THE ROYAL SOCIETY

Estimating population size by genotyping faeces

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Population size is a fundamental biological parameter that is difficult to estimate. By genotyping coyote (*Canis latrans*) faeces systematically collected in the Santa Monica Mountains near Los Angeles, California, we exemplify a general, non-invasive method to census large mammals. Four steps are involved in the estimation. First, presumed coyote faeces are collected along paths or roadways where coyotes, like most carnivores, often defaecate and mark territorial boundaries. Second, DNA is extracted from the faeces and species identity and sex is determined by mitochondrial DNA and Y-chromosome typing. Third, hypervariable microsatellite loci are typed from the faeces. Lastly, rarefaction analysis is used to estimate population size from faecal genotypes. This method readily provides a point count estimate of population size and sex ratio. Additionally, we show that home range use, paternity and kinship can be inferred from the distribution and relatedness patterns of faecal genotypes.

Keywords: *Canis latrans*; faecal DNA; population size; microsatellite; sex ratio; mark–recapture

1. INTRODUCTION

To predict the long-term persistence of animal populations, accurate estimates of population size as a function of environmental change and habitat disturbance are necessary (e.g. Sutherland 1996). Without population size data, conservation management is ineffective (e.g. Wilson *et al*. 1996). However, logistical and ethical problems often hamper efforts to estimate population size (e.g. Sutherland 1996; Bekoff & Jamieson 1996). Census methods based on direct counts may be inaccurate because individuals are difficult to detect. Similarly, the accuracy of trapping-based methods depends on how readily individuals are captured, and the handling of rare or endangered species may cause injury (Greenwood 1996). Data analysis is also problematic; statistical tools to estimate population size based on observation or trapping methods often depend on unrealistic assumptions about density, capture probability, migration and mortality (Greenwood 1996). Consequently, non-invasive methods are needed that combine the strengths of direct count and mark–recapture methods but do not require observation or handling of individuals (Höss *et al*. 1992; Kohn & Wayne 1997; Morin & Woodruff 1996).

New molecular techniques for the analysis of faeces (Höss *et al*. 1992) may offer a means to count individuals in a population (Kohn & Knauer 1997; Kohn & Wayne 1997). Specifically, faeces can be collected systematically across an area and typed for diagnostic markers to confirm species' identity. Thereafter, faeces are typed for several hypervariable microsatellite loci to deduce the number of unique multilocus genotypes in the population. Once a large sample of faeces is typed, the cumulative number of unique multilocus genotypes can be expressed as a function of the number of faeces sampled. The asymptote of this curve can be determined analytically and provides an estimate of local population size. Similarly, mark–recapture models may be useful to analyse faecal genotyping data (Kohn & Knauer 1997). In this approach, faeces with the same multilocus genotype are treated as recaptures. Information on sex ratio and home range is obtained through the use of sex specific markers and analysis of the geographic distribution of genotypes. Finally, data on paternity and relatedness can also be derived from comparison of multilocus genotypes.

Our approach requires that faeces be easily found and recovered and hence is well-suited for large carnivores which often defaecate along trails or territorial boundaries (Macdonald 1980). Consequently, we chose to test the use of faecal genotyping on a population of coyotes from the Santa Monica Mountains National Recreation Area near Los Angeles, California, USA, that was traversed by trails and roads (figure 1*a*). We also initiated a capture and radiotelemetry study concurrent with the faecal collection to obtain independent measurements of population size dynamics and territory and pack structure.

2. METHODS

(a) *Faecal sampling*

During a two-week period beginning 8 July 1997, we removed all 651 recognizable carnivore-like faeces from 381 sites along six transects in a 15 km^2 area (figure 1*b*). Whole faeces

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Received 17 November 1998 *Accepted* 11 December 1998

were picked up using disposable gloves and transferred into ziploc bags. Almost all faeces were already completely desiccated when found in the field and were stored dried at −20 °C until required for analysis (Frantzen *et al*. 1998). For each faeces a Global Positioning System (GPS) reading was taken and transferred into a Geographic Information System (GIS) to establish their map locations. We also collected 50 blood samples from coyotes trapped during an ongoing ecological study in the Santa Monica Mountains that included our study area (Sauvajot *et al*. 1997). For eight coyotes, matched blood and faecal samples were available.

