INTRASPECIFIC VARIATION AND GENETIC DIFFERENTIATION OF THE COLLARED TUCO-TUCO (CTENOMYS TORQUATUS) IN SOUTHERN BRAZIL

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The collared tuco-tuco, Ctenomys torquatus Lichtenstein, 1830 (Ctenomysidae), is a subterranean rodent that occurs in grassland habitats of southern Brazil and northern Uruguay. A population of collared tuco-tucos located in Alegrete Municipality, Rio Grande do Sul State, Brazil, has been proposed as a potential new species because of their remarkable differences in chromosome number and their unique patterns of pelage coloration. The aim of this work was to evaluate the degree of genetic differentiation of this population using mitochondrial DNA (mtDNA) sequences and nuclear microsatellite markers, and to describe spatial patterns of genetic diversity for the other 5 populations of C. torquatus in southern Brazil, focusing on patterns of intra- and interpopulation variation in coat color. The analysis of 1,110 base pairs of the mtDNA cytochrome-\(b\) (Cytb) gene and 9 nuclear microsatellite loci revealed 7 haplotypes (\(n = 65\)) and 48 alleles (\(n = 70\)), respectively. Genetic diversity was moderately low within populations (\(H_E = 0.40–0.56\)), and significantly partitioned among locations (\(R_{ST} = 0.21; P < 0.01\)). Analysis of the microsatellite data suggested that genetic differentiation is consistent with a simple model of isolation by distance (\(r = 0.56, P < 0.05\)), and that the population is in equilibrium between gene flow and local genetic drift. The partially reconstructed phylogeny revealed that the haplotypes derived from the Alegrete population were not reciprocally monophyletic, and that there was a lack of structure for coat color and karyotype variation. Thus, the individuals from the Alegrete population fall within the range of variation for C. torquatus, and should not be considered a new species. We suggest that they be considered a local, specialized lineage that could be treated and managed from a conservation perspective as a Management Unit.

Key words: gene flow, geographic variation, intraspecific phylogeny, microsatellites, mitochondrial DNA

Genetic variation within a species is a fundamental concept in ecological genetics, and has 3 components: genetic diversity, genetic differentiation, and genetic distance (Lowe et al. 2006). A basic issue in the study of intraspecific variation is the analysis of how it is allocated within and between local populations. Variation is the source of evolutionary change, and the description of these patterns is fundamental for identifying evolutionary units in nature (D’Anatro and Lessa 2006). Molecular markers, such as mitochondrial DNA (mtDNA) sequences and nuclear microsatellite loci, enable us to estimate genetic variation among populations and to infer the evolutionary processes responsible for the corresponding intraspecific polymorphism (Hoffman et al. 2006). According to D’Elia et al. (1998), there are at least 3 possibilities that explain differences in population gene frequencies: they have been molded by natural selection, with the selective agents spatially structured; they show the effects of genetic drift; and patterns may result from intermixing of different stocks. These possibilities are not mutually exclusive and thus may operate in combination to produce the observed pattern of geographic variation.

Subterranean rodents, specifically tuco-tucos (Ctenomyidae), are useful organisms for testing hypotheses about processes that lead to evolutionary divergence of lineages (Patton et al. 1996). They generally occur in naturally fragmented environments, forming small genetic units, and in general show extensive karyotypic variation (Patton and Smith 1990; Smith 1998; Wlasiuk et al. 2003). The genus Ctenomys Blainville, 1826, is
distributed throughout the Neotropics, where about 56 species are considered valid (Reig et al. 1990). They form a karyotypically diverse group, with chromosomal diploid numbers ranging from 10 to 70 (Cook et al. 1990; Gallardo 1991; Ortells 1995; Reig et al. 1992). This remarkable karyotypic variation in a single clade has attracted speculation regarding the mechanisms that promoted high chromosomal diversification (Nevo 1999) and tuco-tucos have been proposed as a prime example of chromosomal speciation (Reig and Kiblisky 1969).

Diversification of *Ctenomys* may have been facilitated by the isolation of small demes that characterize population structure in most species (Reig et al. 1990). An assessment of population structure and processes, especially the estimation of critical population parameters such as gene flow, could be of key importance in understanding the evolutionary dynamics of the group (Wlasiuk et al. 2003). According to Freitas (2006), 5 species of *Ctenomys* occur in southern Brazil: *C. flamarioni*, *C. lami*, *C. minutus*, *C. torquatus*, and an undetermined *Ctenomys*; the later 2 are the focus of this study. The undetermined *Ctenomys* (hereafter, *Ctenomys* A) was hypothesized as a separate taxon based on its unique karyotype (2n = 40) and coat-color pattern (Gonçalves et al. 2003). *C. torquatus*, also known as the collared tuco-tuco, is dorsally brown and ventrally white and has a pale collar (Fig. 1A). Like most species of the genus, collared tuco-tucos are highly territorial and build exclusive and large burrow systems in the sandy soils of grasslands. They are distributed from the northern half of Uruguay through the southern half of Rio Grande do Sul State, Brazil (Freitas 1995), an area also known as Campos Sulinos (Fonseca et al. 1996). The chromosomal diploid number of the Uruguayan populations has been characterized as 2n = 44 (Kiblisky et al. 1997), and that of the Brazilian populations of *C. torquatus* as 2n = 46 (Freitas and Lessa 1984).

