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Hybrid zone between *Ctenomys lami* and *C. minutus*: habitat alterations influence the evolutionary history of two burrowing rodent species of southern Brazil

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Hybridization events provide insights into central questions in evolutionary biology regarding the role of reproductive isolation in speciation mechanisms. Because of that, there is increasing interest in understanding the patterns and processes underlying hybrid zones. The sister species *Ctenomys minutus* (2n = 48a) and *Ctenomys lami* (2n = 56) are burrowing rodents endemic to the southern Brazilian coastal plain and collectively exhibit high karyotype polymorphism. We analyzed chromosomal and mitochondrial DNA (mtDNA) variation to assess their evolutionary history and confirm and characterize an interspecific hybrid zone between the species. Despite the absence of reciprocal monophyly of mtDNA lineages, *C. minutus* and *C. lami* maintain distinct geographical distributions as well as cytogenetic and morphological differences, suggesting that these incipient species are in the early stages of evolutionary differentiation. The 19 hybrid individuals we include in this analysis show intermediate karyotypic forms between the parental types. These admixed individuals all shared the same mtDNA haplotype that is otherwise only found in *C. minutus.* Although *C. minutus* and *C. lami* are incipient species, their unique differences require distinct conservation efforts. Conservation efforts should focus on maintaining the integrity of pure populations of both species and minimizing anthropogenically induced hybridization.

Los eventos de hibridación brindan información sobre cuestiones centrales en biología evolutiva con respecto al papel del aislamiento reproductivo en los mecanismos de especiación. Debido a eso, existe un creciente interés en comprender los patrones y procesos subyacentes a las zonas híbridas. Las especies hermanas *Ctenomys minutus* (2n = 48a) y *Ctenomys lami* (2n = 56) son roedores excavadores endémicos de la llanura costera del sur de Brasil y colectivamente exhiben un alto polimorfismo cariotípico. Analizamos la variación cromosómica y del ADN mitocondrial (ADNmt) para evaluar su historia evolutiva y confirmar y caracterizar una zona híbrida interespecífica entre las especies. A pesar de la ausencia de monofilia recíproca de linajes de ADNmt, *C. minutus* y *C. lami* mantienen distribuciones geográficas distintas, así como diferencias citogenéticas y morfológicas, lo que sugiere que estas especies incipientes están en las primeras etapas de la diferenciación evolutiva. Los 19 individuos híbridos que incluimos en este análisis muestran formas cariotípicas intermedias entre los tipos parentales. Todos estos individuos mezclados compartían el mismo haplotipo de ADNmt que, de otro modo, solo se encuentra en *C. minutus*. Aunque *C. minutus* y *C. lami* son especies incipientes, sus diferencias únicas requieren esfuerzos de conservación específicos. Los esfuerzos de conservación deben centrarse en mantener la integridad de las poblaciones puras de ambas especies y minimizar la hibridación inducida antropogénicamente.

Keywords: Allopatry; chromosomal polymorphism; cyto-nuclear genome discordance; hybrid zone; incomplete lineage sorting.

Introduction

The concepts of species, species boundaries, and the process of speciation remain the central focus of evolutionary biology. Hybridization may influence the evolutionary process, and its consequences will be determined by the genetic, morphological, chromosomal, and ecological characteristics of the species that come into contact (Barton 2001; Mallet *et al.* 2008). Because of that, hybrid zones are considered laboratories for evolutionary studies, providing insights into the patterns and processes of geographical genetic variation among taxa and the mechanisms of reproductive isolation and speciation (Dowling and Secor 1997).

Hewitt (1988) considered a contact or hybridization zone a region where different forms meet, mate, and produce hybrids. Contact zones can be classified as (a) primary if the populations are parapatric and exhibit a cline in terms of their genetic differences (Hewitt 1988) or (b) secondary if intermittent gene flow occurs between two otherwise allopatric populations (Hewitt 1988). In hybrid zones where hybrids survive beyond the F_1 generation, interbreeding between them and backcrossing with their parental types may lead to a population with a wide variety of recombinant types (Harrison 1993).

The assessment of individuals' karyotypic information can be a helpful starting point for identifying a hybrid zone since diploid numbers and chromosomal rearrangements are commonly variable among species or even at the intraspecific level and may also substantially influence inter-breeding outcomes. The well-documented examples in the literature of hybrid zones among chromosomal races of mammals were reported for the *Sorex araneus* group (Searle and Wójcik 1998) and *Mus musculus domesticus* (Piálek *et al.* 2005), which were used as models for investigations on the role of chromosomal rearrangements in the speciation process.

Among subterranean rodents, the most studied groups regarding hybridization associated with chromosomal variation have been *Spalax, Thomomys,* and *Geomys* (Nevo 1986; Patton 1973; Patton *et al.* 1979, 1984; Baker *et al.* 1989). More recently, studies conducted on intraspecific and interspecific hybrid zones of subterranean rodents from the genus *Ctenomys* (Gava and Freitas 2002; Gava and Freitas 2003; Castilho *et al.* 2012) have provided important insights into the geographical genetic structure among populations with fixed chromosomal differences (Gava and Freitas 2003, Tomasco and Lessa 2007; Fernandez *et al.* 2012; Lopes and Freitas 2012; Lopes *et al.* 2013; Kubiak *et al.* 2020).

Ctenomys minutus and *Ctenomys lami* are sister species of burrowing rodents from the Torquatus group in the genus *Ctenomys* (Freitas 2001; Parada *et al.* 2011; De Santi *et al.* 2024). Both species are endemic to the coastal plain of southern Brazil (Figure 1). The species are characterized by significant Robertsonian chromosomal polymorphisms (Freitas 2006, 2001). *Ctenomys minutus* has a narrow distribution along the first dune line and sand fields near the coast in Santa Catarina (SC) and Rio Grande do Sul (RS) Brazilian states (Figure 1; Lopes *et al.* 2013; Freitas 2021). This species has diploid numbers ranging from 2n = 42 to 50 and autosomal arm numbers (AN) ranging from 68 to 80, comprising 45 karyotypes. The main karyotypes (2n = 42, 46a, 46b, 48a, 48b, 48c, 50a, and 50b) are distributed parapatrically. Between each pair of parapatric karyotypes, with one exception, there is an intra-specific hybrid zone (Freitas 1997; Gava and Freitas 2002, 2003; Freygang *et al.* 2004; Castilho *et al.* 2012; Lopes *et al.* 2013; Freitas 2021).