(b) *DNA extractions*

DNA was extracted from the faeces using a commercially available extraction kit (IsoQuick, ORCA Research Inc., Bothell, WA). Briefly, about 60 mg were removed from the outside of the faeces and added to 200 µl of a 1:1 mixture of sample buffer and lysis buffer. After a brief vortex and 15 min of incubation at room temperature the extract was centrifuged at 12 K for 10 min and the supernatant (*ca*. 150 µl) was purified following the rapid protocol as described by the supplier. DNA was dissolved in 100 µl of sterile water and kept at −4 °C. DNA from blood was extracted using a standard proteinase K digest followed by phenol–chloroform purification and ethanol precipitation (Sambrook *et al*. 1989). All faecal extractions were done in an isolation facility designated for research projects dealing exclusively with diluted and degraded DNA. Controls in which no faeces were added to the extraction were included to monitor for contamination.

(c) *Faecal typing*

Each DNA extract was first screened for species-diagnostic *Mva* I restriction sites located in the mitochondrial DNA (mtDNA) control region (Pilgrim *et al*. 1998). Coyote-derived faeces were sexed based on the presence of PCR product from the Y-chromosome *SRY* gene (Meyers-Wallen *et al*. 1995). PCR of mtDNA and *SRY* used 50 ng of DNA isolated from blood or 5 µl of DNA extracted from faeces in a 20 µl reaction volume containing 1.0 mM $MgCl_2$, 1.0 mg ml^{-1} bovine serum albumin (BSA), 0.05 mM of each dNTP, 5.0 pmoles of each primer, 1 × PCR reaction buffer and 0.3 units of *Taq*. DNA polymerase (Perkin Elmer, Emeryville, CA). Amplification was done in a programmable thermocycler (Perkin Elmer Cetus 9600) as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50–57 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Amplification of three canid-specific tetranucleotide repeat microsatellite markers (*CXX2001*, *2062*, and *2140*; Mellersh *et al*. 1997), which are unlinked according to the dog genome map (Mellersh *et al*. 1997), followed the same protocol but 0.5 mM $MgCl_2$ was used. Forward primers were labelled with ^{32}P (Sambrook *et al*. 1989). Mitochondrial DNA and *SRY* products were separated on 6% polyacrylamide minigels and visualized under UV light after ethidium bromide staining. Microsatellite products were separated on 6% polyacrylamide sequencing gels and visualized through autoradiography. Allele sizes were compared to the DNA sequence of M13mp18 (USB, Cleveland, Ohio). PCR reagents were always tested for contamination.

When pipetting dilute and degraded DNA, stochastic pipetting errors, PCR-jumping, and sporadic contamination may lead to PCR-artefacts such as allelic drop-out and the generation of false alleles (Gerloff *et al*. 1995; Taberlet *et al*. 1996). The frequency of these artefacts was estimated by comparing genotypes from multiple amplifications from pairs of matched blood and faeces, and repeated amplifications from faecal extracts. Each of the three loci was amplified in a separate PCR. For each unique three-locus microsatellite genotype the sequential probability of it being the result of stochastic PCR-artefacts (P_{artefact}) was computed as $[K \times (K/7)^{n-1}]^f$ (modified from Gagneux *et al*. (1997)), where K denotes the frequency of observed PCR artefacts, n denotes the number of repeated PCRs from an extract with $n \geqslant 2$, and f denotes the number of occurrences of the genotype in the sample. Note that the value of seven is used in the denominator because for each three-locus genotype there are seven possible incorrect outcomes, each with a different probability. Computations of population genetic parameters and mean relatedness values and their standard deviations were implemented in computer programs by Marshall *et al*. (1998) and Queller & Goodnight (1989), respectively. The combined exclusion power of the loci *XX2001*, *2062* and *2140* was calculated following Paetkau *et al*. (1995).

