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Assessing reliability of microsatellite genotypes from kit fox faecal samples using genetic and GIS analyses

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Abstract

Noninvasive faecal DNA sampling has the potential to provide a wealth of information necessary for monitoring and managing endangered species while eliminating the need to capture, handle or observe rare individuals. However, scoring problems, and subsequent genotyping errors, associated with this monitoring method remain a great concern as they can lead to misidentification of individuals and biased estimates. We examined a kit fox scat data set (353 scats; 80 genotypes) for genotyping errors using both genetic and GIS analyses, and evaluated the feasibility of combining both approaches to assess reliability of the faecal DNA results. We further checked the appropriateness of using faecal genotypes to study kit fox populations by describing information about foxes that we could deduce from the 'acceptable' scat genotypes, and comparing it to information gathered with traditional field techniques. Overall, genetic tests indicated that our data set had a low rate of genotyping error. Furthermore, examination of distributions of scat locations confirmed our data set was relatively error free. We found that analysing information on sex primer consistency and scat locations provided a useful assessment of scat genotype error, and greatly limited the amount of additional laboratory work that was needed to identify potentially 'false' scores. 'Acceptable' scat genotypes revealed information on sex ratio, relatedness, fox movement patterns, latrine use, and size of home range. Results from genetic and field data were consistent, supporting the conclusion that our data set had a very low rate of genotyping error and that this noninvasive method is a reliable approach for monitoring kit foxes.

Keywords: endangered species, faecal analysis, faecal genotypes, kit fox, noninvasive sampling, scats, *Vulpes macrotis mutica*

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Introduction

Noninvasive faecal DNA sampling is an attractive technique for obtaining information on wild mammal populations that are increasingly elusive as their numbers decline (Kohn & Wayne 1997), and this monitoring method has become more common. Recent studies using faecal DNA have identified species and individuals in an area, evaluated distribution, sex ratio, and kinship of wild populations, and estimated population size (Taberlet *et al.* 1997; Kohn *et al.* 1999; Ernest *et al.* 2000; Lucchini *et al.* 2002; Eggert *et al.* 2003; Bellemain *et al.* 2005). Overall, this technique offers great promise to researchers because it has the potential to provide necessary information for assessment of management and conservation strategies while eliminating the need to capture, handle or observe individuals (Kohn & Wayne 1997; Kohn *et al.* 1999).

Despite the benefits of this noninvasive approach, faecal DNA sampling has several limitations and difficulties associated with its use. In particular, the low amounts and poor quality of DNA contained in faeces (scats) can lead to

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critical genotyping errors (primarily allelic dropout and false alleles), and thus, misidentification of individuals and biased estimates (Taberlet *et al.* 1996, 1999). For example, Creel *et al.* (2003), in a study on wolves recently reintroduced to Yellowstone National Park, found that such errors led to a genetic population estimate more than five times greater than the actual number of wolves known to be present. Such findings have caused wildlife managers to be concerned about the reliability of genetic data, especially when genetic results are not compared to, or combined with, field data such as behavioural observations and radiotelemetry information.

Solutions for addressing and reducing the severity of genotyping errors in noninvasive DNA-based studies include protocols for replicating amplification and programmes for determining the magnitude of error in a data set (Taberlet *et al.* 1996; Miller *et al.* 2002; Bonin *et al.* 2004; McKelvey & Schwartz 2004). Additionally, checking geographical consistency of scat locations with telemetry home range data has been used to verify genotype accuracy (Kohn *et al.* 1999; Bellemain *et al.* 2005). However, to our knowledge, no one has yet combined many of these solutions with GIS information on scat locations in the field to assess the reliability of genotype results.

Because traditional methods of monitoring endangered San Joaquin kit foxes (*Vulpes macrotis mutica*) such as trapping, nocturnal spotlight surveys, and scent-stations can be problematic, costly, or have a risk of injury (Ralls & Eberhardt 1997; Warrick & Harris 2001; Harrison *et al.* 2002; Schauster *et al.* 2002), the effectiveness of a noninvasive DNA-based sampling approach to monitor these foxes merits exploration. Here, we examine a kit fox scat data set (353 scats; 80 genotypes) for genotyping errors by applying (i) five proposed recommendations for reducing and tracking error, (ii) information on the location where each scat was collected, and (iii) consistency of sex determination. Our primary objective was to evaluate the feasibility and utility of using both genetic and GIS analyses to assess the reliability of the DNA results. As a final check on the feasibility of using faecal genotypes to study kit fox populations, we describe the information about foxes that we could deduce from the 'acceptable' scat genotypes, and compare it to information gathered with traditional field techniques.

Materials and methods

Study species

The biology of kit foxes has been reviewed most recently by Cypher (2003) and Moehrenschlager *et al.* (2004). Kit foxes are socially monogamous, living primarily as unrelated mated pairs with or without pups (Ralls *et al.* 2001). They mate once a year in December and January and an average of 3–4 (range 1–7) pups are born in mid-February to mid-March. The average age at dispersal is about 8 months (Koopman *et al.* 2000) but occasionally pups remain on their natal territories into adulthood, leading to the formation of social groups with three or more adults (Ralls *et al.* 2001). Members of a family group have similar home ranges, with an average of 70% overlap in one study (White & Ralls 1993). Home ranges of adjacent kit fox family groups frequently overlap, e.g. an average of 10% for females and 19% for males belonging to adjacent groups (White & Ralls 1993) but core areas of concentrated use are generally occupied exclusively by a family group (White & Ralls 1993; Spiegel 1996; Murdoch 2004). Kit foxes frequently defecate along trails, at latrines (accumulations of three or more scats), and on or near conspicuous objects such as bones and fence posts (Murdoch 2004; Ralls & Smith 2004).

Study site

Scats of kit fox were collected in the northwestern portion of the Carrizo Plain National Monument (CPNM), California. As described in Smith *et al.* 2003, the CPNM lies adjacent to the southwestern edge of the San Joaquin Valley, is one of the largest continuous habitats (> 750 km^2) for San Joaquin kit foxes, and is one of three 'core areas' considered essential for conservation of this species (USFWS 1998). The principal habitat types included non-native annual grassland, alkali sink and saltbush scrub, and upper Sonoran subshrub scrub. The climate is semi-arid with hot, dry summers and cool, wet winters. Approximate summer high and winter low temperatures are 40 °C and −10 °C, respectively. Average yearly precipitation is 26 cm, occurring primarily as winter rains.

Sample collection

We collected scats during July–August 2000. We established 23.2 km of transects for scat collection along (i) the main unpaved road that crossed through our study site, and (ii) several unpaved roads that branched off of the main road. Additionally, we established 48 km of transects in vegetated area that was adjacent to the main unpaved road. Transect length varied from 0.5 to 2.0 km (see details Smith *et al.* 2003).

Because scats of kit foxes are small (~1–3 cm) and cryptic, and we wanted to ensure a sufficient sample of scats for analyses, we used dogs trained to detect scats of kit foxes in addition to human observers visually locating scats along transects. Previously, we reported that dogs found numerous scats along both unpaved roads and transects through vegetation, were capable of distinguishing kit fox scats from scats of sympatric carnivores, and were far more effective at locating scats than were human observers (Smith *et al.* 2001, 2003, 2005).

We used two detection dog-handler teams and two human observers to locate scats. Each dog indicated that it had found one or more scats by sitting or lying down next to them, allowing the handler to view the location and number of scats present and recover the scat sample. Each human observer was trained in kit fox scat identification (e.g. Halfpenny & Biesiot 1986), and visually located scats present on transects.

Because fresh scats yield higher quality DNA (Lucchini *et al.* 2002; Prugh *et al.* 2005; J. E. Maldonado, unpublished), dog-handler teams removed presumed kit fox scats from all transects 8 days before beginning the experiment to maximize the likelihood that only fresh scats would be collected. Then, systematic scat searches were conducted on all transects within an 8-day period, and repeated alternating dog-handler teams and human observers on all established routes so that the search effort by each dog and human was equal. Average temperature during the hours of scat collection was 23 °C (range 14–32 °C) (Smith *et al.* 2003).

The location of each scat collected was geo-referenced using a Global Positioning System (GPS) unit (Garmin GPS III +). Scats were stored in plastic bags containing one teaspoon of silica gel for desiccation (Fisher Scientific) and shipped within 7 days to the National Zoological Park/National Museum of Natural History's Molecular Genetics Laboratory (Washington, D.C.) for storage at –4 °C.

