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ANCIENT DNA EVIDENCE OF PROLONGED POPULATION PERSISTENCE WITH NEGLIGIBLE GENETIC DIVERSITY IN AN ENDEMIC TUCO-TUCO (*CTENOMYS SOCIABILIS*)

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We traced a population of *Ctenomys sociabilis,* a highly endemic South American tuco-tuco, through 1,000 years to assess its response to climatic change and recent human disturbance. Samples were obtained from a late-Holocene raptor roost in Parque Nacional Nahuel Huapi, Argentina, which produced a diverse and abundant rodent fauna, with >10 genera extending from the present to 950 ± 50 years ago (CAMS-45936). The site (Estancia Nahuel Huapi locality 1) was located near the center of the present geographic range of *C. sociabilis,* which occurs throughout 8 of 9 stratigraphic levels in the site. To examine genetic structure through time, we extracted ancient DNA from 16 teeth at those levels and from 1 modern tooth at the surface for a total of 17 specimens. Cytochrome-*b* sequences from ancient and modern specimens were compared with a modern tuco-tuco sequence from the extant local population. Our results show that of those 17 specimens, all but 1 had identical sequences. Further, these sequences were identical to a representative of the modern population. Thus, that population has remained genetically identical for at least 1,000 years in the face of climatic change, human disturbance, and proximity of other tuco-tuco species (*C. haigi*, *C. maulinus*) with adjacent geographic distributions. Our findings indicate that a population bottleneck contributing to low genetic diversity of *C. sociabilis* occurred before 1,000 years ago and that late-Holocene climatic change occurred without a corresponding impact on the genetic diversity of this species.

Key words: aDNA, climatic change, endemism, Holocene, Patagonia, population bottleneck

Loss of genetic diversity caused by extreme population decline is hypothesized to heighten extinction risk because of limits to genetic flexibility in the face of threats such as pathogens, disease, or rapid environmental change (Amos 1999; O'Brien 1994). However, few empirical studies assess effects of extreme population declines on genetic diversity over time, and fewer still test theoretical expectations of persistence through time with data from populations with low genetic diversity (cf. Hadly et al. 1998). Typical studies infer a population bottleneck from limited present genetic diversity, such as is the case with the cheetah (*Acinonyx jubatus*—O'Brien 1994), although few studies actually track genetic diversity through time. An exceptional example of such a study is provided by assessments of demography and genetic diversity of *Ctenomys maulinus brunneus* before and after a volcanic eruption (Gallardo et al. 1995). The eruption caused a 91% reduction in population size in 3 years and led to severe reductions in average heterozygosity. However, the long-term effects of such diversity loss are not well documented with empirical data.

Similarly, there are few studies documenting effects of limited genetic variation on survival of populations over the short term (Bouzat et al. 1998; Keller 1998; Sac-

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cheri et al. 1998). As a result, it can be difficult to provide a context for interpreting the significance of limited genetic variation in species over the longer term. One strategy is to compare closely related species in similar environments (Amos 1999). These studies can characterize consequences of past demographic events to present genetic diversity and expectations for persistence of populations in the future, as has been accomplished for elephant seals (*Mirounga angustirostrus*—Hoelzel et al. 1993; Slade et al. 1998). Another example of such a study is provided by tuco-tucos from Patagonia: the social tuco-tuco (*C. sociabilis*) and the Patagonian tuco-tuco (*C. haigi*—Lacey 2001). These species are found in close proximity in similar habitats but have significant differences in average heterozygosity, microsatellite allelic sizes and frequencies, and demographic structures. Evidence suggests that repeated population bottleneck events significantly reduced genetic variation in *C. sociabilis*, whereas *C. haigi* did not experience such events. Although these modern genetic data suggest relatively recent bottleneck events, there is no evidence suggesting how long the population has existed with low levels of genetic variation.

The advent of technological advances in acquiring DNA from historic samples now allows us to investigate genetic diversity in populations through long periods of time, such as hundreds to thousands of years. We studied tuco-tucos (*Ctenomys*) excavated from a late-Holocene paleontological site in northern Patagonia at 41°S latitude. The genus *Ctenomys* includes 56 species of small to large (100–750 g) subterranean rodents that occupy much of South America south of 12°S latitude (Lacey et al. 2000; Reig et al. 1990). Tuco-tucos are typical subterranean rodents with allopatric geographic ranges presumably related to intense competition in the subterranean niche (Cameron 2000). Each species also tends to be associated with particular habitat characteristics, many of which are determined by climate (Pearson 1984). Thus, this genus offers the chance to investigate ecological and evolutionary responses to environmental change within a comparative perspective.

Three allopatric species of tuco-tucos occur at 41°S latitude: *C. sociabilis*, *C. haigi*, and *C. maulinus* (Christie 1984; Pearson and Christie 1985). Although the exact nature and extent of the geographic ranges of these species are not well known, in general, they are separated by differences in elevation that determine local vegetation types, including grassland characteristics such as grass density, soil color, and moisture (Pearson and Christie 1985). *C. maulinus*, a relatively large tuco-tuco, has been trapped in open, rich grassland habitat from 1,000 to 1,600 m above mean sea level in the Andes (Christie 1984; Pearson 1984; Pearson and Christie 1985), divided from the other species by forests of *Nothofagus*. *C. sociabilis* is found in and around mesic meadows about 1,000 m in elevation (Pearson and Christie 1985), whereas *C. haigi* is widely distributed and is found to the east in drier grassland scrub at 400–1,000 m (Christie 1984; Pearson and Christie 1985). Populations of different subterranean species are known to replace each other geographically as the environment fluctuates (Gallardo and Köhler 1992; Nevo 1979).

