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MICROSATELLITE ANALYSIS OF A HYBRID ZONE BETWEEN CHROMOSOMALLY DIVERGENT POPULATIONS OF *CTENOMYS MINUTUS* FROM SOUTHERN BRAZIL (RODENTIA: CTENOMYIDAE)

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This paper describes variation at 6 microsatellite loci in 107 specimens of *Ctenomys minutus* from a chromosomal hybrid zone on the coastal plain of southern Brazil. Of the 56 alleles uncovered in this study, 39.2% are exclusive to alternative cytotypes or the contact populations. Clinal variation is not obvious because there are no microsatellite fixed differences among chromosomally divergent populations, but variation at Hai 2 locus is gradual across the zone. The local populations are highly differentiated and structured. The moderate estimated values of gene flow follow an isolation-by-distance model, which predicts concentration of an allele or a homozygous genotype in patches with generations of individuals with limited dispersal and mating by proximity. Analysis of populations distantly localized from the contact zone probably will provide further insights in the genetic relationship among cytotypes.

Key words: *Ctenomys minutus*, hybrid zone, microsatellites, population differentiation, subterranean rodent

The subterranean rodent *Ctenomys minutus*, the tuco-tuco, inhabits the sandy fields and dunes of coastal plains of the Brazilian states of Santa Catarina and Rio Grande do Sul (de Freitas 1995). Populations of the species possess an impressive karyotypic variation never recorded before for a species of tuco-tuco. At least 5 different karyotypes exist (de Freitas 1997): diploid chromosome number (2n) = 46a, 46b, 48, and 50 (autosomal arm number [AN] = 76) and 2n = 42 (AN = 74). This variability is primarily distributed among populations rather than within them, with the exception of polymorphisms detected in zones of contact between the cytotypes 2n = 46a × 48 and 2n = 42 × 48, and between *C. minutus* and *C. lami* (Gava and de Freitas 2002, 2003).

Chromosomal analysis of the 46a × 48 contact suggests that this zone is the result of secondary contact of populations after divergence in allopatry (Fig. 1). The parental populations have large distributions along the coastal plain (110 km for 2n = 48 and 135 km for 2n = 46a—Gava and de Freitas 2003) and are differentiated by a single Robertsonian rearrangement that shows clinal variation inside the 10-km contact zone.

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Individuals with heteromorphic karyotypes comprise 37.8% of the total number of specimens from polymorphic sites (Gava and de Freitas 2003). The lack of evidence for heterozygote deficiency or excess in polymorphic populations suggests that the rearrangement is not underdominant. The width of the zone is 100 times the estimate of dispersal for the individual organisms—0.1 km per generation per year as calculated for *C. talarum* (Pearson et al. 1968) and *Thomomys* (Howard and Childs 1959)—which suggests that near-neutral diffusion, after recent contact, of parental chromosomes may be occurring. This diffusion is constrained by the fragmented nature of suitable habitats occupied by subterranean rodents (Busch et al. 2000) and the nearly 1-dimensional coastal habitat used by *C. minutus*.

Chromosomal hybrid zones are common among eutherian mammals (Searle 1993). They have been studied with regard to the meiotic consequences of structural heterozygosity for individual fitness (Nachman 1992; Nachman and Searle 1995; Wallace et al. 1992) and the nature of differences associated with the hybridizing taxa (Baker et al. 1989; Saïd et al. 1999). The population structure context under which chromosomal differences are fixed has mostly been studied by using protein electrophoresis (Brünner and Hausser 1996; Hafner et al. 1987). However, some of these studies failed to add data concerning parameters such as gene flow and genetic structuring within these zones, because the markers used (allozymes) normally show low genetic variation (Wójcik and

