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Genetic variation over 10 000 years in *Ctenomys*: comparative phylochronology provides a temporal perspective on rarity, environmental change and demography

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Abstract

An understanding of how ecological traits influence past species response to environmental change can aid our future predictions of species persistence. We used ancient DNA and serial coalescent modelling in a hypothesis-testing framework to reveal differences in temporal genetic variation over 10 000 years for two species of subterranean rodents that currently differ in rarity (abundance, range size and habitat specificity) and mating system, but that reside in the same volcanically active region. Comparative phylochronologic analyses indicated little genetic change and suggest genetic stability in the solitary widespread *Ctenomys haigi* over thousands of years. In contrast, we found a pattern of haplotypic turnover in the rare and currently endangered *Ctenomys sociabilis*. Serial coalescent modelling indicated that the best-fit models of microevolutionary change included gene flow between isolated populations for this species. Although *C. haigi* and *C. sociabilis* are congeners that share many life history traits, they have behavioural, habitat-preference and population-size differences that may have resulted in contrasting patterns of temporal variation during periods of environmental change.

Keywords: ancient DNA, *Ctenomys haigi*, *Ctenomys sociabilis*, serial coalescent, subterranean rodents, temporal variation

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Introduction

As conservation biologists seek to prevent further extinctions and to preserve biodiversity, effective conservation hinges on the availability of scientific knowledge to evaluate the population status and rates of change of rare and endangered species. This goal is inherently temporal, as we gain greater understanding of the past to predict and prevent future extinctions. Faunal analyses and ancient DNA extracted from subfossil material are particularly useful, as they enable the tracking of species abundance and genetic variation over long timescales, thus providing greater insight into species persistence and response to environmental change (Leonard 2008; Campos *et al.* 2010; Prost *et al.* 2010).

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We used ancient DNA from subfossil teeth excavated from a late-Quaternary site at the base of the Patagonian Andes (Pearson & Pearson 1981; Montero *et al.* 1983; Chan *et al.* 2005) to contrast changes in relative abundance and genetic variation from 12 225 to 1894 calibrated years before present (cal yr BP) in two species of subterranean rodents in the widespread South American family, Ctenomyidae, *Ctenomys haigi* (Haig's tuco-tuco) and *C. sociabilis* (Colonial tuco-tuco). These congeners are predicted to share many life history traits based on their body size and fossorial habits, such as number of offspring per litter, longevity, limited dispersal and high amounts of population structure (Lacey & Wieczorek 2004).

However, behavioural and demographic studies (Lacey *et al.* 1997; Lacey 2004; Lacey & Wieczorek 2004) have revealed that modern populations differ in three important aspects of rarity: abundance, geographic range and habitat specialization (Rabinowitz 1981).

C. sociabilis, currently listed as Critically Endangered by the IUCN (Bidau *et al*. 2008b), is endemic to 1400 km^2 of Reserva Nacional del Parque Nacional Nahuel Huapi, Neuquéun Province, Argentina (Woods & Kilpatrick 2005). In contrast, *C. haigi* is listed as Least Concern (Bidau *et al*. 2008a) with a larger range that encompasses Chubut and Rio Negro Provinces (Woods & Kilpatrick 2005). They are parapatric, currently occupying precordilleran steppe grassland in the same climatic region of southwestern Argentina. However, they do not co-occur, with geographic ranges presently separated by the Traful and Limay rivers (Lacey & Wieczorek 2003; Woods & Kilpatrick 2005). The two species also differ in habitat specificity. Within the arid precordilleran steppe grassland are wet meadow habitats known as mallínes. *C. sociabilis* is confined to these mesic mallínes, while *C. haigi* is found in both the arid and wet mallín habitats (Lacey & Wieczorek 2003). Finally, behaviourally, *C. haigi* is solitary (Lacey *et al*. 1998), while *C. sociabilis* is the only species in the genus *Ctenomys* known to be fully social (Lacey 2004).

Differences in demography, habitat and behaviour are predicted to lead to differences in both spatial and temporal structuring of genetic variation. The degree to which mutation, drift, migration and selection shape patterns of genetic variation within and among populations is influenced by the interaction of species ecology and demography with these evolutionary forces. Species characteristics such as vagility, mating system and local abundance affect migration rates and effective population size, and as a result, the degree to which drift influences genetic diversity and structure. Ecological specialization and habitat preferences can also influence gene flow and effective population size by facilitating or inhibiting dispersal between populations and by determining abundance and distribution through habitat availability and stability. In addition to ongoing demographic and ecological factors, genetic diversity and structure is also strongly affected by the historical demography of populations and particular events, such as population bottlenecks, recent colonization or population expansion.

Molecular studies of *C. sociabilis* have revealed extremely low existing genetic variation at 15 microsatellite loci (Lacey 2001). Such low levels of variation could result from spatial and temporal aspects of their population structure such as small populations in isolated habitat patches, high rates of extinction and colonization and/or frequent population bottlenecks (Steinberg & Patton 2000; Lacey 2001). In contrast, *C. haigi* has much higher levels of genetic variation at those same microsatellite loci (Lacey 2001). However, population history has a large influence on levels of genetic variation and should be considered when examining the potentially confounding influence of modern life history traits. Previous studies comparing the prehistoric levels of genetic variation in *C. sociabilis* to genetic variation in the modern populations found that low variation persisted for at least 1000 years (Hadly *et al*. 2003), yet surprisingly high levels of diversity were present in the species prior to approximately 2900 years ago (Chan *et al*. 2005, 2006). Although the modern demography of *C. sociabilis* (i.e. low dispersal distances and isolated, disjunct populations) is concordant with low genetic variation, the high genetic variation at that time suggested that the species had experienced a bottleneck immediately afterwards (Chan *et al*. 2005, 2006) and underscored the importance of history and temporal genetic structure in interpreting the patterns of genetic variation that we see today.

Molecular markers are used to measure levels of variation and patterns of genetic structure, and those patterns are commonly used to infer microevolutionary forces and to estimate population genetic parameters such as effective population size and migration rates. The reliability of such estimates depends on how well a sampling of modern populations reflects the genetic variability over longer time periods as well as on our ability to separate current ecological and demographic factors from the influence of historical demography (Lessios *et al*. 1994). Because separating current and historical factors is difficult, many studies implicitly assume temporal stability and equilibrium between drift and gene flow. Knowing which ecological traits enhance the reliability of these estimates makes the study of temporal variation relevant to modern population genetic and phylogeographic studies.

