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High genetic variation in leopards indicates large and long-term stable effective population size

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Abstract

In this paper we employ recently developed statistical and molecular tools to analyse the population history of the Tanzanian leopard (*Panthera pardus*), a large solitary felid. Because of their solitary lifestyle little is known of their past or present population dynamics. Eighty-one individuals were scored at 18 microsatellite loci. Overall, levels of heterozygosity were high (0.77 ± 0.03), with a small heterozygote deficiency (0.06 ± 0.03). Effective population size (N_e) was calculated to be 38 000–48 000. A N_e:N ratio of 0.42 (average from four cat studies) gives a present population size of about 100 000 leopards in Tanzania. Four different bottleneck tests indicated that this population has been large and stable for a minimum of several thousand years. F_{ST} values were low and no significant genetic structuring of the population could be detected. This concurs well with the large migration values (N_m) obtained (>3.3 individuals/generation). Our analysis reveals that ecological factors (e.g. disease), which are known to have had major impact on other carnivore populations, are unlikely to have impacted strongly on the population dynamics of Tanzanian leopards. The explanation may be found in their solitary life-style, their often nonconfrontational behaviour toward interspecific competitors, or that any bottlenecks have been of limited size, localized, or too short to have affected genetic variation to any measurable degree. Since the genetic structuring is weak, gene flow is not restricted to within protected areas. Local loss of genetic variation is therefore not of immediate concern.

Keywords: conservation, microsatellites, *Panthera pardus*, population dynamics, population genetics

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Introduction

The ongoing advancement of molecular techniques is providing the scientific community with a wealth of tools for revealing the demographic history of a population without the need for data on past population numbers. In particular, past bottlenecks, population expansions or declines, as well as current and past effective population (N_e) sizes can now, by a variety of molecular and statistical methods, be estimated for natural populations (see reviews by Estoup & Angers 1998; Luikart & England 1999; but cf. Lavery *et al*. 1996; Hedrick 1999). Knowledge of past events is crucial for the understanding of present genetic variation in any population, and hence its potential to respond to evolutionary changes [e.g. (Menotti-Raymond & O'Brien 1993; Frankham 1996)]. For example, a currently large population may have undergone a recent expansion, and thus the long-term effective population size may be substantially different from the current census size (N_c) (Vucetich & Waite 1998). This may have important implications for conservation efforts (O'Brien 1994; Menotti-Raymond & O'Brien 1995). A population may decline drastically and enter a bottleneck for a variety of reasons. Causes include, but are not limited to, natural disasters, interspecific competition (including human hunting or persecution) and disease. Interspecific competition between top-level predators often takes dramatic forms and is a limiting factor for many carnivore species (Creel *et al*. 1999). Another well-known example affecting a large number of species today is habitat loss. This is also a common limiting factor for large carnivores since they range widely. Furthermore, they often live at low population densities and thus need large areas in order to attain viable

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population sizes (Primm & Clark 1996; Woodroffe & Ginsberg 1998).

Despite the fact that the importance of genetic variation, or rather lack, may have been overstated for some species (Caro & Laurenson 1994; Gilligan *et al*. 1997; Merola 1998), inbreeding depression remains a serious threat for some populations, and may ultimately impede recovery schemes (Barone *et al*. 1994; Frankham 1995a). Increased confinement to protected areas places many species at risk of losing genetic variation because gene flow between such areas is often restricted or completely absent. Again, this is particularly true for large carnivores since they, by their way of life, conflict with human interests, and consequently their survival prospects outside protected areas are often low. A population, or species, deprived of genetic variation may be more susceptible to disease (O'Brien 1994; Sanjayan *et al*. 1996), a growing concern for many conservation programmes of endangered carnivores in Africa and elsewhere (e.g. Macdonald 1993; Young 1994; Alexander *et al*. 1996; Goltsman *et al*. Macdonald 1996; see review by Murray *et al*. 1999). Infectious diseases such as canine distemper viruses (CDV), canine parvovirus (CPV), rabies, or anthrax can all cause epizootics in mammals. Since they occasionally emerge in highly virulent forms (Carpenter *et al*. 1998) they all have the potential to cause rapid reductions in population size. For example, in 1994 the lion (*Panthera leo*) population in the Serengeti, Tanzania, suffered a severe epidemic of CDV, which killed about 30% of the population. As this pathogen is not species specific, other species, such as hyenas (*Crocuta crocuta*), bat-eared foxes (*Otocyon megalotis*) and leopards (*Panthera pardus*), were also affected (Roelke *et al*. 1996). CDV and rabies have also impacted on species in the Serengeti region in the past (Alexander & Appel 1994; Kat *et al*. 1995). Since many carnivores are difficult to monitor and census, little is generally known about the past or present population dynamics, and large population declines, and subsequent recoveries, may well have passed unnoticed.

