

Habitat barriers limit gene flow and illuminate historical events in a wide-ranging carnivore, the American puma

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Abstract

We examined the effects of habitat discontinuities on gene flow among puma (*Puma concolor*) populations across the southwestern USA. Using 16 microsatellite loci, we genotyped 540 pumas sampled throughout the states of Utah, Colorado, Arizona, and New Mexico, where a high degree of habitat heterogeneity provides for a wide range of connective habitat configurations between subpopulations. We investigated genetic structuring using complementary individual- and population-based analyses, the latter employing a novel technique to geographically cluster individuals without introducing investigator bias. The analyses revealed genetic structuring at two distinct scales. First, strikingly strong differentiation between northern and southern regions within the study area suggests little migration between them. Second, within each region, gene flow appears to be strongly limited by distance, particularly in the presence of habitat barriers such as open desert and grasslands. Northern pumas showed both reduced genetic diversity and greater divergence from a hypothetical ancestral population based on Bayesian clustering analyses, possibly reflecting a post-Pleistocene range expansion. Bayesian clustering results were sensitive to sampling density, which may complicate inference of numbers of populations when using this method. The results presented here build on those of previous studies, and begin to complete a picture of how different habitat types facilitate or impede gene flow among puma populations.

Keywords: gene flow, landscape connectivity, microsatellites, population genetics, population structure, *Puma concolor*

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Introduction

The puma (*Puma concolor*) is a large and adaptable American felid, and the sole remaining large predator in many parts of the western United States and Canada. Pumas were the most widely distributed mammal in the Western Hemisphere at the time of European settlement, but have since been eliminated from nearly all of eastern North America and portions of their range in South America (Young & Goldman 1946; Anderson 1983). Pumas persist in western North America, but some populations are becoming isolated due to habitat fragmentation (e.g. Beier 1996; Loxterman 2001; Ernest *et al.* 2003).

Highly mobile carnivores often exhibit rates of gene flow sufficient to limit the accumulation of genetic differences between subpopulations (Wayne & Koepfli 1996). Pumas have been known to disperse large distances (e.g. Anderson *et al.* 1992), even in the presence of large discontinuities in habitat (Ruth *et al.* 1998; Logan & Sweanor 2001), suggesting that gene flow should be high and genetic differentiation minimal across large areas. Genetic data have been equivocal, however. Two studies have demonstrated low population subdivision across distances spanning several hundred kilometres (Sinclair *et al.* 2001; Anderson *et al.* 2004), while others have found substantial structuring at similar scales where suitable habitat is less contiguous (Walker *et al.* 2000; Loxterman 2001; Ernest *et al.* 2003), suggesting that habitat barriers may play a role in structuring populations.

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Questions also remain about the puma's evolutionary history in North America. Although fossil evidence of pumas in North America spans 300 000 years, Culver *et al.* (2000) found genetic evidence that extant populations may be much younger. They hypothesized that pumas in North America were eliminated at the end of the Pleistocene, and replaced by a handful of immigrants from South America within the last 10 000–12 000 years.

The naturally patchy distribution of puma habitat in the southwestern United States provides a unique opportunity to explore relationships between habitat pattern and gene flow. Areas of high quality puma habitat, including woodland and chaparral vegetation, are interspersed in varying configurations with nonhabitat, including open desert and expansive grasslands. The region also forms the only mountainous connection between large blocks of puma habitat in the United States and Mexico, thus providing an opportunity to investigate the post-Pleistocene recolonization hypothesis because it covers the likely path of a northward range expansion. Here we address questions of fine-scaled phylogeography and population history across the southwestern United States relative to gaps in suitable puma habitat. Our results build on the range-wide work by Culver *et al.* (2000), complement more fine-scaled puma studies, and provide unique insight relevant to puma conservation and management.

Materials and methods

Sample collection and laboratory procedures

We collected muscle samples of 540 legally hunted pumas from hunters, taxidermists, and state game management agencies in Arizona, Colorado, New Mexico, and Utah (Fig. 1). All samples were from free-ranging pumas killed between 1999 and 2002, with known relatives excluded. Sample locations were distributed in all suitable puma habitats where hunting occurred; puma habitat was fairly contiguous throughout Utah, Colorado, and northern New Mexico, while habitat in portions of Arizona and southern New Mexico was more fragmented by grassland and low desert vegetation types (Fig. 1).

We extracted DNA from samples using the Puregene Genomic DNA Isolation Kit (Gentra Systems). We amplified 16 microsatellite loci originally developed for the domestic cat (*FCA026*, *FCA035*, *FCA043*, *FCA052*, *FCA057*, *FCA077*, *FCA082*, *FCA090*, *FCA096*, *FCA098*, *FCA132*, *FCA144*, *FCA176*, *FCA221*, *FCA229*, and *FCA290*; Menotti-Raymond *et al.* 1999, 2003) by polymerase chain reaction (PCR) under conditions described in Menotti-Raymond *et al.* (1999). These loci occurred on nine chromosomes, with a minimum of 22 centimorgans separating loci on the same chromosome (Menotti-Raymond *et al.* 1999, 2003). PCR products were electrophoresed using an ABI 377 sequencer and data were

analysed using GENESCAN and GENOTYPER software (Applied Biosystems).

Individual-based analyses

We used a Bayesian model-based clustering method (STRUCTURE 2.1, Pritchard *et al.* 2000) to infer numbers of populations and assign individuals to populations based only on multilocus genotype data (i.e. without knowledge of sample origin). For K population clusters, the program estimates the probability of the data, $\Pr(X|K)$, and the probability of individual membership in each cluster using a Markov chain Monte Carlo (MCMC) method under the assumption of Hardy–Weinberg equilibrium within each cluster. The estimated number of subpopulations is taken to be the value of K at which $\Pr(X|K)$ plateaus. When the F model assuming correlated allele frequencies among populations is used, STRUCTURE also estimates allele frequencies of a hypothetical ancestral population (Falush *et al.* 2003). For each population cluster k , the program estimates F_k , a quantity analogous to Wright's F_{ST} , but describing the degree of genetic differentiation of population k from the ancestral population. Each F value can thus be interpreted as the degree of genetic drift undergone by population k following population subdivision (Falush *et al.* 2003). We ran STRUCTURE assuming correlated allele frequencies and admixture, and conducted five independent runs of $K = 1$ –20 to estimate the true number of clusters with 10^6 MCMC cycles each for burn-in and data collection.

