multilocus microsatellite genotypes. Considering the range of one to three potential populations, the probability value obtained for three populations (mean value of ln likelihood = - 1085.7 ± 8.0) was higher than two (- 1160.3 ± 6.5) and one population (- 1454.1 ± 3.8).

The allele frequency distributions showed differences among populations (Fig. 2). The calculations were done excluding the loci from HWE (Hai 12 for XA and Hai 6 for PI).

The distribution of allele frequencies for PI was clearly L-shaped, with the largest proportion of alleles at low frequencies (0.01 - 0.20). In contrast, the distribution of allele frequencies from the XA and RE datasets showed the largest proportion of alleles in the class of intermediate frequencies (0.21 - 0.80), with a consequent diminution in the class of low-frequency alleles. The shape of the distribution of allele frequencies in the latter populations was more clearly shifted toward a lower number of more-abundant alleles, as expected in bottlenecked populations; even so, the statistical test showed significant mode-shift distribution only for XA population.

Kolmogorov-Smirnov tests showed significant differences in the distribution of allele frequencies between the XA and PI populations (χ^2 = 13.47, *d.f.* = 2, *P* = 0.001) and the RE and PI populations (χ^2 = 10.40, *d.f.* = 2, *P* = 0.006), but not between the XA and RE populations (χ^2 = 1.30, *d.f.* = 2, *P* = 0.520).

The test for excess heterozygosity produced non-significant *p*-values for the PI population (0.230 based on TPM and 0.677 based on SMM), and different significant *p*-values depending on the mutation model for the other two populations (0.027 based on TPM and 0.045 based on SMM for XA; 0.011 based on TPM and 0.048 based on SMM for RE).

Genetic signatures of bottlenecks estimated using the ratio *M*, the mean ratio of the number of alleles to the range of sizes of alleles, were not observed. For the polymorphic loci in HWE analyzed, were obtained: *M* = 0.73 for XA (seven loci), *M* = 0.76 for RE (eight loci), and *M* = 0.79 for PI (eight loci). All *M* values were higher than the critical value (0.68) indicated by Garza and Williamson (2001).

Genetic differentiation and gene flow.–The estimates of interpopulation differentiation measured by F_{ST} and by ρ_{ST} were similar, and thus only the F_{ST} values are shown (Table 2).

The values revealed significantly high levels of genetic differentiation among PI and the other populations. The pairwise F_{ST} between RE and XA was lower, but still indicated significant population structure. These results are in concordance with the numbers of migrants per generation calculated using Slatkin's private alleles method for males, females, and all individuals among the populations (Table 2).

Estimates of intrapopulation structure calculated using the inbreeding coefficient (F_{IS}), indicated a higher value for XA (F_{IS} = 0.193, P < 0.01) than for RE (F_{IS} = 0.077, P = 0.13) and PI (F_{IS} = 0.042, P = 0.17; Table 1). However, when the locus in disequilibrium of HW (Hai 12) was removed, the inbreeding coefficient for XA decreased to F_{IS} = 0.118 (P = 0.06).

Analyzing the pairwise values of F_{ST}, we found that the overall patterns for males and females were concordant with those observed for the pairwise F_{ST} across populations for all individuals: lower values for XA - RE, and higher for the other pairwise comparisons (Table 2). However, the male F_{ST} value obtained for XA - RE was lower and not different from zero at the 0.05 level after Bonferroni correction for multiple tests (F_{ST} = 0.086, P = 0.019, α_c = 0.016). All the other measures of pairwise F_{ST} were highly significant (P < 0.0001). Similarly, the number of migrants per generation using the private alleles method was less than one for all comparisons except between XA and RE male migrants (Table 2).

From Amova tests, the proportion of the genetic variation apportioned within populations was higher (near 75%) than that apportioned among populations in the three tests performed (all individuals, males, and females). Overall obtained F_{ST} values were high and significant: females, F_{ST} = 0.238; males, F_{ST} = 0.258; and all individuals, F_{ST} = 0.248; P < 0.0001). The amova performed to assess differences in the genetic variation

apportioned among sex, among samples within sex, and within samples showed non-significant differences only for the first hierarchical level (among sex; Table 3).

Values of F_{IS} for both males and females were not significantly different from zero for all three populations (Table 4). Both the *mAIc* and the *vAIc* values obtained for each population showed non-significant differences between the sexes (Table 4).

When tested for differences among populations, the assignment-index values for XA and RE showed significant differences when compared with PI, but not from each other.

DISCUSSION

Hardy-Weinberg equilibrium, genetic variability and linkage disequilibrium.– Significant linkage disequilibrium was observed only between Hai 6 and Hai 9 in the XA population. Evidence for linkage disequilibrium can be caused either by real association between alleles at these loci (by physical linkage or selection on multilocus genotypes), or may be an artifact of substructure (i.e., the presence of subgroups or inbreeding within some samples; Otha 1982). Because other studies with these loci in the genus *Ctenomys* did not show linkage disequilibrium between Hai 6 and Hai 9 (El Jundi and Freitas 2004; Gava and Freitas 2004; Lacey 2001; Wlasiuk et al. 2003), linkage because of close proximity on the chromosomes seems less likely.

Levels of microsatellite variability in populations of *C. flamarioni* were intermediate compared with other species of the genus *Ctenomys*. In *C. flamarioni* we detected 38 alleles for the 9 polymorphic loci analyzed, with a mean of 4.2 (ranging from 3 to 6) per locus (Table 1). Mean numbers of alleles per locus reported for other species are: 2.5 (range 2 to 3) for *C. lami* (El Jundi and Freitas 2004); 8.3 (range 6 to 14) for *C. rionegrensis* (Wlasiuk et al. 2003); 7.5 (range 3 to 13) for *C. haigi* (Lacey 2001); and 9.3

(range 5 to 15) for *C. minutus* (Gava and Freitas 2004). In the social species *C. sociabilis*, the mean number of alleles per locus was 2.3 for the polymorphic loci, ranging from 1 to 3 (Lacey 2001). From a populational analysis in *C. talarum* was reported 2 to 6 and 2 to 9 alleles per locus in each of two populations studied (Cutrera et al. 2006).

Even though the estimated allelic richness values (A and A_c) for the XA and RE populations were lower than that found for PI, the tests applied to search for differences in number of alleles among population indicated non-significant differences among them. Likewise, no significant interpopulation differences in expected heterozygosity were detected. Although there is no evidence of significant quantitative differences in the number of alleles and heterozigosity, the existence of a higher number of private alleles in the PI population (12 alleles) than RE (2 alleles) and XA (no alleles) evidences qualitative differences among them (see below).

A global exact test of the Hardy-Weinberg equilibrium (HWE) using all loci revealed a deviation from equilibrium only for the XA population. This deficit of heterozygotes was suggested by the significant values of F_{IS} obtained in this population. Several possibilities may account for the observed deviation from the HWE: nonrandom mating, inbreeding, null alleles, and population structure (e.g., the Wahlund effect). Because no blank genotypes (putative null homozygotes) were observed, and the loci were polymorphic and in HWE in other populations studied (data not shown), the null-allele hypothesis could be rejected.

Differences in population demographic history: origins and evolutionary processes.– The characteristics of the process of population formation and the history of reductions in population size may play an important role in the evolution of the patterns of genetic variation. Populations that originated from a small number of individuals will show

reductions in genetic variability compared with populations without strong founder effects. These low levels of initial variability can only be reversed by sufficient increases in the population size, gene flow, and periods of stability over time. Likewise, populations that were subject to past reductions in population size will undergo different degrees of reduction in variability (Avise 1994). Although we do not know the demographic history of these populations of *C. flamarioni*, the observed patterns of genetic variation suggest different rates of gene flow, genetic drift, and variation in population size.

Many different approaches to detect the genetic signatures of a recent bottleneck have been proposed (Beaumont 1999; Cornuet and Luikart 1996; Garza and Williamson 2001; Leberg 2002; Luikart et al. 1998a, 1998b; Spencer et al. 2000), with different limitations on their predictive power. We combined three methods to obtain more powerful estimates. The Wilcoxon signed rank test for excess of heterozygosity and the test for fit to L-shaped allele's frequency distribution showed differences between the PI and the other populations. These tests detected a pattern of recent bottleneck for the more-disturbed locations, XA and RE populations, but not for PI. The pattern was confirmed by the Kolmogorov-Smirnov test, to compare the distribution of allele frequencies among populations.

The *M*-statistic, which has been shown to be a good predictor of the demographic history for the population in which this information was available (Garza and Williamson 2001; but see Whitehouse and Harley 2001), did not detect a pattern of bottlenecked population as suggested by the former test. If the bottleneck effectively existed in the demographic past of the XA and RE populations, some possible causes may be responsible for the higher than expected *M*-values: 1. the effect of entrance of new alleles into the populations by immigration; 2. a low ratio of multi-step to one-step mutations in the tuco-

tuco microsatellite dataset that produced a more restricted (and bell-shaped) size distribution of alleles; 3. the effect of mutation and genetic drift acting together. Because the XA and RE populations occupy a very fragmented area, the effect of entrance of new alleles as the main cause for the observed increase of *M*-values seems improbable. A more plausible explanation may be the combined action of the process of allele generation, with a high proportion of one-step mutations and the genetic drift guiding the loss of alleles. This could increase the chance of losing the largest or smallest allele, which in turn would reduce the allele size range and offset any reduction in *M*. On the other hand, the evidence from the private alleles suggests that PI, with 12 private alleles and all of the 9 loci studied in the polymorphic state, could effectively have a demographic history of more stability than RE and XA, with 2 private alleles and none respectively, and 1 of 9 loci in the monomorphic state.

However, an additional point to be considered is the action of non-human factors in the decrease of genetic variability. First, a genetic bottleneck can also occur in the absence of a demographic bottleneck, when there are few breeders of one sex because of a skewed sex ratio or a polygynous (or polyandrous) breeding system (Luikart et al. 1998b; but see Dobson et al. 2004). In this sense, the polygyny evidenced in *C. flamarioni* from field data could produce or act with the bottleneck, contributing to the observed patterns in the smaller (XA and RE) populations. Second, the known instability of the coastal environment, both historical and contemporary, could have played an important role in the patterns of differentiation and genetic variation observed nowadays in the tuco-tuco populations. Again, the smaller and more-isolated populations of XA and RE should have suffered more than PI, the action of genetic drift on their variability. Analyses for two demographically distinct populations of the talar tuco-tuco (*C. talarum*) had shown that

despite their differences in the population density, degrees of polygyny, patterns of population structure and genetic variability, two of the three procedures used to detect historical reductions in population size failed to reveal significant evidence of bottleneck (Cutrera et al. 2006). The test for excess heterozygosity, the most powerful test used in our study, was the only one that produced significant *p*-values for the *C. talarum* population that exhibit less variability, stronger pattern of polygyny, more subdivision and more population density than the other.

Genetic variation and sex-biased dispersal pattern.– Despite the short distance between XA and RE, our results showed genetic divergences among all three populations studied, as evidenced by the significant values of pairwise genetic differentiation (F_{ST}). The choice of F_{ST}-based estimates was supported by the close similarity of the pairwise values obtained from the analogue ρ_{ST} (assuming a stepwise mutation model), and because when sample sizes are moderate or small ($n_s \leq 10$) and the number of loci scored is low ($n_l < 20$), F_{ST} provides more precise estimates than ρ_{ST} (Gaggiotti et al. 1999). On the other hand, F_{ST} can be used simply as a relative measure of population divergence, and to suggest tendencies in the current gene flow (Neigel 2002).

The gene flow estimates based on private alleles showed low numbers of migrants among the population, although sustaining the same pattern that was observed from F_{ST} estimates with a higher number of migrants per generation for RE and XA ($Nm \geq 1$) than that observed between these populations and PI ($Nm < 1$).

Analyzing the population structure and considering males and females independently, the divergence values between populations (F_{ST}) were non-significant only for males between the nearest populations (RE and XA; Table 2), a pattern also shown in the *Nm* estimates. Because the degree of genetic structure is expected to decrease with an increase

in dispersal distance (Perrin and Mazalov 2000), these results suggest the existence of a subjacent pattern of male-biased dispersion. This pattern could be expected from the evidence of polygyny in *C. flamarioni* (sexual dimorphism and significant deviations from a 1:1 adult sex ratio). The reason is that male-biased dispersal has been reported to be stronger in polygynous than in monogamous mammals (Dobson 1982), because males show strong local mate competition, which results in pressure for male dispersal (Perrin and Mazalov 1999, 2000). Evidences of polygyny in the genus were reported from morphological and demographical data for *C. australis* (Malizia et al. 1991; Zenuto and Busch 1998) and *C. talarum* (Busch et al. 1989; Malizia and Busch 1991, 1997). In the latter species, polygyny was also confirmed from behavioral data (Zenuto et al. 2002) and molecular data (DNA fingerprinting; Zenuto et al. 1999). On the other hand, the fact that the pattern of male-biased dispersion was observed only among XA and RE populations could be explained by a stepping-stone model, in which we would expect to see differences between F_{ST} males and females from neighboring populations, but these differences will vanish as populations are farther and farther apart (Goudet et al. 2002). For this reason, the F_{ST}-based test should be more powerful to detect short-distance than long-distance sex-biased dispersal.

However, when we tested for between-sex differences by the assignment index (*mAIc* and *vAIc*) and F_{IS}, these differences were not significant for any population, nor were they when all populations were pooled together. This pattern of no differences among the apportioned variation by sex was also supported by the AMOVA analysis.

The differences observed through the different methods applied may be related to the problem that sex bias and dispersal rate need to be very high to be detected by any of these methods (Goudet et al. 2002), and therefore the absence of patterns of biased dispersal

(principally when it is small) might be shaped by limitations of the statistical method, rather than by behavioral or ecological causes.

Nevertheless, in subterranean rodents, dispersal by both sexes appears to be very frequent (Busch et al. 2000), and was reported in populations of *C. talarum* (Malizia et al. 1995; Zenuto and Busch 1998). Likewise, other studies estimating dispersal from capture-mark-recapture field data for *C. flamarioni* have shown no significant sex-biased dispersal (JFBS pers. comm.).

Inter-population differences were observed in XA and RE, which are denser and more human-impacted, versus PI. Even with only three populations analyzed, we expected a different dispersal pattern between saturated versus non-saturated populations (Perrin and Mazalov 2000), with more evidence of dispersal (and more clear for females than for males) in the low-density population. Comparisons from behavioral and genetic data reported differences in sex-biased dispersal patterns among high- versus low-density populations of *C. talarum*, with more male-biased dispersal in high-density populations, and bigger dispersal distances for males and females in low-density population (Cutrera et al. 2005).

In conclusion, based on our analyses, the *C. flamarioni* populations showed no unequivocal pattern of dispersal by sex, nor by populations. The former can be explained through the differences in the robustness of the methods employed and the low degree of philopatry of females. The significant differences in the dispersal patterns observed among the populations can be better explained because of the qualitative differences among the areas and the capability of individuals to disperse in high-density versus low-density areas.

Even though the common hypotheses to explain natal dispersal in mammals include inbreeding avoidance, competition with close relatives, and variation in territory quality

(Dobson 1982; Greenwood 1980), this study did not attempt to test this hypothesis directly, and further investigations are necessary to resolve the question of which of these factors may be affecting dispersal.

Knowledge of these and other genetic and demographic characteristics is fundamental for endangered and threatened species, such as *C. flamarioni,* which urgently require decisions on appropriate management to counteract the damage caused by human action. This damage could be increased by population characteristics such as their breeding system, and stochastic factors such as the natural instability of the coastal habitats of Rio Grande do Sul. Therefore, populations that presently show signs of loss of genetic variability must be more carefully managed, through action to protect not only the natural habitats but the variability of the processes within them.

RESUMO

O tuco-tuco-das-dunas, *Ctenomys flamarioni*, é um roedor endêmico do ecossistema de dunas costeiras do litoral sul do Brasil. No presente trabalho foram estudadas três populações com diferente grau de impacto antrópico, e usados métodos diretos e indiretos para acessar informações demográficas e genéticas das mesmas. A partir de estudos de campo, foi observada uma tendência à desvios da proporção sexual em favor das fêmeas, e dimorfismo sexual com machos maiores tanto para a variável peso quanto para comprimento do corpo. Estas evidências apóiam a hipótese de poliginia em *C. flamarioni*. Através do uso de 9 locos de microssatélites foram analisados os padrões de variação e estrutura genética populacional. Para Xangri-lá e Remanso, que habitam os ambientes mais alterados, é sugerido um padrão de redução recente do tamanho populacional não observado para a população de Pinhal. Todavia, outros fatores tais como o sistema

poligínico de cruzamento e a instabilidade ambiental que caracteriza as dunas costeiras, poderiam estar influenciando os padrões observados. As análises de estrutura genética revelaram forte diferenciação entre as populações, mas não dentro elas. Valores não significativos obtidos para os testes de *assignment* (F_{ST}, F_{IS}, *mAIc*, *vAIc*) em cada população, indicam um padrão de dispersão similar entre machos e fêmeas. O mesmo foi observado a partir dos testes de AMOVA. Todavia, os menores valores de F_{ST} e maiores de *Nm* obtidos para os machos das populações de Xangri-lá e Remanso, sugerem um padrão fraco de dispersão predominante dos machos. Diferenças nos padrões de dispersão entre as populações foram detectadas entre as fêmeas: maior dispersão para as fêmeas de Pinhal do que para as de Remanso e Xangri-lá.

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FIGURE LEGENDS

Fig. 1. The locations of the three populations of *Ctenomys flamarioni* studied on the coastal plain of Rio Grande do Sul, Brazil: Xangri-lá (XA; 29°47'S, 50°01'W; *n* = 24), Remanso (RE; 29°49'S, 50°02'W; *n* = 30) and Pinhal (PI; 30°18'S, 50°15'W; *n* = 31). The geographic distances between the populations are 2.5 km for XA - RE, 60 km for RE - PI, and 62.5 km for XA - PI.

Fig. 2. Allele frequency distributions in the three populations of *Ctenomys flamarioni* studied. The bars represent the percentage of all alleles detected (only for the loci in HW equilibrium), in each allele frequency class calculated for each population sampled: Xangri-lá (*n* = 24), seven loci; Remanso (*n* = 24), eight loci; and Pinhal (*n* = 33), eight loci.

TABLES

Table 1. Microsatellite genetic variation in *C. flamarioni*. Total number of alleles per locus (*N*), number of alleles per population (N_i), observed heterozygosity (H_o), Nei´s estimated heterozygosity (H_e) and inbreeding coefficient values (F_{IS}) per locus for each population (Xangri-lá, Remanso, and Pinhal). *n*, sample size; mean, average of polymorphic loci; *A*, mean number of alleles per population (allelic richness); $A_{c[B]}$ and $A_{c[J]}$, allelic richness corrected by bootstrapping and jackknifing respectively; %*P*, percentage of polymorphic loci.

		Xangri-lá (*n* = 24)				Remanso (*n* = 27)				Pinhal (*n* = 30)			
Locus	*N*	*Ni*	H_o	H_e	F_{IS}	*Ni*	H_o	H_e	F_{IS}	*Ni*	H_o	H_e	F_{IS}
Hai3	5	3	0.54	0.55	-0.07	2	0.37	0.51	0.32	4	0.67	0.59	-0.16
Hai4	3	2	0.04	0.08	0.00	2	0.04	0.07	0.00	3	0.40	0.47	0.11
Hai5	4	1	0	0	-	1	0	0	-	4	0.50*	0.62	0.13
Hai6	3	2	0.46	0.50	0.25	2	0.15	0.17	-0.06	3	0.10**	0.35	0.69**
Hai7	4	4	0.63	0.62	0.07	4	0.70*	0.65	-0.07	4	0.67	0.65	-0.06
Hai9	5	3	0.58	0.60	0.25	3	0.44*	0.50	-0.06	5	0.53	0.58	0.02
Hai12	4	4	0.13**	0.49	0.87**	3	0.63	0.61	0.00	2	0.57	0.51	-0.12

Table 1. Continued.

		Xangri-lá (n = 24)				Remanso (n = 27)				Pinhal (n = 30)			
Locus	N	Ni	H_o	H_e	F_{IS}	Ni	H_o	H_e	F_{IS}	Ni	H_o	H_e	F_{IS}
Soc2	6	3	0.50	0.64	0.08	4	0.70	0.66	-0.08	5	0.77	0.78	0.05
Soc3	4	2	0.38*	0.61	0.14	3	0.33*	0.54	0.44	2	0.23	0.23	-0.08
mean			0.36**	0.45	0.19**		0.37	0.41	0.08		0.49*	0.53	0.04
A	4.2	2.7				2.7				3.6			
$A_{c[B]}$		2.5				2.5				3.4			
$A_{c[J]}$		2.7				2.6				3.5			
%P		88.8				88.8				100			

* Deviations between observed and expected levels of heterozygosity at this locus were found to be significantly marginalized by the exact test for HWE ($0.01 < P < 0.05$), but the deviations were not significant when α-levels were corrected for multiple test.