Leopards are solitary carnivores, only associating with mating partners and dependent young (Bailey 1993). They are mainly nocturnal and hunt primarily ungulate prey. Due to their great adaptability they inhabit virtually every African habitat and are only absent where they have been extirpated by man (Martin & De Meulenaer 1988). The leopard is a highly important species in both ecological and economic terms, and knowledge of its population dynamics is therefore desirable for many reasons. In many areas it is an abundant predator and plays an important role in terms of predator–prey interactions and interspecific competition with other carnivores, as well as acting as a large potential reservoir for pathogens. For the trophy hunting industry the leopard is one of the most valuable species; and setting sustainable levels of off-take is therefore of great importance. For example, selective shooting of large adult males by the hunting industry may distort the effective sex ratio, causing genetic depletion in the long run (Ginsberg & Milner 1994). A strongly skewed sex ratio, demographic or effective, may cause a genetic bottleneck in the absence of a demographic bottleneck. For threatened, ecologically or commercially important species, management should therefore include genetic monitoring (Luikart *et al*. 1998), especially when the species is difficult to monitor using regular census methods (e.g. strip transects, aerial surveys, or capture–recapture). Despite this, in the absence of detailed information about population numbers, quotas are generally set by educated guesswork.

The leopard's solitary lifestyle and secrecy have made previous research on this species difficult, but the introduction of new molecular methods provides an opportunity to increase our knowledge of this species vastly and with relative ease. In this paper we investigate the historical population dynamics of Tanzanian leopards by employing recently developed statistical tools to analyse nuclear molecular data. This approach mitigates the need for historical records on population sizes, relieving the problem of effective population sizes being, in most cases, only fractions of censused population sizes (Frankham 1995b). Our approach, although unable to measure population size directly, estimates the past effective population size, an important parameter from a conservation, as well as evolutionary, aspect.

We first examine the pattern of heterozygosity levels, and use these and theta (θ) estimates for present population size estimates. We then invoke four different statistical tests to check for signs of bottlenecks. Lastly, we use *F*-statistics to describe the genetic structure and create a dendrogram to display visually the genetic structure of the population.

Materials and methods

Specimens

A 5 × 10-mm piece of skin was cut from leopards shot by trophy hunters in hunting reserves throughout Tanzania between the years 1995 and 1998 (Fig. 1). Most were taken from dried and treated (salt or insecticide) skins, but a few samples were from fresh skins. The tissue was stored in 1.5 mL 95% ethanol buffer, containing 100 mM ethylenediaminetetraacetic acid (EDTA), at ambient temperature for up to 3 months in the field, and from then on at –20 °C.

Molecular analysis

DNA was extracted from the tissue by proteinase K-mediated lysis, followed by standard phenol–chloroform extraction. DNA was precipitated and washed with ethanol and then left to air dry for a few hours until the alcohol

Fig. 1 Map of Tanzania showing sample locations. The numbers in parentheses indicate the number of samples from each area. All samples came from protected areas of some form (game reserves or game-controlled areas). Five samples had unknown Tanzanian origin. Makao and Masai were assumed to centre on the Serengeti, since their exact location data was unavailable. Numbers 1–4 in Selous refer to a subdivision made of samples from this reserve.

had evaporated. The dried DNA was then reconstituted in double-distilled H_2O and the DNA concentration was measured in a spectrophotometer. Extracted samples were kept at –20 °C.