Ctenomys lami is geographically restricted to an area of 78 x 12 km, corresponding to inland sandy fields of the RS coastal plain (Figure 1; Freitas 2001). This species has five different diploid numbers (2n = 54, 55, 56, 57, 58) and 10 AN (from 74 to 82 and 84), which combined form 26 karyotypes. Four karyotypic blocks, named Blocks A, B, C, and D, were described for this species considering the Robertsonian rearrangements (Freitas 2007). Two intra-specific hybrid zones were reported for this species, one between blocks A x B and another between blocks C x D (Figure 1 inset; Freitas 2007, 2021).

These species are indistinguishable by external morphology. However, *C. lami* was described as a separate species from *C. minutus* due to differences in their karyotypes (mainly regarding the chromosomal forms and diploid numbers), the distinct habitats that the species occupy, and differences in skull morphology (Freitas 2001). The current hypothesis about the process of speciation between *C. minutus* and *C. lami* is based on the role of chromosomal rearrangements and geographical barriers, resulting in an allopatric model of speciation followed by chromosomal differentiation (Freitas 2006).

Cytogenetic data from six individuals sampled in the western margin of Barros Lake, in the state of RS (Figure 1, inset), demonstrated diploid numbers and chromosomal rearrangements intermediate between specimens of *C. lami* (2n = 56) and *C. minutus* (2n = 48a) that inhabit the areas surrounding this region. These individuals have been considered inter-specific hybrid forms between *C. lami* and *C. minutus* (Gava and Freitas 2003). The authors suggested that this hybrid zone is a product of secondary contact. Historically, a wide marsh area west of Barros Lake represented a geographical barrier isolating *C. minutus* and *C. lami*. However, around the 1950s, the introduction of rice cultivation in the region completely drained the marsh, exposing a sandy area, which allowed interspecific contact, mating, and, consequently, the establishment of hybrids. The extent and consequences of that hybrid zone for both species are uncertain and require further investigation.

The goals of the present article are to i) describe the hybrid zone between both species, ii) describe the evolutionary history of *C. lami* and *C. minutus* in the southern Brazilian coastal plain, and iii) provide information to help future conservation decisions, and management strategies for both species. To address these issues, we examine mitochondrial DNA (mtDNA) control region and cytochrome c oxidase subunit I sequences coupled with cytogenetic data for each sampled individual.

Materials and methods

Sampling, species identification and study overview. Sampling covered the entirety of the currently known distribution of *C. lami* and *C. minutus* in the coastal plain of southern Brazil. Both species occur in completely different areas, so initial identification was based on their geographic location of capture. The karyotype determined by Freitas (1997, 2001, and 2007) was examined for each specimen.

A total of 166 individuals of *C. lami* were previously sequenced for mitochondrial DNA (mtDNA), comprising 28 sampling sites (Lopes and Freitas 2012). For *C. minutus,* 244 were sequenced from 30 sites (Lopes *et al.* 2013).

Nineteen new individuals were collected from three distinct sites within the area of secondary contact previously described by Gava and Freitas (2003; Table 1, Figure 1). Trapping was conducted using Oneida-Victor n°0 snap-traps.

Cytogenetics. For the 19 possible hybrids, we determined the diploid and autosomal arm numbers through analyses of at least 20 metaphase spread cells stained with Giemsa following Ford and Hamerton (1956). Meiotic analyses were conducted for males using the technique of verifying the behavior of chromosomes and Robertsonian rearrangements during meiosis (Ford and Evans 1969).

DNA amplification and sequencing. We extracted DNA from the 19 possible hybrids following a modified phenol-chloroform protocol (Sambrook and Russel 2001). Two fragments of mitochondrial DNA (mtDNA) were analyzed. A segment of the HVS1 control region (CR) was amplified using the primers TucoPro (Tomasco and Lessa 2007) and TDKD (Kocher *et al.* 1989). The amplification of cytochrome c oxidase subunit I (COI) followed the protocols suggested by Lopes *et al.* (2013), using the primers LCO-1490 and HCO-2198 (Folmer *et al.* 1994). PCR products were visualized on 1 % agarose gels and purified using Exonuclease I and Shrimp Alkaline Phosphatase (GIBCO-BRL Life Sciences/Invitrogen, Carlsbad, California), following the guidelines of the suppliers. Sanger sequencing was conducted on an ABI3730 automated sequencer, using the forward primers TucoPro and LCO-1490 for CR and COI, respectively. The electropherograms were visually inspected using Chromas 2.33 (http://www.technelysium.com.au/chromas.html), and ambiguous sequences were reamplified and resequenced. The sequences were aligned using the Clustal W algorithm with default options implemented in Mega 5.2.1(Tamura *et al.* 2011). Alignments were checked and edited by hand.

Genetic variability and genetic differentiation between species and populations. Mitochondrial sequence analyses were performed using CR and COI data sets separately, but most results were achieved using a concatenated data set of CR+COI (CC). Measures of mtDNA genetic diversity, such as the number of polymorphic sites, the mean number of pairwise differences (π), the average number of nucleotide differences (k), the number of haplotypes (H), and haplotype diversity (*Hd*), were calculated using DNAsp 5.10.01 (Librado and Rozas 2009).

Phylogenetic analyses and divergence time estimates. The appropriate model of nucleotide sequence evolution to be applied in the phylogenetic analysis was determined for each mtDNA dataset separately, using the Bayesian Information Criterion (BIC) estimated in jModelTest 2.1.4 (Posada 2008). The HKY + I + Γ and KHY + I provided the best fit for CR and COI data sets, respectively. We conducted Maximum Likelihood (ML) analyses in PhyML 3.0 (Guindon *et al.* 2010). Analyses were seeded with a Neighbor-Joining tree, followed by nearest-neighbor interchange branch-swapping. We evaluated nodal support using 1,000 bootstrap replicates with random taxon addition.

