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ORIGINAL PAPER

An efficient method for screening faecal DNA genotypes and detecting new individuals and hybrids in the red wolf (*Canis rufus*) experimental population area

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Abstract Previously, sequencing of mitochondrial DNA (mtDNA) from non-invasively collected faecal material (scat) has been used to help manage hybridization in the wild red wolf (*Canis rufus*) population. This method is limited by the maternal inheritance of mtDNA and the inability to obtain individual identification. Here, we optimize the use of nuclear DNA microsatellite markers on red wolf scat DNA to distinguish between individuals and detect hybrids. We develop a data filtering method in which scat genotypes are compared to known blood genotypes to reduce the number of PCR amplifications needed. We apply our data filtering method and the more conservative maximum likelihood ratio method (MLR) of Miller et al. (2002 Genetics 160:357–366) to a scat dataset previously screened for hybrids by sequencing of mtDNA. Using seven microsatellite loci, we obtained genotypes for 105 scats, which were matched to 17 individuals. The PCR amplification success rate was 50% and genotyping error rates ranged from 6.6% to 52.1% per locus. Our data filtering method produced comparable results to the MLR method, and decreased the time and cost of analysis by 25%. Analysis of this dataset using our data filtering method verified that no hybrid individuals were present in the Alligator River National Wildlife Refuge, North Carolina in 2000. Our results demonstrate that nuclear DNA microsatellite analysis of red wolf scats provides an efficient and accurate approach to screen for new individuals and hybrids.

Keywords *Canis rufus* · Faecal DNA · Genotyping errors · Hybrid detection

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Introduction

In the last five years, the use of non-invasive genetic sampling (NGS) of faeces (scat) has increased because of its applicability to a variety of research questions. Some uses of scat DNA sampling include identifying the presence of a rare species (Paxinos et al. 1997; Hansen and Jacobsen 1999; Farrell et al. 2000; Davison et al. 2002; Palomares et al. 2002; Dalén et al. 2004), detecting hybrids (Adams et al. 2003), elucidating paternity and kinship relationships (Gerloff et al. 1995; Launhardt et al. 1998; Gerloff et al. 1999; Constable et al. 2001), and estimating population size (Kohn et al. 1999; Banks et al. 2002; Eggert et al. 2003; Frantz et al. 2003; Flagstad et al. 2004; Piggott et al. 2006). The latter two applications involve generating multi-locus genotypes from nuclear DNA for individual identification. This can be difficult because of the low quantity, degraded DNA found in scats (Taberlet et al. 1996). Poor DNA quality often leads to two types of genotyping errors. Allelic dropout occurs when one allele of a heterozygous locus does not amplify (Taberlet et al. 1996). False alleles are artifacts of the PCR process and can cause a heterozygous result at a homozygous locus or three alleles at a heterozygous locus (Taberlet et al. 1996).

To minimize genotyping errors, a multiple tubes genotype screening approach was developed in which an allele had to be observed at least twice and homozygous results were replicated up to seven times to account for false alleles and allelic dropout (Taberlet et al. 1996). However, fulfilling these requirements is costly and time consuming and can deplete the DNA template before genotyping is complete. As a result, other studies have developed new data filtering methods that require fewer PCR amplifications but still produce relatively error free genotypes (Banks et al. 2002; Miller et al. 2002; Frantz et al. 2003). In most cases, the new data filtering methods were developed for situations in which little is known about the number of individuals within a population. However, when the genotypes of many individuals are known, this data can potentially be used to improve the efficiency of non-invasive genotype data filtering methods.

One example is the endangered red wolf (*Canis rufus*) population in northeastern North Carolina. Red wolves were reintroduced to the wild in 1987 (USFWS 1989) and have increased in number and expanded their range to include five counties (6,000 km^2, personal communication, Buddy Fazio, Project Leader, Red Wolf Recovery Program, USFWS). This population is heavily managed and extensively trapped to prevent hybridization with coyotes (*Canis latrans*). Thus, approximately 80% of the wild red wolf population has been captured and sampled for genetic material (personal communication, Buddy Fazio). However, because of the risk of hybridization with coyotes the 20% of the population that hasn't been sampled poses the greatest threat to red wolf recovery if these individuals are hybrids or coyotes. Non-invasive scat sampling can succeed where trapping efforts have failed by locating the presence of new potentially hybrid individuals or coyotes.