We used 18 primers (Table 1), constructed for domestic cats (Menotti-Raymond *et al*. 1999) on 81 individuals. Most loci were dinucleotide repeats, except Fca115, Fca391 and Fca37 which were trimeric. We ran multiplex polymerase chain reaction (PCR) amplification in 10 μL reactions containing: 1– Perkin Elmer (PE) PCR buffer, 2.5 nmol dNTPs, 15–20 pmol of $MgCl_2$, 4 pmol of each primer, 0.4 U of AmpliTaq DNA polymerase and between 3 and 50 ng of target DNA. The PCR amplifications were performed in either an Eppendorf Mastercycler Gradient or a GeneAmp® PCR System 9700 under the following conditions: one cycle of 3 min at 94 °C; 25–35 cycles of 94 °C for 30 s, 49–52 °C for 45 s, 72 °C for 1 min 30 s; and finally 72 °C for 10 min (ramp speed 0.8 °C/s or 3 °C/s). Most samples worked with only 25 cycle repeats, but some failed to amplify unless subjected to 35 repeats. After amplification, 2 μL of the PCR product was mixed with 6 μL of formamide and 0.03 μL PE Tamra size standard and was denatured at 95 °C for 2 min. The denatured products were then analysed in an ABI Prism™ 310 Genetic Analyser. Fluorescently labelled primers in different colours (dyes Hex, Tet and Fam; PE Biosystems) enabled scoring of a minimum of three loci simultaneously.

Statistical analyses

Observed and expected levels of heterozygosity were calculated using standard methods in the software GENEPOP (Raymond & Rousset 1995). Testing for Hardy–Weinberg equilibrium was also performed within the GENEPOP software. The proportion of null alleles was calculated as $(H_E - H_O)/(1 + H_E)$ after Brookfield (1996).

Effective population size was calculated for each locus as $N_e = (1/[1 - H_E]^2 - 1)/8\mu$ (Lehmann *et al*. 1998), where μ is the mutation rate. This model assumes a strict single stepwise mutational model. As an estimate of mutation rate we used the value 2.05×10^{-4}, which is the average of three published mammalian studies (Rooney *et al*. 1999).

Single-locus estimates of θ and proportion of multistep mutations were obtained from MICROSAT 1.0 (http://ib.berkeley.edu/labs/slatkin/software.html) (Nielsen 1997). Using the expression $\theta = 4N_e\mu$, we also made single locus estimates of effective population sizes from the θ-values obtained.

We searched for signs of bottlenecks using four different statistical methods.

First, the T2 test, which is a test for recent bottlenecks. It is based on the principle that following a bottleneck the allelic diversity is reduced more than heterozygosity, leading to a relative excess of heterozygotes (Cornuet & Luikart 1996). The effect is transient and disappears relatively shortly after the bottleneck. We tested for this using the program BOTTLENECK (http://www.ensam.inra.fr/URLB) (Cornuet & Luikart 1996).

Second, the allele frequency distribution test was used. A further indication that a bottleneck may have occurred at some point is an observed deviation from the L-shaped allele frequency distribution that normally arises in a population at mutation-drift equilibrium. The program BOTTLENECK estimates this frequency distribution and defines the curve shape (Cornuet & Luikart 1996).

Third, the within locus kurtosis test is designed to detect population expansions (Reich & Goldstein 1998; Reich *et al*. 1999) based on the distribution of allele sizes. The test is based on the observation that the allele distributions of an expanding population and that of a population that has been stable for a long time differ. In an expanding population the kurtosis (k), or rather a combination of the variance and kurtosis (Reich *et al*. 1999), of the allele size distribution is positive. The method uses a binomial test of the number of positive k-values based on the expectation of an almost ($P = 0.515$) equal probability of negative and positive k-values.

Table 1 Summary statistics of the 18 loci used in the analysis

Locus	Chromosome	No. of. indiv.	No. of. alleles	Size range	H_O	H_E	H_E under T.P.M†	Prop. null alleles‡	θ §	N_e /1000¶	N_e /1000**	Prop. multisteps§
Fca001	A2	76	17	136–170	0.68	0.90	0.90	0.11	38	60	46	0.13
Fca008	A1	81	13	104–134	0.78	0.82	0.86	0.02	16	16	20	0.06
Fca026	D3	68	17	112–158	0.91	0.92	0.90	0.01	30	95	37	0.13
Fca031	E3	75	14	235–261	0.71	0.85	0.88	0.12	15	26	18	0.06
Fca045	A1	75	15	126–162	0.87	0.90	0.88	0.03	37	60	45	0.08
Fca077	C2	76	14	119–161	0.79	0.87	0.87	0.07	20	35	24	0.11
Fca126	B1	76	17	131–165	0.91	0.93	0.90	0.02	50	124	61	0.13
Fca149	B1	65	10	114–134	0.80	0.79	0.82	0.01	12	1	15	0.03
Fca223	B3	61	10	171–203	0.69	0.78	0.82	0.08	19	12	23	0.08
Fca232	B4	78	16	96–140	0.78	0.79	0.89	0.01	29	13	35	0.06
Fca272	A3	73	13	99–129	0.85	0.84	0.87	0.01	15	23	18	0.08
Fca275	B2	79	14	107–143	0.53	0.73	0.87	0.17	18	8	22	0.11
Fca391	B3	66	9	189–219	0.80	0.82	0.80	0.02	12	18	15	0.01
Fca506	F2	79	23	174–246	0.92	0.93	0.93	0.01	128	124	156	0.08
Fca567	E1	81	13	76–102	0.83	0.89	0.86	0.06	18	50	22	0.06
Fca628	D2	69	12	91–113	0.88	0.89	0.85	0.01	21	50	26	0.06
F37	C1	58	5	212–224	0.33	0.47	0.63	0.12	3	2	4	0.01
F115	B2	73	22	184–250	0.83	0.93	0.93	0.09	63	124	77	0.08
Average*		72.7	14.3		0.77	0.84	0.86	0.05	31.1	48.4	38.0	0.077