To visualize relationships among samples from different habitat blocks, we used nonmetric multidimensional scaling (NMDS) ordinations of individuals, with dissimilarity matrices consisting of shared allele distances ($1 - D_{PS}$) calculated using MICROSAT (Minch 1997). The technique plots samples such that distances between samples in ordination space best reflect the rank order of genetic distances among them. NMDS is more robust than other ordination techniques because it does not assume linearity (Minchin 1987), an assumption often violated in analyses based on frequency data. Following the ordination, we assessed differences between groups of samples from different portions of our study area using analysis of similarities (ANOSIM; Clarke and Green 1988). We ranked dissimilarities among samples to assess differentiation between groups using the ANOSIM test statistic, R , which ranges from zero (no difference in the rank of dissimilarities within and between groups) to one (all samples within groups more similar to each other than to those from different groups; Clarke & Green 1988). We assessed statistical significance by randomly assigning samples to groups 999 times and determining whether the R value of the original data set was greater than that obtained by chance alone. We conducted ordinations and ANOSIM tests using PRIMER version 5.2.8 (Primer-E Ltd).

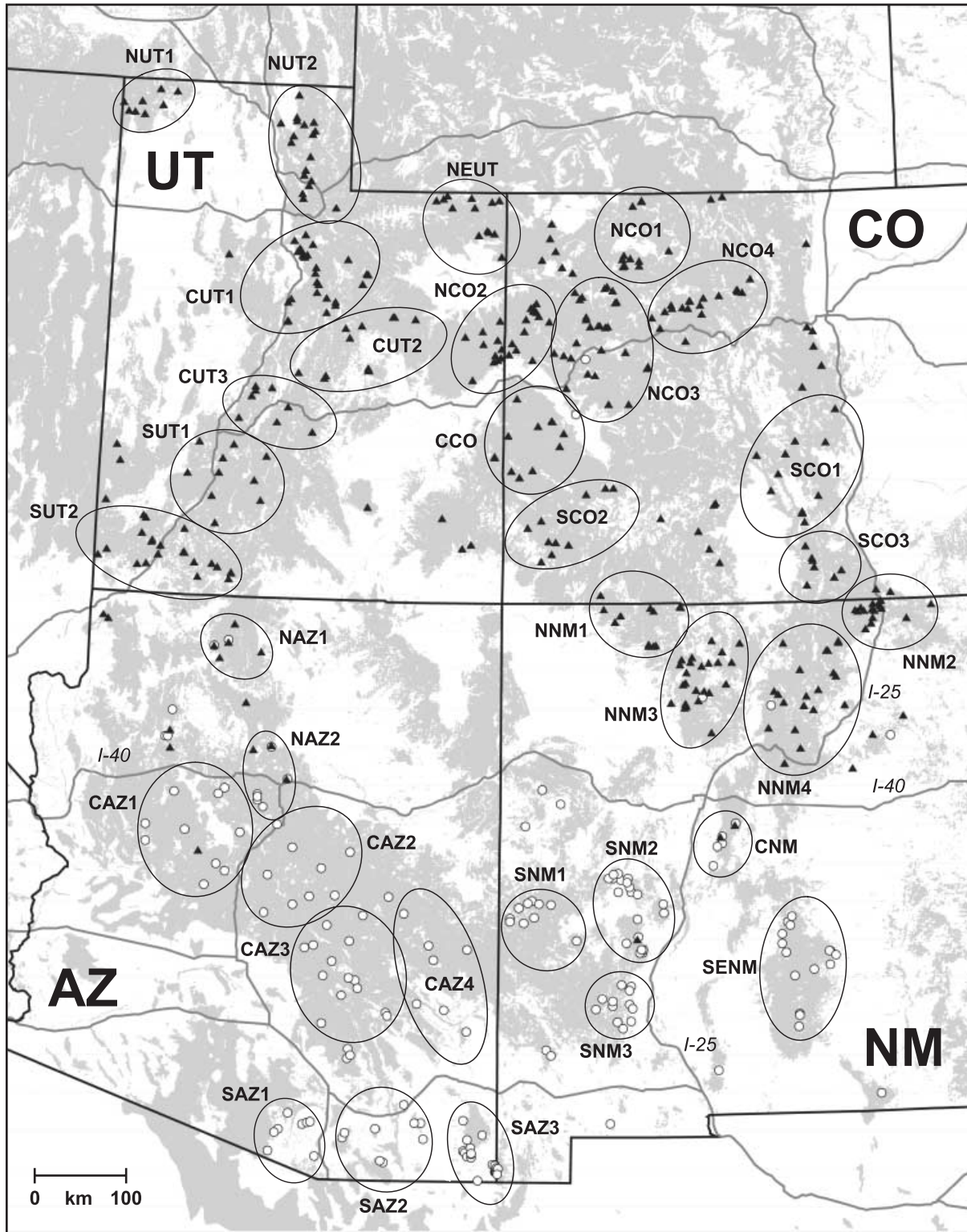


Fig. 1 Map of study area and locations of 540 puma samples. Shading indicates puma habitat which includes woodland, forest, chaparral, and montane and desert shrub cover types, while nonhabitat includes low desert, grassland, tundra, urban, agriculture, and nonvegetated cover types (McRae 2004). Ellipses indicate sample membership in 34 sample groups for population-based analyses; sample groups were obtained by clustering samples based on geographical proximity alone. State abbreviations and interstate highways (with I-40 and I-25 labelled) are also shown. Black triangles denote samples assigned to northern cluster using *STRUCTURE* with $K = 2$; open circles denote samples assigned to southern cluster.