Individuals collected at a locality in western Rio Grande do Sul State (Alegrete Municipality) that were assigned to *Ctenomys* A display a brown coat color containing large white areas on the posterior body parts (Fig. 1B). This locality is geographically nested within the distribution range of *C. torquatus*. Because the ranges of 2 different species of *Ctenomys* do not generally overlap, an assessment of the taxonomic status of the population with 2n = 40 is warranted. The integrative approach of mitochondrial and nuclear markers allows inferring whether individuals of Alegrete form an independent lineage (new species) or constitute a population of *C. torquatus*. Thus, to determine the degree of genetic differentiation from other populations of *C. torquatus*, we conducted a phylogenetic analysis of this group using nucleotide sequences of the mitochondrial cytochrome-*b* gene (Cytb). We also conducted analysis of microsatellite allele frequencies to estimate the relationship between genetic and geographic distance and to investigate the patterns of genetic structure of these populations.

**Materials and Methods**

*Study area and sampling.*—Tuco-tucos were sampled from 6 locations in Rio Grande do Sul State (Fig. 2), including populations of the undetermined *Ctenomys* from Alegrete and *C. torquatus* with karyotype and coat color previously reported (Gonçalves 2007). Polytypic populations from 4 localities (Alegrete [ALE], Catimbau [CAT], Caverá [CAV], and Rosário [ROS]) were sampled from 2005 to 2007 to describe intra- and interpopulation phenotypic and karyotypic variation. The habitat is similar for all sample sites and is mainly characterized by sandy soil grasslands. Although restricted to areas unfavorable to agriculture, this habitat type can be continuously distributed throughout the corresponding tuco-tuco geographic ranges. We obtained tissue samples (toe snips, preserved in 95% ethanol for subsequent DNA extraction and genetic analyses) from a total of 85 individuals of *Ctenomys*, which were live-trapped with Oneida Victor No. 0 snap traps (Oneida Victor, Inc., Ltd., Euclid, Ohio) with a rubber cover to avoid injuring animals. After collection of tissue samples, animals were immediately released back within the same burrow system where they had been originally captured, but some individuals (X = 5 individuals/locality) were killed to obtain karyotypes and museum specimen vouchers. Chromosome preparations were made from bone marrow according to Ford and Hamerton (1956). Voucher specimens were prepared and deposited in the mammalian collection of Laboratório de Citogenética e Evolução, Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. All portions of the study involving capture, handling, and euthanasia

![Fig. 1.—A schematic illustration of coat-color pattern of *Ctenomys torquatus*. A) Uniformly brown, B) brown with white areas on the posterior body parts, and C) gray.](https://academic.oup.com/jmammal/article-abstract/90/4/1020/847453/10412f0b847a3)
DNA extraction, polymerase chain reaction amplification, and sequencing.—Genomic DNA was extracted following a modified protocol described by Medrano et al. (1990). Two overlapping fragments spanning the entire Cytb gene were amplified by polymerase chain reaction using the following primers: MVZ05 (5'-CGAAGCTTATATGAAAAACCAT-CGTT-3'—Smith and Patton 1993), TUC006 (5'-GTGAAATG-GAATTTCGTCGTA-3), TUC007 (5'-TACAGCAATAGTAA-TAAT-3), and TUC014 (5'-CCAATGTTATTTTATAC-3—Wlasik et al. 2003). Polymerase chain reaction amplifications were carried out in a 20-μl reaction volume, and amplification conditions were the same as those described by Lessa and Cook (1998). Polymerase chain reaction products were examined on a 1% agarose gel stained with ethidium bromide and purified with Shrimp Alkaline Phosphatase and Exonuclease I (Invitrogen, Carlsbad, California). Purified polymerase chain reaction products were sequenced using the Big Dye chemistry (ABI) and subsequently analyzed in an ABI PRISM 3100 automated sequencer (Applied Biosystems Inc., Foster City, California). All the sequences reported herein were deposited in GenBank under accession numbers EF372280–EF372291. Sequence electropherograms were visually inspected and edited using CHROMAS 1.45 (Technelysium Pty. Ltd., Tewantin, Australia) software, and aligned using the CLUSTALW algorithm implemented in MEGA 3.0 (Kumar et al. 2004). Alignments were checked and edited by eye.