Ctenomys sociabilis is an endemic tuco-tuco confined to the eastern edge of Nahuel Huapi Parque Nacional in southern Argentina (Fig. 1). The geographic range of *C. sociabilis* is very small, about 700 km^2. The species is bounded to the east by the Rio Limay, to the north by the Rio Traful, and to the south by Lago Nahuel Huapi (Pearson and Christie 1985). To the east and north, *C. sociabilis* is replaced in as small a distance as 500 m by *C. haigi* (Lacey 2001). Unlike other tuco-tucos, *C. sociabilis* has life-history traits indicative of social behavior, such as adult burrow sharing, female philopatry, and possibly alloparental care (Lacey 2000). Concordant with its sociality, *C. sociabilis* exhibits much lower genetic diversity and higher genetic relat-

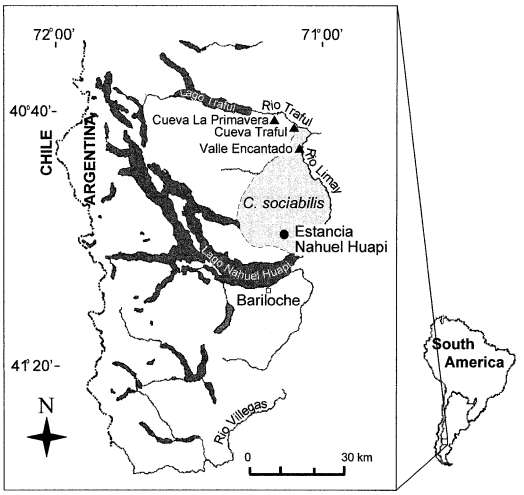

FIG. 1.—Species range of *Ctenomys sociabilis*, indicated by light gray shading, and the location of the late-Holocene raptor roost, Estancia Nahuel Huapi (ENH) locality 1. Locations of 2 rodent middens, Cueva La Primavera and Valle Encantado, where indications of late-Holocene warming were found, are shown.

edness among individuals within populations relative to neighboring *C. haigi*, which shows higher genetic diversity within populations and larger dispersal distances (Lacey 2001). Dispersal distances in *C. sociabilis* are very low (Lacey 2001), leading to speculation about the ability of the species to persist with perturbations that lead to habitat loss.

Investigation of the genetics of populations through longer time periods than that studied by Gallardo and colleagues (Gallardo and Köhler 1994; Gallardo et al. 1995) is rare (cf. Barnes et al. 2002; Hadly et al. 1998; Leonard et al. 2000). The long-term record presented here provides an empirical test of how species respond to perturbations (cf. Barnosky et al. 2003; Hadly 1996, 1997), thus aiding in assessing evolutionary models and predictions about how species might respond to future events. Such perturbations may have been particularly important in Patagonia, which has witnessed very recent historic environmental perturbations that contributed to vegetation shifts, including fluctuations in fire frequency (Kitzberger et al. 1997; Markgraf and Anderson 1994), grassland degradation due to overgrazing (Aagesen 2000; Veblen et al. 1989), volcanic eruptions (Paskoff 1977), and climatic change (Bianchi et al. 1999; Heusser 1995; Mancini 1998; Margraf 1983; Stine 1994).

Our purpose is to determine whether such historic perturbation events influenced the range of tuco-tucos in the area and whether there is genetic evidence from *C. sociabilis* demonstrating a bottleneck effect from population size reduction that is coincident with environmental change. Data for this study include mitochondrial cytochrome-*b* sequences from modern *C. sociabilis* specimens and from fossils excavated from a late-Holocene raptor roost (Estancia Nahuel Huapi [ENH] locality 1) within the range of *C. sociabilis*.

MATERIALS AND METHODS

Study area.—Estancia Nahuel Huapi locality 1 is at 41°S latitude located 60 km E of the Andean crest, 20 km NE of San Carlos de Bariloche, and 10 km W of the Rio Limay at an elevation of 1,000 m (UTM 19G 5461.75 S, 1565.5 E) in Argentina. ENH locality is 4 km SW of the type locality of *C. sociabilis* (Pearson and Christie 1985). The site is under an overhang of rhyolitic volcanic bedrock with a north-trending aspect. The cliff face has several overhangs and larger caves, some of which were occupied by barn owls (*Tyto alba*) during excavation in November 1996. The excavation site was chosen because it appeared to have some depth and was well protected from rain, but it was small enough to rule out past human occupation. The cliff and overhang around the site were coated with whitewash from raptors, and the surface of the excavation was littered with raptor pellets and bones. A single 1- by 1-m pit was excavated in natural stratigraphic units not exceeding 10 cm in depth. Unit numbers from 1 to 9 were assigned from top to bottom. Maximum depth was 81 cm below datum, where the deposits rested on volcanic bedrock. All excavation was done with a trowel, and all material except a single bulk sample per level was dry screened through a series of 3 stacked screens of decreas-

ing mesh size (4, 8, and 16 squares per inch [2.5 cm]). Large organic fragments, including bone, charcoal, and plant material were collected in the field and bagged separately. Preservation at all levels was generally excellent. The complete fauna from this site will be described elsewhere (E. A. Hadly et al., in litt.)

Genetic assessment.—We extracted DNA from 17 teeth from 8 of 9 stratigraphic levels from ENH to identify tuco-tuco species and assess genetic variation through time. We chose to sequence cytochrome *b* because it is optimal for detecting variation from the genus level to the population level in tuco-tucos (D'Elia et al. 1996; Lessa and Cook 1998) and other mammals (Bradley and Baker 2001). Because the mitochondrial genome is present in extremely high copy numbers per cell (generally 1,000 times more abundant than nuclear DNA), it is ideal for ancient DNA (aDNA) studies of populations and species (Hadly et al. 1998; Wayne et al. 1999).