Population-based analyses

Delineation of sample groups from geographical coordinates. We grouped individuals into local clusters of samples (sample groups) to permit population-based analyses. These analyses complemented individual-based approaches, and also facilitated comparison with previous studies using pairwise F_{ST} values. Our goal was to create sample groups based solely on geographical distance between samples (i.e. without using genetic data, and without imposing our own notions of population boundaries on the data). We used a simple hierarchical clustering algorithm (UPGMC; Sneath & Sokal 1973) to group nearest individuals and nascent clusters based on Euclidean distance between individuals and cluster centroids. At each step the geographically closest pair of individuals, pair of clusters, or individual-cluster pair was combined. A new centroid location representing the geographical centre of all individuals in the combined cluster was then calculated. We allowed individuals and clusters to merge at distances of 80 km or less because this distance approximated average dispersal distances reported in the literature for male pumas (e.g. 86.2 km reported by Anderson *et al.* 1992; 101.3 km reported by Logan & Sweanor 2001). We did not assume resulting sample groups to necessarily be discrete populations, but instead treated them as local clusters of samples with allele frequencies assumed to be representative of those at cluster centroids. We retained only those sample groups of ≥ 7 individuals for subsequent analyses, because a higher threshold would have eliminated sample groups of particular interest (e.g. the Manzano Mountains, NM). We assigned individuals in sample groups with < 7 individuals to the closest larger cluster when it was within 80 km, and excluded them from population-based analyses otherwise. Software used to perform the clustering is available at <http://www.kgl.nau.edu/epi/bmcræ>.

Descriptive statistics. After defining our sample groups, we calculated descriptive statistics for each group using FSTAT 2.9.3 (Goudet 2001), including mean number of alleles per locus, expected and observed heterozygosities, and multilocus F_{IS} . We used GENEPOP version 3.3 (Raymond & Rousset 1995) to test for significant departure from Hardy-Weinberg equilibrium (HWE) within sample groups using the Markov chain method of Guo & Thompson (1992) to estimate exact P values for each sample group at each locus and across all loci. We also used GENEPOP to test for linkage disequilibrium among all pairs of loci using the Markov chain method and Fisher exact test. Genetic distances between sample groups were measured by calculating pairwise F_{ST} values (Weir & Cockerham 1984) using FSTAT, and the log-likelihood ratio distance (D_{LR} ; Paetkau *et al.* 1997; program available at <http://www2.biology.ualberta.ca/jbrzusto/Doh.php>). D_{LR} has been shown to perform particularly

well in studies of fine-scaled population structure (Paetkau *et al.* 1997). Statistical significance of pairwise F_{ST} values was tested using permutations in FSTAT. We corrected for multiple comparisons in all statistical tests using a sequential Bonferroni adjustment (Rice 1989).

Neighbour-joining trees and ordinations. We used the NEIGHBOR subroutine in PHYLIP version 3.5c (Felsenstein 1993) to construct neighbour-joining (NJ) trees based on D_{LR} among sample groups with ≥ 7 individuals. We evaluated robustness of the tree topology by generating 100 bootstrap replicates. We also visualized relationships among the sample groups using NMDS ordinations. For summarizing relationships among populations undergoing recurrent gene flow, ordination complements tree construction because it does not assume a bifurcating evolutionary history among populations (Paetkau *et al.* 1999). To ensure that unequal sample sizes were not biasing our results, we repeated tree construction and ordinations with genetic distances calculated using seven individuals randomly selected from each sample group.

Mantel tests. To test for correlations between geographical and genetic distance matrices we conducted Mantel tests (Mantel 1967) using the R Package (Legendre & Vaudor 1991). We included only sample groups with ≥ 10 individuals in order to minimize error in characterizing patterns of isolation by distance. We assumed a two-dimensional habitat model, and tested for correlations between log-transformed geographical distance and $F_{ST}/(1 - F_{ST})$ as advocated by Rousset (1997).

Based on indications of a split between northern and southern regions using individual-based analyses, we tested for a barrier between northern sample groups (Utah, Colorado, northern New Mexico) and southern sample groups (Arizona and New Mexico south of Interstate 40) using a partial Mantel test (Smouse *et al.* 1986). This test allowed us to assess how much additional genetic differentiation could be attributed to a barrier after controlling for effects of geographical distance.

Results

Individual-based analyses

For 540 pumas, results of our STRUCTURE analyses showed dramatic improvement in $\Pr(X|K)$ when two population clusters were assumed rather than one, and more modest improvement for higher numbers of clusters (Fig. 2). The probability of the data reached a plateau after $K = 9$ populations, and alpha, a measure of admixture between populations, was lowest at $K = 9$. Assignment to clusters with $K = 2$ is shown in Fig. 1; all runs with $K > 1$ shared the same abrupt north-south division, with clusters residing

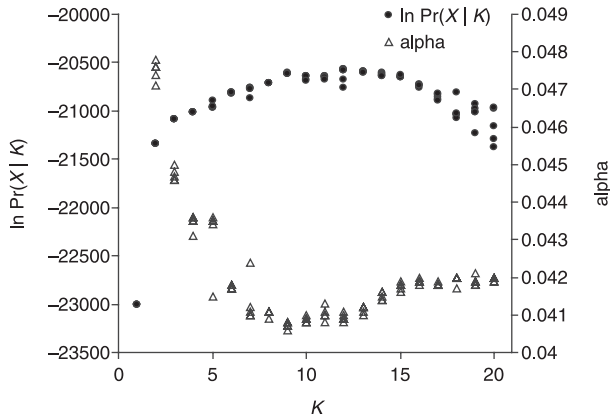


Fig. 2 Results of Bayesian clustering analysis for 540 pumas. For each number of population clusters tested (K), $\Pr(X|K)$ is the probability of the data, and alpha is a measure of admixture among populations.

almost entirely within northern or southern portions of the study area. A narrow transitional zone between the two regions was evident in the $K = 2$ case (Fig. 1), with individuals from Arizona north of Interstate 40 and the Manzano Mountains in central New Mexico assigning to both northern and southern clusters in roughly equal numbers. Northern Arizona was also transitional for the $K = 9$ case, but individuals from the Manzano Mountains assigned exclusively to southern clusters (Supplementary material, Fig. S1).

For all values of $K > 2$, STRUCTURE identified a greater number of clusters in the northern portion of the study area than in the south; for example, six clusters occurred in the north and three in the south for the $K = 9$ case. To determine whether the larger number of northern clusters was an artefact of the larger sample size in the northern region,

we randomly discarded northern samples to achieve a sample size equal to that in the southern region (159). After discarding northern samples, $\Pr(X|K)$ peaked at $K = 6$ populations, five of which consisted entirely or predominantly of samples from the south, and one which contained 156 northern samples and four southern samples.

Across five runs, northern F values averaged 0.20, 0.16, and 0.23 for the $K = 2$, $K = 9$, and $K = 6$ (reduced northern data set) cases, respectively, while southern F values averaged 0.036, 0.12, and 0.074, respectively. In each case, every southern population cluster had a lower F value than did every northern cluster, suggesting that northern populations had undergone greater genetic drift following subdivision of the ancestral population.