We continue the study of these two species through time, but here, we consider the relative roles of demography and behaviour on their patterns of temporal genetic variation. We used a hypothesis-testing, serial coalescent modelling approach to closely examine the influence of microevolutionary forces in shaping the contrasting temporal patterns of genetic variation. Furthermore, we consider how the differences in range and abundance, habitat specificity and behaviour may have shaped the cause of the decline of *C. sociabilis*. Putative causes of the bottleneck in *C. sociabilis* include environmental change resulting from a decline in steppe grassland habitat with a subsequent increase in *Nothofagus* forest, a volcanic eruption approximately 1950–2500 cal yr BP (Montero & Silveira 1983; Villarosa *et al*. 2006), and/or competition from *C. haigi* (Chan *et al*. 2005, 2006). Comparison of ancient DNA between two species inhabiting the same area through several thousand years reveals a unique perspective on the microevolutionary, environmental and demographic forces generating and maintaining variation and further yields insight

into the causes of past decline in *C. sociabilis* and its present and future persistence.

Methods

Study site, sampling and sequencing

Cueva Traful (40°43′ S, 71°07′ W, elevation 760 m) is a late-Quaternary, mostly Holocene, archaeological site and barn owl (*Tyto alba*) roost excavated during 1973–1978 (Montero *et al.* 1983). Because of the deposition of owl pellets, material excavated from the cave provides a continuous temporal sequence of small mammal bones and teeth dating from 12 381 to 1894 cal yr BP (radiocarbon ages shown in Table 1) (Pearson & Pearson 1981; Montero *et al.* 1983; Chan *et al.* 2005). Pearson & Pearson (1981) morphologically identified 3174 small mammal specimens from 1 marsupial and 18 rodent species in the 19 stratigraphic levels from Cueva Traful. The cave is located in the forest-steppe ecotone approximately 6 km north of the current northern distributional limit for *C. sociabilis*. The cave is separated from modern *C. haigi* populations by the approximately 100-m-wide Traful River.

DNA sequences included in our analyses comprise an expanded data set from Chan *et al.* 2005; consisting of 368 base pairs (bp) (previously 253 bp) of cytochrome *b* amplified in three fragments from *C. sociabilis* ($n = 34$) and *C. haigi* ($n = 31$). The protocol of Hadly *et al.* (2003) was used to extract DNA recovered from eight

Table 1 Data were grouped into three temporal intervals based on radiocarbon dates for Cueva Traful (Chan *et al.* 2005; Supporting information). Radiocarbon dates and sample size for each level are shown below. Serial coalescent simulations included the number of samples for each dated stratigraphic level, and summary statistics were calculated based on the temporal interval

Stratigraphic level	Sample	Radiocarbon date BP	Calibrated age BP (2 sigma range, relative area)	*Ctenomys sociabilis* (*N*)	*Ctenomys haigi* (*N*)	Temporal Interval	Time period covered
Capa 3 Hu	AA57248*	2033 ± 43	1894–2114 (1.000)		8	A	4412–1894 cal yr BP
Capa 3 Iu, Hu	LJ-5130†‡	2230 ± 40	2152–2336 (1.000)			A	
Capa 4 Hu	LJ-5131†‡	2720 ± 40	2753–2883 (0.980) 2909–2920 (0.020)			A	
Capa 1 Ir	AA57246*	3293 ± 49	3403–3432 (0.046) 3437–3637 (0.954)	4	7	A	
Capa 2 Ir	AA57247*	3749 ± 94	3885–4412 (1.000)	2	8	A	
Capa 5						Tephra	
Capa 6 Ir	AA57249*	5906 ± 84	6504–6521 (0.013) 6529–6937 (0.987)	2	3	B	7274–6302 cal yr BP
Capa 8 Hu	AA57250*	5655 ± 73	6302–6570 (0.933) 6583–6631 (0.067)	8	2	B	
Capa 9 Hs, Is	I-11304†§	6030 ± 115	6572–6581 (0.003) 6633–7177 (0.988) 7215–7241 (0.009)	5	3	B	
Capa 9 Im	LJ-5132†‡	6240 ± 60	6979–7274 (1.000)			B	
Capa 13 In	LJ-5133†‡	7850 ± 70	8460–8467 (0.006) 8477–8496 (0.017) 8512–8812 (0.804) 8878–8978 (0.112)	4		C	12381–7574 cal yr BP
Capa 13 In	LP-62†¶	9285 ± 313	9561–9572 (0.002) 9582–11260 (0.998)			C	
Capa 13 In	LP-8113†¶	7308 ± 285	7574–8718 (1.000)			C	
Capa 14 Ho	AA57251*	10209 ± 96	11406–11453 (0.018) 11471–11559 (0.039) 11595–12225 (0.890) 12257–12381 (0.053)	9		C	

*NSF-Arizona AMS Laboratory.
†indicates samples from (Montero *et al.*, 1983).
‡Mt Soledad Radiocarbon Laboratory.
§Teledyne Isotopes.
¶Laboratorio de Tritio y Radiocarbono LATYR (La Plata, Argentina).

stratigraphic levels (12 381–1894 cal yr BP). Levels were combined into three temporal intervals based on the stratigraphy and 17 radiocarbon dates (Montero *et al.* 1983; Chan *et al.* 2005), which we calibrated with CALIB Rev. 5.0 (Stuiver & Reimer 1993); Interval A (levels dated 4412–1894 cal yr BP, *C. sociabilis* $n = 6$, *C. haigi* $n = 23$), Interval B (levels dated 7274–6302 cal yr BP, *C. sociabilis* $n = 15$, *C. haigi* $n = 8$) and Interval C (levels dated 12 381–7574 cal yr BP, *C. sociabilis* $n = 13$, *C. haigi* $n = 0$) (Table 1, Fig. 2). Despite differences in the time periods covered by each interval, these were the clearest breaks for grouping levels based on the stratigraphy, dating and paleoenvironment. Interval B encompasses a mid postglacial interval of favourable conditions during a time of increasingly deteriorating conditions resulting from an increase of *Nothofagus* tree cover and decline overall of steppe habitat (Markgraf 1983). Interval A contains a tephra layer marking a volcanic eruption of the Puyahue-Cordón complex (Villarosa *et al.* 2006).

Strict ancient DNA protocols were followed, as described in Chan *et al.* (2005; Supporting information) and Hadly *et al.* (2003). These included DNA extraction and PCR set-up in a specially designed and dedicated ancient DNA facility that is spatially and temporally separated from post-PCR amplification and sequencing (Cooper & Poinar 2000; Hadly *et al.* 2003). All extraction and PCR amplification experiments included multiple negative controls and positive controls were only from ancient sources. Both forward and reverse strands were sequenced. We reamplified and resequenced 60% of the fragments to detect cytosine deamination (Hofreiter *et al.* 2001). Validity of our sequences was also investigated with independent replication of five teeth that were broken in half and extracted and amplified in a separate laboratory and cloning of PCR products from three teeth from levels of different ages (29 clone sequences total, data in Chan *et al.* 2005; Supporting information) to detect damage and nuclear pseudogenes. Simulations of DNA damage were conducted (Ho *et al.* 2007), and our data were found to possess very low damage and high fidelity.