The following primers were used to obtain 3 adjacent ~150 base pair (bp) stretches of cytochrome *b*: forward primers (5′–3′), CTENOMYS1 CTT TGG CTC TTT ATT AGG AGT ATG; CTENOMYS3 GGA GCA TCA ATA TTC TTC ATT TT; CTENOMYS5 ATA GGY TAC GTA CTA CCA TGA GG; reverse primers (5′–3′), CTENOMYS2 GGA GCA TCA ATA TTC TTC ATT TTC; CTENOMYS4 TAC CAT GAG GAC AAA TAT CYT TAT G; CTENOMYS6 ATT AAC CCG ATT CTT TGC TTT CC. Primer position in *Mus musculus* (NC-001569) of 1st base of primer: CTENOMYS1 14234, CTENOMYS2 14394, CTENOMYS3 14394, CTENOMYS4 14536, CTENOMYS5 14523, CTENOMYS6 14660. Primers were designed based on publicly available *Ctenomys* and *Octodon* cytochrome-*b* sequences (GenBank, version 125.0) and a *C. sociabilis* sequence from E. Lacey (University of California, Berkeley). Because aDNA generally is degraded into short fragments (Pääbo 1989), we chose highly informative short regions to characterize fossil and recent populations.

Several precautions were taken to minimize contamination risk (Cooper and Poinar 2000; Handt et al. 1994), and several controls were included at each step to monitor contamination: primers were designed specifically only for octodontid species; the lab had no prior or concurrent history of working with modern *Ctenomys* DNA; the aDNA extraction and polymerase chain reaction (PCR) setup were performed in a facility physically and temporally isolated from the PCR, cloning, and sequencing facility, and no researchers were permitted entry to this facility after recent contact (within 24 h) with PCR products; bleach (10–30%) and UV-light sterilization procedures were performed regularly to destroy nontarget DNA in the aDNA area (Ou et al. 1991; Sarkar and Somer 1993); and the aDNA extraction facility had key features for ensuring cleanliness of samples: positive airflow, which minimized extraneous airborne particulate matter from entering (air entering the room is either from the outside or through a HEPA filter but never from the other molecular facility), and the room was equipped with UV lamps that were used to irradiate all surfaces and materials before extraction. The extraction process was performed in a Class II laminar flow hood, preventing particulate matter from contaminating air in the clean room.

Before DNA-containing materials were brought into the aDNA extraction room, all surfaces were cleaned with Alconox detergent (VWR Scientific Product, West Chester, Pennsylvania) and a bleach solution, and the room (including tubes and digest reagents excluding proteinase K) was UV irradiated overnight. Teeth were chosen, photographed, weighed, placed in a vial, and brought into the sterile aDNA room. Experiments were performed in small batches of ≤5 samples at a time. Samples (*Ctenomys* molariform teeth) ranged from 3.7 to 4.7 mg (±0.2 *SD*). Two types of extractions were performed; one extraction generally followed Höss and Pääbo (1993), with solutions modified from Boom et al. (1990). In this protocol, the extraction buffer consisted of 10 M GuSCN; 0.1 M Tris-HCl, pH 6.4; 0.02 M ethylenediaminetetraacetic acid (EDTA), pH 8.0; and 2.3% Triton X-100. Because we obtained limited success (3 of 17 teeth yielded amplification products) with this extraction, a 2nd extraction type was undertaken on remaining teeth. This extraction was modified from the protocol described by Yang et al. (1998); the extraction buffer was based on digestion of proteins with proteinase K (0.5 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate; and 100 μg/ml proteinase K). After 24 h, the digest was purified with Qiaquick PCR purification kit silica columns (Qiagen Inc., Valencia, California—Yang et al. 1998).

Both protocols used silica-based DNA separation and liquid nitrogen to assist in pulverizing the tooth inside sterile foil. The powdered material was then digested at 55°C for 24 h on a shaker. To insure that materials and reagents were not compromised, we included 1 negative control throughout the extraction (controlling for air in the tooth-collection room and the powdering phase), negative controls at each addition of buffer in the 1st protocol, and a negative control during the extraction phase in the 2nd protocol, which allowed us to monitor any contamination in digest and Qiaquick PCR purification kit reagents.

The PCR reactions were prepared in the laminar flow hood of the same aDNA room, but no thermocycling was ever performed in this room. The PCR tubes and solutions (except *Taq* DNA polymerase, primers, and template) were irradiated with UV. Final reaction concentrations were *AmpliTaq* Gold polymerase (0.035 units/ml), *Taq* Gold buffer (1×), $MgCl_2$ (5 mM), deoxynucleoside triphosphates (1 mM each), sterile water, and bovine serum albumin (BSA, 1.3 mg/ml—Fisher Scientific, Fair Lawn, New Jersey) in a total volume of 25 or 50 μl. For the latter stages of this project, spermidine (1 mM—Wan and Wilkins 1993) was substituted for BSA, resulting in greater amplification success. A negative control (reagents without template) was included in every PCR reaction to detect contamination in the reagents. Assembled PCR reactions were then brought into the PCR room and run on a Gene Amp PCR system 9700 (ABI, Applied Biosystems Division of Perkin–Elmer, Inc., Foster City, California) thermal cycler under the following conditions: 95°C for 10 min and 45 cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s. Aliquots of PCR products were visualized on 2–4% agarose gels stained with ethidium bromide and documented with a digital camera system (Kodak Digital Science, Electrophoresis Documentation and Analysis System 120), using the Kodak 1D version 3.0 Scientific Imaging Systems software (Eastman Kodak Company, New Haven, Connecticut). Successfully amplified fragments were cleaned with the Qiagen Qiaquick PCR purification kit protocol. The resulting product served as template in the sequencing thermocycling reaction together with 2.5 μM of the forward or reverse primer and BigDye Terminator Ready Reaction mix (version 1.0, ABI) with standard parameters (25 cycles: 96°C for 10 s, 50°C for 5 s, 60°C for 4 min). After cycle sequencing, excess dye terminators, primers, buffer, and salts were removed using G-50 Sephadex and Centri-Sep columns (Princeton Separations—Sambrook et al. 1989), dried on a speedvac, resuspended in deionized formamide, denatured, and cooled on ice. Samples were run on an ABI PRISM 310 Genetic Analyzer. To corroborate results and resolve ambiguous sites on single strands, fragments were sequenced in both directions. Sequences were deposited in GenBank under accession numbers AY211323–AY211325.