Ordination using all 540 individuals (Supplementary material, Fig. S2) also showed strong separation between northern and southern pumas (ANOSIM $R = 0.67$, $P < 0.001$). Pumas from transitional areas indicated by STRUCTURE analyses (AZ north of Interstate 40; Manzano Mountains, NM), clustered to the north and south in roughly equal numbers. Figure 3 illustrates ordination results at the sharpest interface between the two regions – the split between northern and southern New Mexico. The figure includes individuals from all New Mexico sample groups, with northern Colorado (NCO) individuals included for comparison. All groups were significantly differentiated ($P < 0.05$ after Bonferroni correction), with the strongest separation evident between northern and southern sample groups (Fig. 3). For example, NNM individuals exhibited strong separation from SNM individuals ($R = 0.71$, $P < 0.001$), yet they overlapped substantially with more geographically distant but better-connected NCO individuals ($R = 0.14$, $P < 0.001$). SENM and SNM also overlapped considerably ($R = 0.21$, $P = 0.0014$), but were more differentiated from each other than were NCO and NNM.

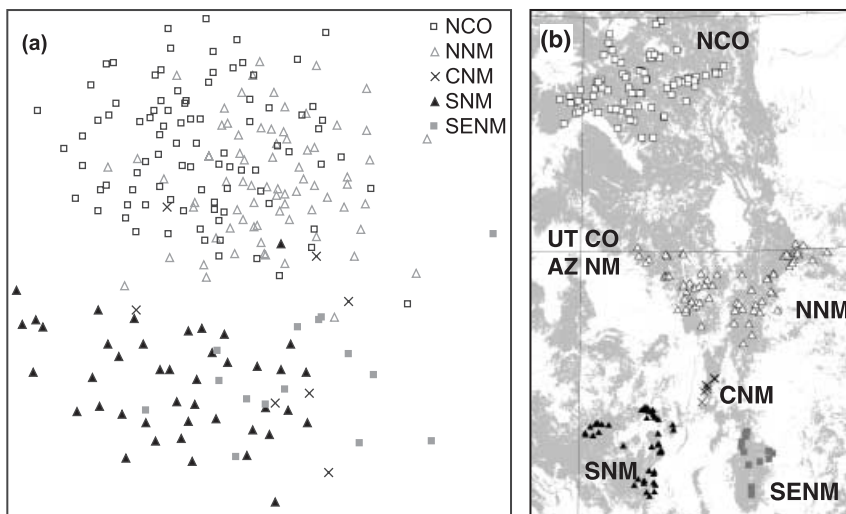


Fig. 3 (a) NMDS ordination of individuals from all New Mexico sample groups with NCO individuals included for comparison. Ordination is based on genetic distances ($1 - D_{PS}$) between individuals. Northern and southern pumas formed distinct clusters, while pumas from the Manzano Mountains (CNM) fell in both northern and southern clusters. (b) Location of the five sample groups included in the ordination, including state abbreviations and boundaries.

Table 1 Descriptive statistics for 34 geographically delineated puma sample groups, including group identification (ID), approximate location (Locale), sample size (n), mean number of alleles per locus (Alleles), average expected heterozygosity (H_E), average observed heterozygosity (H_O), and F_{IS} (values in bold differ significantly from Hardy–Weinberg equilibrium). See Fig. 1 for geographical locations of sample groups

| ID | Locale | n | Alleles | H_E | H_O | F_{IS} |
|------|------------------------------|-----|---------|-------|-------|--------------|
| NAZ1 | Kaibab plateau | 8 | 4.13 | 0.70 | 0.66 | 0.056 |
| NAZ2 | Flagstaff, AZ area | 9 | 4.06 | 0.67 | 0.52 | 0.237 |
| CAZ1 | Prescott National Forest | 12 | 4.00 | 0.66 | 0.69 | -0.039 |
| CAZ2 | W. Mogollon Rim | 9 | 4.31 | 0.68 | 0.69 | -0.004 |
| CAZ3 | Pinal Mountains | 14 | 4.19 | 0.62 | 0.61 | 0.024 |
| CAZ4 | San Carlos Indian Res. | 7 | 3.50 | 0.61 | 0.63 | -0.031 |
| SAZ1 | Baboquivari/Sierrita Mts. | 9 | 3.50 | 0.59 | 0.60 | -0.015 |
| SAZ2 | Huachuca/Dragoon Mts. | 9 | 4.31 | 0.68 | 0.70 | -0.032 |
| SAZ3 | Chiricahua/Peloncillo Mts. | 25 | 5.06 | 0.66 | 0.67 | -0.013 |
| NCO1 | Elkhead/Williams Fork Mts. | 11 | 3.50 | 0.60 | 0.59 | 0.019 |
| NCO2 | Book Cliffs | 25 | 4.06 | 0.61 | 0.60 | 0.018 |
| NCO3 | White River National Forest | 29 | 4.19 | 0.63 | 0.63 | 0.012 |
| NCO4 | Vail Pass area | 20 | 3.75 | 0.61 | 0.57 | 0.061 |
| CCO | Uncompahgre Plateau | 13 | 4.06 | 0.63 | 0.64 | -0.010 |
| SCO1 | N. Sangre de Cristo/Wet Mts. | 12 | 3.44 | 0.61 | 0.59 | 0.021 |
| SCO2 | W. San Juan Mts. | 10 | 3.63 | 0.61 | 0.57 | 0.076 |
| SCO3 | Spanish Peaks area | 8 | 3.38 | 0.58 | 0.61 | -0.054 |
| NNM1 | Navajo Lake area | 12 | 3.88 | 0.60 | 0.59 | 0.028 |
| NNM2 | Abiquiu Lake area | 18 | 3.56 | 0.57 | 0.61 | -0.085 |
| NNM3 | Raton, NM area | 25 | 4.13 | 0.63 | 0.65 | -0.017 |
| NNM4 | S. Sangre de Cristo Mts. | 21 | 4.06 | 0.58 | 0.60 | -0.030 |
| CNM | Manzano Mts. | 7 | 3.88 | 0.68 | 0.71 | -0.049 |
| SNM1 | Reserve, NM area | 10 | 4.00 | 0.66 | 0.63 | 0.039 |
| SNM2 | San Mateo/Gallinas Mts. | 21 | 5.00 | 0.65 | 0.66 | -0.020 |
| SNM3 | Black Range | 12 | 4.44 | 0.64 | 0.63 | 0.012 |
| SENM | Sacramento Mts. | 15 | 4.38 | 0.61 | 0.61 | -0.008 |
| NUT1 | Grouse Creek Mts. | 8 | 3.25 | 0.55 | 0.60 | -0.102 |
| NUT2 | Wasatch/Monte Cristo Ranges | 18 | 3.81 | 0.62 | 0.61 | 0.011 |
| NEUT | Flaming Gorge area | 13 | 3.81 | 0.59 | 0.66 | -0.120 |
| CUT1 | S. Wasatch Range | 27 | 4.06 | 0.63 | 0.66 | -0.058 |
| CUT2 | Price, UT area | 12 | 3.63 | 0.64 | 0.63 | 0.026 |
| CUT3 | Pahvant Range/Sevier Plateau | 8 | 3.75 | 0.63 | 0.69 | -0.101 |
| SUT1 | Beaver, UT area | 10 | 3.44 | 0.57 | 0.57 | -0.003 |
| SUT2 | Southeastern UT | 21 | 4.00 | 0.61 | 0.62 | -0.016 |