Species identification and fossil abundance

Teeth can only be identified morphologically to genus *Ctenomys;* therefore, phylogenetic analysis (Chan *et al.* 2005; Supporting information) after sequencing was used to identify samples to species. We used ModelTest, version 3.7 (Posada & Crandall 1998) to determine the best-fit model of DNA substitution for a data set of 25 *Ctenomys* species and two *Octodon* species from Genbank based on 257 bp. Using AIC, the best-fit model of molecular evolution was HKY (Hasegawa *et al.* 1985) with the among-site rate variation, (I) proportion of invariable sites = 0.4138 and variable sites (G) gamma distribution shape parameter = 0.8423.

Once samples were identified to species, we determined the relative abundance of *C. sociabilis* and *C. haigi* based on their genetic proportion for each level. The relative abundance of *Ctenomys* was first determined from the proportion of minimum number of individuals (MNI) morphologically identified as *Ctenomys* out of the total MNI for every species found in Cueva Traful (Pearson & Pearson 1981). Then, the proportion of samples genetically identified as *C. sociabilis* or *C. haigi* was used to determine relative fossil abundance for each species in each stratigraphic level. This method allowed a fossil abundance of each species through time independent of genetic diversity estimates.

Genetic diversity

A minimum spanning network and set of unique haplotypes for each species were identified using TCS version 1.21 (Clement *et al.* 2000), and a temporal haplotype figure was drawn in R using TempNet (Prost & Anderson 2011). Levels of divergence within and between species and standard errors based on 1000 bootstrap replicates were calculated using MEGA version 3.1 (Kumar *et al.* 2004). We calculated nucleotide diversity and segregating sites for each time Interval A through C using Arlequin 2.000 (Excoffier *et al.* 2005) to be used in the serial coalescent modelling. To compare levels of diversity between species and across time within species, we calculated a temporally unbiased statistic of diversity, π_h that is the average number of pairwise differences adjusted for heterochrony (Depaulis *et al.* 2009).

We used an analysis of molecular variance (AMOVA) to partition genetic variation temporally using Arlequin 2.000 (Excoffier *et al.* 2005). Samples from each stratigraphic level were grouped by temporal interval to examine the amount of variation accounted for within stratigraphic levels, among levels within temporal intervals and among intervals. Allele frequencies between temporal intervals were compared with Fisher's exact tests (Raymond & Rousset 1995) using GENEPOP version 3.4.

Coalescent modelling

Bayesian skyline plots implemented in BEAST v1.6.1 using an uncorrelated lognormal relaxed clock (Drummond & Rambaut 2007) were used to reconstruct historical demography. An HKY model of nucleotide substitution was specified with gamma distributed rate variation among sites and a proportion of invariant sites based on results from ModelTest. We chose a piecewise-constant model with two (*C. haigi*) or three

(*C. sociabilis*) groups for comparison to the temporal Intervals A, B and C, and with 10 groups. The MCMC chains were run for 100 000 000 iterations to ensure effective sample sizes for parameter estimates.

We coupled the Bayesian skyline approach with hypothesis-testing and serial coalescent modelling in a phylochronologic statistical framework (Ramakrishnan & Hadly 2009) and using the program Bayes Serial SimCoal (Anderson *et al.* 2005). In this approach, we used the observed summary statistics, nucleotide diversity and segregating sites calculated for each temporal interval, and F_{ST} between temporal intervals to calculate probabilities for 125 different population models based on 5000 simulations. In all models, we used a Kimura 2-parameter mutation model with a gamma parameter of 0.8423 and a mutation rate of 2.26×10^{-5} based on results from Model Test and previous serial coalescent simulations (Chan *et al.* 2006) and assumed a generation time of 2 years (Chan *et al.* 2006).

First, we tested models of closed constant populations with effective population sizes ranging from N_e = 1000–200 000. We rejected single population models with constant population size for *C. sociabilis* and so explored more complex models incorporating demographic change and migration. Because the calculation algorithm for F_{ST} in Arlequin sometimes results in negative values or NA values if the sample intervals being compared have zero diversity, we forced those F_{ST} estimates to zero.

Model probabilities were based on the frequency of simulated values that were less than or equal to the observed (if the mean of the simulated values was greater than the observed) or that were greater than or equal to the observed summary statistic (if the mean was less than the observed). Because distributions of the summary statistics were often skewed, we used the distance from the mean of the observed value on both sides of the distribution to calculate a two-tailed probability.

When the observed summary statistic fell outside the range of simulated values, we set P = 0.0005 as a conservative estimate (Belle *et al.* 2006) or P = 0.9995 if the estimate was one. Probabilities of the two summary statistics for each temporal interval and F_{ST} estimates between intervals were combined for each model using Fisher's method (Sokal & Rohlf 1995) and Stouffer's method (Z-transform test; Whitlock 2005).

Results

Fossil abundance

Overall relative abundance of *Ctenomys* spp. out of all small mammal species in Cueva Traful was highest during the oldest level, Capa (Level) 14 (12 381–11 406 cal yr BP) and Capa 8 (6631–6302 cal yr BP; during Interval B; Fig. 1). Relative abundances of the two species changed through time (Chan *et al.* 2005) (Fig. 1). *Ctenomys sociabilis* comprised 100% of the two oldest levels (12 381–11 406 and 11 260–7574 cal yr BP). By Capa 9 (7274–6572 cal yr BP), *C. haigi* appeared, although *C. sociabilis* was still the most abundant ctenomyid (70%) in Interval B (7274–6302 cal yr BP). By Interval A (4412–1894 cal yr BP), the relative abundance of *C. sociabilis* had declined to 30%. *Ctenomys sociabilis* is entirely absent from the level dated 2336–1894 cal yr BP or later, and the species is not found near the cave today: its closest occurrence is >5 km to the south.

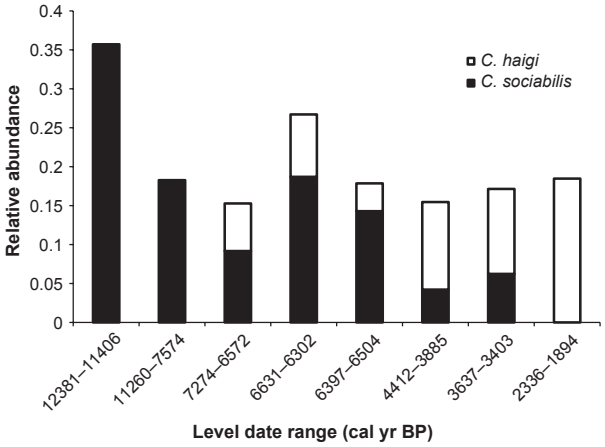

Fig. 1 Height of bars indicates relative abundance of *Ctenomys* spp. (based on minimum number of individuals) out of all small mammal species found in Cueva Traful. Black and white indicate relative abundance of *Ctenomys sociabilis* and *Ctenomys haigi* identified genetically, respectively. While *C. sociabilis* declines towards the present and is absent from the most recent temporal level, *C. haigi* is absent from the oldest levels, appears at 6135 ybp and increases in relative abundance over time. The modern geographic range of *C. sociabilis* does not encompass Cueva Traful, although the range of *C. haigi* does.