*The average is weighed by the number of individuals.
†Two-phased mutation model, see Cornuet & Luikart (1996).
‡Estimated by the expression $(H_E - H_O)/(1 + H_E)$ (Brookfield 1996).
§Estimated by the program MICROSAT 1.0 (Nielsen 1997).
¶Estimated by the expression $(1/[1 - H_E]^2 - 1)/8\mu$ (Lehmann *et al*. 1998).
**Estimated by the expression $\theta = 4N_e\mu$ (Nielsen 1997).

Fourth, the variance test is an interlocus test which tests the observation that in stable populations the variance of the allele sizes is highly variable among loci, whereas in an expanding population this variance is more even. Thus, sufficiently low variances in allele sizes may be taken as evidence for an expansion, and we used cut-off values given in Reich *et al*. (1999). It should be noted that this test was the most powerful of six tests evaluated by Luikart *et al*. (1998).

To display the genetic distance among individuals we used the Δ distance, which is the squared difference in allele size between individuals (Goldstein *et al*. 1995), to create a dendrogram using UPGMA.

Finally, we tested the degree of geographical structuring by calculating F_{ST}-values using the different areas of sampling as subpopulations. F_{ST} has been shown to perform better than R_{ST} for small sample sizes ($n \leq 10$) scored for a low number of loci ($n \leq 20$) (Gaggiotti *et al*. 1999). A further advantage of F_{ST}-values is that they exhibit a smaller variance than R_{ST}-values do (Estoup & Angers 1998). For comparative reasons calculations of the *F*-statistics and migration rates (N_m) were carried out using both the ARLEQUIN software (http://anthropologie.unige.ch/arlequin) (Schneider *et al*. 1996) and in GENEPOP 3.1 (Raymond & Rousset 1995). Throughout this paper, means are given followed by the standard error of the mean.

Results

The different loci were all highly variable with on average 14 ± 1 alleles (Table 1). Heterozygosities were consistently high, but slightly lower than expected under Hardy–Weinberg equilibrium. Testing each locus in GENEPOP under the null hypothesis of no heterozygote deficit the test failed to reject this for eight loci at $P < 0.05$, and if correcting for multiple tests, only four loci are likely to deviate from Hardy–Weinberg equilibrium.

The estimation of N_e based on the strict stepwise mutation model and using the expression $1/[1 - H_E]^2 - 1)/8\mu$ (Lehmann *et al*. 1998) gave an average N_e of 48 000 ± 10 000 for all loci. Based on single locus estimates, θ was found to be in the range of 3–132 (31 ± 7). From our θ estimates and the following expression, $\theta = 4N_e\mu$, we generate an average N_e of 38 000 ± 8000. The actual mutation rate (μ) of the different loci used in this analysis is unknown, but we have assumed μ to be 2.05×10^{-4} [which is an average of three mammalian studies (Rooney *et al*. 1999)].

