Differentiating Mexican gray wolf and coyote scats using DNA analysis

Janet E. Reed, Robert J. Baker, Warren B. Ballard, and Brian T. Kelly

Abstract Mexican gray wolves (*Canis lupus baileyi*) are the smallest subspecies of North American gray wolves (*Canis lupus*), and identification of Mexican wolf scats could be confused with those of sympatric coyotes (*Canis latrans*). We used DNA analysis (molecular scatology) to differentiate scats (n=203) of free-ranging Mexican gray wolves and coyotes and compared the results to traditional field methods (i.e., diameter, location, and sign) and odor used for identifying scats of the 2 species. We then used the scats whose species identifications were confirmed with DNA analysis to evaluate discriminant analysis for classifying scats using 3 measurements—diameter, mass, and length. Forty-nine (24%) of the field-collected scats (n=203) tested provided amplifiable DNA and were determined to comprise 28 scats deposited by Mexican wolves and 21 deposited by coyotes. Scats identified with DNA analysis to the 2 species had a 79% diameter overlap (Mexican wolf 16.3–35.8 mm; coyote 17.4–27.8 mm), and scats ≥28 mm in diameter were Mexican wolf scats. There was a significant difference (t=–2.28; P<0.05) between diameter means for the 2 species (Mexican wolf $\bar{x}$=26.0 mm; coyote $\bar{x}$=22.8 mm). Of 45 scats that would have been field-identified as deposited by Mexican wolves based on location and odor criteria, DNA analysis indicated that 19 (42%) were deposited by coyotes; of 41 scats that would have been field-identified as deposited by coyotes based on diameter <30 mm criterion, 20 (49%) were deposited by Mexican wolves. Halfpenny's (1986) suggested diameter criterion for field identification of scats identified 3 of the scats as gray (*Urocyon cinereoargenteus*) or red fox (*Vulpes vulpes*; 0% correct), 24 as coyote (62% correct), and 20 as Mexican wolf (75% correct). Discriminant analysis indicated that diameter and mass of scats offered the best results for accurately classifying coyote scats (86%) but provided relatively low accuracy for classifying Mexican wolf scats (65%). Our results suggest that previous diet studies using traditional identification methods may have misrepresented the diets of both the North American gray wolf and the coyote when the 2 species were sympatric. Molecular scatology appears to be a more definitive scat-identification technique than traditional field methods or odor for these canids.

Key words *Canis latrans*, *Canis lupus baileyi*, coyote, DNA, Mexican wolf, scats

Fecal material often is the most common sign and easily collected source of information for rare, secretive carnivores (Putman 1984), and scat analysis appears to provide the best noninvasive sampling method for determining diets of free-ranging carnivore species. However, which species actually deposited a scat must be accurately determined for the method to be valid. Traditional scat-identification criteria have been based primarily on morphology (Halfpenny 1986, Foran et al. 1997), which can be subjective and confounded by sympatric species that are comparably sized and share similar diets (Weaver and Fritts 1979, Green and Flinders 1981, Danner and Dodd 1982, Foran et al. 1997).

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Scott (1941, 1943) reported that fecal passage sizes varied approximately in proportion to the amount of food consumed. Weaver and Fritts (1979) reported that scat diameter might be influenced by diet composition and suggested that canid scats could not be identified to species based on diameter alone. Halfpenny (1986) reported that visual identification of scat to species by experienced naturalists had error rates approaching 50–66%. Furthermore, previous dietary analysis based on scat identification using diameter alone may have biased results in favor of prey items that produce larger scat diameters (Danner and Dodd 1982).

Halfpenny (1986) reported that scat diameter and length values did not provide positive identification of a species because both values were too variable to be used as adequate diagnostic criteria. Green and Flinders (1981) reported that the dry mass of scats varied considerably for coyotes (*Canis latrans*) and red foxes (*Urocyon cinereoargenteus)* and suggested that diameter used in conjunction with mass could be used for identifying scats of the 2 species.

No scientific studies were conducted on the Mexican gray wolf (*Canis lupus baileyi*) before it was extirpated from the United States by the late 1960s; therefore, little is known of the subspecies' natural history (Brown 1983). Previous studies indicated the Mexican wolf is the smallest (Hall and Kelson 1959, Bogan and Mehlhop 1983), the most genetically distinct (Wayne et al. 1992, Nowak 1995, Garcia-Moreno et al. 1996), and the most endangered subspecies of North American gray wolf (*C. lupus*) (McBride 1980, Brown 1983, Bednarz 1988, Ginsberg and Macdonald 1990). In April 1998 the United States Fish and Wildlife Service (USFWS) began releasing captive Mexican wolves into the Blue Range Wolf Recovery Area (BRWRA) in Arizona and New Mexico to restore the subspecies into a portion of its historical range.