** Significant heterozygote deficiency after Bonferroni corrections ($P < 0.003$).

Table 2. Pairwise fixation indices (F_{ST}, upper half matrix) and pairwise estimates of gene flow by the private alleles method (*Nm*, lower half matrix) between three *Ctenomys flamarioni* populations (Xangri-lá, XA; Remanso, RE and Pinhal, PI) based on nine microsatellite loci and calculated for (adult) females, males, and all individuals. Asterisks indicate significance levels for the fixation index, F_{ST}.

Population		XA	RE	PI
XA	Females	-	0.165**	0.252**
	Males	-	0.086*	0.283**
	All individuals	-	0.139**	0.283**
RE	Females	0.201	-	0.281**
	Males	5.470	-	0.307**
	All individuals	1.620	-	0.298**
PI	Females	0.203	0.189	-
	Males	0.152	0.134	-
	All individuals	0.286	0.208	-

**$P < 0.00001$; *$P = 0.019$, not significant after the Bonferroni-corrected $\alpha = 0.016$

Table 3. Analysis of molecular variance (AMOVA) among *Ctenomys flamarioni* male and female samples based on *F*-statistics. The test was performed considering two groups (males and females) with three samples of each (XA, RE and PI samples) in order to assess differences in the genetic variation apportioned between sex, among samples within sex, and within samples. d.f., represents the degrees of freedom in each analysis; %, percentage of total variance explained by each hierarchical level; *P*, probability that any random value obtained after 1000 permutations is > observed value.

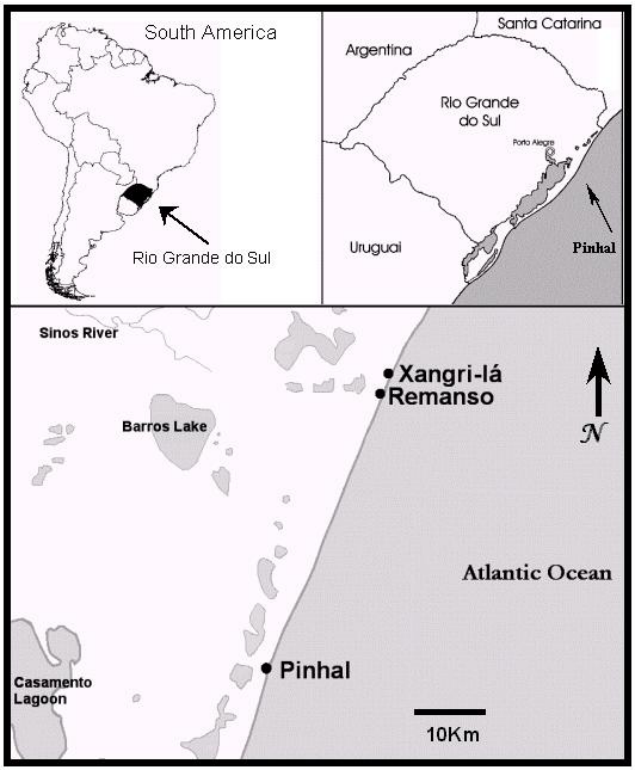
Variance components	*d.f.*	%	*F*-statistic	*P*
Between sexes	1	-9.1	F_{CT} = -0.09	0.8
Among samples within sex	4	27.3	F_{SC} = 0.25	0.00
Within samples	156	81.8	F_{ST} = 0.18	0.00

Table 4. Inbreeding coefficient values (F_{IS}), mean corrected assignment index (*mAIc*), variance of corrected assignment index (*vAIc*) and interpopulation comparison were obtained for (adult) females, males, and all individuals in each population (XA, Xangri-lá; RE, Remanso and PI, Pinhal). Significance levels for each intrapopulation indices were all not significantly diferent from zero, nor for between sexes (*p*-values not shown); *n*, sample size. ns, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; †calculated from seven polymorphic loci in HWE.

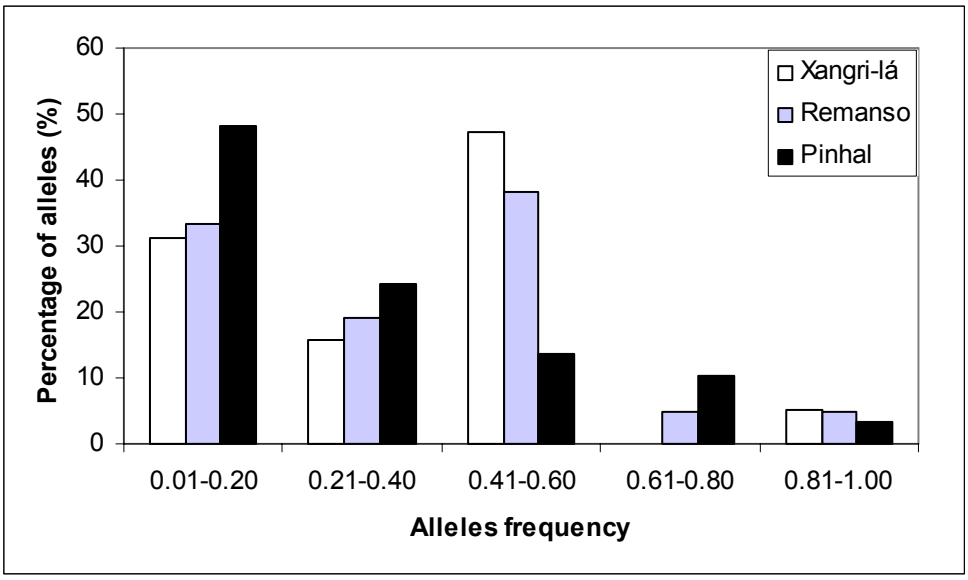
					Interpopulation comparison (*p*-values)		
		XA	RE	PI	XA vs. RE	RE vs. PI	XA vs. PI
F_{IS}	Females	0.086† (n = 15)	0.101 (n = 17)	0.071 (n = 15)	ns	ns	ns
	Males	0.080† (n = 9)	-0.027 (n = 10)	0.021 (n = 15)	ns	ns	ns
	All individuals	0.118† (n = 24)	0.077 (n = 27)	0.042 (n = 30)	ns	ns	ns
mAIc	Females	0.087	-0.076	-1.114	ns	**	**
	Males	-0.145	0.129	1.114	ns	ns	ns
	All individuals	-0.367	0.326	-1.146	ns	**	**
vAIc	Females	2.0	2.5	11.1	ns	*	**
	Males	5.2	3.0	5.5	ns	*	ns

Table 4. Continued.

				Interpopulation comparison (*p*-values)		
	XA	RE	PI	XA vs. RE	RE vs. PI	XA vs. PI
All individuals	5.4	3.4	11.4	ns	**	*


South America
Rio Grande do Sul
Argentina
Santa Catarina
Rio Grande do Sul
Porto Alegre
Uruguai
Pinhal
Sinos River
Xangri-lá
Remanso
Barros Lake
N
Atlantic Ocean
Pinhal
Casamento Lagoon
10Km

[Figure 1]


60
50
40
30
20
10
0
Percentage of alleles (%)
Xangri-lá
Remanso
Pinhal
0.01-0.20
0.21-0.40
0.41-0.60
0.61-0.80
0.81-1.00
Alleles frequency

[Figure 2]

IV. CAPÍTULO II

PATTERNS OF PHYLOGEOGRAPHY AND POPULATION HISTORY OF THE TUCO-TUCO-DAS-DUNAS (*CTENOMYS FLAMARIONI*): THE GENETIC FOOTPRINTS OF THE QUATERNARY SEA-LEVEL CHANGES IN THE COASTAL PLAIN OF SOUTHERN BRAZIL

GABRIELA P. FERNÁNDEZ-STOLZ, J. F. B. STOLZ AND THALES R. O. DE FREITAS

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Gabriela Paula Fernández-Stolz

Departamento de Genética, Avenida Bento Gonçalves 9500, Prédio 43323

Caixa postal 15053 - CEP 91501-970, Bairro Agronomia, Porto Alegre,

Rio Grande do Sul, Brazil.

gabriela.fernandez@ufrgs.br

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GABRIELA P. FERNÁNDEZ-STOLZ [1, 2], J. F. B. STOLZ [3, 4] AND THALES R. O. DE FREITAS [1, 3, 5, 6]

[1] *Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre 91501-970, Rio Grande do Sul, Brazil*

[2] *E-mail: gabriela.fernandez@ufrgs.br*

[3] *Programa de Pós-Graduação em Ecologia, Universidade Federal do Rio Grande do Sul, Porto Alegre 91540-000, Rio Grande do Sul, Brazil*

[4] *E-mail: tucotuco@pop.com.br*

[5] *Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre 91501-970, Rio Grande do Sul, Brazil*

[6] *E-mail: thales.freitas@ufrgs.br*

Abstract.--- Knowledge of the patterns of distribution of the genetic variation over the landscape provides valuable information to infer the evolutionary scenarios related to the evolution of a species. The tuco-tuco-das-dunas, *Ctenomys flamarioni*, has a distribution limited to the most recent Quaternary sand dunes on the southern coast of Brazil, and its evolutionary history has been linked to the geomorphologic evolution of the coastal plain. The main goal of this study was to assess the extent of this relationship. Nucleotide sequence data from the mitochondrial control region and cytochrome-*b* (cyt-*b*) were used in a phylogeographic context to investigate the history of the populations of *C. flamarioni*. AMOVA analyses showed the substantial relationship between the haplotype partitioning and the two of three hypothesized historical barriers to gene flow. Positive correlation between the genetic and geographic distances (isolation-by-distance pattern) was found when the total sampled range was considered, but not when only central populations were tested. Weak evidence of recent population expansion was obtained from the mismatch distribution, Tajima and Fu's tests, and using an exponential growth model. The lack of power of the applied tests may result from a complex pattern of the species' occupation of its range of distribution, in which historical and current reductions in the population size possibly had a primary role in the loss of genetic variability. Further analysis from nonparametric and semiparametric methods had estimated a divergence time of haplotypic lineages for the species of 90,000 years ago (YA), in the Late Pleistocene, and a rate of cyt-*b* evolution $\mu = 1.9\%$ per million of years. Using this rate and assuming a stepwise scenario, the major increase in effective population size was estimated at 60,000 YA. However, this time, based on a particular rate of mutation and evolutionary scenario, is better seen as a series of reductions and expansions at different temporal scales. This pattern, and the observed low variability of the mitochondrial molecular markers, was

hypothesized to have been principally caused by the transgression-regression marine cycle in the Holocene, with a maximum 5,000 YA, and accentuated by more recent stochastic events at a smaller geographic scale.

Key words.--- Phylogeography, *Ctenomys flamarioni*, genetic variability, range expansion, control region, cytochrome-*b*, tuco-tuco.

Beginning as a formal discipline in 1980, and growing explosively during the 1990s, phylogeography is one of the most important conceptual tools for understanding the historical components of the contemporary spatial distributions of gene lineages (Avise 2000).

Historical and contemporary characteristics, such as topographic or climatic changes in the habitat, can affect the spatial structure of the populations and then act on the genealogical relationship patterns observed. Thus, the phylogeographic approach aids in characterization of the geographical distribution, phylogenetic relationships, genetic distances, and time of divergence among evolutionary lineages. Moreover, phylogeographic analysis allows us to test the congruence between the evolution of landscapes, and the demographic and distributional histories of one or more taxa spread across that landscape (Avise 1998; Bermingham and Moritz 1998; Conroy and Cook 2000; Lessa et al. 2003; Whorley et al. 2004). In other words, this approach can test the strength of the historical biogeographical factors as shapers of a species' distribution patterns.

Knowledge of how genetic variation is partitioned among populations in the landscape may have important implications for conservation biology (Manel et al. 2003). Phylogeography can be of great utility in developing conservation strategies, through the

identification of the population genetic resources and any evolutionarily isolated areas (Avise and Hamrick 1996; Moritz and Faith 1998; Firestone et al. 1999), mainly in threatened and endangered species.

The tuco-tuco-das-dunas *Ctenomys flamarioni* (Travi 1981) inhabits the littoral sand dunes of the State of Rio Grande do Sul in southern Brazil. Its geographical distribution extends from Arroio Texeira in the north, to the mouth of the Chuí River in the south. The species is thought to have originated from the Argentinean species *C. australis*, through migration and subsequent isolation (Freitas 1994; Massarini and Freitas 2005), then spreading across the most recent marine and eolian deposits of the Coastal Plain of Rio Grande do Sul (CPRS; Freitas 1994). This hypothesis is supported by a combination of cytogenetic evidence (the same karyotype with $2n = 48$ and a large amount of heterochromatin), the simple-asymmetrical form of the spermatozoa (which is also shared with another species of the mendocinus-group; Massarini et al. 1991; Freitas 1994 and 1995; D'Elia et al. 1999; Slamovits et al. 2001; Massarini and Freitas 2005) and shared morphological traits (larger size and pale color, cryptic in the substrate) and ecological characteristics related to its exclusive occupancy of coastal sand dunes (Busch 1989; Malizia et al. 1991; Freitas 1994 and 1995; Mora et al. 2006; Fernández-Stolz et al. in press). This limited habitat, added to the urban development in coastal environments and the progressive destruction of the natural landscape (Tomazelli and Villwock 2000; Esteves et al. 2002) have made *C. flamarioni* a threatened species (Fontana et al. 2003).

The geomorphological evolution of the CPRS was described by Villwock et al. (1986) and was well characterized in several stratigraphic and paleontological studies (Tomazelli and Villwock 1996, 2000; Villwock and Tomazelli 1998; Tomazelli et al. 2000). The coastal plain comprises two main depositional systems, both formed during Quaternary

glacio-eustatic fluctuations in sea level: the alluvial fan deposits in the west, and the barrier-lagoon system in the east. The latter was created by at least four successive transgression-regression cycles, which formed four barrier-lagoon systems at the landward limit of a marine transgression and preserved it because of the subsequent regression of the shoreline. The fourth and latest interglacial sea-level event was responsible for the formation of the most recent barrier-lagoon system about 5,000 years ago (YA).

The Rio Grande do Sul shoreline is 620 km long, extending between 29° and 34° S, and is one of the longest sandy-beach coastlines in the world, with only two permanent discontinuities (the Tramandaí Lagoon and Patos Lagoon inlets). In the last 5,000 years, the sand budget and thus the coastal morphology of the Rio Grande do Sul coast has been mainly controlled by alongshore gradients of wave energy (Esteves et al. 2002).

Considering the lack of genetic information on the tuco-tuco-das-dunas and the need for action to promote its conservation, our goals were the examination of its phylogeographical structure, its genetic diversity, the divergence times between the main phylogenetic lineages, and the congruence (or lack thereof) between these patterns and the geological evolution of the CPRS. We used haplotype variants of fragments of the mitochondrial cytochrome-*b* and control region, and analyzed these two combined datasets, to obtain more information about the evolutionary history of this species.

Materials and Methods

Sampling

The entire range of *C. flamarioni* in the shoreline sand dunes of the Coastal Plain of Rio Grande do Sul, Brazil (nearly 600 km long) was studied. The populations sampled were selected by taking into account four regions limited by three potential natural barriers

to gene flow: two of these are permanent discontinuities (Tramandaí Lagoon inlet, barrier A; and Patos Lagoon inlet, barrier C), and the third is the principal temporary washout (Peixe Lagoon inlet, barrier B; Fig. 1). We obtained tissue samples of 89 individuals from the following regions and localities (*n* = sample size): Region I (*n* = 22): Xangri-lá (XA; 29°47'S, 50°01'W; *n* = 7), Remanso (RE; 29°49'S, 50°02'W; *n* = 9) and Imbé (IM; 29°55'S, 50°06'W; *n* = 6); Region II (*n* = 23): Pinhal (PI; 30°18'S, 50°15'W; *n* = 12) and São Simão (SS; 30°58'S, 50°40'W; *n* = 11); Region III (*n* = 16): Bujurú (BJ; 31°39'S, 51°22'W; *n* = 9) and São José do Norte (SJN; 32°03'S, 51°59'W; *n* = 7); Region IV (*n* = 28): Cassino (CASS; 32°09'S, 52°06'W; *n* = 6), Taim (TA; 32°43'S, 52°26'W; *n* = 15) and Chuí (CHU; 33°44'S, 53°22'W; *n* = 7). The individuals were trapped using Oneida Victor No. 0 snap-traps, wrapped with rubber strips to avoid injury and overstress to the animals. For each specimen collected, ear biopsies or tail skin tissue were taken and preserved in absolute ethanol. Skin samples were deposited in the collection of the Cytogenetic and Evolution Laboratory of the Genetics Department of the Federal University of Rio Grande do Sul. All individuals included in this analysis were adults, based on the age criteria taken from Wilks (1963). Their reproductive condition and body weight were measured as described by Fernández-Stolz et al. (in press).

Molecular Methods

Mitochondrial DNA was extracted following Medrano et al. (1990). We examined variation in the hypervariable 5´region of the control region (391 base pairs, bp) in 89 individuals of *C. flamarioni*. Polymerase chain reaction (PCR) amplification of the region between the tRNApro locus and the central conserved domain was done using primers TucoPro (5´- ACT TTC GTT TAT TGC TTA ATT - 3´; Tomasco and Lessa in press) and TDKD (5´- CCT GAA GTA GGA ACC AGA TG -3´; Kocher et al. 1989). PCR

amplifications were carried out in a reaction volume of 20 µl containing 25-100 ng of DNA, 0.2 µM of each primer, 0.2 µM dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California). The thermocycling profile included an initial denaturing at 94ºC for 5 min, followed by 30-34 cycles of denaturing at 94ºC for 30 s, annealing at 45ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min.

We also amplified 665 bp of the mitochondrial cytochrome-*b* gene for a subsample of 43 individuals. The primers used in the fragment amplifications were MVZ 05 (5´- CGA AGC TTG ATA TGA AAA ACC ATC GTT - 3´), - TUCO 06 (5´- GTG AAA TGG AAT TTT GTC TGA - 3´) and TUCO 07 (5´- ATT ACA GCA ATA GTA ATA AT - 3´), - TUCO14 (5´- CCA ATG TAA TTT TTA TAC - 3´). The conditions of PCR amplification were the same used for the set of samples from the control region, with only one change in the thermocycling conditions for the fragment amplified by the MVZ 05 - TUCO 06 pair of primers: the annealing temperature was 48ºC.

PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California) performed following the guidelines of the suppliers and sequenced in an ABI Prism 3100 (Applied Biosystem) automated sequencer, using the forward primer used in the PCR. For the samples with unclear sequences, the procedures were also done with the reverse primer.

Statistical Analyses

Molecular diversity, population divergence, and phylogeographic structure

Standard genetic diversity indices as haplotype diversity (*Hd* - Nei 1987) and mean number of pairwise differences (π - Tajima 1983) were estimated for both mitochondrial

datasets using ARLEQUIN 3.0 (Excoffier et al. 2005). The population parameter theta (θ = 2Mu, where M is equal to N for the haploid population, and u is the overall mutation rate at the haplotype level) was estimated based on the number of segregating sites ($\theta_s = \theta_w$ - Watterson 1975).

An analysis of molecular variance (AMOVA – Excoffier et al. 1992) implemented using the program ARLEQUIN 3.0 (Excoffier et al. 2005) was used to estimate the partitioning of genetic variation and to test the hypothesis that the present geographical barriers provide a good explanation for the genetic divergence observed in *C. flamarioni*. With this aim, we ran three analyses: (1) considering all populations subdivided into three groups, taking into account two permanent geographical discontinuities in the range (the Tramandaí Lagoon [A] and Patos Lagoon [C] inlets); (2) all populations separated into four groups within each region limited by the two permanent discontinuities, plus a potential third barrier (Peixe Lagoon [B] inlet); (3) and on a subset including only populations of regions II and III arranged in two groups within each region, determined by the potential barrier (B; Fig. 1).

In order to test for positive correlations between genetic and geographical distances (isolation by distance; Slatkin 1993), we used a Mantel test (Mantel 1967) as implemented in ARLEQUIN 3.0 (Excoffier et al. 2005). Statistical significance was tested using 10,000 random permutations. This test was performed for the entire range of the distribution of *C. flamarioni*, and also only for the central region, delimited by the two permanent washouts (regions II and III).