(d) *Population size estimation*

Each faeces to be typed was drawn at random from the total sample of collected faeces. To reduce effort, we had decided arbitrarily that when only one new genotype was discovered in 30 consecutively analysed faeces, we would stop typing further faeces. The multilocus genotype of each randomly drawn sample was compared to all those drawn previously and the total number of genotypes was incremented if the newly found genotype was unique (figure 2*a*). Assuming that each unique genotype represents a different coyote, the population size was projected as the asymptote, a, of the function $y = (ax)/(b + x)$, where y equals the cumulative number of unique genotypes, x is the number of faeces sampled, and b is the rate of decline in the value of the slope. Estimates of a and b and their 95% confidence intervals (CI) were obtained through iterative nonlinear regression using the program JMP IN 3 (SAS Institute, Inc.). Furthermore, to test if the order in which faeces were drawn for analysis affected a, the random sampling of the 115 multilocus genotypes was repeated 1000 times after the matrix was randomized using a program written in Mathematica v. 3 (Wolfram 1996). Alternatively, data were analysed using the mark–recapture model by Burnham & Overton (1979) which allows for different capture probabilities of individuals. The estimate and 95% CI were computed as outlined in Greenwood (1996, pp. 29–32).

(e) *Rate of disappearance of faeces on and off trails*

Forty-eight samples of faeces were equally distributed in vegetation near trails, in the middle of trails, and in tyre tracks of dirt roads at eight locations in the study area. Faeces were scored once per week and classified as disappeared when physical damage rendered them non-identifiable as putative coyote faeces. Time until complete disappearance and 95% CI were extrapolated by straight-line least-squares regression and ANOVA (Sokal & Rohlf 1995).

3. RESULTS AND DISCUSSION

(a) *Species verification, sexing and microsatellite typing*

Coyote faeces can be confused with those from dogs, grey foxes, bobcats and badgers. Therefore, we verified putative coyote faeces through PCR-amplification by canid-specific primers followed by *Mva* I restriction