In June 2000 we began a study to determine diets of captive-released and wild-born Mexican gray wolves in the southwestern United States. From April 1998 through October 2001, we collected carnivore scats (n = 1,682) from the BRWRA on the Apache and Gila National Forests and Wilderness areas of Arizona and New Mexico and identified them to species using traditional field methods (i.e., diameter, location, and sign) and odor. Since Mexican wolves are the smallest subspecies of the North American gray wolf, we hypothesized that traditional scat-identification techniques and odor might have a high error rate in separating Mexican wolf and coyote scats collected where the 2 species were sympatric.

There are no published reports using fecal DNA analysis (i.e., molecular scatology) to differentiate between wolf and coyote scats. The purpose of this paper was to validate methods used to accurately differentiate Mexican wolf and coyote scats. Accurate identification of scats would be beneficial for determining the level of dietary overlap of wolves and coyotes in areas where they are sympatric, managing wolf and prey populations, and investigating livestock depredation incidents in areas where wolves have been reintroduced or are recovering.

Study area

We conducted research in the BRWRA, a 17,820-km^2 area that included the Apache National Forest (NF) in east-central Arizona and the Gila National Forest (NF) and Wilderness area in west-central New Mexico. Our focus was the 2,600-km^2 primary recovery zone where captive Mexican wolves were released and wild-born since March 1998. The Gila Wilderness within the Gila NF of New Mexico was included beginning in spring 2000 after translocations of Mexican wolves to this area from those previously released in Arizona. The BRWRA was bordered on the west by the White Mountain (or Fort Apache) and San Carlos Indian Reservations, while private lands were scattered within and bordered the east, north, and south. Almost all areas were grazed by domestic livestock.

Elevations within the study area ranged from <1,220 m along the San Francisco River to 3,350 m on Mount Baldy, Escudilla Mountain, and the Mogollon Mountains (United States Fish and Wildlife Service 1996). Rolling hills with moderately steep-walled canyons and sandy washes characterized lower elevations, while rugged slopes, deep canyons, elevated mesas, and rock cliffs typified the higher elevations. Major vegetation included ponderosa pine (*Pinus ponderosa*), aspen (*Populus tremuloides*), fir (*Abies* spp.), juniper (*Juniperus* spp.), piñon (*Pinus cembroides*), mesquite (*Prosopis* spp.), evergreen oaks (*Quercus* spp.), and a variety of grasses and forbs. Annual temperatures reported for the BRWRA averaged 16.4°C maximum and –3.1°C minimum. Annual rainfall averaged 52.1 cm, and annual snowfall averaged 139.3 cm (Western Region Climate Center, Alpine Station, Alpine, Ariz., unpublished data).

From April 1998 through October 2001, ≥87 Mexican wolves were either released from captivity or born in the wild within the BRWRA. As of October 2001, ≥37 Mexican wolves resided in the BRWRA and 31 of those were fitted with radiocollars (B. T. Kelly, personal communication). Other predators resident in the BRWRA included coyote, red fox (*Vulpes vulpes)*, gray fox, bobcat (*Lynx rufus*), mountain lion (*Puma concolor*), and black bear (*Ursus americanus*) (United States Fish and Wildlife Service 1993, Arizona Game and Fish Department 1994). Density estimates for these predators were unavailable, but densities were thought to be high (United States Forest Service and Arizona Game and Fish Department, unpublished data). These sources also reported that neither feral nor stray dogs were common residents within the BRWRA; therefore, we assumed a low likelihood of collecting dog scats.

Methods

Sample collection

Carnivore scats (n=1,682) were collected opportunistically from the BRWRA by the Interagency Field Team (IFT; United States Fish and Wildlife Service, Arizona Game and Fish Department, New Mexico Department of Game and Fish, USDA APHIS Wildlife Services, United States Forest Service, and White Mountain Apache Tribe) from April 1998–October 2001. We actively collected scats from June–August 2000 and March–October 2001 in areas known to be frequented by captive-released, translocated, and wild-born Mexican wolves. The sampling strategy (Frenzel 1974) consisted of following Mexican wolves as they moved within the study area, as reported by the USFWS. This included driving forest roads; hiking or horseback riding forest trails, ridgelines, and riparian areas; and searching campsites, opened release pens, den sites, and kill or carcass sites.

We collected scats using disposable rubber or food-preparation gloves and placed them in brown paper bags labeled with date and location. We allowed the scats to air-dry in the brown paper bags before storing them in large plastic containers at room temperature until analyzed.

We aged scats at time of collection as old, recent, or fresh according to appearance, exposure of deposition site, and weather conditions (Ciucci et al. 1996). We identified scats to species using traditional scat-identification techniques (i.e., location, diameter, and sign) and odor as described below.