In this study, our main goal is to develop a scat genotype data filtering method for use in the reintroduced red wolf population, which incorporates a database of reference genotypes from captured individuals. We compare the results of our data filtering method to those generated using the maximum likelihood based data filtering method of Miller et al. (2002). We calculate genotyping error rates for the microsatellite loci used in this study and compare these rates to those of other scat genotyping studies. Finally, we discuss an application of our data filtering method to scat samples collected as part of a previous study (Adams et al. 2003) to identify new red wolf, hybrid or coyote individuals within the Alligator River National Wildlife Refuge, North Carolina (ARNWR).

Methods

Sample collection, DNA extraction and microsatellite analysis

Scats were collected along all roads in the ARNWR using all terrain vehicles during April and May in 2000 as described in Adams et al. (2003). Scats were transferred within 12 h to a 95% EtOH solution in a 1:4 volume scat to EtOH ratio, to preserve DNA for extraction (Murphy et al. 2002; Adams et al. 2003).

Samples of whole blood stored in lysis buffer (Longmire et al. 1991) from wild red wolves known to inhabit the ARNWR during the study ($n = 15$) were extracted using a QIAmp^{TM} blood protocol (Qiagen, Valencia, CA) provided in the tissue kit. DNA was extracted from scats using a modified QIAmp^{TM} tissue protocol (Murphy et al. 2000) in a separate laboratory reserved for low quality DNA extraction. One extraction negative was included in each extraction. All scats were assigned to species using mitochondrial DNA sequencing (Adams et al. 2003). Scats containing the red wolf mitochondrial haplotype ($n = 210$) were analyzed further. Previously, nineteen microsatellite loci were screened for variability in red wolves (Miller et al. 2003). The probability of identity (PID_{THEO}), observed probability of identity (PID_{OBS}) and the expected probability of identity between siblings (PID_{SIBS}) were calculated according to Waits et al. (2001) for 175 wild red wolves (unpublished). Seven loci were chosen for further analysis based upon their PID values, their size, and their ability to be multiplexed with other loci (AHT103, Holmes et al. 1995, CXX.172, C09.173, CXX.20, CXX.225, Ostrander et al. 1993: FH2054, Mellersh et al. 1997, CXX.403, Ostrander et al. 1995).

The seven microsatellite loci were combined into four PCR reactions. The 15 μl PCR mixture for multiplex one consisted of 0.2 μM of primers CXX.225 and CXX.403, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 1 mg/ml BSA, 1× Gold *Taq* buffer supplied by the manufacturer (Applied Biosystems), 1.2 μl DNA extract and 0.5 U of Gold *Taq* DNA polymerase (Applied Biosystems). The PCR profile for multiplex 1 was 55 cycles of 30 s at 95°C, 30 s at 53°C, and 1 min at 72°C, following an initial denaturation step of 10 min at 95°C. All other PCR reactions contained 1.5 mM $MgCl_2$ and differed in the annealing temperature used. Multiplex two contained 0.2 μM of primers AHT103 and CXX.20, with 1.5 mM $MgCl_2$, and had an annealing temperature of 55°C. Multiplex three contained 0.2 μM of primers CXX.172 and C09.173 with 1.5 mM $MgCl_2$, and had an annealing temperature of 60°C. Singleplex one

contained 0.2 μM of primer FH2054 with 1.5 mM $MgCl_2$, and had an annealing temperature of 63°C. All blood PCR amplifications were carried out as in Miller et al. (2003). Each PCR reaction tray contained one negative control to monitor for contamination and one positive control to determine if the reaction worked. Samples were run on an ABI 377 automated sequencer following the manufacturers' protocols. Data were extracted from the gels using Genescan 3.1.2 (Applied Biosystems Inc.) and allele sizes were called using Genotyper 2.5 (Perkin Elmer).

