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Phylogeny and historical biogeography of African ground squirrels: the role of climate change in the evolution of *Xerus*

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Abstract

We used phylogenetic and phylogeographical methods to infer relationships among African ground squirrels of the genus *Xerus*. Using Bayesian, maximum-parsimony, nested clade and coalescent analyses of cytochrome *b* sequences, we inferred interspecific relationships, evaluated the specific distinctness of Cape (*Xerus inauris*) and mountain (*Xerus princeps*) ground squirrels, and tested hypotheses for historical patterns of gene flow within *X. inauris*. The inferred phylogeny supports the hypothesized existence of an 'arid corridor' from the Horn of Africa to the Cape region. Although doubts have been raised regarding the specific distinctness of *X. inauris* and *X. princeps*, our analyses show that each represents a distinct well-supported, monophyletic lineage. *Xerus inauris* includes three major clades, two of which are geographically restricted. The distributions of *X. inauris* populations are concordant with divergences within and disjunctions between other taxa, which have been interpreted as results of Plio–Pleistocene climate cycles. Nested clade analysis, coalescent analyses, and analyses of genetic structure support allopatric fragmentation as the cause of the deep divergences within this species.

Keywords: climate change, nested clade analysis, phylogeography, Sciuridae, southern Africa, *Xerus*

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Introduction

Africa has undergone several major episodes of climate change since the Pliocene (van Zinderen Bakker 1978; deMenocal 1995), which are likely to have caused shifts in vegetation. Several authors have hypothesized that an 'arid corridor' stretched from the Horn of Africa to the Cape of Good Hope during drier periods, providing a route by which xeric flora and fauna dispersed from central to southern Africa (Knoch & Schulze 1956; van Zinderen Bakker 1969, 1978). This hypothesis is supported by the disjunct distributions of several arid-adapted plant taxa found in both southern and eastern Africa (Werger 1978; Jürgens 1997).

The ground squirrel tribe Xerini (Sciuridae) is an example of an arid-adapted animal taxon with a disjunct distribution in Africa (Moore 1959). Two of the three extant genera in this tribe are endemic to Africa and are thought to derive from a single invasion in the Early to Middle Miocene (Mercer & Roth 2003). The Barbary ground squirrel, *Atlantoxerus getulus*, is restricted to the extreme northwest of Africa, while the genus *Xerus* is broadly distributed across the arid and semiarid regions of sub-Saharan and southwest Africa. The third member, the long-clawed ground squirrel (*Spermophilopsis leptodactylus*), occupies a range east of the Caspian Sea in central Asia (Wilson & Reeder 1993).

The four species making up the genus *Xerus* occupy two disjunct ranges separated by nearly 2000 km (Fig. 1). With the exception of *Xerus erythropus*, which is a widely distributed habitat generalist (Rosevear 1969; Linn & Key 1996), *Xerus* is restricted to arid semidesert and savannah habitats. The striped ground squirrel (*X. erythropus*) and the unstriped ground squirrel (*Xerus rutilus*) occupy overlapping ranges in sub-Saharan Africa, while the Cape ground squirrel (*Xerus inauris*) and the mountain ground squirrel (*Xerus princeps*) occupy overlapping ranges in southern Africa. Pleistocene changes in precipitation patterns may have fragmented arid habitats in southern Africa (Matthee & Flemming 2002), thus present patterns of genetic structure

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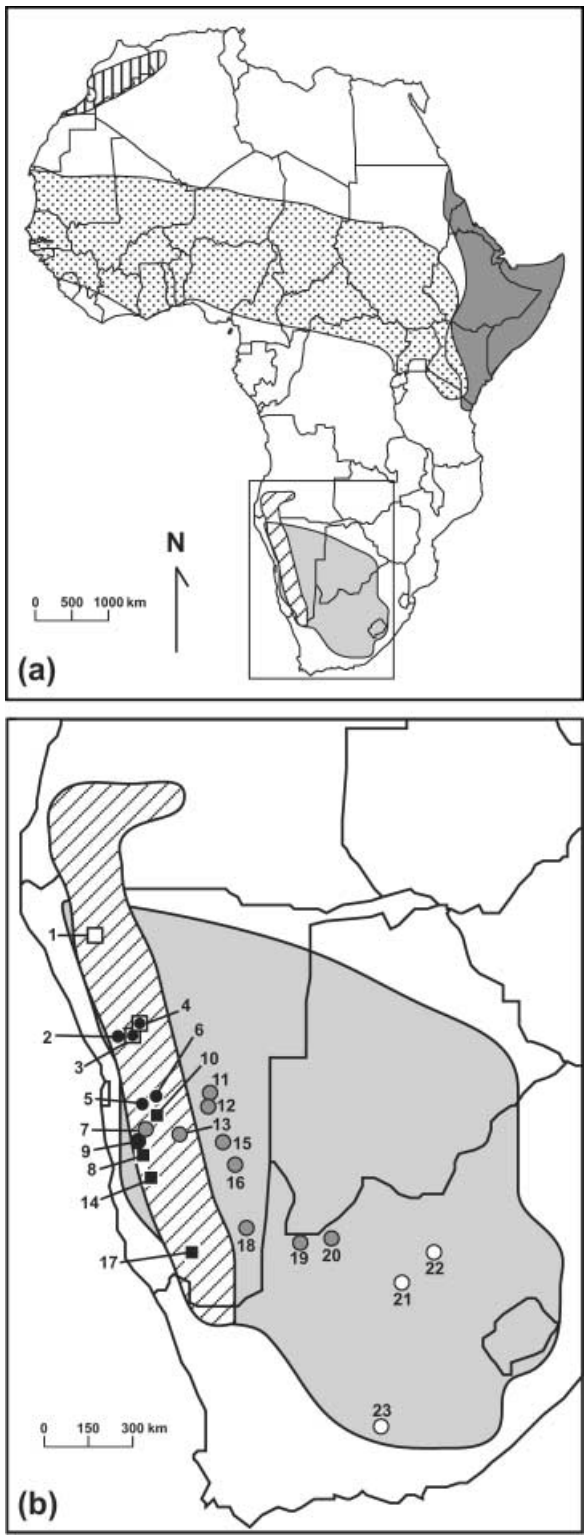

Fig. 1 (a) Map of Africa showing ranges of *Atlantoxerus getulus* (vertical stripes), *Xerus erythropus* (dots), *Xerus inauris* (light grey), *Xerus princeps* (diagonal stripes) and *Xerus rutilus* (dark grey). (b) Southern Africa with ranges and sampling localities for *X. inauris* (circles) and *X. princeps* (squares). Populations of *X. inauris* are indicated by black (northwestern), grey (central), and white (southeastern) circles. Clades of *X. princeps* are indicated by white (northern) and black (southern) squares. Note that both species were collected from localities three and four.

in arid-adapted taxa may have been significantly impacted by past isolation of populations in xeric refugia. Alternatively, current patterns of genetic structure may have been more heavily impacted by isolation in warm refugia during cold periods tied to glacial cycles (Jürgens 1997).

