



A hybrid zone of the genus *Ctenomys*: A case study in southern Brazil

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Abstract

We describe variation at microsatellite loci and the chromosomal polymorphisms of a hybrid population, and hybridizing populations of *Ctenomys minutus* (the minor tuco-tuco) from the coastal plain of Rio Grande do Sul, southern Brazil. Cytogenetic analysis and a survey of six microsatellite loci included 101 specimens of *C. minutus* from the parental populations ($2n/AN = 42/74$ and $48a/76$) and their contact zone. Cytogenetic analysis recorded 26 different karyotypes exhibited by 50 individuals from the hybrid population. Of the 26 karyotypes, only 14% presented a parental-like configuration, and none had the combinations of $2n$ and AN expected for an F_1 hybrid. The remaining karyotypes were alternative hybrid forms, with $2n$ varying from 42 to 46 and AN from 68 to 80. These results suggest chromosomal rearrangements are only of minor significance in the establishment of reproductive barriers for this species.

Keywords: chromosome polymorphism, subterranean rodent, karyotypes, microsatellite.

Introduction

Hybrid zones occur when genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry (Barton and Hewitt, 1989; Harrison, 1993). They have attracted the attention of evolutionary scientists because the extensive genetic variation that they contain helps to understand evolutionary processes that contribute to their origin and maintenance (Harrison, 1993). Hybrid zones have had a major importance in studies of speciation, especially for evolutionary models, patterns of introgression and species integrity (Hewitt, 1988; Harrison, 1993; Gava and Freitas, 2003, 2004; Trigo *et al.*, 2008). The hybridizing populations may be from the same or different species (Hewitt, 1988; Barton and Hewitt, 1989; Harrison, 1993).

In southern Brazil there is an exceptional opportunity to study hybrid zones in the genus *Ctenomys*, as there are intra- and inter-specific hybrid zones described here for *C. minutus* (Gava and Freitas, 2002, 2003; Freygang *et al.*, 2004; Gava and Freitas, 2004; Castilho C, 2004, Master's thesis, Universidade Federal de Rio Grande do Sul, Porto Alegre, RS, Brazil). Hybrid zones can also have different forms of variation, for example, a "hybrid swarm", when a diverse array of recombinant types exist, or in other situa-

tions when only F_1 hybrids (in addition to parental types) are found (Harrison 1993). Both of these situations are described along the distribution range of *C. minutus* in southern Brazil (Gava and Freitas, 2002, 2003; Freygang *et al.*, 2004; Gava and Freitas, 2004; Castilho C, 2004, Master's thesis, Universidade Federal de Rio Grande do Sul, Porto Alegre, RS, Brazil).

Hybrid zones that include chromosomal variation, as is the case in several species of *Ctenomys*, are of major interest because of the continuing controversy regarding the role of chromosomal rearrangements in speciation (Sites and Moritz, 1987; Hewitt, 1988; Barton and Hewitt, 1989). Despite the knowledge that chromosomal changes may cause phenotypic changes, with consequences for the adaptation of organisms to their environment and for changes in interactions between individuals of the same species (summarized by White, 1978), their role as reproductive barriers is still controversial (King, 1987; King, 1993; Rieseberg, 2001; Navarro and Barton, 2003). The idea is that if underdominance of heterozygotes for chromosomal rearrangement is strong, it would be very unlikely that new rearrangements could become established. On the other hand, if underdominance is weak enough for fixation to occur, chromosomal rearrangements would be very poor barriers to gene flow and, thus, unlikely to contribute to speciation (Navarro and Barton, 2003).

Microsatellite markers have been used in studies of hybrid zones, where they have provided insights into introgression, population structure and gene flow (Goodman *et al.*, 1999; Dallas *et al.*, 1998; Gava and Freitas, 2004; Trigo

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et al., 2008) and have also revealed hybrid fertility in backcrosses of hybrids or crosses between hybrids (Harrison, 1993).

