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Heather E. Watts
Loyola Marymount University, heather.watts@lmu.edu

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Genetic diversity and structure in two spotted hyena populations reflects social organization and male dispersal

H. E. Watts[1,2], K. T. Scribner[2,3], H. A. Garcia[1] & K. E. Holekamp[2]

1 Department of Biology, Loyola Marymount University, 1 LMU Drive, Los Angeles, CA, USA
2 Department of Zoology, Michigan State University, 203 Natural Science Building, East Lansing, MI, USA
3 Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI, USA

Keywords
carnivore; *Crocuta crocuta*; genetic diversity; population structure; relatedness; spotted hyena.

Correspondence
Heather E. Watts, Department of Biology, Loyola Marymount University, 1 LMU Drive, Los Angeles, CA 90045, USA.
Email: hwatts1@lmu.edu

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Abstract

Populations of large carnivores are particularly vulnerable to demographic changes that can reduce genetic diversity and threaten the persistence of these species. Although the spotted hyena *Crocuta crocuta* is the most abundant large carnivore in Africa, it has been extirpated locally from many areas. In this study, we compare genetic diversity, patterns of relatedness and genetic structure in spotted hyenas, in order to investigate whether social structure and male dispersal patterns may serve to buffer this species from potential losses of genetic diversity. Using 10 microsatellite markers, we compared two Kenyan populations of spotted hyenas that have experienced different recent population histories. The Masai Mara population has remained large and stable, whereas the Amboseli population has recently recovered from a demographic bottleneck. Despite these historical differences, we found no difference in genetic diversity between the two populations (H_O, Mara: 0.598 ± 0.060; Amboseli: 0.577 ± 0.071; $P = 0.76$). Patterns of relatedness within and between clans were similar in both populations, except that immigrant males appeared to be more closely related to one another in Amboseli than in the Mara. This difference in relatedness among immigrant males appears to reflect differences between populations in patterns of immigration. Hierarchical analysis of the population genetic structure revealed significant genetic differentiation among spotted hyena clans within populations ($F_{SC} = 0.055$, $F_{ST} = 0.108$) and among spotted hyena study populations ($F_{CT} = 0.057$). We suggest that behavioral traits of the spotted hyena, particularly the predominance of male dispersal, were important in the maintenance of genetic variation in the Amboseli population.

Introduction

Large carnivore populations around the world are declining and becoming locally extinct, due largely to direct and indirect effects of human activity (Woodroffe & Ginsberg, 1998; Woodroffe, 2001; Cardillo *et al*., 2004). Large carnivores appear to be particularly vulnerable to demographic changes that may in turn reduce population levels of genetic diversity because they tend to live at low densities, have large home ranges and pose a threat to humans and livestock (Woodroffe & Ginsberg, 1998; Mills, Freitag & Van Jaarsveld, 2001; Woodroffe, 2001). Indeed, many large carnivores now persist in relatively small and fragmented populations (e.g. Vilà *et al*., 1999; Bauer & Van Der Merwe, 2004). The viability of such small populations is of particular concern to conservation biologists (Lande, 1988; Frankham, 2005). From a genetic perspective, small populations may experience inbreeding and the loss of genetic variation due to genetic drift, which may in turn lead to reduced fitness and limited evolutionary potential (Lynch, 1996; Lacy, 1997; Keller & Waller, 2002; Reed & Frankham, 2003). Alarmingly, even if populations that were once small are able to recover demographically, the genetic legacies of small ancestral populations (i.e. population bottlenecks, including founder events) may still threaten their persistence. For example, African lions *Panthera leo* in the Ngorongoro Crater, Tanzania, exhibit reduced levels of genetic diversity together with reduced reproductive performance as a result of historical bottleneck(s) (Packer *et al*., 1991).

Interestingly, however, such threats to small or bottlenecked populations do not appear to be uniform among vertebrates. Recent studies have revealed that some populations have maintained high levels of genetic diversity despite demographic bottlenecks (Kuo & Janzen, 2004; Aspi *et al*., 2006; Hailer *et al*., 2006; Kaeuffer *et al*., 2007), and that other populations have lost genetic diversity but without evidence of reduced fitness (Wisely *et al*., 2002). Numerous aspects of a species' biology, particularly its behavior, are likely to influence genetic and fitness outcomes in small and bottlenecked populations. For example, social organization,

mating system, dispersal patterns and vagility may all influence population genetic dynamics (Sugg *et al*., 1996; Keller *et al*., 2001; Vilà *et al*., 2003), and ultimately population persistence. Consequently, in order to properly assess the health and viability of carnivore populations, we must understand not only demographic processes, but also patterns of genetic diversity and genetic structuring in these populations. Data from free-living populations are therefore useful as we endeavor to elucidate general principles (Amos & Balmford, 2001), as well as providing information for species-specific conservation planning.

