Using faecal DNA sampling and GIS to monitor hybridization between red wolves (Canis rufus) and coyotes (Canis latrans)

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Abstract

The US Fish and Wildlife Service’s (USFWS) Red Wolf Recovery Program recognizes hybridization with coyotes as the primary threat to red wolf recovery. Efforts to curb or stop hybridization are hampered in two ways. First, hybrid individuals are difficult to identify based solely on morphology. Second, managers need to effectively search 6000 km² for the presence of coyotes and hybrids. We develop a noninvasive method to screen large geographical areas for coyotes and hybrids with maternal coyote ancestry by combining mitochondrial DNA sequence analysis of faeces (scat) and geographic information system (GIS) technology. This method was implemented on the Alligator River National Wildlife Refuge (1000 km²) in northeastern North Carolina. A total of 956 scats were collected in the spring of 2000 and 2001 and global positioning system (GPS) coordinates were recorded. Seventy-five percent of the scats were assigned to species and five coyote/hybrid scats were detected. Placement of scat location coordinates on a map of the experimental population area revealed that four of the coyote/hybrid scats were detected within the home ranges of sterilized hybrids. The other coyote/hybrid scat indicated the presence of a previously unknown individual. We suggest this method be expanded to include more of the experimental population area and be optimized for use with nuclear markers to improve detection of hybrid and back-crossed individuals.

Keywords: Canis latrans, Canis rufus, faecal DNA, GIS, hybridization, mtDNA

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Introduction

The refinement of noninvasive genetic techniques is providing valuable new approaches for studying populations of threatened or endangered species. The ability to isolate and analyse DNA from hair or faeces has allowed scientists to identify whether a particular species is present in a given area (Foran et al. 1997; Paxinos et al. 1997; Hansen & Jacobsen 1999; Farrell et al. 2000), evaluate the phylogenetic relationships of taxa (Kohn et al. 1995), produce a genetic identity for each individual encountered in a population (Taberlet et al. 1997; Woods et al. 1999; Ernest et al. 2000; Lathuilliere et al. 2001; Ernest et al. 2002; Lucchini et al. 2002), determine paternity and kinship relationships (Gerloff et al. 1995; Launhardt et al. 1998; Constable et al. 2001) and to estimate population sizes (Kohn et al. 1999; Mowat & Strobeck 2000). All these data were gathered without capturing or handling the study species, which is particularly useful if the species is highly endangered, difficult to capture, ranges over a large area or if the loss of an individual to trap injuries cannot be tolerated.

Noninvasive genetic sampling may also be very useful in detecting hybridization within a population. Hybridizing populations are becoming more common as human habitat modification brings previously isolated species into contact (Allendorf et al. 2001). If the hybrid individuals are fertile, continued hybridization could allow the genes of one species to introgress into the other and cause genetic swamping (Allendorf et al. 2001). Detection of hybridization would be especially important in small, reintroduced populations where introgression could threaten the gene
pool. A classic example of an endangered species threatened with introgression is the reintroduced red wolf (*Canis rufus*), which hybridizes with coyotes (*C. latrans*).

Historically, the red wolf ranged across the southeastern United States; however, the ecology of this species was not studied until human persecution and habitat loss had drastically reduced its numbers (Nowak 1979). Therefore, very little is known about the natural history of the red wolf or its previous interactions with other canids (USFWS 1989). For example, it is not clear whether the red wolf interbred historically with the coyote where their ranges overlapped or whether there were biological or behavioural barriers to breeding that broke down as European settlement spread across the southern United States (Nowak 1979). Furthermore, molecular investigations into the origin of the red wolf suggest that it may not represent a distinct species, but instead is a hybrid species formed by interbreeding of grey wolves (*C. lupus*) and coyotes (Wayne & Jenks 1991; Roy *et al.* 1994a; Roy *et al.* 1994b; Roy *et al.* 1996; Reich *et al.* 1999). By the time the red wolf received the attention of the scientific community in the early 1960s, it had hybridized extensively with the coyote and a hybrid swarm was threatening the species with extinction (McCarley 1962).