The genus *Ctenomys* comprises approximately 60 living species of rodents and shows impressive intra- and inter-specific karyotypic variation (Reig *et al.*, 1990). *C. minutus* inhabits sandy fields and dunes on the southern Brazilian coastal plain (Freitas, 1997; Gava and Freitas, 2002; Freygang *et al.*, 2004). It is polytypic, possessing 11 distinct karyotypes: $2n = 42, 46a, 46b, 47a, 47b, 48a, 48b, 49a, 49b, 50a,$ and $50b$, and arm numbers (AN) varying from 74 to 78 (Freitas, 1997, 2006; Gava and Freitas, 2002, 2003, 2004; Freygang *et al.*, 2004). The chromosomally divergent populations have a parapatric distribution across the coastal plain, and four intraspecific hybrid zones are known in the contact regions of parapatric karyotypes: i) $46a \times 48a$; ii) $46b \times 48b$; iii) $42 \times 48a$; and iv) $50b \times 48b$ (Gava and Freitas, 2002, 2003, 2004; Freygang *et al.*, 2004; Castilho C, 2004, Master's thesis, Universidade Federal de Rio Grande do Sul, Porto Alegre, RS, Brazil). Two of these intraspecific hybrid zones and an interspecific one (between *C. minutus* and *C. lami*) have been recorded and studied with respect to chromosomal dynamics and genetic structures (Gava and Freitas, 2002, 2003).

The survey of *Ctenomys* hybrid zones included contacts between populations with different degrees of chromosomal divergence (Gava and Freitas, 2003). The cytotype $2n = 48a$ has a wide distribution across 110 km of the coastal plain. Its northernmost populations interact in a 10-km-wide zone of contact with cytotype $2n = 46a$. The cytotypes are differentiated by a single Robertsonian rearrangement, and the contact zone has been studied with regard to details of the origin, evolution and adaptive relationships of the cytotypes (Gava and Freitas, 2002), cranial morphology (Marinho and Freitas, 2000), and genetic differentiation and structure (Gava and Freitas, 2004). In the southernmost part of the distribution of *C. minutus*, Gava

and Freitas (2003) identified a hybrid population in a contact zone between two chromosomal races, $2n = 42$ and $2n = 48a$. Cytotype $2n = 48a$ interacts with populations with $2n = 42$. The latter is a cytotype differentiated by a tandem fusion of two acrocentrics, plus a Robertsonian fusion that created a new, readily distinguishable, large chromosome (Freitas, 1997; Gava and Freitas, 2003). The $2n = 42$ karyotype is also differentiated from the $2n = 48$ cytotype by another Robertsonian fusion of 2 acrocentrics. The geographical width of the hybrid zone has not been determined, but the northern ($2n = 48a$) and southern ($2n = 42$) parental-like populations recorded were 14 km apart (Gava and Freitas, 2003).

Although chromosomal rearrangements are considered to have played a major role in the diversification and speciation of the genus *Ctenomys* (Reig *et al.*, 1990; Lessa and Cook, 1998; Wlasiuk *et al.*, 2003; Tomasco and Lessa, 2007), this role is still controversial for *C. minutus*.

Considering that chromosomal rearrangements have been of major interest in studies in the genus *Ctenomys*, and their role being controversial in *C. minutus* hybrids, we used this contact zone between two parental forms with parapatric distribution and different chromosomal rearrangements as an evolutionary model to study the impact of chromosomal rearrangements as reproductive barriers, and their effect on gene flow between populations with different karyotypes in *C. minutus*.

Material and Methods

Samples

We collected tissue and blood samples from 50 individuals on a gradient consisting of four different localities in the contact region between the chromosomal races $2n =$ and $2n = 48a$ (Figure 1; coordinates $30^{\circ}59'S$ and $50^{\circ}49'W$), in order to determine diploid number and microsatellite variation. A complete map with details on the



Figure 1 - Sampling sites of individual *Ctenomys minutus* from populations fixed for $2n = 48a$ (gray squares) or $2n = 42$ (white triangles), both collected by Gava and Freitas (2003), and polymorphic populations $2n = 42-46$ (black circles), collected for this study from a contact zone on the coastal plain of southern Brazil. Abbreviations for sampling locations are shown in Table 1.

distribution of *Ctenomys minutus*, karyotypes, hybrid zones and parental chromosomal rearrangements can be found in Fornel *et al.* (2010).