All sequences were aligned using Sequencher software (version 3.1.1, Gene Codes, Corporation, Ann Arbor, Michigan), checked by eye for potential polymorphic sites, and translated to check for possible stop codons. Alignment was straightforward because the functional cytochrome *b* does not contain insertions or deletions. The aligned sequences were imported into PAUP* 4.0 beta version software (Swofford 2002) and analyzed with other representative *Ctenomys* sequences from GenBank using an exhaustive maximum parsimony search and several neighbor-joining models.

Although we did not expect to pick up nuclear cytochrome-*b* copies, we nonetheless investigated all sequences for the presence of stop codons, deletions, and insertions. This was following Mirol et al. (2000), who described the abundance of nuclear inserts in tuco-tucos and showed evidence for at least 3 independent migration events to the nucleus in the history of Ctenomyidae (although *C. sociabilis* was not included in that study). Specifically, 1 clade of nuclear inserts described showed close sequence similarity to the functional mitochondrial copy and perhaps could be mistaken for the functional sequence and subsequently yield an overestimation of genetic variation. To assign an age to ENH deposits, we submitted 1 piece of charcoal from level 5 and 1 piece of charcoal from level 9 to Stafford Research Laboratories, Inc. (Boulder, Colorado) for accelerator mass spectrometry radiocarbon dating.

RESULTS

The radiocarbon age calibration of the deposits at ENH indicate a maximum age of 1,000 years. Two charcoal samples from level 5 at 40 cm below datum and level 9

at the bottom of the deposit 75 cm below datum gave the ages of 360 ± 40 years ago (CAMS-45935) and 950 ± 50 years ago (CAMS-45936), respectively. There was no indication that there was a hiatus in sedimentation at the site; accumulation was therefore thought to be relatively constant throughout the deposit. The sedimentation rate between levels 9 and 5 was 0.08 cm/year, and that between levels 5 and 1 was 0.06 cm/year. Thus, the deposit extended from about 1,000 years ago to the present and was still actively accumulating specimens during excavation.

Bones at the site were deposited mainly by raptors, probably barn owls. Other raptors (*Polyborus plancus*, *P. chimango*, and *Falco*) were seen frequently during excavation, and individual owls were observed several meters away from the site in other cliff recesses. All recesses along the cliff face contained large piles of raptor pellets. As expected in owl-pellet collections, preservation of the cranial material was excellent with some intact skulls, complete with teeth (Dodson and Wexlar 1979). Specimens were of the size that raptors could carry, and there were few large mammalian remains. Raptor pellets were present at the surface before excavation, and a few accumulated at night during the excavation. Raptors generally forage within 4 km of their roost (Johnsgard 1990), so raptor deposits are mostly of local origin. Ongoing research in 2 late-Holocene caves in Yellowstone National Park has demonstrated that even over several thousands of years, raptors and carnivore accumulations are local, generally within ~7 km (Hadly 1999; Porder et al. 2003). Although raptors are able to forage more widely, the preliminary analyses of the ENH fauna suggest that it is of local origin.

Deposits of ENH contained thousands of mammalian bones. The complete description of the fauna is under way and is beyond the scope of this article. Preliminary analysis of the ENH fauna includes several mammalian, avian, and herpetological taxa. Small mammal genera identified to date include *Loxodontomys*, *Phyllotis*, *Chelemys*, *Reithrodon*, *Euneomys*, *Octodon*, *Eligmodontia*, *Ctenomys*, *Cavia*, and *Irenomys*.

Cranial material from *Ctenomys* is distinctive and relatively large compared with other rodents in the ENH deposit. *Ctenomys* can be easily distinguished from *Octodon* and *Aconaemys*, the other large hystricognath rodents, by the simplicity of its molar morphology (Pearson 1995). From the faunal sorting accomplished to date, 98 cranial and dental specimens were attributed to *Ctenomys* (Table 1). Specimens included representatives from the surface and from levels 1 through 8. Only level 9, which overall had sparse faunal material and rests on bedrock, contained no *Ctenomys* fossils. A selection of 17 molariform teeth from the 98 specimens was chosen for DNA analyses (Table 1). The specimens were selected from stratigraphic levels that included additional *Ctenomys* material. Isolated teeth subjected to genetic analyses were not always identifiable to a specific molar or premolar in the jaw and were photographed before analysis; thus, a minimal amount of morphologic information was lost.