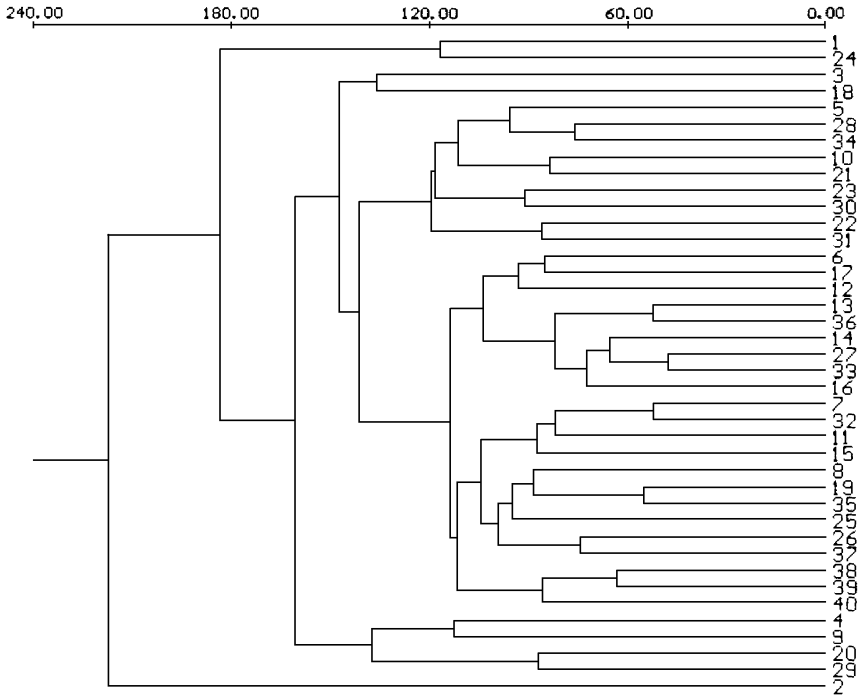

Fig. 2 Dendrogram of squared allele size differences between individuals. The uniform distribution of branching points indicates a long-term stable effective population size. Note that only 51 of the 81 individuals were used in order to make the figure easier to read.

Applying a strict single stepwise mutation (SMM) model for the T2 bottleneck test we found a significant heterozygosity deficiency, rather than the excess expected if the population had experienced a bottleneck. Of 18 loci, 12 showed a deficiency when the expected number of loci showing a deficit was 7.34 ($P = 0.02$). Allowing for an 8% multistep mutation rate (the average of our multistep estimates), the bias disappeared with 10 loci showing a deficiency (expected number of loci with deficiency 7.49, $P = 0.17$).

In the second test, the distribution of allele frequencies was L-shaped which supports the conclusion of a long-term stable population size.

The kurtosis test (Reich *et al*. 1999) concurred with the notion of no bottleneck: the test revealed that 13 out of 18 loci had a positive kurtosis, which is not significantly different from binomial distribution ($\varphi = 0.062$).

Finally, the variance test estimates the variance of the variance of allele sizes to 1.11. For this test to indicate a bottleneck the value has to be below 0.24 at an α-level of 0.05 ($n = 160$) as tabulated by Reich *et al*. (1999).

Since all four bottleneck tests failed to reject the null hypothesis of a long-term stable population size in the examined leopard population a dendrogram of pairwise differences between individuals should have branching points scattered over the entire tree. In a bottlenecked population most branching events would be expected near the root of the dendrogram. To visualize the population structure we therefore used 51 individuals to construct such a dendrogram (Fig. 2), where indeed the branching points were evenly distributed over the tree. Although displaying the data in this way cannot be regarded as a formal test, it reaffirms the results obtained from the tests above.

Although the tests carried out are presumed to be robust to minor deviations from the assumed single stepwise mutation model (Cornuet & Luikart 1996; Kimmel *et al*. 1998; Reich *et al*. 1999), large deviations may still cause a problem. However, the estimation of the proportion of multistep mutations (Nielsen 1997) indicates that this proportion is low for most loci (0.08 ± 0.01), even though five loci show values over 10% (Table 1). We compared the observed heterozygosity levels with the expected heterozygosity levels under two different models, the single step mutation model and the two-phased mutation model. The latter allows multistep extensions but only single step deletions (Di *et al*. 1994). The expected heterozygosity levels under the two-phased mutation model (assuming the proportion of multistep to be 8%, see Table 1) showed a close absolute fit, deviating by an average of 0.04 ± 0.01, to the observed heterozygosity levels. The single stepwise mutation model also showed a close fit with a deviation of 0.05 ± 0.01, so the difference between the models was very small, indicating that the deviation from the SMM was also very small. Lastly, varying the proportion of multistep mutations in the T2 test did not change the prediction of no bottleneck. Considering all the above points we feel confident that potential deviations from the assumed mutation models are unlikely to have biased the results.