We surveyed 9 microsatellite loci isolated from the Argentinean species C. haigi (Hai 2, Hai 3, Hai 4, Hai 5, Hai 6, Hai 9, and Hai 10—Lacey 2001) and C. sociabilis (Soc 1 and Soc 2—Lacey et al. 1999) that also proved to be polymorphic in C. torquatus. The polymerase chain reaction amplifications were carried out in a reaction volume of 20 μl, containing 25–100 ng of DNA, 0.2 mM of each primer, 0.2 mM of deoxynucleoside triphosphates, 2× polymerase chain reaction buffer, 1.6 mM of MgCl2, and 0.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, California) following procedures of Wlasik et al. (2003). To confirm amplification, 5 μl of each product were mixed with 5 μl of stop solution (bromophenol blue, xylene cyanol, and deionized formamide) and electrophoresed on a 6% nondenaturing polyacrylamide gel for 120 min. Then, 1 μl of polymerase chain reaction product was added to 9 μl of formamide/ROX solution (Applied Biosystems), electrophoresed, and detected on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Fragment size analysis was performed using GeneScan 2.1 (Applied Biosystems).

Data analysis.—Genetic diversity within each population was estimated using the number of alleles per locus (N), the number of alleles per locus per population (Nt), the mean number of alleles per locus (allelic richness [A]), the percentage of polymorphic loci (%P), observed heterozygosity (H0), and the heterozygosity expected from Hardy–Weinberg equilibrium (Hp—Nei 1978), using FSTAT 2.9.1 (Goudet 1995) and GENEPOP 3.4 (Raymond and Rousset 1995). Linkage disequilibrium and deviations from Hardy–Weinberg equilibrium (Guo and Thompson 1992) were tested using ARLEQUIN 3.0 (Excoffier et al. 2005). Sequential Bonferroni corrections were applied to correct for multiple comparisons (Rice 1989), with α = 0.05 to adjust the statistical significance levels. Standard genetic diversity indices such as haplotype diversity (Hd—Nei 1978) and mean number of pairwise differences (π—Tajima 1989) were estimated for mtDNA sequences using ARLEQUIN 3.0.

Genetic differentiation between populations was characterized by exact tests (Raymond and Rousset 1995) and by estimating pairwise FST (Weir and Cockerham 1984) and RST
(Slatkin 1995) values using GENEPOP 3.4 and ARLEQUIN 3.0. The levels of significance for multiple tests were adjusted by the sequential Bonferroni method (Rice 1989). We investigated the relationship between genetic and geographical distances using Mantel tests (Mantel 1967), based on mtDNA sequences and microsatellite loci. The significance of isolation by distance values was assessed by the Mantel procedure (1,000 randomizations) using ARLEQUIN 3.0.

To investigate the existence of hierarchical levels of population structure, an analysis of molecular variance (AMOVA) was performed using allele frequencies with ARLEQUIN 3.0. Populations with similar karyotypes were grouped as follows: ALE, 2n = 40; CAT, 2n = 42; and CAV and ROS, 2n = 44.

We looked for a genetic signature of past demographic events (e.g., population expansion or decline) based on mismatch distribution analyses (Rogers and Harpending 1992). To detect the genetic signatures of bottlenecks we used a simple graphical method based on changes in allele frequencies 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). Bottlenecks 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.

To determine the appropriate model of nucleotide sequence evolution we used the Akaike information criterion (AIC) as implemented in MODELTEST 3.6 (Posada and Crandall 1998). The phylogenetic relationships among *Cytb* haplotypes of *Ctenomys* A and *C. torquatus* were inferred using PAUP* 4.0b10 (Swofford 1998) adopting 2 criteria: maximum parsimony, with heuristic searches using 10 replicates of random taxon addition; and maximum likelihood, incorporating the JC69 model (Jukes and Cantor 1969). In each case, 100 bootstrap replicates were used to evaluate nodal support. A separate phylogenetic analysis using Bayesian inference was performed with MRBAYES 3.0b4 (Huelsenbeck and Ronquist 2001), incorporating the JC69 model (Jukes and Cantor 1969).

RESULTS

Phenotypic and karyotypic variation.—We found high levels of variation in pelage coloration both within and among the populations sampled. Three primary color patterns were recognized: uniform brown (Fig. 1A); brown with white areas on the posterior body parts (Fig. 1B); and uniform gray, described for the 1st time for the species (Fig. 1C). In all populations, individuals uniformly brown were at a greater frequency (Fig. 3). Populations ALE and CAT also included brown individuals with white spots and those of CAV and ROS included uniformly gray individuals (Fig. 3). We identified a new diploid number (2n = 42) in 11 individuals karyotyped, found only in the Catimbau locality (Fig. 2).