DNA extractions and typing

As described in Smith *et al.* 2003, DNA was extracted from every scat sample using a QIAGEN DNeasy™ DNA extraction kit following a modified protocol as in Eggert *et al.* (2005). Extractions were carried out in a separate room under quasi-clean conditions to prevent contamination. Each sample was isolated a minimum of two times and then subjected to a species identification test based on mitochondrial DNA. Negative controls (no scat material added to the extraction) accompanied each set of extractions and were used to check for contamination. Once DNA was extracted, polymerase chain reaction (PCR) amplification and restriction enzyme analyses were performed using a modified version of the protocol and reagents described in Paxinos *et al.* (1997) as follows: a 350-bp fragment of the mitochondrial cytochrome *b* gene was amplified using a canid specific light STRAND primer (Canid L1, Paxinos *et al.* 1997) and a universal heavy STRAND primer (H15915, Irwin *et al.* 1991) in a 50-μL PCR including 0.5 U AmpliTaq Gold (PerkinElmer), 2.5 mM $MgCl_2$, 1× reaction buffer (PerkinElmer) 200 μM each dNTP, 1.0 mg/mL Fraction-V BSA, and 1 μM each primer. Reactions were run for 30 cycles (1-min denaturing at 95 °C, 1-min annealing at 55 °C and 2-min extension at 70 °C) in a PTC-100 programmable thermocycler (MJ Research Corp.). PCR products were screened with three species-diagnostic restriction enzymes (*Alu*I, *Hin*fI, *Taq*I) as specified in Paxinos *et al.* (1997). Positive controls for kit fox, coyote (*Canis latrans*), domestic dog (*Canis familiaris*), red fox (*Vulpes vulpes*) and grey fox (*Urocyon cinereoargenteus*) were used for comparison in the restriction analysis. Scat samples that failed to produce PCR amplification products after the second extraction attempt were deemed unusable for genetic analyses. All scat samples identified as a kit fox were subsequently genotyped to the individual level and sexed.

Six tetranucleotide microsatellite primers (FH 2140, FH 2535, FH 2137, PEZ19, FH 2226, FH 2561) were originally obtained from the canine genome map (Francisco *et al.* 1996; Mellersh *et al.* 2000), and adapted for use with kit foxes. Using microsatellites developed for one species in a related species can give misleading answers to some questions, such as the relative levels of polymorphism in the two species. However, the possible negative bias in estimated polymorphism levels in kit foxes resulting from our use of dog microsatellites (Ellegren *et al.* 1995) did not pose a problem for our study because we used microsatellites only to distinguish among individuals.

PCR conditions were optimized for kit foxes using the following protocol: each DNA extract was subjected to at least three independent PCR amplifications for each locus for allele size verification. PCR amplifications were carried out in a programmable thermocycler (MJ Research PTC-200 DNA engine). Final amplification reagents in 25-μL volumes were 1× reaction buffer (PerkinElmer), 2.5 mM $MgCl_2$, 200 μM each deoxynucleotide (dNTP), 1.7 mg/mL Fraction-V BSA, 0.25 U *Taq*Gold DNA polymerase (Applied Biosystems), and 1 μM of each primer. The forward primer of each pair of microsatellite DNA primers was labelled with a fluorescent dye (FAM, HEX or TET) to allow detection during electrophoresis. The reaction for scat DNA extracts as well as DNA extract and PCR negative controls (reaction reagents without template) were cycled 35 times following an initial 10 min at 94 °C hot start using the following profile: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min. For gel analysis, amplified products were multiplexed in two batches by pooling 1 μL of PCR product for each of the three loci labelled with three different fluorescent dyes (Batch 1: FH2140-FAM, FH2531-HEX, FH2137-TET; Batch 2: PEZ 19-FAM, FH2226-HEX, FH2561-TET) and dried together and resuspended with a loading mixture (ratios 1:1:6 standard GeneScan-500 ROX, loading buffer and deionized formamide), denatured by incubation at 95 °C for 5 min, loaded on a 6% Long Range gel (Applied Biosystem) and separated on an ABI PRISM 377 sequencer. Microsatellite allele sizes were estimated by comparison to the size standard and using the GENESCAN ANALYSIS® 3.1 and GENOTYPER® 2.5 software (Applied Biosystems).

We used restriction fragment length polymorphism (RFLP) analysis of the zinc finger (ZF) protein genes for sex

identification (Fernando & Melnick 2001). We used a primer set that amplifies a short (195 bp) fragment of the zinc finger (*Zfx* and *Zfy*) protein genes in kit foxes and other canids (Ortega *et al.* 2004). This fragment contains a *Taq*I digestion site unique to the *Zfy* gene in the endangered San Joaquin kit fox. PCR and *Taq*I restriction enzyme reactions were performed following conditions as stipulated in Ortega *et al.* (2004).

Reliability of the genotyping results

Proposed recommendations for tracking error. We followed five recommendations for reducing and tracking error in our data set. First, we strictly adhered to the protocol outline of Bonin *et al.* (2004) to limit potential errors in the genotyping process. All of their suggestions from sampling to analysis were followed including blind samples and automation, experience and rigour for the laboratory work and scoring, and precautions for preventing contaminations and technical artefacts.

Second, we followed a multiple tubes approach as suggested by Taberlet *et al.* (1996) that used Lucchini *et al.*'s (2002) protocols for microsatellites with some modifications. Each scat sample was isolated a minimum of two times. All extraction samples were amplified at least three times, those loci that were heterozygous in all replicates were scored as reliable, all homozygote and uncertain genotypes were additionally replicated up to eight times, and all samples that could not be reliably typed at all loci for at least three of eight amplifications were treated as missing data and discarded.

Third, after each scat sample was successfully typed at all loci, the reliability of each observed multilocus score was determined using the program RELIOTYPE (Miller *et al.* 2002). RELIOTYPE is a program for assessing the reliability of an observed multilocus genotype using a maximum-likelihood approach for minimizing genotyping errors. The program estimates how reliable a particular genotype is and strategically directs replication at loci most likely to contain errors. The model used in this program assumes that false and contaminant alleles can be removed from the data set and that the rate is even across loci. To be conservative, only scores in our data set with ≥ 95% reliability were considered 'acceptable' without further data analysis, i.e. examination of information on scat sex and location (see below).

Fourth, to further identify any genotyping errors and the relative magnitude of a problem within our multilocus scores, we performed the proposed tests of McKelvey & Schwartz (2004): Examining Bimodality (EB) and Difference in Capture History (DCH).

Finally, we determined genotypic mismatches between all unique scores with the program CERVUS 2.0 (Marshall *et al.* 1998), and identified those scat samples with a greater likelihood of error (i.e. where there was only one mismatch for one allele at one locus).

Examining scat location information. We determined the difference in metres between the locations of each scat sample using GEOTRANS 2.2.2 (http://mac01.eps.pitt.edu/courses/GEO1445/Geotrans2_assignment.htm). If one or more scats within a set of identical genotypes were found at the same latrine, only one scat from this latrine was chosen for this distance analysis. We then examined the distribution of distances between collection locations for the following five categories of scats: (i) randomly chosen pairs, (ii) pairs of scats with the same microsatellite genotype, (iii) pairs of scats with a microsatellite genotype considered 'unacceptable' by RELIOTYPE and its most similar genotype, (iv) pairs of scats with the same microsatellite genotype where all scats belonging to that genotype were considered 'acceptable' by RELIOTYPE, and (v) pairs of scats with the same microsatellite genotype where some scats belonging to that genotype were considered 'unacceptable' by RELIOTYPE. A Kolmogorov–Smirnov test was used to compare the mean distance between locations of all scats within a set of identical genotypes and the distance between scats selected at random. Additionally, we compared mean distance between locations of scats with the same microsatellite genotype where either (i) all scats were considered 'acceptable' by RELIOTYPE or (ii) one or more scats were considered 'unacceptable' by RELIOTYPE. Finally, we examined the mean distance between locations of all scats considered 'unacceptable' by RELIOTYPE and its most similar genotype and the distance between scats selected at random.