The analysis of population structure revealed no significant differentiation by distance, despite the sampling

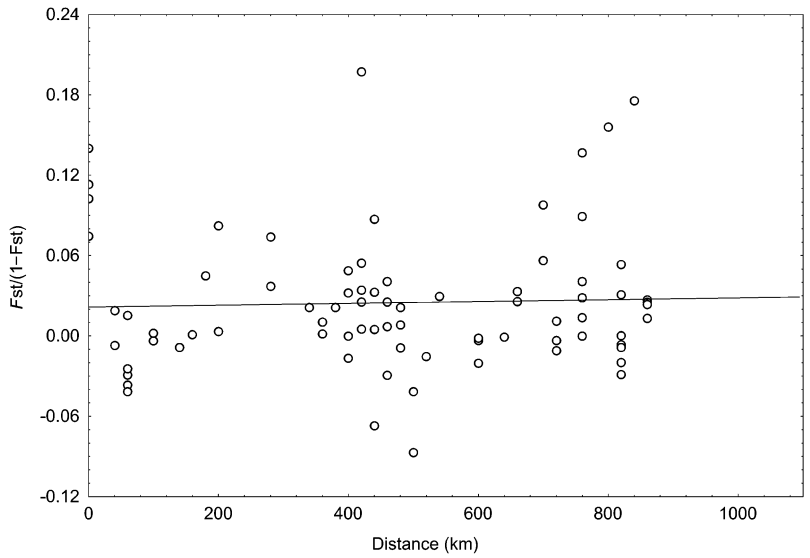

Fig. 3 Pairwise $F_{ST}/(1-F_{ST})$ values plotted against distance (Mantel test $r = -0.07$, $P = 0.27$). F_{ST} values from GENEPOP.

spanning almost all of Tanzania (Figs 1 and 3). The observed small level of genetic differentiation indicates a high level of gene flow. Our findings thus support the large dispersal distances of leopards found in earlier studies (Bailey 1993; Bothma *et al*. 1997). Estimates of N_m varied depending on the numbers of subpopulations created, but were all high. For example, dividing the Tanzanian population into four groups (A, Selous; B, Serengeti, Maswa, Masai, Makao; C, Ugalla, Kigosi; and D, Rungwa, Kisigo) the N_m estimate, using the private allele method (Barton & Slatkin 1986) and correcting for size, was 3.3 migrants/generation. In the light of this it is hardly surprising that we fail to detect any large differentiation of the population. The ARLEQUIN software uses the expression $N_m = (1 - F_{ST})/2F_{ST}$ to estimate migration rates. Because of the very low F_{ST} values obtained in this study, the N_m estimates using this method are very large, and for many pairwise comparisons, approach infinity.

Discussion

The authors of a previous study on the bowhead whale proposed five different explanations for the heterozygote deficiencies they found (Rooney *et al*. 1999), namely: population subdivision, strong genetic drift, null alleles, synteny and hitchhiking. In our study, several of these explanations can be discounted. There is no significant subdivision in the studied population (Fig. 3), so this alone would not explain the observed deficit. Genetic drift may be excluded based on the results from the bottleneck tests (see below). Null alleles seem to be rare (Table 1). In our maximum estimates of null alleles only four out of 18 showed values over 10%. It is unlikely that synteny is important in this study since the loci were deliberately distributed with a maximum of two loci per chromosome (Table 1) and for loci that have been shown to recombine (Menotti-Raymond *et al*. 1999). While we cannot rule out hitchhiking as a possible cause, we wish to stress that our estimation of expected heterozygosity is dependent on the mutational model. Using a strict SMM we have 12/18 loci with a heterozygote deficit, and allowing for 10% multistep mutations 10/18 loci show a small deficit. Finally, errors when scoring might also result in an apparent deficit, and although some samples were re-run several times, allelic drop-out remained a problem for some individuals and loci. So although largely unexplained, the average deficit is very small, and the additive effect of a weak population structuring, scoring mistakes, null alleles and allelic drop out, probably explains the observed deficit.