Genetic diversity.—In the alignment of the 1,110-base-pair sequence of the mtDNA *Cytb* gene, we found a total of 11 variable sites: 3 singletons and 8 parsimony-informative sites. In total, we identified 7 unique haplotypes (Table 1). All the observed polymorphisms were single base-pair mutations and all 11 were transitions. No gaps (insertions–deletions) were found among sequences. Haplotype diversity values range from 0 (CAV, ROS, and Fronteira [FRO]) to 0.6 (Quaraí [QUA]), with an average value of 0.679 (Table 2). All 9 microsatellite loci that were surveyed were polymorphic (Table 2). The total number of alleles per locus ranged from 2 (Hai 5) to 12 (Hai 6), with an average of 4.2 alleles. Allelic diversity (A) varied from 2.6 (CAT) to 5.2 (CAV). Values of expected heterozygosity ($H_e$; averaged across loci) ranged from 0.40 (ROS) to 0.54 (CAV; Table 2). Exact tests of genotypic linkage disequilibrium (either global or for subpopulations) yielded no significant values ($P > 0.05$), suggesting that loci are independent. Significant positive departures from Hardy–Weinberg equilibrium were found in 3
Genetic differentiation and isolation by distance.—Estimates of genetic structure among all localities were moderate for microsatellite and sequence data ($R_{ST} = 0.21$ and $F_{ST} = 0.03$, respectively). In addition, pairwise estimates of gene flow based on sequence data showed values ranging from 0.006 between ALE and ROS to 0.06 between ALE and CAV. The corresponding values from allele frequency ranged from 0.14 and 0.37, respectively (Table 3). The AMOVA showed that variation was evenly distributed among hierarchical levels; within populations (31.75%), among populations within groups (34.31%), and among groups (33.94%). All these components of variance ($F_{CT} = 0.321$, $F_{SC} = 0.291$, $F_{ST} = 0.344$) were statistically significant. Mantel tests did not show a significant correlation between geographic distance, based on sequence data, and estimates of gene flow, thus failing to support a pattern of isolation by distance ($r = 0.40$, $P > 0.05$). However, when the test was performed using microsatellite data, it resulted in a marginally significant positive correlation ($r = 0.56$, $P = 0.04$).

Population decline or expansion.—Neutrality tests using Tajima’s $D$ and Fu’s $F_S$ were not significant for any population. The majority of localities showed positive values for these statistics (Table 2). The $F_S$-test was positive and not significant ($F_S = 2.38$, $P > 0.10$). The overall $D$-test was both negative and nonsignificant ($D = -0.86$, $P > 0.10$). The mismatch distribution analysis was not consistent with a recent demographic expansion, depicted by a multimodal distribution and a low average number of nucleotide differences ($k = 2.23$). Genetic signatures of bottlenecks estimated using the distribution of microsatellite allele frequencies showed clearly normal L-shaped distributions for ALE, CAT, and ROS, with the largest proportion of alleles at low frequencies (0.1–0.20; Fig. 4). In contrast, the distribution of allele frequencies from CAV showed the largest proportion of alleles in the class of intermediate frequencies (0.2–0.80), with a consequent decrease in the class of low-frequency alleles. The shape of the distribution of allele frequencies in the latter populations was clearly shifted toward a lower number of more-abundant alleles, as expected in bottlenecked populations (Fig. 4).

Principal component analysis.—Patterns of genetic differentiation were represented by a multivariate PCA plot (Fig. 5). The PCA individual scores, plotted into the first 2 principal component axes, PC-I and PC-II, explained 19.43% and 10.53%, respectively, of the total genetic diversity. Samples from ALE and ROS grouped separately from the other populations on the upper part of the PCA plot. Samples from CAT and CAV clustered into the lower part, although with a lot of mixture in the central area of the PCA plot.

Bayesian admixture analyses.—The Bayesian clustering procedure detected the maximum likelihood for a model of 4 genetically distinct populations (log-likelihood [lnL] = $-1,950.0$). The proportion of individual genotypes ($q$) belonging to each of the 4 inferred clusters showed that samples from ALE, CAT, CAV, and ROS were assigned to distinct clusters, because $q > 0.7$. Individual assignment did not vary among 5 independent simulations. Results showed that all samples were correctly assigned to a single population with probability $q_i \geq 0.7$ (Table 4).

Phylogenetic relationships.—Parsimony, maximum-likelihood, and Bayesian analyses resulted in topologically equivalent trees that differed only at nodes with bootstrap values below 50%. Maximum-likelihood distances were conservatively chosen because their bootstrap values were

![Fig. 3.—Coat-color frequency in 4 populations of Ctenomys torquatus from Rio Grande do Sul State, Brazil. The bars represent the percentage of all hair pigmentation detected in each population sampled. Abbreviations for populations are given in Fig. 2.](image)