Genetic diversity

We extracted DNA from 80 *Ctenomys* teeth: eight teeth yielded no amplifiable DNA, 1 tooth yielded only one of the three fragments, and five teeth were not included because the entire extraction appeared contaminated by *Canis* DNA, leaving 66 usable samples (extraction success of 81%).

Analyses of the transition : transversion ratios and the codon positions of mutations from the longer data set were comparable to cytochrome *b* from other *Ctenomys* (D'Elia *et al.* 1998; Gimenez *et al.* 2002; Castillo *et al.* 2005). For *C. sociabilis*, there were 18 variable sites (14 transitions, 4 transversions), 12 of which were parsimony informative. For *C. haigi*, there were only six variable

sites (all transitions), only one of which was parsimony informative. The longer data set (368 bp) increased the number of haplotypes from earlier analyses (253 bp) in *C. sociabilis* from 8 to 12 (Chan *et al.* 2005) with new haplotypes added to the two more recent temporal intervals in Cueva Traful. Variation at the third, second and first codon positions overall for both species was 66.7%, 8.3% and 25%, respectively.

Because substitution of C to T and G to A comprises the most common form of damage (Hofreiter *et al.* 2001), we closely examined 8 of 18 variable sites in *C. sociabilis* and four of six variable sites in *C. haigi.* All of these sequences were resequenced, replicated in the forward and reverse direction, over half were confirmed from multiple independent amplifications, and a subset was cloned. Sequence damage for these specimens was estimated to be extremely low (<0.00033%; Ho *et al.* 2007). Haplotype sequences can be found under Genbank accession numbers DQ402060–DQ402066 and GU433041–GU433046 for *C. sociabilis* and *C. haigi*, respectively.

Net uncorrected sequence divergence between *C. haigi* and *C. sociabilis* was 11.4% (SE 1.5%). Mean sequence divergence within species was much lower for *C. haigi* (0.22% SE 0.1%) than *C. sociabilis* (1.4% SE 0.36%). Higher sequence divergence within *C. sociabilis* was because of the presence of two previously identified clades (net divergence 2.0% SE 0.6%, maximum sequence divergence 4.2%), which were referred to as the modern clade (M) and ancient clade (A) (Chan *et al.* 2005; Fig. 2).

Temporally unbiased levels of diversity were higher overall in *C. sociabilis* than in *C. haigi* for the most recent temporal intervals B and A, but were comparable to *C. haigi* for the oldest interval C (Fig. 2, *C. sociabilis*, Interval A: $\pi_h = 6.126 \pm 3.390$, Interval B: $\pi_h = 5.495 \pm 2.797$, Interval C: $\pi_h = 0.7343 \pm 0.5728$, *C. haigi*, Interval A: $\pi_h = 0.6265 \pm 0.5040$, Interval B: $\pi_h = 1.306 \pm 0.9044$). Higher levels of diversity in the more recent temporal intervals were because of the appearance of the modern *C. sociabilis* clade in Intervals A and B.

The AMOVA for *C. haigi* indicated that 97% of the variation was within levels, and only 3% of the variation ($P > 0.05$) could be accounted for among temporal intervals. In contrast, for *C. sociabilis*, only 60% of the variation was within levels, and 33% was among temporal intervals ($P < 0.01$) (Table 2). The difference between the temporal intervals in *C. sociabilis* was also apparent from the Fisher's exact tests, where the null hypothesis of homogeneity of haplotype frequencies was rejected between the oldest Interval C (12 381 and 7574 cal yr BP) and the two more recent Intervals A and B (dated 4412–1894 cal yr BP, $P < 0.00009$; and 7274–6302 cal yr BP, $P < 0.00517$, respectively).

Coalescent modelling

Bayesian skyline plots had large confidence intervals and showed little change over time for *C. haigi* and a slight increase in effective population size for *C. sociabilis*, which is concordant with the increase in observed nucleotide diversity and segregating sites for *C. sociabilis*, but

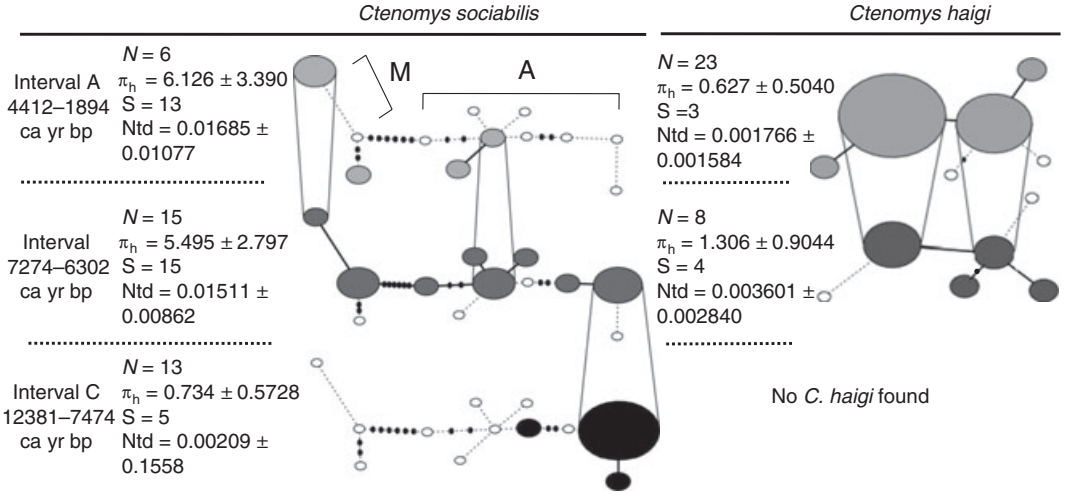

Fig. 2 Haplotype network with change in frequency of cytochrome *b* haplotypes (368 bp) through time, as well as sample size (*N*) and time-adjusted pairwise differences (π_h). Lines between circles indicate a base pair change with a solid dot indicating each additional base pair change. Open circles indicate haplotypes that were not found in that temporal interval. Also shown are observed number of segregating sites (S) and observed nucleotide diversity (Ntd) for each temporal interval used in the serial coalescent simulations. The absence of *Ctenomys haigi* haplotypes from the oldest time period is because none were sampled. M (Modern) and A (Ancient) clades for *Ctenomys sociabilis* are also indicated.