Theory predicts genetic variation to be correlated with effective population size, and empirical observations strongly support this contention (Frankham 1996). However, the exact relationship between these two parameters is likely to vary across species and other external factors. Extrapolating population sizes from data derived in this manner should therefore be treated with some caution. The average of our two estimates (average for all loci) of effective population size is 43 000 leopards. Due to fluctuation in numbers, reproductive skew, overlapping generations, etc., N_e is always considerably lower than the actual population size. Few studies have estimated the N_e:N ratio, something that requires good long-term demographic data and information of total population numbers. But, the N_e:N ratio has been estimated for four cat species, namely the ocelot, *Leopardis pardalis* (Ludlow & Sunquist 1987); tiger, *Panthera tigris* (Smith & Mcdougal 1991); Florida panther, *Felis concolor coryi* (Seal *et al*. 1989); and puma,

Felis concolor (Dueck 1990 in Nowell & Jackson 1996). In his study of pumas, Dueck (1990), found a ratio of 0.64, but later work has shown that the formulae he used tends to overestimate the ratio by about 60% (Harris & Allendorf 1989). Correcting for this we get a ratio of 0.40, giving an average for all studies of 0.42. All species are solitary, and all but the ocelot are large [the ocelot weighs about 10 kg (Ludlow & Sunquist 1987)]. The two puma studies were conducted on pumas living under very different conditions and in completely different habitats, and therefore both ratios are used for the average. Pooling and extrapolating our N_e estimates give a population size of about 100 000 leopards in Tanzania. Based on rainfall data and estimates of available habitats the number of leopards has been estimated to be between 10 000 and 100 000 in Tanzania by Martin & De Meulenaer (1988). Our analyses indicate that the higher end of this range estimate is likely to be closest to the true number. A population of 100 000 leopards would result in an average density of leopards across Tanzania of about 0.1 individuals/km^2. In prime habitat leopard populations often reach densities of 0.3 individuals/km^2 (Bailey 1993), showing that this estimate is well within possible limits.

While our approach to estimating N is effective in many aspects, it suffers from several important caveats. First, Tanzania is an arbitrary unit and not biologically relevant in terms of gene flow. Thus, the sampled population experiences influx of genes from outside Tanzania, boosting genetic variation and causing estimates of population size based on this variation to be overestimates. We have no measure of by how much we have overestimated the population size, but an overestimate by as much as 10–20% does not alter the fact that the number of leopards in Tanzania is still high. Second, the assumed mutation rate may be incorrect for the used loci and may therefore bias the extrapolated population size. If the actual mutation rate is twice as high as the assumed, the population estimate would be halved. For example, in humans the average mutation rate has been estimated at 1.2×10^{-3} (Weber & Wong 1993). Assuming the same rate for leopards would result in a population estimate of about 18 000 individuals. Third, the uncertainty of the N_e:N ratio, as mentioned earlier, is a potential problem. Lastly, primers were chosen for their ease of use and for loci that had been shown to be highly variable in the domestic cat, and this may have biased the results. On the other hand, allelic dropout or null alleles would bias the population size estimate toward smaller than actual size.

All the bottleneck tests carried out concur with the conclusion that the Tanzanian leopard population has not suffered from any serious bottlenecks during the last 5000 years (at a minimum). Therefore, the data indicate that the long-term effective population size has been in the order of tens of thousands at a minimum. Leopards thus seem largely to have escaped from large-scale declines, unlike some other African mammals that have declined drastically due to epizootics in particular. One potential explanation for this may be the strictly solitary life of the leopard (Bailey 1993; Stander *et al*. 1997) in contrast to the highly social life of both lions and wild dogs, both species that have been struck by epizootics in the recent past. Leopards associate with other leopards only during courting and mating, probably making lateral transmission rarer than in more social species. In addition they have the ability to seek refuge in trees from competitors such as lions, hyenas and wild dogs, species which commonly drive leopards up trees (personal observations, Bailey 1993; Creel & Creel 1996), and this nonconfrontational behaviour is likely to limit the spread of viruses.

While our data suggest that the leopards have escaped epizootics in the past, it should be noted that the recent large-scale epizootics might be a modern phenomenon. Due to high densities of carnivores in protected areas and an often large number of domestic dogs bordering these areas, unprecedented conditions favouring the spread of pathogens might occur nowadays, e.g. the Serengeti in 1994 (Roelke *et al*. 1996). Historic outbreaks of diseases to which leopards are susceptible may have been localized, rare, or have involved only a few animals. Following this line of argument avoidance of epizootics would not be unique to leopards; other carnivores may also have been largely exempted from dramatic population declines in the past.