Tests based on sex primer consistency and scat location. The reliability of an observed multilocus score was further evaluated by incorporating information on sex primer consistency and scat location in the field. For all scat genotypes identical to genotypes of different scat samples, yet considered 'unacceptable' by RELIOTYPE, we examined the following three variables: (i) whether or not there was consistency of sex determination for scats belonging to the same microsatellite genotype (sex agreement), (ii) whether or not the scat with the 'unacceptable' genotype was collected at a latrine containing another scat sample of identical genotype (latrine agreement), and (iii) whether or not the scat with the 'unacceptable' genotype was collected within a distance equal to or less than 2107 m (the value at which 99% of scat locations for genotypes with all scats considered 'acceptable' by RELIOTYPE were found; see below) of all scat samples with the same microsatellite genotype (distance agreement). Because scat samples that were in latrine agreement were automatically in distance agreement, we selected either sex and latrine agreement, or sex and distance agreement, as our final tests of genotype reliability. If the scat genotype in question passed two of the three above

agreement tests, we choose to consider that scat genotype 'acceptable', despite the original RELIOTYPE determination.

Feasibility of the genotyping results

We employed two approaches to estimate if the overall number of unique genotypes identified in our data set was a reasonable number of foxes to be found through scat collection on our transects. First, we used accumulation curves in which the number of scat samples genotyped was plotted against the number of unique genotypes found in order to determine what proportion of the population we potentially sampled (Kohn *et al.* 1999). Two common accumulation curve techniques, one of which assumes a hyperbolic form, and the other an exponential form, were applied. The hyperbolic function, '*Hyp* ()', was similar to the method used by Kohn *et al.* (1999) where '$E(x) = ax/(b + x)$' ['x' is the number of genotyped samples, '$E(x)$' is the cumulative number of unique genotypes found in 'x' genotyped samples, 'a' is the asymptote of the function and thus the expected number of genotypes that would be found with complete sampling, and 'b' is the nonlinear slope of the function which declines as 'x' becomes large]. The exponential function, '*Exp* ()', was similar to the method used by Eggert *et al.* (2003), where '$E(x) = a(1 - e^{(bx)})$'. As the order of addition of samples can affect the estimation of the shape of the resulting accumulation curve, each data set was randomized 1000 times and the value of 'a' was estimated each time. Estimates of 'a' were obtained through iterative nonlinear regression using the program JMP IN 3 (SAS Institute, Inc.).

Second, to identify the number of foxes that were potentially present in the survey area, we simulated fox territories at the site. The average mean 95% fixed kernal nocturnal home range size for male and female foxes in the CPNM near our study site is 2490 m² (Bean 2002). Thus, we laid a grid of square, hypothetical kit fox home ranges over a map of our transects. A random point was chosen to position this grid, and the exercise was repeated 10 times. The average number of home ranges, and foxes, possibly encountered during scat collection was then calculated.

Error rates, P_{ID} (Polymorphism) and Hardy–Weinberg equilibrium

To calculate a genotyping error rate, we used the approach similar to Bellemain *et al.* (2005) and randomly chose about 5% of the successfully genotyped samples to be amplified another three times, and then compared to the first typing. Previously, Ortega *et al.* (2004) examined a set of scats from 16 foxes of known sex and found our sex primer yielded reliable results. Here, we calculated the error rate of the sex primer by checking the consistency of sex determination within a set of identical genotypes for all scat samples in our data set (Bellemain *et al.* 2005).

To determine the ability of our six microsatellites to distinguish between individuals, the probability of identity (P_{ID}) (i.e. the probability of different individuals sharing an identical genotype at random; Mills *et al.* 2000; Waits *et al.* 2001) and the P_{ID} between siblings was estimated in a set of 56 tissue samples from live-trapped foxes using methods of Waits *et al.* (2001).

Departures from Hardy–Weinberg equilibrium (HWE) were tested using the probability test by Guo & Thompson (1992) as implemented in GENEPOP version 3.2 (Raymond & Rousset 1995). Additionally, tests for genotypic linkage disequilibrium among pairs of loci were performed in GENEPOP using Fisher's exact tests (Raymond & Rousset 1995), with unbiased P values derived by a Markov chain method and the following parameters were employed: 10 000 dememorizations, 1000 batches, and 10 000 iterations/batch. The significance value for multiple significance tests was set using the sequential Bonferroni procedure (Rice 1989).

Relatedness estimation

Values of relatedness (r; Queller & Goodnight 1989) among all 'acceptable' multilocus faecal genotypes were determined using the software KINSHIP version 1.2 (Goodnight & Queller 1999). Similar to Lucchini *et al.* (2002), we tested a primary hypothesis of first-order relationship (i.e. full-sibs or parental–offspring have an expected relatedness $r = 0.5$) vs. a null hypothesis of no relationship ($r = 0.0$).

Results

DNA extractions and typing

During the fresh scat collection, detection dogs and humans recovered 469 and 123 presumed kit fox scats, respectively (total scats = 592). DNA was successfully isolated from 448 scats (76%), of which 447 yielded kit fox mtDNA and 1 yielded coyote mtDNA. The coyote scat was collected by a human.

Of the 447 scat samples identified as kit fox, 353 (79%) were successfully amplified for all six loci and met the scoring requirements of our strict multiple tubes approach protocol. From these 353 scats, we identified 80 unique genotypes of which 61 were found multiple times. Each multilocus genotype was found on average 4.4 ± 4.2 times (range: 1–20 scats). We successfully assigned a sex to 72 (90%) of the 80 unique genotypes.

Reliability of the genotyping results

Proposed recommendations for tracking error. Examination of our data set with RELIOTYPE, revealed 69 of the 353 (19.55%) scat genotypes were deemed < 95% reliable and thus

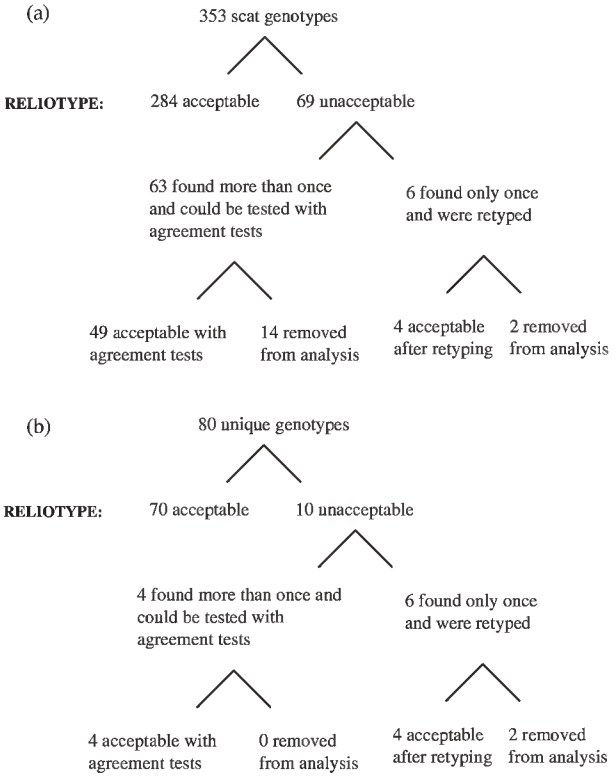

Fig. 1 The number of scat genotypes (a) and unique genotypes (b) that were found acceptable with RELIOTYPE, agreement tests and retyping.

'unacceptable' (Fig. 1a). The average estimated reliability of these 'unacceptable' genotypes was 84.7 ± 20.7%. Because the majority of these scat genotypes were within a set of scats with identical genotypes, some of which were deemed reliable, only 10 of the 80 (12.5%) unique genotypes were represented entirely by scats with 'unacceptable' scores and therefore considered 'unacceptable' by RELIOTYPE (Fig. 1b).

The EB test examined the distribution of the genetic differences between samples (McKelvey & Schwartz 2004). The distribution of the minimum number of loci at which individuals differed was unimodal: no individuals differed at all loci, and none differed at zero loci (Fig. 2). The average number of loci at which individuals differed was 2.45 ± 0.745 loci. This lack of any bimodal structure strongly indicated a low degree of error within the data set. The DCH test determined if the number of new individuals in the sample increased faster than would be expected through the removal of the shadow effect when additional loci were added to the genetic tag (McKelvey & Schwartz 2004). No locus added a significant number of individuals, although locus 2137 and locus 2561 increased the number of unique individuals by 1 and 3, respectively. Again, this result indicated that our data set had a low rate of genotyping error.

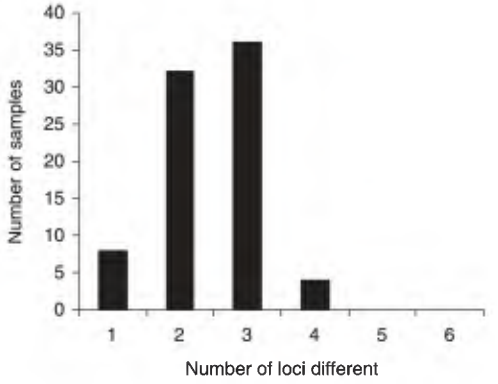

Fig. 2 The minimum number of loci at which one individual differed from all other individuals in our data set (353 scats, 80 genotypes) based on six heterozygous loci.