The phylogenetic tree based on a Bayesian Inference (BI), as well the time of the most recent common ancestors (tMRCA) of the main mtDNA clades of *C. lami* and *C. minutus,* were estimated using Beast 1.7.5 (Drummond *et al.* 2012), employing a coalescent prior of constant population size model, under a strict molecular clock, with substitution rates estimated for ctenomyids by Roratto *et al.* (2015). For CR, the rate applied was 2.96×10^{-8} site/year (95 % confidence interval: 1.65×10^{-8} - 4.42×10^{-8}), and for COI, the substitution rate was 2.13×10^{-8} site/year (95 % confidence interval: 1.42×10^{-8} - 2.87×10^{-8}). Both substitution rates and evolution models were applied separately to each partition of the mtDNA data. Analyses were performed following 100 million iterations of MCMC, sampling trees every 10,000 steps and discarding the first 10 % of trees as burn-in. Results were visually inspected using the program Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and summarized in TreeAnnotator 1.6.1. The BI phylogenetic tree and the inferred tMRCAs for the main clades were recovered using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Trees were outgroup-rooted with homologous sequences of *Ctenomys torquatus*, *Ctenomys pearsoni*, and *Ctenomys ibicuiensi*. Finally, the topological relationships among haplotypes were estimated using Network 4.5.1.0 (http://www.fluxux-engineering.com) with the median-joining approach for the CC data set.

Results

Cytogenetic data. Of the 19 individuals sampled on the western banks of Barros Lake, 18 were successfully karyotyped, 12 of which were hybrid individuals. Comprising six diploid numbers (2n = 48, 50, 53, 54, 55, and 56) and four autosomal arm numbers (AN = 74, 76, 78, and 80), a total of 10 different karyotypes were recovered. The karyotypes for each sampling site are described in Table 2 and in Figure 2. The karyotypic forms of 2n = 56, AN = 80; 2n = 56, AN = 78; and 2n = 55, AN = 76 were previously recorded for *C. lami*, and the form of 2n = 48, AN = 76 was previously described for *C. minutus*. The karyotypes of the putative hybrids were geographically distributed in a gradient through the landscape, ranging from 2n = 56 in individuals from sampling site 1, near populations of *C. lami*. Karyotypes of hybrids were progressively reduced to 2n = 48 in sampling site 3, near populations of *C. minutus* (Figure 1 and Table 2). The sex chromosome pair, with a sub-metacentric X and an acrocentric Y chromosome, was the same for all karyotypes.

In Figure 2, the karyotypes 2n = 56 AN = 80 and AN = 78 belong to the parental forms of *C. lami*. The forms 2n = 55 AN = 76 originated from the crossing of 2n = 54 AN = 80 (backcrossing between 2n = 52 and 2n = 56) and 2n = 56 (*C. lami*). The karyotypes 2n = 54 AN = 80 and AN = 78 originated from the crossing between 2n = 52 and 2n = 56 (*C. lami*). 2n=53 AN=78 and AN=74 originated from the crossing between 2n=52 and 2n=54 (backcrossing 2n=52 x 2n=56). The forms 2n=50 AN=74 and AN=76 originated from the backcrossing between

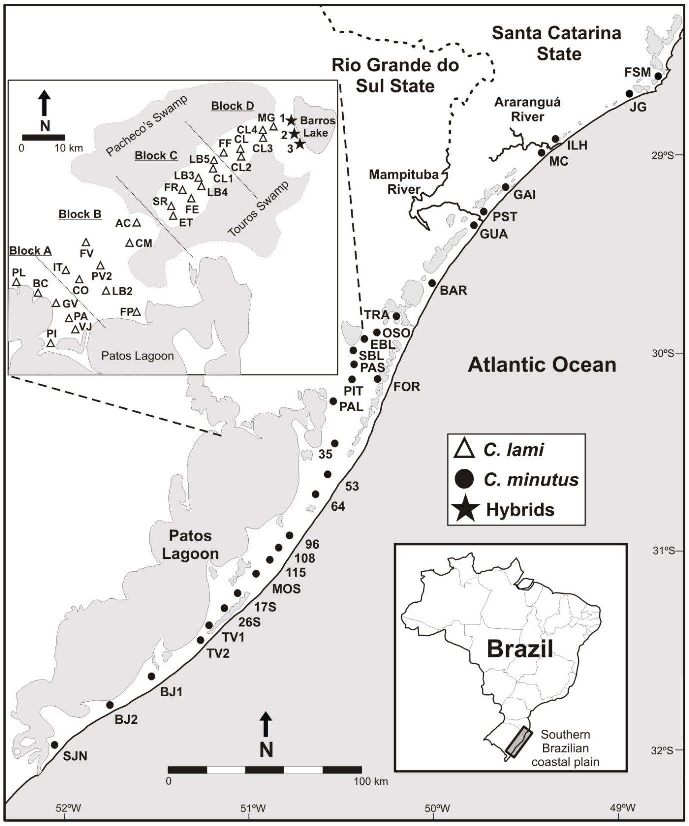

Figure 1. Sampling sites of *C. lami*, *C. minutus*, and interspecific hybrids along the coastal plain of southern Brazil. Locality abbreviations correspond to those in Table 1.

Table 1. Sampling sites, karyotypic and mitochondrial variation observed for *C. lami,* interspecific hybrids, and *C. minutus* and mtDNA clusters. CC haplotype designations follow those shown in the haplotype network (Figure 4).