In 1973 the red wolf received federal protection under the Endangered Species Act, and a recovery plan was developed by the USFWS (Carley 1975). The remaining wild red wolves were captured and placed in a captive breeding programme to protect the red wolf from extinction and provide animals for release back into the wild (USFWS 1989). In 1984, the Alligator River National Wildlife Refuge (ARNWR) in northeastern North Carolina was identified by the USFWS as a potential release site (Parker 1987). This refuge represented a large piece (approximately 1000 km²) of federally controlled land that was believed to be uninhabited by coyotes (Parker 1987). In 1987, the first captive-bred wolves were released onto the refuge (USFWS 1989). Since then, the wild red wolf population has grown to approximately 100 individuals and expanded its range to include 6000 km² in portions of five counties in North Carolina (personal communication, Buddy Fazio, Project Leader, Red Wolf Recovery Program, Fig. 1). This expansion, as well as an eastwardly expanding coyote front (Parker 1995), has brought the red wolf into contact with coyotes resulting in hybridization between the two species (Kelly *et al.* 1999).

The Red Wolf Recovery Program currently recognizes hybridization as the primary threat to the recovery of the species (Kelly *et al.* 1999). As a result, an adaptive management plan was drafted in 2000 with the goal of curbing or stopping hybridization in the experimental population area (Kelly 2000). In trying to attain the goal of minimal coyote introgression, the red wolf programme faces two problems. First, wolf/coyote hybrids are difficult to distinguish based solely upon morphology, therefore managers risk mistaking both hybrids for red wolves which could lead to a higher degree of introgression in the population, and red wolves for hybrids which in turn would decrease the number of red wolves. Second, the size of the experimental population area makes it difficult to pinpoint exactly where hybridization events are occurring as the number of personnel are limited and trapping can have sporadic results. As part of the adaptive management plan, the Red Wolf Recovery Program intends to maintain the ARNWR, approximately 20% of the entire experimental population area, as a coyote- and hybrid-free area (Fig. 1), and build the wild red wolf population westward across three management zones (Kelly 2000). Therefore, it is crucial to screen the ARNWR and other locations effectively for nonred wolf canids. The use of molecular techniques to distinguish between red wolves and hybrids, coupled with noninvasive sampling, would allow the Red Wolf Recovery Program to screen vast areas effectively for the presence of hybrids. We develop a technique that uses genetic analysis of faecal material (scat) to address the question of whether or not nonred wolf canids are present in the ARNWR. We then evaluate whether this technique is able to detect the presence of known sterilized hybrids located south of the ARNWR. Finally, we assess the feasibility of expanding this technique to the rest of the experimental population area.

**Methods**

**Scat collection procedures**

Most of the roads in the ARNWR are surrounded by water canals, which make it difficult to access off-road areas. Therefore, to expedite and simplify the sampling process, scats were collected only on roads. There were three possible ways to survey and collect scats on roads in the ARNWR—walking, using all-terrain vehicles (ATVs) or by truck. A pilot study was performed to test the reliability and efficiency of each method. Two transects, 2 km in length, were searched first by walking, then by truck with different volunteers and a total of 10 of the 16 scats observed in foot-searching were missed using the truck method. Two more transects, 2 km in length, were searched first by walking then by ATV with different volunteers, and three of the 24 scats observed by walking were missed using the ATV method. ATVs were chosen as the most efficient way to survey roads for scat because more scats were found when compared with truck surveys, and they were faster than walking.

Scats were collected using ATVs along all existing roads on the ARNWR in both April and May in 2000 and 2001. Roads were either dirt or gravel and ranged from high traffic use to restricted traffic use. All scats that were potentially canid were collected regardless of age or condition.
When encountered, each scat was placed into a zip-lock bag by turning it inside out and picking up the scat to prevent contamination by the collector or cross-contamination from other samples. UTM coordinates were recorded with a Trimble ProXRS GPS, Trimble Geoexplorer or by Garmin hand-held GPS units. Scats were transferred within 12 h to a 95% EtOH solution in a 1:4 volume scat to EtOH ratio, to preserve DNA (Murphy et al. 2002).