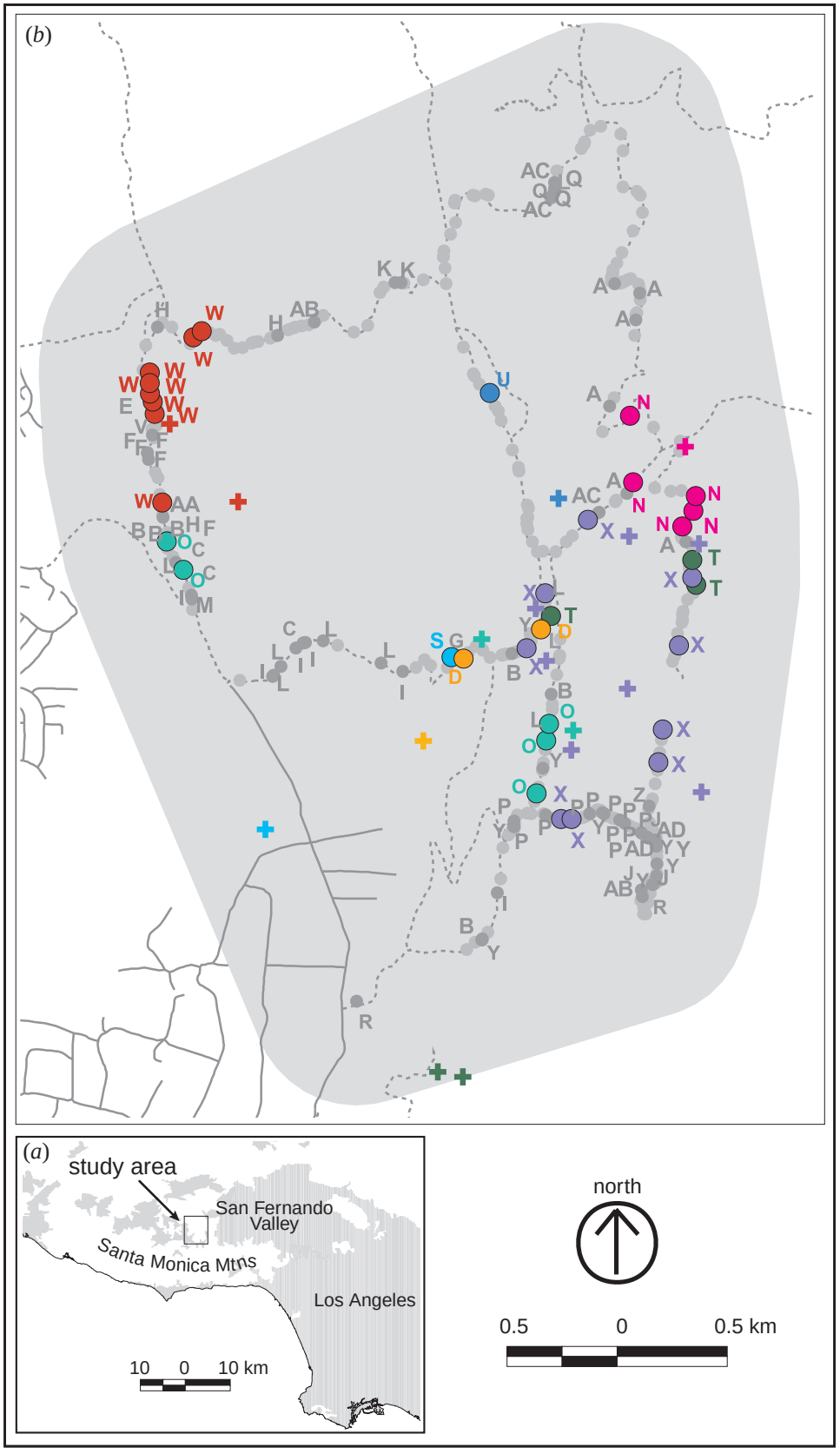

Figure 1. Location of coyotes deduced by faecal genotyping and radiotelemetry. (*a*) Location of the study area (box) in the Santa Monica Mountains in Southern California, USA. (*b*) The 15 km^2 study area including the 500 m perimeter (shaded) where faeces were collected. Multilocus faecal genotypes that matched those of captured coyotes and their nearest telemetry locations are presented as coloured circles identified with letters and matching colour symbols, respectively. Dark-grey circles represent faeces with genotypes that did not match any of the captured coyotes. Light-grey circles represent the remaining sites where faeces were collected. Sex and estimated P_{artefact}-values for genotypes are as follows: A ♂, B ♂, C ♀, D ♀, F ♂, H ♂, I ♀, J ♀, K ♂, L ♂, N ♀, O ♀, P ♂, Q ♀, R ♀, T ♂, W ♂, X ♂, Y ♀, AB ♂, AC ♂, and AD ♂, $P_{\text{artefact}} \leqslant 0.00001$; and for E ♀, G ♀, M ♂, S ♂, U ♀, V ♀, Z ♀, and AA ♀, $P_{\text{artefact}} = 0.0032$.