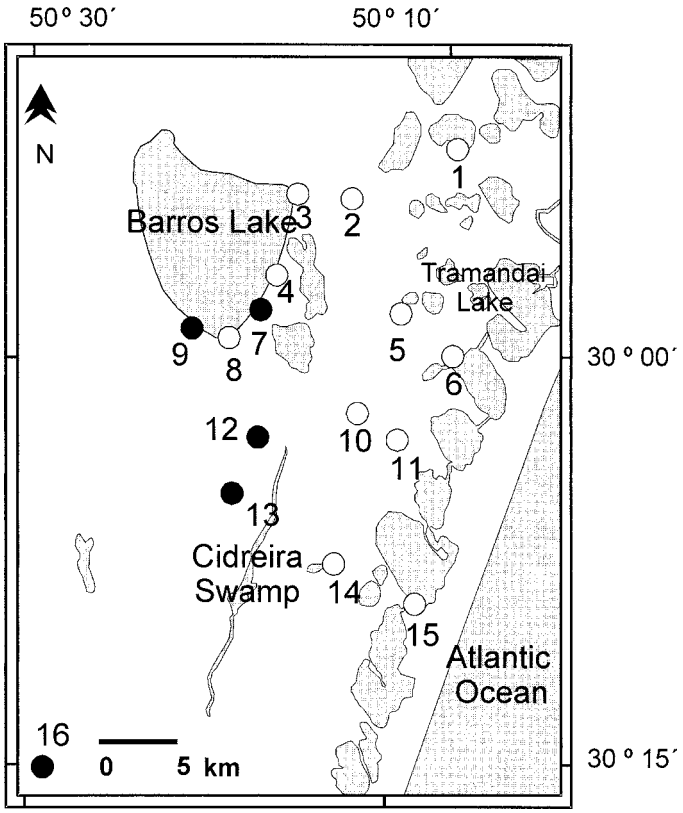

FIG. 1.—Trapping sites of individual *Ctenomys minutus* from populations fixed for 2n = 46 (white circles) or 2n = 48 (black circles) and polymorphic populations (gray circles) from a contact zone on the coastal plain of southern Brazil.

Wójcik 1994). More recently, microsatellite markers have provided data concerning degree of introgression (Goodman et al. 1999), population structuring, gene flow, and isolation inside hybrid zones (Dallas et al. 1998; Wyttenbach et al. 1999a, 1999b).

The development of polymorphic microsatellites for *C. haigi* and *C. sociabilis* (Lacey 2001; Lacey et al. 1999) provides new opportunities for the study of genetic divergence and population aspects of the chromosomal hybrid zone in *C. minutus*. Our study presents microsatellite analysis of the contact zone between the cytotypes 2n = 46a and 48 of *C. minutus* and discusses the maintenance of the chromosomal polymorphism. Microsatellite-based analyses are used to assess a hypothesis of a secondary origin of this hybrid zone, followed by neutral diffusion of chromosomal rearrangements (Gava and de Freitas 2002, 2003).

MATERIALS AND METHODS

A sample of 107 specimens of *C. minutus* (52 females and 55 males) was livetrapped with Oneida Victor number 0 traps (Cumberland's Northwest Supply, Inc., Owatonna, Minnesota) from 16 sites within the chromosomal contact zone and surrounding areas (29°45′N, 30°15′S and 50°30′W, 50°00′E; Fig. 1). Skulls and skins were deposited in the collection of the Departamento de Genética, Universidade Federal do Rio Grande do Sul. Six populations are fixed for 2n = 46, 5 are polymorphic, and 5 are fixed for 2n = 48 (Fig. 1). The study area possesses extensive grasslands and pockets of forest characteristic of the southern border of Brazil (Joly et al. 1999). The natural physiognomy of this area has been extensively altered by fields of rice, *Pinus*, and *Eucalyptus*; most of the extensive grasslands have been used as pastures, with the introduction of foreign grasses. The region has a subtropical climate, and rain is more or less evenly distributed during the year. Areas of intermittent flooding are common and a geological depression is located near the center of the hybrid zone.