<table>
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<th>0219</th>
<th>0247</th>
<th>0312</th>
<th>0337</th>
<th>0338</th>
<th>0461</th>
<th>0546</th>
<th>0611</th>
<th>0951</th>
<th>1091</th>
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<td>9</td>
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</tr>
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<td>CAT</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>10</td>
<td>--</td>
</tr>
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<td>--</td>
<td>--</td>
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<td>--</td>
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<td>--</td>
<td>1</td>
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<tr>
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<td>--</td>
<td>7</td>
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<td>3</td>
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**Table 1.**—Polymorphic sites (except ambiguous ones) at the mitochondrial cytochrome- $b$ gene for populations of Ctenomys torquatus ($n = 65$). Nucleotides are numbered from 1 to 1110. Each haplotype distribution is represented per locality and by the total number of individuals per haplotype. Abbreviations for populations are given in Fig. 2; periods represent an absence of the correspondent haplotype.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>ALE</th>
<th>CAT</th>
<th>CAV</th>
<th>ROS</th>
<th>QUA</th>
<th>FRO</th>
<th>Total</th>
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<tr>
<td>H1 (EF372280)</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
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<tr>
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<td>T</td>
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<td>T</td>
<td>C</td>
<td>G</td>
<td>T</td>
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<tr>
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<td>C</td>
<td>G</td>
<td>T</td>
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<td>.</td>
</tr>
<tr>
<td>H6 (EF372290)</td>
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<td>G</td>
<td>T</td>
<td>C</td>
<td>G</td>
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the lowest (Fig. 6). Maximum-likelihood analysis produced a score of \(-\ln L = 2,497\). One monophyletic group of mtDNA lineages of \(C. \) torquatus was strongly supported, including samples of \(C. \) torquatus from Uruguay. Also, there was no support for geographic subdivision within the clade in relation to karyotype or coat-color pattern. The haplotype networks produced with NETWORK and TCS were nearly identical, and only the former is shown here (Fig. 7). It indicates haplotypes with high frequencies in central and peripheral positions, connected by short branches (1 mutational step for all except 1 case). A conspicuous starlike pattern was not observed, which is congruent with other results, such as the neutrality tests and mismatch distribution, indicating no signal for a recent sudden expansion. Three haplotypes (H1, H3, and H4) were shared between \(C. \) torquatus and Ctenomys A.

**DISCUSSION**

**Genetic diversity.**—Levels of microsatellite variability in \(C. \) torquatus were intermediate compared with other species of the genus Ctenomys. In \(C. \) torquatus we detected 48 alleles for the 9 polymorphic loci analyzed, with a mean of 4.2 per locus. Mean numbers of alleles per locus reported for other species are 5.1 for \(C. \) lami (Fernández-Stolz et al. 2007), 2.5 for \(C. \) lami (El Jundi and Freitas 2004), 8.3 for \(C. \) rionegrensis (Wlasiuk et al. 2003), 7.5 for \(C. \) haigi (Lacey 2001), and 9.3 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 (Lacey 2001). A population analysis in \(C. \) talarnum, an Argentinian species that shows a remarkable variation in chromosome number along its northern costal distribution (Massarini et al. 2002) but that does not present a marked variation in pelage coloration, reported 2–9 alleles per locus (Cutrera et al. 2006). Although polymorphic for all loci, \(C. \) CAT showed the lowest allelic richness. In addition, this population is invariant in the surveyed mtDNA sequences, possibly due to genetic drift in the small population.

The mtDNA diversity reflects a low genetic variability, similar to that based on microsatellite data. We found only 7 mtDNA haplotypes, a small number of nucleotide polymorphisms, and only 3 polymorphic populations. The number of \(Cytb \) haplotypes identified in \(C. \) torquatus can be considered moderately low when compared to that of \(C. \) rionegrensis (15) reported by Wlasiuk et al. (2003).

This low haplotype diversity found in all populations surveyed suggests a possible reduction in the ancestral population size. Considering that the populations show moderate levels of genetic structure, it is possible that genetic

**TABLE 2.**—Summary for genetic variability of populations of Ctenomys torquatus based upon 9 microsatellite loci and mitochondrial DNA sequences. \(n, \) sample size; \(A, \) average number of alleles per locus; \(\%P, \) percentage of polymorphic loci; \(H_o, \) observed (average) heterozygosity; \(H_e, \) expected (average) heterozygosity; \(n_h, \) number of haplotypes; \(H_d, \) haplotype diversity; and \(\pi, \) nucleotide diversity. Abbreviations for locations (populations) are given in Fig. 2.

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<td>All populations</td>
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* Significant heterozygote deficiency after Bonferroni corrections, \(P < 0.01\).

**TABLE 3.**—Pairwise estimates of gene flow based on \(FST_{\text{up}}\) (upper half matrix) and \(RST_{\text{up}}\) (lower half matrix) for cytochrome-\(b\) gene sequences and microsatellites, respectively. An asterisk (*) indicates statistical significance; \(P < 0.01\). Abbreviations for locations (populations) are given in Fig. 2.

<table>
<thead>
<tr>
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<th>CAV</th>
<th>ROS</th>
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<tr>
<td>CAT</td>
<td>0.003</td>
<td>0.003</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>CAV</td>
<td>0.144*</td>
<td>0.156*</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>0.377*</td>
<td>0.355*</td>
<td>0.239*</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 4.**—Allele frequency variation in 4 populations of Ctenomys torquatus from Rio Grande do Sul State, Brazil. The line represents the frequency of all alleles detected in each allele class, calculated for each population sampled. Abbreviations for populations are given in Fig. 2.
drift acting in small subpopulations randomly eliminated some rare haplotype variants. However, the patterns of diversification at a smaller geographical scale remain to be investigated.