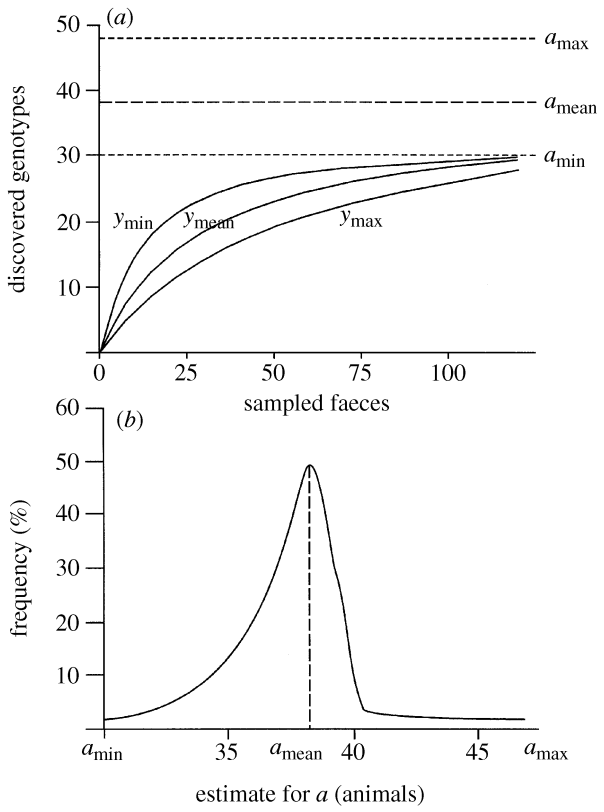

Figure 2. Population size estimation based on the faecal genotype rarefaction curve. (*a*) Plot of the average number of unique genotypes, y, discovered as a function of the number of sampled faeces, x. A curve defined by the equation $y = (ax)/(b + x)$ was fitted to the data. The process was repeated 1000 times to deduce the simulated distribution of curves and asymptotes. Mean (y_{mean}), minimum (y_{min}), and maximum (y_{max}) of the rarefaction curves are shown. Broken lines indicate fitted values of mean (a_{mean}), minimum (a_{min}), and maximum (a_{max}) values of corresponding asymptotes. (*b*) Frequency distribution for possible values for the asymptote, a, as deduced from simulations.

digest of a segment of the mtDNA control region. To confirm the accuracy of classification, we tested DNA from the blood of 15 resident coyotes, one dog and five grey foxes. As found previously (Pilgrim *et al*. 1998), coyotes had no *Mva* I restriction sites whereas dogs and grey foxes had one and two sites, respectively. No PCR products were obtained from amplifications of bobcat and badger control DNA. Finally, amplifications of rat and mouse DNA did not result in PCR products implying that rodent DNA in faeces would not cause a false positive (Kohn *et al*. 1995).

Out of the 238 faeces randomly drawn for analysis, 188 (79%) yielded coyote mtDNA and 115 (48%) of these were successfully typed for three canid-specific microsatellite loci. We found 30 unique three-locus genotypes designated A through to AD. The map locations of faeces were determined from their GPS readings (figure 1*b*). Polymorphism information content (PIC) values for loci were high (between 0.60 and 0.85) and the probability of a random match between multilocus genotypes was 0.0065, i.e. about 1 in 154. For populations with lower genetic variability additional loci should be added (e.g. Paetkau *et al*. 1995).

To determine the sex ratio, each of the 115 faeces was typed by sex-specific PCR (Meyers-Wallen *et al*. 1995; see figure 1*b* legend). The sex of eight matched blood and faecal samples were the same in two independent rounds of extraction and amplification. However, for six out of the 115 faeces the sex could only be established after a third amplification was done. The female to male ratio was 1:1.14 and was not, therefore, significantly different from 1:1 (Windberg 1995) or from our own estimates produced from live trapping ($\chi^2 = 0.14$ and 0.03, respectively, data not shown).