These analyses were performed for the obtained sequences organized in two datasets: control region and control region plus cyt-*b* (expanded mitochondrial).

The median-joining network method (Bandelt et al. 1999) was performed for two datasets in the NETWORK program (http://www.fluxus-engineering.com/netwinfo.htm).

This method, using a parsimony criterion, combines the minimum-spanning trees (MSTs) with a single network, allowing more detailed populational information than do strictly bifurcating trees (see review in Posada and Crandall 2001).

Historical population dynamics

We used a distance-based method (mismatch distribution) based on an assumed stepwise growth model (Roger and Harpending 1992; Rogers 1995) to evaluate the hypothesis of recent population growth performed through the program ARLEQUIN 3.0 (Excoffier et al. 2005). This method evaluates the distribution of the observed number of pairwise differences between haplotypes. Under a scenario of recent demographic expansion, a unimodal distribution of pairwise differences and a low mean number of nucleotide differences (k) is expected. The program uses a non-linear least-squares approach to estimate growth parameters (Schneider and Excoffier 1999): $\theta_0 = 2\mu N_0$ (before expansion), $\theta_1 = 2\mu N_1$ (after expansion) and $\tau = 2ut$ (time of expansion), where $u = m_\tau\mu$ is the mutation rate for the entire haplotype, m_τ is the number of nucleotides of the sequence, and μ is the mutation rate per nucleotide (Rogers and Harpending 1992). N_0 and N_1 are the effective population size of females before and after expansion, respectively. Approximate confidence intervals for growth parameters are obtained by a parametric bootstrap approach (1,000 replicates). The validity of the estimated stepwise expansion model is tested using the same parametric bootstrap approach by a goodness-of-fit test between the observed distribution of the pairwise differences pattern and the simulated one based on the estimated model parameters.

Two tests of selected neutrality based on the infinite-site model without recombination were implemented using ARLEQUIN 3.0 (Excoffier et al. 2005): Tajima's *D* test (Tajima

1989) and Fu's *Fs* (Fu 1997). The former compares two estimators of the mutation parameter θ through the test statistic $D = (\theta_\pi - \theta_s) / \sqrt{(\text{var}\ (\theta_\pi - \theta_s))}$, where $\theta_\pi = \pi$ (based on the number of nucleotide differences per site between any two sequences; Tajima 1983) and $\theta_s = \theta_w$ (based on the number of segregating sites; Watterson 1975).

Fu's *Fs* test takes into account the polarity of mutations, and it estimates θ based on the number of derived unique mutations (singletons). It appears to be a very sensitive test for population demographic expansion, and a more powerful detector of population growth than other tests (Fu 1997; Ramos-Onsins and Rojas 2002).

The significance of the *D* and *Fs* statistics is tested by generating random samples under an assumption of selective neutrality and population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990).

Additional estimates of population expansion were performed using LAMARC 2.0.2 (Kunher et al. 1998; Beerli and Felsenstein 2001), based on coalescence, to generate maximum-likelihood estimates of the present-day value of θ ($\theta = 2\mu N_F$) and the exponential growth rate (*g*), where μ is the mutation rate per nucleotide, and N_F is the present-day effective population size of females. This analysis, using a Markov Chain Monte Carlo approach with Metropolis-Hastings algorithm to search through genealogies, was performed for each mitochondrial dataset (control region, cyt-*b*, and expanded dataset). Based on the Akaike Information Criterion as implemented in the MODELTEST 3.7 program (Posada and Crandall 1998), we determined the substitution model that provided the best fit to each dataset, and used the empirical Ti/Tv ratio and base frequencies obtained from the program. The TrN model of substitution (Tamura and Nei 1993) was determined for the control region and the expanded dataset, and the HKY model (Hasegawa et al. 1985) for cyt-*b*. The nucleotide substitution rates under the TrN model

were [A-C] = [A-T] = [C-G] = [G-T] = 1.00; [A-G] = 409031808.00 and [C-T] = 60238004.00 for the control region, and [A-C] = [A-T] = [C-G] = [G-T] = 1.00; [A-G] = 18059036672.00 and [C-T] = 6524471296.00 for the expanded dataset. Under the HKY model, the Ti/Tv ratio was 4.9399e+14 for cyt-*b*. The Markov Chain Monte Carlo parameters for all three datasets were 10 short chains of 1,000 steps each and 2 long chains of 20,000 steps.

Because recent bottlenecks can affect the ability to detect historical population size changes, we tested for population bottlenecks using a graphical method to detect a deficit in rare haplotypes in the sample (Luikart et al. 1998). Therefore, we plotted the frequency distribution of the control region and cytochrome-*b* haplotypes in classes of frequencies over all the populations.

Coalescence Time and Mutation Rate

In order to investigate the divergence time and rates of substitution from the cyt-*b* haplotypes of *C. flamarioni*, additional complete sequences were obtained from GeneBank (1140 bp) for 20 caviomorphs (from the South American group), and sequences from *Ctenodactylus vali* and *Heterocephalus glaber* were used as the outgroup (see Appendix I). We used the program r8s version 1.70 (Sanderson 2003) to estimate these parameters. The program emphasizes nonparametric and semiparametric methods that relax the stringency of the clock assumption, using smoothing methods to obtain better estimates of rates and times. Its starting point was taken from a phylogenetic tree for caviomorphs with estimated branch lengths obtained by PAUP* 4.0b10 (Swofford 2002). We used the empirical parameters and base frequencies obtained from MODELTEST 3.7 for the GTR model with a proportion of invariable sites (I = 0.436), nucleotide substitution rates ([A-C] = 0.87; [A-

G] = 4.56; [A-T] = 1.40; [C-G] = 0.27; [C-T] = 13.88; [G-T] = 1.00) and $\alpha = 0.76$. Calibration points were used to permit scaling of rates and times to absolute units: one was assigned as a fixed age to the node, and the others as minimum or maximum age constraints to a node. The fixed age used corresponds to the radiation of the Caviomorpha, previously dated between 31 and 37 million years ago (MYA; Wyss et al. 1993), and estimated as 33.8 ± 1.8 MYA in a molecular timescale for caviomorph rodents (Opazo 2005). The minimum age and maximum age constraints were based on the same molecular timescale developed by Opazo (2005) (except when indicated) and on data from two fossil records (*). The time constraints used in our infile dataset were: 1. Ctenomyidae-Octodontidae split: min_age = 9.8 MYA (*) (Quintana 1994; Verzi 2002) and max_age = 17.5 MYA; 2. Octodontidae: min_age = 6.3 MYA, max_age = 9.3 MYA; and 3. *Ctenomys*: min_age = 3.0 MYA (*) (Verzi 2002). For the estimation of times and rates, we used two different approaches: the nonparametric rate smoothing (NPRS) method (Sanderson 1997) that relaxes the assumption of the molecular clock by using a least-squares smoothing of local estimates of substitution rates; and the penalized likelihood (PL) method, a semiparametric approach that combines a parametric model having a different substitution rate on every branch with a nonparametric penalty when rates change too quickly from branch to branch. The set of search strategies, optimization methods, and other algorithmic issues used to perform the program were based on recommendations given by the author in the User's Manual. Therefore, we used the optimal smoothing parameter value $S = 1000$, rates gamma distributed across sites and shape parameter $\alpha = 0.76$, number of searches from random starts = 5, number of restarts after each search = 5, and the other options by default.

Results

Molecular diversity, population divergence, and phylogeographic structure

In our control-region set of sequences of *C. flamarioni*, a total of 391 bp were aligned in 89 samples containing six variable and parsimony informative sites and a total of seven haplotypes (H1 - H7; GeneBank accession numbers AF000000-AF000000). From the 665 bp sequenced for the cyt-*b* subsample ($n = 43$) we obtained five variable sites, four parsimony informative, one singleton, and five haplotypes (C1 - C5; GeneBank accession numbers AF000000-AF000000). In the extended data matrix (two datasets together) from 42 sequences and a total of 1051 bp, we observed nine haplotypes (E1 – E9). The haplotype sequences for each dataset, and their frequencies and distribution through the ten populations studied are shown in Table 1. Measures of genetic variability in all populations sampled as haplotype diversity (*Hd*), nucleotide variation estimated based on the number of segregating sites (θ_w), and the mean number of pairwise differences for sampled populations (π) are shown in Table 2. The polymorphism observed within each population was very low, with only three of ten populations exhibiting two or three different haplotypes, and the rest being monomorphic for one haplotype. The polymorphic populations belonged to regions II (PI) and III (BJ) from control-region data, and regions III (BJ) and IV (CASS) from the cyt-*b* dataset. The values of diversity observed between sequences in PI (from the control region) and in BJ (from cyt-*b*) were higher than those obtained for the total set of sequences (Table 2).

Mantel's test detected a significant, positive correlation between genetic and geographic distances for both the control-region set of sequences ($r = 56\%$; $P < 0.001$) and the expanded mitochondrial dataset ($r = 46\%$; $P = 0.008$). However, when the test was

performed only for the central populations (regions II and III), the *p*-values were not significant.

The AMOVA showed that a statistically significant amount of the molecular variance could be attributed to each level tested (among groups, among populations within groups, and within populations, $P < 0.001$; Table 3). Nevertheless, the proportion of variance explained by intrapopulation divergence was lowest (≤ 20%) in the tests applied, as expected given the absence of polymorphism and low divergence among all the populations studied. Regardless of how the total pool of populations was organized (three or four groups), the greatest source of variation was found at the most inclusive hierarchical level (among groups). The proportion of variance explained by the divergence of haplotypes among populations belonging to the same group, was higher for the test of three groups than the four groups (Table 3). When only populations of regions II and III were used for the AMOVA test, the component of the variance explained by the two-group division was small and not significant for all datasets ($P > 0.1$; Table 3). All these results, obtained using pairwise difference values, were concordant with that obtained using Kimura-2-parameters, and computing *F*-statistics from haplotype frequencies only (data not shown).

The genealogical relationships between haplotypes were estimated in the median-joining networks illustrated in Figure 2. The networks from both the control region and the expanded dataset revealed a shallow pattern of phylogeographic structure. Haplotypes 1 for the control region and 6 for the expanded dataset had the highest probability of representing ancient forms because of their central position, and they differ from some other derived haplotypes by only one or a few nucleotide changes. Despite the observed

low phylogeographic structure, a group of populations belonging to region IV constituted an evolutionarily independent group.

Historical population dynamics

In spite of the shallow pattern of phylogeographic structure and the low number of nucleotide differences among haplotypes (Fig. 2), the different tests performed to test for demographic expansion indicated different conclusions about the subjacent demographic processes. The mismatch distribution analysis only supported the recent demographic expansion hypothesis for the expanded dataset when all populations were combined into a single one. The observed distribution of pairwise differences did not differ significantly from the simulated pattern based on the sudden expansion model ($P = 0.231$), indicating recent demographic expansion. Table 4 shows the parameters estimated under this model.

Neither Tajima's nor Fu's test for the control region data supported the demographic expansion hypothesis: the Tajima's D value was positive and nonsignificant, $D = 0.156$, $P = 0.571$; and the Fs was negative but likewise nonsignificant, $Fs = -0.519$, $P = 0.424$. From our expanded mitochondrial dataset of sequences, both Tajima's and Fu's test showed negative although nonsignificant values: $D = -0.273$; $Fs = -1.275$; $P > 0.10$ (Table 4).

The expansion test based on the coalescent and an exponential growth obtained from LAMARC 2.0.2 (Kunher et al. 1995; Beerli and Felsenstein 2001) for the three datasets again provided support for a population expansion hypothesis, although with wider confidence intervals, as shown in Table 4.

The results obtained for the observed haplotype frequency distribution (Fig. 3) showed a pattern different from the L-shaped one expected when no bottleneck effects on the genetic variability are observed.

The divergence time estimated using nonparametric methods for the *Ctenomys* node, obtained from our 908 bp cyt-*b* dataset, was 3.30 MYA, and for *C. flamarioni* was 0.09 MYA. The rate of substitution per site per unit of time was 0.019 for both nodes. The obtained rate of substitution ($\mu = 0.019$⁄site⁄million years [MY]) and the value of $\tau = 2.634$ previously estimated were used as an alternative method to calculate the time of expansion for the species. Using these values, the formulas $\tau = 2ut$, and $u = m_\tau\mu$, with $m_\tau = 1{,}037$ and assuming a generation time of one year, the value of the expansion time obtained was $t = 66{,}000$ YA (or 0.06 MYA).

Discussion

Taken as a whole, our results suggest a complex pattern of occupation of *C. flamarioni* in its range of distribution, with few signs of demographic expansion.

When all populations were considered together, the AMOVA analysis showed that the most variation was found at the among-groups hierarchical level despite the alternatives tested and the methods used. These results suggest the substantial relationship between the haplotype partitioning and the hypothesized historical barriers to gene flow. On the other hand, the proportion of variance explained by the divergence of haplotypes among populations belonging to the same group, was larger for the test of three groups than the four groups, suggesting the hypothesis that the present temporary geographic barrier (Peixe Lagoon inlet) should provide a good explanation for the genetic divergence observed between the *C. flamarioni* populations belonging to regions II and III. However, when only

these regions (II and III) were examined with the AMOVA test, the component of the variance explained by the population in two groups was small and not significant. This indicates that the Peixe Lagoon inlet probably contributed to the divergence observed nowadays, but does not constitute an important barrier to gene flow.

In accordance with our results, other genetic studies have shown the strength of barrier C (Patos Lagoon inlet) in the genetic differentiation between the northern and southern populations. An electrophoretic analysis based on ten polymorphic loci revealed a Roger's similarity coefficient ($S = 0.773$) between the northern and southern populations lower than those commonly found for conspecific populations (Moreira et al. 1991). On the other hand, Freitas (1994) found a negative correlation between the amounts of constitutive heterochromatin present in the *C. flamarioni* karyotype and latitude along the seashore of Rio Grande do Sul, explained principally by the loss of the short arm of chromosome 1 for populations below the Rio Grande Bar (barrier C in our study).

Evidence for the positive correlation between the genetic and geographical distances over the total range sampled from the Mantel test supports the hypotheses of restricted gene flow (linear pattern of differentiation). However, when the Mantel test was performed only for the populations belonging to region II and III, the lack of expected correlation did not support the hypothesis of stable occupation along the range. These results suggest that even though insufficient time has passed since *C. flamarioni* occupied its current distribution, the vicariant processes that isolated regions I and IV may be determining the observed pattern of isolation by distance, mimicking a situation of gene flow-genetic drift equilibrium.

On the other hand, the observed pattern of low nucleotide and haplotype diversity from both molecular markers used, suggests a recent reduction in the population size, with

weak signs of later demographic expansion. This scenario was suggested from the mismatch distribution test from our expanded dataset and the exponential growth. Fu's *Fs* test and the more conservative Tajima's *D* test did not detect significant disequilibrium among recent and ancient mutations.

The lack of agreement among the tests employed could be explained by the recent establishment of the *C. flamarioni* population in its present range but with slight demographic expansion, and it is probably masked by the new differentiation of allopatric populations (produced by the combination of recent and effective geographical barriers) and the existence of other processes shaping the demographic traits of the populations. The low number of haplotypes observed from three datasets, as well as the presence of only one haplotype (from the control region) and two haplotypes (from cyt-*b*) in samples over the Region IV range (about 214 km), suggest and support the hypotheses of a recent colonization by the species of its present range, and the existence of one (or more) reductions in population size and genetic drift masking the merger of new haplotypes. This was supported by the haplotype frequency distribution from two sets of data (control region and cyt-*b*) that showed low frequencies for the haplotypes, with frequencies between zero and one. This pattern differs from the L-shaped distribution expected from populations that did not experience some kind of reduction in size (Luikart et al. 1998). In this sense, both the characteristics of the process of population formation and the history of the reduction in population size may have acted to reduce the variability to the observed level. Both factors may have played an important role in the patterns of differentiation and genetic variation in the southern part of the distribution of *C. flamarioni* (region IV).

This explanation is supported by the well-reported instability of the coastal plain from geomorphologic evolutionary data, and determined by the periodic oscillations in climate

and habitat availability because of the associated glacio-eustatic sea-level fluctuations. In the last 7,000 YA the coastline of Rio Grande do Sul has experienced several changes in sea level, with a maximum nearly 5,100 YA situated at +5, followed by a minimum at 3,500 YA situated between -6 and -10, a later transgressive-regressive oscillation near +4 and from 2,300 YA, decreasing to the current one (Corrêa et al. 1992). There is evidence from palynological data about the marine ingression in Tramandaí and Patos lagoons during the Late Holocene (Medeanic et al. 2000, 2001; Marques-Toigo et al. 2002). Likewise, sedimentary structures and radiocarbon dating of marine fossils in Mirim Lake (southern distribution of the species; Buchmann et al. 1998) support the hypothesis of important reductions in the potentially occupied areas and a related reduction in population size.

On the other hand, the restricted distribution of *C. flamarioni* to Quaternary dunes suggests that this species is not much older than the dunes themselves; however, the lack of fossils and comparative phylogeography studies preclude the gathering of some evidence of the time of occupation of the sandy substrate and the entrance of ancestral forms onto the coastal plain of Rio Grande do Sul.

Our estimated divergence time for the species *C. flamarioni*, both from nonparametric and semiparametric methods that relax the stringency of the clock assumption (Sanderson 2002), was 90,000 YA. This suggests that the *C. flamarioni* clade originated within the Late Pleistocene, during the third Quaternary glacio-eustatic fluctuation of the sea level between the last Pleistocene maximum transgression (approximately 125,000 YA) and the later maximum regression (at about 18,000 YA). These estimates are concordant with the hypothesis of the entrance of *C. flamarioni* onto the southern Brazilian coastal plain when it was wider than the present and the Plate River was not such an important geographical

barrier (Corrêa et al. 1992), as proposed by Freitas (1994) and later by Massarini and Freitas (2005).

Using the distance-based method (mismatch distribution) based on an assumed stepwise growth model (Roger and Harpending 1992; Roger 1995), we obtained ~ 60,000 YA, results that suggest that the larger increases in effective population size of *C. flamarioni* were related to an older period of geological history than the most recent marine regression, which took place after the largest Holocene marine transgression about 5,000 YA (Tomazelli et al. 2000; Esteves et al. 2002), as we hypothesized. Our estimates are related to a Pleistocene period with an arid climate, in which the discharge of the most important rivers may have been many times less than at present (Latrubesse et al. 2005; De Oliveira et al. 2005) and the present geographic barriers to gene flow did not exist. It should be emphasized that the obtained estimate of expansion time was based on our particular mutation rate of 1.9%/MY, and the stepwise scenario represents a simplified reflection of demographic history. Furthermore, the expansion indicated by the models may be better seen as a series of demographic fluctuations over hundreds of thousands of year, than as a punctual event (see discussion in Zheng et al. 2003).

All these results permit us to reconstruct the probable evolutionary scenario that involves the origin and distribution of the species: entrance, expansion, isolation, and genetic-drift events, and stochastic events from marine transgression-regression cycles on reductions in population size (founder effects and bottleneck) acting differentially over the range of the species.

Among the species of the genus *Ctenomys*, some of those belonging to the *mendocinus* group (phylogenetically related to *C. flamarioni*) are also associated with friable Quaternary soils, and have demographic histories related to a process of recent expansion

in their range of distribution. These studies permit us to corroborate a pattern observed in recent studies with *C. rionegrensis* in Uruguay (Wlasiuk et al. 2003) and *C. australis* in Argentina (Mora et al. 2006) which demonstrated the association of these species with the formation of Quaternary dunes not older than 6,000 YA.

Intriguing questions have arisen from these findings, such as the comparison of the local evolutionary scenarios that have led to the origin and genetic differentiation of each of these phylogenetically related species. Further studies using more-variable molecular markers (as microsatellite *loci*) will provide a fine-scale genetic pattern for the historical patterns found in this study.

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APPENDIX I

GeneBank accession numbers for the complete mitochondrial DNA cytochrome-*b* sequences (and the publications that report each of them) used in our estimates of the time and rates of diversification of *C. flamarioni*.