Table 2 Analysis of molecular variance of temporal variation in 368 bp of cytochrome *b*, divided into temporal intervals, among stratigraphic levels within intervals and within stratigraphic levels

	Variance components	% Total	*F*-statistics	*P*
Ctenomys sociabilis				
Among intervals	0.98287	33.02	F_{SC} = 0.09901*	0.00196
Among strata within intervals	0.19742	6.63	F_{ST} = 0.39650	0.26979
Within strata	1.79650	60.35	F_{CT} = 0.33018	0.09971
Ctenomys haigi				
Among intervals	0.01217	3.01	F_{SC} = −0.00759	0.39198
Among strata within intervals	−0.00298	−0.74	F_{ST} = 0.02270	0.70870
Within strata	0.39548	97.73	F_{CT} = 0.03006	0.09677

P is the probability of having a more extreme variance component and phi-statistic than observed by chance alone.

not for *C. haigi* (Fig. 3). This analysis may not be appropriate because of the limited timescale (approximately 10 000 years), lower mutation rate of cytochrome *b* compared with control region and the resulting low genetic variation that places this data set just at the edge of a measurably evolving population for which this type of analysis was designed (Drummond *et al.* 2003). Therefore, we used serial coalescent modelling to further explore change in effective population size through time as well as changes in genetic structure and gene flow.

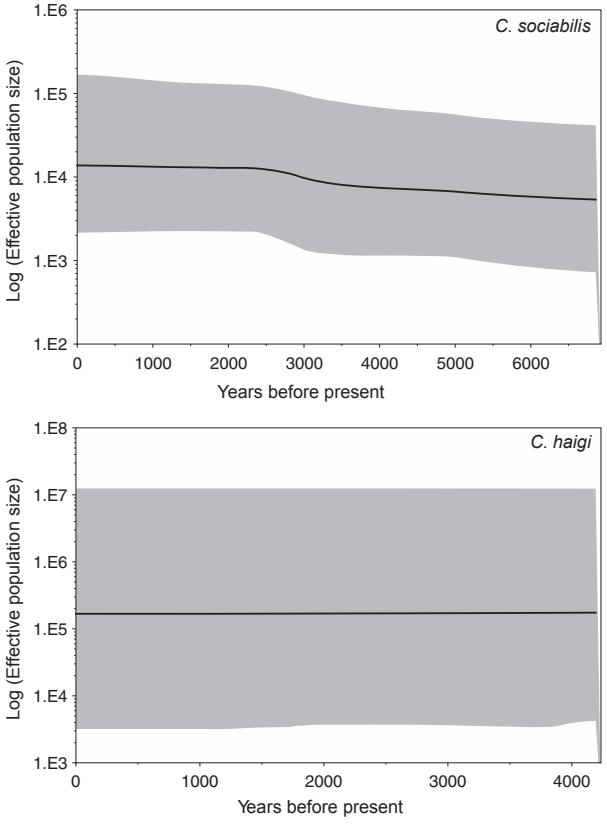

Fig. 3 Bayesian skyline plots of median effective population size (assuming a 1 year generation time) and showing wide confidence intervals, little change in historical population demography for *Ctenomys haigi* and a slight increase in effective population size for *Ctenomys sociabilis*. Note the *Y*-axis is on a log scale. Results are shown for 10 groups, although the 2–3 group analysis was similar.

Serial coalescent modelling of effective population sizes, N_e = 1000, 2000, 5000, 10 000, 20 000, 50 000, 100 000 and 200 000, indicated that we could not reject a closed constant population size model for *C. haigi*. Highest probabilities were for effective population sizes of N_e = 10 000 and N_e = 20 000 (Fig. 4). We did reject a closed constant population for N_e = 1000–200 000 for *C. sociabilis* when we combined probabilities over all three statistics (nucleotide diversity, segregating sites and F_{ST} between temporal intervals) for all three temporal intervals (Fig. 4). This led us to examine more complex models that incorporated demographic change and migration.

Because genetic diversity in *C. sociabilis* increased from the oldest to the most recent time period, we modelled closed populations that increased based on the highest probability N_e from the constant population size models. For the oldest interval C, the highest probability for nucleotide diversity was N_e = 10 000 and for segregating sites was N_e = 20 000. For Intervals A and B, the highest probability was N_e = 100 000 for both statistics. Neither of the closed increasing population size models fit the data when we combined probabilities over all three statistics (Fig. 5).

The low dispersal distances of these subterranean rodents suggest significant population structuring. Furthermore, the observed pattern of haplotypic turnover (Fig. 2) indicated the possibility of migration from another differentiated population in *C. sociabilis*. Therefore, we examined multiple population models with a single migration event midway between dated levels 13 and 9 (concurrent with the appearance of novel clade M) between two or three previously closed populations

C. haigi		1000	2000	5000	10000	20000	50000	100000	200000
Interval A	SegSites	0.0072	0.0326	0.4400	0.9995	0.4792	0.0546	0.0192	0.0038
	NucltdDiv	0.0126	0.0426	0.5558	0.8884	0.3884	0.0966	0.0572	0.0352
Interval B	SegSites	0.0005	0.0012	0.0288	0.1448	0.9995	0.3068	0.0968	0.0340
	NucltdDiv	0.0002	0.0034	0.0374	0.3646	0.9402	0.2582	0.1002	0.0518
A vs B	Fst	0.9154	0.8640	0.7430	0.7118	0.7258	0.8018	0.8324	0.8370
Stouffer's	Combined	2.88E-06	0.0001	0.0871	0.9493	0.9889	0.0756	0.0093	0.0009
Fisher's	Combined	1.73E-07	3.41E-05	0.0727	0.7442	0.9414	0.0996	0.0098	0.0006

C. sociabilis		1000	2000	5000	10000	20000	50000	100000	200000
Interval A	SegSites	0.0005	0.0005	0.0005	0.0005	0.0036	0.3334	0.8068	0.2444
	NucltdDiv	0.0005	0.0005	0.0005	0.0005	0.0040	0.1724	0.8998	0.2974
Interval B	SegSites	0.0005	0.0005	0.0005	0.0005	0.0048	0.4798	0.4478	0.0598
	NucltdDiv	0.0005	0.0005	0.0005	0.0005	0.0042	0.2554	0.7278	0.1646
Interval C	SegSites	0.0005	0.0006	0.0190	0.1218	0.9995	0.2630	0.0580	0.0170
	NucltdDiv	0.0110	0.0334	0.4770	0.9858	0.4846	0.1192	0.0662	0.0386
A vs B	Fst	0.1546	0.9264	0.8462	0.1932	0.1576	0.1206	0.1006	0.0984
A vs C	Fst	0.0206	0.0134	0.0052	0.0010	0.0005	0.0002	0.0002	0.0005
B vs C	Fst	0.0802	0.0734	0.0618	0.0462	0.0314	0.0204	0.0126	0.0142
Stouffer's	Combined	5.17E-15	4.66E-12	4.52E-10	1.56E-09	3.57E-06	0.000192	0.006143	7.72E-07
Fisher's	Combined	1.14E-13	9.88E-13	5.77E-11	2.91E-11	2.84E-08	0.000308	0.000508	5.17E-06