All 17 specimens yielded cytochrome-*b* sequences of between 136 and 449 bp (inclusive of the primers); our longest informative fragments are 368 bp (exclusive of the primers; Table 2). Although the probability of obtaining nuclear sequences from ancient specimens was quite low, 1 of the 17 specimens showed a sequence divergence of 33.1% from *C. sociabilis* in 2 extractions. This sequence, identical in the 2 extractions, also differed from those of the other tuco-tuco species in the area (*C. maulinus* and *C. haigi*) and from that of *C. tuconax* by ≥37%. No obvious sequence similarity to other potential mammalian contaminants was observed. Translation of the 136-bp fragment showed 2 stop codons for a vertebrate reading frame, indicating that the sequence was nonfunctional. However, translation within a fungal reading frame indicated no stop codons. Sequence from

TABLE 1.—Numbers of *Ctenomys* specimens by stratigraphic level from Estancia Nahuel Huapi locality 1, Argentina. Total number of teeth is given before the parentheses; values in parentheses are the numbers of left premolar 4 and molars 1–3 followed by right premolar 4 and molars 1–3. DNA specimens used in this study were teeth taken from each stratigraphic level containing *Ctenomys* fossils and include the following specimens: surface—EH-96-6; level 1—EH-96-7; level 2—EH-96-24, level 3—EH-96-39, EH-96-36; level 4—EH-96-42; level 5—EH-96-50, EH-96-50; level 6—EH-96-34, EH-96-36, EH-96-36, EH-96-36; level 7—EH-96-64, EH-96-65, EH-96-65; level 8—EH-96-76; undifferentiated—EH-96-61. Number of asterisks denotes specimen(s) from which DNA was extracted.

Level	Lower teeth	Upper teeth	Molariform teeth	Jaws	Number of identified specimens	Minimum number of individuals
Surface	7 (1,1,1,0,2*,1,1,0)	0	0	3	10	2
1	2 (0,0,1*,0,1,0,0,0)	1 (0,0,0,0,1,0,0,0)	0	1	4	1
2	17 (1,2,3,0,4,3,3,1)	15 (3,3,1,0,2,3,3,0)	1*	4	37	4
3	9 (1,0,3,0,1,2,2,0)	10 (2,1,2,0,0,1*,0,0)	2**	8	29	3
4	2 (1,0,0,0,1,0,0,0)	3 (0,1,1,0,0,1*,0,0)	1*	0	6	1
5	6 (1,2,1,0,1,0,1*,0)	3 (1,0,0,0,2,0,0,0)	1*	3	13	2
6	8 (2,1,1,0,1,1,2,0)	14 (2,4*,2,0,2,1*,2,0)	2**	2	26	4
7	9 (0,1,2,0,2,2,2*,0)	7 (2,2**,1,0,0,1,1,0)	0	3	19	2
8	1 (0,0,0,0,1,0,0,0)	2 (1,0,0,0,0,0,1,0)	1*	0	4	1
9	0	0	0	0	0	0
Undifferentiated	2 (1*,0,0,0,1,0,0,0)	2 (2,0,0,0,0,0,0,0)	0	0	2	2
Total	63	57	8	24	150	22

this specimen (EH-96-36 from level 6) appeared to have been derived from fungal cytochrome-*b* contamination and was confirmed with BLAST analyses in GenBank. The closest match for this specimen was with fungi (14–18% sequence divergence). We cloned and sequenced 18 samples from this anomalous specimen with the hope of finding the *C. sociabilis* functional copy. None of them showed the *Ctenomys* sample, suggesting extensive degradation of source DNA in this tooth.

We also cloned 16 samples from a level 3 specimen that yielded the *C. sociabilis* sequence to see if we could detect the anomalous sequence in more than 1 individual. All gave the same mitochondrial copy; none of them showed the ''fungal'' sequence (Table 3). All 16 remaining sequences were *C. sociabilis*, which were identical to a sequence from the modern population near the type locality (unpublished sequence provided by E. Lacey, Museum of Vertebrate Zoology, University of California, Berkeley; Table 2). *C. sociabilis* differs from *C. haigi* and *C. maulinus* in the study area by ≥12% uncorrected sequence divergence in this 368-bp region (Table 4). The smallest sequence variation found between *C. sociabilis* and any other *Ctenomys* sequences available from GenBank was with *C. tuconax*, which occurs in northern Argentina. There were 37–39 base differences in the 368-bp region, amounting to 10% uncorrected sequence divergence between these 2 species (Table 2).

Lack of genetic variation as a major result of an aDNA study could be unnatural and due to several recurrences of a single contamination source. We believe contamination to be unlikely due to our stringent aDNA protocol with multiple controls and overlapping sequence fragments. To produce our results, we ran 99 negative controls. Of these 99 controls, 10 were positive and the rest were negative. The 10 positives were sequenced, and none came out as *Ctenomys*; all were miscellaneous sequences.

An alternative is that the cytochrome-*b*

TABLE 2.—All variable sites from 368 base pairs of cytochrome-*b* for *Ctenomys sociabilis* (E. Lacey, pers. comm.), *C. maulinus*, *C. tuconax*, and *C. haigi*. Labels in parentheses refer to laboratory extraction annotation or GenBank accession numbers. Position 270 is located within the primer region.