Little is known regarding the phylogeny of the Xerini, and several studies have presented conflicting hypotheses for their phylogenetic position within the Sciuridae (Pocock 1923; Moore 1959; Mercer & Roth 2003; Herron *et al*. 2004; Steppan *et al*. 2004). Denys *et al*. (2003) inferred a monophyletic genus *Xerus* with *X. erythropus* sister to the southern African species, based on osteological and dental characters. Their study was based on few characters, and the only relationship that was well supported was a sister group relationship between *X. inauris* and *X. princeps*. No study using molecular sequence data has explored the relationships within or among the four species of the genus *Xerus*.

Questions have been raised regarding the specific distinctness of *X. inauris* and *X. princeps* (de Graaff 1981; Robinson *et al*. 1986), and Allen (1939) considered *X. princeps* a subspecies of *X. inauris*. A single dental synapomorphy (entoconid on m1–m2 oblique to longitudinal) distinguishes *X. inauris* from *X. princeps* in the analysis of Denys *et al*. (2003), and they are so similar in appearance that they are difficult to distinguish in the field (Herzig-Straschil & Herzig 1989). Although *X. princeps* generally prefers rockier, more mountainous terrain, its habitats overlap those of *X. inauris*, and Haim *et al*. (1987) reported capturing individuals of both species from burrows within 200 m of each other. It is not known what mechanisms keep them separated when living in this proximity (Herzig-Straschil & Herzig 1989).

In spite of their morphological similarities and overlapping ranges, *X. inauris* and *X. princeps* exhibit strikingly different social behaviours. The mountain ground squirrel is essentially asocial (Herzig-Straschil & Herzig 1989), while both male and female Cape ground squirrels are highly social (Waterman 1995). This dramatic difference in social systems provides an opportunity to investigate the potential adaptive significance of social behaviours. Interpreting the results of such an investigation will, however, require an understanding of the evolutionary relationship between the two taxa.

Resolution of the taxonomic validity of *X. inauris* and *X. princeps* also carries relevance for conservation. *Xerus princeps* is uncommon (Griffin 1999) and restricted to the western escarpment (Fig. 1). This narrow strip of land running from southern Angola through Namibia to northwestern South Africa is high in endemism of birds (Robertson

Table 1 Primers used in this study

Primer name	Reference	Sequence
L14724	Irwin *et al*. (1991)	5′-TYTYCWTYTTNGGTTTACAARAC-3′
H15915	Irwin *et al*. (1991)	5′-TGAAAAAYCATCGTTGT-3′
XerusREV	This study	5′-TTNGGTTTACAAGACCAAGT-3′
HtRNA-GluScB	This study	5′-TTCATTWYWGGTTTACAAGACCARAG-3′
LtRNA-ThrScB	This study	5′-ACYAATGACATGAAAAATCATCGTTG-3′
XerusRctb	This study	5′-CAGAAGGATATYTGYCCYCATGG-3′
XerusFctb	This study	5′-TGAGGRCAAATATCCTTCTGAGG-3′

et al. 1998) and plants (Maggs *et al*. 1998). Barnard *et al*. (1998) recommended protecting the Namibian portion. If *X. princeps* is a unique evolutionary lineage, its restricted distribution through this region would further underscore the western escarpment as a priority for conservation.

Here, we (i) infer the evolutionary relationships among the four currently recognized species of the genus *Xerus*, (ii) investigate the historical biogeographical patterns that have led to the current distributions of *Xerus* species, (iii) investigate the roles of gene flow and climate change in the phylogeographical and demographic history of *X. inauris*, and (iv) assess the validity of the specific distinction between *X. inauris* and *X. princeps* by testing whether or not they represent distinct, reciprocally monophyletic lineages.

Materials and methods

Sampling

We collected *Xerus inauris* and *Xerus princeps* (Fig. 1) by live trapping using Tomahawk 15 × 15 × 48 cm traps and either plucked several hairs with tweezers or clipped 2–3 mm of the tail tip with sterile scissors. Additional samples from road kills were opportunistically collected by removing muscle and/or liver tissue with sterile scissors. All samples were preserved in 95% ethanol. For two *Xerus erythropus* (Xe 113 and Xe 114) and one *X. inauris* (Xi 115), we used 5 × 5 mm pieces of tissue from dried museum specimens from the Museum of Vertebrate Zoology, University of California, Berkeley (MVZ 186199, MVZ 101021, and MVZ 117287, respectively). For one *X. erythropus* (Xe 67), we received blood drawn from a live captive animal from the Baltimore Zoo (BZ91116). We vouchered two *X. inauris* (Xi 55 and Xi 57) and one *X. princeps* (Xp 24) to the National Museum of Namibia and one *X. inauris* (Xi 76) to the McGregor Museum, Kimberly.

DNA extraction, amplification and sequencing

We extracted total DNA from tissue, blood, and most hair samples using standard proteinase K digestion followed by phenol–chloroform–isoamyl alcohol (PCI) organic separation methods as described in Sambrook & Russell (2001). We extracted the remaining hair samples using 5% Chelex-100 as described in Goossens *et al*. (1998). We PCR amplified the cyt *b* gene (Saiki *et al*. 1988) using combinations of the following primers (Table 1): L14725 (Irwin *et al*. 1991), H15915 (Irwin *et al*. 1991), XerusREV (this study), HtRNA-GluScB (this study), and LtRNA-ThrScB (this study). As a secondary evaluation of our sterilization and DNA extraction protocols for hair samples, DNA from two individuals (Xi 55 and Xi 57) was extracted, amplified, and sequenced separately from hair and tissue samples.

We extracted DNA from dried museum skins using PCI methods after soaking overnight in TE buffer (10 mM Tris, 1 mM EDTA). Full-length cyt *b* products could not be amplified from museum specimen DNA, so we amplified a smaller (*c*. 420 bp) fragment using the primers L14725 and XerusRctb (this study; Table 1).

We PCR amplified template DNA using an initial denaturation at 94 °C for 3.75 min; 45 cycles of denaturation at 94 °C for 1 min, annealing at 45–60 °C for 1 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 7 min. PCR products were excised from 1.4% agarose electrophoresis gels and purified using the MinElute Gel Extraction Kit (QIAGEN). When necessary, purified PCR products were cloned with the TOPO TA Kit (Invitrogen), and plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN). We sequenced purified PCR products in both directions using the amplification primers and the internal primers XerusFctb (this study; Table 1) and XerusRctb. We sequenced purified plasmid DNA with external M13 primers and internal primers XerusFctb and XerusRctb. All sequencing reactions were run using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter), electrophoresed on a Beckman CEQ8000 automated sequencer, according to the manufacturer's protocols.

Phylogenetic analyses

We edited raw sequence chromatographs in SEQUENCHER 4.1.2 (Gene Codes Corp.). For specimens sequenced from plasmid DNA, sequences were compiled from the consensus

of three independent clones. All sequences were aligned manually in GENEDOC 2.6.002 (Nicholas & Nicholas 1997), translated to amino acid sequences to confirm alignment, and checked for stop codons or shifts in open reading frame which may indicate amplification of nuclear pseudogenes.

We were only able to amplify and sequence a portion of the cyt *b* gene for the three *X. erythropus* samples. To ensure that missing data did not noticeably bias phylogenetic estimates including incomplete sequences, we constructed two data sets: (i) the full data set, which included all available sequence data for all specimens sequenced (1140 aligned positions), and (ii) a reduced data set, which included 420 bp of the 5′ end of the cyt *b* gene.