Data filtering method

Because we had the option of comparing scat genotypes to blood genotypes, our objective was to design a data filtering method that would provide accurate genotypes with fewer PCR replicates. Our method, the reference genotype method, is similar to previously published methods in that an allele was accepted only after it was observed twice, and two PCRs per locus were initially performed (see Fig. 1). A maximum of seven positive PCRs were performed per locus. The reference genotype method differs from the other methods because homozygous results were provisionally accepted after two replicates (Fig. 1).

Once a consensus genotype was obtained at six loci it was compared to reference blood genotypes in order to identify matches using the program GIMLET (Valiere 2002). To be certain that using six versus seven loci would not affect our ability to differentiate between individuals, we calculated PID_{OBS} and PID_{SIBS} for each combination of six loci (Waits et al. 2001). All scat genotypes that were identical to a blood genotype were scored as originating from that individual. When a scat genotype differed by one allele from a blood genotype and the mismatch could have been due to allelic dropout the scat genotype was

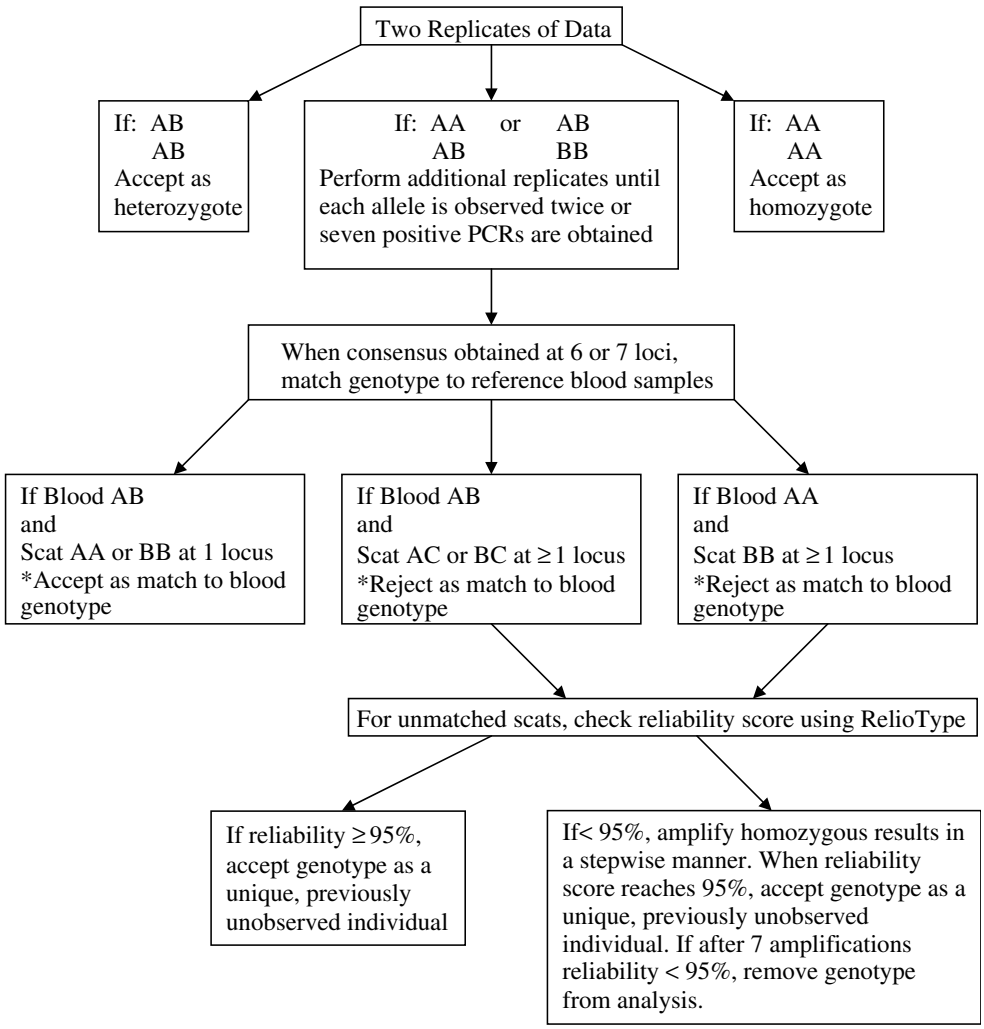

Fig. 1 A schematic for the comparative reference genotype data filtering method used in this study which is based upon the multiple tubes approach (Taberlet et al. 1996), the MLR method (Miller et al. 2002) and the comparative multiple-tubes approach (Frantz et al. 2003)

scored as having originated from that individual. All other cases involving one mismatch were rejected as a match to the blood genotype (Fig. 1).

All scat genotypes that were rejected as matches to blood genotypes were analyzed further using the maximum-likelihood based method (MLR) of Miller et al. (2002) to assess their reliability. Allele frequency distributions used in the calculations were generated from red wolf field samples ($n = 200$, unpublished data). Genotypes that received a reliability score of 95% or greater were accepted as having originated from unique, never before documented individuals. Genotypes that had a reliability score of less than 95% were replicated further. One PCR replicate was added to each homozygous result until the reliability score for the genotype reached 95% or until seven homozygous results were obtained (Fig. 1). Once the genotype received a reliability score of 95% it was accepted as unique. If, after seven PCR replicates the reliability score was still less than 95%, the sample was removed from the analysis (Fig. 1). The ARNWR represents only one sixth of the experimental population area, therefore all genotypes that did not match individuals known to inhabit the ARNWR were compared with genotypes from known individuals in the rest of the experimental population area (unpublished data).