Capromys pilorides, AF422915 (Leite and Patton 2002); *Cavia porcellus*, AF490405 (Spotorno et al. 2002); *Coendou bicolor*, U34852 (Lara et al. 1996); *Ctenodactylus vali*, AJ389532 (Montgelard et al. 2002); *Ctenomys australis*, AF370697 (Slamovitz et al. 2001); *C. boliviensis*, AF007038 (Lessa and Cook 1998); *C. flamarioni*, AF119107 (D'Elia et al. 1999); *C. haigi*, AF007063 (Lessa and Cook 1998); *C. mendocinus*, AF007062 (Lessa and Cook 1998); *C. rionegrensis*, AF538374 (Wlasiuk et al. 2003); *C. leucodon*, AF007056 (Lessa and Cook 1998); *C. steinbachi*, AF007043 (Lessa and Cook 1998); *Dasyprocta fuliginosa*, AF437784 (Jansen van Vuuren et al. 2004); *Euryzygomatomys spinosus*, U34858 (Lara et al. 1996); *Heterocephalus glaber*, AY425944 (Faulkes et al. 2004); *Myocastor coypus*, AF422919 (Leite and Patton 2002); *Myoprocta pratti*, U34850 (Lara et al. 1996); *Octodon degus*, AF007059 (Lessa and Cook 1998); *Octodontomys gliroides*, AF370706 (Slamovitz et al. 2001); *Spalacopus cyanus*, AF007061 (Lessa and Cook 1998); *Trinomys setosus*, U34857 (Lara et al. 1996); *Tympanoctomys barrerae*, AF007060 (Lessa and Cook 1998).

Figure Legends

Fig. 1. Distribution of the tuco-tuco-das-dunas (*Ctenomys flamarioni*) along the seashore of Rio Grande do Sul. The ten populations sampled and the four regions studied (I, II, III, IV) are indicated. Barriers to gene flow tested are identified with capital letters: A. Tramandaí Lagoon; B. Peixe Lagoon; and C. Patos Lagoon inlets.

Fig. 2. Genealogical relationships between *C. flamarioni* haplotypes obtained from the median-joining network method for (A) Control region (H1 – H7) and (B) Expanded mitochondrial (E1 – E9) datasets. Circle areas are proportional to haplotype frequencies. Crosshatches represent nucleotide substitutions between haplotypes. Shadings represent each of the four regions studied (I, II, III and IV). On the right, each of the ten populations belongs to each region from the North (top) to the South (bottom). Haplotype numbers and populations correspond to those in Table 1. In (C) is summarized the geographical distribution of the expanded mitochondrial haplotypes by region, without specifying genealogical relationships among populations.

Fig. 3. Mitochondrial DNA haplotype distribution. Bars represent the absolute frequency of all haplotypes detected for the control region and cytochrome-b sets of data (in all populations) falling in each of five haplotype frequency classes. Based on Luikart et al. (1998), the obtained distribution indicates that *C. flamarioni* populations have experienced one or more recent reductions in size (bottlenecks).

TABLES

Table 1. Variable position of the haplotypes obtained from three different datasets for the populations of *Ctenomys flamarioni*: (a) Control region (CR), (b) Cytochrome-*b* (Cyt-*b*) and (c) Expanded dataset (Expanded). Dots represent match with nucleotides present in the haplotype 1. Distribution of haplotypes through populations (XA, Xangri-lá; RE, Remanso; IM, Imbé; PI, Pinhal; SS, São Simão; BJ, Bujurú; SJN, São José do Norte; CASS, Cassino; TA, Taim; CHU, Chuí) and the total numbers of individuals by haplotype and by population are shown.

Dataset	Haplotypes	Nucleotide position						Haplotype frequencies by population										
								XA	RE	IM	PI	SS	BJ	SJN	CAS	TA	CHU	Total
		0	0	0	0	1	1											
		0	3	4	8	6	9											
		8	0	5	3	8	7											
CR	H1	T	A	A	G	A	A	7	9	6	-	-	2	-	-	-	-	24
	H2	.	.	.	A	.	.	-	-	-	-	11	-	-	-	-	-	11
	H3	.	.	G	.	.	.	-	-	-	-	-	-	-	6	15	7	28
	H4	.	.	.	.	.	G	-	-	-	-	-	7	7	-	-	-	14
	H5	.	.	.	.	G	.	-	-	-	3	-	-	-	-	-	-	3
	H6	.	G	.	.	.	G	-	-	-	6	-	-	-	-	-	-	6
	H7	C	.	.	.	.	.	-	-	-	3	-	-	-	-	-	-	3
	Total							7	9	6	12	11	9	7	6	15	7	89

Table 1. Continued.

Dataset	Haplotypes	Nucleotide position										Haplotype frequencies by population										
												XA	RE	IM	PI	SS	BJ	SJN	CAS	TA	CHU	Total
		1	3	3	3	4																
		8	0	1	7	6																
		6	9	9	5	9																
	C1	T	C	T	C	A						-	-	-	-	-	-	-	4	3	6	13
Cyt-*b*	C2	.	.	.	T	.						3	3	3	8	3	1	4	-	-	-	25
	C3	.	T	.	T	.						-	-	-	-	-	2	-	-	-	-	2
	C4	.	.	C	T	G						-	-	-	-	-	2	-	-	-	-	2
	C5	C	.	.	.	.						-	-	-	-	-	-	-	1	-	-	1
	Total											3	3	3	8	3	5	4	5	3	6	43
		1	3	3	3	4	6	7	7	8	8											
		8	0	1	7	6	9	0	4	3	6											
		6	9	9	5	9	4	9	7	2	0											
	E1	T	C	T	C	A	A	G	G	A	A	-	-	-	-	-	-	-	4	3	6	13
	E2	.	.	.	T	.	G	A	.	.	G	-	-	-	6	-	-	-	-	-	-	6
	E3	.	T	.	T	.	.	A	.	G	.	-	-	-	1	-	-	-	-	-	-	1
Expanded	E4	.	.	.	T	.	.	A	A	.	.	-	-	-	-	3	-	-	-	-	-	3
	E5	.	T	.	T	.	.	A	.	.	.	-	-	-	-	-	2	-	-	-	-	2
	E6	.	.	.	T	.	.	A	.	.	.	3	3	3	-	-	-	-	-	-	-	9
	E7	.	.	.	T	.	.	A	.	.	G	-	-	-	-	-	1	4	-	-	-	5
	E8	.	.	C	T	G	.	A	.	.	G	-	-	-	-	-	2	-	-	-	-	2
	E9	C	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	1	-	-	1
	Total											3	3	3	7	3	5	4	5	3	6	42

Table 2. Summary of genetic variability in populations of *C. flamarioni* over the entire range of the species. Localities sampled by regions, sample size (*n*), number of haplotypes (*nhap*), haplotype diversity (*Hd*), nucleotide variation estimated based on the number of segregating sites (θ_w), and mean number of pairwise differences for sampled populations (π). Data are based on 391 bp sequences from a control-region fragment and 665 bp sequences from a cyt-*b* fragment.

		Control Region					Cytochrome-*b*				
Region	Locality	*n*	*nhap*	*Hd*	θ_w	π	*n*	*nhap*	*Hd*	θ_w	π
I	XA	7	1	0.000	-	-	3	1	0.000	-	-
	RE	9	1	0.000	-	-	3	1	0.000	-	-
	IM	6	1	0.000	-	-	3	1	0.000	-	-
II	PI	12	3	0.682	1.325	1.909	8	1	0.000	-	-
	SS	11	1	0.000	-	-	3	1	0.000	-	-
III	BJ	9	2	0.390	0.368	0.390	5	3	0.800	1.437	1.801
	SJN	7	1	0.000	-	-	4	1	0.000	-	-
IV	CASS	6	1	0.000	-	-	5	2	0.400	0.483	0.398
	TA	15	1	0.000	-	-	3	1	0.000	-	-
	CHU	7	1	0.000	-	-	6	1	0.000	-	-
	All pops.	89	7	0.790	1.186	1.267	43	5	0.579	1.156	0.768

Table 3. Analysis of molecular variance (AMOVA) among *Ctenomys flamarioni* mtDNA haplotypes from the control region and expanded dataset performed for each of three hierarchical geographic levels: (1) Three groups: considering all populations subdivided into three groups taking into account two permanent geographical discontinuities in the range; (2) Four groups: all populations subdivided into four groups taking into account two permanent, plus one temporary geographical discontinuity; (3) Two groups: only populations belonging to regions II and III. AG: among groups; AP/WG: among populations/ within groups; WP: within populations.

		Percentage of variability (Fixation Index)		
		All populations		Central pops.
	Hierarchical level	Three groups	Four groups	Two groups
Control Region	AG (F_{CT})	49.04 (0.49)*	57.61 (0.58) *	11.65 (0.12)
	AP/WG (F_{SC})	31.70 (0.62) **	22.32 (0.53) **	50.35 (0.57) **
	WP (F_{ST})	19.26 (0.81) **	20.08 (0.80) **	38.00 (0.62) **
Expanded dataset	AG (F_{CT})	63.00 (0.63) *	65.78 (0.66) *	-3.64 (-0.04)
	AP/WG (F_{SC})	21.41 (0.58) **	17.68 (0.52) *	63.07 (0.61) *
	WP (F_{ST})	15.59 (0.84) **	16.55(0.84) **	40.57 (0.59) **

* $P < 0.05$; ** $P < 0.001$

Table 4. Estimated parameters related to population expansion: Tajima's *D*, Fu's *Fs*, and parameters estimated under the Exponential expansion model and the Stepwise expansion model (†).

		Dataset		
		Control Region	Cytochrome-*b*	Expanded
Tajima's test	*D*	0.156 ^{ns}	-0.839 ^{ns}	-0.273 ^{ns}
Fu's test	*Fs*	- 0.519 ^{ns}	-0.966 ^{ns}	-1.275 ^{ns}
Exponential	θ_w	0.004 [0.001 – 0.010]	0.005 [0.001 – 0.058]	0.004 [0.002 – 0.014]
model †	*g*	81.1 [-704 – 3,473]	5,647 [-1,429 – 19,187]	1,281 [-691 – 5,774]
	$\theta_0 = 2u N_0$	-	-	0.000 [0.000 – 1.431]
Stepwise model †	$\theta_1 = 2u N_1$	-	-	8.684 [1.926 – 449.622]
	$\tau = 2ut$	-	-	2.634 [1.040 – 5.338]

† parameters under the exponential and stepwise models are given as estimates [95% confidence limits]; ns, not significant.

Figures

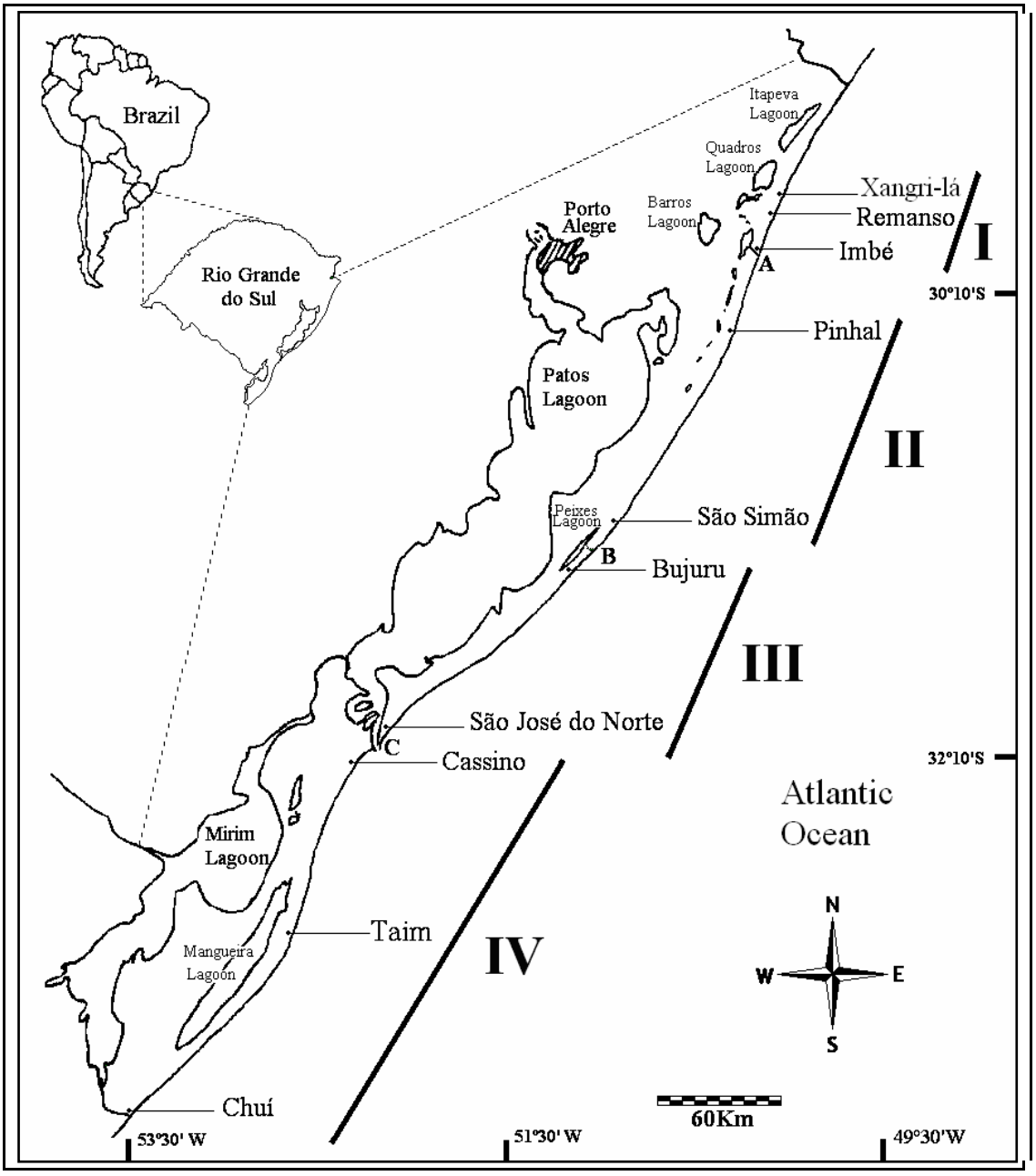
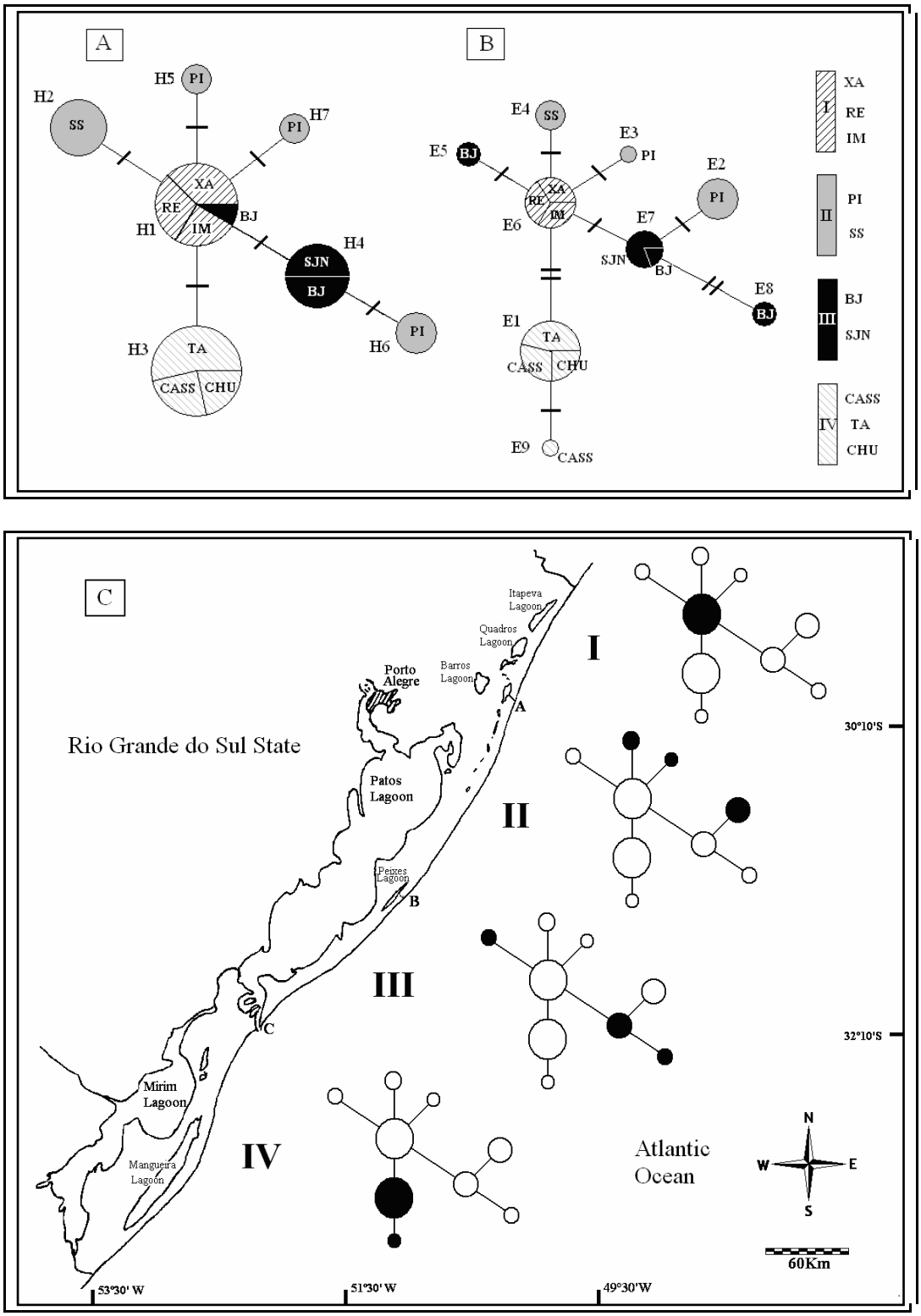


Figure 1.


A
B
C
H1
H2
H3
H4
H5
H6
H7
SS
PI
XA
RE
IM
BJ
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CASS
CHU
E1
E2
E3
E4
E5
E6
E7
E8
E9
I
II
III
IV
Rio Grande do Sul State
Itapeva Lagoon
Quadros Lagoon
Barros Lagoon
Porto Alegre
Patos Lagoon
Peixes Lagoon
Mirim Lagoon
Mangueira Lagoon
Atlantic Ocean
30°10'S
32°10'S
53°30' W
51°30' W
49°30'W
N
W
E
S
60Km

Figure 2.

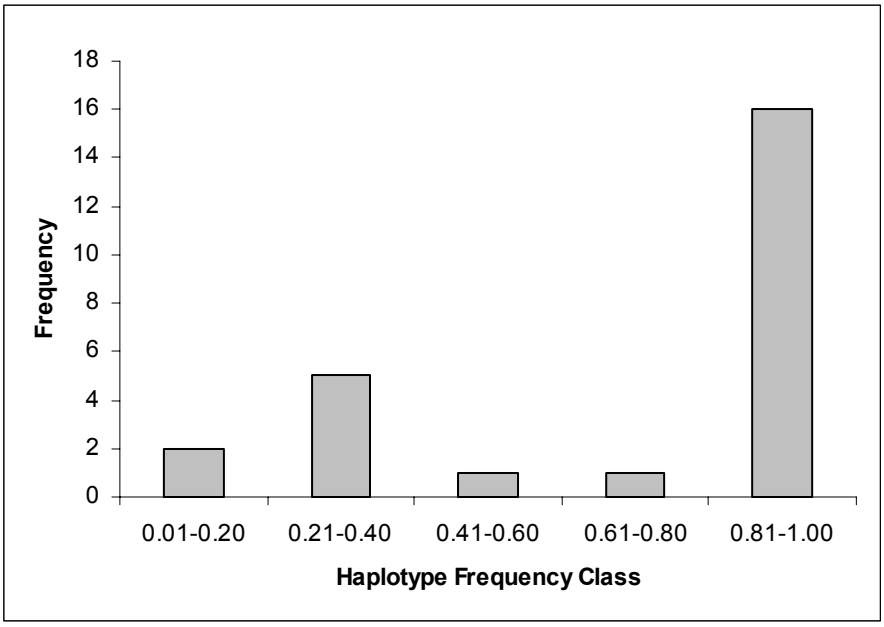
18
16
14
12
10
8
6
4
2
0
Frequency
0.01-0.20
0.21-0.40
0.41-0.60
0.61-0.80
0.81-1.00
Haplotype Frequency Class

Figure 3.