We estimated phylogenetic relationships using both Bayesian Metropolis-coupled Markov chain Monte Carlo (MCMC) and maximum-parsimony (MP) phylogenetic methods. A cyt *b* sequence from *Spermophilopsis leptodactylus* (GenBank Accession no. AF157865), the hypothesized sister group to the African Xerini (Mercer & Roth 2003) was the outgroup for all analyses. MODELTEST version 3.0 (Posada & Crandall 1998) was used to infer the simplest, best-fit model of evolution for the MCMC analyses based on hierarchical log-likelihood-ratio tests comparing successively more complex models (Huelsenbeck & Crandall 1997; Posada & Crandall 2001). All MCMC phylogenetic reconstructions were conducted in MRBAYES version 3.0b4 (Ronquist & Huelsenbeck 2003) with vague priors and model parameters estimated as part of the analyses as per the program's defaults. We used three heated chains and a single cold chain in all MCMC analyses, and initiated runs with random trees. We sampled trees every 100 generations and assembled majority rule consensus phylograms and posterior probabilities (PP) for nodes from all sampled post burn-in trees. We conducted six independent MCMC runs with 3.5 million generations per run for both full and reduced data sets. The first 500 000 generations from each run were discarded as burn-in. Convergence of posterior probability estimates among runs was tested by comparing posterior probability estimates from subsets of the sampled trees using the 'Comparetrees' function in MRBAYES. Runs were considered to have adequately sampled solution space if posterior probability estimates were 99% correlated between each pair of runs (Smith & Farrell 2005).

We conducted equally weighted MP searches in PAUP* version 4.0b10 (Swofford 2002) with gaps treated as missing. We used a heuristic search strategy to search for optimal MP trees with 200 random taxon addition sequence replicates and starting trees obtained via stepwise addition. Settings for MP analyses included tree-bisection–reconnection branch swapping, steepest descent off, and MULTREES option on (Swofford 2002). Nodal support for the full data set was assessed with 500 bootstrap (BP) pseudoreplicates with 10 random addition sequence replicates per BP pseudoreplicate. Nodal support for the reduced data set was assessed with 1000 pseudoreplicates, each with 200 random addition sequence replicates. Additional settings employed for the bootstrap analysis were identical to those described above. We tested the molecular clock hypothesis using a likelihood-ratio test (Felsenstein 1981) implemented in PAUP* 4.0b10 (Swofford 2002).

Nested cladistic analysis of Xerus inauris

We constructed a statistical parsimony network for *X. inauris* using a 95% confidence limit in TCS version 1.13 (Clement *et al*. 2000). We excluded incomplete sequences (haplotypes F, M, O, and AA) from the nested cladistic analysis. We nested the resulting haplotype network following the procedures outlined in Templeton *et al*. (1987), Templeton & Sing (1993) and Crandall (1996). We performed nested contingency analysis and nested geographical distance analysis using GEODIS version 2.0 (Posada *et al*. 2000) and interpreted the results using the inference key of Templeton (2004).

Genetic diversity and genetic structure of Xerus inauris

We used the ARLEQUIN 2.000 software package (Schneider *et al*. 2000) to estimate genetic diversity within and among groups of *X. inauris* and to infer population genetic structure. We assigned sampling localities to populations (populations 1–3 in Fig. 1) based on a general correspondence with clades recovered in phylogenetic analyses (clades I–III in Fig. 2) and tested the inferred genetic structure using an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) and an exact test of population differentiation (10 000 permutations; Raymond & Rousset 1995). We estimated geographical distances between pairs of sampling localities using great circle distance (Kern & Bland 1948) and used a Mantel test (100 000 permutations; Mantel 1967) to evaluate correlation between genetic and geographical (ln km) distance matrices in ARLEQUIN (Schneider *et al*. 2000).

We estimated absolute numbers of female migrants exchanged between pairs of populations assuming migration–drift equilibrium using the formula $M_f = N_f m_f = (1 - \Phi_{ST})/2\Phi_{ST}$ with Φ_{ST} values estimated using the substitution model of Tamura & Nei (1993) in ARLEQUIN. We obtained coalescent-based estimates of effective population size ($\Theta = 2\mu N_f$) and migration rates ($M = 2mN_f$) between each pair of populations using MIGRATE 1.7.6 (Beerli & Felsenstein 2001). For this analysis, we estimated the transition/transversion ratio using maximum likelihood in PAUP* (Swofford 2002) and used empirical base frequencies, Watterson (1975) estimate of initial theta, and the heating option with temperatures of 1.0, 1.2, 1.5 and 3.0. We initially performed three independent runs in MIGRATE, each with 10 short chains of 5000 sampled genealogies, three long chains of

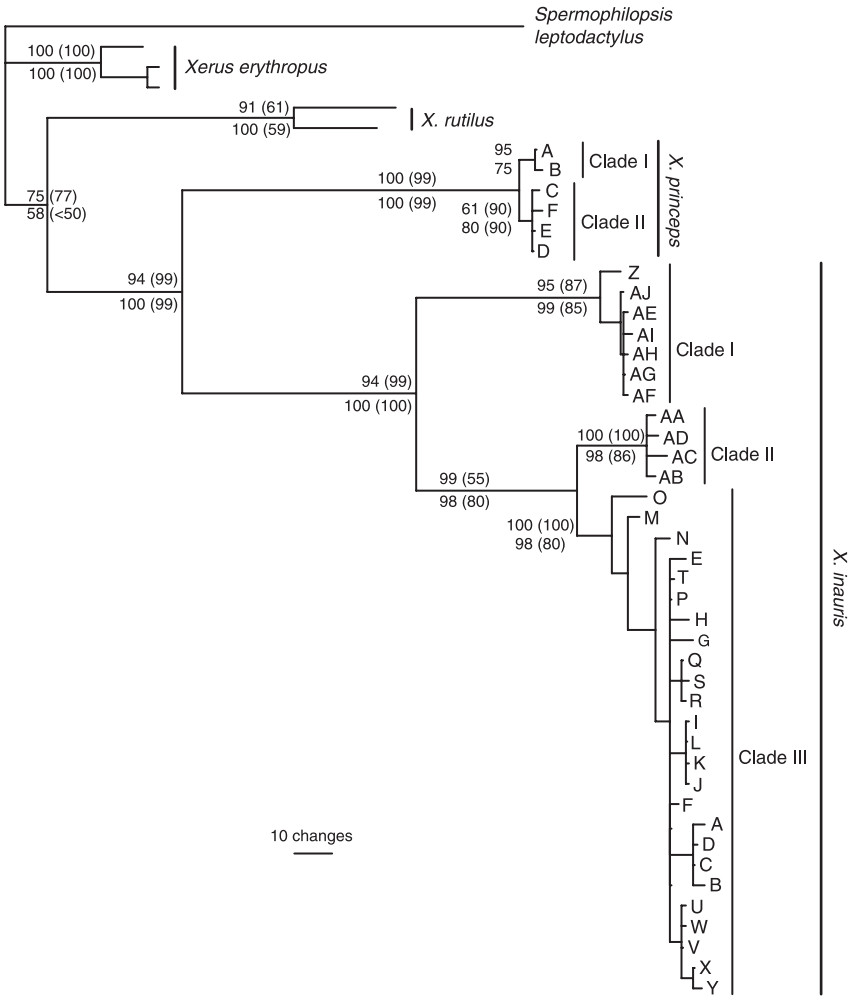

Fig. 2 Phylogram resulting from maximum-parsimony phylogenetic analysis of the full (1140 bp) data set. Posterior probabilities (PP) estimated from a total of 18 million post burn-in generations (from six independent MCMC runs) are indicated above bars. Bootstrap (BP) support values from 500 pseudoreplicates are indicated below branches preceding nodes. Corresponding PP and BP values for the reduced data set are shown in parentheses. Vertical bars identify major clades of *Xerus princeps* and *Xerus inauris*. This is one of many equally parsimonious trees.