Fig. 4 Probabilities (binned into four categories with darker coloured cells representing higher probability) for segregating sites, nucleotide diversity and Fst (between temporal intervals) for *Ctenomys haigi* and *Ctenomys sociabilis* as a function of effective population size for a closed constant population size model. A closed single constant population size model of N_e = 10 000–20 000 cannot be rejected for *C. haigi* (indicated by bold combined probabilities). None of the constant effective population sizes fit the data for *C. sociabilis*.

with per cent migration between populations of m = 0.05, 0.25, 0.5, 0.75 and 0.95. We explored models of migration from two or three different populations to see whether migration could account for the increase in diversity and change in F_{ST} between temporal intervals. Best-fit models included models with migration (Table 3). Two population and three population models did not differ statistically and were dependent on the rate of the migration (Table 3).

Finally, because estimates of fossil abundance suggested a population decline for *C. sociabilis* through the time spanned by the deposit, we examined declining population sizes coupled with migration to see whether migration could offset the decline in variation from the decrease in population size. We examined models with most recent population sizes of N_e = 10 000, 20 000, 50 000, 100 000 and 200 000, with older population sizes larger by the same percentage as the change in subfossil-relative abundance over time and levels of migration ranging from 0.05 to 0.95. The declining population size models matched the percentage change in *C. sociabilis* over time at different ending values of N_e. Depending on the level of migration, those models were not statistically different from the constant population size models (Δ AIC < 2, Table 3).

Discussion

Our comparison of temporal variation from approximately 12 000 to 2000 cal yr BP in *C. sociabilis* and *C. haigi* revealed marked differences between these congeners. Concurrent with a decline in fossil abundance, we observed an increase in genetic diversity in *C. sociabilis*. The pattern of haplotypic turnover and our modelling results suggest that this results from gene flow between genetically differentiated populations. Modelling temporal variation showed that even if population size declines over time, migration could be sufficient to increase genetic diversity to the level observed in *C. sociabilis*. In *C. haigi*, despite an increase in relative abundance, we observed little change in genetic variation, with almost all of the variation (97%) accounted for within temporal intervals. For *C. haigi*, we could not reject a closed, constant, population model. Species-relative abundance also indicates that while *C. sociabilis* disappears from the record after a volcanic event, *C. haigi* does not. *Ctenomys haigi* is currently also found near the cave, while Cueva Traful is no longer within the geographic range of *C. sociabilis*.

The contrasting patterns of temporal variation between these two species reveal the possible influence of differences in range size and abundance, habitat specificity and behaviour on temporal stability. We will discuss each of these factors in the context of the microevolutionary forces at work over the last several thousand years in *C. sociabilis* and *C. haigi*, which may shed further light on the causes of decline in *C. sociabilis* and its implications for conservation and management.

C. sociabilis		10000-100000	20000-100000
Interval A	SegSites	0.8052	0.8152
	NucltdDiv	0.8912	0.8946
Interval B	SegSites	0.447	0.4498
	NucltdDiv	0.7274	0.7242
Interval C	SegSites	0.0544	0.0588
	NucltdDiv	0.0654	0.064
A vs B	Fst	0.0005	0.0005
A vs C	Fst	0.3158	0.5038
B vs C	Fst	0.0005	0.0005
Stouffer's method	Combined	0.00560	0.00947
Fisher's method	Combined	0.00022	0.00032

Fig. 5 Probabilities (binned into four categories with darker coloured cells representing higher probability) for segregating sites, nucleotide diversity and F_{ST} (between temporal intervals) for *Ctenomys sociabilis* as a function of effective population size for a closed increasing population size model. The increasing population size model was based on genetic effective size as estimated from the highest probabilities for the constant population size models. The declining population size was based on fossil abundance through time. None of the closed population models fit the data for *C. sociabilis*.

Temporal stability

We found no evidence for population or genetic change in *C. haigi* over thousands of years. The pattern of stasis in *C. haigi*, like in European rabbits (Hardy *et al.* 1995) and in bowhead whales (Poulsen *et al.* 2005), indicates that for some species, equilibrium can be maintained over millennia. Genetic temporal stability has also been observed in salmonid populations (Nielsen *et al.* 1999b; Tessier & Bernatchez 1999), leopard frogs (Hoffman *et al.* 2004), marine isopods (Lessios *et al.* 1994), and kangaroo rats (Thomas 1990) over annual or decadal time spans. Temporal stability is critical for interpreting population genetic studies. If populations are in genetic equilibrium and therefore demonstrate genetic stability over time, then the assumptions of most genetic models are fulfilled. Determining the factors responsible for stability or instability in a species has implications for the reliability of measurements taken from a single modern time point, and the subsequent measurements of gene flow, genetic structuring in space and reconstructions of demographic history (Lessios *et al.* 1994).

However, ancient DNA studies often find dynamic population histories, such as those found in bison (Shapiro *et al.* 2004), bears (Barnes *et al.* 2002; Valdiosera *et al.* 2008), mammoths (Gilbert *et al.* 2008), cave hyenas and neandertals (Hofreiter *et al.* 2004). In contrast to the lack of evidence for change observed in *C. haigi*, *C. sociabilis* showed a pattern of instability of haplotype frequencies over time with turnover of haplotypes and a large portion of the variance accounted for between temporal intervals rather than within intervals. Genetic instability and nonequilibrium dynamics over time have been found in species with metapopulation structure, such as snails (Arnaud & Laval 2004) and other small mammals (ground squirrels, O'Keefe *et al.* 2009 and voles, Hadly *et al.* 2004). The large amount of temporal variation in genetic diversity in *C. sociabilis* suggests that for this species, a single measurement of genetic diversity in time would not accurately represent the dynamics of the species. We rejected models of a single closed population and found that models incorporating migration were a better fit.

The contrasting patterns of temporal stability, stasis and equilibrium in *C. haigi*, and haplotypic turnover and instability in *C. sociabilis* could result from the differences in rarity and behaviour between these two parapatric ctenomyid species.