Spotted hyenas *Crocuta crocuta* are the most abundant large carnivore in Africa. In contrast to many other terrestrial large carnivores, spotted hyenas remain widespread on the continent, with numerous large, viable populations (Höner, Holekamp & Mills, 2008). Interestingly, spotted hyenas exhibit several behavioral attributes that may help buffer their populations from potential losses of genetic diversity. Spotted hyena populations are socially structured; spotted hyenas live in large social groups, called clans, in which individuals cooperate to defend group territories (Kruuk, 1972). Whereas female spotted hyenas spend their lives in their natal clan, male hyenas disperse from their natal clan once they reach adulthood (Boydston *et al*., 2005). Consequently, hyena clans are typically composed of natal females, their offspring, and immigrant males who sire most offspring (Engh *et al*., 2002; Höner *et al*., 2007). Furthermore, patterns of female mate choice result in low reproductive skew among immigrant males (Engh *et al*., 2002). Finally, male spotted hyenas have high rates of dispersal (97–100% of males disperse; East & Hofer, 2001; Boydston *et al*., 2005), are capable of moving over large distances (Hofer & East, 1993), and may be particularly well-suited to disperse across potential barriers, including areas of human development, due to their behavioral plasticity. Hyenas can utilize a wide range of habitats and food resources, and have been found to quickly modify their behavior in response to human activity (Mills & Hofer, 1998; Boydston *et al*., 2003; Hayward, 2006; Kolowski *et al*., 2007).

In this study, we examine population genetic diversity, patterns of relatedness and genetic structure in two populations of free-living spotted hyenas, to inquire whether social structure and male dispersal patterns of spotted hyenas might buffer them from potential losses of genetic diversity. Specifically, we focus on two populations that differ in their recent demographic histories: Amboseli National Park and the Masai Mara National Reserve. Although spotted hyenas remain relatively abundant, they have undergone local extinctions in many areas (Mills & Hofer, 1998). In Amboseli National Park, a small reserve (390 km^2) in southern Kenya, the spotted hyena population recently rebounded from a demographic bottleneck. The Masai Mara National Reserve (hereafter Mara) is a large reserve (1530 km^2) located *c*. 250 km west of Amboseli. Spotted hyenas in the Mara have been monitored continuously since 1979 (e.g. Frank, Holekamp & Smale, 1995), and in contrast to Amboseli, the Mara hyena population is known to have remained large and stable since at least the late 1970's.

Materials and methods

Study populations

Amboseli National Park is comprised primarily of semi-arid savanna. It is now largely surrounded by habitat dedicated to agriculture and pastoralism (Campbell *et al*., 2005), although dispersal corridors persist, as indicated by occasional sightings in the park of certain rare carnivores (e.g. African wild dogs, *Lycaon pictus*; Watts & Holekamp, 2005). Early reports make clear that spotted hyenas were once abundant in the area that is now Amboseli National Park (e.g. Thompson, 1885). However, in the early 1970's, the Amboseli hyena population experienced a major decline, possibly due to disease (Faith & Behrensmeyer, 2006) or local anthropogenic activity. Subsequently, hyena sightings were rare (Faith & Behrensmeyer, 2006) throughout the 1970's and 1980's, and the park's entire hyena population was estimated at 50 animals (C. Moss, pers. commun.). The period spanning the 1970's and 1980's coincided with growth of the local pastoralist population and expansion of agricultural practices, leading to habitat loss and fragmentation in the ecosystem (Campbell *et al*., 2003, 2005); these changes were presumably detrimental to many animal populations. Then, in the 1990's, the hyena population underwent a rapid expansion (Faith & Behrensmeyer, 2006), which was likely related to two other changes that occurred in Amboseli during the same period: a dramatic reduction in the lion population (a major competitor with hyenas) due to human persecution (Behrensmeyer, 1993; Chardonnet, 2002), and an increase in the abundance of prey species such as wildebeest and zebra due to the conversion of woodland habitat to grassland habitat (Western, 1989). Regardless of the cause of the marked population increase, by the early 2000's the hyena population in Amboseli was estimated to contain 300–400 individuals (Watts & Holekamp, 2005).

The Masai Mara National Reserve is characterized primarily by rolling savanna habitat. It borders the Serengeti National Park to the south, whereas other surrounding habitat is largely pastoral rangeland with some agricultural lands (Homewood *et al*., 2001). Spotted hyenas in this population have been monitored continuously since the late 1970's, and the population has remained large and stable since at least that time. Detailed surveys of the hyena population in 1991 and 2003 estimated the Mara hyena population at 620 and 617 hyenas, respectively (Ogutu, Bhola & Reid, 2005).

Sampling

As part of a larger study on the behavior and ecology of spotted hyenas in Amboseli (e.g. Watts & Holekamp, 2008), we monitored hyenas belonging to two clans, the Airstrip and Ol Tukai clans, from July 2003 to July 2005 (Fig. 1). During this period we collected fecal and tissue samples for genetic analysis from 80 individually recognized hyenas (Watts & Holekamp, 2008). Blood and tissue samples were

collected from the Mara population, as described by Engh *et al.* (2002). Briefly, most samples originated from venous blood collected from hyenas that were anesthetized with Telazol. To ensure similarity in sampling intensity and timing of sample collection between Amboseli and the Mara populations, samples were taken from 72 known individuals belonging to two intensively studied Mara clans between July 2002 and July 2004 (Fig. 1). Study clan sizes were similar in both populations (see Table 1) and covered similar geographic sampling areas. The Amboseli clans defended adjacent territories covering 28.0 km^2 (Airstrip clan) and 26.4 km^2 (Ol Tukai clan), whereas the Mara clans were separated by a single territory; these clans defended territories covering 28.4 km^2 (Talek clan) and 31.0 km^2 (Mara River clan; Kolowski *et al.*, 2007). In both, populations genetic samples were obtained from 70–80% of the study hyenas.