**Genetic differentiation and geographic patterns of variation.**—Gene flow may either constrain evolution by preventing adaptation to local conditions or promote it by spreading new genes throughout a species’ range (Slatkin 1987). Continuously distributed populations may be genetically structured either if gene flow is restricted or if they are under diversifying local selection (Congdon et al. 2000; Hudson et al. 1992). Some species are restricted to a nearly linear spatial distribution because of a strict association with particular habitat conditions (Mora et al. 2006). This is the case for some populations of *C. torquatus* in the westernmost part of Rio Grande do Sul State, which are restricted to sandy soils unfavorable for agriculture. Our results indicated incongruent patterns of genetic structure estimated from mtDNA data ($F_{ST}$) and microsatellites ($R_{ST}$). According to the mtDNA data, ALE and ROS represent a single population, despite the karyotypic variation among individuals. These findings could be interpreted as evidence that the mtDNA haplotypes are too old to uncover the recent population biological processes in the area under study. In contrast, pairwise comparison of $R_{ST}$ indicates moderately high genetic differentiation among populations (Hartl and Clark 1997; Hudson et al. 1992; Wright 1978). The resolving power of microsatellites, which have a higher mutation rate than mtDNA, seems to be high enough to detect genetic variability even in populations with low haplotypic variation, as is exemplified by CAT and ROS.

The AMOVA showed that the genetic variation is evenly distributed among groups, within populations between groups, and within populations. The highly significant fixation indices indicated that genetic structuring is as strong between populations within a karyotypic group as it is between all populations.

The significant correlation between pairwise estimates of gene flow and geographic distance, based on microsatellites, clearly indicated a pattern of isolation by distance, reflecting limited dispersal and reduced gene flow. A species with limited dispersion will show such a pattern, when migration is stable for enough time to reach the equilibrium (Slatkin 1993). The pattern of isolation by distance observed here suggests stability and equilibrium between drift and migration.

Additional evidence for such equilibrium and population size stability came from the negative results for both Fu and Tajima neutrality tests. The ALE population has a negative value for Tajima’s $D$, indicating the absence of neutrality, but

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**Table 4.**—Results of a Bayesian assignment test. The number and percentage of individuals assigned to their original population with a probability threshold of 70% are indicated. Abbreviations for locations (populations) are given in Fig. 2.

<table>
<thead>
<tr>
<th>Population</th>
<th>ALE</th>
<th>CAT</th>
<th>CAV</th>
<th>ROS</th>
<th>Unassigned</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALE</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>92.0</td>
</tr>
<tr>
<td>CAT</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CAV</td>
<td>0</td>
<td>3</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>76.4</td>
</tr>
<tr>
<td>ROS</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>15</td>
<td>0</td>
<td>88.2</td>
</tr>
</tbody>
</table>

---

**Fig. 5.**—Scores of microsatellite genotypes for individuals of *Ctenomys torquatus* plotted on the first 2 axes (PC-I and PC-II) of a principal component analysis. Abbreviations for populations are given in Fig. 2.

**Fig. 6.**—Maximum-likelihood (ML) tree of *Ctenomys* mitochondrial DNA haplotypes identified in this study, based on 1,110 base pairs of the cytochrome-*$b$ gene. Values above branches indicate support for each node based on parsimony/ML/Bayesian inference. Asterisks indicate bootstrap support below 50%. Triangles on the right indicate diploid number: black, 2n = 44; white, 2n = 40; and gray, 2n = 42. The schematic illustration represents the coat-color variation (uniformly brown, brown with white areas on the posterior body parts, and gray). Closed squares represent *Ctenomys torquatus* and open squares the undetermined *Ctenomys* A.
these values are not statistically significant. Such a result may be due to the large number of divergent haplotypes among populations, which may represent recent local admixture.

**Historical demographic events that could have affected genetic variation of C. torquatus.**—Although the internal phylogeny topology composed of short, inconsistent branches suggests a recent population expansion (Avise 1994), it can also result from high levels of gene flow. The moderate number of nucleotide differences between haplotype pairs and a multimodal curve indicate that population expansion is unlikely to have occurred. According to Rogers and Harpending (1992) and Tajima (1989), bottlenecks may generate waves in the distribution of pairwise nucleotide differences. However, contrary to expansion, a population contraction leads to maintenance of allele diversity through time. In a bottleneck model individuals differ in the average number of nucleotide changes when taken randomly from a given population. Such an effect leads to multi-peak nucleotide distributions, as well as large pairwise differences between them (Harpending et al. 1998; Rogers and Harpending 1992). When estimated by median-joining, the haplotype topology also did not support the hypothesis of population expansion, because it did not fit into a typical starlike model (Harpending et al. 1998; Slatkin and Hudson 1991).