We found that eight individuals differed from another individual by one allele at one locus and performed three additional amplifications on the 19 scat samples that corresponded to these eight suspect genotypes to check the reliability of the scores. In all cases, the new allele scores were identical to the first typing indicating a lack of genotyping error. Thus, we considered these eight genotypes to be different individuals.

Examining scat location information. The distribution of distances for pairwise comparisons of scats chosen at random revealed a bimodal structure with many scat locations found within a fairly close range (0–10 000 m) and many scat locations found a great distance apart (20 000–30 000 m) (Fig. 3a). In contrast, distances between pairs of scats with the same genotype revealed a unimodal structure with the majority of scats found within a comparatively short distance of each other (0–5000 m) (Fig. 3b). Distances between scats of the same genotype considered 'unacceptable' by RELIOTYPE and scats with the most similar genotype showed a bimodal distribution similar to that for pairs of scats chosen at random, suggesting that these genotypes were not erroneous scores but in fact different individuals from the next nearest genotype (Fig. 3c). Finally, both distributions of distances for scats belonging to the same putative individual, whether all scats within the set of identical genotypes were considered 'acceptable' by RELIOTYPE (Fig. 3d), or some scats within the set of identical genotypes were considered 'unacceptable' by RELIOTYPE (Fig. 3e) showed a unimodal structure. However, for genotypes with all scats considered 'acceptable' by RELIOTYPE, 99% of scat locations were found within a very narrow range (0–2107 m) compared to those genotypes with some scats considered 'unacceptable' by RELIOTYPE (0–5000 m). This greater difference in distances among scat locations for genotypes with some scats considered 'unacceptable' by RELIOTYPE was due to scats from only 6 of 32 genotypes. These six presumptive individuals (genotypes 14, 29, 39,

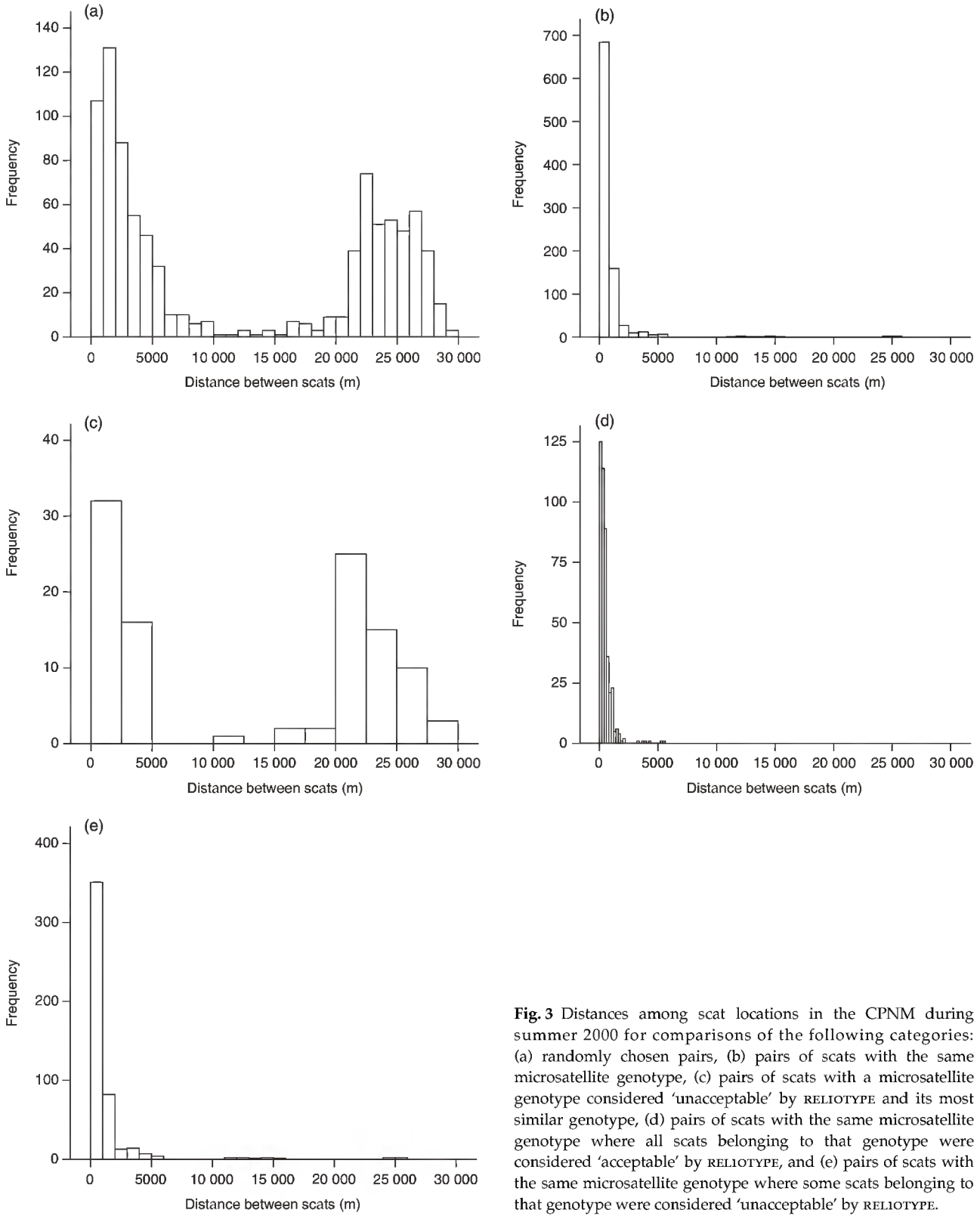

Fig. 3 Distances among scat locations in the CPNM during summer 2000 for comparisons of the following categories: (a) randomly chosen pairs, (b) pairs of scats with the same microsatellite genotype, (c) pairs of scats with a microsatellite genotype considered 'unacceptable' by RELIOTYPE and its most similar genotype, (d) pairs of scats with the same microsatellite genotype where all scats belonging to that genotype were considered 'acceptable' by RELIOTYPE, and (e) pairs of scats with the same microsatellite genotype where some scats belonging to that genotype were considered 'unacceptable' by RELIOTYPE.

41, 43, 46; Appendix) deposited scats both less than 2107 m, and up to 5000 m apart.

The mean distance between locations of all scats within a set of identical genotypes was significantly different than scats selected at random ($Z = 15.50$, $P = 0.00$). Additionally, the mean distance between locations of scats within a set of identical genotypes where all scats were considered 'acceptable' by RELIOTYPE was significantly different than cases where one or more scats were considered 'unacceptable' by RELIOTYPE ($Z = 3.50$, $P = 0.00$). However, when we removed the six foxes that deposited scats > 2107 m apart from the analysis, we found there was no difference in the mean distance between locations of scats within a set of identical genotypes where all scats were considered 'acceptable' by RELIOTYPE or where one or more scats were considered 'unacceptable' by RELIOTYPE ($Z = 1.45$, $P = 0.07$). Finally, there was no difference in the mean distance between locations of all scats considered 'unacceptable' by RELIOTYPE and its most similar genotype and scats selected at random ($Z = 0.84$, $P = 0.20$).

Tests based on sex primer consistency and scat location. We were able to apply the sex, latrine, and distance agreement tests to 63 of 69 scat genotypes that were considered 'unacceptable' by RELIOTYPE (Appendix). Of these 63 scats, 49 passed two of the three agreement tests, and thus we considered their scores 'acceptable', or reliable, after all (Fig. 1a). Moreover, of the 10 unique genotypes considered 'unacceptable' by RELIOTYPE, four changed to an 'acceptable' rating (Fig. 1b). The other six genotypes were not testable because they were only found once. We performed three additional amplifications on each of these scat samples to check the reliability of these remaining six scores. We found four of these samples had allele scores identical to the first typing. Because of this, and because these samples differed from other genotypes at two or more loci, we decided that they were unique individuals rather than genotyping errors. The remaining two samples revealed faint bands that were difficult to score during the second typing (i.e. poor quality samples, Prugh *et al.* 2005). Even though these two samples were successfully genotyped with our multiple tubes approach in the first typing, we decided not to use them to deduce information about the population of kit foxes, thus leaving us with 78 rather than 80 individual fox genotypes in our sample.