Blood samples were collected from hybrid animals using a capillary plus a syringe inserted into the side of the ocular globe of sedated animals. After blood and tissue collection the animals were released at the same spot as they were captured. All tissue samples were deposited in the

Laboratório de Citogenética e Evolução, Departamento de Genética, Universidade Federal do Rio Grande do Sul following the sample number of this collection. The permits for this research were obtained from IBAMA (Permit number 14690-1). For information about sample collection in parental populations see Gava and Freitas (2003).

A total of 51 reference specimens with $2n = 48a$ (25 specimens) and $2n = 42$ (26 specimens; see Freitas, 1997

Table 1 - Sample localities of 101 specimens of *Ctenomys minutus*. (LN=locality number, N =sample size, $2n/NA$ =diploid number and autosomal arm numbers, P = frequency of karyotypes) and Reference= sample collection reference.

Locality	LN	N	$2n/NA$	p	Reference
Palmares do Sul	1	6	48/76	1.0	Gava and Freitas 2003
km 35 Capivari-Tavares Road	2	4	48/76	1.0	Gava and Freitas 2003
km 53 Capivari-Tavares Road	2	5	48/76	1.0	Gava and Freitas 2003
km 64 Capivari-Tavares Road	2	3	48/76	1.0	Gava and Freitas 2003
km 96 Capivari-Tavares Road	3	4	48/76	1.0	Gava and Freitas 2003
km 101 Capivari-Tavares Road	3	3	48/76	1.0	Gava and Freitas 2003
Capivari-Tavares Road	4-5	8	44/74	0.25	This study
			45/76	0.25	
			43/70	0.125	
			44/73	0.125	
			45/74	0.125	
			46/76	0.125	
Capivari-Tavares Road	6	7	42/74	0.44	This study
			42/73	0.14	
			42/71	0.14	
			42/69	0.14	
			43/74	0.14	
Capivari-Tavares Road	7	6	45/76	0.17	This study
			42/74	0.17	
			45/80	0.17	
			45/74	0.17	
			46/71	0.17	
			46/76	0.17	
Capivari-Tavares Road	8	29	42/74	0.10	This study
			46/74	0.07	
			43/75	0.07	
			42/72	0.07	
			46/77	0.07	
			44/76	0.07	
			44/75	0.07	
			45/78	0.07	
			44/74	0.07	
			43/73	0.07	
			42/68	0.03	
			46/78	0.03	
			42/71	0.03	
			43/72	0.03	
			44/72	0.03	
			45/76	0.03	
			42/70	0.03	
			43/74	0.03	
km 115 Capivari-Tavares Road	9	3	42/74	1.0	Gava and Freitas 2003
km 120 Capivari-Tavares Road	10	23	42/74	1.0	Gava and Freitas 2003

for more details on parental karyotypes). In this work we used the same set of microsatellite loci as previously employed by Gava and Freitas (2003) (Table 1).

Cytogenetic methods

The blood was seeded in AIM-V culture medium (Gibco) and incubated at 37 °C for 70 h. 50 mL of colchicine (0.025%) was then added and the solution incubated for 2 h at 37 °C. The cultures were treated with hypotonic solution (0.075 M KCl) for 15 min and then fixed with methanol:acetic acid (3:1). Slides were prepared and conventionally stained with Giemsa for determining diploid numbers.

DNA extraction and microsatellite loci analysis

DNA from individuals of mixed ancestry was isolated from the tissue samples using the method described by Medrano *et al.* (1990) with modifications. Six polymorphic microsatellites isolated from *C. haigi*, with dinucleotide (Hai2; Hai3; Hai4; Hai5; and Hai6) and trinucleotide repeats (Hai12) were employed (Lacey *et al.*, 1999). Amplification reactions were performed in volumes of 20 µL (50 ng of genomic DNA, 0.4 µM of each primer, 0.4 mM dNTP, 1x *Taq* Buffer, 1.5 mM MgCl₂ and 0.8 U *Taq* DNA Polymerase). The conditions used were: denaturation at 94 °C for 5 min, 30 cycles with annealing temperatures from 52-60 °C for the different primer sets, ending with a final extension of 5 min at 72 °C. PCR products were separated in 8% denatured polyacrylamide gels and stained with silver nitrate. A 25 bp DNA ladder was used to score the genotypes, and individuals were replicated in different gels to certify the allele lengths.