The tests for recent population bottlenecks did not show statistical significance for most of the populations investigated. Only the population from Cavera showed a shifted pattern of allele frequency distribution. However, these findings suggest that current genetic structure of tuco-tuco populations may not be explained by the most recent population decline, but is likely to be the consequence of more ancient historical processes.

**Implications for the taxonomic status of the 2n = 40 population.**—The phylogenetic analysis of the mtDNA haplotypes clearly shows that the 2n = 40 population does not belong to a reciprocally monophyletic clade and, therefore, our results do not support the recognition of this population as a distinct species from *C. torquatus*. Neither form is reciprocally monophyletic, an essential condition to characterize an evolutionarily independent unit. The haplotypes of the 2n = 40 population are nested in the large *C. torquatus* clade, and both the maximum-likelihood tree and the network topology resulted in polyphyletic populations supporting this result. *C. torquatus* is sister to a clade composed of *C. pearsoni* and *C. perrensi*, consistent with expectations based on the literature (Castillo et al. 2005; D’Elía et al. 1999; Slamovits et al. 2001). Consequently, the differences in chromosome numbers are the result of intraspecific variation in karyotype.

The classic ideas of chromosomal speciation are based on the principle that different rearrangements represent distinct biological units, and new chromosome arrangements act as a postzygotic reproductive barrier (Lande 1984). Our results do not support the basic requirement of classical chromosomal speciation because we did not observe the different rearrangements of *C. torquatus* fixed in small, local populations. In other words, we conclude that there is no genetic support to recognize different species among these collared tuco-tucos, because only *C. torquatus* constitutes a genetic unit, albeit phenotypically variable. Estimates of gene flow and geographic isolation show different patterns of genetic structure using mtDNA sequences and microsatellite loci. This suggests that the populations of *Ctenomys* in question may be
differentiating, but not yet isolated enough to reach reciprocal monophyly. Tomasco and Lessa (2007) suggest that to understand the mechanisms by which rearrangements are fixed, it is necessary 1st to elucidate the genetic basis of the chromosomal modifications. Thus, it is essential to understand the influence of such karyotypic rearrangements in speciation.

Implications for chromosomal speciation theory and pelage color evolution in Ctenomys.—The expected time for a given lineage to reach reciprocal monophyly is 4 times longer for chromosomal rearrangements (autosomal) than for mtDNA (Lande 1984). Thus, it is expected that derived karyomorphs are more likely to be monophyletic at mtDNA markers than for autosomal rearrangements. However, the karyomorphs of *C. torquatus* do satisfy this prediction. In addition, a careful review of the chromosomal literature suggests that *C. torquatus* is not unique in this regard within *Ctenomys*. Other surveyed species of tuco-tucos characterized by high karyotypic variation, such as *C. boliviensis*, *C. perrensi*, and *C. pearsoni*, also are not monophyletic at mtDNA markers (Giménez et al. 2002; Mascheretti et al. 2000; Tomasco and Lessa 2007). Future studies are needed to understand why the chromosomal variation is geographically arranged whereas mitochondrial variation is not.

The PCA based on microsatellite data showed that populations at Rosário and Alegrete are genetically distinct, whereas those from Catimbau and Caverá appeared partly admixed or not clearly differentiated from each other. These results suggest that when populations are moderately structured, multivariate analyses are not appropriate for assessing interindividual relationships (Randi et al. 2001). Probabilistic approaches, on the other hand, are more sensitive and can be used to infer either the number of distinct populations in a sample or to assign individuals to populations (Pritchard et al. 2000). For example, Bayesian analysis identified genetic structure; 4 population units were identified based upon 4 sample sites. The high percentage of individuals assigned to their correct populations (based on sampling locale) is consistent with high levels of genetic differentiation.

The PCA plots show a low degree of concordance with the Bayesian assignments. The individuals with the highest percentage of correct assignment (CAT and CAV) were grouped in similar clusters in the PCA plotting. All CAT samples were correctly assigned to the site from which they were sampled. However, it is possible that the low number of individuals sampled from this locality may have influenced our analysis. The ability to detect substructuring using Bayesian inference increases with the number of loci and individuals investigated (Pritchard et al. 2000). Thus, the results obtained by these tests should be interpreted with some caution, because some of the sample sizes were small, and collected over areas of different sizes.

Our results indicate that individuals from Catimbau may represent 2, not mutually exclusive, possibilities: hybrids between the parental forms 2n = 40 and 2n = 44 (or a new chromosomal rearrangement), or a neutral and transient polymorphism. To test this hypothesis, a detailed study of chromosomal homology among different karyotypes of *C. torquatus* is needed.

Despite predictions based on geographic distance, estimates of gene flow from microsatellites reveal higher values between ALE and CAT than between ALE and CAV. We suggest that individuals with 2n = 40 are more closely related to those with 2n = 44, rather than those with 2n = 42. This hypothesis also is supported by the PCA results, where samples with 2n = 40 grouped with samples with 2n = 44.