Feasibility of the genotyping results

Using simulated fox territories, we found the average number of fox home ranges potentially encountered during surveys was 30.40 ± 2.72 (range: 27–34). Because fox core areas are used by resident family groups including a mated pair, their offspring, and occasionally juveniles of the previous season (Ralls *et al.* 2001; Cypher 2003; Moehrenschlager *et al.* 2004) and our surveys were conducted in late July and early August when many adult-sized young of the year were still on their natal territories, we considered that at least two, and possibly up to seven, foxes could be detected per home range. Thus, our results fall well within the range of potential foxes available to be sampled based on reproductive success that year, and suggest that encountering scats of 78 unique foxes during transect surveys is very feasible.

We used accumulation curves to estimate how many more unique genotypes might have been found if we had performed more sampling. Models, E_{hyp} and E_{exp}, indicated that there were approximately 104 ± 2.8 (SD) and 81 ± 1.3 (SD) individuals, respectively. Thus, we sampled about 75% and 96%, respectively, of the estimated number of foxes in the area. Again, our identification of 78 unique foxes in the area is very plausible.

Error rates, P_{ID}, polymorphism, and Hardy-Weinberg equilibrium

Using the approaches of Bellemain *et al.* 2005, amplification another three times of about 5% of the successfully genotyped samples showed that 204 alleles were identical and 5 alleles were different from the first typing. Thus, the error rate is ~2.5%. Additionally comparisons of the consistency of sex determination within a set of identical genotypes found 6 incompatibilities, corresponding to 5 individuals in 349 comparisons. Thus, the error rate is < 2%.

With six microsatellites in a tissue sample set of 56 foxes, we estimated that the probability of a random match between unrelated individuals for all multilocus genotypes was $2.03\ 10^{-6}$ ($P_{\text{ID unbiased}}$), and the probability of a random match between siblings for all multilocus genotypes was $7.95\ 10^{-3}$ ($P_{\text{ID sibs}}$). Thus, the overall PI was low suggesting our selected microsatellites were adequate to differentiate between individual foxes, including relatives, in the CPNM.

Rates of misprinting and dropout increase significantly with the number of loci screened (Creel *et al.* 2003). In our case, the estimate of $P_{\text{ID sibs}}$ suggested six loci were needed as the conservative upper bound necessary to distinguish relatives (Waits *et al.* 2001). However, with our two most polymorphic loci (16 and 14 alleles), we identified 76 (95%) of the 80 unique genotypes originally obtained, and by combining sex determination with these two loci, we identified 79 (99%) of these 80 genotypes. Thus, screening at the additional four loci did not appear to increase the error rate or substantially bias the unique number of genotypes.

We detected a total of 56 alleles at the six loci, with an average of 9.33 alleles per locus (range: 4–16 alleles) (Table 1). The average heterozygosity value of our six loci was 0.682 (range: 0.438–0.863). None of our loci were in linkage disiquilibrium and five of the six loci were in Hardy–Weinberg equilibrium with the exception of locus FH2140 that yielded a deficiency of heterozygous genotypes.

Table 1 Microsatellite loci used in this study. These tetranucleotide primers were originally obtained from the canine genome map (Francisco *et al.* 1996; Mellersh *et al.* 2000)

Locus	No. of alleles	H_E	H_O
FH 2140	5	0.542	0.438
FH 2535	7	0.534	0.563
FH 2137	16	0.903	0.863
PEZ19	4	0.638	0.788
FH 2226	10	0.524	0.588
FH 2561	14	0.782	0.850
Mean	9.3	0.654	0.682

Information about kit foxes deduced from 'acceptable' scat genotypes

Sex ratio. Of the 78 unique genotypes with a final 'acceptable' rating, 70 were successfully sexed. We found a male to female ratio not significantly different from 1:1 (38 males and 32 females) ($\chi^2_{0.05,1} = 0.271$, $P = 0.603$). Additionally, there was no difference between the average number of multiple occurrences of female (mean 5.72 ± 4.96, $n = 25$) or male (mean 5.23 ± 3.71, $n = 31$) genotypes ($t = 0.426$, d.f. = 54, $P = 0.672$), suggesting that both sexes defecate equally.

Relatedness. The average relatedness estimated from the overall area surveyed was $r = 0.1 \pm 0.24$, showing that the study area included many unrelated individuals. Each fox had on average $r = 6.1 \pm 2.8$ putative first-order relatives assigned (i.e. parent–offspring and sib relationships), and the majority of these relatives were found within an approximate home range area (2490 m², Bean 2002). Additionally, many foxes (43%) had one or more relatives located at a distance greater than one home range away (> 2490 m², Bean 2002).

Movements. Of the 61 individual foxes detected multiple times, 42 had deposited scats within an approximate home range area (2490 m², Bean *et al.* 2002). Fifteen individuals deposited scats both within and on approximate, adjacent home range areas. Additionally, two individuals were detected from scats that were deposited ~5 km apart, and one individual, that was trapped and radio-collared the previous year on a different study (Bean 2002), was detected from scats deposited ~6 km from where it was originally captured.

Latrine use. We found 43 latrines (a site with an accumulation of at least three kit fox scats, Ralls & Smith 2004). There was no difference in the number of male ($n = 30$) and female ($n = 24$) foxes, or the number of related ($n = 13$) and unrelated ($n = 23$) individuals that deposited scats at latrines ($\chi^2_{0.05,1} = 0.334$, $P = 0.563$; $\chi^2_{0.05,1} = 1.416$, $P = 0.234$). However, there were significantly more latrines consisting of scats from more than one individual ($n = 24$) than only one individual ($n = 8$) ($\chi^2_{0.05,1} = 4.267$, $P = 0.039$). Within the 16-day scat collection period, the mean number of scats deposited at latrines by a particular individual was 1.6 ± 1.2 scats (range: 1–8 scats), and the mean number of individuals contributing to a latrine was 2.2 ± 1.2 individuals (range: 1–6 individuals).

Home range size. The distribution of distances for scats belonging to the same individual where all scats within a set of identical genotypes were considered 'acceptable' by RELIOTYPE (Fig. 3d), is unimodal, and shows that 75 of the 78 'acceptable' unique individuals (96%) deposited scats within a distance no greater than 2107 m apart. Thus, examination of the distribution of distance comparisons for this category of foxes allowed us to predict the probable diameter of a fox's home range in the area surveyed.

Discussion

Applying genetic and GIS analyses to our kit fox scat data set provided insights into testing the reliability of genotyping results. By combining several genetic methods for tracking and reducing error, we were able to thoroughly assess the level of error in the data set. Furthermore, tests based on sex primer consistency and scat location information proved extremely useful in verifying how 'acceptable' a scat genotype was. Our study demonstrates that both genetic and GIS approaches can be important in ensuring data accuracy, and after such analyses are incorporated, faecal DNA sampling can offer relevant information in studies of wild populations.

The reliability of the genotyping results

We had a low probability of identity, genotyping error rate (~2.5%), and sex primer error rate (~2%). Unlike other studies (Bellemain *et al.* 2005; Prugh *et al.* 2005), we did not consider genotypes with one genetic mismatch at one allele for one locus as belonging to the same individual, and thus may have slightly overestimated the number of individuals present. However, blind re-amplification and rescoring of the eight genotypes identical to another genotype in sex and at all six microsatellite loci except for one allele confirmed identical scores to the first typing indicating that these eight genotypes were most likely 'real' and represented foxes present on our transects.

Critical scoring errors due to the 'shadow effect' (i.e. underestimating the real number of unique individuals) (Mills *et al.* 2000) or genotyping error (i.e. falsely identifying unique individuals) (Taberlet *et al.* 1996, 1999; Waits & Leberg 2000) can create serious bias in scat data sets. Genotyping error in particular can have a great impact on

estimates, and rates of error can increase significantly with the number of loci screened (Waits & Leberg 2000; Creel *et al.* 2003). We believe that following the proposed recommendations of Bonin *et al.* (2004) and adhering to a strict multiple tubes approach protocol led to an extremely low chance of error in our scat data set, and we were able to confirm that screening with six loci did not bias the total number of 78 individuals found. Finally, by applying the EB and DCH tests of McKelvey & Schwartz (2004) after genotype scores were assigned, we were able to determine that our data set indeed had a low rate of genotyping error. Although, six markers is a relatively small number of loci for running these proposed tests [i.e. McKelvey & Schwartz (2004) suggest a minimum of eight loci], if there was a high rate of genotyping error it would likely have appeared (M. Schwartz, personal communication).