In 2000, scat collection was divided into three sampling periods to allow for a mark–recapture estimate of population size as part of a separate research project. Persistence time of scats was evaluated by placing scats on roads with varying levels of vehicle use and recording the time to disappearance. The results suggested that the average number of days between sampling periods should be 19, which allowed time for more scats to be deposited onto the roads but also ensured that scats did not disappear due to degradation (unpublished data). The first sampling period was a comprehensive sample of all the refuge roads. The second and third sampling periods were carried out on a subset of 22 roads designated as transects. Data for the scat survey were pooled across all three sampling periods.

In 2001, the mark–recapture method of sampling scats was not performed and each road on the ARNWR was sampled once. Scats also were collected along a few roads south of the ARNWR (Fig. 1) within the home ranges of sterilized red wolf/coyote hybrids.

**DNA extraction**

Whole blood samples representing the four red wolf matrilines from the red wolf captive breeding programme ($n = 8$) and a Kentucky coyote ($n = 1$) were extracted using a phenol/chloroform protocol (Vardenplas et al. 1984).
Samples of whole blood stored in lysis buffer (Longmire et al. 1991) from California coyotes \((n = 7)\) and a random sample of known red wolf hybrids \((n = 40)\), captured in the experimental population area and identified by multilocus microsatellite genotype analysis (Paul Wilson, unpublished data) were extracted using a QIAamp™ blood protocol in the Qiagen Tissue Kit (Qiagen, Valencia, CA, USA). Seventeen of the known red wolf hybrids had been captured and removed from the population. The other 23 known hybrids were captured, sterilized and then released back into the experimental population area to serve as territory holders. Tissue samples stored in lysis buffer (Longmire et al. 1991) from a Nebraska coyote \((n = 1)\), North Carolina coyotes \((n = 24)\), Texas coyotes \((n = 7)\) and Virginia coyotes \((n = 26)\) were extracted using a QIAamp™ tissue kit (Qiagen). For 2000, collected scats were extracted using a modified QIAmp™ tissue protocol (Murphy et al. 2000). For 2001, collected scats were extracted using the QIAamp™ stool protocol (Qiagen). In order to reduce contamination risk, all scat extractions were performed in a separate laboratory for low-concentration degraded DNA which contains no concentrated DNA [polymerase chain reaction (PCR) product or blood and tissue extracts] and is separated physically from other laboratories. One or more negative controls were included in each extraction to monitor for contamination.

**Restriction enzyme analysis**

In order to reduce sequencing costs, the scats collected in 2000 were analysed first using restriction enzyme analysis to remove all nonred wolf, hybrid or coyote scats from further analysis. We modified the restriction enzyme system of Paxinos et al. (1997) by designing primers that amplified 200 base pairs (bp) of the cytochrome b region of mitochondrial DNA and retained most diagnostic cut sites. Each scat sample was amplified by PCR in PTC-100 (MJ Research, Inc.) machines in 36 cycles (95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, with a final extension of 72 °C for 2 min) after an initial denaturation step of 95 °C for 2 min using primers L16345 and H16751 from Ward et al. (1991). The PCR mix consisted of 20 pmol of each primer, 0.2 mM dNTPs, 1x buffer, 2.5 mM MgCl₂, and 0.5 µM AmpliTaq (Applied Biosystems) in 15 µL with 150 ng of DNA extract. Each PCR reaction was run with a negative control to monitor for contamination.

For the scat samples, a 200-bp fragment of the mitochondrial DNA control region was amplified by PCR in PTC-100 (MJ Research, Inc.) machines in 40 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, with a final extension of 72 °C for 2 min) after an initial denaturation step of 95 °C for 2 min using newly designed primers (ScatSeqF: 5′-CCATGCATATAAGGCATGTACAT-3′, ScatSeqR: 5′-AGATGC-CAGGTATAGTTCAA-3′). The PCR mix was the same as the mix for the restriction enzyme analysis, except that it was in a 15-µL reaction volume with 1.5 µL of DNA extract. Each PCR was run with an average of five negative controls to monitor for contamination and five positive controls to determine whether the PCR reaction worked.