MLR method

To assess the accuracy and efficiency of the comparative blood method, we generated scat genotypes using a data filtering method with more stringent acceptance criteria, the MLR method of Miller et al. (2002). The MLR method uses allele frequencies from a population and produces a maximum likelihood estimate of the allelic dropout rate per locus and uses that value to estimate the reliability of a genotype (Miller et al. 2002). If the reliability of the genotype is below an arbitrary threshold, further PCR replications are suggested at the most erroneous loci (Miller et al. 2002). The MLR method assumes equal dropout rates across loci, but is robust to moderate deviations from this assumption (Miller et al. 2002). First, two replicates of data for each scat sample were analyzed and a reliability score and replication strategy were obtained. Scat genotypes that received a reliability score of 95% or greater were accepted as reliable. For scat genotypes that received a reliability score of less than 95%, we followed the suggested replication strategy by performing the necessary number of PCR replicates. The maximum number of amplifications attempted for each locus was arbitrarily chosen as seven. If 95% reliability was not reached or could not be reached after seven replicates, the genotype was dropped from further analysis. Genotypes that received a reliability score of 95% or greater were compared using GIMLET (Valiere 2002), and all non-matching genotypes were considered unique.

We tallied the total number of PCR reactions, the number of scat genotypes obtained, and the number of individuals observed for both methods. The genotyping error rate was calculated per locus by counting the number of allelic dropout events observed at heterozygous loci and the number of false alleles detected at both homozygous and heterozygous loci as in Broquet and Petit (2004). False alleles were detected at heterozygous loci by the amplification of a third allele in one PCR replicate and at homozygous loci by the amplification of a second allele in one PCR replicate. To determine whether there was a difference in dropout rates between alleles, we recorded the number of times the long or short allele dropped out for each locus and then performed a Mann–Whitney *U*-test in SAS 8.0 (SAS Institute). Similar to Frantz et al. (2003), we fitted a generalized linear mixed model using SAS to determine whether there was a difference in dropout rates between loci.