Species	1111111111 2222445566 0167141925	1111222222 6779112235 8478362575	2222222233 7888899900 0256814736	3333333333 0112234445 9281212595	3333333444 6778999122 9281067706	4444444444 3444556677 8147062513	44444555 77799011 47828139
C. sociabilis (E. Lacey)	TCACAATATT	CTTCATTTTT	CCCCTCCATT	CCTCAGTCAT	TGTTTGCTTA	TACCCTTCAC	TCCCGTCT
Surface (C1.1)	....	....	N.........	....	....	....	....
Unit1 (C1.2/3.1)	....	....	N.........	....	....	....	....
Unit2 (C1.3)	....	....	N.........	....	....	....	....
Unit3 (C1.4)	....	....	N.........	....	....	....	....
Unit3 (C5.2)	....	....	N.........	....	....	....	....
Unit4 (C1.5/3.2)	....	....	N.........	....	.......???	??????????	????????
Unit5 (C2.1/3.3)	....	....	N.........	....	.......???	??????????	????????
Unit5 (C4.2)	....	....	N.........	....	.......???	??????????	????????
Unit6 (C4.1)	....	....	N.........	....	.......???	??????????	????????
Unit6 (C4.5)	....	....	N.........	....	.......???	??????????	????????
Unit6 (C5.1)	....	....	N.........	....	....	....	....
Unit7 (C2.3/3.5)	....	....	N.........	....	....	....	....
Unit7 (C4.3)	....	....	N.........	....	.......???	??????????	????????
Unit7 (C4.4)	....	....	N.........	....	.......???	??????????	????????
Unit8 (C2.4/3.6)	....	....	N?????????	??????????	??????????	??????????	????????
Undifferentiated (C2.5/3.7)	....	....	N.........	....	.......???	??????????	????????
C. tuconax (AF370693)	.T...TC.C.	.CCTTCCC.C	.T.TC...A.	TTCT..C..C	CACC.A...G	...TT..T.T	C..T.CTC
C. tuconax (AF370684)	.T...TC.C.	.CCTTCCCCC	.T.TC...A.	TTCT..C..C	CACC.A...C	...TT..T.T	C.TT.CTC
C. tuconax (AF370683)	.T.T.TC.C.	.CCTTCCCCC	.T.TC...A.	TTCT..C..C	CACC.A...C	...TT..T.T	C..T.CTC
C. maulinus (AF370702)	..GTTGCGCC	.CCTTCCCCC	TTTTCT.GA.	.TCT...TGC	.ACCCATC..	.GTT.CC.T.	.T.TA.TC
C. maulinus (AF370703)	...TTGCGCC	.CCTTCCCCC	TTTTCTT.A.	.T.T...TGC	.ACCCATC..	.GTT.CC.T.	.T.T..TC
C. sociabilis[a] (U34853)	C..TTCCGCC	A.CATCCCCC	.TTT.T..AC	.T.TTA.TGC	CACCCA.CC.	C..T.CC...	..T.A.T.
C. haigi (AF007063)	C..TTCCGCC	A.CATCCCCC	.TTT.T..AC	.TCTT..TGC	CACCCA..CG	...T.CC...	....A.TC

[a] *C. haigi* (according to E. Lacey, pers. comm.); concordant with analyses herein.

TABLE 3.—Variation observed in 16 clones of the 1st polymerase chain reaction fragment from a level 3 *Ctenomys* sequence. Variable sites were observed in low frequency and were equally distributed across 2nd and 3rd codon positions, suggesting that this variation was due to random *Taq* errors. The majority of clones showed sequences identical to *C. sociabilis*. Furthermore, nucleotide ambiguity observed in 9 (126, 134, 145, 157, 174, 177, 210, 213, and 228) sites by as many as 44% of the clones was indicative of DNA damage in the level 3 tooth.

	Position
	111222
	488144
Number of clones	039137
12	CCCCTC
2	T.....
1	.TTGC.
1	.....T

fragment we chose did not contain enough variation at the population level in octodontid rodents. However, a review of publicly available cytochrome-*b* sequences from other *Ctenomys* species (Table 4) showed absolute genetic variation displayed in 16 of 18 *Ctenomys* species in the 368-bp fragment used in this study, while also showing that the relative genetic variation in this fragment was similar to the observed genetic variation from the entire cytochrome-*b* gene. Moreover, lack of genetic variation agreed with the modern genetic structure of *C. sociabilis* (Lacey 2001) and thus extended this scarcity of genetic variation to 1,000 years ago.

DISCUSSION

Lack of genetic variation in *C. sociabilis* is particularly striking given the late-Holocene history of this region. Today, between 35° and 55°S latitudes, strong westerly winds dominate. Northeastern Patagonia thus lies in the rain shadow of the Andes, resulting in one of the world's steepest moisture gradients from the Andean crest eastward (Hoffman 1975; Veblen et al. 1996). At 41°S latitude, mean annual precipitation declines about 65 mm/km, with a decrease from 4,500 mm at Puerto Blest to 600 mm at the San Carlos de Bariloche airport, a distance of 60 km. Precipitation then declines more gradually eastward to ≤100 mm on the Patagonia steppe proper (Hoffman 1975). This abrupt moisture gradient is clearly reflected in the vegetation. Rainfall and elevation patterns in Patagonia are concordant with rapid vegetation shifts (Veblen et al. 1996; Villalba et al. 1992). From the Andean crest east, these include tundra; temperate, mostly evergreen, rainforests (*Nothofagus*) with an understory of bamboo (*Chusquea*); temperate, mostly deciduous, woodlands (*Nothofagus* and evergreen *Austrocedrus chilensis*); and the Patagonian steppe of shrubs and bunchgrasses. Local moist meadows (maillins) within the grassland steppe are dominated by sedges, rushes, and grasses.