Examining the frequency distributions of scat locations allowed us to have even more certainty that our data set was relatively error free. Comparison of distances among scats belonging to the same individual revealed most individuals deposit scats within a limited area. The majority of scats 'recaptured' from one individual should fall within this range, and will be clearly separated from a random distribution. In our case, the random distribution of scats showed a bimodal structure which may have been partly the result of our survey design where transects searched for scats ranged from adjacent transects to transects that were ~28 km apart. Use of similar home ranges by individual foxes belonging to the same family group probably also contributed to this bimodal structure.

By further comparing scat genotypes deemed 'acceptable' or 'unacceptable' by RELIOTYPE, we could shed more light onto the accuracy of our multilocus scores. Distributions of distances for scats belonging to the same individual where all scats within a set of identical genotypes were considered 'acceptable' by RELIOTYPE revealed a considerably narrower range of distances in comparison to those genotypes with some scats considered 'unacceptable' by RELIOTYPE. This difference in distances among scat locations appeared to be the result of six individual foxes who deposited scats both within and above the narrow range of 0–2107 m. Thus, in cases where mean range size of an individual is known, scat genotypes belonging to one individual that fall within this estimated range size are likely very 'acceptable' genotypes. Scat genotypes that fall outside of this range may not necessarily be erroneous. However, additional laboratory work to test the reliability of such genotypes should be considered. Finally, comparisons of distances between locations of scats from unique genotypes considered 'unacceptable' by RELIOTYPE and their most similar unique genotype can provide additional evidence that both genotypes are different individuals. In our case, distribution results of this category of comparisons showed a distribution similar to that of random comparisons, indicating these unique genotypes were not genotyping errors but rather different individuals. If these genotypes were from the same individuals, we would expect their scat locations to fall with the same range more often than not.

Through the use of the program RELIOTYPE, we were alerted to specific scat genotypes that might be 'false'. Thus, we could focus on these samples as potential scoring errors and carefully scrutinize them. Rather than immediately resorting to additional laboratory work, however, we found that analysing information on sex primer consistency and scat locations proved extremely helpful. We were able to apply the sex, latrine and distance agreement tests to 91% of these questionable scat genotypes allowing us to verify if a 'real' scoring problem existed. Also, this allowed us to limit the number of samples ($n = 6$) that needed to be retyped three times at all six loci for score verification to those unable to be examined under the agreement tests because they were found only once.

Based on our results, we suggest that the sex primer consistency and scat location agreement tests can provide a useful assessment of scat genotype error. When these tests cannot be applied because a genotype is represented by only a single scat, we recommend retyping that scat by repeating the initial multiple tubes approach protocol. Of course, the usefulness of these tests will depend on the study species but for species such as canids with specific home ranges that defecate at prominent sites, these agreement tests offer a useful tool for identifying scats with potentially erroneous scores.

By using simulated fox territories and accumulation curves, we demonstrated that encountering scats of 78 different individuals in the area we surveyed was highly probable, and that these 78 unique genotypes are likely a reasonable representation of the minimum number of foxes in the study area at the time we sampled. Although we did not formally estimate population size, this would be possible from faecal samples using DNA-based capture–recapture models (Lukacs & Burnham 2005). It should be noted, however, that estimates of population size based on DNA from scats will not always agree with estimates based on other techniques, particularly if the different methods sample different time periods or if scats from different sex/age classes are not equally likely to be found. The number of kit foxes in an area varies considerably over the annual cycle. For example, spotlight surveys during the summer, when large pups are still present on their natal territories, often detect about twice as many foxes as similar surveys during the winter, when the population consists mainly of adult pairs (Ralls & Eberhardt 1997). Fox population size also varies greatly from year to year (Moehrenschlager *et al.* 2004). Little is known about the likelihood of finding scats from different age/sex classes but it seems likely that it may not be equally probable to find the scats of all individuals at all times of year.

For example, it might be less likely to find scats from adult females in February, when females with young pups remain in or near their dens. Noninvasive sampling may be most appropriately used as a way to obtain an independent estimate of population size in addition to field estimates. Then both types of estimates can be combined in order to gain new information about a natural population as demonstrated by Bellemain *et al.* (2005).

Information deduced from 'acceptable' scat genotypes

A systematic collection of scats followed by molecular typing allowed us to confirm scats at the species level, identify individuals on the study site, and determine sex ratio. Furthermore, genetic data allowed us to obtain information on relatedness, fox movements, latrine use, and home range size that was consistent with results of other field studies collected with traditional techniques. Thus, faecal DNA sampling can provide a reliable way to obtain information on kit fox populations. Moreover, the consistency between results of our genetic data with previous collected field data further supports the conclusion that our data set had a low rate of genotyping error.

We determined that kit foxes in this study area have a 1:1 sex ratio which agrees with results of previous field studies using other techniques (Cypher 2003; Moehrenschlager *et al.* 2004). Additionally, both sexes appear to defecate equally, similar to other canids such as coyotes (Gese & Ruff 1997). Again, it is important to note that if we had conducted surveys at a different time of year (e.g. when females had young pups and were remaining in or near their dens), we may not have found an equal sex ratio.

Foxes tended to have a high number of relatives found within an approximate home range area, as well as one or more relatives located at a distance greater than one home range away. This was expected as scats for the analysis were collected during the summer months (July–August) when kit foxes exist in family groups that contain a mated pair, their offspring (usually 1–6 pups) of that season, and occasionally juveniles of the previous season that have not dispersed (Ralls *et al.* 2001; Cypher 2003; Moehrenschlager *et al.* 2004). Furthermore, adult female foxes on adjacent home ranges are often closely related (Ralls *et al.* 2001). Finally, kit foxes have been found to disperse on average 8 km from their natal home ranges (Koopman *et al.* 2000), and long-distance dispersal, although infrequent, is known to occur (Schwartz *et al.* 2005).

Results on fox movement patterns also agreed with prior field observations. The majority of foxes deposited scats within an approximate home range area, some foxes deposited scats on adjacent home ranges, and a few individuals deposited scats from ~5–6 km away. Because kit fox social groups occupy distinct core areas within larger home range areas that overlap considerably with home ranges of adjacent, social groups (Ralls *et al.* 2001), we expected to detect the majority of scats of an individual within a small area and occasionally locate some scats at farther distances. Also, because annual dispersal rates can be as high as 52% for juveniles (Cypher 2003) with average dispersal distances of 8 km from their natal home ranges (Koopman *et al.* 2000), we expected to locate scats of a few foxes at great distances.

Extensive use of latrines by kit foxes has recently been documented (Murdoch 2004; Ralls & Smith 2004). This study provided us with additional behavioural information on latrine use. Direct observations of kit foxes showed that all focal foxes used latrines, and mated pairs shared between three and five latrines throughout their respective ranges (Murdoch 2004). Our study confirmed that both males and females deposit scats at latrines but provided new information on equal frequency of deposition, suggesting both sexes actively use faeces as a form of chemical communication. Furthermore, the majority of latrines found in our study contained scats from more than one individual (i.e. a latrine contained scats from an average of two and a maximum of six individuals). We found unrelated individuals of the same sex [which strongly indicates they were from different social groups (Ralls *et al.* 2001)] sometimes used the same latrine. In European badgers, neighbouring groups tend to place a similar number of scats at shared latrines near territory boundaries (Stewart *et al.* 2001). Because we collected and analysed only fresh scats for this study, we were unable to determine the total quantity of scats from an individual or group at a latrine. However, our findings indicate that several individuals can share a latrine, and that many of the individuals in the area will contribute to a latrine. This supports the hypotheses that latrines allow information to be transmitted among individuals (Roper *et al.* 1993; Stewart *et al.* 2001), and may convey important intraspecific messages between neighbouring social groups (Murdoch 2004).

Finally, we discovered faecal genotypes can be useful in deducing the probable diameter of a fox home range in the area surveyed. By examining the distribution of distances among scats considered 'acceptable' by RELIOTYPE and belonging to the same genotype, we found 96% of foxes deposited scats within a distance no greater than 2107 m. We suggest that the distribution of distances among scats with the same genotype may offer a new approach to estimating home range size with noninvasive faecal sampling. However, this method requires further testing with several populations of various species.