Microsatellite genotyping

Most DNA samples from the Amboseli population were obtained from feces, all of which were collected from known hyenas within 10 min of defecation. Samples were then either (i) extracted shortly after collection (2–6 h); or (ii) immediately frozen or stored in >95% ethanol for later extraction

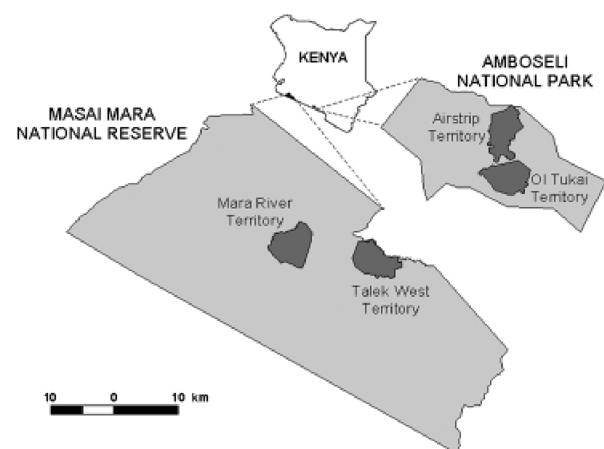

Figure 1 Map of the study sites and spotted hyena *Crocuta crocuta* clan territories.

(usually 18–36 h, but some samples were stored up to 28 months). Fecal DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol for a 1 g sample. Rather than originating from feces, most DNA samples from the Mara population originated from blood samples. DNA was extracted from blood samples shortly after collection using a Puregene kit (Gentra Systems, Minneapolis, MN, USA). For tissue samples from both populations, DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen).

We amplified 10 microsatellite loci (CCr01, CCr04, CCr07, CCr11, CCr12, CCr13, CCr14, CCr15, CCr16, CCr17) described previously for this species (Libants *et al.*, 2000; Engh, 2002; Van Horn *et al.*, 2004). The polymerase chain reaction conditions for DNA extracted from blood and tissue followed Libants *et al.* (2000) and Engh (2002). For DNA extracted from feces, these conditions were modified as follows: (i) reactions contained either 1 μL (CCr11), 3 μL (CCr07, CCr15) or 5 μL (CCr01, CCr04, CCr12, CCr13, CCr14, CCr16, CCr17) of DNA extract; (ii) 0.25 μg of bovine serum albumin was added to reactions; (iii) 1U ChromaTaq DNA polymerase (Denville Scientific, Meutuchen, NJ, USA) was used for each reaction. Additionally, the number of PCR cycles was increased to 40 for amplification of all loci.

To genotype individuals using DNA extracted from fecal samples, we used a modified multi-stage, multiple-tubes approach (Navidi, Arnheim & Waterman, 1992; Taberlet *et al.*, 1996). First, a single positive amplification was carried out for one extract from each individual. For individuals scored as heterozygous from this first amplification, no further amplifications were performed. In cases where only one allele was scored (homozygotes) on the first amplification, a second amplification was performed. If the second amplification produced a heterozygous genotype sharing one allele with the initial amplification, the individual was scored as heterozygous. If the second amplification was homozygous for the same allele as in the first amplification, the individual was scored as homozygous for that allele. Cases in which the second amplification was homozygous for a different allele than the first amplification were scored as heterozygous for those two alleles. In cases ($n = 3$ single locus amplifications) where more than two alleles were

Table 1 Comparison of genetic diversity between the Amboseli and Mara spotted hyena *Crocuta crocuta* populations

Population and clan[a]	N	A[b]	H_O[b]	H_E[b]	H_I	IR	F	R
Amboseli	80	4.6 ± 0.7	0.577 ± 0.071	0.548 ± 0.069	0.575 ± 0.016	−0.049 ± 0.026	−0.015 ± 0.012	
Airstrip	45	4.2 ± 0.7	0.593 ± 0.069	0.548 ± 0.059	0.592 ± 0.023	−0.070 ± 0.038	−0.023 ± 0.016	0.018 ± 0.011
Ol Tukai	35	4.3 ± 07	0.556 ± 0.091	0.512 ± 0.081	0.553 ± 0.023	−0.021 ± 0.035	−0.005 ± 0.019	0.156 ± 0.016
Mara	72	4.8 ± 0.6	0.598 ± 0.060	0.600 ± 0.046	0.593 ± 0.015	0.017 ± 0.027	0.021 ± 0.017	
Talek	49	4.5 ± 0.5	0.603 ± 0.053	0.590 ± 0.046	0.601 ± 0.020	0.007 ± 0.038	0.031 ± 0.022	0.028 ± 0.010
Mara River	23	4.4 ± 0.5	0.574 ± 0.095	0.586 ± 0.072	0.577 ± 0.018	0.041 ± 0.027	−0.001 ± 0.026	0.146 ± 0.025

[a]Clan sizes for the study period are as follows: Airstrip: 42–64, Ol Tukai: 32–48, Talek: 47–55, Mara River: 28–38. Data from Kolowski & Holekamp (2008) and Watts & Holekamp (2008).