Results

Probability of identity

Expected heterozygosities ranged from 0.58 to 0.72 and the total number of alleles per locus ranged from three to seven (Table 1) for the microsatellite loci used. The observed probability of identity (PID_{OBS}) and the probability of identity for siblings (PID_{SIBS}) for the seven loci chosen for this study were 0.000197 and 0.00725 respectively. We allowed scat with genotypes based on six loci of data to remain in the analysis. The PID_{OBS} ranged from 0.000197 to 0.000591 and the PID_{SIBS} ranged from 0.014 to 0.015 for each of the combinations of six loci.

Amplification success and genotyping errors

Of the 210 scats assigned to red wolf with mitochondrial DNA sequencing (Adams et al. 2003), we obtained genotype data at 105 (50%). Genotyping error rates per locus ranged from 6.6% to 52.1% (Table 2). When a genotyping error occurred, allelic dropout was much more common (97.1%) than false alleles (2.9%) across loci (Table 2). There was no difference in dropout rates between the long and short allele (Mann–Whitney *U*; $N1 = 7$, $N2 = 7$; $Z = 1.0$; $P = 0.949$; Table 2). The generalized linear mixed model indicated there was a

Table 1 The expected (H_e) and observed (H_o) heterozygosities, total number of alleles in wild red wolves (# alleles), the theoretical probability of identity (PID_{THEO}), the observed probability of identity (PID_{OBS}), and probability of identity for siblings (PID_{SIBS}) for the seven microsatellite loci used

Locus name	H_e	H_o	# alleles	PID_{THEO}	PID_{OBS}	PID_{SIBS}
CXX.172	0.58	0.55	3	0.26	0.25	0.53
AHT103	0.61	0.62	4	0.21	0.21	0.50
CXX.20	0.58	0.61	4	0.26	0.28	0.53
C09.173	0.72	0.78	6	0.11	0.11	0.42
FH2054	0.59	0.66	5	0.21	0.23	0.51
CXX.225	0.59	0.65	4	0.23	0.24	0.51
CXX.403	0.63	0.66	7	0.16	0.16	0.48
Average	0.61	0.65	4.7	0.21	0.21	0.50

Table 2 The size (in base pairs), total number of PCR amplifications (# PCR), the number of correct heterozygous amplifications (correct), the number of incorrect homozygous results with the long allele missing (long), the short allele missing (short), the number of false alleles (false) and the genotyping error rate (error rate) for each locus

Locus	Size bp	# PCR	Correct	Long	Short	False	Error rate
225	162–166	190	91	39	59	1	52.1%
403	267–287	144	85	33	22	4	41.0%
103	72–80	121	113	2	5	1	6.6%
20	122–132	138	87	28	21	2	37.0%
2054	145–161	114	102	6	6	0	10.5%
173	97–111	165	146	14	5	0	11.5%
172	142–156	137	116	9	12	0	15.3%
Total		1009	740	131	130	8	26.6%

significant difference in error rates between loci ($P < 0.001$).

Reference genotype method results

Under this data filtering method, we obtained genotypes for 105 scats. Forty-nine scat genotypes matched blood genotypes at all seven loci. Forty-four scat genotypes matched blood genotypes at six loci (only data from six loci were obtained for these genotypes). Twelve scat genotypes matched blood genotypes at six loci with a homozygous mismatch at the seventh locus. Seventeen individuals were detected, with the number of observations per individual ranging from 1 to 21 (Table 3). Fourteen of the 15 individuals known to inhabit the ARNWR were detected. Three scat genotypes did not match any of the known 15 individuals. The total number of PCR amplifications performed for this method was 1,773. The average number of PCR amplifications per locus ranged from 2.1 to 2.9 with an overall average number of amplifications per locus of 2.4 (Table 3).