Conclusions

Both genetic and GIS analyses can address issues of faecal DNA sample reliability. The screening of multilocus scores with both approaches provides a careful assessment of a

scat data set and produces information that can be used to judge whether individual scat genotypes are 'acceptable'. An important conclusion of this study is that GIS analyses can greatly compliment, or even be substituted for, particular genetic methods that test reliability. In any case, a combination of the two approaches could allow significant improvement in the quality of the noninvasive genetic data.

We found that overall noninvasive faecal DNA sampling offers an effective way to obtain information on kit foxes without the need to capture or observe individuals. After appropriate steps are taken to ensure genotype reliability, this molecular method can be used to reveal details on minimum number of animals in an area, sex ratios, genetic relatedness, movement patterns, scent-marking behaviours, and home range size. This noninvasive genetic approach is quite applicable to studies of other elusive or rare animal populations.

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This study is part of the PhD research of Deborah Smith that focuses on the use of noninvasive methods to obtain information on rare species. Aimee Hurt is a research biologist, and Megan Parker and Brice Adams are completing their PhDs at the University of Montana, Missoula, and the University of Louisiana, Lafayette, respectively. Deborah, Aimee, and Megan are also cofounders of wildlife detection dog organizations based out of California and Montana. Katherine Ralls is a Senior Scientist at the Smithsonian's National Zoological Park. Dr Jesús Maldonado is a Research Geneticist at the Genetics Program at Smithsonian's Institution.

Appendix

Scat genotypes identical to genotypes of different scat samples, yet considered 'unacceptable' by RELIOTYPE (bold) were examined under the sex, latrine, and distance agreement tests. If a scat genotype passed two of the three agreement tests it was considered 'acceptable' despite the original RELIOTYPE determination. Scat samples, unique fox genotypes, final determination of the genotype sex, and the sex of each scat sample are listed below. 'L' indicates a latrine location, followed by an alphabetical code (e.g. a, b, c, d) which indicates the specific latrine the scat was found in for scat samples of identical genotype. Average distances between locations of all scat samples with the same microsatellite genotype were calculated (below) and compared to the value at which 99% of scat locations for genotypes with all scats considered 'acceptable' by RELIOTYPE were found (2107 m)

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
A003 21	1	M	M	La	157				
A007 21			M	La					
B001 23			M		313				
D027 22	2	M	M		singleton				
A009 21	3	?	M	La	1513				
A010 21			M	La					
A005 31			M		1962				
B005 14			F		1663				
A012 21	4	?	F	La	441				
A013 21			**F**	**La**		**unacceptable**	**no**	**latrine**	**no**
A015 21			F		401				
A017 21			**F**	**Lb**	**401**	**unacceptable**	**no**	**latrine**	**no**
A018 21			F	Lb					
D030 22			**F**		**428**	**unacceptable**	**no**	**distance**	**no**
D040 22			F		466				
D001 32			M		1418				
AO10 62			**M**		**806**	**unacceptable**	**no**	**distance**	**no**
AO11 62	5	M	M	La	0				
AO12 62			**M**	**La**		**unacceptable**	**yes**	**latrine**	**yes**
D022 11	**6**	**F**	**F**		**382**	**unacceptable**	**yes**	**distance**	**yes**
D030 11			**F**		**442**	**unacceptable**	**yes**	**distance**	**yes**
D017 13			**F**		**280**	**unacceptable**	**yes**	**distance**	**yes**
D005 u1	7	M	M		singleton				
A008 11	8	F	F		141				
A009 11			F		213				
A011 12			F		265				
B003 13			F		201				
B004 13			F	La	119				
B005 13			F	La					
B006 13			F		129				
D032 13			F		260				
D033 13			F	Lb	121				
B004 14			F		168				
B007 14			F	Lb					
B008 14			F		217				
D039 11	9	M	M		287				
D044 11			M		212				
D048 11			M		212				
D053 11			M		501				
A021 02	10	F	F		487				
A027 02			F		639				
D012 O4			F		446				
D013 O4			F		443				
A020 12	11	M	M	La	6				
A021 12			M	La					
A023 12			M	La					
A025 12			M	La					
A026 12			M	La					
A027 12			**M**	**La**		**unacceptable**	**yes**	**latrine**	**yes**

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
A028 12			M	La					
A029 12			M	La					
A031 12			M		38				
D012 22	12	M	M		757				
A009 62			**M**		**793**	**unacceptable**	**yes**	**distance**	**yes**
AO13 62			**M**		**536**	**unacceptable**	**yes**	**distance**	**yes**
AO14 62			M		606				
D015 22	13	F	F		412				
D016 22			F		280				
D010 24			**F**		**381**	**unacceptable**	**yes**	**distance**	**yes**
T004 51	14	F	F		1413				
T006 51			F		1390				
T007 51			F		1401				
T009 51			F		1463				
D002 11			**F**		**5317**	**unacceptable**	**yes**	**distance**	**no**
A017 12	15	F	F		486				
A030 12			F		486				
D034 11	**16**	**F**	**F**		**426**	**unacceptable**	**yes**	**distance**	**yes**
D036 11			F		241				
D042 11			**F**		**328**	**unacceptable**	**yes**	**distance**	**yes**
D025 13			F		244				
B001 14			F		318				
D001 M1	17	F	F		1305				
A002 P2			**F**		**895**	**unacceptable**	**yes**	**distance**	**yes**
D008 O4			F		921				
D018 O4			F		1024				
D020 O4			F		731				
D004 P4			F		741				
A007 02	18	M	M	La	599				
A010 02			M	La					
D001 M3			M		1784				
B001 O3			M		602				
T003 21	19	M	M		singleton				
T001 51	**20**	**F**	**F**		**1167**	**unacceptable**	**yes**	**distance**	**yes**
D004 u1			F		1167				
T003 71	21	M	M		289				
T004 71			M		629				
T001 74			**M**	**La**	**289**	**unacceptable**	**yes**	**latrine**	**yes**
T002 74			M	La					
A020 02	**22**	**M**	**M**		**1832**	**unacceptable**	**yes**	**distance**	**yes**
T002 83			M		1832				
D043 11	**23**	**F**	**F**	**La**	**155**	**unacceptable**	**yes**	**latrine**	**yes**
D045 11			**F**	**La**		**unacceptable**	**yes**	**latrine**	**yes**
D052 11			**F**		**308**	**unacceptable**	**yes**	**distance**	**yes**
A001 p1	24	F	F	La	436				
A002 p1			F	La					
A029 02			**F**		**450**	**unacceptable**	**yes**	**distance**	**yes**
A031 02			**F**		**344**	**unacceptable**	**yes**	**distance**	**yes**
A032 02			F		345				
A035 02			**F**		**381**	**unacceptable**	**yes**	**distance**	**yes**
A040 02			F	Lb	393				
A052 02			F	Lb					
A005 P2			F	Lc	402				
A006 P2			F		451				
D015 O4			**F**		**375**	**unacceptable**	**yes**	**distance**	**yes**
D006 P4			F	Lc					

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
D007 M1	25	M	M		3816				
D004 M2			M		3816				
D018 11	26	M	M		353				
D021 11			M		391				
D007 13			**M**		**458**	**unacceptable**	**yes**	**distance**	**yes**
D008 13			M		435				
D010 13			M		352				
D021 13			M		642				
A018 02	**27**	**M**	**M**	**La**	**1**	**unacceptable**	**yes**	**latrine**	**yes**
A019 02			**M**	**La**		**unacceptable**	**yes**	**latrine**	**yes**
D003 u1	28	M	M		2793				
D001 U3			M		2784				
T003 53			M		2511				
B002 14			M		4711				
A008 P2	**29**	**F**	**F**		**16371**	**unacceptable**	**yes**	**distance**	**no**
A001 Q2			**F**		**10927**	**unacceptable**	**yes**	**distance**	**no**
A003 Q2			**F**		**10829**	**unacceptable**	**yes**	**distance**	**no**
D009 64			**F**		**14813**	**unacceptable**	**yes**	**distance**	**no**
D005 24			F		15242				
D014 O4			F		15751				
T002 22	30	F	F	La	378				
T003 22			F	La					
T007 22			F		378				
T009 22			F		443				
T010 22			F		511				
D008 64			F		656				
A001 11	31	M	M	La	418				
A002 11			M	La					
D019 21			M		322				
T004 22			M		392				
AO16 62			M		370				
B006 23			M		383				
B007 23			M		356				
A004 31	32	F	F		469				
A008 31			F	La	404				
A009 31			F	La					
T001 31			F		264				
T003 31			F		206				
T002 32			F		378				
T005 32			F	Lb	274				
TO11 32			F		209				
TO12 32			F		204				
B003 33			F	Lc	245				
T001 33			F	Lb					
D014 34			F	Ld	247				
D015 34			F	Ld					
D016 34			F	Lc					
D018 34			F		284				
T006 34			F		283				
T009 34			F		272				
T012 34			F		227				
T014 34			F		203				
D015 11	33	M	M		1009				
D026 11			M		431				
D013 13			M		424				
D016 13			M		468				