We measured the maximum diameter of each dried scat as described by Scott (1943), Weaver and Fritts (1979), Green and Flinders (1981), and Danner and Dodd (1982); however, we took 2 measurements at the maximum diameter to the nearest 0.1 mm with a 152-mm dial caliper and used the average for diameter size. Minimum diameter for identifying northern gray wolf scats in the field was established as ≥24 mm by Thompson (1952) and has been the accepted criterion for several studies (Mech 1970, Stephenson and Johnson 1972, Peterson 1974, Van Ballenberghe et al. 1975). However, Weaver and Fritts (1979) suggested that a ≥30-mm minimum diameter be used for identifying wolf scats, and this was used by Arjo et al. (2002). Halfpenny (1986) reviewed 3 studies and suggested ≥25 mm diameter for identifying wolf scats. We chose the more conservative ≥30-mm-diameter criterion for field-identifying Mexican wolf scats. Sign criteria included tracks and visual observations of Mexican wolves. Mexican wolf recovery field team personnel provided instruction for identifying Mexican wolf scats by odor. We were given scats identified as Mexican wolf scats by wolf biologists and were instructed that the odor (i.e., sweet, musky) of those scats was that of wolf. No odor description was available for identifying coyote scats.

Genetic identification

We used fecal DNA analysis to identify scats deposited by either Mexican wolves or coyotes. Our design was to isolate and analyze Mexican wolf and coyote DNA from shed epithelial cells from the intestinal lining found on scats (Kohn et al. 1995, Foran et al. 1997, Kohn and Wayne 1997, Reed et al. 1997, Frantzen et al. 1998). We examined a portion of the mitochondrial DNA (mtDNA) control region (D-loop) to differentiate Mexican wolf scats from coyote scats collected from the field (Pilgrim et al. 1998).

We took 2–6 subsamples from each scat (n=203) for DNA analysis by scraping the surface to decrease the possibility of removing undigested prey parts from the scat and to increase the probability of obtaining sloughed epithelial cells (Reed et al. 1997). Each of the subsamples, comprising ≤1 g of fecal material, was placed in a 1.5-mL micro centrifuge tube and stored at room temperature (Taberlet et al. 1997). We used the remainder of each scat for diet analysis. To optimize laboratory

efforts, we did not randomly subsample scats but primarily selected them from scats field-identified as deposited by Mexican wolves (n = 169). We sorted the master database for scats identified as probably deposited by Mexican wolves (n = 1,111) based on traditional field methods, then selected every fifth scat to be subsampled for DNA analysis.

DNA isolation

Although fecal DNA often is degraded and scarce (Gerloff et al. 1995, Kohn et al. 1995), mtDNA is likely to be present in greater quantity than single-copy nuclear DNA (Reed et al. 1997, Woods et al. 1999). Polymerase chain reaction (PCR) amplification of short mtDNA fragments results in more consistent amplifications than longer fragments (Kohn et al. 1995). We sought to identify and establish a simple, rapid, and reliable protocol that extracted as much DNA as possible from degraded samples and removed any potential PCR inhibitors (Boom et al. 1990, Deuter et al. 1995, Kohn et al. 1995, Reed et al. 1997). We used the highest extraction method (GuSCN and silica method) as reported by Höss and Pääbo (1993) and Reed et al. (1997).

We generated a reference collection for genetically identifying target species (Foran et al. 1997) using genomic DNA from: whole blood (n = 38) collected from previously released Mexican wolves; frozen coyote tissue (liver; n = 4); and fresh scats collected from captive Mexican wolves (n = 13), coyotes (n = 5) where they were not sympatric with wolves along the New Mexico and Texas border, and domestic dogs (n = 5). We used standardized methods, and details of DNA isolation, PCR amplification, and DNA analysis were provided by Reed (2004).

Statisical analyses

Based on the measurement values (i.e., diameter, mass, and length) of scats, we used discriminant analysis (Williams 1982) to classify scats as either Mexican wolf or coyote. We weighed 47 of the scats identified with DNA analysis as deposited by Mexican wolf or coyote to the nearest 0.1 g (dry weight) on a OHAUS Precision Plus TP4000 scale (OHAUS Corporation, Florham Park, N. J., USA); we measured total length of each scat with a metric straight edge to the nearest 0.1 cm. We used discriminant analysis to classify scats based on 1) diameter, 2) diameter and mass, 3) diameter and length, and 4) diameter, mass, and length. Since coyote density was unknown within the study area, we accepted prior probability (q_i) = 0.50. We calculated differences between scat-diameter means for Mexican wolf and coyote using a standard t-test (Ott 1988) and deemed them significant if $P < 0.05$.

Results

Genetic identification

We were able to isolate and analyze DNA from 49 (24%) of 203 scats tested. We identified 28 as Mexican wolf scats and 21 as coyote scats with restriction fragment analysis (n = 18), sequence analysis (n = 24), or both (n = 7). Two of the wolf scats were uncollectibles (Floyd et al. 1978) that could not be measured for diameter or length, and we excluded them from further analysis.