During the last 1,000 years, the Patagonian region of South America experienced both warming and cooling periods, which are thought to correlate with El Nino Southern Oscillation events (Villalba 1994). Particularly notable episodes include a warmer interval in AD 1080–1250, which occurred in the Andean region between 38° and 50°S latitude, and a cooler, wetter interval in AD 1340–1640, which coincides with the Little Ice Age (Villalba 1994). The warmer, drier interval in AD 1080–1250 is concordant with the Medieval Warm Period documented in Europe and North America (Broecker 2001; Hughes and Diaz 1994; Lamb 1965, 1977). Whereas the Medieval Warm Period apparently is time transgressive globally (Broecker 2001; Hughes and Diaz 1994), Southern Hemisphere records of the Medieval Warm Period are almost synchronous. These records include glacial, dendrochronologic, palynological, and ice-core evidence from South America (Pendall et al. 2001; Stine 1994; Villalba 1994), Tasmania (Cook et al. 1991, 1992), and Antarctica (Masson et al. 2000). During this warm period, sea-surface temperatures were about 1°C warmer than they are today (Keigwin 1996).

TABLE 4.—*Ctenomys* species, number of available sequences, number of variable sites per 368 base pairs (bp) of cytochrome-*b* and total cytochrome-*b* sequence, and collection localities (URU = Uruguay, ARG = Argentina, CHI = Chile, BOL = Bolivia).

Ctenomys species	Number of available sequences	Number of variable sites, 368 bp (frequency)	Number of total variable sites, 1,140 bp (frequency)	Species locality (individuals per locality)
boliviensis	5	20 (0.054)	67 (0.059)	Santa Cruz, 1 km SE Puerto Pacay (3), Robore (2), BOL
mendocinus	3	10 (0.027)	36 (0.032)	Tupungato (2) and San Isidro (1), Mendoza Province, ARG
rionegrensis	5	10 (0.027)	30 (0.026)	Rio Negro, El Abrojal (1); Rio Negro, Los Arrayanes (1); Rio Negro, Las Canas (1), URU; Entre Rios, Parana (1); Entre Rios, Ibicuy (1), ARG
haigi–"*sociabilis*"	2	7 (0.019)	25 (0.022)	13.5 km E Perito Moreno, Rio Negro Province (*haigi*); Reserva Nacional Nahuel Huapi, Estancia Rincon Grande, ARG ("*sociabilis*," MVZ-ELL1)
opimus	4	7 (0.019)	27 (0.024)	Tres Cruces Jujuy Province (1); Parinacota (1) Province, CHI; Oruro, 3.5 km E Huancarona (2), BOL
maulinus	2	5 (0.014)	20 (0.018)	Rio Colorado, Caracautin, Malleco Province, CHI
fulvus	4	6 (0.016)	19 (0.017)	San Pedro de Atacama, El Loa Province, (1); Vegas de Turi, Antofagasta Province (2); Salar de Atacama, Región de Antofagasta (1), CHI
torquatus	3	6 (0.016)	13 (0.011)	Tacuarembo, Ipora (1); Rio Negro, El Trillo (2), URU
talarum	3	3 (0.009)	25 (0.022)	Necochea, Buenos Aires Province, ARG
porteousi	2	6 (0.016)	18 (0.016)	Bonifacio, Buenos Aires Province, ARG
goodfellowi	2	4 (0.011)	14 (0.012)	Santa Cruz, San Ramon, BOL
tuconax	3	4 (0.011)	7 (0.006)	El Infiernillo, Tucumán Province, ARG
tucomanus	3	2 (0.005)	6 (0.005)	Ticucho, Tucumán Province, ARG
latro	2	4 (0.011)	6 (0.005)	Tapia, Tucumán Province, ARG
coyhaiquensis	3	1 (0.003)	2 (0.002)	Region XI, CHI
steinbachi	2	1 (0.003)	1 (0.001)	Santa Cruz, 6 km N Buen Retiro, BOL
frater	2	0 (0)	2 (0.002)	Tarija, 8 km W Rancho Tombo, BOL
conoveri	2	0 (0)	0 (0)	Chuquisaca, 9 km E Carandayti, BOL
sociabilis	16	0 (0)	NA	Reserva Nacional Nahuel Huapi, Estancia Rincon Grande, ARG: surface to ~900 years ago

The Little Ice Age in Patagonia is documented by tree rings and glacial advances in the Andes (Villalba 1990, 1994). In the northern Patagonian region, most of the evidence for the Little Ice Age suggests increased precipitation rather than a decrease in temperature. Forest expansion since the Little Ice Age may be documented by increased pollen percentages in the forest-steppe ecotone since the end of the 19th century (Veblen and Lorenz 1988). During the end of the Little Ice Age, temperatures may have been as much as 1°C cooler than at present (Keigwin 1996).

Subtle differences in precipitation can result in significant changes in vegetation in areas where moisture gradients are already extreme. The paleoecological record indicates that the ecotone between forest and the Patagonian steppe, which is maintained by the steep Patagonia moisture gradients, is very sensitive to past climatic variation (Markgraf 1983). Evidence from rodent middens 60 km N of ENH, located in a sim-

ilar ecotone between steppe and forest that ENH spans, indicates that late-Holocene warming between 1,300 and 1,800 years ago may have been coincident with increased human disturbance, mainly due to anthropogenic burning of forests (Markgraf et al. 1997). However, that conclusion is far from firm because the 2 sites (Cueva La Primavera and Cueva Valle Encantado; Fig. 1) on which inferences are derived occur in slightly different parts of the forest–grassland ecotone and do not overlap in time. Additionally, rodent middens can be difficult to interpret and constrain temporally unless they are stratified or characterized by multiple radiocarbon dates (Webb and Betancourt 1990), neither of which was true for these 2 sites.