Population-based analyses

Geographic sample clustering. Solely on the basis of geographical proximity, the UPGMC method allocated 488 individuals into 34 sample groups of ≥ 7 individuals each (Fig. 1, Table 1). Individuals were always within 150 km of other sample group members. Sample groups tended to be within single habitat blocks, although nine straddled interstate highways. Six appeared to be somewhat isolated from others by habitat discontinuities: CNM (Manzano Mountains), SENM (Sacramento Mountains), NAZ1 (Kaibab Plateau), and the three ‘Sky Island’ region sample groups, SAZ1, 2, and 3.

Allele frequencies and genotypic equilibrium. No single locus deviations from HWE were detected after Bonferroni

correction, and only one sample group (NAZ2) was found to be out of HWE (before or after correction) using tests across all loci (Table 1). NAZ2 had a deficit of heterozygotes, suggesting possible subdivision within the sample group. No pair of loci exhibited linkage disequilibrium in any sample group after Bonferroni correction; approximately as many comparisons were significant prior to correction as would be expected under H_0 (3.2% at $\alpha = 0.05$), with no loci exhibiting consistent patterns.

Pairwise F_{ST} values between the 34 geographically delimited sample groups (see Supplementary material, Table S1) ranged from essentially zero to 0.24; 95% of the 561 pairwise comparisons were statistically significant prior to Bonferroni correction, and 70% remained significant after correction. Excluding NAZ1, NAZ2, and CNM, pairwise F_{ST} values averaged 0.041 among the 20 northern sample

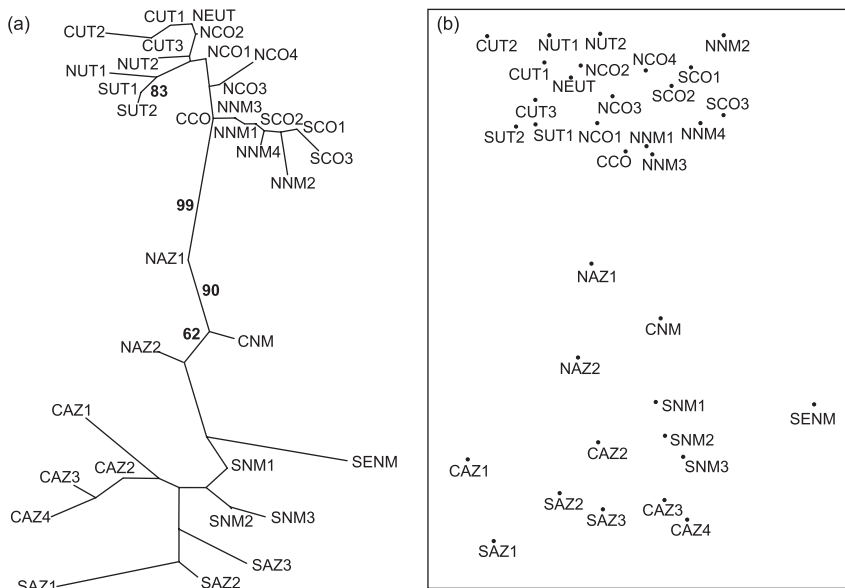


Fig. 4 (a) Unrooted neighbour-joining tree based on D_{LR} between 34 sample groups, with bootstrap values over 60% shown. (b) NMDS ordination based on $D_{LR'}$ summarizing relationships among sample groups without restriction to a bifurcating tree. See Fig. 1 for geographical locations of sample groups.

groups (range -0.016 – 0.13), 0.052 among the 11 southern sample groups (range 0.0091 – 0.11), and 0.15 in 220 comparisons (all significant) between northern and southern sample groups (range 0.074 – 0.24).

Neighbour-joining analyses and ordinations. Figure 4a shows the tree based on D_{LR} . Northern and southern sample groups were separated into distinct clades, while NAZ1, NAZ2, and CNM appeared to be intermediate. Sample groups tended to cluster according to geography, particularly in the south. Despite the smaller geographical extent of the southern region, southern sample groups (especially SENM) appeared to be more differentiated from one another than northern sample groups, as indicated by longer branch lengths in the southern clade. Similarly, the ordination (Fig. 4b) showed strong separation between northern and southern sample groups ($R = 0.998$, $P < 0.001$), with northern groups clustering more tightly than their southern counterparts, NAZ1, NAZ2, and CNM falling between the two regions, and SENM separated from neighbouring populations. All of these properties were reflected in trees and ordinations constructed with seven individuals per sample group, indicating that differences in sample group sizes did not bias our results.