Locality name	No. S	2n(AN)	CC haplotypes (CR+COI)	mtDNA clusters
Ctenomys lami				
Parque Itapuã (PI)	15	54(76,78)/55a(78,80)	CC55(14)/CC56(1)	BL
Passo da Areia (PA)	3	54(76,78)/55a(76)	CC55(2)/CC57(1)	BL
Varzinha do Jacaré (VJ)	4	54	CC23(4)	BL
Gravatá (GV)	6	56a	CC55(3)/CC23(1)/CC58(2)	BL
Beco do Cego (BC)	11	54(76,82)/55a(76,78,80)/56a(76,78,80)	CC53(10)/CC54(1)	BL
Praia do Lami (PL)	8	-	CC53(8)	BL
Itapuã (IT)	4	58(78,80,83)	CC56(4)	BL
Costa do Oveiro (CO)	9	58(78,79)	CC23(2)/CC54(4)/CC56(1)/CC60(2)	BL
Passo do Vigário 2 (PV2)	2	58(80,82)	CC60(1)/CC61(1)	BL
Fervura (FV)	7	54/58	CC62(7)	BL
Lombas 2 (LB2)	4	57(77)/58(78,80,86)	CC58(3)/CC59(1)	BL
Fazenda Pimenta (FP)	7	58(78)	CC54(2)/CC59(5)	BL
Beco da Macega (BM)	6	58	CC23(2)/CC62(4)	BL
Águas Claras (AC)	3	58(78,80)	CC23(1)/CC59(2)	BL
Estiva (ET)	6	54(74,76,77,78,79)/55a(76)	CC64(4)/CC65(2)	BL
Sanga da Rapadura (SR)	9	54	CC23(3)/CC63(2)/CC64(4)	BL
Fazenda do Estácio (FE)	2	54	CC23(1)/CC66(1)	BL/BC+D
Fazenda Rita Maria (FR)	12	54	CC23(9)/CC66(3)	BL/BC+D
Lombas 4 (LB4)	5	54(75,76,77)	CC23(3)/CC66(2)	BL/BC+D
Lombas 3 (LB3)	6	54(75,76,78)	CC23(1)/CC66(5)	BL/BC+D
Chico Lomã 1 (CL1)	4	54	CC23(1)/CC66(3)	BL/BC+D
Lombas 5 (LB5)	4	54(75,76,78)	CC23(1)/CC66(3)	BL/BC+D
Fazenda dos Freitas (FF)	11	56b(78,80,82)	CC66(7)/CC67(4)	BC+D
Chico Lomã 2 (CL2)	4	-	CC69(4)	BC+D
Chico Lomã (CL)	3	56b(80)	CC66(1)/CC68(2)	BC+D
Chico Lomã 3 (CL3)	6	56b	CC68(6)	BC+D
Chico Lomã 4 (CL4)	-	56b	-	BC+D
Morro Grande (MG)	5	54	CC69(5)	BC+D
1	8	53(74)/54(78)/55(76)/56(78,80)	CC26(8)	BL
2	8	50(74,76)/53(74,80)/54(78,80)	CC26(8)	BL
3	3	48(76)/50(76)	CC26(3)	BL
Ctenomys minutus				
Farol de Santa Marta (FSM)	15	50a(76)	CC48(2)/CC49(8)/CC50(3)/CC51(1)/ CC52(1)	N2
Jaguaruna (JG)	17	48c(76)/49a(76)/50a(76)	CC46(14)/CC47(3)	N1/N2
Ilhas (ILH)	15	48c(76)	CC45(15)	N2
Morro dos Conventos (MC)	12	46a(76)	CC43(6)/CC44(6)	N1
Gaivota Beach (GAI)	10	46a(76)	CC42(10)	CO
Passo de Torres (PST)	3	46a(76)	CC39(1)/CC40(1)/CC41(1)	CO
Guarita Beach (GUA)	12	46a(76)	CC38(12)	CO
Barco Beach (BAR)	7	46a(76)	CC35(3)/CC36(1)/CC37(3)	CO

Table 1. Continuation.

Locality name	No. S	2n(AN)	CC haplotypes (CR+COI)	mtDNA clusters
Tramandaí (TRA)	2	46a(76)	CC31(1)/CC34(1)	BL
Osório (OSO)	9	46a(76)	CC25(3)/CC31(2)/CC32(3)/CC33(1)	BL
East Barros Lake (EBL)	5	46a(76)/47a(76)/48a(76)	CC27(5)	BL
South Barros Lake (SBL)	11	47a(76)/48a(76)	CC26(9)/CC28(2)	BL
Passinhos (PAS)	4	48a(76)	CC26(4)	BL
Pitangueira (PIT)	5	48a(76)	CC24(2)/CC25(3)	BL
Fortaleza Lake (FOR)	12	47a(76)/48a(76)	CC26(1)/CC29(1)/CC30(10)	BL
Palmares do Sul (PAL)	7	48a(76)	CC21(5)/CC23(1)/CC24(1)	BL
Road km 35 (35)	4	48a(76)	CC20(3)/CC22(1)	BL/ MO
Road km 53 (53)	5	48a(76)	CC17(2)/CC18(2)/CC19(1)	MO
Road km 64 (64)	3	48a(76)	CC16(2)/CC17(1)	MO
Road km 96 (96)	6	48a(76)	CC13(4)/CC15(2)	MO
Road km 108 (108)	15	42(68,69,70,71,72,73,74)/ 43(70,72,73,74,75)/ 44(72,73,74,75,76)/ 45(74,75,76,78,80)/ 46(71,74,76,77,78)	CC11(15)	MO
Road km 115 (115)	3	42(74)	CC11(2)/CC13(1)	MO
Mostardas (Mos)	16	42(74)	CC11(5)/CC12(1)/CC13(6)/CC14(4)	MO
17 Km south of Mostardas (17S)	10	42(74)/43(74)	CC9(8)/CC10(2)	TA
26 Km south of Mostardas (26S)	15	42(74)/43(70,72,74)/44(74)/46b(76)	CC4(10)/CC8(3)/CC9(2)	TA
Tavares 1 (TV1)	14	46b(76)	CC4(10)/CC5(1)/CC6(3)	TA
Tavares 2 (TV2)	6	46b(76)/47b(76)	CC4(2)/CC6(3)/CC7(1)	TA
Bujuru 1 (BJ1)	12	48b(76,78)	CC3(12)	SO
Bujuru 2 (BJ2)	9	49b(76,77)/50b(76)	CC2(9)	SO
São José do Norte (SJN)	12	50b(76,77)	CC1(12)	SO

No. S – number of samples sequenced; 2n(AN) – diploid and autosomal arm numbers; CC – concatenated mtDNA haplotypes and corresponding number of individuals in parentheses; mtDNA clusters – BL: Barros Lake; BC+D: Blocks C+D; N2: North 2; N1: North 1; CO: Coastal; MO: Mostardas; TA: Tavares; SO: South. Abbreviations of locality names are in parentheses. *Ctenomys lami* localities are in italic font, interspecific hybrids in bold font, and *C. minutus* localities are in normal font.

2n=52 and 2n=48 (*C. minutus*). Cytotype 2n=48 AN=78 is the parental form of *C. minutus*. Pair 1 of *C. minutus* is the largest submetacentric pair of the karyotype in all karyotypes studied in this species (2n=48 AN=76) and is a marker pair for *C. minutus* in this system. It is worth mentioning that this pair does not appear in 2n=56; AN=80 and AN=78 and in 2n=55; AN=76. In karyotypes 2n=54; AN=78, 2n=53; AN=80 and 74 and in 2n=50; AN=76, these two chromosomes appear in pair 1 in the homozygous form. On the other hand, in the forms 2n=54; AN=80 and 2n=50; AN=74 only one chromosome is observed. Two factors reinforce the hybridization between *C. minutus* and *C. lami*: the occurrence of hybrid karyotypes, observed in the 13 animals, different from 2n=48 and 2n=56, and the occurrence of homozygotes for the two submetacentric chromosomes that are the marker pair of *C. minutus*. We obtained cells in spermatogenesis from males collected in the sampling sites 2 and 3. The cells showed meiosis with normal behavior and proper segregation of chromosomes. Two males were analyzed from locality 2, one showed 2n = 50, NA = 74, and gametic cells with n = 25, and another with 2n = 53, AN =

Table 2. Karyotypes of individuals sampled in western banks of Barros Lake.