Microsatellite analysis.—Six polymorphic microsatellites, isolated from the Argentinean species *C. haigi*, with 2 (Hai 2, Hai 3, Hai 4, Hai 5, and Hai 6) and 3 (Hai 12) base pair (bp) motifs, were employed (Lacey et al. 1999). The loci were amplified in 10-µl reaction volumes containing 25–100 ng of DNA, 0.2 µM of each primer, 0.2 mM of deoxynucleoside triphosphate, 1 × *Taq* buffer (1.5 µM of $MgCl_2$, 10 mM of Tris-HCl, and 50 mM of KCl), and 0.75 units of *Taq* DNA polymerase (Gibco-BRL Life Sciences/Invitrogen, Carlsbad, California). The thermocycling profile included an initial denaturing at 94°C for 5 min, followed by 30–35 cycles of denaturing at 94°C for 30 s, annealing at 54–64°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 1 min. Polymerase chain reaction products were resolved by nondenaturing 6% polyacrylamide gel eletrophoresis and visualized by silver nitrate staining. Differences of 1 bp motif could be resolved, and alleles were scored from heteroduplex patterns and alleles were sized by using a 25-bp pair ladder as reference. Allele sizes were tentatively scored in an initial set of specimens and compared with those obtained by Lacey et al. (1999). The remaining samples were scored by using an allelic ladder constructed from the initial genotypes.

Statistical methods.—We carried out Fisher's exact tests for linkage disequilibrium for each pair of loci across samples as implemented in GENEPOP version 3.3 (Raymond and Rousset 1995). Observed allele frequencies for each population were calculated and compared to values expected under Hardy–Weinberg equilibrium by using Arlequin version 2000 software (Schneider et al. 2000). F_{IS} values for each population and locus were calculated as implemented in Fstat software (Goudet 1995). Genetic diversity was quantified as the total number of alleles at each locus and over all loci. Gene diversities (Nei 1987) and observed heterozygosities were estimated for every locus across populations by using Fstat.

Population structure.—Tests of allelic differentiation for all pairs of populations were carried out by using GENEPOP to verify dependence among local populations, that is, that allelic distribution is identical in paired populations. Pooled populations with 2n = 46, those that were polymorphic, or those with 2n = 48 also were tested for dependence. We performed a locus by locus analysis of molecular variance (AMOVA—Michalakis and Excoffier 1996) to infer population genetic structure through the fixation indexes F_{IS}, F_{ST}, and F_{IT} (computed by following Weir and Cockerham [1984]). An unbiased single-locus and overall ρ_{ST} was computed by using the program RSTCalc (Goodman 1997). Effective number of migrants per generation (N_em) was estimated based on ρ_{ST} because migration rates based on F_{ST} for microsatellite may be overestimated (Slatkin 1995). A 2nd overall estimate of migration was derived from the frequency of private alleles (Barton and Slatkin 1986; Slatkin 1985), as implemented in GENEPOP. The significance of *F*-statistics and ρ_{ST} were tested by a permutation approach that assesses the probability of obtaining the observed level of differentiation by chance (Excoffier et al. 1992).

An AMOVA with a hierarchical layout was designed to test the partitioning of variation in allelic content (distance method: number of different alleles) among and within local populations, regardless of karyotype, and the partitioning of variation among and within metapopulations fixed for either 2n = 46 or 2n = 48, or those that

TABLE 1.—Microsatellite diversity values in *Ctenomys minutus* from a hybrid zone in southern Brazil. Localities are shown in Fig. 1. Asterisks indicate significance levels for the deviation from Hardy–Weinberg equilibrium.[a]