All samples were sequenced using the Big Dye kit version 2.0 (Perkin Elmer) with the upstream primer and run on an ABI 377 automated sequencer following the manufacturer’s protocols. Sequences containing unresolved bases were then sequenced with the downstream primer.

Sequences were analysed using Sequencher 3.0 (Gene Codes Corporation, Inc.). Sequences from blood and tissue samples were aligned by eye in PAUP 4.0b10 (Swoford 1998) and then imported into MacClade 3.07 (Sinauer Associates) to determine the number of unique haplotypes. Unique haplotypes were then compared to previously published coyote, grey wolf and dog haplotypes (Vila et al. 1997; Pilgrim et al. 1998; Vila et al. 1999). In order to compare the phylogenetic relationship of generated haplotypes with previously published haplotypes we first used the program MODELLTEST 3.06 (Posada & Crandall 1998) to determine the model of DNA substitution that best fit the phylogenetic data. The program selected the DNA sequencing

For the blood and tissue samples a 360-bp fragment of the mitochondrial DNA control region was amplified by PCR in PTC-100 (MJ Research, Inc.) machines in 40 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, with a final extension of 72 °C for 2 min) after an initial denaturation step of 95 °C for 2 min using primers L16345 and H16751 from Ward et al. (1991). The PCR mix consisted of 20 pmol of each primer, 0.2 mM dNTPs, 1x buffer, 2.5 mM MgCl₂, and 0.5 µM AmpliTaq (Applied Biosystems) in 15 µL with 150 ng of DNA extract. Each PCR reaction was run with a negative control to monitor for contamination.

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HKY + I + G model of substitution (Hasegawa et al. 1985) with the proportion of invariable sites (I) = 0.5916 and a gamma shape parameter (G) = 0.6463. The transition/transversion ratio (ti/tv) was 14.53 using this model. To determine the phylogenetic placement of the new coyote haplotypes a neighbour-joining tree was constructed in PAUP 4.0b10 (Swofford 1998) under the HKY + I + G model of evolution using the parameters given above, and using a previously published Ethiopian wolf sequence (Vila et al. 1999) as an outgroup. The neighbour-joining tree was constructed using a total of 340 bases from the previously published haplotypes and from the 28 new haplotypes. Bootstrap analysis was performed using 1000 replicates.

Scats were compared first to the generated red wolf haplotype in Sequencher 3.0 (Gene Codes Corporation, Inc). Non-red wolf sequences were then compared to other known sequences using a blast search on the NCBI website (www.ncbi.nlm.nih.gov). Scats were assigned to species based upon the sequence found to have the closest match, with the minimum requirement of at least 85% of the base pairs matching. Because most of the nonred wolf or coyote sequences were poor in quality, we wanted to reduce the possibility of sequences matching by chance and yet still find the closest match. We therefore set the threshold to 85% of base pairs matching.

GIS analysis

The boundary of the ARNWR and surrounding experimental population area was created with data obtained through the National Wetlands Inventory (www.nwi.fws.gov) and through USGS topographic maps (1:24000) obtained from the GIS Data Depot (www.spatialnews.com) using Arcview 3.2 (ESRI). Arcview shapefiles containing all the roads in the experimental population area were obtained from the North Carolina Department of Transportation website (www.ncdot.org/planning/statewide/gis/) and then projected onto the boundary layer. Finally, UTM coordinates collected for each scat were projected onto the previous two layers. Coordinates were not differentially corrected because it was not necessary to show the exact location of each scat.

Results

Sequencing results

Sequencing 340 bp of the mitochondrial DNA control region of the four red wolf matrilines and 66 coyote samples yielded a total of 28 unique haplotypes. There were 60 variable sites: 12 indels (insertions or deletions), 46 transitions and two transversions. The four red wolf matrilines had identical control region sequences; therefore, assuming no mutations, all descendants of the founding captive stock of 14 red wolves should have the same haplotype for this 340 bp segment of the control region. When compared to the haplotypes generated from coyotes, the red wolf haplotype was unique. The 27 coyote haplotypes (Cla1–Cla27) differed from the red wolf haplotype (Cru) by 4–34 substitutions with an average divergence of 3.24%. The 27 coyote haplotypes differed from one another by 1–38 substitutions with an average divergence of 2.79%.