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

No.S - number of specimens per karyotype; M – male; F – female; 2n - diploid numbers; AN - autosomal arm numbers; Bi - number of autosomal biarmed chromosomes; Ac - number of autosomal acrocentric chromosomes. *Karytotypic forms previously described for *C. lami*.

$^{\pm}$ Karytotypic form previously described for *C. minutus*.

Figure 2. Karyotypes obtained in the interspecific hybrid zone between *C. lami* and *C. minutus.*

80, and gametic cells with n = 26 or 27. In locality three only one male was collected with 2n = 48, AN = 76, and gametic cells with n = 24.

Genetic diversity and population differentiation. Mitochondrial DNA sequencing resulted in 398 bp of the control region (CR) and 620 bp of COI, comprising 1,018 bp for CC (CR+COI) data. Measures of genetic variability were higher in *C. minutus* than in *C. lami*, especially those related to the number of nucleotide differences (k and π; Table 3).

Phylogenetic and genealogical relationships among haplotypes. The phylogenetic trees generated by ML and BI analyses showed similar topologies (Figure 3). The main difference was an apparent paraphyly in the ML tree among the clades Coast, Barros Lake, Mostardas, and Block C+D (data not shown). Nonetheless, the low nodal value, even in the BI analysis (gray arrow), confirms the lack of certainty in this portion of the tree.

We found eight well-supported clades based on posterior probabilities (81 – 100) and bootstrap values (63 – 100), six of which exclusively contain haplotypes of *C. minutus* (indicated by a capital M at each tip, Figure 3), one of which exclusively contained haplotypes of *C. lami* (capital L after the tip, Figure 3), and one of which contained haplotypes of both parental species and hybrids (Barros Lake clade, Figure 3). The North 1 (MC and JC) and North 2 (FSM, JG, and ILH) clades correspond to the northern sampling sites of *C. minutus*, and the clades South (BJ2, BJ1, and SJN) and Tavares (26S, 17S, TV1, and TV2),

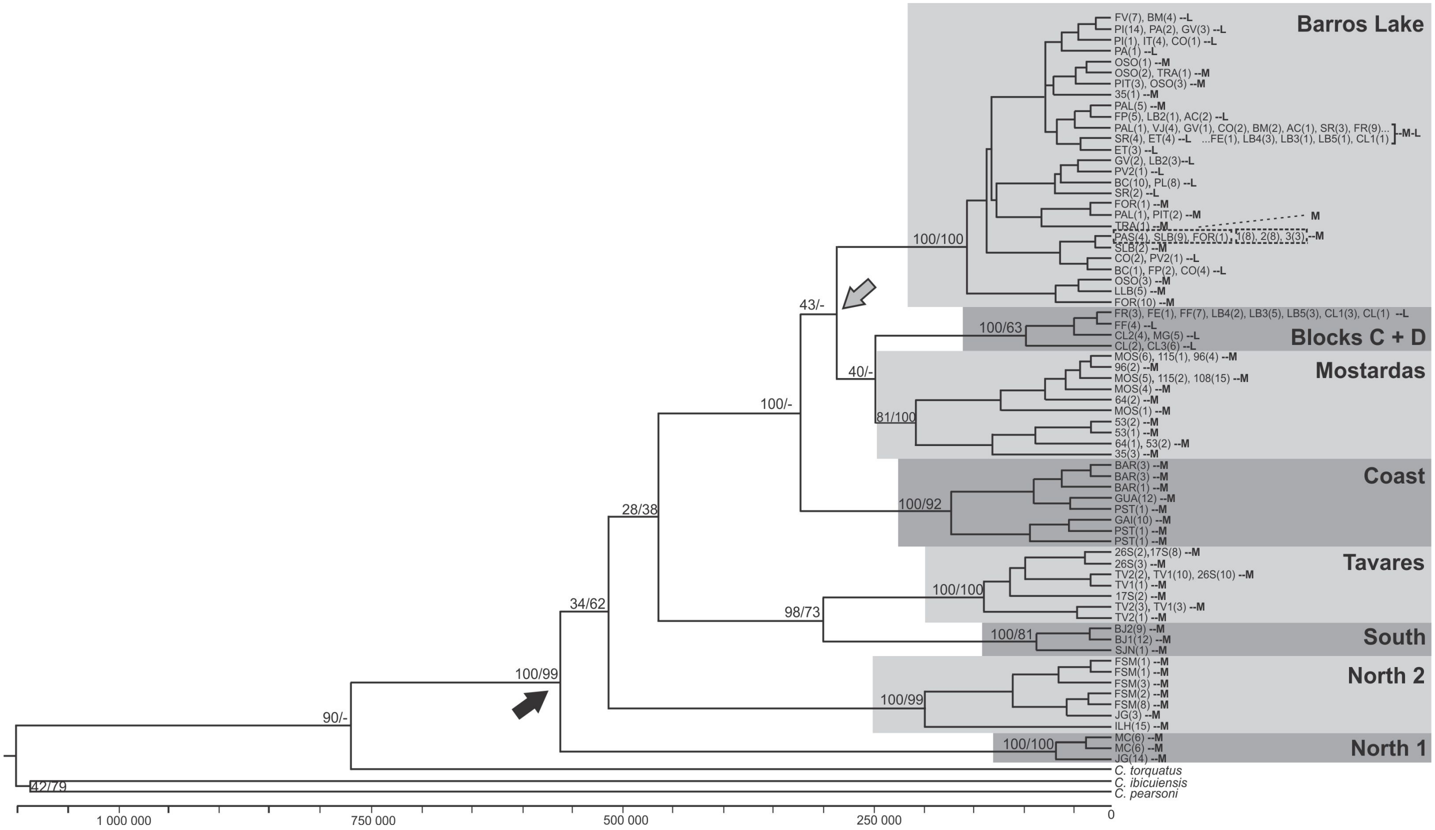

Figure 3. Bayesian phylogenetic tree of mitochondrial DNA concatenated data. Sampling sites are abbreviated according to Table 1 and are followed by the corresponding number of individuals in parentheses as well as a species or hybrid designation: M = *C. minutus*, L = *C. lami*, and H = hybrid. Nodal support is indicated by values above branches for Bayesian Inference and Maximum Likelihood, respectively, - indicates absence of the branch according to each analysis. The eight main mtDNA clades are highlighted by gray squares. Black arrow indicates the most recent common ancestor of all *C. minutus* and *C. lami* individuals, and the grey arrow denotes the node potentially representing the beginning of the divergence process between the two species. Bottom ruler corresponds to the divergence time in years.