Rarity in Ctenomys sociabilis

The use of ancient DNA to track changes in genetic variation in *C. sociabilis* and its comparison to *C. haigi* provided a unique opportunity to examine a temporal dimension to rarity, yielding insight into the cause of the decline in *C. sociabilis* and its response to environmental change. Modern *C. sociabilis* possess the type of rarity typified by small population size, low abundance, high endemism and habitat specificity. The possible gene flow and migration observed in *C. sociabilis* could be a reflection of their current greater habitat specificity, lower dispersal and the resulting genetic population structure. It is also consistent with their current social structure, although it is unknown whether they were social in the past as they are now (Chan *et al.* 2006). It is important to note that the pattern observed here is from matrilineally inherited mitochondrial DNA. The social structure of *C. sociabilis* with its characteristics of female-biased philopatry, polygamy and female-biased sex ratio is the one that could increase the effective population size of the mitochondrial locus relative to an autosomal locus (Chesser & Baker 1996). Therefore, the levels of diversity observed here at cytochrome *b* may not reflect adaptive potential at the remainder of the genome. Whether the pattern we observe here is representative of the species as a whole needs to be confirmed with other loci, as well as from future comparisons of other sites within the species' range.

Fluctuation in relative abundance and reconstruction of effective population size for the two species revealed dynamic and individualistic responses to environmental change. The earliest temporal interval C (12 381–7574 cal yr BP) contained the highest relative abundance of *Ctenomys* spp. (Fig. 1) and our genetic analysis detected only *C. sociabilis*. Environmentally, the steppe habitat that both species prefer and that dominated dur-

Table 3 Model testing of top fifteen best models for *Ctenomys sociabilis* as well as negative log likelihood and ΔAIC values for best models with constant and declining population size and no migration. The top ten models show that we do not have the statistical power to distinguish between a constant or declining population with a high migration rate. However, closed populations with no migration are statistically worse (ΔAIC > 2)

Rank	Migration	PopSize	N_e	NoPops	K-parameters	Probability	−LN (likelihood)	AIC	ΔAIC
1	0.5	Constant	10 000	2	2	0.9979	0.002	4.004	0.00
2	0.75	Constant	20 000	2	2	0.9901	0.010	4.020	0.02
3	0.75	Constant	10 000	2	2	0.9838	0.016	4.033	0.03
4	0.25	Constant	10 000	2	2	0.9824	0.018	4.036	0.03
5	0.25	Constant	20 000	2	2	0.9744	0.026	4.052	0.05
6	0.75	Constant	10 000	3	2	0.7190	0.330	4.660	0.66
7	0.75	Constant	20 000	3	2	0.5811	0.543	5.086	1.08
8	0.5	Constant	20 000	2	2	0.4702	0.755	5.509	1.50
9	0.75	Declining	10 723*	2	3	0.9906	0.009	6.019	2.01
10	0.25	Declining	10 723*	2	3	0.9861	0.014	6.028	2.02
11	0.05	Constant	20 000	3	2	0.3619	1.016	6.033	2.03
12	0.5	Declining	10 723*	2	3	0.9628	0.038	6.076	2.07
13	0.75	Declining	5361†	2	3	0.7917	0.234	6.467	2.46
14	0.5	Declining	5361†	2	3	0.7872	0.239	6.479	2.47
15	0.5	Declining	20 000	3	3	0.7840	0.243	6.487	2.48
22	0	Declining	53 617‡	1	2	0.1194	2.126	8.251	4.25
45	0	Constant	100 000	1	1	0.0061	5.092	12.185	8.18

*Mean population size over time because of decline from initial N_e of 20 000.
†Mean population size over time because of decline from initial N_e of 10 000.
‡Mean population size over time because of decline from initial N_e of 100 000.

ing the late-glacial period declined from approximately 12 000 years before present as indicated by the increase in *Nothofagus* spp. pollen and probable expansion of the forest (Markgraf 1983). During Interval B (7274–6302 cal yr BP), relative abundance of *Ctenomys* spp. slightly increases (Fig. 1) and our genetic analysis revealed a time of inter- and intraspecific flux, with the first appearance of *C. haigi* and of a novel modern clade M in *C. sociabilis*. The pattern of haplotypic turnover in *C. sociabilis* and our modelling suggest gene flow between genetically differentiated populations. In *C. haigi*, despite an increase in relative abundance, we observed little change in genetic variation, with almost all of the variation (97%) accounted for within temporal intervals. For *C. haigi*, we could not reject a closed, constant, population model. A mid-postglacial interval occurred approximately 8500–5000 ybp, evidence for which was seen in the pollen record of Cueva Traful which was dominated by steppe habitat at this time (Heusser 1983; Markgraf 1983). It is possible the intra- and interspecific time of flux resulted from this short increase in favourable environmental conditions. In Interval A (4412–1894 cal yr BP), *Nothofagus* spp. again increased (Heusser 1983). From 2859 to 2053 ybp, volcanic eruptions from the Puyehue–Cordón Caulle complex covered the present range of *C. sociabilis* with between 10 and 200 cm of ash (Villarosa *et al.* 2006). Subsequent to these events, *C. sociabilis* disappeared from the Cueva Traful record while *C. haigi* returned and is found near the cave today.

Species traits such as widespread geographical range, wide niche breadth and morphological and behavioural simplicity have been found to increase resistance, defined as the 'ability of a species to remain at its current abundance despite an environmental change' (McKinney 1997). These are all traits that *C. haigi* possesses in contrast to *C. sociabilis* and, therefore, may have contributed to the resistance of *C. haigi* to environmental change through time.

Reconstruction of demography for *C. haigi* from serial coalescent modelling showed the best-fit effective population size to be 20 000 for Interval B (levels dated 7274–6302 cal yr BP) and 10 000 for Interval A (levels dated 4412–1894 cal yr BP). Although neither constant population size model was statistically rejected, the slight decline in genetic diversity provides some evidence that *C. haigi* may also have been responding to changes in the deteriorating environment with a decrease in population size, perhaps due to the probable contraction of steppe vegetation, and like *Thomomys talpoides* (Hadly *et al.* 1998) remained a closed population through this time.

The pattern in *C. sociabilis* is quite different. Overall genetic variability, as measured by total segregating sites and nucleotide diversity, was higher in ancient *C. sociabilis* than in ancient *C. haigi*. This is in contrast

to molecular studies at microsatellite loci for modern samples, which showed much greater diversity in *C. haigi* than *C. sociabilis* (Lacey 2001). Interval B (levels dated 7274–6302 cal yr BP) shows an increase in diversity because of the appearance of a novel clade (M clade). Our serial coalescent modelling indicates that this is unlikely to be due to an increase in census population size, but rather, it is concordant with migration between populations during this interval. The greater habitat specialization of *C. sociabilis* may have resulted in the greater genetic structuring over time (Kelley *et al.* 2000; Brouat *et al.* 2004), with migrants moving during periods of instability or climatic change (Hadly *et al.* 2004).