Each multilocus genotype was found on average 3.8 ± 2.9 times (figure 1*b*). There was no significant difference between the average number of multiple occurrences of female (mean 3.6 ± 2.7, $n = 14$) or male (mean 4.1 ± 3.3, $n = 16$) genotypes (Wilcoxon–Kruskal–Wallis ranked-sums test, $p = 0.76$). Thus, as suggested by field observations (Gese & Ruff 1997), both sexes defaecate equally on trails. Unlike spraint marking, defaecation rates in coyotes may also be independent of social status and age such that a faecal survey of coyotes should be generally unbiased in respect to these parameters (Gese & Ruff 1997). However, coyote pups were only about three months of age when sampling was conducted and are probably rare in our sample (e.g. Bekoff & Wells 1982). Thus, repeated sampling over time is advocated to monitor reproduction and population dynamics (Kohn & Wayne 1997).

To estimate microsatellite-typing error, 118 amplifications from two independent extracts from each of 59 faeces samples were scored. Six out of the 118 amplifications did not agree because of allelic drop-out ($n = 3$) and false alleles ($n = 3$) and four did not amplify. Similarly, 92 single-locus genotypes from 23 extracts of eight faeces samples were compared to those from matched blood samples. Five out of 92 genotypes did not match the corresponding blood genotypes (three allelic drop-out, two false alleles) and four did not amplify. Thus, in both cases, the frequency of error of microsatellite typing was about 0.05 per locus. Each multilocus genotype was replicated at least once and ambiguous genotypes were replicated for at least a third time to reduce the cumulative probability of error per three-locus genotype to $\leqslant 0.0032$ (see figure 1*b* legend). Mean heterozygosity was 76%, no loci deviated from Hardy–Weinberg expectations, and the estimated frequency of null alleles was 0.003. In summary, these results suggest that the presence of null alleles or faecal PCR-artefacts did not substantially bias our analysis.

(b) *Population size estimation*

As deduced from the asymptote of the rarefaction curve shown in figure 2*a*, population size was approximately 38 individuals (95% CI, 36–40). The order in which samples were analysed could potentially affect the population size estimate. Results from simulations show that population size estimates vary between 30 and 47 (figure 2*a*). Values below 36 and above 40 were supported in only about 20% of simulations (figure 2*b*). The number of multilocus genotypes actually observed in our sample of 115 faeces was 30, about 80% of the estimated population size. To sample 90% of the genoytpes estimated in the population, the rarefaction regression predicts that at least another 220 faeces samples would need to be typed. Consequently, the

Table 1. *Rate of disappearance of faeces on and off trails and dirt roads*

(Faeces were scored once per week and classified as disappeared when physical damage (e.g. flattening) rendered them non-identifiable as putative coyote faeces. Unrecognizable faeces and off-trail faeces in vegetation (low detectability) were not collected during the faecal survey. Time until complete disappearance and 95% CI was extrapolated by least-squares linear regression. ANOVA statistics: off-trail, $F=88.6$, $R^2=0.89$, d.f. $=12$, $p<0.0001$; middle of trail, $F=123.1$, $R^2=0.92$, d.f. $=12$, $p<0.0001$; and tracks, $F=14.5$, $R^2=0.57$, d.f. $=12$, $p=0.003$.)

	faeces remaining		
week(s)	off trails	middle of trails	tracks on trails
0	16	16	16
1	16	14	9
2	16	11	5
3	15	9	2
4	15	7	1
5	14	7	1
6	14	6	0
7	13	6	—
8	12	6	—
9	12	5	—
10	9	2	—
11	7	1	—
12	7	1	—
persistence (95% CI)	22 (17–31) weeks	12 (9–17) weeks	6 (2–9) weeks

rarefaction curve provides both an estimate of population size and the effort required to sample a specified proportion of the population. Finally, population size was estimated as about 41 (95% CI, 38–45) using the mark–recapture model by Burnham & Overton (1979). The model allows for animals to differ in probability of capture, which was evident in our sample (mean 3.83, range 1–11; figure 1*b*).