represent the southern-most localities of *C. minutus*. The Coast (GAI, PST, GUA, and BAR) and Mostardas (MOS, 115, 96, 108, 64, 53, and 35) clades were also exclusively represented by *C. minutus* specimens. In contrast, the Block C+D clade was comprised only of specimens of *C. lami* sampled in the karyotypic Blocks C (CL1, FR, FE, LB3, LB4, and LB5) and D (FF, CL2, CL3, CL4, CL, and MG, Figure 1).

The Barros Lake clade contains haplotypes common to both species and hybrids. In this group we find all individuals of *C. lami* sampled southeast of the connection between Pachecos and Touros swamps (corresponding to karyotypic Blocks A and B) and some specimens sampled north of this connection (from karyotypic Block C), plus all individuals of *C. minutus* surrounding the Barros Lake region, ranging from sampling sites TRA south to 35, and also the 19 possible hybrids, sampled at the three-starred localities near Barros Lake (Table 1, Figures 1 and 2). The Barros Lake clade showed a star-like shape in the median-joining network (Figure 4), with several low-frequency haplotypes connected by a few mutational steps to a central one (CC23), which is the only haplotype shared between both species. Moreover, all 19 possible hybrids share the same haplotype (CC26), with *C. minutus* specimens sampled from SBL, PAS, and FOR localities.

Divergence time estimates. The estimated divergence time between our in-group and the sister species *C. torquatus* was 762,754 years ago (ya; 95 % Highest Posterior Density (HPD): 413,172 – 1,073,748 ya), and the tMRCA for the in-group was estimated to be 561,666 ya (95 % HPD: 326,433 – 794,706 ya). The divergence times and the 95 % HPD among haplotypes of each of the eight main clades were: North 1: 80,543 ya (8,949 – 112,513 ya); North 2: 206,936 ya (77,502 –301,077 ya); Coast: 182,106 ya (69,535 – 251,475 ya); Barros Lake 166,963 ya (71,030 – 218,147 ya); Blocks C+D: 109,542 ya (17,814 – 159,096 ya); Mostardas: 216,059 ya (96,948 – 301,746 ya); Tavares: 150,457 ya (52,319 – 208,226 ya); and South: 99,300 ya (11,599 – 151,258 ya).

Discussion

Evolutionary history of C. minutus and C. lami in the coastal plain of southern Brazil. Only recently have *Ctenomys minutus* and *C. lami* been considered two separate species due to differences in their karyotypes, areas of occurrence, and skull morphology (Freitas 2001). Our phylogenetic reconstruction based on mtDNA variation shows that the species are not reciprocally monophyletic. Instead, the main clades highlighted the clustering of individuals following a strong pattern of geographic subdivision. This strong geographic association in both species was previously identified in phylogeographical studies (Lopes and Freitas 2012 and Lopes *et al.* 2013). For *C. lami*, mtDNA haplotypes were partially isolated between population blocks

B and C due to the connection between the Pachecos and Touros swamps (Lopes and Freitas 2012). For *C. minutus*, the main subdivisions of mtDNA haplogroups and population clusters were associated with geographic discontinuities throughout the landscape, represented by rivers, paleochannels, and the transition between sandy fields and dunes (Lopes *et al.* 2013, Freitas 2021).

The species from the genus *Ctenomys* are commonly characterized by low rates of adult dispersal among relatively small and fragmented populations, which promote the establishment of small genetic units where genetic variation is low and interpopulation divergence is high (Reig *et al.* 1990; Lessa and Cook 1998; Wlasiuk *et al.* 2003; Gaspareto *et al.* 2024). Considering that the mtDNA has a non-recombinant maternal inheritance and that females of ctenomyids commonly show low rates of dispersal (Malizia and Busch 1991; Cutrera *et al.* 2006), it is expected that the power of taxonomic resolution of the mtDNA is limited when the process of speciation is a recent event, accounting for a pattern of incomplete lineage sorting, in which the gene genealogy may differ from the species phylogeny (Pagès *et al.* 2013).

The estimation of the tMRCA among all individuals of *C. minutus* and *C. lami* was around 562 thousand years ago (kya; black arrow in Figure 3). However, this node age does not necessarily match the starting point of the process of speciation between these species. While the basal relationships among the in-group clades are uncertain, it is possible that the divergence process between *C. minutus* and *C. lami* began at approximately 292 kya, which corresponds to the age estimation of the node that includes both species and hybrids, represented by the clades of Barros Lake, Blocks C+D, and Mostardas (grey arrow in Figure 3). Nonetheless, distinguishing gene tree divergence from population divergence at this stage of species differentiation remains difficult.

Another well-studied complex of species in the genus *Ctenomys* is the Perrensis group, which, like *C. minutus* and *C. lami*, shows discordance in species delimitations across different datasets. Traditionally, based on geographic range, morphology, and chromosomal variation, the Perrensis group is described as formed by three species (*Ctenomys roigi*, *Ctenomys perrensi,* and *Ctenomys dorbignyi*) and several forms of uncertain taxonomic status (*Ctenomys* sp.; Ortells 1995; Caraballo *et al.* 2012; Caraballo and Rossi 2017). Later studies based on chromosomes and mtDNA cytochrome b sequences suggested the existence of two other species in addition to the first three, despite the absence of reciprocal monophyly among clades (Giménez *et al.* 2002). More recently, Fernández *et al.* (2012) analyzed microsatellite and mitochondrial data, which revealed an even more complex evolutionary scenario than previously described. The authors suggested that populations were not sufficiently isolated to complete speciation, and thus, when they come into contact and hybridization takes place, even between populations with different chromosomal numbers, this leads to incongruence among character sets. A similar result was found by Patton and Smith (1994) between *Thomomys bottae* and *T. townsendii,* wherein incongruence among character sets reflected the known complexity of the divergence process, especially in the presence of hybridization. For *C. minutus* and *C. lami*, despite the absence of mtDNA monophyletic clades separating both species and the presence of a hybrid zone, *C. minutus* and *C. lami* maintain distinct geographic distributions as well as cytogenetic and morphological differences, suggesting that these incipient species are at the early stages of the differentiation process.