When the 28 haplotypes were compared to previously published coyote (n = 14), grey wolf (n = 34) and dog (n = 24) haplotypes (Vila et al. 1997; Pilgrim et al. 1998; Vila et al. 1999), 26 haplotypes were unique and two matched published data. Haplotype Cla15 matched la12 (Vila et al. 1999), a haplotype found in Florida coyotes, and haplotype Cla24 matched la6 (Vila et al. 1999), a haplotype found in Michigan coyotes. Furthermore, phylogenetic analysis showed that two of the haplotypes found in coyotes clustered with grey wolves and dogs with 95% bootstrap support. One of these haplotypes, Cla17 (la24, from Adams et al. 2003) found in coyotes from North Carolina and Virginia (n = 7), clustered with clade I dogs from Vila et al. (1997). The other haplotype, Cla12, found in one coyote from Texas, was related most closely to haplotype lu32 (Vila et al. 1999). Coyote haplotypes formed three clades, with 56% bootstrap support for clade II and 73% bootstrap for clade III (Fig. 2). The red wolf haplotype falls in coyote clade I (Fig. 2), but bootstrap support is very low (less than 5%).

Sequencing of the 40 known red wolf hybrids produced eight different haplotypes. One haplotype (n = 14) was identical to the red wolf haplotype. The other seven haplotypes differed from the red wolf sequence by five to eight substitutions with an average divergence of 2.07%. Two nonred wolf haplotypes were unique; the other nonred wolf haplotypes matched coyote haplotypes Cla15 (n = 6), Cla23 (n = 10), Cla24 (n = 5) and Cla26 (n = 1), all from coyotes from North Carolina and Virginia, and la9 (n = 2) found in a coyote from Louisiana. All hybrid haplotypes fall into coyote Clade I (Fig. 2).

Restriction enzyme results

Restriction enzyme analysis using the new ScatID primers resulted in unique restriction fragment patterns for dogs, bobcats, red foxes and grey foxes, and identical cut patterns for red wolf and coyote samples. Dogs and red foxes appear to have indistinguishable restriction fragment patterns on a 3% agarose gel because of low band resolution. These species are not the focus of our study and no further analyses were performed to distinguish between the two. The black bear samples amplified poorly with the ScatID primers and so no restriction fragment

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patterns were identified. Analysis of the 20 blind samples revealed correct identification of seven red wolves or coyotes, three grey foxes, five bobcats and five dogs or red foxes.

Scat results 2000

During the 2000 field season 404 scats were collected (Fig. 1). Faecal DNA amplification success rates were high using
both the ScatID (83%) and ScatSeq (89%) primers and of the 335 amplified scats, 306 were identified to species using the restriction enzyme analysis (Table 1). Restriction enzyme analysis identified 237 red wolf or coyote scats, 65 bobcat scats and four dog scats. The 237 scats identified as possible red wolves or coyotes and the 29 scats that could not be assigned to species through the restriction enzyme analysis were then amplified using the ScatSeq primers. Of the 266 amplified scats, 221 were assigned to species after DNA sequencing; 210 red wolves, eight prey species (Leporidae), two black bears (Ursus americanus) and one dog (Table 1, Fig. 3a). The black bear and dog results were from the 29 scats that could not be assigned to species using restriction enzyme analysis. No scats were identified as originating from a coyote or hybrid.

**Scat results 2001**

During the 2001 field season 552 scats were collected (Fig. 1), no restriction enzyme analysis was performed; instead, all samples were sequenced to minimize cost and effort (see Methods). Amplification success rates with the ScatSeq primers were 84%. Of the 463 amplified scats, 430 were assigned to species after DNA sequencing; 399 red wolves, eight prey species (Leporidae), two black bears (Ursus americanus) and one dog (Table 1, Fig. 3a). The black bear and dog results were from the 29 scats that could not be assigned to species using restriction enzyme analysis. No scats were identified as originating from a coyote or hybrid.