Statistical analysis

The number of polymorphic loci, number of alleles per locus and number of exclusive alleles per population determined by Arlequin 3.1 (Schneider *et al.*, 2000) were used to estimate genetic variability. Observed heterozygosity (H_o), expected heterozygosity (H_e) and Hardy-Weinberg equilibrium were estimated for all loci and all populations using the Markov Chain Method (Guo and Thompson, 1992) implemented in Genepop 3.4 (Raymond and Rousset, 1995) and Arlequin 2.1. Linkage disequilibrium was tested between loci in all populations using Genepop 3.1. These results were adjusted with a Bonferroni correction (Rice, 1989). Values of F_{IS} were calculated using the method of Weir and Cockerham (1984) as in Genepop. The probability of the presence of null alleles, allele dropout, and scoring errors due to stutter was tested in global data set using MicroChecker 2.2.3 (Van Oosterhout *et al.*, 2004). A locus-by-locus Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) was performed to infer population genetic structure through the fixation index F_{ST} implemented in Arlequin 2.1.

To assess the existence of population structure, we used a Bayesian model-based clustering method implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000), using all 101 samples and six microsatellite loci. We conducted five independent runs for each number of clusters (k) between 1 and 15, using the admixture model, no prior population information, 500,000 burn-in and 10⁶ replications. We also used a principal components analysis (PCA) of the autosomal gene-frequency data to investigate spatial patterns of genetic variation, using PCAGEN.

Results

Chromosomal polymorphism

We recorded variation in 2n from 42 to 46 and AN from 68 to 80, distributed across 26 different karyotypes of 50 individuals from the contact zone (Table 1). None of the karyotypes showed 2n = 48a and AN = 76, typical for the northern sampling sites or the F1 configuration 2n = 45 and AN = 75, expected in crosses between parental types. The 2n = 42 and AN = 74 configuration was found in 14% of the specimens. Homokaryotypes comprised 46% of the sample, whereas 54% of it was composed of heterozygotes for one (28% fusion/fission) or another rearrangement (20% of pericentric inversions) or both rearrangements (6% fusion/fission and pericentric inversions).

Microsatellite polymorphism, heterozygosity and linkage disequilibrium

The six loci analyzed were highly polymorphic. The number of alleles scored was 67 for all loci in the three populations (both parental and hybrid), and 36 (54%) of them were exclusive to one of the populations. Forty-eight alleles were found in the reference 2n = 48a population, of which 18 were not found in the hybrid or 2n = 42 population. Twelve of 43 alleles found for the reference 2n = 42 population were not found in either the 2n = 48a or the hybrid population. Forty-eight alleles were found in the hybrid population, of which six were not found in either of the parental populations (Table 2). The most variable locus was Hai6, with 19 alleles (allele size ranges: 120-154 bp). The least variable loci were Hai2 (169-181 bp) and Hai5 (201-211 bp) with 7 and 6 alleles, respectively. Hai4; Hai3 and Hai12 showed 15 (158-160/164-184/188-190 bp), 11 (150-170/174 bp), and 9 alleles (120-144 bp) respectively. Analysis of linkage disequilibrium, after Bonferroni correction, suggested lack of genetic linkage among pairs of loci across all populations.

The contact population showed values of heterozygosity that deviated from Hardy-Weinberg equilibrium ($p < 0.05$; after Bonferroni correction), with a heterozygote deficit in five of six loci analyzed (except for Hai5). A heterozygote deficit was also detected in most loci of the parental populations, except for Hai5 and Hai12 for population 2n = 48a, and Hai3, Hai4, Hai5, and Hai12 for popula-

Table 2 - Microsatellite diversity in a hybrid zone of *C. minutus*. 2n = number of chromosomes, n = number of individuals, NA = number of alleles found, PA = Number of alleles found in only one population (Private Alleles), H_E shows the expected heterozygosity, and H_o the observed heterozygosity. F_{is} is the inbreeding index calculated for each population.