Table 3. Genetic diversity for *C. lami*, *C. minutus* and interspecific hybrids using the mtDNA concatenated data.

Identification	***N***	**No.H**	**Pol. Sites**	***k***	***π***	***Hd***
C. lami	166	18	21	4.63	0.00456 (±0.00021)	0.908 (±0.009)
C. minutus	276	52	87	15.59	0.01533 (±0.00025)	0.965 (±0.003)
Hybrids	19	1	0	0.00	0.00000 (±0.00000)	0.000 (±0.000)

N - number of samples; No.H - number of haplotypes; Pol. Sites - polymorphic sites; k - average nucleotide differences; π - nucleotide diversity; *Hd* - haplotype divergence.

The hybrid zone. The results of mtDNA and cytogenetic data presented here strongly support the hypothesis of a region of hybridization around the western banks of Barros Lake, in RS state, between *C. minutus* and *C. lami* (Figure 1).

In this work, of the 19 individuals sampled in the hybrid zone region, 13 presented chromosomal rearrangements and intermediate diploid numbers between the karyotypic forms of the parental types involved (*C. lami* 2n = 56b, and *C. minutus* 2n = 48a; see Table 2, and Figure 1). Gava and Freitas (2003) found hybrids for the first time in five animals and in another population 5 km further north with 2n = 50 FN = 78, 2n-51 FN = 77, FN =7 8 and FN=80 and 2n = 52 FN =7 9. The wide range of karyotypic forms found among hybrids may be derived from several crossings beyond the F1 generation, as well as backcrossing with parental forms. The supposed low effectiveness of chromosomal differences as barriers to gene flow may be caused by a neutral or weak under-dominant condition of the chromosomal rearrangements, and thus, they may not substantially reduce hybrid fitness (Rieseberg 2001). The heterozygous carriers of chromosomal rearrangements may maintain fertility by means of mechanisms that suppress partial or total recombination with harmful effects during meiosis (Rieseberg 2001).

Regarding mtDNA data, all 19 hybrids shared the same haplotype (CC26; see Figure 3) with *C. minutus* from SBL, PAS, and FOR sampling sites located near the region of hybridization (Figure 1). Since the mitochondrial genome is only maternally inherited, the pattern of mtDNA recovered in hybrids provides evidence that *C. minutus* females are the main, if not the only, donors of the mitochondrial genome to hybrids (Figure 4).

The pattern observed in the hybrid zone described here, in which hybrids carry the mtDNA of *C. minutus,* may have been reached by means of pre and/or post-zygotic isolation

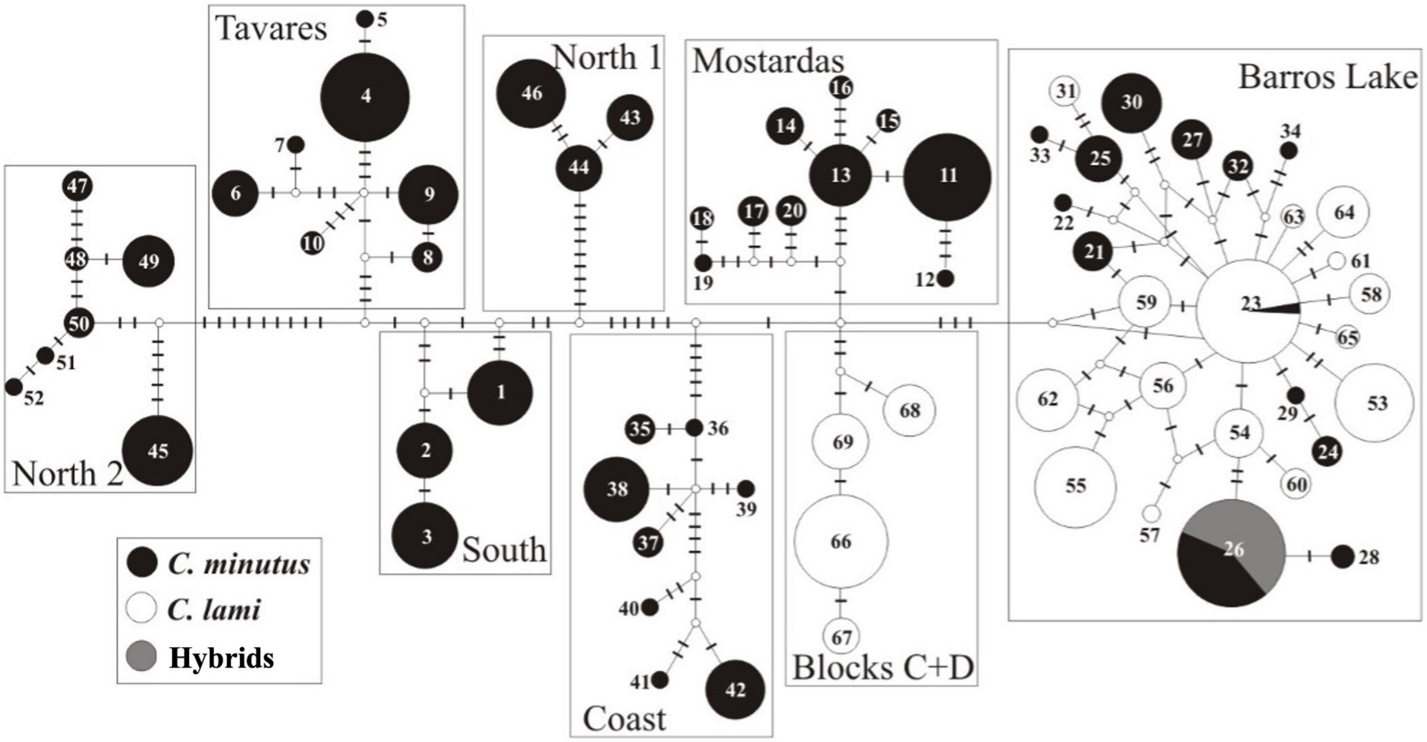

Figure 4. Median-joining haplotype network of mitochondrial DNA concatenated data. The colors represent the species and hybrids following the legend. The area of the circle is proportional to the haplotype frequency, each bar represents one mutational step, and small white dots indicate unsampled haplotypes. Haplotype numbers correspond to those in Table 1. The eight main haplogroups are highlighted by squares.