[b]Values are calculated across loci (N = 10).

Values are presented as mean ± 1 SE for the number of alleles (A), observed and expected heterozygosities (H_O and H_E), individual heterozygosity (H_I), internal relatedness (IR), individual inbreeding coefficient (F), and within-clan relatedness of natal animals (R).

observed in either replicate, the sample was presumed to have been contaminated by more than one individual and was discarded.

Reliability of genotypes obtained from fecal DNA

The use of fecal DNA with microsatellite markers typically results in a higher frequency of genotyping errors than with other source materials such as blood or tissue, which generally yield a higher quality and quantity of DNA (Bayes *et al.*, 2000). To assess error rates for genotypes derived from fecal samples, we randomly selected a subset of individuals for each locus. Our goal was to sample approximately 10% of individuals (i.e. eight to nine individuals per locus). This subset of individuals was genotyped from fecal DNA using the traditional multiple-tubes approach described by Taberlet *et al.* (1996), with each individual assigned a consensus genotype that was assumed to be the true genotype. All positive amplifications performed for an individual were then used to estimate the rate of allelic dropout and the probability of a false allele following Broquet & Petit (2004).

Additionally, for four individuals from the Mara population, DNA samples derived from both blood and feces were available. For these individuals, genotypes obtained based on one to three amplifications of fecal DNA per individual were compared with genotypes obtained using blood samples.

Measures of genetic diversity, relatedness and population structure

We used the program CERVUS 3.0 (Kalinowski, Taper & Marshall, 2007) to estimate the number of alleles (A), allele frequencies, observed and expected heterozygosities (H_O and H_E), and to test for deviations from the Hardy–Weinberg equilibrium. Genetic diversity was compared between populations using loci-based measures (A, H_O, and H_E), and individual-based measures: individual heterozygosity (H_I), internal relatedness (IR), and individual inbreeding coefficient (F). H_I is the proportion of heterozygous loci per individual. Due to differences in allele frequencies between the two populations, we were unable to standardize H_I to account for missing data (Coltman *et al.*, 1999; Lukas *et al.*, 2004). Nevertheless, because missing data were similarly distributed in both populations, they should not have biased this measure. IR (Amos *et al.*, 2001) was calculated as

$$\frac{(2H - \sum f_i)}{(2N - \sum f_i)}$$

where N is the total number of loci, H is the number of loci that are homozygous and fi is the frequency of the ith allele in the genotype across all loci. IR is analogous to Queller & Goodnight's (1989) relatedness coefficient (R), but reflects genetic correlation between a pair of alleles at a locus. Thus, it can be thought of as reflecting the degree of relatedness between an individual's parents. More negative IR values indicate greater genetic diversity and suggest that an individual is more 'outbred'. Finally, F was estimated from microsatellite genotypes using Ritland's (1996) moment estimator implemented in COANCESTRY (Wang, 2011). Only individuals genotyped at 5 or more loci ($\bar{X} \pm$ standard error (SE) $= 9.5 \pm 0.1$ loci) were included in these analyses. The Mann–Whitney U-test was used to test for differences in genetic diversity between populations, except for comparisons of F. To test for differences in F, 95% confidence intervals were generated using the bootstrapping method (1 000 000 replicates) in COANCESTRY. Unless otherwise indicated, statistical analyses were performed in STATISTICA (StatSoft, 2002). Differences between groups were considered significant when $P < 0.05$. Mean values are presented $\pm$ 1 SE.

We used two methods to test for a genetic signature of a population bottleneck. First, the program BOTTLENECK (Cornuet & Luikart, 1996) was used to test for an excess of heterozygosity, which is expected to occur when genetic drift at small population sizes leads to a loss of rare alleles. Heterozygote excess was tested using a Wilcoxon signed-rank test with a two-phased mutation model and 95% probability of single-step mutations, as recommended for microsatellites (Piry, Luikart & Cornuet, 1999). Second, we used the M-ratio test (Garza & Williamson, 2001), which is a measure of the number of alleles relative to the range in allele sizes. As recommended by Garza & Williamson (2001), values < 0.68 were considered indicative of a deficit in the total number of alleles relative to the range in allele size resulting from a bottleneck.

To asses whether patterns of relatedness differed between our two study populations, pairwise relatedness values (R) were estimated for individuals sampled from each population using program RELATEDNESS 5.0 (Queller & Goodnight, 1989). As the inclusion of related animals to estimate population allele frequencies can bias R (Queller & Goodnight, 1989), only adults were used to estimate allele frequencies. Analyses were conducted separately for each population. Only individuals genotyped at 8 or more loci were included in relatedness analyses. As pairwise relatedness values are not independent, two-sample randomization tests (9999 iterations) implemented in POPTOOLS (Hood, 2006) were used to test for differences in mean relatedness between groups.