**Discussion**

A large number of haplotypes was observed in our sample of coyotes compared to a previous study, which found 32 coyote haplotypes in 327 coyote samples using restriction enzyme analysis of the mitochondrial DNA molecule (Lehman & Wayne 1991). One haplotype, Cla17, was related closely to dog haplotypes and may represent a past hybridization event between dogs and coyotes (Adams et al. 2003). Another haplotype (Cla12, sampled in Texas) is related closely to grey wolf haplotypes. This sample was provided by the USFWS forensic laboratory in Ashland, Oregon, and no morphological differences were reported. We hypothesize that this sample represents an animal that either escaped from captivity or was a grey wolf hybrid pet, rather than a representative of a grey wolf or Mexican grey wolf population in Texas. We cannot exclude the possibility that this haplotype represents a past hybridization event, because we sampled only seven coyotes from Texas and the haplotype could be present in other Texas coyotes.

By sampling coyote populations near the red wolf experimental population area we were able to demonstrate that none of the coyote haplotypes found in the southeast (n = 14) or in published datasets (n = 12) matched the red wolf haplotype. Mitochondrial DNA sequencing of scats provides a useful method for differentiating between red wolves and coyotes or hybrids with maternal coyote ancestors. However, this method is unable to detect hybrids or back-crosses with maternal red wolf ancestry and paternal coyote ancestry. DNA sequence analysis of 40 known red wolf–coyote hybrids indicated that our current genetic test would identify all coyotes, but potentially miss 35% of the hybrids.

Detecting all hybrids would require a genetic test that uses nuclear DNA analysis. This approach was considered but abandoned for multiple reasons. First, there are no
known nuclear DNA markers that definitively differentiate red wolves and coyotes. Thus, a nuclear DNA test for hybrid individuals would need to be based on differences in allele frequencies and an assignment test-based approach (Paetkau et al. 1995; Cornuet et al. 1999; Pritchard et al. 2000). Second, we needed to develop a test quickly, and creating an allele frequency database for a multilocus assignment-based approach would require many months of work. Third, nuclear DNA amplification success rates from faecal material will be lower than mitochondrial DNA amplification success rates as seen in previous studies on scats in grey wolves (53%, six loci, Lucchini et al. 2002), mountain lions (63%, 12 loci, Ernest et al. 2002), coyotes (48%, three loci, Kohn et al. 1999), seals (19.1%, five loci, Reed et al. 1997) and bears (20%, six loci, Taberlet et al. 1997; 89%, one locus, Murphy et al. 2000). Finally, nuclear DNA analysis of faecal samples at multiple microsatellite loci can take considerable amounts of time and money when PCR analysis is repeated multiple times to avoid genotyping errors (Taberlet et al. 1999). When weighing these considerations, mitochondrial DNA analysis provided the best alternative for the current research and management needs.

Restriction enzyme analysis of mitochondrial DNA PCR products was used in 2000 to separate red wolf and coyote scats from all other carnivore scats in an effort to minimize the time and cost of sequence analysis. However, only a small proportion (17%) of samples was obtained from other carnivores, and the restriction enzyme analysis added a day to the overall analysis process. The amount of time and money saved by dropping the restriction enzyme analysis outweighed the cost of sequencing extra scats and the restriction enzyme analysis was omitted in 2001.

In 2000, eight samples that were identified as red wolf/coyote with the restriction enzyme analysis appeared to be Leporidae when sequenced with the ScatSeq primers. All collected scats were carnivore in origin (based upon their size and shape) therefore the leporid results are probably instances where the ScatSeq primers amplified prey DNA. Although the ScatSeq primers were designed from the red

Fig. 3 (a) Locations of scats assigned to red wolf and bobcat for 2000. Approximate pack locations are shown. Pack polygons are not a representation of home range size, but are an indication of where each pack resides. (b) Locations of scats assigned to hybrid/coyote, red wolf and bobcat for 2001. Numbers represent locations of different hybrid/coyote mitochondrial haplotypes.
wolf control region haplotype, they appear to amplify other species as well (bobcat, red fox, otter and Mustelidae) as shown by the 2001 sequencing results. The scats identified as black bear and dog through sequencing were scats that could not be assigned to species with the restriction enzyme analysis. Therefore, these scats probably originated from a black bear and a dog, and do not represent instances of red wolves or coyotes preying upon these species.