V. CAPÍTULO III

FINE-SCALE GENETIC PATTERNS AND CONSERVATION ISSUES IN THE TUCO-TUCO-DAS-DUNAS (*CTENOMYS FLAMARIONI*), AN ENDANGERED SPECIES OF THE SAND DUNES OF RIO GRANDE DO SUL, BRAZIL

GABRIELA P. FERNÁNDEZ-STOLZ, J. F. B. STOLZ, T. N. S. FORNEL AND THALES R. O. DE FREITAS

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GABRIELA P. FERNÁNDEZ-STOLZ, [*] J. F. B. STOLZ, [§] T. N. S. FORNEL [†] and THALES R. O. DE FREITAS [‡]

[*]*Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre 91501-970, Rio Grande do Sul, Brazil,* [§]*Programa de Pós-Graduação em Ecologia, Universidade Federal do Rio Grande do Sul, Porto Alegre 91540-000, Rio Grande do Sul, Brazil,* [†]*Curso de graduação em Biologia, Universidade Federal do Rio Grande do Sul, Porto Alegre 91501-970, Rio Grande do Sul, Brazil,* [‡]*Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre 91501-970, Rio Grande do Sul, Brazil*

Correspondence: Gabriela. P. Fernández-Stolz. *Present adress: Departamento de Genética, Avenida Bento Gonçalves 9500, Prédio 43323. Caixa postal 15053 - CEP 91501-970, Bairro Agronomia, Porto Alegre, Rio Grande do Sul, Brazil. Fax: 55 51 33166727; E-mail: gabriela.fernandez@ufrgs.br

Running title: Genetic and conservational issues in *Ctenomys flamarioni.*

Keywords: *Ctenomys flamarioni*, mitochondrial DNA, microsatellites, genetic structure, isolation by distance, conservation genetics, evolutionarily significant units.

Abstract

The tuco-tuco-das-dunas, *Ctenomys flamarioni*, is an endangered subterranean rodent with a distribution limited to the coastal sand dunes of southern Brazil. This habitat is a dynamic system that has undergone historical and contemporary natural changes, and during the last century has been impacted by human modifications of the landscape. We examined the variation of nine populations over the range of the species, in the mitochondrial control region and cytochrome-b DNA (mtDNA), and at nine microsatellite loci, in order to examine the patterns of genetic variation at the populational and phylogeographic levels, and to attempt to formalize the conservation status of *C. flamarioni*. Both microsatellites and mtDNA have low within-population genetic variation, but this is more critical for mtDNA. The three populations in the southern part of the range showed the lowest values of diversity, with most loci monomorphic and a small number of haplotypes for both mtDNA datasets. The lower number of polymorphic loci in these populations precluded the use of statistical tests for bottleneck examination. However, for three of six populations examined, a genetic signature of recent reduction in population size was observed. Among-population levels of genetic differentiation were higher for microsatellites than for mitochondrial loci. The results from the AMOVA test showed a positive relationship between the allelic frequency distribution at nuclear loci, and the two hypothesized barriers to gene flow, grouping populations into three regions (I, II + III and IV), concordant with that previously obtained for mtDNA. Evidence of positive correlation between genetic and geographical distances over the entire range sampled supports the hypothesis of a linear pattern of differentiation with restricted gene flow. Despite the lack of phylogeographic information to define Evolutionarily Significant Units (ESUs), based on the patterns of variation and divergence from two kinds of markers and previous allozyme and karyotype

studies, we propose (1) two ESUs, the first including the north (I) and central (II and III) regions, and the second including all populations in the southern part of the range (IV); (2) each of the populations sampled constitutes a separate MU.

Introduction

Complex interactions among ecological factors, demographic characteristics, and the footprint of the geological and microgeographic processes determine the evolutionary direction of intraspecific lineages. Historical and contemporary losses of suitable habitat are important issues affecting the destiny of a species, especially when it has a limited geographical distribution.

The subterranean rodent *Ctenomys flamarioni* (Travi 1981) occupies the most recent depositional system on the Rio Grande do Sul coastal plain (southern Brazil; Freitas 1994 and 1995; Massarini and Freitas 2005; Fernández-Stolz et al. in press). The geomorphological features of this coastal plain represent a significant record of late Quaternary climate changes with their associated glacio-eustatic sea-level fluctuations. Periodic oscillations in climate and habitat availability caused by glaciations have profoundly influenced population subdivisions and genetic divergence in some species and many biological groups (Avise 2000; Beheregaray 2001; Hewitt 2001).

The increasing interest in coastal processes since the late 1980s has been impelled mainly by the rapid growth of coastal populations and the economic importance of beach-related tourism. Several studies have addressed coastal erosion in Rio Grande do Sul, indicating both natural and human-induced contributing factors in the long and the short terms (Tomazelli and Villwock 2000; Esteves et al. 2002; Medeanic and Dillenburg 2005). Anthropogenic changes such as urbanization in active dune areas, shoreline reinforcement,

sand mining, jetty construction, and the presence of certain kinds of domestic animals has modified the natural landscape for years. As a result of urban development in coastal environments, the native habitats have become more fragmented or have even been destroyed. Several endemic species, including *C. flamarioni*, that are linked to the destiny of the dune ecosystem have been included as "vulnerable" in the National List of Endangered Species of the Brazilian Fauna (Ibama 2004) and the List of Endangered Fauna in Rio Grande do Sul (Fontana et al. 2003).

In spite of the debate in recent years about the best method to distinguish the units for conservation below the species level, the most widely applied concept has been the *evolutionarily significant unit* (ESU). Since the original definition of the ESU by Ryder (1986), the concept has been modified and shaped by the necessity to find the operational criteria that aid managers to propose more accurate conservation plans for a given situation (e.g., Waples 1991; Dizon et al. 1992; Avise 1994; Moritz 1994; Vogler and DeSalle 1994; Crandall et al. 2000; Fraser and Bernatchez 2001). Despite the points of disagreement, the conflicting concepts of ESU all aim to define segments of species whose divergence can be measured in the light of the evolutionary forces acting on them at different temporal scales (see Fraser and Bernatchez 2001 for a review). The latest and most integrative framework for defining conservation units is proposed under the term *adaptive evolutionary conservation* (AEC), which emphasizes flexibility in the use of criteria to guide conservation efforts, constituting a consensus on the available proposed criteria that contribute to the conservation goals. In this sense, the ESU definition under AEC is, ‘a lineage demonstrating highly restricted gene flow from other such lineages within the higher organization level (or lineages) of species’ and its application in conservation

decisions is suggested based on integrative approaches on a case-by-case basis (Fraser and Bernatchez 2001).

Nuclear microsatellites and mitochondrial DNA (mtDNA) are both proven, powerful, and widely used tools for examining population-level variation, structure, and differentiation in the subterranean-rodent genus *Ctenomys* (Lacey 2001; Wlasiuk et al. 2003; El Jundi and Freitas 2004; Gava and Freitas 2004; Mora et al. 2006; Tomasco and Lessa in press; Fernandez-Stolz et al. submitted). When used in combination, they also permit sensitive definition of conservation units.

In this study we proposed (1) to examine and compare the patterns of genetic variation and divergence within the species *C. flamarioni* at two levels of variation, evolutionary divergence (phylogeographic variation) and the patterns of distribution of the variability at a less-inclusive level (fine-scale) (2) to attempt the first formalization of the conservation status of *C. flamarioni* below the species level.

Materials and Methods

Sampled localities

A total of 160 individuals were captured, and their tissue was collected, at nine localities covering the entire distributional range of *C. flamarioni* (shoreline sand dunes of the coastal plain of Rio Grande do Sul, Brazil). The populations sampled included representatives of each of four regions, which are demarcated by three potential natural barriers to gene flow, as tested in previous studies: two of those represent permanent discontinuities (the Tramandaí Lagoon inlet, barrier A; and the Patos Lagoon inlet, barrier C), and the third is one of the main temporary washouts (Peixe Lagoon inlet, barrier B) (Fernandez-Stolz et al. submitted, Fig. 1). The populations by regions and localities were

(*n* = sample size): Region I (*n* = 57): Xangri-lá (XA; 29°47'S, 50°01'W; *n* = 24), Remanso (RE; 29°49'S, 50°02'W; *n* = 27), and Imbé (IM; 29°55'S, 50°06'W; *n* = 6); Region II (*n* = 43): Pinhal (PI; 30°18'S, 50°15'W; *n* = 30) and São Simão (SS; 30°58'S, 50°40'W; *n* = 13); Region III (*n* = 22): Bujurú (BJ; 31°39'S, 51°22'W; *n* = 10) and São José do Norte (SJN; 32°03'S, 51°59'W; *n* = 12); Region IV (*n* = 38): Cassino (CASS; 32°09'S, 52°06'W; *n* = 8), Taim (TA; 32°43'S, 52°26'W; *n* = 22) and Chui (CH; 33°44'S, 53°22'W; *n* = 8). The individuals were trapped using Oneida Victor No. 0 snap-traps, as described by Fernández-Stolz et al. (in press). Skin samples (ear or tail tissue) were taken from each specimen, preserved in absolute ethanol, and deposited in the collection of the Cytogenetic and Evolutionary Laboratory of the Genetics Department of the Federal University of Rio Grande do Sul, stored at -20°C. The spatial positions of the tuco-tucos and localities were recorded with a global positioning satellite (GPS), to use the distances for later analysis. All the individuals included in this analysis were adults, based on the age criteria taken from Wilks (1963), using reproductive condition and body weight as described by Fernández-Stolz et al. (in press).

Genetic Methods

Microsatellite data

Molecular genetic analyses were performed on a subsample (*n* = 154) from nine polymorphic microsatellite loci, isolated from the Argentinean species *C. haigi* (Hai 3, Hai 4, Hai 5, Hai 6, Hai 7, Hai 9, and Hai 12; Lacey 1999) and *C. sociabilis* (Soc 2 and Soc 3; Lacey et al. 2001).

PCR amplifications were carried out in a reaction volume of 20 µl containing 25-100 ng of DNA, 0.2 µM of each primer, 0.2 µM dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$ and 1.0

unit of Taq DNA polymerase (GIBCO-BRL Life Sciences/ Invitrogen, Carlsbad, California). Amplifications were achieved with the following conditions: initial denaturing at 94ºC for 5 min, 30-34 cycles of denaturing at 94ºC for 30 s, annealing at 50-64ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 1 min.

The products were run in non-denaturing 6% polyacrylamide gels and 8% polyacrylamide - 40% urea denaturing sequencing gels at 1000V for 2-4 hrs, as dictated by the size of the amplification products for each locus. The products were developed with silver-nitrate stain.

Mitochondrial data

A subsample of individuals of *C. flamarioni* was screened for variation at two mitochondrial loci: 391 base pairs (bp) of the hypervariable 5´ region of the control region (CR, $n = 89$) and at 665 bp of the mitochondrial cytochrome-*b* gene (cyt-*b*, $n = 45$).

Polymerase chain reaction (PCR) amplification for the control region was achieved using primers TucoPro (5´- ACT TTC GTT TAT TGC TTA ATT - 3´, Tomasco and Lessa in press) and TDKD (5´- CCT GAA GTA GGA ACC AGA TG -3´, Kocher et al. 1989). The primers used in the cytochrome *b* fragment amplifications were MVZ 05 (5´- CGA AGC TTG ATA TGA AAA ACC ATC GTT - 3´, Smith and Patton 1993) - TUCO 06 (5´- GTG AAA TGG AAT TTT GTC TGA - 3´) and TUCO 07 (5´- ATT ACA GCA ATA GTA ATA AT - 3´) - TUCO14 (5´- CCA ATG TAA TTT TTA TAC - 3´) as described by Wlasiuk et al. 2003.

PCR amplifications were carried out in the same reaction volume and with the same concentrations of reagents used for the microsatellite amplifications. The thermocycling profile of the control-region fragment included an initial denaturing at 94ºC for 1 min,

followed by 30-34 cycles of denaturing at 94ºC for 30 s, annealing at 45ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min. The conditions of PCR amplification for the cyt-*b* fragment were the same used for the control-region set of samples, except for the annealing temperature (48 ºC) used for the partial fragment amplified by the MVZ 05 - TUCO 06 pair of primers.

PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California), performed following the guidelines of the suppliers and sequenced in an ABI Prism 3100 (Applied Biosystem) automated sequencer, using the forward primer used in the PCR. For the samples with unclear sequences, the procedures were also carried out with the reverse primer.

Microsatellite data analyses

For each population, the genetic diversity was estimated from the mean number of alleles per locus (*A*), percentage of loci that were polymorphic (%*P*), mean observed heterozygosity (*Ho*), and the mean expected heterozygosity from Hardy-Weinberg proportions (*He* – Nei 1978). Analysis of linkage disequilibrium based on a likelihood ratio test (Excoffier and Slatkin 1998) and deviations from the Hardy-Weinberg equilibrium (Guo and Thompson 1992) were tested using ARLEQUIN 3.0 (Excoffier et al. 2005).

Levels of genetic divergence among populations were analyzed by computing F_{ST} according to Weir and Cockerham (1984) implemented in FSTAT (Goudet 1995). The significance of F_{ST} for all pairwise population comparisons was assessed by permutating the values 1,000 times.

Sequential Bonferroni corrections were applied to correct for multiple simultaneous comparisons (Rice 1989) with $\alpha = 0.05$ to adjust the statistical significance levels.

An analysis of molecular variance (AMOVA, Excoffier et al. 1992) was used to estimate the partitioning of genetic variation and to test the hypothesis that present geographical barriers provide a good explanation for the genetic divergence observed in *C. flamarioni* performed by the program ARLEQUIN 3.0 (Excoffier et al. 2005). With this aim, we ran two tests: (1) considering all populations subdivided into three groups, taking into account two permanent geographic discontinuities in the range (the Tramandaí Lagoon [A] and Patos Lagoon [C] inlets; Fig. 1); (2) and only populations of regions II and III arranged in two groups within each region determined by the potential third barrier (the Peixe Lagoon [B] inlet; Fig. 1).

A Mantel test (Mantel 1967) was implemented to test for positive correlations between genetic and geographical distances (isolation by distances) (Slatkin 1993) as performed by ARLEQUIN 3.0 (Excoffier et al. 2005). Statistical significance was tested using 10,000 random permutations.

With the aim of detecting the genetic signatures of bottlenecks, we used the program BOTTLENECK (Cornuet and Luikart 1996) to test for excess of heterozygosity compared to the expected values from the observed number of alleles at each locus and population, assuming mutation-drift equilibrium. As recommended by the authors, we used the Wilcoxon sign-rank test to determine whether a population exhibits a significant number of loci with excess heterozygosity. The computations were based on both stepwise mutation (SMM) and two-phase mutation (TPM) models (Di Rienzo et al. 1994).

Mitochondrial data analysis

Sequences were aligned using CLUSTAL X (Thompson et al. 1997), manually corrected and excluding ambiguous sites for subsequent analysis.

We applied the neighbor-joining, maximum parsimony, and maximum likelihood methods, to reconstruct phylogenetic relationships among haplotypes and populations for both the control-region and cytochrome-*b* data, using the computer program PAUP* 4.0b10 (Swofford 2002). Based on the Akaike Information Criterion as implemented in MODELTEST 3.7 program (Posada and Crandall 1998), we determined the substitution models that provided the best fit to our datasets. The HKY model (Hasegawa et al. 1985) with a proportion of invariable sites ($I = 0.897$) and Ti/Tv ratio = 7.82 provided the best fit to the control-region dataset. For cyt-*b* the same model (HKY) and Ti/Tv ratio = 6.94 provided the best fit to this dataset. We used the bootstrap resampling technique with 1000 replicates to evaluate support for the internal nodes. Homologous sequences of other species of the genus (*C. australis*, *C. rionegrensis*, and *C. pearsoni*) were used as outgroup taxa in each dataset. As an alternative approach, we applied a median-joining network method (Bandelt et al. 1999) for our complete (expanded) set of data as implemented by the NETWORK program (http://www.fluxus-engineering.com/netwinfo.htm). This method, through a parsimony criterion, combines the minimum-spanning trees (MSTs) with a single network, and, like other methods to construct networks, allows a more detailed population information than do strictly bifurcating trees (see review in Posada and Crandall 2001).

Genetic diversity within each population studied was measured as the number of haplotypes (*NH*), haplotype diversity (*Hd* - Nei 1987), and the mean number of pairwise differences (π - Tajima 1983) for the sequences obtained from the three mitochondrial datasets.

To test the hypotheses of restricted gene flow in *C. flamarioni*, we obtained estimates of genetic divergence between populations (pairwise F_{ST}; Weir and Cockerham 1984) and

studied their correlations (or lack thereof) with the geographical distances, applying a Mantel test (Mantel 1967). Statistical significance was tested using 10,000 random permutations. These analyses were performed from the complete set of mitochondrial data (expanded matrix) and implemented in ARLEQUIN 3.0 (Excoffier et al. 2005).

Results

Microsatellite and Mitochondrial loci variation

The nine microsatellite loci surveyed were polymorphic, with a variable number of alleles per locus, ranging from three (for Hai 4) to eight (for Soc 2), with a mean value of 5.3 alleles (Table 1). The mean number of alleles per locus observed for each population varied between 1.1 (Chui) and 3.6 alleles (Pinhal). Mean observed heterozygosity across all loci ranged from 0.21 (Cassino) to 0.70 (Bujurú). The lowest levels of variability were obtained for populations belonging to region IV (Cassino, Taim, and Chui) as observed from both the mean number of alleles (1.1 to 1.4) and the percentage of polymorphic loci (11 to 33%; Table 1). The microsatellite allele frequencies across populations are shown in Appendix I.

Three of nine loci showed significant deviations from the expected genotype proportion according to the Hardy-Weinberg equilibrium (HWE) after the Bonferroni correction for multiple tests: Hai 12 for XA, Hai 6 for PI, and Hai 9 for SJN. However, a global test of HWE using all loci showed a deviation from HWE equilibrium only for XA and SJN, and it disappeared when Hai 12 (for XA) and Hai 9 (for SJN) were excluded from the analysis.

A likelihood-ratio test used for pairwise comparisons of allele frequencies revealed significant linkage disequilibrium between Hai 6 and Hai 9 in XA, after α-levels were corrected for multiple tests ($P < 0.0006$).

For the mitochondrial control-region set of *C. flamarioni* sequences, we obtained a total of 391 bp for the 89 samples (GeneBank accession numbers AF000000-AF000000), containing six variable and parsimony informative sites and a total of seven haplotypes (H1 - H7). From the 665 bp sequenced for 45 cyt-*b* samples (GeneBank accession numbers AF000000-AF000000), five variable sites were identified, four parsimony informative, one singleton, and a total of five haplotypes (C1 - C5). Finally, for the two mitochondrial datasets together (the extended matrix of data) for 44 sequences and a total of 1054 bp, we obtained nine haplotypes (E1 – E9). All these haplotype data and their frequencies and geographical distributions are summarized in Table 2. Only three of nine populations exhibited more than one haplotype: PI, CASS, and BJ (Table 1 and 2). The haplotype diversity and other estimates of variability for the three mitochondrial datasets are shown in Table 1. Global values of variability (obtained from the extended mitochondrial dataset) were $Hd = 0.833$ when all populations were pooled together and ranged from 0.285 in Pinhal, to 0.800 in Bujuru, whereas $\pi = 0.002$, locally ranged from 0.0004 in Cassino to 0.0023 in Bujuru.

Genetic Structure and Isolation by distance

Significant population structure was observed from the microsatellite dataset, with all but one value of pairwise F_{ST} significantly different from zero (Table 3). The only non-significant value corresponded to the CASS-CHU comparison. In contrast, the mtDNA

dataset showed lower genetic structure, with high but non-significant F_{ST} values after a Bonferroni correction for multiples test.

No single haplotype was broadly shared across the range of the species. However, one haplotype was the only (or most frequent) one found in the populations of region I (H1 for control region, and E6 for the expanded dataset) and region IV (H3 for control region and E1 for the expanded set of sequences).

The phylogenies of the haplotypes obtained were analogous despite the method used. Figure 2 illustrates two outgroup rooted neighbor-joining trees for the control-region and cyt-*b* sequence data. The results from parsimony and maximum-likelihood analysis largely followed the same pattern, although some internal nodes were collapsed (data not shown). These phylogenies clearly show the shallow relationship between the haplotypes, as also visualized from the network approach in the expanded dataset (Fig. 3).

From microsatellite data, the AMOVA tests performed to test differences in the geographical apportionment of the variance at two levels of regional arrangements (considering three and two groups of populations) showed significant *p*-values at all levels analyzed, except for the among-groups level when the localities were assembled in two groups (Table 4).

Positive correlation between genetic and geographical distance was observed from both nuclear and mitochondrial sets of data, with low but significant coefficients of correlation ($r = 0.46$, $P = 0.008$ for microsatellites, and $r = 0.52$, $P = 0.004$ for mtDNA) indicating that large-scale gene flow in *C. flamarioni* showed an isolation-by-distance pattern (Fig. 4).