To evaluate population structure, F-statistics were calculated using ARLEQUIN 3.5.1.1 (Excoffier & Lischer, 2010). Hierarchical F-statistics were calculated by specifying four levels of genetic structure: individual (I), clan (S), study population (C) and total population (T). Pairwise F_{ST} values were estimated among all clans. P-values for F-statistics were generated by permutation tests (10 000 permutations) in ARLEQUIN. For pairwise estimates of F_{ST} a Bonferroni correction was applied to correct for multiple tests.

Finally, in order to assess potential population structure that was not evident from our hierarchical analysis that was conducted based on pre-defined groups, we used STRUCTURE 2.3.3 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2003) to identify clusters of genetically similar individuals in the absence of any *a priori* information

on group membership. Using multilocus genotypes, STRUCTURE identifies clusters and assigns individuals to clusters so as to minimize deviations from the Hardy–Weinberg equilibrium and linkage equilibrium within clusters. We were particularly interested to use this approach to gain additional insight into the structure of the Amboseli population and its recent history. To determine the number of clusters (K) within in our sample, we conducted eight independent runs for values of K from 1 to 9 using the admixture model, correlated allele frequencies and a burn-in period of 50 000 followed by 100 000 Markov chain Monte Carlo (MCMC) steps. The optimal value of K was determined by evaluating the log probability of the data $\Pr(X|K)$ for each value of K (Pritchard *et al.*, 2000). Using this optimal K value, we then carried out a final run of the model (100 000 burn-in, 1 000 000 MCMC) to determine individual membership in clusters. Individuals were assigned to clusters based on the highest percentage of membership (Q).

Results

Reliability of genotypes

For fecal DNA, across all loci the rate of allelic dropout was 0.027 and the probability of a false allele was 0.010. Error rates varied among loci (see supporting information Appendix S1) though performance did not appear to reflect allele size (range 95–199 bp). Agreement between genotypes generated from both feces and blood was 100% (39/39 genotypes, four individuals).

Genetic diversity and test for bottleneck

All loci were in the Hardy–Weinberg equilibrium in both populations, except for CCr16 in the Mara population ($\chi^2 = 9.68$, d.f. = 1, $P < 0.05$), which exhibited a deficiency of heterozygotes. However, when the larger available Mara dataset was examined ($n = 377$ hyenas sampled since 1990), all loci were in the Hardy–Weinberg equilibrium (Van Horn *et al.*, 2004). All loci were retained for analyses.

Using several measures of genetic diversity, we found no evidence for lower genetic variation in the Amboseli population than in the Mara population. Neither the mean number of alleles (Mann–Whitney $U = 46.5$, $n_1 = n_2 = 10$, $P = 0.79$) nor observed heterozygosity ($U = 46.0$, $n_1 = n_2 = 10$, $P = 0.76$) differed significantly between Amboseli and the Mara populations (Table 1). Similarly, H_I did not differ significantly between populations ($U = 2667.5$, $n_1 = 80$, $n_2 = 72$, $P = 0.43$). There was a trend for IR to be higher in the Mara ($U = 2406.0$, $n_1 = 80$, $n_2 = 72$, $P = 0.08$). But, we found no significant differences in F between the two populations (95% confidence interval $= -0.042$ to 0.041, observed difference $= -0.036$).

Consistent with these results, we found no genetic signature of a population bottleneck in Amboseli. There was no excess of heterozygosity, which would result from a loss of rare alleles during a bottleneck, in either Amboseli (whole population: $P = 0.5$, Ol Tukai clan: $P = 0.5$, Airstrip clan: $P = 0.3$) or in the Mara (whole population: $P = 0.4$, Talek clan: $P = 0.5$, Mara River clan: $P = 0.7$). In both the study populations, the M-ratio indicated no deficit in the number of alleles relative to the range in allele size (Amboseli: $M = 0.798$, variance across alleles = 0.039; Mara: $M = 0.835$, variance across alleles = 0.029). There was also no significant difference in M-ratio between populations across alleles ($U = 45.5$, $n_1 = n_2 = 10$, $P = 0.73$).

Relatedness

Overall, pairwise estimates of relatedness showed similar patterns in the Mara and Amboseli. Mean R-values for known mother-offspring pairs (Mara: 0.45 ± 0.04, $n = 27$ pairs; Amboseli: 0.42 ± 0.04, $n = 32$ pairs) were only slightly lower than the expected value of 0.5. In both populations relatedness was significantly higher between natal animals belonging to the same clan, than between natal animals from different clans (Fig. 2a; Amboseli, $n_1 = 1041$, $n_2 = 1039$ pairs, $P < 0.0001$; Mara, $n_1 = 786$, $n_2 = 592$ pairs, $P < 0.0001$). Patterns of within-clan relatedness for natal animals differed significantly between clans in both populations (Amboseli, $n_1 = 666$, $n_2 = 375$ pairs, $P < 0.0001$; Mara, $n_1 = 666$, $n_2 = 120$ pairs, $P < 0.0001$), with higher relatedness observed in the smaller clans (Table 1). One difference between populations was that relatedness among adult female clan-mates was significantly higher than among immigrant male clan-mates in the Mara (Fig. 2b; $n_1 = 132$, $n_2 = 48$ pairs, $P = 0.004$), but not in Amboseli (Fig. 2b; $n_1 = 173$, $n_2 = 30$ pairs, $P = 0.20$). Mean pairwise relatedness among all immigrant males was 0.043 ± 0.029 in Amboseli ($n = 66$ pairs) and -0.034 ± 0.030 in the Mara ($n = 78$ pairs).