Locality number	Locality	*n*	Hai 2		Hai 3		Hai 4		Hai 5		Hai 6		Hai 12		F_{IS} value
			Ho	*Hs*	*Ho*	*Hs*	*Ho*	*Hs*	*Ho*	*Hs*	*Ho*	*Hs*	*Ho*	*Hs*	
1	South Traíras Lake	4	0.250	0.644	0.500	0.929	0.750	0.929	0.750	0.5236	0.50	0.857	0.500	0.679	0.226
2	Northeast Barros Lake 1	6	Monomorphic		0.500	0.621	0.333**	0.910	0.333	0.454	0.667	0.758	0.667	0.803	0.279
3	Northeast Barros Lake 2	7	0.571	0.44	0.714	0.747	0.286	0.604	0.714	0.67	0.286*	0.692	0.715	0.714	0.220
4	East Barros Lake	6	0.333	0.561	1.000	0.712	0.667	0.682	0.500	0.561	0.333	0.636	0.833	0.924	0.067
5	South Emboaba Lake	9	0.444	0.451	0.889	0.771	0.778	0.765	0.625	0.592	0.889	0.863	0.444	0.627	−0.015
6	Osório Park	4	0.750	0.750	0.250	0.643	0.250	0.786	0.500	0.857	0.500	0.607	0.250	0.536	0.362
7	Southeast Barros Lake	6	0.167	0.167	1.000	0.621	0.333	0.697	Monomorphic		1.000	0.711	0.833	0.833	0.009
8	South Barros Lake	11	0.273	0.507	1.000**	0.567	0.636	0.636	0.182	0.255	0.910	0.710	0.818	0.775	−0.015
9	Southwest Barros Lake	10	0.600	0.505	1.000*	0.610	0.125*	0.600	0.429	0.472	0.625	0.675	0.900	0.821	0.059
10	South Índios Lake	4	1.000	0.571	0.250*	0.964	Monomorphic		0.250	0.250	0.750	0.607	0.750	0.786	−0.029
11	Estância Velha	6	0.500	0.621	0.600**	0.867	0.600	0.800	0.200	0.378	0.500	0.788	0.800	0.644	0.0302
12	East Manoel Nunes Lake	8	0.125*	0.575	0.571	0.527	0.714*	0.912	0.429	0.725	0.571	0.813	0.375	0.683	0.307
13	Pitangueira	5	0.800	0.533	1.000	0.689	0.600	0.511	0.800	0.800	0.800	0.711	Monomorphic		−0.246
14	Fortaleza	9	0.222	0.503	0.889	0.725	0.444***	0.915	0.333*	0.804	0.778	0.830	0.444	0.765	0.322
15	South Fortaleza Lake	6	0.167	0.682	1.000	0.758	0.500*	0.955	0.333	0.561	0.500	0.758	0.167	0.318	0.282
16	Palmares do Sul	6	0.333	0.318	0.167	0.318	0.667*	0.818	0.167	0.454	0.667	0.681	0.500	0.530	0.045
		107	0.408	0.446	0.466	0.578	0.480	0.696	0.409	0.452	0.642	0.694	0.562	0.613	

[a] *Ho* = gene diversity; *Hs* = observed heterozygosity.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

were polymorphic. These calculations were performed by using Arlequin software. The relationship between genetic subdivision and geographical distance also was examined. A regression of multilocus values of F_{ST} ($F_{ST}/(1 - F_{ST})$) and ρ_{ST} ($\rho_{ST}/(1 - \rho_{ST})$) to the natural logarithm of geographic distances larger than 1 km was tested (5,000 permutations) for the null hypothesis of independence between genotype counts and geographic location, as implemented in GENEPOP. The geographical distances among all pairs of sample sites were obtained as straight-line measures taken from 1:250,000 scale maps.

RESULTS

Polymorphism, heterozygosity, and linkage disequilibrium.—The 6 loci analyzed were highly polymorphic: the most variable locus was Hai 6 with 15 alleles (allele size range 124–150 plus 1 allele with 170 bp) and the least variable were Hai 2 (169–177 bp) and Hai 5 (203–211 bp), both with 5 alleles. Hai 4, Hai 3, and Hai 12 presented 12 (158–160 plus 164–180 and 1 allele with 184 bp), 10 (152–164, 168–170, and 174 bp), and 9 alleles (123–147), respectively. The total number of alleles scored for all loci and populations was 56. An overall analysis of linkage disequilibrium suggested no genetic linkage among pairs of loci across all populations (results not shown). Significant ($P < 0.05$) linkage disequilibrium was found between Hai 3 and Hai 4, Hai 3 and Hai 12, and Hai 4 and Hai 12 from a polymorphic population (sample 14; Fig. 1).

Values of heterozygosity deviated from Hardy–Weinberg equilibrium in 6 populations (2 polymorphic) in at least 1 locus, and in 3 populations (1 polymorphic) at 2 loci (Table 1). Biases because of the small local sample sizes are expected, but amalgamation of populations resulted in further deviations. F_{IS} values for all loci in each population suggested heterozygote excess in 1 polymorphic population and 3 pure populations; all the other values were positive (Table 1). Observed heterozygosities for each locus over populations are high (0.408–0.642, average 0.495) and close to the gene diversity values (0.446–0.696, average 0.580; Table 1).