Evidence of recent bottlenecks

Despite the model of microsatellite mutation considered (SMM or TPM), a test for excess heterozygosity produced significant *p*-values only for the XA, RE (region I), and BJ (region III) populations (Table 5). For the populations belonging to region IV, the analysis was not applied because of the low number of polymorphic loci (less than four).

Discussion

Levels of genetic variability

Levels of molecular diversity within populations of *C. flamarioni* were moderate to low compared with those reported for other species of tuco-tucos. We detected a total of 48 alleles for the 9 polymorphic loci analyzed, with a mean of 5.3 (ranging from 3 to 8) per locus over all populations. Mean numbers of alleles per locus reported for other species are 2.3 (range 1 to 3) for *C. sociabilis* (Lacey 2001), 2.5 (range 2 to 3) for *C. lami* (El Jundi and Freitas 2004), 7.5 (range 3 to 13) for *C. haigi* (Lacey 2001), 8.3 (range 6 to 14) for *C. rionegrensis* (Wlasiuk et al. 2003), and 9.3 (range 5 to 15) for *C. minutus* (Gava and Freitas 2004).

Estimation of microsatellite variation resulted in the lowest estimates for the populations belonging to Region IV (southern part of the distribution), with a variable but high number of monomorphic loci.

These results were concordant with the lower variation obtained for the three mtDNA datasets with few haplotypes, a small number of nucleotide changes, and only three polymorphic populations. Compared to other species of the genus *Ctenomys*, the number of haplotypes (for an equivalent mitochondrial control-region set of data) was lower for *C. flamarioni* (six haplotypes) than *C. australis* (24 haplotypes; Mora et al. 2006) and *C.*

pearsoni (22 haplotypes; Tomasco and Lessa in press). The same is observed when comparing the cytochrome-*b* variation to the Uruguayan species *C. rionegrensis*, from which 15 different haplotypes have been reported (Wlasiuk et al. 2003)

Among-population levels of genetic differentiation

Ctenomys flamarioni populations were highly differentiated from one another according to the microsatellite analysis, as shown by the high pairwise F_{ST} values obtained even among populations that were in close geographical proximity. The only pairwise comparison that gave divergence estimates not significantly different from zero (after Bonferroni adjustment) was CASS – CHUI, although these populations are separated by a large geographical distance (213 km). One probable explanation for this fact is the smaller sample size (n = 8) of both the CASS and CHUI populations, which may by chance have led to erroneous interpretations. However when the mtDNA data were examined, another picture was observed, with a less obvious pattern of differentiation among populations and significant *p*-values obtained only in four of 36 pairwise comparisons involving populations of different regions.

The AMOVA test applied to the microsatellite data when all nine populations were considered, showed that approximately 50% of the variation can be explained by the population level, although significant *p*-values were observed for each hierarchical level. This last indicates the positive relationship between the allele frequency distribution and the hypothesized historical barriers to gene flow.

From the AMOVA performed to test the strength of the Peixe Lagoon inlet as a conspicuous barrier to gene flow (between regions II and III), the component of the variance explained by the populations organized in two groups was small and not

significantly different from zero. For this test, the proportion of the variance explained by the differences within populations was high (73%).

These results were concordant with those obtained by Fernandez-Stolz et al. (submitted) from the mitochondrial dataset, supporting the hypothesis that the permanent inlets (Tramandaí Lagoon [A] and Patos Lagoon [C] inlets) provide a good explanation for the genetic divergence observed in *C. flamarioni*, but not the temporary barrier in the middle of the species' distribution (Peixe Lagoon [B] inlet). Evidence of the strength of the Patos Lagoon inlet in causing genetic differentiation between the northern and southern populations was observed from two kinds of markers in previous studies. From an electrophoretic analysis (based on ten polymorphic loci), the Roger's similarity coefficient obtained between the northern and southern populations ($S = 0.773$) was lower than those commonly found for conspecific populations (Moreira et al. 1991). From karyotype analysis, Freitas (1994) found a negative correlation between the amounts of constitutive heterochromatin present in the karyotype of *C. flamarioni* and the latitude along the seashore of Rio Grande do Sul, explained principally by the loss of the short arm of chromosome 1 in populations below the Rio Grande Bar.

The low number of haplotypes observed for the mtDNA datasets, in samples over the range of region IV (about 214 km) was suggested to have been caused by one (or probably more) reductions in the population size and genetic drift that prevented the merger of new haplotypes, in other studies with the species (Fernández-Stolz et al. in prep). On the other hand, the results from a phylogeographic approach support the hypothesis that this species has only recently colonized its present range.

The results generated from our microsatellite data showed that populations of region IV had the lowest variability, and are concordant with that expected for populations that have

experienced recent reductions in size, although statistical tests could not be applied because of the low proportion of polymorphic loci (fewer than the recommended four). For the rest of the distribution, the genetic signature of bottlenecks was identified in the XA, RE, and BJ populations. Because of the geographical location of XA and RE on the northern coast of the state (where the first line of coastal dunes has nearly disappeared beneath the dense human settlement), we may expect a recent bottleneck in these populations, but not in the BJ population, which is very little affected by humans. This leads us to suppose that other factors besides humans may be modifying the genetic stock of the species. Another possible explanation is based on knowledge of the strongly unstable characteristics of the coastal environment, as pointed out by Fernandez-Stolz et al. (submitted) which may have caused some populations (by chance) to undergo critical reductions in population size.

Two important issues that must be considered at large-scale geographic patterns are the process of origin of the population of *C. flamarioni* (after invasion and later demographic expansion through the vacant coastal dunes) and the subsequent history of the reduction (or reductions) in population size caused by the periodic oscillations in climate and habitat availability because of the associated glacio-eustatic sea-level fluctuations. All these historical and contemporary factors may have played an important role in the patterns of differentiation and genetic variation that are observed nowadays in the species.

Despite the phylogeographic and fine-scale genetic patterns that show moderate to strong geographic structure, and the processes of population reduction at different temporal scales, the evidence of the positive correlation between the genetic and geographical distances over the total sampled range supports the hypotheses of restricted gene flow (linear pattern of differentiation).

Identification of conservation units

The examination and comparison of the genetic variation and divergence patterns within the species at macro- and micro-geographic scales was one of the aims of this study, and is closely related to the second objective of contributing to conservation definitions for the tuco-tuco *C. flamarioni.*

This is a first attempt to delineate the conservation units for a species that, only 25 years after its description, has been listed on the national and regional lists of endangered fauna. We began by considering the binary approach proposed by Moritz (1994), which seeks to identify (1) populations that have been historically isolated (Evolutionarily Significant Units, ESUs), which, sensu Moritz (1994), are sets of populations distinguished by strongly phylogenetic structuring of mtDNA variation (reciprocal monophyly) and divergence in the frequencies of nuclear alleles; and (2) populations that are currently isolated, or effectively so (divergent nuclear allele frequencies), but were connected to others historically (Management Units, MUs).

Application of the above criteria is difficult for *C. flamarioni* because of the low variation in mtDNA (few haplotypes and changes between them) together with the presence of unresolved nodes at the shallow mtDNA phylogeny (Fig. 2). Because of the lack of reciprocal monophyly as evidence of historical (long-term) separation between the four areas considered (regions I to IV), our tentative conclusion is that *C. flamarioni* currently represents a single ESU. However, despite the lack of phylogeographical information to define ESUs, the patterns of divergence from the AMOVA test showed a clear and significant geographical distribution of the variation in three divergent regions (I, II + III, and IV). Likewise, the patterns of variation and divergence from nuclear

microsatellite loci indicated the existence of the same three divergent regions at the macro-geographical scale, as shown by the AMOVA tests.

On the other hand, most of the populations examined were structured based on pairwise comparisons of nuclear allele frequencies, thus suggesting that each of these must be treated as a separate MU.

At this point, based on the information presented herein and on knowledge of the differences in the allozyme and karyotype traits between populations north and south of the Patos Lagoon inlet, we propose the next conservation units for the tuco-tuco-das-dunas: two ESUs, the first including the north (region I) and central part of the distribution (region II + III), and the second including all populations south of the Patos Lagoon inlet (region IV). We also propose that each of the populations sampled constitutes a separate MU. The later proposal was guided and supported by the spatial and genetic patterns observed for the tuco-tuco-das-dunas: variably continuous distribution of populations in the one-dimension (linear) available habitat, high interpopulation structure, and large-scale gene flow showing an isolation-by-distance pattern (suggesting not a present but a historical connection among populations).

To conclude, we strongly believe that the designation of ESUs in order to assess the conservation status of a species, should be viewed as a dynamic and multidisciplinary criterion, based not only on the information from molecular markers, but rather as an essential complement to other methods of assessment.

In the case of *C. flamarioni*, the knowledge provided by other genetic methods, such as the patterns of enzyme polymorphism and the geographical distribution of the karyotype variants, and the contribution of the geomorphological coastal plain evolution are powerful aids to defining conservation units based on an integrative approach.

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Appendix I Allele frequencies of nine microsatellite loci in nine populations of *Ctenomys flamarioni*. For each population, in brackets, is shown the number of individuals analyzed by locus.

Locus - allele	Population								
	Xangri-lá	Remanso	Pinhal	S. Simão	Bujurú	S.J.Norte	Cassino	Taim	Chuí
Hai 3	**(24)**	**(27)**	**(30)**	**(13)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
168	0.000	0.000	0.217	0.000	0.000	0.000	0.063	0.000	0.000
170	0.458	0.519	0.000	0.000	0.100	0.000	0.313	0.000	0.000
172	0.000	0.000	0.000	0.423	0.250	0.208	0.625	1.000	1.000
174	0.000	0.000	0.017	0.346	0.400	0.750	0.000	0.000	0.000
176	0.521	0.482	0.600	0.231	0.150	0.000	0.000	0.000	0.000
178	0.021	0.000	0.167	0.000	0.100	0.042	0.000	0.000	0.000
Hai 4	**(24)**	**(27)**	**(30)**	**(13)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
152	0.979	0.982	0.017	0.000	0.000	0.000	0.000	0.000	0.000
154	0.021	0.019	0.650	1.000	1.000	1.000	1.000	1.000	1.000
158	0.000	0.000	0.333	0.000	0.000	0.000	0.000	0.000	0.000
Hai 5	**(24)**	**(27)**	**(29)**	**(13)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
209	0.000	0.000	0.086	0.000	0.350	0.000	0.000	0.000	0.000
211	0.000	0.000	0.276	0.000	0.000	0.000	0.000	0.000	0.000
213	1.000	1.000	0.569	0.885	0.650	1.000	1.000	1.000	1.000
215	0.000	0.000	0.069	0.000	0.000	0.000	0.000	0.000	0.000
217	0.000	0.000	0.000	0.115	0.000	0.000	0.000	0.000	0.000
Hai 6	**(24)**	**(27)**	**(30)**	**(13)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
128	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000
134	0.583	0.926	0.817	1.000	0.750	1.000	0.875	0.114	1.000
136	0.000	0.000	0.000	0.000	0.250	0.000	0.125	0.886	0.000
138	0.417	0.074	0.133	0.000	0.000	0.000	0.000	0.000	0.000
Hai 7	**(24)**	**(27)**	**(30)**	**(12)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
196	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.000	0.000
198	0.021	0.074	0.317	0.808	0.600	0.042	0.000	0.000	0.000
200	0.396	0.500	0.517	0.115	0.050	0.083	0.000	0.046	0.000

Appendix I *Continued*

Locus - allele	Population								
	Xangri-lá	Remanso	Pinhal	S. Simão	Bujurú	S.J.Norte	Cassino	Taim	Chuí
202	0.479	0.315	0.067	0.000	0.350	0.708	1.000	0.955	1.000
204	0.104	0.111	0.100	0.000	0.000	0.167	0.000	0.000	0.000
Hai 9	**(24)**	**(26)**	**(30)**	**(13)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
223	0.354	0.018	0.117	0.000	0.000	0.000	0.000	0.000	0.000
227	0.000	0.000	0.017	0.000	0.000	0.458	0.000	0.000	0.000
229	0.000	0.000	0.167	0.885	0.800	0.542	0.000	0.000	0.000
231	0.563	0.269	0.633	0.115	0.200	0.000	1.000	1.000	1.000
233	0.083	0.712	0.067	0.000	0.000	0.000	0.000	0.000	0.000
Hai 12	**(24)**	**(27)**	**(30)**	**(13)**	**(9)**	**(12)**	**(8)**	**(22)**	**(8)**
126	0.000	0.000	0.000	0.307	0.056	0.083	0.000	0.000	0.000
132	0.125	0.000	0.483	0.654	0.444	0.458	1.000	1.000	1.000
135	0.083	0.130	0.000	0.000	0.444	0.000	0.000	0.000	0.000
138	0.750	0.407	0.517	0.039	0.000	0.125	0.000	0.000	0.000
141	0.042	0.463	0.000	0.000	0.056	0.250	0.000	0.000	0.000
144	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.000
Soc 2	**(24)**	**(27)**	**(30)**	**(12)**	**(10)**	**(12)**	**(8)**	**(22)**	**(8)**
139	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000
141	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125
143	0.458	0.204	0.200	0.042	0.450	0.000	0.000	0.000	0.000
145	0.167	0.278	0.333	0.375	0.550	0.833	1.000	1.000	0.875
147	0.375	0.482	0.217	0.250	0.000	0.167	0.000	0.000	0.000
149	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000
151	0.000	0.000	0.167	0.083	0.000	0.000	0.000	0.000	0.000
153	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000
Soc 3	**(21)**	**(27)**	**(27)**	**(13)**	**(8)**	**(12)**	**(8)**	**(22)**	**(8)**
134	0.595	0.426	0.000	0.000	0.000	0.000	0.000	0.000	0.000
140	0.000	0.000	0.000	0.192	0.000	0.000	0.000	0.000	0.000
142	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000

Appendix I *Continued*

Locus - allele	Population								
	Xangri-lá	Remanso	Pinhal	S. Simão	Bujurú	S.J.Norte	Cassino	Taim	Chuí
144	0.405	0.537	0.907	0.000	0.375	1.000	0.125	0.341	0.000
146	0.000	0.037	0.000	0.308	0.563	0.000	0.875	0.659	1.000
148	0.000	0.000	0.093	0.500	0.000	0.000	0.000	0.000	0.000

Figure Legends

Figure 1 Geographic distribution of the tuco-tuco-das-dunas (*Ctenomys flamarioni*) along the coast of Rio Grande do Sul. The ten populations sampled and the four regions studied (I, II, III, IV) are indicated. Barriers to gene flow tested are identified by capital letters: A. Tramandaí Lagoon; B. Peixe Lagoon; and C. Patos Lagoon inlets.

Figure 2 Neighbor-joining trees for mitochondrial-DNA sequence data from (A) control region (CR) haplotypes based on HKY + I model of substitution (I = 0.897 and Ti/Tv ratio = 7.82 and (B) Cytochrome-*b* (cyt-*b*) gene based on HKY model (Ti/Tv ratio = 6.94). Parsimony-based network approach from the total mitochondrial dataset (RC plus cyt-*b*).

Figure 3 Parsimony-based network approach from the expanded mitochondrial dataset. Circle areas are proportional to haplotype (E1 – E9) frequencies. Crosshatches represent nucleotide substitutions between haplotypes. Shadings represent each of the four regions studied (I, II, III, and IV). On the right, each of the ten populations belongs to each region from north (top) to south (bottom). Haplotype numbers and populations correspond to those of Table 1.

Figure 4 Isolation-by-distance analysis showing the positive correlation between the pairwise geographical (km) and genetic distances (F_{ST}-values) estimated from nine microsatellite loci (A); and from the mtDNA dataset (B). *P*-values obtained by the Mantel test (10,000 permutations) were significant.

Table 1 Genetic variability in *Ctenomys flamarioni* from nine microsatellite loci, mitochondrial control region, cytochrome-*b* and expanded (control region plus cytochrome-*b*) datasets. *n*, sample size; *A*, mean number of alleles per locus; %*P*, percentage of polymorphic loci; *Ho*, mean observed heterozygosity; *He*, mean expected heterozygosity; *nh*, number of haplotypes; *Hd*, haplotypic diversity and π, nucleotide diversity. Regions (Reg.) and populations (Pop.) correspond to those of Figure 1 (XA, Xangrilá; RE, Remanso; PI, Pinhal; SS, São Simão; BJ, Bujurú; SJN, São José do Norte; CASS, Cassino; TA, Taim; CHU, Chuí).

Reg.	Pop.	Microsatellites					Control Region				Cytochrome-*b*				Expanded			
		n	*A*	%*P*	H_o	H_e	*n*	*nh*	*Hd*	π	*n*	*nh*	*Hd*	π	*n*	*nh*	*Hd*	π
I	XA	24	2.7	89	0.36**	0.45	7	1	0.000	-	3	1	0.000	-	3	1	0.000	-
	RE	27	2.7	89	0.37	0.41	9	1	0.000	-	3	1	0.000	-	3	1	0.000	-
	IM	-	-	-	-	-	6	1	0.000	-	3	1	0.000	-	3	1	0.000	-
II	PI	30	3.6	100	0.49*	0.53	12	3	0.682	0.005	8	1	0.000	-	7	2	0.285	0.0008
	SS	13	2.6	78	0.41	0.51	11	1	0.000	-	3	1	0.000	-	3	1	0.000	-
III	BJ	10	2.7	89	0.70	0.76	9	2	0.390	0.001	7	3	0.800	0.0027	7	4	0.800	0.0027
	SJN	12	2.2	56	0.30	0.46	7	1	0.000	-	4	1	0.000	-	4	1	0.000	-
IV	CASS	8	1.4	33	0.21**	0.41	6	1	0.000	-	5	2	0.400	0.0006	5	2	0.400	0.0004
	TA	22	1.3	33	0.36	0.48	15	1	0.000	-	3	1	0.000	-	3	1	0.000	-
	CHU	8	1.1	11	0.25	0.23	7	1	0.000	-	6	1	0.000	-	6	1	0.000	-

Table 1 *Continued*

Reg.	Pop.	Microsatellites					Control Region				Cytochrome-*b*				Expanded			
		n	*A*	%*P*	H_o	H_e	*n*	*nh*	*Hd*	π	*n*	*nh*	*Hd*	π	*n*	*nh*	*Hd*	π
	All Pop	154	5.3	64.2	0.30	0.47	89	7	0.790	0.003	45	5	0.579	0.0012	44	9	0.833	0.0020

* Deviations between observed and expected levels of heterozygosity at this locus were found to be significantly marginalized by the exact test for HWE ($0.01 < P < 0.05$), but the deviations were not significant when α-levels were corrected for multiple test.

** Significant heterozygote deficiency after Bonferroni corrections.

Table 2 Variable position of the haplotypes obtained from three different datasets for populations of *Ctenomys flamarioni*: (a) Control region (CR), (b) Cytochrome-*b* (Cyt-*b*) and (c) Expanded set of data (Expand). Dots represent match with nucleotides present in the haplotype 1. The distribution of haplotypes by population and the total number of individuals by haplotype and by population are shown. Populations correspond to those of Figure 1 and Table 1.