(c) *Comparison to field data*

During a 21-month period beginning January 1996, 37 coyotes were captured in our study area. The capture effort was considerable, with 2131 attempts to trap individuals. However, out of the 37 live-caught coyotes, only eight had genotypes that matched those in the faeces. The difference in individuals detected in the trapping and faecal studies reflect the difference in the time-scale of observation. The trapping survey is longitudinal and provides a cumulative estimate of population size that includes individuals which are now deceased or have migrated. In contrast, the faeces-based estimate is a relatively instantaneous point count as faeces usually decay rapidly (Wallmo *et al*. 1962).

To assess the site-specific decay rates of faeces in our study area we placed 48 faeces on trails and monitored their loss over a 12-week period (table 1). An equal number of faeces were placed in three locations on trails and dirt roads: in the bordering vegetation, in the middle, and in worn tyre tracks. Vehicles, horses, bicycles and people frequent these trails and dirt roads. Faeces on trails and dirt roads disappear rapidly and by 12 weeks only one remained on the trails and none on the tracks. In vegetation, persistence time was estimated as between 17 and 31 weeks. We have only collected on trails, hence, all faeces collected represent a maximum time window of 9–17 weeks (table 1).

Consequently, the lack of agreement of individuals detected in the trapping and faecal survey suggest high rates of population turnover. In fact, annual mortality rates for adults and juveniles in the study population are high (0.25 and 0.85, respectively; Sauvajot *et al*. 1997). Radiotelemetry of the 37 live-caught animals show that 15 have died, five have dispersed, the fate of five is unknown, and 12 remain in the area. Of the latter, eight had genotypes that matched those in faeces suggesting that at least 22 individuals were not captured. Finally, only four out of 41 captures were a recapture and zero out of nine coyotes photographed at 133 remote camera stations in our study area were collared. Therefore, a large fraction of the population avoided detection by conventional means.

Telemetry observations document a minimum of 12 coyotes in our study area during the period of faecal collection, of which the multilocus genotypes of eight individuals matched those in the faeces (figure 1*b*). On average, $61\pm32\%$ of all telemetry locations of these 12 coyotes were within the area of faecal collection, the remaining telemetry locations were found outside the study area. However, coyotes used the area to different degrees. The eight coyotes common to the radiotelemetry and faecal studies had an average of $70\pm27\%$ of their telemetry locations in the study area whereas three missed coyotes had less than $26\pm20\%$ and one had 95% of telemetry locations within the study area. For each of the 12 radio-collared coyotes, there was a significant positive relationship between the percentage of telemetry locations within the study area and the number of their matched faeces that were recovered during the survey (figure 3). Thus, the number of faeces deposited by individuals is correlated with their relative use of the study area. This result supports the use of the mark–recapture model by Burnham & Overton (1979) for the population size estimation as it specifically incorporates variation in capture probabilities between individuals.

The observed distribution of faecal genotypes is consistent with radiotelemetry locations. On average, the faeces of matched coyotes were found within 610 ± 265 m ($n=34$) of their nearest telemetry locations (figure 1*b*). Of the matched faeces, 50% were less than 350 m from a telemetry location, 25% were between 350 and 570 m, 15% were between 570 and 2000 m, and 10% were more than 2 km away. Therefore, faecal DNA analysis may be useful to identify core areas used by individuals as well as long-distance excursions. However, the precise mapping of individual home ranges will require substantial collection efforts (Taberlet *et al*. 1997).