Isolation by distance. Isolation by distance was evident at several scales. Among the 24 sample groups with ≥ 10 individuals across the study area, genetic and geographical distances were strongly correlated ($R^2 = 0.51$, $P < 0.001$) (Fig. 5). Figures 1, 3, and 4 indicate a strong division into two regions, and partial Mantel tests confirmed a strong barrier between northern and southern sample groups ($r =$

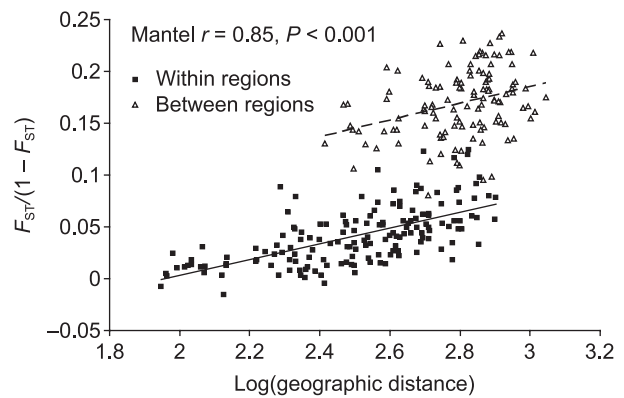


Fig. 5 Genetic distance plotted against log-transformed geographical distance for 24 sample groups with ≥ 10 individuals. Within region points represent distances between pairs of sample groups falling within northern or southern region, while between region points represent pairs of sample groups falling across boundaries separating the regions. Results of partial Mantel tests for the effect of a barrier separating northern and southern sample groups and regression lines for comparisons within and between regions are shown.

0.85 , $P < 0.001$). Among seven sample groups with $n \geq 10$ in the southern region, correlations between genetic and geographical distance remained strong ($R^2 = 0.54$, $P = 0.003$), as they did among 17 northern sample groups ($R^2 = 0.46$, $P < 0.001$). We also observed significant relationships between genetic and geographical distance within each of the three states with enough sample groups with ≥ 10 individuals to conduct Mantel tests (UT, $n = 6$, $R^2 = 0.60$, $P = 0.011$; CO, $n = 7$, $R^2 = 0.16$, $P = 0.035$; NM, $n = 8$, $R^2 = 0.75$, $P = 0.0010$). Additionally, we observed significant isolation

by distance among all nine sample groups with ≥ 7 individuals in Arizona ($R^2 = 0.27$, $P = 0.002$). Mantel tests and partial Mantel tests conducted at the individual level supported the above results.

Patterns of allelic richness

In addition to strong differentiation between pumas sampled in the northern and southern portions of the study area, there were considerable differences in genetic diversity between these two regions. Excluding transitional zones (AZ north of Interstate 40; CNM), we found substantially greater numbers of alleles in 159 southern pumas than in 353 northern pumas; out of 107 alleles in the entire population, 103 were found in the south and only 81 in the north. There were also six times as many unique alleles in the south (26) as in the north (4). Further emphasizing these patterns, we found fewer alleles in the entire northern region than in portions of the southern region with much smaller sample sizes (e.g. SAZ1-3, 90 alleles, $n = 43$; CAZ1-4, 84 alleles, $n = 42$; and SNM1-3, 86 alleles, $n = 44$). Average numbers of alleles per locus, allele size ranges, and variances across all loci were all significantly higher in the south based on Wilcoxon signed rank tests ($P < 0.05$).

Discussion

Spatial scale and pattern of genetic differentiation

The most striking result was a strong north–south division of pumas (Figs 1, 3, 4, and 5). Results using STRUCTURE, ordinations, neighbour-joining trees, and partial Mantel tests all indicated greater differentiation between northern and southern pumas than could be explained by distance alone. For example, although NNM individuals are geographically closer to SNM than to NCO, they show strong genetic overlap with NCO individuals, and almost none with SNM individuals (Fig. 3). Genetically similar NNM and NCO individuals are connected by wide swaths of habitat, while genetically distinct but more geographically proximate NNM and SNM individuals are separated by considerable habitat gaps. Similarly, SENM individuals show greater separation from neighbouring sample groups than do more distant but well-connected sets of samples (Fig. 3). The consistent result in these areas, and throughout the study area, is that those populations which are most strongly differentiated are those which are poorly connected by habitat, regardless of distance.

Strong differentiation at such scales is surprising given the dispersal capabilities of pumas. In a classic example of their long-distance movement ability, Ruth *et al.* (1998) translocated 13 pumas from the San Andres Mountains in southern New Mexico to northern New Mexico. After

relocation, two males returned to their original home ranges, travelling a distance of 465–490 km. Although pumas can make the journey from northern to southern New Mexico (and presumably the reverse), our results suggest that such movements are rare under natural circumstances, or that dispersers between the two regions rarely breed.

Although narrow habitat corridors appear to connect the northern and southern regions through northern Arizona and central New Mexico (Fig. 1), these corridors are bisected by several potential barriers, ranging from the ancient to the recent. In northern Arizona, historical barriers may include grasslands north of Flagstaff, the Colorado River and the Grand Canyon, and desert areas north of the Kaibab Plateau, while recent barriers may include the Flagstaff metropolitan area and the Interstate 40 transportation corridor. Potential historical barriers in central New Mexico include large expanses of grasslands, while the Albuquerque metropolitan area and both Interstates 25 and 40 may now impede movement between northern and southern New Mexico. In these portions of our study area, grasslands likely formed much more substantial habitat gaps prior to their recent invasion by woodland types (Archer 1994; Miller & Wigand 1994). Reduction of grasslands and introduction of anthropogenic barriers likely means that the locations and types of barriers in this landscape have changed considerably in the past century.

The timescales on which these barriers have operated obviously span decades (Interstate 40) to millennia (Colorado River). It is likely that the strong north–south subdivision in our study area is due to natural habitat barriers that have existed for centuries (a point we return to later), with recent anthropogenic barriers superimposed upon, and interacting with, a previously structured population. For example, NAZ2 was the only sample group to exhibit a significant deficit of heterozygotes, despite its relatively small geographical extent. Moreover, NAZ2 was the only sample group bisected by a metropolitan area (Flagstaff, AZ), and one hypothesis explaining the deficit could be that anthropogenic barriers, such as urban development and Interstate 40, have subdivided the group. It seems unlikely, however, that a subdivision so recent in origin could have resulted in allele frequencies differing enough on either side to result in the disequilibrium we detected. Alternatively, since NAZ2 appears to lie in a narrow transitional zone between historically divergent northern and southern populations (Figs 1 and 4), it may contain individuals from both. Either hypothesis would predict a heterozygote deficit due to a Wahlund effect. A third hypothesis involves an interplay between these historical and contemporary factors, with the superimposition of a ‘hard’ anthropogenic barrier upon a zone that was already transitional between divergent northern and southern populations. This could quickly result in a sharp genetic discontinuity because continued gene flow from the north and south would reinforce

differences on either side of the barrier (i.e. animals just north of the barrier become more northern in their genetic composition, and vice versa). Although our sampling effort was unable to explicitly address these three hypotheses, additional sampling and/or tracking studies in this area could further elucidate the relative effects of, and interactions between, natural and anthropogenic barriers with respect to individual movements and genetic structuring.