Where they are sympatric, species of tuco-tucos exhibit preferences for specific microhabitats differentiated by density of vegetation and soil properties such as color, hardness, moisture, and sediment size (Comparatore et al. 1992). Indeed, morphologic and behavioral traits of some species correlate with different substrates (Madoery and Roig 1995; Vassallo 1998). Within species, local population abundance is determined by density of grasses and soil characteristics of the habitat (Comparatore et al. 1991; Gallardo et al. 1995). The distribution of tuco-tuco species in Andean South America conforms to the island model of isolation (Gallardo and Köhler 1992), with species exhibiting small allopatric ranges tied to a particular climate-controlled vegetation zone. If historic climate fluctuations influence the geographic range of tuco-tucos by movement of vegetation zones (not contraction of them), we would expect to see movement of species with time. If properly situated, a paleontological site spanning these climatic fluctuations should record species replacement. There is evidence of replacement of tuco-tuco species during the early Holocene near Cueva Traful (Pearson 1984; Pearson and Pearson 1993). Changes in morphology suggest that before about 7,000 years ago, *C. sociabilis* was present in Cueva Traful and subsequently was replaced by *C. haigi* (Pearson and Pearson 1993). *C. haigi* is present today north of the Traful River and east of the Limay River across the valley from Cueva Traful (Pearson 1984).

The paleontological site at ENH is positioned at the ecotones between mesic and xeric grassland steppe to the east and grassland and *Nothofagus* forest to the west. Although optimally situated to record any species replacement with a shift in these 2 ecotones, the deposit at ENH records no such replacement during the past 1,000 years. All 16 of the specimens with ancient mitochondrial sequence were *C. sociabilis*, demonstrating that this species was present at this location at the center of the species' present geographic range throughout the past 1,000 years. Thus, the deposit at ENH provides no evidence of ecological response to environmental change by tuco-tucos.

If, instead, response to environmental perturbation in this species occurs at the population level, we might expect to see variation in genetic diversity through time as individuals of different lineages disperse across the landscape or population reduction decreases levels of genetic variation. In fact, a 1988 eruption of the Lonquimay volcano in the southern Andes led to effective reductions of 3 populations of *C. maulinus* of up to 91% (Gallardo et al. 1995). This extreme population reduction resulted in significant decreases in heterozygosity in these populations (57–100%) and was correlated with a disruption of microspatial breeding and demographic units.

The tuco-tucos found in the ENH site exhibit no evidence of genetic change through the past 1,000 years. Our results are concordant with a modern microsatellite study of *C. sociabilis*, which indicated a recent population bottleneck (Lacey 2001). In her study of *C. sociabilis* 15 km E on the edge of the geographic range of *C. sociabilis*, Lacey (2001) found low levels of heterozygosity and bimodal allelic distributions at 15 microsatellite loci, suggesting a recent

population bottleneck. Furthermore, lack of variation at 15 other loci makes it unlikely that the lack of variation shown in the mitochondrial DNA was due to a selective sweep.

Lack of variation through the past 1,000 years also shows no support for population replacements or evidence of dispersal between populations in *C. sociabilis*. However, if the bottleneck that Lacey (2001) found extended throughout the species' range, detection of dispersal between populations would be difficult unless each population maintained genetic diversity patterns and haplotypes that were unique. The paleontological and genetic evidence of social tuco-tucos at ENH suggests that this bottleneck must have occurred before 1,000 years ago. Furthermore, although the population, and perhaps the species, has limited genetic diversity, the species has persisted in the area through that time. Although it is possible that other, more genetically diverse populations are present elsewhere, the available genetic (Lacey 2001), population (Lacey et al. 1997; Pearson 1959; Reig et al. 1990), and historic evidence suggests that this is unlikely.

The persistence of this species through the past 1,000 years with no genetic variation is even more remarkable because of the number of environmental perturbations in the late Holocene. Additionally, very recent disturbances include the effect of humans, which has threatened other Patagonian species. European colonization and expansion during the past 400 years are deemed to be responsible for a decrease in Patagonian range quality because of overgrazing by sheep and cattle (Aagesen 2000) and retreat of the forest boundary because of clearing and burning (Margraf and Anderson 1994; Veblen et al. 1996). Tuco-tucos in southern Patagonia purportedly have been affected by intensive open-range sheep grazing and other agricultural activity (Miller et al. 1983), to the extent that *C. magellanicus*, confined to extreme southern Argentina and Chile, has declined in numbers and has been listed as rare and endangered (Hilton-Taylor 2000). Other tuco-tuco species, including *C. sociabilis*, have been listed as near threatened (Hilton-Taylor 2000). Habitat degradation is particularly severe in wet Patagonian meadows, the edges of which are preferred by *C. sociabilis* (Lacey et al. 1997).

Although our data illustrate that populations can persist for hundreds of years with limited genetic variation, the population is still at risk. Theoretical work suggests that species with life histories including low vagility are particularly susceptible to local extinction (Lande 1988). Extinction vulnerability is further increased in species that occupy small geographic ranges (Jablonski 1991), maintain low effective population size, and have low genetic variation (McKinney 1997; O'Brien et al. 1985). When these traits are correlated, extinction likelihood is greatly magnified (Gilpin and Soulé 1986). In the social tuco-tuco, these traits are correlated, suggesting that this species may be particularly susceptible to extinction. However, our results indicate that this species is able to persist for long periods of time with limited genetic variation, suggesting that efforts to conserve this species are likely to be successful as long as environmental perturbation does not exceed that witnessed in the past millennium. Although assessing geographic variation in abundance, genetic diversity, and species richness are objectives of many ecological and evolutionary studies, few studies provide a temporal context to these investigations. Our ability to predict the effects of future environmental change increasingly relies on evidence of the impact of past events on species, a primary objective of our focus on the genetics of past populations.

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