Population structure

Hierarchical analysis indicated significant genetic differentiation among clans within each study population, and among study populations (Table 2); the magnitude of this differentiation was low to moderate. Using AMOVA, we found that 5.9% of genetic variation was partitioned among study populations and 4.9% of variation was partitioned among clans within populations. Pairwise comparisons of F_{ST} showed significant differentiation at the clan level, among all clans sampled (Table 3). The magnitude of differentiation appeared to be greater among clans from different study populations (mean $F_{ST} = 0.1025$) than among clans from the same study population (mean $F_{ST} = 0.0485$). F_{IS} and F_{IT} values did not differ significantly from zero (Table 2).

Analysis in STRUCTURE indicated the highest likelihood of $K = 7$ clusters in our sample (Fig. 3). When we examined individual membership in these clusters, we found that three clusters corresponded strongly (>80% membership of cluster) to a particular clan: Talek, Airstrip and Ol Tukai (Fig. 4). Three additional clusters corresponded primarily to a specific clan (60–80% membership of cluster), but included an additional 20–40% of members drawn primarily

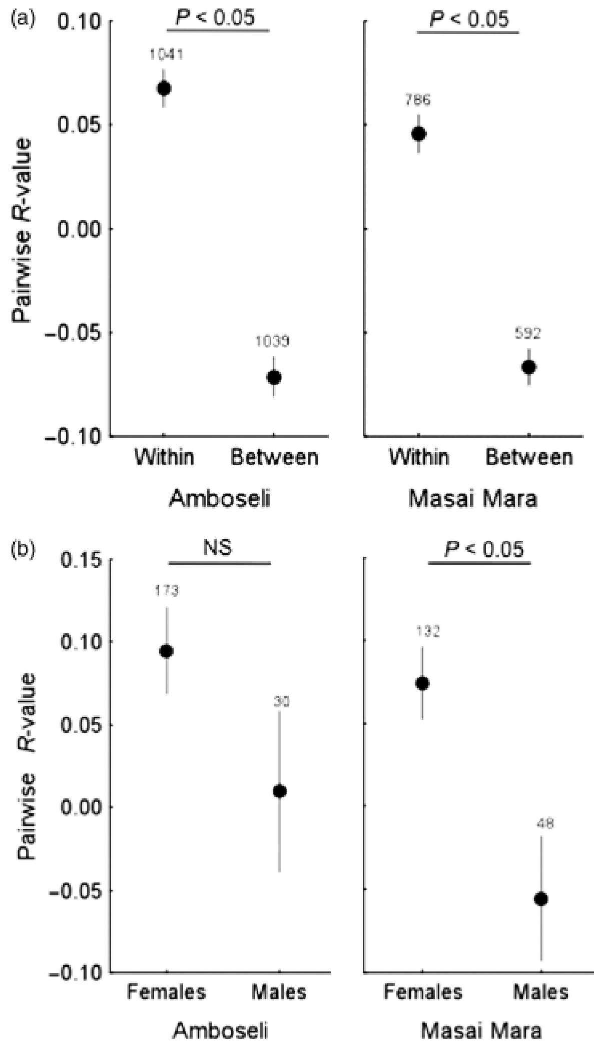

Figure 2 Pairwise *R*-values (a) among natal animals belonging to the same clan or belonging to different clans within the Amboseli and Mara spotted hyena *Crocuta crocuta* populations, and (b) among adult female clan-mates and among immigrant male clan-mates for the Amboseli and Mara populations. Sample sizes indicate number of *R*-values. Values are presented as $\bar{X} \pm$ SE. Results of statistical comparisons between groups are reported as significant ($P<0.05$) or not statistically significant.

Table 2 Hierarchical estimates of population differentiation in spotted hyenas *Crocuta crocuta*

	Variance component	*F*-statistic	*P*-value
F_{IS}	Individual within clan	−0.050	0.997
F_{IT}	Individual within total pop	0.063	0.103
F_{SC}	Clan within study pop	0.055	<0.0001
F_{ST}	Clan within total pop	0.108	<0.0001
F_{CT}	Study pop within total pop	0.057	0.018

I, individual; S, clan; C, study population; T, total population.

Table 3 Pairwise estimates of F_{ST} among spotted hyena *Crocuta crocuta* clans

	Mara		Amboseli	
	Mara River	Talek	Airstrip	Ol Tukai
Mara River	–			
Talek	0.043*	–		
Airstrip	0.089*	0.088*	–	
Ol Tukai	0.120*	0.113*	0.054*	–

*$P<0.05$ after the Bonferroni correction.