Comparisons with other studies

Levels of genetic differentiation between northern and southern portions of our study area (average pairwise F_{ST} = 0.15) are high compared to those reported in studies of microsatellite variation in other large carnivore species. Among five Canadian lynx (*Lynx canadensis*) populations spanning from Alaska to eastern Canada, Rueness *et al.* (2003) reported pairwise F_{ST} values of 0.0017–0.024. Although these values may be artificially low due to the large geographical extent of individual populations, Schwartz *et al.* (2002) found pairwise F_{ST} values that did not exceed 0.07 among 17 lynx populations of smaller geographical extent sampled from Alaska to Wyoming. Among polar bear (*Ursus maritimus*) populations sampled across the species' circumpolar range (Paetkau *et al.* 1999), pairwise F_{ST} values (0.0020–0.11) were also lower than in this study. Similarly, among seven mainland populations of grey wolves (*Canis lupus*), Carmichael *et al.* (2001) reported pairwise F_{ST} values from 0.015 to 0.097. Our high pairwise F_{ST} values relative to these other large carnivore studies are surprising, especially given that all of the above sampled populations that were separated by greater geographical distances than those reported here.

Although high for large carnivores, our pairwise F_{ST} values were similar to those reported elsewhere for pumas. This study is unique in that it contrasts situations in which suitable habitat is well connected across large regions with those in which habitat barriers may limit movement within smaller areas. In the former case, high rates of gene flow apparently occur at scales larger than individual states. Although we found evidence for isolation by distance in both Utah and Colorado, sample groups within Utah (average pairwise F_{ST} = 0.031, range 0.0046–0.068) and Colorado (average pairwise F_{ST} = 0.025, range –0.0048–0.065) were weakly differentiated compared with those separated by habitat gaps elsewhere in our study area. Sinclair *et al.* (2001) also found relatively little differentiation among 10 populations in Utah, and no pattern of isolation by distance, presumably due to smaller sample sizes than those reported here. Anderson *et al.* (2004) found relatively low levels of genetic structuring among pumas in Wyoming and western South Dakota. Pairwise F_{ST} values among populations separated by the Wyoming Basin did not

exceed 0.051, and STRUCTURE suggested a single population across their study area, even when 15 pumas from southern Colorado (480–820 km from other sampled areas) were included.

Where habitat barriers were present, we found greater population structuring than could be explained by distance alone. Similarly, Walker *et al.* (2000) reported a pairwise F_{ST} value of 0.11 between two populations separated by 550 km of grassland and desert scrub in Texas. In California, Ernest *et al.* (2003) found substantial structuring among 12 puma populations (average pairwise F_{ST} = 0.12, range 0.010–0.37). They concluded that the Central Valley, San Francisco Bay and Delta, Los Angeles Basin, and Mojave and Sonoran Deserts were likely responsible for the observed subdivisions. Finally, greater differentiation among sample groups in the patchier southern portion of our study area (Fig. 4a, b) is similar to patterns observed in Idaho. There, Loxterman (2001) found greater structuring among populations isolated by agriculture and other sources of human-induced fragmentation in southern portions of the state than in the more contiguously forested northern portion. Populations separated by agricultural development on the Snake River Plain showed average pairwise F_{ST} values of 0.11 (range 0.049–0.19).

Combined with the results of the studies discussed above, our findings begin to complete a picture of how landscape connectivity affects genetic differentiation and population structuring in pumas. Low levels of differentiation among populations in Utah, Colorado, northern Idaho, Wyoming, portions of California, and along the Mogollon Rim in Arizona and New Mexico indicate that large, contiguous areas of suitable habitat (e.g. forest, woodland, and sagebrush vegetation) permit high rates of gene flow. Greater structuring between northern Arizona and Utah, between northern and southern New Mexico, between the Sacramento Mountains and other mountain ranges in New Mexico, and in portions of California, southern Idaho, and Texas indicate that low desert, grasslands, agriculture and other human development may impede gene flow.

These patterns have important implications for puma management and conservation. We agree with Sinclair *et al.* (2001) that high levels of gene flow at large scales mean that effective puma conservation will require an integrated approach, involving multiple state agencies. However, significant differentiation between nearly all sample group pairs and strong patterns of isolation by distance within states suggest that considerable population structuring can occur within only a few hundred kilometres. Thus, although state agencies may be managing relatively unstructured populations within individual hunting units (each < 15 000 km² in Arizona), populations cannot be assumed to be panmictic across much larger areas. Furthermore, evidence for substantial structuring also means that populations

within some states may not be connected by frequent dispersal and should be divided into separate, intrastate management units. Such divisions would define populations with allele frequencies divergent enough to indicate functional independence (Moritz 1994). For example, our results suggest that pumas in northern New Mexico share weaker genetic ties with neighbouring pumas in southern New Mexico than with more distant populations in Colorado and Utah.

North–south differences in genetic diversity and evidence for a post-Pleistocene range expansion

Two lines of evidence suggest that the north–south split between pumas in our study area may reflect historical events, and not contemporary dispersal barriers alone. First, we found substantially fewer numbers of alleles in the northern portion of our study area than in the south, even though we sampled more than twice as many pumas in the north. Alleles detected in the northern portion of the study area were largely a subset of those detected in the south. Second, analyses of *F* values from all STRUCTURE runs suggest that northern pumas have undergone greater rates of genetic drift following subdivision of the ancestral population. These results echo the larger patterns found by Culver *et al.* (2000), in which substantially lower levels of microsatellite and mitochondrial diversity were detected in North American pumas than in those from South America. If their hypothesis that pumas were eliminated from North America at the end of the Pleistocene is correct, then patterns detected in our study area may reflect the subsequent recolonization, with a historical range expansion from the south resulting in decreasing diversity in more northern populations due to serial founder events (Mayr 1942). Other studies have documented a loss of genetic diversity in populations newly established as a result of range expansions in humans and other animals (e.g. Rendine *et al.* 1986; Sokal *et al.* 1991; Hewitt 1993), especially when dispersal distributions include rare long distance dispersal events (Nichols & Hewitt 1994; Ibrahim *et al.* 1996). A handful of documented movements > 400 km (e.g. Ruth *et al.* 1998) suggest this may be the case with pumas.