50 000 sampled genealogies, and a sampling increment of 100. We obtained final estimates from a single long run with 10 short chains of 50 000 sampled genealogies and three long chains of 500 000 sampled genealogies.

Historical demography of Xerus inauris

We plotted the frequencies of nucleotide differences between pairs of haplotypes (mismatch distributions) globally and for each clade using ARLEQUIN to evaluate the hypothesis of recent population growth. Fit to the expectations of the stepwise expansion model was tested using the sum of squared deviations between the observed and expected mismatch from 1000 parametric bootstrap replicates. We also used ARLEQUIN to estimate parameters $\Theta_0 = 2\,\mu N_0$ (before expansion), $\Theta_1 = 2\,\mu N_1$ (after expansion), and $\tau = 2ut$ (time of expansion) for a stepwise growth model using a nonlinear least squares approach.

We tested the hypothesis of exponential growth globally and for each population using an MCMC approach in FLUCTUATE 1.4 (Kuhner *et al*. 1998). For this analysis, we used the same estimate of transition/transversion ratio described earlier for the MIGRATE analysis and empirical base frequencies. We checked for consistency of results by performing three independent runs for each analysis and obtained maximum-likelihood estimates of effective population size ($\Theta = 2\mu N_f$) and exponential growth rate (g) from final FLUCTUATE runs with five short chains of 1000 steps each, three long chains of 15 000 steps each, and a sampling increment of 20. We tested the significance of g in each case using a likelihood-ratio test comparing the log-likelihood of a model in which g was allowed to vary to that of a model in which g was constrained to zero.

We compared the results of three methods implemented in ARLEQUIN to estimate the time of divergence between each pair of populations. We used the method of Gaggiotti and Excoffier (Gaggiotti & Excoffier 2000) to estimate $\tau = 2Tu$, where T = generations and u = per sequence mutation rate. For this and all subsequent time estimates, we used a mutation rate estimate of 4% per million years (Myr) (Arbogast

& Slowinski 1998; Arbogast 1999). We estimated corrected mean molecular diversities between each pair of clades using $D_{xy} = D - 0.5(D_x + D_y)$ (Edwards 1997; Zheng *et al*. 2003), with mean nucleotide diversities within and between clades estimated using the substitution model of Tamura & Nei (1993) and gamma parameter estimated using maximum likelihood in PAUP* (Swofford 2002). Finally, we estimated divergence times based on corrected means of pairwise differences between clades using the substitution model of Tamura & Nei (1993) with gamma parameter estimated using maximum likelihood in PAUP* (Swofford 2002).

To test the hypothesis that the genetic structure among the three *X. inauris* populations we defined resulted from allopatric fragmentation, we used a coalescent simulation (Knowles 2001; Knowles & Maddison 2002; Kotlík *et al*. 2004) implemented in MESQUITE, version 1.04 (Maddison & Maddison 2004). To test the null hypothesis of no population fragmentation, we simulated a null distribution of 10 000 trees by neutral coalescence. The simulated trees were contained within the alternative model of three ancestral refugia and the number of deep coalescences was measured. The number of deep coalescences in the reconstructed gene tree was compared to the null distribution, and the null hypothesis was rejected if less than 5% of the simulated trees had a number of deep coalescences equal to or less than that of the reconstructed tree. We repeated the test for a range of branch lengths (time in generations since population divergence; 10^3, 10^4, 10^5 and 10^6) and effective population sizes (10^2, 10^3, 10^4, 10^5 and 10^6).

Results

We obtained partial or complete cyt *b* sequences for 3 *Xerus erythropus*, 66 *Xerus inauris*, 9 *Xerus princeps* and 2 *Xerus rutilus* (Appendix). Separate extractions from hair and tissue samples of two individuals in each case yielded two identical full-length sequences. We found 36 haplotypes among *X. inauris* (Table 2) and 6 among *X. princeps* (Table 3). Individuals identified as *X. inauris* never shared haplotypes with those identified as *X. princeps*.

Indels and internal stop codons were not found within the 1140 aligned bases in the full data set. Of the 1140 positions, 762 were invariant, 153 variable characters were parsimony uninformative, and 225 were parsimony informative. Uncorrected pairwise sequence divergences within and among species are shown in Table 4.

Phylogenetic analyses

The simplest, best-fit model for both full and reduced data sets was TVM + G, a restriction of the GTR model with a gamma distribution of among-site rate variation. Because MRBAYES is limited to models with one, two, or six base substitution rate matrices, we used the GTR + G model for both sets of MCMC analyses (TVM has five parameters).

All six independent MCMC runs for the full data set converged with regard to chain likelihood scores and topology (50% majority rule consensus identical among runs). Posterior probability estimates for clade support were 99% correlated in each of the 10 pairwise comparisons between runs by the 500 000th generation. Estimates of substitution model parameters for all six independent runs also converged at stationarity. Based on this evidence for convergence among runs, we pooled all post burn-in trees (representing a total of 18 million post burn-in generations) to estimate the posterior probabilities for clades.

Equally weighted MP reconstruction for the full data set resulted in 363 747 equally parsimonious trees consisting of 568 steps with CI = 0.7764, RI = 0.9225, RC = 0.7162, and HI = 0.3128. The MCMC and MP analyses of the reduced data set yielded an interspecific topology identical to those based on the full data set, and we therefore base our phylogenetic discussion on the results of the full data set. The molecular clock hypothesis was rejected by the likelihood-ratio test ($\chi^2 = 77.838$; d.f. = 46; $0.001 < P < 0.01$).

A maximum-parsimony phylogram showed the most basal divergence within *Xerus* was between *X. erythropus* and the remaining taxa (Fig. 2). The sister group to (*X. inauris* + *X. princeps*) was inferred to be *X. rutilus* (PP = 75, BP = 58). *Xerus inauris* was resolved as the sister to *X. princeps* (PP = 94, BP = 100), and each was resolved as an exclusive monophyletic group (PP = 94, BP = 100 for *X. inauris*; PP = 100, BP = 100 for *X. princeps*). Within *X. princeps*, a northern (PP = 95, BP = 75) and a southern (PP = 61, BP = 90) clade were resolved. Three major clades were resolved within *X. inauris:* clade I (PP = 95, BP = 99), restricted to west-central Namibia; clade II (PP = 100, BP = 98), restricted to South Africa east and south of the Kalahari, and clade III (PP = 100, BP = 98), widely distributed through the Kalahari and overlapping the distributions of clades I and II (Fig. 2).