Dataset	Haplotypes	Nucleotide position						Haplotype frequencies by population										
								XA	RE	IM	PI	SS	BJ	SJN	CAS	TA	CHU	Total
		0	0	0	0	1	1											
		0	3	4	8	6	9											
		8	0	5	3	8	7											
CR	H1	T	A	A	G	A	A	7	9	6	-	-	2	-	-	-	-	24
	H2	.	.	.	A	.	.	-	-	-	-	11	-	-	-	-	-	11
	H3	.	.	G	.	.	.	-	-	-	-	-	-	-	6	15	7	28
	H4	.	.	.	.	.	G	-	-	-	-	-	7	7	-	-	-	14
	H5	.	.	.	.	G	.	-	-	-	3	-	-	-	-	-	-	3
	H6	.	G	.	.	.	G	-	-	-	6	-	-	-	-	-	-	6
	H7	C	.	.	.	.	.	-	-	-	3	-	-	-	-	-	-	3
	Total							7	9	6	12	11	9	7	6	15	7	89
		1	3	3	3	4												
		8	0	1	7	6												
		6	9	9	5	9												
Cyt-*b*	C1	T	C	T	C	A		-	-	-	-	-	-	-	4	3	6	13
	C2	.	.	.	T	.		3	3	3	8	3	3	4	-	-	-	27
	C3	.	T	.	T	.		-	-	-	-	-	2	-	-	-	-	2
	C4	.	.	C	T	G		-	-	-	-	-	2	-	-	-	-	2
	C5	C	.	.	.	.		-	-	-	-	-	-	-	1	-	-	1
	Total							3	3	3	8	3	7	4	5	3	6	45

Table 2 *Continued*

Dataset	Haplotypes	Nucleotide position										Haplotype frequencies by population										
												XA	RE	IM	PI	SS	BJ	SJN	CAS	TA	CHU	Total
		1 8 6	3 0 9	3 1 9	3 7 5	4 6 9	6 9 4	7 0 9	7 4 7	8 3 2	8 6 0											
Expand	E1	T	C	T	C	A	A	G	G	A	A	-	-	-	-	-	-	-	4	3	6	13
	E2	.	.	.	T	.	G	A	.	.	G	-	-	-	6	-	-	-	-	-	-	6
	E3	.	T	.	T	.	.	A	.	G	.	-	-	-	1	-	-	-	-	-	-	1
	E4	.	.	.	T	.	.	A	A	.	.	-	-	-	-	3	-	-	-	-	-	3
	E5	.	T	.	T	.	.	A	.	.	.	-	-	-	-	-	2	-	-	-	-	2
	E6	.	.	.	T	.	.	A	.	.	.	3	3	3	-	-	2	-	-	-	-	11
	E7	.	.	.	T	.	.	A	.	.	G	-	-	-	-	-	1	4	-	-	-	5
	E8	.	.	C	T	G	.	A	.	.	G	-	-	-	-	-	2	-	-	-	-	2
	E9	C	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	1	-	-	1
	Total											3	3	3	7	3	7	4	5	3	6	44

Table 3 Pairwise fixation indices (F_{ST}) between nine populations of *Ctenomys flamarioni* from nine microsatellite loci (lower half of matrix) and from mitochondrial DNA expanded dataset haplotypes (upper half of matrix). Populations correspond to those of Figure 1 and Table 1. *P* is the probability that any random value obtained is > observed value (10,000 permutations).

Populations									
	XA	RE	PI	SS	BJ	SJN	CASS	TA	CHU
XA	-	0.000^{ns}	0.675†	1.000^{ns}	0.205^{ns}	1.000†	0.881†	1.000^{ns}	1.000†
RE	**0.137***	-	0.675†	1.000^{ns}	0.205^{ns}	1.000†	0.881†	1.000^{ns}	1.000†
PI	**0.272***	**0.294***	-	0.784†	0.416†	0.537†	**0.837***	0.838†	**0.879***
SS	**0.475***	**0.473***	**0.285***	-	0.469†	1.000†	0.918†	1.000^{ns}	1.000†
BJ	**0.405***	**0.416***	**0.225***	**0.146***	-	0.195^{ns}	0.650†	0.601†	**0.713***
SJN	**0.478***	**0.459***	**0.295***	**0.372***	**0.254***	-	0.929†	1.000†	**1.000***
CASS	**0.515***	**0.548***	**0.383***	**0.489***	**0.375***	**0.544***	-	-0.132^{ns}	0.040^{ns}
TA	**0.631***	**0.664***	**0.504***	**0.652***	**0.552***	**0.684***	**0.466***	-	0.000^{ns}
CHU	**0.565***	**0.594***	**0.431***	**0.530***	**0.444***	**0.630***	0.172†	**0.590***	-

* Significant values are in bold ($P < 0.001$); † non-significant values after Bonferroni correction ($0.05 < P < 0.006$); ns, not significant ($P \geq 0.05$).

Table 4 Analysis of molecular variance (AMOVA) for *Ctenomys flamarioni* populations based on nine loci of microsatellites performed for two different arrangements of localities: (a) grouped in three regions (I; II + III; IV) defined by geographic barriers to gene flow (A and C; following Fernández-Stolz et al. in prep); (b) and two regions (II; III) separated by a hypothesized geographical barrier to gene flow (B). Region numbers and barrier letters correspond to those of Figure1.

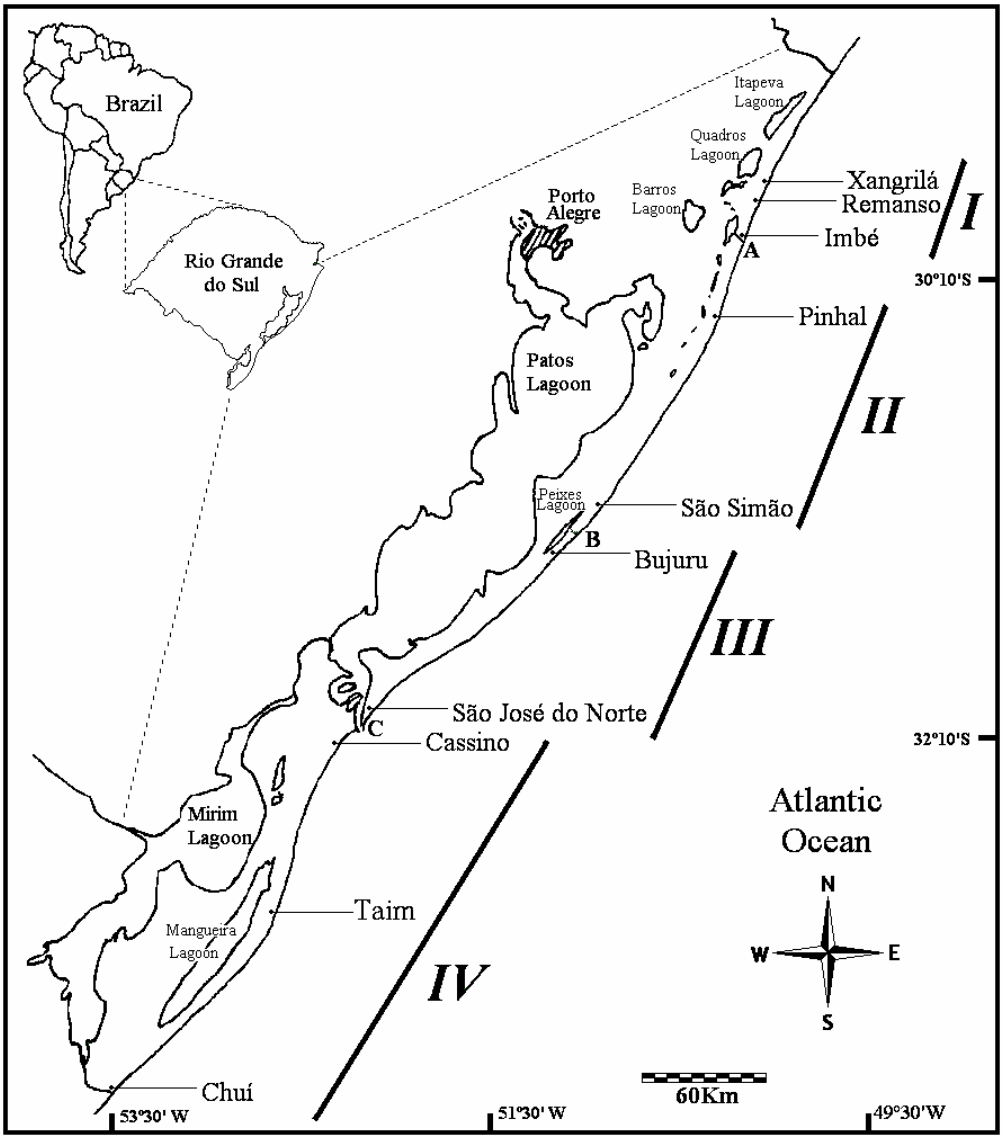
Hierarchical level	% variation	Fixation Indices
a. Three groups		
Among groups	31.55	F_{CT} = 0.315
Among populations within groups	17.31	F_{SC} = 0.253
Within populations	51.14	F_{ST} = 0.488
b. Two groups		
Among groups	-0.22	F_{CT} = -0.002 ^{ns}
Among populations within groups	27.43	F_{SC} = 0.274
Within populations	72.79	F_{ST} = 0.272

Each level was significantly different from zero ($P < 0.05$) unless indicated by ^{ns}.

Table 5 Test for excess heterozygosity for *Ctenomys flamarioni* populations. The reported loci used are polymorphic and in HWE at each population. M.l., monomorphic loci; TPM, Two-phased mutation model; SMM, Stepwise mutation model; *p*-val, probability of significant heterozygosity excess calculated using a Wilcoxon sign-rank test.

Region	Population	Sample size	Loci used (M.l.)	*p*-val	
				TPM	SMM
I	Xangri-lá	24	7 (1)	0.027*	0.045*
	Remanso	27	8 (1)	0.011*	0.048*
II	Pinhal	30	8 (0)	0.230	0.677
	São Simão	13	7 (2)	0.289	0.594
III	Bujurú	10	8 (1)	0.004*	0.013*
	São José do Norte	12	5 (4)	0.500	0.890
IV	Cassino	8	0 (6)	-	-
	Taim	14	0 (6)	-	-
	Chui	8	0 (8)	-	-

* Significant *p*-values ($p < 0.05$).


Brazil
Rio Grande do Sul
Itapeva Lagoon
Quadros Lagoon
Barros Lagoon
Porto Alegre
Xangrilá
Remanso
Imbé
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Pinhal
Patos Lagoon
II
Peixes Lagoon
São Simão
B
Bujuru
III
São José do Norte
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Cassino
32°10'S
Atlantic Ocean
N
W
E
S
Mirim Lagoon
Taim
Mangueira Lagoon
IV
Chuí
60Km
53°30' W
51°30' W
49°30'W

Figure 1

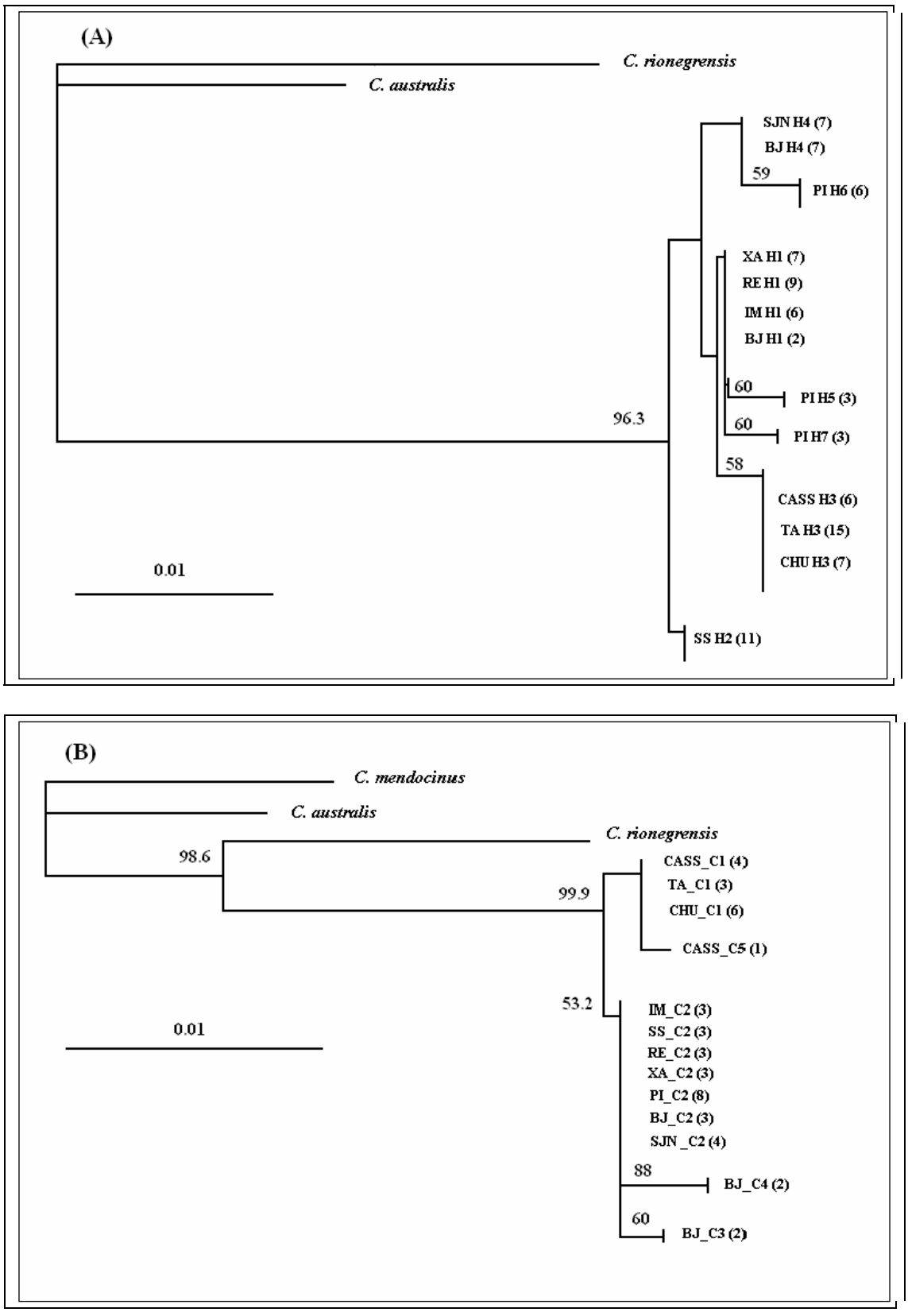

(A)
C. rionegrensis
C. australis
SJN H4 (7)
BJ H4 (7)
59
PI H6 (6)
XA H1 (7)
RE H1 (9)
IM H1 (6)
BJ H1 (2)
60
PI H5 (3)
96.3
60
PI H7 (3)
58
CASS H3 (6)
TA H3 (15)
CHU H3 (7)
0.01
SS H2 (11)
(B)
C. mendocinus
C. australis
C. rionegrensis
98.6
CASS_C1 (4)
TA_C1 (3)
99.9
CHU_C1 (6)
CASS_C5 (1)
53.2
IM_C2 (3)
SS_C2 (3)
0.01
RE_C2 (3)
XA_C2 (3)
PI_C2 (8)
BJ_C2 (3)
SJN _C2 (4)
88
BJ_C4 (2)
60
BJ_C3 (2)

Figure 2

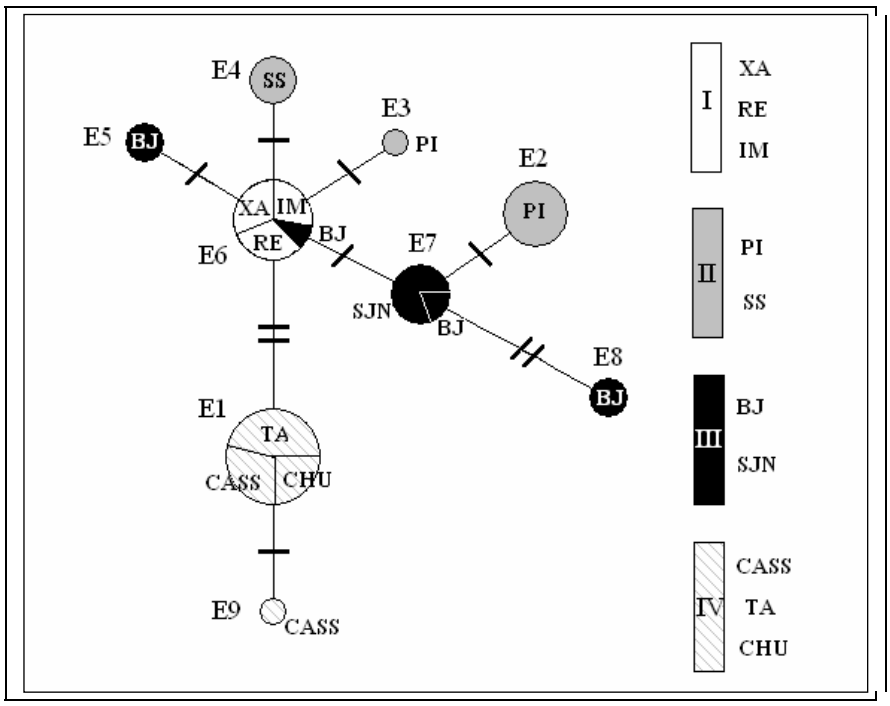
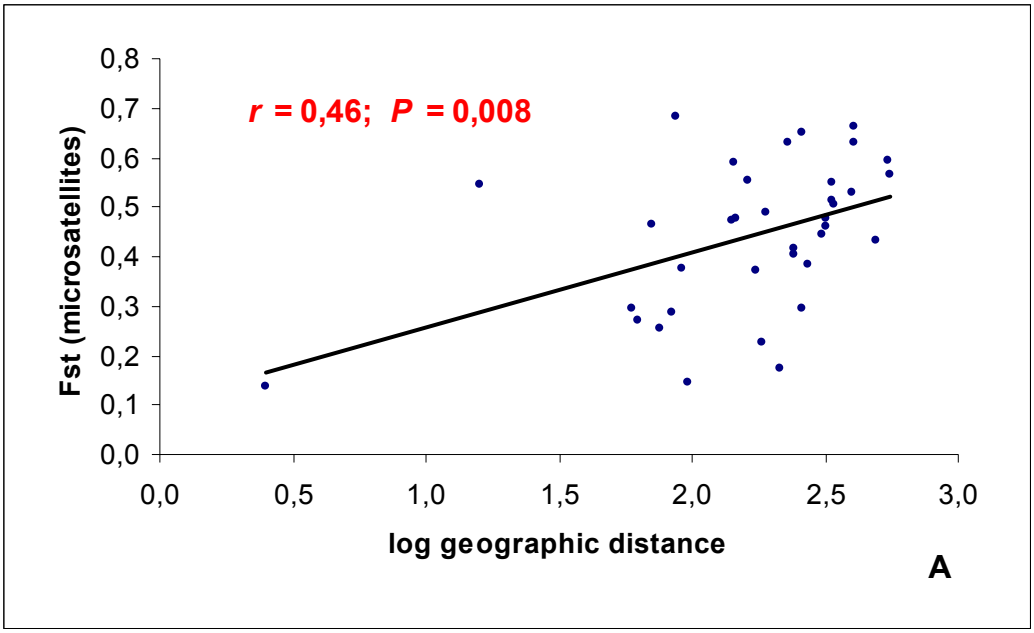

E4
SS
E3
PI
E5
BJ
E2
PI
XA
IM
RE
BJ
E6
E7
SJN
BJ
E8
BJ
E1
TA
CASS
CHU
E9
CASS
I
XA
RE
IM
II
PI
SS
III
BJ
SJN
IV
CASS
TA
CHU

Figure 3


r = 0,46; P = 0,008
Fst (microsatellites)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0,0
0,5
1,0
1,5
2,0
2,5
3,0
log geographic distance
A

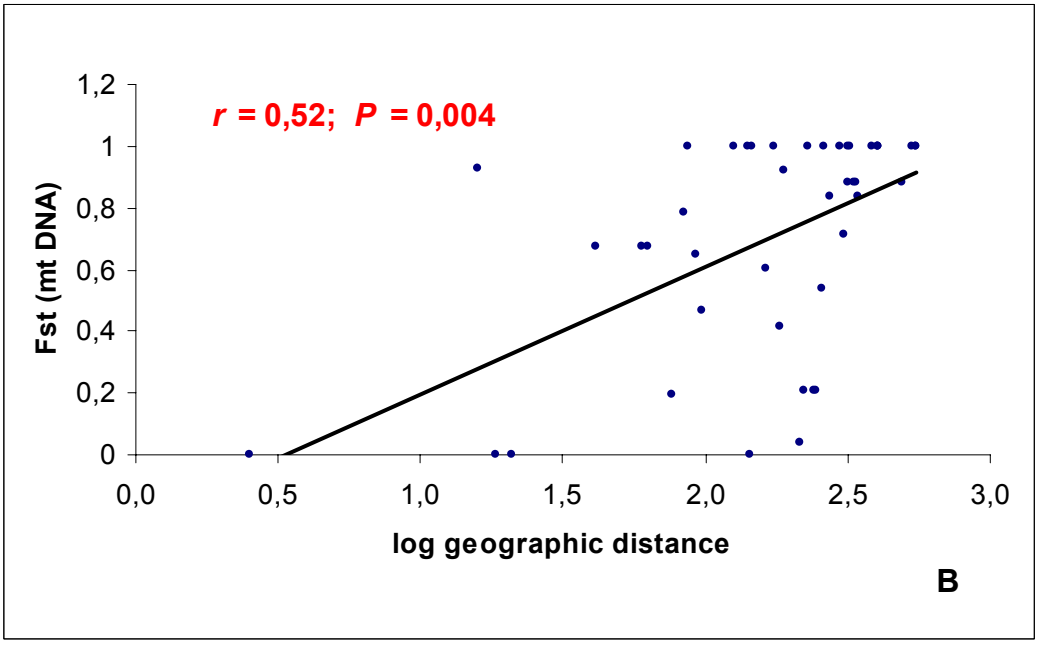

r = 0,52; P = 0,004
Fst (mt DNA)
1,2
1
0,8
0,6
0,4
0,2
0
0,0
0,5
1,0
1,5
2,0
2,5
3,0
log geographic distance
B

Figure 4

VI. DISCUSSÃO

Através deste estudo foi caracterizada tanto a variabilidade quanto os padrões genéticos subjacentes em *C. flamarioni* e, a partir destes, feitas inferências a respeito da história evolutiva da espécie, identificando a existência de eventos demográficos acontecidos no passado (tanto recente quanto histórico), a distribuição espacial das linhagens filogenéticas encontradas, e os principais fatores que teriam propiciado a divergência genética tanto nas populações locais quanto num contexto filogeográfico. Esta informação, assim como a disponível na bibliografia para esta espécie ameaçada, tem sido aplicada num contexto de conservação.