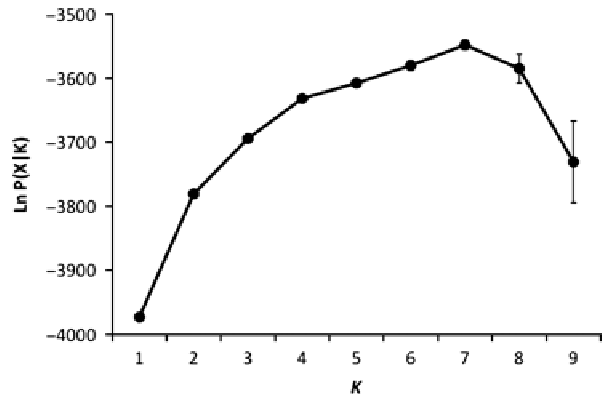

Figure 3 Results of STRUCTURE analysis indicating the likelihood of subdivision of the total population of sampled hyenas into *K* clusters [Ln Pr(*X*|*K*)] for values of *K* from 1 to 9. Data are presented as mean ± 1 standard deviation.

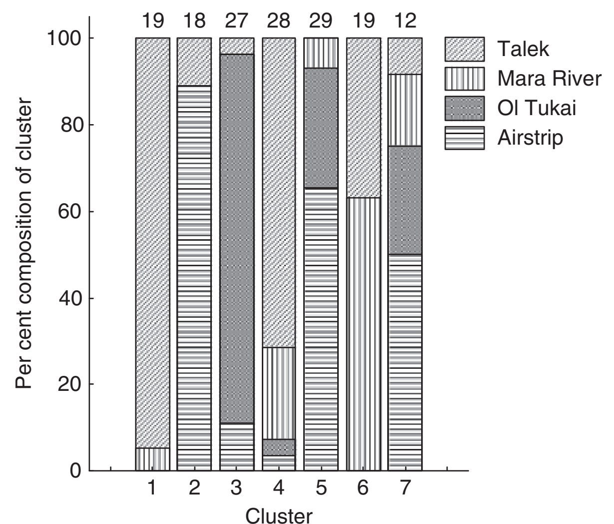

Figure 4 Composition by clan of seven clusters from STRUCTURE analysis. The number of hyenas assigned to each cluster is given above the bars. The Talek and Mara River clans belong to the Mara population. The Ol Tukai and Airstrip clans belong to the Amboseli population.

from within the same study population; these were a Mara River cluster, a second Talek cluster and a second Airstrip cluster. The final cluster was the smallest, and was less clearly associated with a particular clan. It included hyenas from all four clans, with no clan comprising more than 50% of the cluster. Instead, immigrant males made up more than half of the cluster, with most of those males (five of seven) belonging to the Airstrip clan. Thus, this final cluster may be best characterized as representing immigrants from outside the study areas. We know from behavioral observations that males in this cluster did not arrive in the Amboseli in a single dispersal event; at least one male was already present in the Airstrip clan in July 2002, and one male did not join the clan until January 2004.

Discussion

Overall, we found similar patterns of genetic diversity, relatedness and population genetic structure in our two spotted hyena populations. Both populations exhibited patterns of relatedness and genetic structure consistent with social organization and mating patterns described from detailed behavioral studies of spotted hyenas in the Mara and northern Tanzania (e.g. Frank, 1986; East & Hofer, 2001; Engh *et al.*, 2002; Boydston *et al.*, 2005; Höner *et al.*, 2007). However, subtle differences in genetic structure between the Amboseli and the Mara point to differences in patterns of male immigration into the two populations. Characteristics of spotted hyena behavior and life history, particularly patterns of male dispersal, appear to have been important in the maintenance of the relatively high levels of genetic diversity observed in the Amboseli population.

Amboseli and Mara populations compared

Levels of genetic diversity were similar in the Amboseli and Mara populations, despite differences in demographic history between the two populations. Although, populations that have experienced a demographic bottleneck are expected to show reduced genetic variation, this was not the case in Amboseli.

The Amboseli and Mara populations exhibited similar patterns of relatedness among natal animals. As in a previous study of Mara hyenas (Van Horn *et al.*, 2004), relatedness was higher among clan-mates than among animals born in different clans within a single reserve, though the absolute relatedness values within clans were relatively low overall. These findings and relative levels of *F*-statistics describing variance in allele frequency within and between clans within populations in the Mara and Amboseli indicate that social organization and mating patterns in Amboseli are similar to those described in the Mara. Moreover, similarities in patterns of relatedness within and between clans indicate that the current Amboseli population is not likely the result of a fission event within a single matriline, which would generate high levels of relatedness between clans (Van Horn *et al.*, 2004).

Notably, the Amboseli and Mara populations differed with respect to relatedness among immigrant males. In the Mara, but not Amboseli, relatedness was significantly lower among immigrant males than among adult females. The Mara pattern is consistent with immigration of males from multiple neighboring clans. Immigrant males appear to be more closely related in the Amboseli than in the Mara, although our sample of immigrant males was relatively small ($n = 11$ and $n = 12$, respectively), so this finding should be interpreted cautiously. Results of the STRUCTURE analysis also suggest differences in patterns of immigration into the two populations. Whereas immigrant males in the Mara population were assigned to clusters associated with Mara clans, a group of Amboseli immigrant males formed their own cluster that was not associated with natal Amboseli hyenas. These results suggest that there was immigration into the Amboseli population by several males from single source outside the study area. Such a pattern of immigration could generate the observed pattern of relatively high relatedness among Amboseli males, as well as the trend toward more negative IR values (indicating more outbred individuals) among the Amboseli hyenas as these immigrants bred with natal females.