Color polymorphism has been defined as the presence of 2 or more distinct, genetically determined color morphs within a single interbreeding population, the rarest of which is too frequent to be solely the result of recurrent mutation (Huxley 1955). Examples of such intraspecific variation in rodents include *Peromyscus crinitus*, *Peromyscus maniculatus*, *Peromyscus polionotus* (Hoekstra 2006), *Thomomys b. baccatus*, *Chaetodipus intermedius* (Hoekstra and Nachman 2005), *C. rionegegensis* (Altuna et al. 1985), and *C. pearsoni* (G. D’Elía, Universidad de Concepción, pers. comm.).

The populations of *C. torquatus* reported here show variation in pelage color. Certain pelage types are fixed in some populations, whereas other populations are polymorphic. The intraspecific phylogeny showed that coat color is polyphyletic. Reig et al. (1965) suggested that pelage color is not a reliable character for delimiting tuco-tuco species, because most species show the same agouti pelage, whereas others are highly polymorphic. Hoekstra et al. (2004) analyzed the phenotype–genotype association in *C. intermedius* using sequences of “neutral” genes (not related to pelage color; *COIII* and *ND3*) and candidate genes for pigmentation (*MCIR*). Their reconstructed phylogenies from mtDNA sequences showed polyphyletic phenotypes. However, the *MCIR* allele phylogeny resulted in a strong correlation between phenotype and genotype. Thus, polyphyletic phenotypes are not unexpected in *C. torquatus*, because we used a neutral gene for phylogenetic reconstruction. To resolve questions about the evolution and maintenance of coat-color variation in *C. torquatus* it is necessary to reconstruct the phylogeny using genes responsible for the chromatic polymorphism, such as *MCIR* and *Agouti ASIP* (see Eizirik et al. 2003).

Managements units.—By analyzing the genetic structure of *C. torquatus*, we found that the ALE population shows significant divergence in allele frequencies at both nuclear and mitochondrial loci, regardless of the phylogenetic distinctiveness of those alleles. The PCA, corroborated by the Bayesian assignment test, identified genetic structure in our samples. According to Moritz (1994), populations that do not show reciprocal monophyly for mtDNA alleles, but have diverged in allele frequency, are important from a conservation perspective. Populations that have low levels of gene flow can be considered functionally independent. The geographic distribution of *C. torquatus* in Rio Grande do Sul State coincides closely with the distribution of coal reserves and reforestation areas. Although collared tuco-tucos are not included in the National List of Species of the Brazilian Endangered Fauna, they have been used as sentinel organisms for coal-mining hazards. Silva et al. (2000)
demonstrated, through comet and micronucleus assays, that coal and its derivatives induced DNA damage and chromosomal lesions in tuco-tuco cells. Considering the economic interest of reforestation in the westernmost part of Rio Grande do Sul, we suggest that the population of tuco-tucos from Alegrete (with a different karyotype and coat color) should be considered as a local, specialized lineage, to be treated and managed from a conservation perspective as a Management Unit.

RESUMO

O tuco-tuco-de-colar, *Ctenomys torquatus* Lichtenstein, 1830 (Ctenomyidae), é um roedor subterrâneo que ocorre nos campos arenosos do sul do Brasil e norte do Uruguai. Devido à marcada variação no número cromossômico e na coloração da pelagem foi sugerido que os indivíduos de uma dada população, localizada no Município de Alegrete, Rio Grande do Sul, Brasil, compreenderiam uma nova espécie. O principal objetivo deste estudo é avaliar a identidade genética e a singularidade desta população utilizando sequências de DNA mitocondrial e microsatélites. Além disso, visa descrever os padrões espaciais de diversidade genética para outras cinco populações de *C. torquatus* no Sul do Brasil, focando no padrão de variação da coloração da pelagem intra e interpopulacional. Em uma análise de 1,110 pares de base encontramos sete haplótipos (*n* = 65), e 48 alelos de microsatélites (*n* = 70). A diversidade génica foi moderadamente baixa dentro de populações (*H*<sub>E</sub> = 0.40–0.56), e significativamente fracionada entre locais (*R*<sub>ST</sub> = 0.21, *P* < 0.01). Os dados de microsatélites indicaram que a diferenciação genética é consistente com um modelo simples de isolamento pela distância (*r* = 0.56, *P* < 0.05), sugerindo equilíbrio entre fluxo gênico e deriva genética local. A filogenia parcialmente reconstruída demonstrou populações polifiléticas, e ausência de estruturação para coloração da pelagem e variação cariotípica. Considerou-se esta linhagem uma variação intra-específica de *C. torquatus*. As populações apresentaram estruturação (*R*<sub>ST</sub> = 0.21), mas não demonstraram estar isolada a tempo suficiente para atingir a monofília. A despeito da filogenia, os resultados apontam que a população de Alegrete pode ser considerada uma linhagem evolutiva diferenciada. Dessa forma, é sugerido tratar a população correspondente como uma Unidade de Manejo.

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