An alternative explanation for reduced diversity and greater rates of drift in the north may be a more recent genetic bottleneck resulting from widespread persecution of pumas in the last century. However, the rapid loss of alleles from northern pumas in recent times would have required a simultaneous and severe reduction in population sizes over a very large area. The increased drift in surviving populations would have also resulted in rapid differentiation between them – an expectation inconsistent with the relatively small genetic distances among northern sample groups. There is also little evidence to suggest that puma persecution has been any

more intense in the north than in the south. Furthermore, the data of Roelke *et al.* (1993) and Culver (1999) suggest that the pattern observed here may be more general, with pumas sampled in other portions of the United States and Canada consistently exhibiting lower levels of allozyme and microsatellite diversity than those sampled in Arizona.

Interpretation of Bayesian clustering results

Two concerns with the application of STRUCTURE to our data set warrant discussion. First, STRUCTURE indicated radical improvement in $\Pr(X|K)$ when two populations were assumed rather than one, but more modest improvement for higher population numbers, making interpretation of the ‘true’ number of populations difficult. In cases like this, the inferred value of *K* may not always have a clear biological interpretation (Pritchard *et al.* 2000). In particular, clusters may not correspond to ‘real’ populations when allele frequencies vary among sampling locations due to isolation by distance rather than genuine population subdivision (Pritchard *et al.* 2000). In such cases, the smallest value of *K* that captures the major structure in the data is likely the best choice (Pritchard & Wen 2003), and it is possible that there are as few as two relatively discrete populations, with further model improvement being due to the more subtle effects of isolation by distance within populations. Supporting this interpretation, additional subdivisions at values of $K > 2$ were less sharp, with clusters overlapping one another considerably (Supplementary material, Fig. S1). Furthermore, the proportion of the sample assigned to each population in the $K = 9$ case was roughly the same (c. $1/K$), signalling a lack of true structure (Pritchard & Wen 2003).

A second concern was an apparent effect of sampling intensity on the STRUCTURE results. With the full data set, more clusters were identified in the northern portion of the study area than in the south for all $K > 2$. Randomly discarding individuals from the north to equalize sample sizes with the south reproduced the same north–south division, but values of $K > 2$ consistently produced more clusters in the south. Our larger sample size in the north may have provided greater opportunity to generate spurious clusters in the presence of isolation by distance or other departures from model assumptions. The consistent result from our data, however, was a hierarchical pattern of structuring, with clusters residing nearly entirely in northern or southern portions of the study area, regardless of the value of *K* or the number of samples analysed (Fig. 1; Supplementary material, Fig. S1). At a minimum, there appear to be two puma populations with distinct histories, with isolation by distance and habitat barriers contributing to further substructuring within these populations.

Geographic clustering method

Conducting population-based analyses can be problematic when individuals do not naturally fall into discrete groups. Although using the individual as the sampling unit is one way to avoid this problem (Manel *et al.* 2003), clustering individuals into populations allows analyses based on allele frequencies, which can increase resolving power and provide convenient ways to summarize patterns of genetic structuring and compare results among studies. Investigators usually group individuals into populations using their own judgement or nonbiological criteria such as game management units. The UPGMC method offered an alternative that allowed us to objectively delineate local sample groups specifying only biologically reasonable dispersal distances. Individuals were assigned to intuitively reasonable local clusters, the allele frequencies of which could be assumed to approximate average frequencies at cluster centroids.

The method could result in combination of separate populations if individuals are pooled across barriers or if excessively large cut-off distances are used. Other than prohibiting clusters to cross barriers that have been hypothesized a priori, tests for structuring within clusters may be used to evaluate cluster integrity when sample sizes are adequate. We found no departure from HWE within 33 of our 34 sample groups. While these results should be interpreted with caution for small groups (like some of ours), lack of structuring in larger groups suggests that our cut-off distances were not too large in the absence of habitat barriers. In contrast, significant differentiation between 95% of sample group pairs indicates that larger cutoff distances may have combined genetically distinct populations. For example, increasing the cut-off distance to 100 km would have combined eight pairs of sample groups into larger groups (for a total of 26 sample groups). Three of the combined pairs had significant (albeit small) pairwise F_{ST} values between them. However, our conclusions would have changed little had we chosen this larger clustering distance; results of population-based analyses (trees, ordinations, and isolation by distance) repeated with the 26 sample groups were remarkably similar to those with a cut-off distance of 80 km. NAZ2 was combined with CAZ1, and the new sample group remained the only group to deviate significantly from HWE.

Grouping individuals for population-based analyses will always involve some level of arbitrary choice; our purpose here is to argue for methods that are as objective and explicit as possible. Our UPGMC technique had a simple, biological basis: if two samples or clusters were separated by less than the average dispersal distance for male pumas, they were combined. Other approaches to delineating groups without introducing investigator

bias could undoubtedly be used, and we encourage their development and comparison.

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Supplementary material

The following supplementary material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2571/MEC2571sm.htm>

Fig. S1 Assignment of samples to populations using STRUCTURE with $K = 9$. Sample colours indicate assignment to nine population clusters; individuals that did not assign to any population with > 50% probability are shown in grey.

Fig. S2 NMDS ordination of all 540 puma genotypes. Symbols indicate samples from northern region, southern region, and a transitional zone (AZ north of I-40 and the Manzano Mountains in NM), where samples did not consistently assign to northern or southern clusters using STRUCTURE or ordinations.

Table S1 Pairwise F_{ST} (upper diagonal) and D_{LR} values between 34 sample groups. Underlined F_{ST} values were significant before Bonferroni correction ($P < 0.05$), and bold values remained significant after correction.

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This study was part of Brad McRae's doctoral project, which also involved modeling the effects of landscape heterogeneity on gene flow in natural populations. Paul Beier works on ecoregional conservation planning, including collaborative, science-based "missing linkages" efforts in Arizona and California. Laura DeWald conducts research in conservation and ecological genetics to guide decision-making in conservation problems such as fragmentation. Lynn Huynh is interested in ecological and evolutionary dynamics of epidemic infectious diseases and is now working on her PhD at Emory University. Paul Keim is the Cowden Endowed Chair and Director of the NAU Environmental Genetics and Genomics (EnGGEN) center, which specializes in genetic analyses of natural populations of plants, animals and microorganisms.