Allelic frequency distribution for all loci was identical in only 5 paired comparisons (population pairs 1 and 5, 4 and 8, 4 and 9, 7 and 9, and 8 and 9). All the other paired populations had significantly different allelic distributions ($P < 0.05$). Pooled populations (metapopulations) with 2n = 46, 2n = 48, or those that were polymorphic also exhibited independence ($P < 0.0001$).

Allele frequencies in some loci showed clinal variation in local populations when plotted along a linear axis (Figs. 2a–c). Frequency of alleles from each metapopulation revealed, in a visual inspection, that 39.2% of the alleles are exclusive (at frequencies > 0.05) to metapopulations with 2n = 46 (7.1%) or 2n = 48 (5.4%). Some alleles are exclusive to one or another parental metapopulation but also are present in the polymorphic metapopulation (21.4%; 2n = 46 and polymorphic [8.9%] or 2n = 48 and polymorphic [12.5%]). Allele 169, for instance, showed a frequency as high as 0.53 in the metapopulation with 2n = 48 and 0.46 in the polymorphic metapopulation, whereas it had a frequency of 0.03 in the metapopulation with 2n = 46. Only 3 of 56 alleles were exclusive to the polymorphic metapopulation.

Genetic structure.—The genetic structure of populations was investigated by a weighted analysis of variance framework (Weir and Cockerham 1984) and by using ρ_{ST} , an unbiased estimator of Slatkin's R_{ST} (Goodman 1997). The significant F_{IS} value ($P < 0.001$) for Hai 4 and the multilocus F_{IS} value ($P < 0.01$) suggested a deficiency of heterozygotes within populations, whereas the negative (although not significantly different from zero) F_{IS} value in locus Hai 3 suggests a heterozygote excess. All the F_{ST} values were significantly different from zero ($P < 0.001$) and varied from 0.132 to 0.352 (average 0.203). Single-locus ρ_{ST} values were similar to F_{ST} values and were