The above results highlight some concerns when amplifying DNA from scat. Scat samples potentially contain DNA from not only the target individual, but also DNA from prey items, conspecifics (cannibalism or grooming) or bacteria. In most cases, amplifying prey or bacterial DNA can be avoided by using species-specific primers. When amplifying mitochondrial DNA, however, several regions are conserved across species which would result potentially in amplification of prey DNA. To circumvent this, some description of scat morphology would be needed to verify that the scat was not from the prey species. When the presence of conspecific DNA in scats is a concern, nuclear DNA analysis provides the best way to identify this problem. Conspecific DNA is not a concern for the present study because in cases where conspecific aggression has led to death, the carcass has very rarely been consumed (personal communication, Arthur Beyer, Biologist, Red Wolf Recovery Program).

The 83–89% mitochondrial DNA amplification success rates in this study were comparable to success rates from other studies amplifying mitochondrial DNA from scat: 70% in seals (Reed et al. 1997), 79% in coyotes (Kohn et al. 1999), 80–85% in brown bears (Murphy et al. 2000), 59% in felids (Farrell et al. 2000) and 84% in grey wolves (Lucchini et al. 2002). In the brown bear, felid and grey wolf studies listed above scats were collected either from captive animals or were selected for analysis based upon their freshness and likelihood to produce data. Therefore, our success rates using scats of varying age and condition were slightly higher than those in similar studies (Reed et al. 1997; Kohn et al. 1999) and equal to success rates generated from scats collected under ideal conditions. Success rates using the ScatSeq primers fell slightly (5%) from 2000 to 2001 (Table 1). This is due probably to the fact that in 2000 most noncanid scats were filtered out using the restriction enzyme analysis and in 2001 the restriction enzyme analysis was not used. The ScatSeq primers are designed to amplify canid DNA and therefore perform less well on other carnivore scats.

Based upon our results for the 2000 scat sampling, it is unlikely that there were any resident coyotes in the ARNWR. However, we cannot exclude the possibility that we were unable to detect at least one hybrid (based upon the fact that we are likely to miss 35% of the hybrids) with red wolf mitochondrial DNA. In 2001, we detected four coyote/hybrid scats just south of the ARNWR (Fig. 3b). Three of those scats exhibit identical mitochondrial haplotypes and are clustered in one area (Fig. 3b, no. 1). The other scat had a different haplotype and was located further west (Fig. 3b, no. 2). Based upon field data, we identified a sterilized hybrid that was residing in the area where three coyote/hybrid scats were detected (Fig. 3b, no. 1) and a different sterilized hybrid was residing in the area to the west where the other scat was detected (Fig. 3b, no. 2). Sequence analysis revealed that the two haplotypes found in the scats matched the haplotypes found in these sterilized hybrids. We hypothesize that these scats were obtained from these two sterilized hybrids, but nuclear DNA microsatellite analysis will be necessary to confirm this. Haplotypes from the other 21 sterilized hybrids were not detected, most probably because scat samples were not collected in their core use areas.

The one coyote/hybrid scat found in the northern portion of the ARNWR in 2001 is of concern because it represents an unknown individual in the area of an established red wolf pack in the Milltail area (Fig. 3b, no. 3). Additional analyses have demonstrated that the result is not due to contamination or sample mix-up, and the haplotype does not match that of any of the 23 sterilized hybrids in the experimental population area. We suggest that this unknown individual was passing through the area and was not a resident that would be likely to breed for the following reasons. First, we did not detect more than one coyote/hybrid scat in the area. Second, the scat was relatively fresh when collected in March, and the breeding season is in January and February; therefore it is unlikely that the individual mated with the breeders of the Milltail pack. In addition, the red wolf field crew did not detect a second den or litter of pups in the Milltail area (personal communication, Arthur Beyer) in 2001 and so it is unlikely that the unknown individual was a pregnant female. We acknowledge the possibility that the individual could be a resident of adjacent roadless, therefore nonsampled areas; however, multiple howling surveys (Harrington & Mech 1982; Fuller & Sampson 1988), using sirens by both boat and land, have not detected any canids in that area (personal communication, Buddy Fazio).