Table 3 The genotypes and total number of observations of each genotype are listed for the two data filtering methods

Genotypes	Blood comp	MLR
A	10	9
B	6	6
C	14	14
D	2	2
E	5	5
F	8	7
G	12	12
H	21	18
I	4	3
J	2	2
K	1	1
L	4	4
M	2	2
N	11	9
O	1	1
P	1	1
Q	1	1
Total	105	97
Total PCRs	1773	2379
Avg PCR/locus	2.41	3.24

The total number of PCR amplifications necessary to obtain the data and the average number of PCRs per locus (Avg PCR/locus) are provided at the bottom of the table

MLR method results

Genotypes from 97 scats met the 95% acceptance criteria under the MLR method. Seventeen individuals were detected, with the number of observations per individual ranging from 1 to 18 (Table 3). This method identified the same 17 genotypes as the reference genotype method. The total number of PCR amplifications performed for this method was 2,379. The average number of PCR amplifications per locus

ranged from 2.7 to 4.1 with an overall average number of amplifications per locus of 3.2 (Table 3).

Discussion

Amplification success and genotyping errors

The amplification success rate from red wolf scat samples (50%) was similar to success rates from other canids including gray wolves (*Canis lupus*, 53%, 6 loci, Lucchini et al. 2002, 79%, 13 loci, Creel et al. 2003) and coyotes (48%, 3 loci, Kohn et al. 1999). Success rates would likely increase if scats were screened for the probability of amplification success (i.e. only the freshest scats) prior to DNA extraction or if new PCR approaches like preamplification are used (Piggott et al. 2004; Bellemain and Taberlet 2004; Hedmark and Ellegren 2005).

There are several methods of calculating genotyping error rates used in the scat genotyping literature (Broquet and Petit 2004). The method we used to produce our genotyping error rates is the least biased as error rates are calculated across only heterozygous results (Broquet and Petit 2004). Because of the difficulty in making direct comparisons between different methods of calculating genotyping error rates, we restricted our comparisons to studies using the same method. The range of genotyping error rates reported in this study (Table 2) was similar to the range reported by Frantz et al. (2003) for badgers (*Meles meles*, 11–51%), and higher than the range reported by Morin et al. (2001) for chimpanzees (*Pan troglodytes verus*, 12% to 35%) and Prugh et al. (2005) for Alaskan coyotes (1–10%). The higher error rates observed in our study could be explained by a difference in the quality of samples collected. In Prugh et al. (2005), scats were frozen upon defecation due to freezing ambient temperatures while our samples were collected in a humid coastal climate. Morin et al. (2001) collected their samples soon after defecation while our scats were of varying ages. The constant exposure of scat samples to warm, moist conditions in our study area might cause the DNA to degrade at a faster rate than in a cooler, drier climate. In fact, the negative effect of scat age and increased moisture on PCR amplification has been documented in the brush-tailed rock-wallaby (*Petrogale penicillata*) and red fox (*Vulpes vulpes*, Piggott 2004) and gray wolves (Lucchini et al. 2002).

Allelic dropout events are thought to be the result of sampling only one allele from relatively dilute DNA extracts (Taberlet et al. 1996; Gagneux et al. 1997). It has been suggested that the longer allele of a heterozygous individual might be the target for dropout due to the degraded DNA found in scat DNA extracts (Gerloff et al. 1995; Frantzen et al. 1998; Goossens et al. 1998). We found that there was no difference in dropout rates between the longer and shorter allele. The lack of dropout bias may be due to the relatively small size of the alleles at most of the loci used in this study (Table 2). There were fairly large differences in error rates between loci, which violates the MLR method assumption of even dropout rates among loci. Miller et al. (2002) suggests, however, that the MLR method is fairly robust to moderate differences in error rates. Researchers using this approach should evaluate errors rates in a pilot study and choose loci with similar error rates.

Data filtering methods

The ability to match scat genotypes to reference blood genotypes gave us the opportunity to determine if relaxing previously published acceptance criteria would have a significant effect on individual identification. The reference genotype method differed from the MLR method, because we performed fewer replicates at homozygous loci and chose to accept one mismatch due to allelic dropout between scat genotypes and reference blood genotypes. A one locus, one allele mismatch has been allowed in other faecal DNA studies as a method for decreasing labor and supply costs while minimizing the potential to inflate population estimates (Hung et al. 2004; Bellemain et al. 2005).