As diferentes análises aplicadas aos dois tipos de marcadores moleculares utilizados, tanto os de origem mitocondrial (fragmentos da região controladora e do citocromo-b) quanto nuclear (*loci* de microssatélites), assim como os principais resultados obtidos, têm sido descritos e discutidos de forma independente em cada um dos capítulos precedentes. Como uma forma de recopilação e com uma visão unificadora dos mesmos, a seguir serão apresentados os principais pontos discutidos ao longo do trabalho e, finalmente, sumarizadas as principais conclusões e contribuições deste estudo ao conhecimento da espécie.

Ctenomys flamarioni tem uma característica peculiar que compartilha com poucas outras espécies de ctenomídeos: sua distribuição geográfica linear (ou quase) impõe importantes restrições ao fluxo gênico, favorecendo a migração entre demes vizinhos (ver Slatkin & Barton, 1989; Hutchinson & Templeton, 1999). Estimativas de baixo fluxo gênico apoiando este padrão têm sido obtidas para todas as populações de *C. flamarioni*, inclusive para as mais próximas, Xangri-lá e Remanso (separadas por 2,5 km).

Nas populações para as quais se têm estimado diferenças no grau de estruturação genética entre machos e fêmeas, não foram observadas diferenças significativas entre sexos para a maior parte das metodologias aplicadas. Isto permite inferir que não existe estritamente um sexo filopátrico e outro dispersante para *C. flamarioni*. Assim, a partir de estimativas de divergência entre populações por sexo, foi observado um valor de F_{ST} não significativo para os machos (entre as populações de Xangri-lá e Remanso), o que sugere que apesar de ambos os sexos dispersarem, os machos o fazem em maior proporção. Isto poderia ser explicado pelo fato que, para espécies de distribuição linear (com fluxo gênico restrito entre populações), os padrões de dispersão diferencial entre machos e fêmeas são mais fáceis de serem identificados em populações mais próximas, onde é minimizado o efeito da distância na divergência genética (Goudet e cols., 2002).

Na maioria dos mamíferos, os machos constituem o sexo dispersante e as fêmeas o filopátrico (Greenwood, 1980). Porém existem, entre os roedores subterrâneos, numerosos exemplos que mostram dispersão por ambos os sexos, embora os registros encontrem-se limitados a umas poucas espécies (*C. talarum*, Pearson e cols., 1968; Malizia e cols., 1995; *C. australis*, Zenuto & Busch, 1998; *Geomys attwateri*, Williams & Cameron, 1984; *Spalax microphthalmus*, Wei e cols., 1997).

Nas três populações estudadas no Capítulo I, foram observados desvios da proporção sexual em favor das fêmeas e dimorfismo sexual para a espécie, com machos apresentando tanto maior peso quanto comprimento do corpo. Estas evidências apóiam um padrão de poliginia para esta espécie, concordante com a hipótese de que a forte competição pelas fêmeas em espécies poligínicas resulta numa pressão para a dispersão dos machos (Dobson, 1982; Perrin & Mazalov, 1999; 2000).

Poliginia no gênero *Ctenomys* foi descrita para *C. autralis* (Malizia e cols., 1991; Zenuto & Busch, 1998) e para *C. talarum* (Busch e cols., 1989; Malizia & Busch, 1991; 1997; Zenuto e cols., 1999), mostrando concordância com o padrão de poliginia exibido pela maioria dos roedores subterrâneos (ver revisão em Lacey, 2000).

No Capitulo I também foram descritas diferenças interpopulacionais referentes aos padrões demográficos, observando-se evidências de reduções recentes do tamanho populacional (gargalos de garrafa) nas populações das localidades mais impactadas (Xangri-lá e Remanso) através de todos os métodos empregados. Isto faz com que seja sugerido o fator humano como possível causa dos gargalos de garrafa, evidenciado na redução das dunas devido à intensa utilização de areia para construção de moradias, a introdução de espécies exóticas na composição florística e ao aumento de estruturas recreacionais na primeira linha de dunas costeiras, como possível causa dos gargalos de garrafa.

No Capitulo III foram retomados os testes de gargalos de garrafa e incorporadas mais populações às análises. Embora todas as nove populações tenham sido consideradas, seis foram efetivamente adicionadas às análises estatísticas (neste caso através do programa BOTTLENECK, Cornuet & Luikart, 1996) uma vez que três delas apresentaram-se monomórficas para seis ou mais *loci*, impossibilitando a aplicação do teste (não recomendado para menos de quatro *locus*). Os resultados obtidos indicaram que a população de Bujurú (sem evidências de impacto antrópico importante) também teria experimentado reduções do tamanho populacional no passado recente, sugerindo que mais fatores, e não só as mudanças humanas, poderiam estar levando as populações a atravessar gargalos de garrafa.

Somado a esta evidência, a forte redução no polimorfismo dos *loci* de microssatélites das populações ao sul da barra de Rio Grande (Cassino, Taim e Chuí), sugere a existência de processos que teriam levado à perda de variabilidade, tendo a deriva genética um papel importante. Embora este padrão tenha sido sugerido também a partir dos dados de seqüências (DNA mitocondrial), apresentando baixa variabilidade haplotípica e nucleotídica ao longo de grandes distâncias (aproximadamente 214 km desde a praia do Cassino até o Chuí) não pode ser descartada a existência de alelos nulos para um ou vários dos *loci* analisados. Outro ponto que deve ser salientado é a amostragem de poucos indivíduos para as populações do sul da distribuição da espécie, principalmente para Cassino e Chuí (n = 8), o que poderia estar gerando interpretações errôneas dos resultados. Todavia, esta consideração não parece tão relevante, na medida em que outras populações sub-amostradas não apresentaram a perda de variabilidade observada para estas populações (também verificada na riqueza alélica e na heterozigosidade; ver tabela 1, Capítulo III).

Neste ponto, os resultados provenientes do tratamento dos dados sob uma abordagem filogeográfica podem aportar alguns fatores adicionais às explicações dadas. A partir das várias metodologias aplicadas para testar tanto padrões de expansão regionais quanto em escala macro-geográfica, se obtiveram em geral sinais positivos (*mismatch distribution*, Rogers & Harpending, 1992), embora não significativos para alguns testes (*Fs*, Fu, 1997; *D*, Tajima, 1989) ou com intervalos de confiança muito grandes (modelo exponencial, Kunher e cols., 1998). Em geral, populações que têm experimentado expansão demográfica recente apresentam pouca divergência genética, como observado em populações de *C. flamarioni*. Todavia, a não concordância entre os diferentes modelos testados pode estar evidenciando, para a espécie, um padrão mais complexo de ocupação de sua atual área de distribuição. Diversas variáveis como a existência de barreiras

geográficas ao fluxo gênico que, embora incipientes, mostraram-se efetivas, um ou vários eventos de redução populacional em diferentes escalas temporais (como sugerido pelos padrões observados nos dois tipos de marcadores analisados), e posteriormente, vários eventos menores de redução-expansão do tamanho populacional teriam operado sobre a variabilidade, reduzindo-a até os níveis atuais. O padrão de dinâmica populacional obtido para este roedor assemelha-se a um mosaico, no qual fatores históricos e contemporâneos teriam um papel fundamental, com a deriva genética operando como principal força evolutiva.

Um dos principais e melhor documentados fatores históricos está constituído pelo último evento de transgressão marinha ocorrido durante o Holoceno, o qual teria trabalhado o perfil da costa através de sucessivas flutuações do nível do mar, com um máximo situado próximo aos 5.000 anos (Corrêa e cols., 1992; Tomazelli & Villwock, 2000, Tomazelli e cols., 2000; Esteves e cols., 2002), e provavelmente extinguido grande parte das populações de tuco-tucos ao longo da PC. Evidências desta transgressão marinha foram recolhidas tanto a partir de dados palinológicos quanto de datação de fósseis marinhos mediante ^{14}C, nos diferentes corpos de água que ocupam a PC na atualidade: lagunas de Tramandaí e dos Patos (Medeanic e cols., 2000; 2001; Marques-Toigo e cols., 2002), e lagoa Mirim (Buchmann e cols., 1998).

A partir da utilização de duas metodologias, semi-paramétrica e não-paramétrica, que relaxam a necessidade de assumir o ajuste dos dados a um relógio molecular (Sanderson, 2002), foi estimada uma taxa de mutação para o citocromo-b ($\mu = 0.019$ ⁄ sítio ⁄ milhão de anos). O tempo de divergência das principais linhagens filogenéticas de *C. flamarioni* foi estimado em 90.000 anos, indicando assim a origem do clado no Pleistoceno Tardio.

Estas estimativas são concordantes com a hipótese proposta por Freitas (1994) e posteriormente por Masarini & Freitas (2005), na qual a entrada de *C. flamarioni* na costa brasileira teria acontecido quando a PC era mais ampla que atualmente e o Rio da Prata não representava uma barreira expressiva ao fluxo gênico, como sugerido através de evidências paleoambientais (Corrêa e cols., 1992).

A natureza estocástica dos fatores climáticos e ambientais, que determinam e modelam a evolução da Planície Costeira na atualidade, também poderiam dar conta de uma parte importante da perda de variabilidade e dos padrões filogeográficos observados para a espécie. Isso poderia ter acontecido na medida em que eventos de tempestade, que levam ao alagamento de grandes extensões de campos de dunas, seguem a períodos intensos de seca que, principalmente na região sul da distribuição (sul da barra da Laguna dos Patos), têm sido bem reportados (Saint Hilaire, 1974 [apud Gomes e cols., 1987]; Gomes e cols., 1987; Tomazelli & Dillenburg, 2000; Esteves e cols., 2002) assim como observados atualmente através dos trabalhos de campo realizados na região (J. F. B. Stolz, comunicação pessoal). Estes fatores atuais estariam operando em uma escala menor, sendo provavelmente responsáveis por extinções locais em populações atuais, contribuindo com o complexo mosaico de processos populacionais sugerido.

Usando o método baseado em distâncias genéticas (*mismatch distribution*, Rogers & Harpending, 1992) e através do qual foi detectada expansão populacional, foi estimado o momento no qual teria acontecido o evento de expansão mais recente (que em rigor estatístico não é necessariamente o último, mas sim o que deixou o sinal genético mais evidente, ou seja, o que pode ser detectado através dos recursos teórico-práticos disponíveis atualmente). Esta estimativa indica aproximadamente 60.000 anos antes do presente, o que significa que a expansão teria acontecido no Pleistoceno, num período

caracterizado por condições climáticas áridas, nas quais as vazantes dos principais rios da região eram muito menos importantes que na atualidade (De Oliveira e cols., 2005; Latrubesse e cols., 2005).

Todavia, deve ser enfatizado que este valor deve ser considerado com cautela, já que provêm da estimativa de uma taxa de mutação particular, e se baseia num cenário simplificado para representar a história demográfica das espécies (*stepwise growth model*). Além disso, o valor indicado pelo modelo, na luz do nosso conhecimento a respeito da evolução geomorfológica da PC, deve ser visto como uma série de várias flutuações demográficas ao longo do tempo ao invés do que um evento pontual (ver discussão em Zheng e cols., 2003)

Quanto aos padrões filogeográficos, os dados indicam significativa estruturação genética tanto em escala regional (determinada pelas barreiras geográficas) como ao longo de toda a distribuição geográfica da espécie, exibindo um padrão de isolamento pela distância. As desembocaduras das lagunas de Tramandaí e dos Patos foram indicadas através dos dados genéticos (teste de Amova), como barreiras efetivas ao fluxo gênico, explicando grande parte da divergência genética encontrada ao longo da distribuição da espécie.

Evidências provindas de análises cariotípicas e de eletroforese de proteínas sugerem e apóiam o poder da barra da Laguna dos Patos como barreira ao fluxo gênico. Quanto às análises cariotípicas, Freitas (1994) descreve um polimorfismo para o cromossomo 1 autossômico nas populações ao sul desta desembocadura e, a partir de dados de proteínas, Moreira (1991) relata valores baixos de similaridade (S = 0.773) para as populações em ambos os lados da mesma.

A correlação positiva entre as distâncias genéticas e geográficas foi verificada tanto através de dados de seqüências do DNA mitocondrial (região controladora e citocromo-b) como de *loci* de microssatélites nucleares.

Em espécies como *C. flamarioni*, com distribuição associada a um hábitat com características unidimensionais (como é o sistema de dunas costeiro) e com limitada capacidade de dispersão, se espera o estabelecimento de um padrão de isolamento pela distância. Todavia, este padrão não é sempre encontrado, uma vez que numerosas espécies não atingem o equilíbrio entre deriva genética e fluxo gênico, como no caso de aquelas que têm experimentado expansões demográficas recentes (Slatkin, 1993; Hutchinson & Templeton, 1999).

Ctenomys flamarioni apresentou um padrão de isolamento pela distância em uma escala macro-geográfica, o que não foi visto em uma escala regional, quando apenas as populações da região central (II e III) foram analisadas. Isto sugere um padrão mais complexo para *C. flamarioni*, provavelmente relacionado à ocorrência de um, ou vários, eventos de expansão nas áreas de distribuição atual, a existência de gargalos de garrafa na história demográfica das populações, assim como prováveis eventos de extinção local e recolonização que parecem estar acontecendo em algumas das populações desta espécie (e.g., na localidade do Taim, Stolz e cols., *in prep*.). Por outra parte, como indicado por Bossart & Powel (1998), certas populações podem ser fortemente responsáveis pelas correlações entre as distâncias genéticas e geográficas observadas nos padrões de isolamento pela distância, e a não inclusão destas nas análises pode levar ao desaparecimento deste padrão. Por outro lado, quando estimadores da divergência genética (F_{ST} ou seus análogos) entre pares de populações mostram valores significativos associados a uma população ou a certos grupos de populações, a vicariância é sugerida como a

explicação mais viável. Os resultados obtidos a partir dos *loci* mitocondriais sugerem que esta poderia ser a melhor explicação para os padrões encontrados; embora o mesmo não tenha sido observado para as análises realizadas a partir dos *loci* nucleares, onde os valores de divergência foram altamente significativos através de quase todas as comparações.

Um dos objetivos deste estudo foi poder delinear o conjunto de características genéticas de *C. flamarioni*, e como estas foram moldadas pela influência tanto de processos geológicos (ou em escala geográfica) quanto, em um nível mais inclusivo, de processos microgeográficos. Estreitamente vinculado a este objetivo está o de conservar a variabilidade assim como os processos naturais que mantém e estruturam esta variabilidade no espaço geográfico.

Com esta finalidade, foram descritas no Capítulo III as unidades de conservação que, utilizando um critério "adaptativo" (*sensu* Fraser & Bernatchez, 2001), melhor agrupam a diversidade intrapopulacional:

- Duas unidades evolutivamente significativas (ESUs): a primeira incluindo o litoral norte (região I, no nosso estudo) e a região central da área de distribuição de *C. flamarioni*, até a Barra de Rio Grande (regiões II e III); e a segunda incluindo as populações do sul da distribuição até a saída do Arroio Chuí (região IV);

- Cada população deverá ser considerada como uma unidade de manejo (MU, *sensu* Moritz, 1994) baseado na divergência genética encontrada nos *loci* nucleares assim como no padrão de isolamento pela distância, encontrado quando considerada toda a área de distribuição da espécie.

Na definição das unidades de conservação foi levada em consideração não só a informação acessada a partir dos *loci* neutros, mas também a proveniente de outras abordagens genéticas (como os padrões observados para polimorfismos cariotípicos e

enzimáticos) e de áreas do conhecimento como a geologia, paleontologia e palinologia, que tem contribuído para a reconstrução da história evolutiva da espécie.

VII. CONCLUSÕES

1. O limite sul de distribuição geográfica de *C. flamarioni* foi ampliado até a desembocadura do Arroio Chuí no extremo sul do Brasil.

2. A partir de evidências coletadas nos trabalhos de campo (medidas morfológicas e proporção sexual) foi sugerido um padrão de poliginia para a espécie.

3. *Ctenomys flamarioni* apresentou baixa variabilidade tanto para os haplótipos de DNA mitocondrial (região controladora e citocromo-b) quanto para os nove *loci* de microssatélites analisados.

4. As características demográficas da espécie foram determinadas pelo menos por dois fatores: o efeito de um ou vários gargalos de garrafa, tanto históricos quanto recentes, seguidos de um ou vários eventos de expansão populacional de diferente magnitude: 1. próximo à origem da espécie, no Pleistoceno Tardio, aproximadamente há 60.000 anos; 2. relacionados ao quarto evento de transgressão marinha do Quaternário (Holoceno), com um máximo de aproximadamente 5.000 anos; 3. contemporâneo(os), causado(os) por flutuações climáticas modernas e outros fatores, dentre os quais o fator humano parece ter uma importância crescente.

5. A estrutura genética populacional mostrou ser alta a partir dos *loci* de microssatélites, indicando baixo fluxo gênico atual entre elas, mas quando avaliada a partir dos

haplótipos mitocondriais, esta foi baixa, sugerindo assim maior conectividade histórica entre as populações.

6. As desembocaduras das lagunas Tramandaí e dos Patos mostraram ter importância nos padrões filogeográficos observados para *C. flamarioni*, constituindo barreiras eficientes ao fluxo gênico.

7. O teste de Mantel mostrou uma correlação significativa entre as matrizes de distância genética e geográfica para ambos os tipos de marcadores analisados, indicando um padrão global de isolamento pela distância. Todavia, este não foi observado para os *loci* mitocondriais quando as populações do sul da distribuição foram eliminadas das análises, sugerindo o possível efeito da vicariância no padrão observado.

8. As análises de Network sugerem que os haplótipos do norte da distribuição seriam os mais antigos, a partir dos quais, com poucas substituições nucleotídicas, teriam se originado os demais.

9. As reconstruções filogenéticas através dos diferentes métodos (distâncias, parcimônia e máxima verossimilhança) evidenciaram relações pouco profundas entre os haplótipos e nodos não resolvidos na base.

10. A partir dos nossos resultados, e da informação disponível para a espécie, foram propostas duas ESUs, uma formada pelas populações das regiões I, II e III, e a outra

pelas populações pertencentes à região IV. Quanto às MUs foi proposto que cada população seja considerada uma unidade de manejo independente.

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IX. ANEXOS

Anexo I. Critérios de Unidade Evolutivamente significativa (Evolutionarily Significant Unit, ESU) desde Ryder (1986); modificado de Fraser & Bernatchez (2001).

Autor	Critério
Ryder (1986)	Entidades mais inclusivas dentro do nível de espécie que possuem atributos genéticos significativos para as gerações presentes e futuras da espécie em questão.
Waples (1991)	População ou grupo de populações que: (i) é substancialmente isolada reprodutivamente de outras unidades populacionais co-específicas; e (ii) representa um componente importante do legado evolutivo da espécie.
Dizon e cols. (1992)	Populações ou grupos de populações que divergem significativamente nas freqüências alélicas de marcadores genéticos moleculares.
Avise (1994)	Grupos de populações derivados de filogenias consistentes de genes.
Moritz (1994)	Populações que: (i) apresentam monofilia recíproca para alelos do mtDNA; (ii) evidenciam significativa divergência nas freqüências alélicas para *loci* nucleares.
Vogler & DeSalle (1994)	Grupos diagnosticados através de caracteres exclusivos e excludentes.
Crandall e cols. (2000)	Abandona o termo ESU por um conceito mais holístico de espécie, na qual, as populações variam quanto aos níveis de fluxo gênico, evolucionando através de deriva genética e seleção.
Fraser & Bernatchez (2001)	Linhagem mostrando altamente restrito fluxo gênico com outras linhagens co-específicas.