Sociality may also have contributed to greater genetic structuring. Subterranean rodents in general are solitary, similar to *C. haigi*, where males and females maintain exclusive territories (Lacey 2000). In modern populations of *C. sociabilis*, sociality is female kin-based with females being philopatric, and multiple adults (1–4 females, 0–1 males) sharing burrows (Lacey & Wieczorek 2004). All males, however, disperse from their burrows each year. This pattern of female philopatry and male dispersal is the most likely to maximize genetic differentiation among social groups within and between populations, especially for mitochondrial DNA, which detects only female genetic diversity (Storz 1999). Unusually high levels of intraspecific genetic variability have been found within different species of macaque monkeys (>3%) (Hoelzer *et al.* 1998), and high levels of intercolony divergence have been found in naked mole-rats (Faulkes *et al.* 1997), reportedly reaching as high as 5.8% at cytochrome *b.* These estimates are comparable to those found between the two clades in *C. sociabilis* of 2%. When structured populations and episodic migration were included in our models, the effective population size dropped from estimates of approximately 100 000 (Chan *et al.* 2005; Fig. 4) to estimates that are comparable to *C. haigi* of 10 000–50 000. Thus, these two species had very different genetic signatures, due in part to possible differences in population structure.

One hypothesis for the loss of genetic diversity in modern *C. sociabilis* was a series of volcanic eruptions from 2859 to 2053 cal yr BP (Montero & Silveira 1983; Villarosa *et al.* 2006) indicated by a tephra layer in Cueva Traful. *Ctenomys sociabilis* is not found in levels dated after 1950 cal yr BP, although *C. haigi* is. Estimates of the thickness of the volcanic ash for the region vary, but the deposits were probably quite thick in the vicinity of Cueva Traful (approximately 2 m), and clearly, there was enough ash in the immediate area to result in a tephra layer approximately 20 cm inside the cave itself (Villarosa *et al.* 2006). Situation of the geographic range of *C. sociabilis* at the foothills of the Andes makes this species more sensitive to eruptions from the volcanoes immediately to the west. These volcanic centres are still active. Recent eruptions from the same sources that erupted three millennia ago, the Puyehue–Cordón Caulle complex along the border of Chile and Argentina, covered inhabited areas to the south and east of the range of *C. sociabilis* with at least 20 cm of ash. The impacts on tucos of the recent eruption (June, 2011) are as yet unknown. However, volcanic events do impact this group of rodents. Volcanic ash from a 1988 eruption in the Chilean Andes caused a 91.3% reduction in population size of *C. maulinus brunneus*, with a profound influence on heterozogosity in the species (Gallardo *et al.* 1995). Using a parameter estimation approach, Chan *et al.* (2005) dated the timing of bottleneck in *C. sociabilis* to be approximately 1500 generations ago, and given a 1- to 2-year generation time, this is consistent with estimation of the 2859–2053 cal yr BP eruption (Villarosa *et al.* 2006). The genetic data are concordant with the environmental data. While *C. haigi* shows a possible decline in population size, it still persists across the river from the cave, whereas *C. sociabilis* is no longer in the vicinity of the cave. This may be due to the differences in the range of the species at the time of the eruption. While we cannot discern what the range sizes of these species were thousands of years ago, modern estimates indicate very different range size for *C. haigi* and *C. sociabilis*. For *C. haigi,* having a larger range and being widespread, may have reduced the correlation among individuals, enabling a disturbance in one part of range while another part remains unimpacted (McKinney 1997), and perhaps providing source populations for *C. haigi* to return to the vicinity of Cueva Traful. Species with larger ranges are more resilient in the face of stochastic environmental change.

Another hypothesis for the decline in genetic diversity from ancient to modern in *C. sociabilis* is competition from other ctenomyid species, in particular, *C. haigi.* However, this hypothesis is difficult to assess with our simulations and data. Although our data do not show that the effective population size for *C. haigi* has increased over time, as one might expect from one species outcompeting another, *C. haigi* first appears in the record during Interval B (levels dated 7274–6302 cal yr BP) coincident with a decline in *C. sociabilis*. Further research is needed from other caves in the area and also from species interaction studies of modern populations to help to tease apart these factors.

Conservation genetics of temporal stability

Conservation biologists often seek to make predictions for future population viability. With respect to population size, the larger the population size (Purvis *et al.*

2000) and less temporal variability (Inchausti & Halley 2003), the lower the extinction risk. For levels of genetic diversity, extinction risk is lower for higher genetic diversity based on the assumption that current levels of variation represent adaptive potential and that high diversity may buffer species from extinction (Lande 1988; Frankham *et al.* 2002). However, in addition to the amount of genetic variation, this study suggests temporal stability in genetic variation may also be an indication of population resilience. Neutral genetic variation that is stable over time suggests that levels of both migration and drift are relatively low. Stability increases the likelihood of local adaptation that can occur when selection is stronger than both migration ($m/s < 1$; Haldane 1930) and drift ($4N_e s > 1$; Endler 1986 i.e. Nielsen *et al.* 1999a), possibly enabling *C. haigi* to adapt and weather environmental change. In this case, despite lower overall diversity, we observe that the stable equilibrium population structure may be indicative of an ability to adapt to local conditions.

Conclusion

Here, we use ancient DNA to reveal changes in temporal genetic variation for two species of subterranean rodent that differ behaviourally and demographically today. Our results suggest that greater habitat specificity and sociality are linked to a pattern of haplotypic turnover, possibly foreshadowing the extreme loss of genetic diversity observed in modern populations of *C. sociabilis*. In contrast, *C. haigi*, a solitary species, demonstrates remarkable genetic stability over thousands of years. By incorporating historical demography and behaviour into studies of ancient DNA, we gain a temporal perspective on microevolutionary processes not possible from modern reconstructions. We also suggest that the temporal dynamics of genetic diversity can provide insight into our understanding of the impact of life history traits on evolution and adaptability (Lessios *et al.* 1994). The population decline and near extinction for *C. sociabilis*, despite its higher diversity in the past, highlights the importance of considering temporal stability as well as spatial structuring and levels of genetic variation when making predictions either in the future or past about species survival. Population susceptibility to environmental catastrophes such as volcanic eruptions, however, may play out purely based on the historical contingency of location and the sheer numbers of individuals. Comparison of ancient DNA between two species inhabiting the same area through several thousand years of environmental change provides a unique perspective on the demographic forces generating and maintaining variation.

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Y.L.C. is interested in applying genetic and modeling tools to animal conservation and to understand how populations have responded to past environmental change. E.A.H. uses community, species, population and genetic data from fossils to unravel how vertebrate animals have responded to events of the past 20 000 years in order to provide insights about how they will handle Earth's environmental challenges of the future.

Data accessibility

DNA sequences: Genbank accession numbers *C. sociabilis* (DQ402060–DQ402066, JN629090–JN629094) and *C. haigi* (GU433041–GU433046). Individual haplotypes for each sample and stratigraphic level are given in Table S1.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Sample information by stratigraphic level, sample ID, and genbank accession number (dates can be found in Table 1).

Fig. S1 A neighbor-joining tree based on p-distance of 232 bp of cytochrome-*b*.

Table S1 Individual haplotypes for each sample and stratigraphic level.

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