To our knowledge, this is the first study to investigate population genetic structure in spotted hyenas. We found significant genetic differentiation among clans and among study populations using both hierarchical *F*-statistics and a clustering method. Genetic differentiation among clans within study populations is consistent with coancestry resulting from strong female philopatry in spotted hyenas. F_{IS} and F_{IT} values were low, as expected given that female hyenas show strong mate preference for immigrant males over adult natal males (Engh *et al.*, 2002).

Maintenance of genetic diversity in Amboseli

Small populations that are not completely isolated may maintain enough gene flow to retain a large degree of genetic variation (Burns, Eldridge & Houlden, 2004). Several studies have shown that just a few migrants into a small population may be sufficient to maintain or restore the level of genetic variation (Keller *et al.*, 2001; Vilà *et al.*, 2003; Hogg *et al.*, 2006). Given the vagility of spotted hyenas in general, and the high rates of dispersal by males in particular, migration into the Amboseli from outside the study area is likely. Although in the Mara, males typically immigrate into a clan just 8–10 km from their natal clan (Boydston *et al.*, 2005), dispersal over a distance of *c.* 85 km has been observed in the Kalahari (Mills, 1990), and hyenas will travel similar distances while foraging (Hofer & East, 1993). The trend toward more negative IR values in Amboseli and the occurrence of a cluster of the Amboseli immigrants in the STRUCTURE analysis are both consistent with such migration into the population. Thus, migration likely led to a larger effective population size during the Amboseli bottleneck than was indicated by census population estimates, facilitating the maintenance of genetic variation.

Moreover, the prevalence of multiple paternity in spotted hyenas, and female mating preferences that result in low reproductive skew among immigrant males (Engh *et al.*, 2002; East *et al.*, 2003), should have further contributed to the maintenance of genetic diversity.

Although our results point to male dispersal as a key factor in the maintenance of genetic diversity in the Amboseli population, two other aspects of spotted hyena biology may have also played a role. First, models developed by Chesser and colleagues (reviewed by Sugg *et al.*, 1996) demonstrate that socially structured populations (e.g. nonrandom mating, sex-biased dispersal), such as those of many gregarious species including spotted hyenas, have larger effective population sizes, and can maintain higher levels of genetic diversity, than populations of comparable size but which lack social structure. Thus, the social structuring of hyena populations by clan membership and female philopatry (Engh *et al.*, 2002) should have helped maintain genetic diversity (Chesser, 1991; Sugg & Chesser, 1994). Second, species with long generation times should be better able to maintain high levels of genetic variation during a bottleneck, than shorter-lived species (Kuo & Janzen, 2004; Hailer *et al.*, 2006). Based on life table data in Watts & Holekamp (2009) for the Mara population, we estimated a generation time of 5.7 years for spotted hyenas. If the Amboseli population was greatly reduced in size for *c.* 20 years, this would correspond with 3.5 generations. This suggests that the Amboseli bottleneck was not severe in duration with respect to generation time in this species.

It is important to recognize that we do not know the mechanism by which the Amboseli population recovered in size; possible mechanisms operating here are increased recruitment within the Amboseli, immigration of individuals from outside of Amboseli, or both. Results of the STRUCTURE analysis suggest that there was immigration of males from outside of Amboseli. However, it is also noteworthy that at the time of this study, reproductive rates were considerably higher in Amboseli than in the Mara (Watts & Holekamp, 2008), suggesting that increased recruitment might also have contributed to population growth.

In order to successfully manage wild populations in smaller and increasingly fragmented habitats, it is essential to understand the factors influencing their population genetic structure. For spotted hyenas, our results suggest that the predominance of male dispersal, perhaps aided by behavioral plasticity, facilitates the maintenance of genetic diversity, even when census population size is small. Conversely, large carnivores that lack these traits may be more likely to experience reductions in genetic diversity as a result of habitat fragmentation and loss. With respect to the conservation of spotted hyena populations, our findings emphasize the importance of management efforts aimed at facilitating natural dispersal patterns from viable source populations. Our results add to a growing literature indicating that dispersal (either natural or human-mediated) is critical for successful conservation of large carnivores (e.g. Haight, Mladenoff & Wydeven, 1998; Vucetich & Creel, 1999; Sweanor, Logan & Hornocker, 2000; Gusset *et al.*, 2009).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Rates of allelic dropout (ADO) and false alleles (FA) for amplification of fecal DNA collected from spotted hyenas. To estimate error rates, a reference genotype was determined using either a multiple-tubes approach or DNA extracted from a blood sample from the same individual. Sample sizes for the multiple-tubes approach are presented as the number of individuals. Matched blood samples were available for 4 individuals. N/A indicates that the ADO could not be calculated because all individuals sampled were homozygous for this locus.

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