The use of scats deposited on roads offered a very effective way to sample the canid population noninvasively in the red wolf experimental population area. Large canids use roads as travel corridors (Mech 1970), are more likely to defecate when travelling (Wells & Bekoff 1981) and use faeces to mark their territories (Vila et al. 1994). Grey wolves have a tendency to defecate at the intersections of trails (Peters & Mech 1975) and in this study, many red wolf scats were collected at the intersections of roads (Fig. 3). Finally, scats provide a good source of genetic material because defecation rates in canids are independent of social status and scats are found throughout a territory,
not just on the periphery (Gese & Ruff 1997). Therefore, scat sampling throughout territories is likely to pick up most individuals in a pack, not just the dominant ones. One potential limitation of faecal sampling on roads in the ARNWR is that coyote and hybrid scats would not be detected if coyotes and hybrids defecated off the roads. Because we were able to detect the presence of coyote/hybrid scats on the roads in areas where known sterilized hybrids occur (Fig. 3b, no. 1 and no. 2), it is unlikely that coyotes and hybrids avoid defecating on roads. Another potential limitation of faecal sampling on roads in the ARNWR is the lack of roads in some areas. However, as mentioned above, howling surveys have been unable to detect any canids in these roadless, swampy areas (personal communication, Buddy Fazio).

The locations of scats correlate well with areas used by established packs on the ARNWR. Four of the 15 red wolf packs in the experimental population area reside on the ARNWR, the River pack, the Milltail pack, the Gator/Airforce pack and the Pamlico pack (Fig. 3). The River pack animals were released after the 2000 scat sampling period and a subsequent change in abundance of red wolf scats can be observed in the area between 2000 and 2001 (Fig. 3). With the exception of the Pamlico pack, which has few roads in its territory, scats were found on most roads within the areas where each pack resides. Thus, faecal DNA analysis has potential as a noninvasive method to delineate pack territories and identify spatial and habitat use patterns of red wolves and other canids.

Conclusions

The combination of genetic methods with GIS technology provides a better understanding of the spatial distributions of red wolves and coyote/hybrids in the ARNWR. Our results suggest that this method was able to detect the presence of both known sterilized hybrids in the area sampled and identify an unknown coyote/hybrid in the ARNWR. This method documents the presence of several different carnivore species in the ARNWR and provides information on their spatial use patterns. This type of sampling and analysis could be adapted easily for other systems, and provides a timely and cost-effective way to monitor multiple species.

Noninvasive genetic sampling of faecal material has provided an important tool in curbing hybridization in the wild red wolf population and will aid in the recovery of this species. This approach will allow managers to screen a large section of the experimental population area and identify the presence of all coyotes and approximately 65% of the hybrids in a timely and cost-effective manner. This approach will also allow managers to identify quickly the species of faecal samples detected in places outside the red wolf core use areas. In the future, we recommend one extensive scat sampling per year during the breeding season to detect hybrids with potential breeding opportunities, and expanding scat sampling to include more of the red wolf experimental population area. Finally, we recommend evaluating the feasibility of using nuclear DNA analysis to increase the proportion of hybrids that can be detected using this method and to improve our understanding of individual movement patterns.

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References


Jennifer Adams is a PhD candidate studying hybridization between red wolves and coyotes in the southeast United States and this study was part of her Master’s thesis. Brian Kelly was the Coordinator of Field Projects for the US Fish and Wildlife Service’s Red Wolf Recovery Program when this research began and is currently the Service’s Mexican Wolf Recovery Coordinator. Lisette Waits is an associate professor who uses genetic tools to study evolution and conservation of large carnivores.