(d) *Genetic relatedness*

Relatedness between faecal genotypes was calibrated using DNA extracted from 50 blood samples of coyotes captured from throughout the Santa Monica Mountains. The mean relatedness value for eight known full-sib pairs and eight parent–offspring pairs was 0.49 ± 0.22 and 0.67 ± 0.24, respectively, and 85 comparisons of individuals

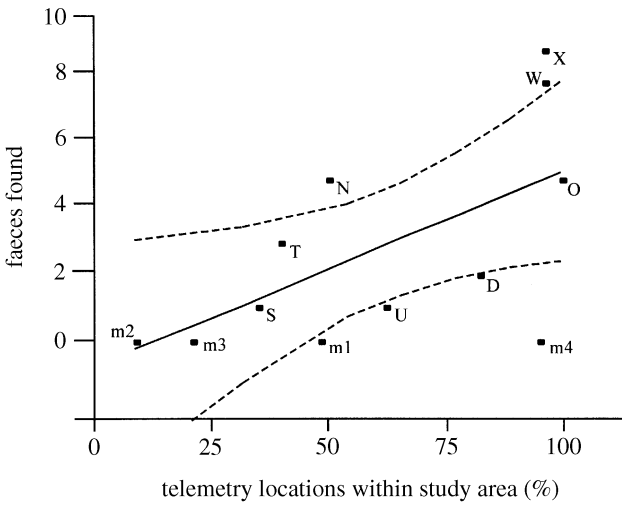

Figure 3. Relationship between number of faeces found and percentage of telemetry locations found within the study area for 12 coyotes. The percentage of telemetry locations of radio-collared coyotes also common to the faecal survey (D, N, O, S, T, U, W, and X) and missed coyotes (m1, m2, m3, m4) found within the area was plotted versus the number of matched faeces recovered in the faecal survey. Coyote identifications as in figure 1*b*, dashed lines denote 95% CI curves ($F = 5.72$, $R^2 = 0.36$, d.f. $= 11$, $p = 0.0377$). Omitting m4 improved the fit ($F = 12.94$, $R^2 = 0.59$, d.f. $= 10$, $p = 0.0058$).

separated by about 10 km and a ten-lane freeway had an average value of relatedness of -0.01 ± 0.24.

We screened a matrix of pairwise comparisons of *r*-values for all 30 multilocus faecal genotypes (data not shown; Queller & Goodnight 1989). The number of individuals that were within one standard deviation of the relatedness values for known parent–offspring and sibling comparisons was determined. Each coyote had on average 6.6 ± 3.4 putative first-order relatives assigned (parent–offspring relationships and sib relationships were pooled), a value consistent with previous field observations which showed that mated pairs, their offspring, and juveniles of the previous season remained in the same area (Bekoff & Wells 1982). We also confirmed a case of suspected paternity; male X and female O, the territories of which overlap, were unrelated and were the parents of a pup found with them at a den (figure 1*b*). The exclusion probability was 0.79 (Marshall *et al*. 1998). The standard deviation of the relatedness values was high owing to the limited number of loci used. More conclusive documentation of kinship patterns in the population requires inclusion of more microsatellite loci and additional behavioural observations (e.g. Girman *et al*. 1997).

4. CONCLUSIONS

We show that the systematic collection of faeces, followed by molecular typing with hypervariable microsatellite markers and sex-specific probes, provides a near-instantaneous estimate of population size and sex ratio that avoids problems of capture and handling. However, the method relies on the assumption that defaecation rates are equal among sexes and age classes, and is independent of social status, as has been shown for coyotes. This assumption should be tested for the species under investigation. Our results suggest that, in our study area, more than two-thirds of the current coyote population may be missed by more long-term ecological surveys. Hence, faecal analysis provides an important independent assessment of population size. Additionally, critical ecological and demographic parameters such as home range use and relatedness can be potentially estimated. For rare and endangered species, faecal typing provides a new window for viewing population dynamics (Kohn & Wayne 1997).

We are indebted to T. K. Fuller and all volunteers involved in the work, especially S. Kim and D. Ortíz. Genetic analysis was supported by a Mildred Matthias grant, Genetic Resources Conservation Programme (GRCP) funds, and by the Southwest Parks and Monuments Association (SPMA) to M.H.K. and R.K.W. Fieldwork was funded by the National Park service Santa Monica Mountains National Recreation Area, the National Park Foundation, and Canon, USA (Expeditions into the Parks Programme).

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