Nested clade analysis

The statistical parsimony haplotype network for *X. inauris* had a 95% parsimony limit of 14 steps (Fig. 3). All haplotypes were included within four three-step clades (3-1 through 3-4 in Fig. 3), of which only 3-3 and 3-4 were connected. Clades 3-1 and 3-2 correspond exactly to clades I and II recovered in the phylogenetic analyses, respectively (Fig. 2). Clades 3-3 and 3-4 correspond to clade III from the phylogenetic analyses, and clade 3-4 corresponds to a subclade of clade III. Seven nested haplotype groups, including the total cladogram, were significantly associated with geography (Table 5). All inferences for lower-level haplotype groups were inconclusive, and geographical sampling was inadequate to infer a pattern for clade 3-4. Within clade 3-3,

Table 2 Haplotype frequencies of *Xerus inauris* by locality

		Haplotype																																					
Locality	Location	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	Total	Lat.	Long.
2	Namib																															1	1	2	1	1		6	–21.803	15.177
3	Ameib 1								1								2																	1				4	–21.803	15.584
4	Ameib 2																1																					1	–21.776	15.655
5	Berghoff																										1										1	2	–23.360	16.000
6	Groot Hakos						1																															1	–23.243	16.299
7	Weldevrede																	6	1	1																		8	–24.177	15.979
9	Oorwinning																	2																				2	–24.395	15.898
11	Eschenhof										4																											4	–23.342	18.015
12	Christirina	1	1	7	1	1		1		1		1	2																									16	–23.413	18.000
13	Namib Rand																								3	1												4	–25.379	16.075
15	Osterodenord																							2														2	–24.382	18.475
16	Gochas																						2															2	–24.998	18.883
18	Gross Aub															1						1																2	–26.716	19.398
19	Kingsrest														1							1	1															3	–27.025	21.054
20	Van Zylsius																				2																	2	–26.890	22.065
21	Benfontein																												2									2	–28.370	24.398
22	SA Lombard													1																1	2							4	–27.614	25.478
23	Vrede																											1										1	–32.242	24.087
	Total	1	1	7	1	1	1	1	1	1	4	1	2	1	1	1	3	8	1	1	2	2	3	2	3	1	1	1	2	1	2	1	1	3	1	1	1	66		

Table 3 Haplotype frequencies of *Xerus princeps* by locality

		Haplotype								
Locality	Location	A	B	C	D	E	F	Total	Lat.	Long.
1	Hobitere	1						1	–19.330	14.375
3	Ameib		1					1	–21.803	15.584
4	Ameib 2		1					1	–21.776	15.655
8	Losberg			1	1			2	–25.046	16.093
10	Nauzerus				1	1		2	–23.872	16.410
14	Kalkrand				1			1	–24.493	17.197
17	Fish River Canyon						1	1	–27.635	17.756
		1	2	1	3	1	1	9		

Table 4 Uncorrected pairwise sequence divergence within and among xerine species

	Minimum	Mean	Maximum
Spermophilopsis vs. *Xerus*	0.182	0.201	0.214
X. erythropus vs. other *Xerus*	0.128	0.153	0.167
X. rutilus vs. *X. inauris* + *X. princeps*	0.103	0.123	0.145
X. inauris vs. *X. princeps*	0.059	0.075	0.122
X. inauris		0.018	0.039
X. princeps		0.006	0.011

Table 5 Nested cladistic analysis results for *Xerus inauris*

Clade	χ^2 statistic	Probability	Inference chain	Inferred pattern*
1-7	6.000	0.0560	1-2-11-17-No	Inconclusive
1-9	5.000	0.2040	1-19-20-2-11-17-No	Inconclusive
1-10	0.625	1.0000	1-2-11-17-No	Inconclusive
1-19	8.400	0.7320	1-2-11-17-No	Inconclusive
3-1	42.000	< 0.0001	1-19-20-No	IGS
3-2	90.900	0.0010	1-2-3-4-9-No	AF
Cladogram	155.930	< 0.0001	1–2-No	Inconclusive
Cladogram†			1-2-3-5-15-No	AF

*AF, Allopatric fragmentation; IGS, insufficient geographical sampling. †Treating clade 3-3 as internal.

allopatric fragmentation was inferred between clade 2-8 (restricted to localities 7 and 9) and clade 2-10 (restricted to localities 11 and 12). The inference for the total cladogram was inconclusive unless clade 3-3 was treated as an interior clade (see Discussion), in which case past habitat fragmentation was inferred.

Genetic diversity and genetic structure of Xerus inauris

We observed 110 substitutions distributed among 108 variable sites within the cyt *b* gene of *Xerus inauris*, of which 94 were transitions and 16 transversions (ti/tv = 5.875). Twenty-five of the substitutions were nonsynonymous, while the rest were synonymous ($K_a/K_s = 0.3012$).

Genetic diversity was highly structured among the three populations we defined. Clade I (see Fig. 2) consisted entirely of individuals from the northwest population, clade II entirely of individuals from the southeast population, and clade III mostly of individuals from the central population. Only six individuals of four haplotypes (F, H, M, and P) from the other populations appeared in clade III, and no haplotype appeared in more than one population. Of the total genetic diversity within *X. inauris*, 56.95% was partitioned among the populations, 22.51% among sampling localities, and 20.54% within sampling localities (AMOVA; $P \leq 0.00001$). All pairwise contrasts of populations were significant (after Bonferroni correction for multiple testing) based on Φ_{ST} values (all $P < 0.00001$). Pairwise contrasts between the northwest and central populations and between the central and southeast populations were significant based on the exact test of population subdivision (both $P < 0.00001$), but the contrast between the northwest