2n	n	NA	PA	Hai2		Hai3		Hai4		Hai5		Hai6		Hai12		Mean H _E	Mean H _o	F _{is}
				H _o	H _E	H _o	H _E	H _o	H _E	H _o	H _E	H _o	H _E	H _o	H _E			
48a	25	48	18	0.28**	0.818	0.60**	0.786	0.625**	0.90	0.40	0.682	0.68**	0.888	0.72	0.789	0.550833	0.8105	+0.310*
42-46	50	48	6	0.48**	0.661	0.76**	0.851	0.64**	0.856	0.46	0.515	0.62**	0.763	0.68**	0.770	0.606667	0.736	+0.174*
42	26	43	12	0.231**	0.399	0.423	0.513	0.769	0.823	0.538	0.578	0.692**	0.899	0.731	0.855	0.564	0.677833	+0.167

** significant for p < 0.05 after Bonferroni correction.

*significant for p < 0.001.

tion 2n = 42 (Table 2). MicroChecker results gave no evidence of allele dropout, although three loci (Hai 2, Hai 4 and Hai 6) presented a general excess of homozygotes for most allele-size classes, thereby implying the possible presence of null alleles. The F_{IS} value calculated for all populations was positive and highly significant (Table 2). Allelic frequencies of 11 alleles from different loci (175 bp-Hai2; 150, 152, 158 and 160 bp-Hai3; 164 bp-Hai4; 203 and 209 bp-Hai5; 134 and 152 bp-Hai6; 120 bp-Hai12) showed clinal variation, with the contact zone showing intermediate frequencies to those of the parental populations.

F_{ST}

The genetic structure of the populations was investigated by the fixation index F_{ST}. The fixation index supported a greater genetic divergence among the parental population 2n = 42 and 2n = 42-46 (F_{ST} = 0.13877; p < 0.001) than among populations 2n = 48a and 2n = 42-46 (F_{ST} = 0.10040; p < 0.001).

Population structure

Bayesian clustering analyses through the software STRUCTURE indicated that there are eight genetic populations in our sample (Figure 2). We could observe that hybrid individuals share more genetic similarity to parental 2n = 48a, especially in sample spots near to the hybrid zone and that parental populations share very little genetic similarities between them. Nonetheless, we found that in hybrid sample site 8 one individual was genetically similar to the parental 2n = 42 genetic populations.

Principal components analysis (PCA)

Patterns of genetic differentiation were represented by a multivariate PCA plot (Figure 3). The individual PCA scores, plotted on the first two principal components axes, PC-I and PC-II, explained 13.54% and 9.54% respectively of the total genetic diversity. Samples from parental 2n = 42 clustered in the upper left part of the PCA plot, whereas samples from parental 2n = 48a were in the lower part. Hybrid samples clustered in the upper part and lower right part, and grouped with 2n = 42 in the upper left and with 2n = 48a in the lower right quadrant (Figure 2).

Discussion

Among the 50 specimens analyzed from the hybrid zone between the parental karyotypes, we found a high percentage of hybrid forms (86%). Considering the variation in AN from 68 to 80 we infer that these variations cannot be produced only by hybrid and parental-like genotypes or parental and parental crosses. Accordingly, in this zone, hybrids would have been able to breed with other hybrids and there is no evidence of hybrid infertility in this hybrid zone in *C. minutus*. This finding is also the first recording of AN less than 74 and higher than 78 for *C. minutus*.

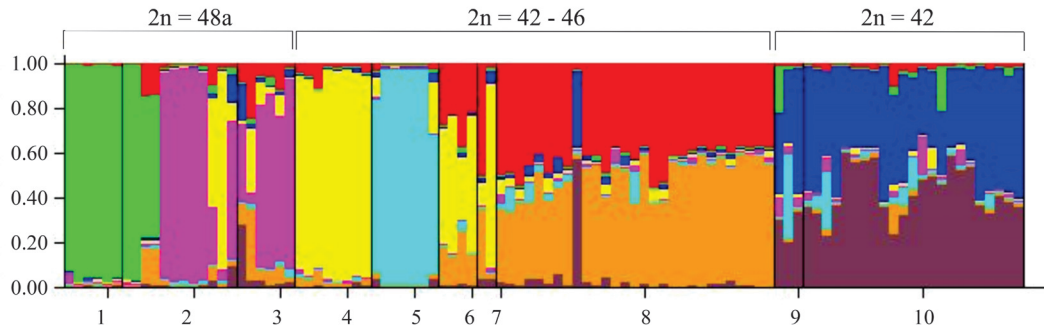


Figure 2 - Structure bars plot showing the eight genetic populations identified by the analysis. The numbers 1-10 correspond to the sample spots in Figure 1 and Table 1.