Our findings suggest that the reference genotype method performed as well as the MLR method. The two methods generated similar numbers of scat genotypes and identified the same 17 individuals. If allowing one mismatch between scat and blood genotypes under the reference genotype method was inaccurate and misclassified new individuals, we would have expected the MLR method to detect these new individuals. This was not the case. In fact, all 12 of the scat genotypes for which we allowed 1 mismatch with a blood genotype, continued to match the blood genotype after further replication under the MLR method (11 with 95% reliability and 1 with 80% reliability).

The filtering methods differed the most in the total number of PCR amplifications needed to generate the genotypes. The reference genotype method required 606 fewer PCR amplifications or 25% less effort than did the MLR method while obtaining results for 8 additional samples (Table 3). Thus, the reference genotype method generated comparable genotype data and required less time and money thereby providing an

alternative when researchers are working in an area where a large percentage of the population has been sampled. The reference genotype method would be useful in reintroduction efforts because a reference genetic sample can be obtained for every individual released. Non-invasive scat genotyping could then be used to track individuals and monitor the growth of the population after the reintroduction occurs. A similar system has been used for the brown bear (*Ursus arctos*) reintroduction in the Italian Alps (De Barba et al. 2005). In addition, this method could be adapted for use in studies without reference genotypes where faecal sampling occurs annually. Baseline genotypes would be established in the first round of sampling using a conservative genotype screening method (Taberlet et al. 1996; Miller et al 2002) and the reference genotype method could then be used in subsequent years. Since the MLR method is used to verify new genotypes under the reference genotype method these new individuals could then be added to the reference database after each sampling year. Thus, the number of reference genotypes would grow as the population increased.

One drawback of the reference genotype method is that efficiency decreases as the number of new genotypes encountered outpaces the number of reference genotypes. This would be the case in a population experiencing rapid growth. However, many non-invasive genetic studies have been applied to either endangered species with small populations (Banks et al. 2002; Hung et al. 2004; Iyengar et al. 2005; Piggott et al. 2006) or populations of large mammals with low reproductive rates (Ernest et al. 2000; Eggert et al. 2003; Flagstad et al. 2004; Goossens et al. 2005).

Individual identification

A total of 17 individuals were identified using scat genotyping. Of the 15 reference individuals known to inhabit the ARNWR, 14 were detected. The one reference individual not detected in the scat sampling by either data filtering method was a breeding female (personal communication, Arthur Beyer, Wildlife Biologist, Red Wolf Recovery Program, USFWS). We believe she may have been in the den whelping during the time of sampling, which would explain why she was not detected. Three individuals who do not reside in the ARNWR were also detected. One individual was a resident of a pack that lives directly south (20 km) of the ARNWR (personal communication, Arthur Beyer). The identification of this individual outside of its home range confirms observations in other studies that wolves make investigative forays into adjacent territories (Messier 1985; Mech 1994). Another individual had been released onto the ARNWR in April 1999 but never established a territory (personal communication, Arthur Beyer). The final individual detected was a resident of the River pack, but died in December 1999 (personal communication, Arthur Beyer). Therefore, we did not expect to sample this individual in April 2000 as scat persistence time had previously been judged to be 21 days (unpublished pilot data). However, our results suggest that in rare cases, scat specimens can last 4–5 months in humid field conditions and still yield workable DNA.

Hybrid detection

As part of a previous study, the scat samples used in this study were analyzed by sequencing the mitochondrial DNA control region in order to detect red wolf/coyote hybrid individuals (Adams et al. 2003). No hybrids were detected during 2000 using this method. However, the drawback of this technique was that F1 hybrids and backcrossed hybrids with red wolf mothers would not be detected. Thus the possibility that hybrids may have been missed could not be ruled out. By optimizing the use of scat genotyping and applying the reference genotype method to this dataset we demonstrated that no hybrid individuals were detected in 2000 on the ARNWR because all scat genotypes matched reference blood genotypes from known red wolves. Optimizing the use of nuclear markers in scat samples, therefore, provides a way to screen large portions of the red wolf experimental population area and identify new, potentially hybrid individuals.

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