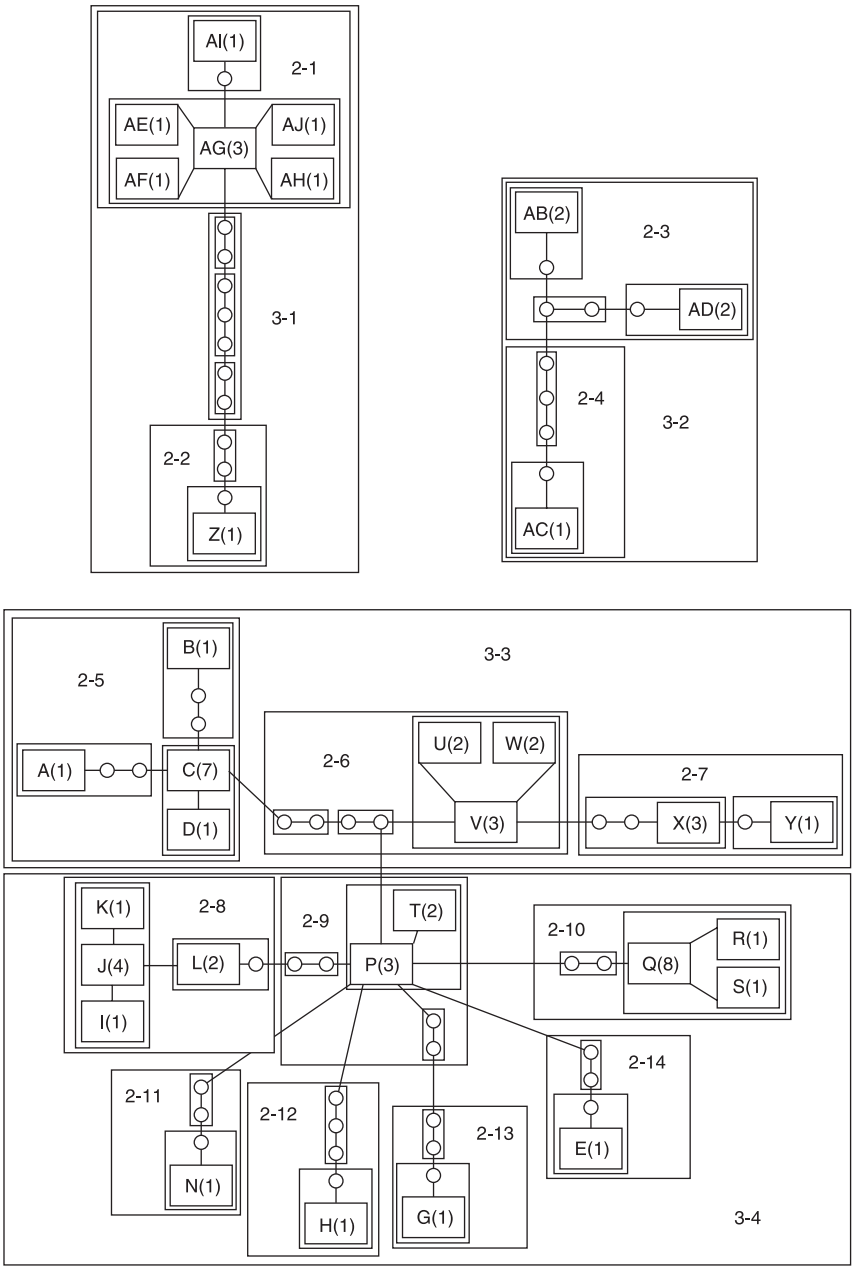

Fig. 3 Nested clade design for the unrooted network of full-length (1140 bp) *Xerus inauris* cyt *b* haplotypes, inferred using statistical parsimony. Incomplete sequences (haplotypes F, M, O, and AA) were excluded from this analysis. Lines represent a single mutational step, with missing haplotypes represented by small circles. Number of individuals represented by each haplotype is given in parentheses. Second and third level clades are labelled 2-1, 3-1, etc.

and southeast populations was not significant after correction for multiple comparisons ($P = 0.02465$; corrected α for three pairwise comparisons = 0.01695). Genetic and geographical distance were significantly correlated based on the Mantel test ($P = 0.05643$).

The two methods used to estimate numbers of female migrants between pairs of populations gave conflicting results (Table 6). Estimates based on pairwise distances show non-negligible numbers of migrants between all three pairs of populations. Coalescent-based estimates show non-negligible migration only between populations 1 and 3 (both directions) and from population 2 to population 1.

Historical demography of Xerus inauris

The histogram of the mismatch distribution for all samples of *X. inauris* shows a trimodal distribution (Fig. 4a). Mismatch distributions of the individual clades are shown as Fig. 4b–d. None of the mismatch distributions are significantly different from the expectations of the stepwise expansion model (all $P > 0.3$), but only that of clade III (Fig. 4d) appears to have a unimodal distribution. All estimates of Θ_1 derived from the stepwise growth model were at least several times higher than the corresponding Θ_0 values (Table 7).

Table 6 Estimates of numbers of migrants between *Xerus inauris* populations based on (a) pairwise distances and (b) coalescent analysis

(a) Estimates based on pairwise distances

		Migration rate ($M_f = N_f m_f$)		
Population	n	1	2	3
1	14	—	—	—
2	45	0.30616	—	—
3	7	0.57415	0.22443	—

(b) Maximum-likelihood estimates from coalescent analysis

		Migration rate ($2mN_f$; × = receiving population)		
Population	Effective population size ($\Theta = 2\mu N_f$)	1,x	2,x	3,x
1	0.01025	—	1.2877	4.8×10^{-12}
2	0.01013	3.2×10^{-12}	—	0.1022
3	0.00099	2.3×10^{-15}	0.9776	—

Table 7 Estimated parameters of population expansion for (a) exponential and (b) stepwise expansion models for *Xerus inauris*

(a) Exponential expansion model

Clade	No. of individuals	No. of haplotypes	$\Theta = 2\mu N_f$ (± SD)	g (± SD)
1	9	7	0.0085 (0.0025)	303.73 (137.59)
2	6	4	0.0146 (0.0062)	517.11 (209.32)
3	51	25	0.0373 (0.0038)	509.06 (69.40)
Total	66	36	0.0417 (0.0046)	126.63 (33.57)

(b) Stepwise expansion model

Clade	$\tau = 2ut$ (95% CI)	Θ_0 (95% CI)	Θ_1 (95% CI)
1	2.027 (0.000, 3.569)	0.000 (0.000, 1.987)	2025.00 (10.015, 7007.500)
2	5.967 (2.381, 11.092)	0.000 (0.000, 4.633)	14.22 (2.798, 6641.719)
3	8.059 (4.336, 11.364)	0.000 (0.000, 2.958)	26.00 (14.629, 3765.995)
Total	4.476 (0.798, 24.552)	9.145 (0.000, 24.462)	35.71 (20.120, 623.838)

Among the g values estimated in FLUCTUATE, only those of clade III and the total population were significantly different from zero according to likelihood-ratio tests (Table 7). Maximum-likelihood estimates of g were positive in both cases, indicating exponential growth of the populations.

Table 8 Divergence time estimates for *Xerus inauris* clades based on (a) mean molecular diversities (b) corrected mean pairwise differences, and (c) τ

(a) Mean nucleotide diversity between clades below diagonal, estimated divergence time (years) above diagonal

Clade	1	2	3
1	—	310 000	370 000
2	0.0231	—	210 000
3	0.0279	0.0156	—

(b) Corrected mean pairwise difference between clades below diagonal, estimated divergence time (years) above diagonal

Clade	1	2	3
1	—	390 000	450 000
2	33.0052	—	280 000
3	38.6256	23.9160	—

(c) $\tau = 2Tu$ below diagonal, estimated divergence time (generations) above diagonal

Clade	1	2	3
1	—	330 000	390 000
2	27.9048	—	210 000
3	32.9199	17.8707	—

Estimates of divergence times resulting from corrected mean molecular diversities and corrected means of pairwise differences were broadly similar (Table 8). Estimates based on τ were also similar to those of the other methods assuming a generation time of approximately 1 year (Table 8). In each case, the oldest inferred divergence time was between clades I and III and the youngest between clades II and III.

In the coalescent simulations, the upper bound of the bottom 5% of the number of deep coalescences ranged from 22 to 31. Four deep coalescences were observed in the reconstructed tree. No combination of branch lengths and effective population sizes included four deep coalescences above the bottom 5%, and the null hypothesis of no population fragmentation was therefore rejected under all sets of conditions.