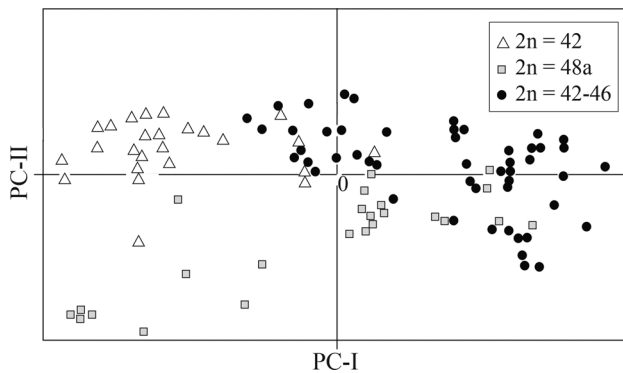


Figure 3 - Scores of individual microsatellite genotypes of *C. minutus* plotted on the first two axes (PC-I, PC-II) of a principal components analysis.

In addition to the chromosomal evidence, the PCA analysis showed overlap between the parental and hybrid populations, as also corroborated by the structure analysis which showed that parental and hybrid populations share genetic similarities. We registered alleles that are shared among populations and 11 alleles on six loci that exhibited a clinal distribution along the zone, indicating gene flow across the hybrid zone and parental individuals. The F_{ST} results indicated that these crosses appear to occur more frequent between hybrids and parental $2n = 48a$ than within parental $2n = 42$. The PCAGEN and Structure results pointed into the same direction.

Gava and Freitas (2003) proposed that the chromosomal variation described for *C. minutus* does not reduce hybrid fitness and acts as a transitory polymorphism in populations. Their conclusion that the role of chromosomal variability for this species as a reproductive barrier and speciation factor is still controversial is also in line with our current data.

The studies of populations and loci showed a significant heterozygote deficit for most loci in all populations, with a highly significant and positive F_{IS} , and significant deviations from Hardy-Weinberg equilibrium. For *C. minutus*, a heterozygote deficit has also been reported in another intraspecific hybrid zone (Gava and Freitas, 2004). In

a study done in Europe on hybridization between wolves and domestic dogs using microsatellites, Randi and Lucchini (2002) found significant deviations from Hardy-Weinberg equilibrium, a heterozygote deficit, and positive values of F_{IS} . Inference was that this occurred through non-random breeding due to inbreeding or geographically structured subpopulations. This may also explain the deficit observed in our study as the species studied live in populations that are structured even on a small geographic scale and dispersed over short distances only. These characteristics may cause population isolation or preferential mating, both resulting in endogamy. Although, these deviations are also to be expected when the analyses are performed in different pooled populations and are explained, most probably, by the Wahlund effect. This would have been the result of an amalgamation of populations with divergence in allelic frequencies, and evidence for this is apparent in the Structure bar plot (Figure 2) where samples sites 1 and 2 are genetically very different from other samples sites. Finally we observed that three of the six loci may have null alleles, this possibly contributing to the deviation from the expected.

Throughout the hybridization zone we found in this study an intermediate distribution of 11 alleles at six loci and a cytotype of $2n = 42/AN = 74$, in addition to the 54% exclusive alleles found in only one or another population. This may indicate a secondary origin for this zone. In an earlier study on a hybrid zone for *C. minutus* using microsatellite loci, Gava and Freitas (2004) found that 39.2% of alleles were present in only one or the other population, as well as the existence of coincident clines in alleles and chromosomes throughout the zone. They concluded that the zone between individuals $2n = 48a$ and $46a$ is secondary in origin.

We can also classify this zone as unimodal (Harrison and Bogdanowicz, 1997) because of the predominance of intermediate hybrid genotypes. Across this hybridization zone no environmental variation was observed between areas occupied by the parent stocks and the hybrids, except for distance and an environment with a patchy distribution

of areas suitable for *Ctenomys* occupation (Gava and Freitas, 2004).

In conclusion, as we observed several intermediate hybrid forms and evidence of gene flow between parental and hybrid individuals, we suggest that chromosomal rearrangements have minor significance on the establishment of reproductive barriers for *Ctenomys minutus*.

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Internet Resources

PCAGEN software, <http://www2.unil.ch/popgen/softwares/pcagen.htm> (July 15, 2003).

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