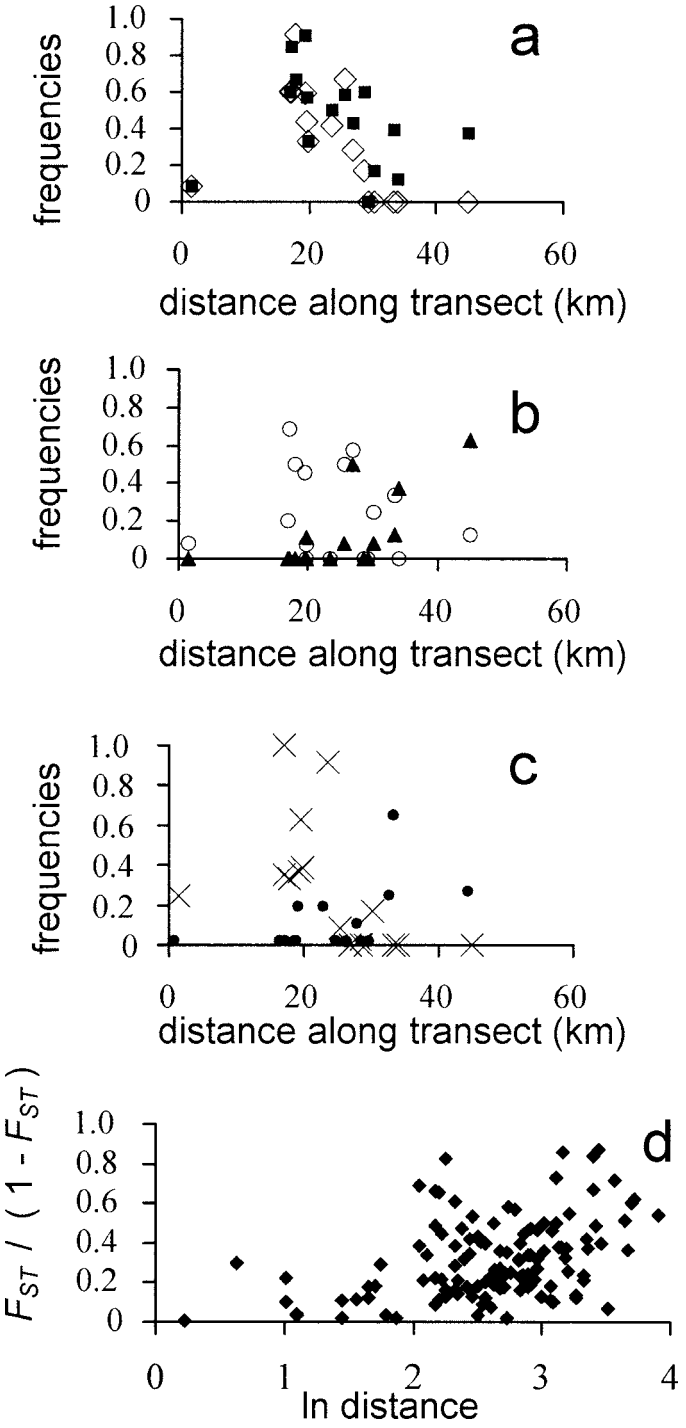

FIG. 2.—Patterns of variation at 6 microsatellite loci of *Ctenomys minutus* from a hybrid zone in southern Brazil: a) clinal variation in the frequency of alleles amplified from Hai 2 (diamond) and Hai 3 (square), b) Hai 4 (circle) and Hai 5 (triangle), and c) Hai 6 (full circle) and Hai 12 (×). d) Plot of F_{ST} ($F_{ST}/(1 - F_{ST})$) values for pairs of samples of *C. minutus* against the natural logarithm of geographic distances between sample sites ($P < 0.01$).

higher in 6 of 7 comparisons including the significant overall value (0.27; Table 2). The migration rate estimated from the overall ρ_{ST} was 0.68 migrants/generation, whereas the estimation of N_em calculated from the private-allele method resulted in a higher value, 1.23 migrants/generation, after correction for sample size.

TABLE 2.—Spatial genetic subdivision in a chromosomal hybrid zone of *Ctenomys minutus* in southern Brazil. Asterisks indicate significance levels for the *F*-statistics and averaged ρ_{ST} value.

Loci	F_{IS}	F_{ST}	F_{IT}	ρ_{ST}	Comparison of F_{ST} and ρ_{ST} values
Hai 2	0.145	0.212**	0.326**	0.312 nt[a]	$F_{ST} < \rho_{ST}$
Hai 3	−0.200	0.144**	−0.0272**	0.081 nt	$F_{ST} > \rho_{ST}$
Hai 4	0.300**	0.176**	0.423**	0.240 nt	$F_{ST} < \rho_{ST}$
Hai 5	0.120	0.352**	0.428**	0.403 nt	$F_{ST} < \rho_{ST}$
Hai 6	0.080	0.132**	0.200**	0.280 nt	$F_{ST} < \rho_{ST}$
Hai 12	0.080	0.223**	0.282**	0.312 nt	$F_{ST} < \rho_{ST}$
All loci	0.0859*	0.203**	0.271**	0.270**	$F_{ST} < \rho_{ST}$

[a] nt = not tested.
* $P < 0.01$; ** $P < 0.00$.

The AMOVA showed that variation in allelic content is distributed predominantly among local populations within metapopulations (14.41%), rather than among individuals within populations (4.61%). Populations were locally differentiated and variation among metapopulations accounted for only 4.42% of the total variation, whereas variation within individuals is 76.56%.

Testing the matrix of geographic distances (natural logarithm of distance) between populations against the genetic distance matrix ($F_{ST}/(1 - F_{ST})$ or $\rho_{ST}/(1 - \rho_{ST})$) through Mantel tests revealed positive and significant associations of these variables ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2d). Spatially proximate populations possess a higher genetic identity, which diminishes with geographic distance, following an isolation-by-distance model (Slatkin 1993).

DISCUSSION

Levels of microsatellite variability in *C. minutus* are high and observed heterozygosity is similar to that obtained in *C. haigi* (Lacey 2001); however, the number of alleles detected per population is lower, averaging 3.5 alleles per population. The results suggest that *C. minutus* is characterized by strong subdivision and differentiation among populations. The significant F_{IS} value indicates a general trend to local inbreeding or the presence of local subdivision; another possibility is the presence of undetected null alleles. Linkage disequilibrium was detected in 1 population from the hybrid zone. The association of loci in hybrid populations may be generated by continual diffusion of parental combinations of genes into the contact zone (Barton and Hewitt 1989).