Discussion

Our results indicate deep divergences among the four described species of *Xerus* consistent with the former existence of an arid corridor extending from the Horn of Africa to the Cape of Good Hope (Knoch & Schulze 1956; van Zinderen Bakker 1969, 1978). The sister group relationship

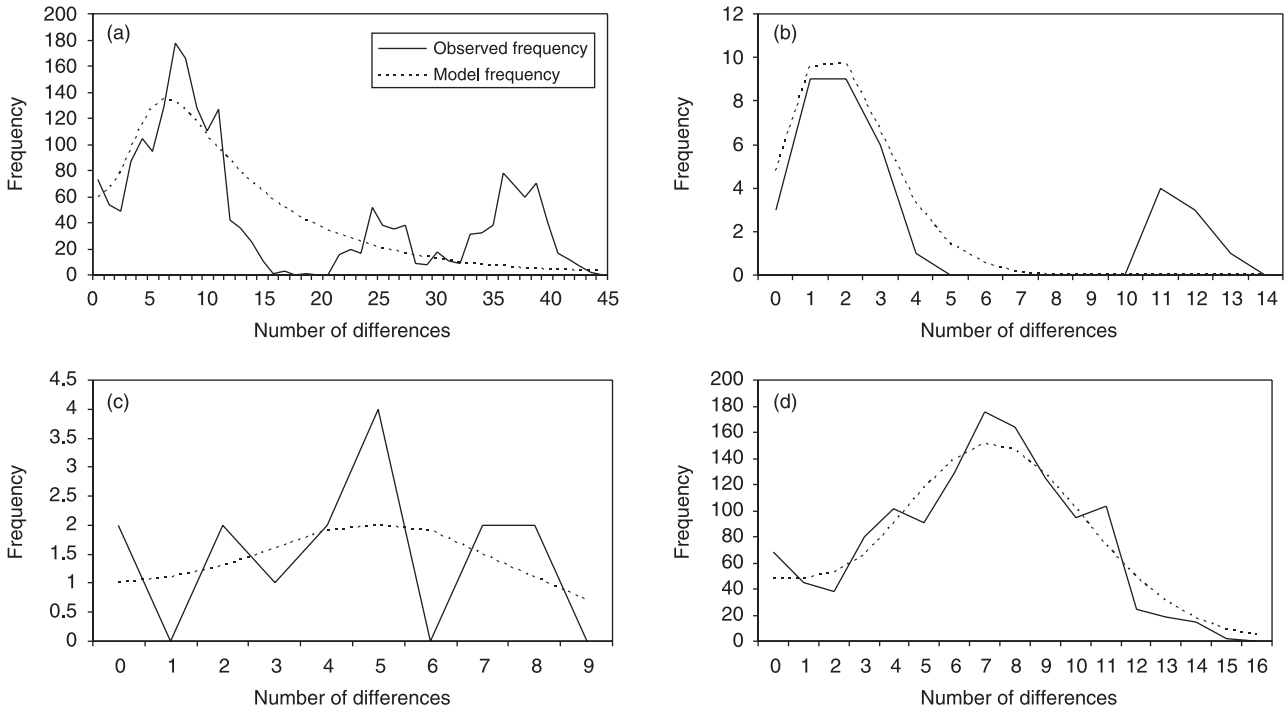

Fig. 4 Nucleotide mismatch frequency distributions for observed data and stepwise expansion model: (a) all *Xerus inauris* samples, (b) clade I, (c) clade II, and (d) clade III.

between *Xerus rutilus* and the southern African species is consistent with the pattern of disjunctions between the arid-adapted plants of the southwest arid zone and eastern Africa (De Winter 1971; Werger 1978). Unfortunately, because the likelihood-ratio test indicated that the cyt *b* gene has not evolved in a clock-like manner in *Xerus*, we cannot infer meaningful estimates of divergence times to relate to climatic events.

Relationships among species of *Xerus* are generally congruent with several characters, including number of mammae (three pairs in *Xerus erythropus*, two pairs in the other species, Moore 1961), bacular morphology (Moore 1959), dental formula (Rosevear 1969; Zumpt 1970; Eisentraut 1975), and karyotype (Robinson *et al*. 1986; Baskevich 1997; Dobigny *et al*. 2002). *Xerus erythropus* and the southern African species are, however, more similar in general appearance than either is to *X. rutilus*, which is smaller and lacks the distinctive white stripe of its congeners (O'Shea 1991).

The two southern African species, *Xerus inauris* and *Xerus princeps*, are almost indistinguishable based on external morphology and occur sympatrically in portions of their respective ranges. Both are terrestrial, semifossorial and diurnal (Dorst & Dandelot 1970). In spite of their morphological and ecological similarities, *X. inauris* and *X. princeps* have strikingly different social behaviours; *X. inauris* forms large bands, whereas *X. princeps* is mainly solitary (Herzig-Straschil & Herzig 1989; Waterman 1995). Female *X. inauris* live in highly social kin clusters of up to three adult females and subadult offspring (Waterman 1995, 1997). Males form social groups composed of unrelated individuals; their range overlaps the ranges of several female social groups (Waterman 1995, 1997). Both males and females forage as groups and share sleeping burrows (Waterman 1995).

Our results indicate that *X. princeps* and *X. inauris* are reciprocally monophyletic lineages. Both clades are well supported in all analyses (PP and BP ≥ 94 for both clades in the full and reduced data sets), and maximum uncorrected pairwise divergence within each taxon is less than the minimum uncorrected pairwise divergence between groups. As pointed out by Funk & Omland (2003), a number of reasons exist that could cause a real biological species to appear paraphyletic or polyphyletic in mitochondrial DNA sequences, and so we consider the reciprocal monophyly of *X. princeps* and *X. inauris* a conservative indicator of specific distinction. Our data are thus consistent with differences already documented between the two taxa. Robinson *et al*. (1986) found a heterochromatic difference in an autosomal chromosome pair, Herzig-Straschil *et al*. (1991) found significant differences in skull morphology, and Haim *et al*. (1987) found significant differences in resting metabolic rate, conductance, and urine and faecal content.

Xerus inauris and *X. princeps* inhabit the southwest arid zone (SWA) of Africa, which includes the Namib Desert

and the savannahs of the Kalahari, Namaland, Western Cape and Karoo Domains. The SWA is characterized by high floral and faunal endemism (Barnard *et al*. 1998; Maggs *et al*. 1998; Robertson *et al*. 1998). Several studies of plants and animals have documented complex patterns of genetic structure, suggesting historic changes in habitat continuity (e.g. Matthee & Flemming 2002; Jacobson & Lester 2003). The distributions of the three *X. inauris* populations bear a striking similarity to patterns of plant disjunctions highlighted by Jürgens (1997), who attributed these disjunctions to the existence of warm refugia during glacial phases. The distributions of the central and southeastern populations also correspond well with mtDNA clades of the lizard *Agama atra* (Matthee & Flemming 2002) and the rock hyrax, *Procavia capensis* (Prinsloo & Robinson 1992).