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
A011 02	**34**	**M**	**M**		**260**	**unacceptable**	**yes**	**distance**	**yes**
A012 02			**M**		**260**	**unacceptable**	**yes**	**distance**	**yes**
A004 21	35	F	F	La	996				
A005 21			F	La					
A003 31			F		1089				
D007 32			**F**		**484**	**unacceptable**	**yes**	**distance**	**yes**
T003 32			F		499				
T004 32			F		453				
TO07 32			**F**		**407**	**unacceptable**	**yes**	**distance**	**yes**
TO08 32			F		427				
TO10 32			F		395				
B001 33			F		478				
T002 33			F		393				
D017 34			F		450				
D019 34			F		488				
T001 34			F		442				
T004 34			F		505				
T005 34			F		520				
T008 34			F		442				
T010 34			F		396				
T013 34			F		399				
D002 74			F		898				
D043 22	36	F	F		92				
D007 64			F		92				
A013 02	37	F	F	La	42				
A014 02			F	La					
A015 02			F		82				
A053 02	38	M	M		485				
A054 02			M		366				
A055 02			M		332				
A059 02			M		411				
A061 02			M		620				
T002 81	39	M	M		1200				
T004 81			M		1212				
D009 M1			**M**		**1282**	**unacceptable**	**yes**	**distance**	**yes**
A005 02			**M**	**La**	**827**	**unacceptable**	**yes**	**latrine**	**yes**
A006 02			M	La					
A008 02			M	La					
A009 02			**M**	**La**		**unacceptable**	**yes**	**latrine**	**yes**
T001 83			M		1215				
B002 O3			M		826				
B003 O3			**M**		**2203**	**unacceptable**	**yes**	**distance**	**no**
DO01 72	40	?	M		518				
D003 34			F		518				
TOO1 72	**41**	**M**	**M**		**3677**	**unacceptable**	**yes**	**distance**	**no**
TO02 82			M		1886				
T002 84			M		1887				
T005 51	42	F	F		singleton				
A010 31	43	M	M		975				
T004 31			M		943				
D002 41			M		1249				
TO06 32			**M**		**992**	**unacceptable**	**yes**	**distance**	**yes**
TO09 32			**M**		**980**	**unacceptable**	**yes**	**distance**	**yes**
DO02 72			**M**		**1145**	**unacceptable**	**yes**	**distance**	**yes**
D001 33			M		1157				
D002 33			M	La	1153				

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
D003 33			M	La					
B013 14			M		4036				
D001 34			M	Lb	1351				
D002 34			M	Lb					
D004 34			M		1293				
T011 34			M		983				
T002 51	44	M	M	La	986				
T003 51			M	La					
D001 u1			M		1970				
A006 21	45	F	F		908				
A011 21			F		1041				
A001 31			F		814				
A012 31			**F**		**876**	**unacceptable**	**yes**	**distance**	**yes**
D024 22			F		739				
T001 32			F		1184				
A008 21	**46**	**F**	**F**		**3261**	**unacceptable**	**yes**	**distance**	**no**
D005 51			F		1263				
D007 51			F		1247				
D003 13			**F**		**1278**	**unacceptable**	**yes**	**distance**	**yes**
B012 14			F		1061				
D004 14			**F**		**1063**	**unacceptable**	**yes**	**distance**	**yes**
D017 22	**47**	**M**	**M**		**516**	**unacceptable**	**yes**	**distance**	**yes**
D007 24			M		504				
D005 64			M		790				
T003 81	**48**	**M**	**M**		**176**	**unacceptable**	**yes**	**distance**	**yes**
T006 81			M		176				
A034 02	**49**	**M**	**M**	**La**	**202**	**unacceptable**	**yes**	**latrine**	**yes**
A036 02			M	La					
A037 02			M	La					
A039 02			**M**	**La**		**unacceptable**	**yes**	**latrine**	**yes**
A041 02			M		224				
A003 P2			M	Lb	592				
A004 P2			**M**	**Lb**		**unacceptable**	**yes**	**latrine**	**yes**
D016 O4			M	La					
A009 01	50	F	F		278				
A058 02			**F**		**316**	**unacceptable**	**yes**	**distance**	**yes**
A060 02			**F**		**266**	**unacceptable**	**yes**	**distance**	**yes**
A007 P2			F		346				
D007 P4			F		350				
D002 M1	51	?	M		114				
D003 M1			**F**	**La**	**57**	**unacceptable**	**no**	**latrine**	**no**
D004 M1			**F**	**La**		**unacceptable**	**no**	**latrine**	**no**
D013 M1	52	M	M		322				
A001 01			M		460				
A002 02			M		457				
D003 N3			M	La	396				
D004 N3			M	La					
D008 N3			M		716				
D009 M3			M		334				
B003 M4			M		319				
D003 O4			M		485				
D006 M1	53	M	M		363				
D006 M3			M		363				
D015 M1	**54**	**M**	**M**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**
D020 11	**55**	**F**	**F**		**861**	**unacceptable**	**yes**	**distance**	**yes**
D006 13			F		861				

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
A003 11	56	M	M		604				
A005 11			M		601				
A006 11			M		640				
D004 11			M		1058				
D009 11			M		1105				
D002 51			M	La	762				
D003 51			M	La					
D006 51			M		757				
B002 13			M	Lb	641				
D027 13			M		1230				
D001 14			M		665				
D002 14			M	Lb					
D003 14			M	Lb					
D005 11	57	M	M		798				
D006 11			M	La	791				
D007 11			M	La					
D008 11			**M**		**803**	**unacceptable**	**yes**	**distance**	**yes**
D005 13			M		795				
D001 53			**M**		**1062**	**unacceptable**	**yes**	**distance**	**yes**
D002 53			M		1142				
D015 24			M		1968				
D002 64			M		2076				
D019 11	58	M	M		204				
D009 13			M		336				
D012 13			M		246				
D001 N1	59	M	M		394				
DO01 M2			M		295				
D012 M1			M		324				
A001 02			M		350				
A004 02			M		537				
B004 O3			M		539				
B005 O3			M		504				
D008 M3			M		373				
D001 N3			M		407				
D002 N3			M		405				
B004 M4			M		297				
B005 M4			M		296				
D001 O4			M		354				
D002 O4			did not amplify		362				
T008 22	60	F	F		126				
T001 24			F		126				
D003 22	**61**	**M**	**M**		**1049**	**unacceptable**	**yes**	**distance**	**yes**
D013 22			M		527				
D014 22			**M**		**530**	**unacceptable**	**yes**	**distance**	**yes**
A007 11	62	F	F		278				
A010 11			F		528				
D055 11			F		248				
A019 12			F		234				
A032 12			F		344				
D028 11	**63**	**?**	**?**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**
D008 M1	64	?	F		1135				
D010 M1			M		719				
D003 M2			F		808				
D001 N2			F		598				
D002 M3			F	La	773				
D003 M3			F	La					

Appendix *Continued*

Scat sample	Fox genotype	Genotype sex	Scat sex	Latrine	Average distance (m)	Reliotype 'unacceptable'	Sex agreement	Latrine/distance agreement	Passed tests
D005 M3			F	La					
D005 N3			F	Lb	677				
D006 N3			F	Lb					
D009 N3			F		882				
B001 M4			F		807				
A011 11	65	F	F		271				
D028 13			F		271				
T005 22	**66**	**M**	**M**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**
D007 M3	**67**	**?**	**?**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**
B001 P3	68	F	F		singleton				
B001 Q3	**69**	**M**	**M**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**
D029 13	70	M	M		58				
B003 14			M		58				
D003 64	71	F	F		singleton				
B006 14	72	F	F		singleton				
T001 84	73	F	F		singleton				
D006 64	74	?	?		singleton				
D003 24	75	F	F		singleton				
D004 24	76	F	F		singleton				
D005 34	77	F	F		1738				
D010 64			F		1738				
D009 O4	78	M	M		singleton				
D019 O4	79	M	M		singleton				
D002 P4	**80**	**F**	**F**		**singleton**	**unacceptable**	**untestable**	**untestable**	**untestable**