Under a stepwise mutation model, values of the *F*-statistics (based on the infinite alleles mutation model) are expected to be smaller than values of the *R*-statistics that have been derived specifically for analysis of microsatellites (Slatkin 1995). This theoretical expectation was confirmed by the results, where ρ_{ST} values were higher in 6 of 7 comparisons. Although F_{ST} is an adequate measure when populations are not greatly differentiated, it seems that ρ-statistics or their unbiased

versions, which take into account the sample sizes (Goodman 1997), perform better when genetic distances are large.

Migration rates obtained by the ρ_{ST} value and through the private-alleles method yield values of $N_em = 0.68$ and $N_em = 1.23$, respectively. These are not too different from 1 and thus do not strongly prevent or impose genetic differentiation by drift alone. Actual migration rates may vary locally in response to population structure and habitat patchiness. The pattern of gene flow fits an isolation-by-distance model, which predicts concentration of an allele or a homozygote genotype in patches with generations of individuals with limited dispersal and mating by proximity (Epperson 1995).

Lower migration levels were obtained for *C. rionegrensis*, a species that exhibits polymorphism in pelage color (Wlasiuk et al. 2003). Mills and Allendorf (1996) estimated, by considering genetic and nongenetic factors, that a minimum of 1 and a maximum of 10 migrants per generation are needed to minimize the loss of polymorphism and heterozygosity within local populations while allowing for divergence in allele frequencies among local populations. Thus, it is possible to reconcile the high degree of allelic differentiation among populations of *C. minutus* with the moderate migration rates estimated with the private-alleles method.

The chromosomal cline detected in the contact zone has been considered the result of secondary contact after divergence of populations in allopatry (Gava and de Freitas 2002). Therefore, some degree of microsatellite differentiation among cytotypes was expected. The existence of 39.2% of alleles presented only in one or another parental or polymorphic metapopulation, as well as the existence of concordant clines in allele and chromosome frequencies, supports this assumption, and argues that the zone is secondary in origin.

The hierarchical analysis of the microsatellite variation suggests that 14.41% of variation should be attributed to differences among local population, whereas 4.42% of variation occurs among metapopulations. This distribution of variation does not argue against secondary contact but suggests shared ancestral polymorphisms due to relatively recent isolation. Alternatively, some shared allele states may be homoplasic.

The hypothesis of secondary contact also is supported by evidence of neutrality of the chromosomal rearrangement (Gava and de Freitas 2002). Furthermore, examination of geological data suggests that drainage systems interrupted the coastal plain in the recent past (Corrêa 1996), potentially facilitating allopatric differentiation. Five thousand years ago, the drainage system began to flow into lagoons that were built up during depositional processes in the Holocene (Côrrea 1996), allowing for a secondary contact between the 2 chromosomal forms.

The high variability at the 6 microsatellite markers employed herein makes them the markers of choice for the study of population genetic structuring of *C. minutus* at a small geographic scale. However, the results obtained in the present study must be considered with caution because of the small local sample sizes. On the other hand, the overall results may truly reflect the propensity of tuco-tucos to be distributed into many, relatively small populations (Gastal 1994; see review by Busch et al. [2000]). As pointed out by Steinberg and Patton (2000), the use of exclusive territories at the individual or colony level (Lacey 2000), limited dispersal, and patchy occupation of the habitat (Busch et al. 2000) may lead to strong geographic structure with variation being partitioned among, rather than within, populations.

Resumo

Este estudo descreve a variação em 6 locos microssatélites de 107 espécimes de *Ctenomys minutus* provenientes de uma zona de contato no sul do Brasil. Dos 56 alelos amplificados, 39,2% são exclusivos dos citótipos alternativos e da zona de contato. A variação clinal na freqüência dos alelos não é óbvia pois não existem diferenças fixadas nos microssatélites entre as populações cromossomicamente divergentes, mas a variação no loco Hai 2, por exemplo, é gradual ao longo da zona. As populações locais são divergentes e estruturadas. O valor moderado de fluxo gênico segue um modelo de isolamento por distância que pressupõe a concentração de um alelo ou genótipo homozigoto. A análise de populações geograficamente distantes da zona de contato será útil para revelar as relações entre os citótipos.

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