mechanisms, *e. g.*, a mate choice system involving females of *C. minutus* with males of *C. lami*. Sexual selection is an important force in reproductive isolation and speciation, and mate preference represents one of the aspects of this force. Mate choice can be influenced by a series of biological characters (Shurtliff 2013), and female preferences have been described in subterranean rodent hybrid zones in the genus *Thomomys* (Patton and Smith 1993) and *Geomys* (Bradley *et al.* 1991). However, it is difficult to distinguish whether the pattern observed here is due to mate choice and/or the consequence of hybrid inviability/sterility from the mating between females of *C. lami* and males of *C. minutus*. Despite our reduced sample size of hybrids and the fact that we were not able to recover cells in spermatogenesis for males from sampling site 1 due to technical problems, the hybrid males collected in sampling sites 2 and 3 presented meiosis with normal behavior and proper segregation of chromosomes, demonstrating that at least some hybrid males seem to be fertile.

Chromosomal rearrangements mainly involve chromosome inversions, fissions, and centromeric fusions between chromosomes. Such rearrangements are mainly observed in *C. lami* (Freitas 2007). Therefore, chromosomal alterations can cause reproductive incompatibility between different individuals (Ferree and Barbash 2009; Nishino *et al.* 2019; Ono and Greig 2020). The occurrence of only one type of crossing may be related to the fact described by Nishino *et al.* (2019) in two subspecies, *Mus musculus domesticus* and *M. m. molossinus*. Between the crossing of *M. m. domesticus* females and *M. m. molossinus* males, the F1 males are sterile, but F1 males are fertile in the reciprocal parental cross. This is because, in sterile males, apoptosis in spermatocytes is considerably higher than in the parental lines and in the fertile hybrids from the opposite parental cross. Further, analysis of metaphase I in this system showed that some chromosomes do not align in the equatorial plane of the cell, indicating the formation of non-viable gametes. Finally, another cause of hybrid sterility in this system is the non-pairing of the X and Y sex chromosomes and, consequently, the lack of formation of the synaptonemal complex during prophase I (Nishino *et al.* 2019).

In *Ctenomys*, a few hybrid zones between different species have been described. Hybrids are known between *C. flamarioni* (2n = 48) and *C. minutus* (2n = 46a). The crosses of these species have a distinct pattern from that described in this work, as they occur in both parental directions. However, unlike what we find between *C. minutus* and *C. lami*, no karyotypes originated from backcrossing between the F1 and either of the parents (Kubiak *et al.* 2020).

De Queiroz (1998) introduced the concept of species as lineages of independent metapopulations that evolve over time. Thus, characteristics that are useful in diagnosing species, such as distinct phenotypic characteristics, ecological differences, and reciprocal monophyly, arise in different ways across the tree of life due to differences in the pace of evolution of morphological, ecological, behavioral, and genetic factors that impact reproductive isolation (de Queiroz 2007). Our analysis of *C. minutus* and *C. lami* shows that the two species are still differentiating and that they are best characterized as incipient species. Thus, conservation efforts should focus mainly on preserving the integrity of pure populations of both species. Furthermore, although *C. minutus* and *C. lami* are incipient species, conservation strategies must be considered separately, respecting their particularities.

Implications for conservation. Hybridization and introgression are not usually listed among the main threats to extinction. If the hybridization process occurs naturally, it is considered part of the evolutionary history of the taxa involved. However, these phenomena can become problematic when they result from human actions (Rhymer and Simberloff 1996). Unnatural hybrid zones created by human interventions deserve special attention from conservationists, as they can compromise the genetic integrity and the evolutionary process of the species involved (Rhymer and Simberloff 1996; Allendorf *et al.* 2001).

Hybridization between *C. minutus* and *C. lami* probably originated approximately 70 years ago through human-mediated habitat alteration associated with the introduction of rice cultivation on the western shores of Lake Barros, which dried up a marsh and allowed contact between both species (Gava and Freitas 2003). Considering that hybridization between these species has become quite advanced, with fertile hybrids even mating with each other or with their parental types, it is a difficult process to stop since the existence of hybrid swarms hinders conservation and the recovery of threatened taxa (Allendorf *et al.* 2001).

Ctenomys lami was recently included in the IUCN Red List of Threatened Species as "vulnerable". Studies have shown that the vulnerability of this species is greater than previously assumed, and its extinction could lead to a major loss of genetic diversity since it represents a unique pool of chromosomal variation among *Ctenomys* species. The results obtained by Lopes and Freitas (2012) supported the designation of an ESU and a MU within the geographic area of *C. lami*, which should be considered in developing conservation strategies and implementing protected areas.

Ctenomys minutus is listed in the IUCN Red List of Threatened Species as data deficient. The data provided in this study and Lopes *et al.* (2013) support that, despite having a larger geographic distribution and higher levels of genetic variability than *C. lami, C. minutus* also deserves attention in conservation efforts. In addition to the consequences that hybridization with *C. lami* and *C. flamarioni* (Kubiak *et al.* 2020) may pose to *C. minutus* populations, these species are subject to threats regarding the vulnerability of the coastal plain of southern Brazil, such as global warming and sea level rise, urbanization, coastal armoring, sand mining, construction of jetties, introduction of domestic animals and exotic vegetation (Fernandes *et al.* 2007).

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Appendix I

Data accessibility

Mitochondrial sequence data are deposited at Genbank under the following accession numbers: *Ctenomys minutus* CR (HM236969 to HM237008), and COI (HM237009 to HM237043); *Ctenomys lami* CR (JQ322885 to JQ322898), and COI (JQ322899 to JQ322907); hybrids CR (HM236991), and COI (HM237016); *Ctenomys torquatus* CR (HM443438), and COI (HM443439); *Ctenomys pearsoni* CR (JQ341031), and COI (JQ341042); and, *Ctenomys ibicuiensi* CR (JQ389108), and COI (JQ389074).