Despite sampling limitations of this study, multiple lines of evidence suggest that the three populations of *X. inauris* that we (albeit somewhat arbitrarily) defined represent biologically meaningful divisions. As we have defined them, no two populations have any haplotypes in common. The exact test of population subdivision and the significance of Φ_{ST} values imply that the populations are significantly differentiated, and the AMOVA shows that the majority of genetic variation within this species is distributed among these three populations. Because our sampling was geographically restricted, however, we cannot definitively determine the geographical extent of these populations or if additional undetected populations may exist in areas outside our sampling. In the nested clade analysis, the inferences of past habitat fragmentation for clade 3-3 and for the total cladogram suggest interruptions of the continuous distribution of suitable *X. inauris* habitat in the SWA, followed by expansion from refugia and eventual secondary contact. This inferred secondary contact of *X. inauris* clades is observed in western Namibia and northern South Africa, where the distribution of clade III overlaps those of clades I and II, respectively. The inference of allopatric fragmentation for the total cladogram is dependent upon treatment of clade 3-3 as an interior clade (the connections of which exceed the 14-step parsimony limit). If the statistical parsimony limit is relaxed, however, clade 3-3 is connected to clade 3-2 by a connection of 22 steps (90% parsimony limit = 21 steps), making clade 3-3 an interior clade (not shown). If the high-level clades diverged due to allopatric fragmentation, this must have occurred early in the species' evolutionary history, but this conclusion lacks statistical support in the nested clade analysis. However, the inference of demographic expansion of clade III from both the mismatch distribution and the coalescent analysis is also consistent with allopatric fragmentation followed by secondary contact.

Allopatric fragmentation in southern Africa is thought to be tied to correlated cycles of temperature and humidity (Matthee & Robinson 1996; Jürgens 1997; Matthee & Flemming 2002). Fragmentation in an arid-adapted species such as *X. inauris* may have resulted in isolation in either warm refugia during cold periods or xeric refugia during periods of higher than contemporary levels of rainfall. Most of the SWA is relatively low in elevation; therefore, even small changes in precipitation would be expected to result in significant changes in vegetation over a large area. In periods of greater than current rainfall, woodland vegetation would likely spread south into what is now savannah, and in fact evidence of woodland vegetation as far south as Cape Town has been dated to the Pleistocene (Axelrod & Raven 1978). *Xerus inauris* is adapted to the open spaces and sparse flora of the savannah, so an expansion of woodland vegetation throughout the Kalahari basin would likely have driven it into xeric refugia, isolating populations.

All three methods of estimating divergence times between *X. inauris* populations indicate roughly similar divergence times. Pleistocene shifts in African aridity were tied to high-latitude glacial cycles, peaking at 100 000 year intervals (deMenocal 2004). Although one or more of these cycles may have contributed to the divergence among *X. inauris* populations, our estimates of divergence times are too approximate (based on poorly known cyt *b* mutation rates in *Xerus*) to resolve whether the divergences among *X. inauris* populations occurred during glacial or interglacial periods. As a result, we cannot distinguish between the hypothesis of warm refugia and that of xeric refugia.

As an alternative scenario to allopatric fragmentation followed by secondary contact, the deep divisions within *X. inauris* could have been caused by isolation by distance. This is consistent with the correlation between genetic and geographical distances identified in the Mantel test, and with the behaviour of this species. Because our analyses relied on a mitochondrial marker, our results must be interpreted in terms of the genetic structure and historical demography of females. In this highly social species, females often remain in their natal burrow clusters, and mitochondrial haplotypes are therefore expected to spread slowly.

Because of recent criticisms of the ability of nested clade analysis to accurately distinguish among historical processes (e.g. Knowles & Maddison 2002), we employed several other methods to assess the inference of allopatric fragmentation. The alternative of isolation by distance is contradicted by the low but non-negligible migration rates indicated by both methods between the central population and the other two populations. The estimates based on pairwise differences are surprising, as populations 1 and 3 seem unlikely to frequently exchange migrants, since they appear to be separated by over 1000 km. Estimates of the numbers of female migrants from MIGRATE are consistent with the geographical locations of the three populations, with substantial migration only between contiguous populations, although this result should be viewed cautiously because

of recent criticisms of the ability of MIGRATE to accurately estimate migration rates (Abdo *et al*. 2004). Even this low level of gene flow is enough to overcome the effect of genetic drift (Slatkin 1987), and it is therefore unlikely that the high levels of divergence among the populations were caused by isolation by distance. Allopatric fragmentation is also supported by the coalescent analysis, in which the number of deep coalescences observed in the reconstructed tree was significantly lower than that of the coalescent simulations across a range of assumed conditions of time since population divergence and effective population size.

The northern and southern clades of *X. princeps* are divided roughly across the same area as the northwestern and central populations of *X. inauris*. Additional sampling will be required to draw any definitive conclusions regarding the genetic structure of this species. *Xerus princeps* is restricted to a narrow range following the western (or Kaoko) escarpment, which parallels the western coast of Namibia. This is an area of high endemism for birds (Robertson *et al*. 1998) and plants (Maggs *et al*. 1998). Barnard *et al*. (1998) listed the Kaoko escarpment as the top priority for habitat protection in Namibia. Restriction of *X. princeps* to this zone underscores the importance of adding all or part of this region to Namibia's network of protected lands.

Xerus inauris and *X. princeps* represent reciprocally monophyletic lineages, supporting their respective recognition as distinct species. Within *X. inauris*, a great deal of genetic structure is apparent, and some of the variation has a clear geographical basis. This research has provided an initial assessment of the phylogeographical basis for genetic structure within this species, and continued studies with better range and resolution of geographical sampling would undoubtedly provide additional information about its evolutionary history. In addition, a comparison of patterns from maternally inherited, paternally inherited, and autosomal markers could provide insights into the effects on genetic structure of the unique social system of this species. Additionally, the fact that *X. princeps* and *X. inauris* are closely related sister taxa with very divergent social systems makes this pair an excellent model for studying the effects of social systems on gene flow, particularly sex-biased gene flow.

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This study represents a major portion of Matthew Herron's master's thesis on sciurid evolution. He now studies evolutionary transitions in individuality, using volvocine algae as a model system, at the University of Arizona. Jane Waterman is a behavioral ecologist whose research focuses on the evolution of social and mating systems. She has studied the behavior of the Cape ground squirrel for over 15 years. Christopher Parkinson is an evolutionary biologist who utilizes molecular techniques to address research questions in biogeography, conservation genetics and processes of speciation in a wide range of vertebrate taxa.

Appendix

GenBank Accession nos of *Xerus* haplotypes

Haplotype	Accession no.	Haplotype	Accession no.
Xerus erythropus		*Xerus inauris*	
A	DQ010400	V	DQ010378
B	DQ010401	W	DQ010379
C	DQ010402	X	DQ010380
Xerus inauris		Y	DQ010381
A	DQ010358	Z	DQ010382
B	DQ010359	AA	DQ010383
C	DQ010360	AB	DQ010384
D	DQ010361	AC	DQ010385
E	DQ010362	AD	DQ010386
F	DQ010363	AE	DQ010387
G	DQ010364	AF	DQ010388
H	DQ010365	AG	DQ010389
I	DQ010366	AH	DQ010390
J	DQ010367	AI	DQ010391
K	DQ010368	AJ	DQ010392
L	DQ010369	*Xerus princeps*	
M	DQ010370	A	DQ010393
N	DQ010371	B	DQ010394
O	DQ010372	C	DQ010395
P	AY452689	D	DQ010396
Q	DQ010373	E	DQ010397
R	DQ010374	F	DQ010398
S	DQ010375	*Xerus rutilus*	
T	DQ010376	A	AY452690
U	DQ010377	B	DQ010399