An alternative explanation is that local leopard populations have in fact undergone bottlenecks but, due to subsequent dispersal from neighbouring populations, the level of genetic variation has been rapidly restored. It is known that males normally leave their natal home ranges around sexual maturity and disperse long distances (Mizutani & Jewell 1998), and the N_m estimates obtained (>3.3) do not contradict this explanation. Even though the population shows a high genetic homogeneity, the actual distribution of leopards may be discontinuous. A fast acting (i.e. highly virulent) epizootic could then 'burn out' in one area before being transmitted to the entire population.

Another important point is that the effect of a population bottleneck is dependent on both the magnitude of the bottleneck and the time spent at low numbers. For example, using the expression $H_t = H_0.e^{-(2t/Ne)}$, where H_t is the heterozygosity at time t and H_0 is the initial heterozygosity (Hartl & Clark 1989), we find that an effective population size of 100 only reduces the initial heterozygosity by as little as 20% over 50 generations. This indicates that shorter and less severe reductions in effective population size may well pass undetected by the above tests.

To summarize our results; (i) we have no evidence to suggest that the sampled areas experience any isolation,

and (ii) this leads to a large effective population size which (iii) explains the high genetic variation, and (iv) the large long-term effective population size may potentially be explained either by escape from severe epizootics, or from quick restoration of depleted areas through unrestricted gene flow.

The above conclusions indicate a healthy and thriving leopard population and, based on these analyses, the leopard population of Tanzania appears to be in no danger of losing any genetic variation. As migration rates are high the leopards in Tanzania may be treated as a continuous population that exchanges genes throughout its range. However, three things are important to emphasize at this point. First, although migration rates are high on an evolutionary timescale, this should not be taken as cause to downplay the dangers of local over-exploitation. Although our results indicate a stable population with a high migration rate, it may not be high enough to compensate for heavy off-takes, and thus hunting quotas should always be set attempting to achieve sustainability on the smallest possible scale. Second, if the leopard population has experienced a recent rapid decline (i.e. in the last decade or two) due to range loss and persecution by man, our analyses may not detect this, since this decline would not yet have impacted on the genetic variation. So, while we may assess the long-term and relatively recent population size reasonably well, any predictions about the present-day population size are based more on the lack of information of large-scale mortality or changes in available habitat for the leopards in Tanzania, rather than the opposite. Martin & De Meulenaer (1988) estimate that even in the absence of outright killing of leopards they would still have declined by up to 50% in many areas of Africa because of habitat loss. We have no information on the rate or extent of habitat degradation in Tanzania, but leopards are highly adaptable and capable of living in close proximity to human habitation, excluding few areas. So, while densities in many areas may be low, leopards occur in most areas of Tanzania, and in protected areas they are generally very common (Martin & De Meulenaer 1988; Bailey 1993). Third, it should be noted that the estimation of N is based on two major assumptions, the mutation rate of the scored loci and the N_e:N ratio. While the latter is likely to be a good predictor of the true ratio, the actual mutation rate remains uncertain. Given these considerations we suggest a cautionary approach in the implementation of this population size estimate. Thus, until these two parameters are firmly established in leopards any management decisions have to be supplemented with further population size estimates.

From a conservation and management perspective a repetition of this approach should be undertaken in the future to monitor any changes in the genetic pattern. Even a relatively small reduction, for example 10%, in population size can be detected by genetic monitoring. This is in part because more animals are remotely censused (e.g. through scats, hairs and other remains or tissue) as compared to other censusing techniques and the addition of more loci will increase the power of any test used (Luikart *et al*. 1998). Demographic monitoring, unless extremely intense, would be unlikely to detect such a small decline in numbers, especially for an elusive species such as the leopard.

This paper shows some of the potential of directly applying genetic methods for monitoring and managing natural populations. As statistical and molecular tools continue to be refined, remote, but accurate, estimates of important population parameters may be achieved. As more studies are completed, calibration of the N_e:N ratio, as well as information on species-specific mutation rates, will increase the accuracy of population size estimates based on molecular data. Estimating N_e from demographic data alone is difficult, and genetic bottlenecks may pass unnoticed simply because the N_c remains large. For endangered populations emerging problems may therefore be detected at an earlier stage using molecular methods. In addition, estimates of effective population sizes may reveal matters that only demographic monitoring in combination with behavioural observations could detect, such as low mating success, strong reproductive skew, etc. For commercially important species such as the leopard, this type of monitoring may become important in setting sustainable quotas and maximizing off-take without jeopardizing the survival or depleting the genetic variation of the species.

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