Mexican wolf scats (n = 26) ranged in diameter from 16.3–35.8 mm, while coyote scats (n = 21) ranged in diameter from 17.4–27.8 mm. We found a 79% overlap in scat diameter for Mexican wolf (n = 16) and coyote (n = 21). There was a significant difference (t = –2.85; $P < 0.05$) between scat-diameter means for the 2 species (Mexican wolf $\bar{x}$ = 26.0 mm, coyote $\bar{x}$ = 22.8 mm). Scat diameters ≥28 mm appeared adequate for identifying Mexican wolf scats without other identification criteria (i.e., location and sign).

Of 45 scats that would have been identified as deposited by Mexican wolves based on location and odor criteria, 19 (42%) were actually deposited by coyotes, and of the 41 scats that would have been identified as deposited by coyotes based on diameter (i.e., < 30 mm), 20 (49%) actually were deposited by Mexican wolves. Using Halfpenny's (1986) suggested diameter criterion for identification of scats of 3 carnivore species, scat diameters <18 mm would have been identified as fox (n = 3; 0% correct), diameters 18–25 mm would have been identified as coyote (n = 24; 62% correct), and diameters ≥25 mm would have been identified as Mexican wolf (n = 20; 75% correct).

Discriminant analysis

For scats identified as Mexican wolf or coyote with DNA analysis, we used discriminant analysis for 4 classifications. Scat diameter (Classification 1) accurately identified 81% of coyote scats but only 50% of Mexican wolf scats. Scat diameter and mass (Classification 2) accurately identified 86% of coyote scats and 65% of Mexican wolf scats. Scat diameter and length (Classification 3) accurately identified 68% of coyote scats and 59% of Mexican wolf

scats. Lastly, a combination of scat diameter, mass, and length (Classification 4) accurately identified 79% of coyote scats and 55% of Mexican wolf scats.

Discussion

Our discriminant analysis results of scats identified with DNA analysis supported Halfpenny's (1986) claim that scat diameter and length were too variable to reliably identify species, but refuted Green and Flinders' (1981) findings that scat diameter and mass could be reliably used to identify species when applied to the identification of Mexican wolf and coyote scats. Discriminant analysis of diameter and mass values of scats (Classification 2) provided the most accurate identification for both species (coyote, 86%; Mexican wolf, 65%). Although diameter and mass values provided a relatively high percentage of accuracy for identifying coyote scats, we considered the results unsatisfactory for identifying Mexican wolf scats. Furthermore, 14% of the scats deposited by coyotes were classified incorrectly as Mexican wolf scats and 35% of the Mexican wolf scats were classified incorrectly as coyote scats. Additionally, had the coyote density been known within the BRWRA, the prior probability could have been adjusted accordingly for predicting scats to the 2 species.

Location of scat deposits has also been used to identify wolf scats. Scats collected from wolf den and rendezvous sites undoubtedly have provided accurate diet information because coyotes rarely attend these areas (W. B. Ballard, Texas Tech University, personal communication). However, the information provides wolf-diet data only for late spring through summer (Murie 1944, Mech 1966, Ballard et al. 1987, Fuller 1989, Spaulding et al. 1997). Identification of scats collected from kill and carcass sites (Thompson 1952, Arjo et al. 2002) may be more problematic in areas where wolves and coyotes are sympatric because coyotes often scavenge from wolf kills (Paquet 1992, Phillips and Smith 1996).

Odor also has been reported as a scat-identification technique, but it is subjective (Stokes and Stokes 1986, Bang 2001) and currently cannot be quantified. The only mention of odor in the literature as an identification technique was for foxes (Scott 1943, Murie 1954, Wilcomb 1956, Korschgen 1980, Turkowski 1980), not for wolves or coyotes. Halfpenny (1986) suggested that odor resulted from the carnivore's diet. However, some wolf biologists have reported through personal communications that they can identify wolf scats by odor, but this claim has not been substantiated and our results suggest that odor is not a reliable method.

Recent noninvasive sampling studies of free-ranging mammals (Foran et al. 1997, Kohn and Wayne 1997, Reed et al. 1997, Ernest et al. 2000, Lucchini et al. 2002) have confirmed that fecal DNA analysis provides a more accurate assignment of the species that deposited a scat than morphology of scats. With non-invasive sampling and fecal DNA analysis, biologists can collect individual fecal samples to study free-ranging species without having to disturb them (Höss et al. 1992, Taberlet and Bouvet 1992, Morin et al. 1993, Kohn and Wayne 1997, Taberlet et al. 1999). We were able to collect scats from the field and isolate DNA for species identification without seeing or disrupting the individuals.

Species identification of scats using DNA-based assays provides an accurate method that is rapid (3–4 days), repeatable, and relatively inexpensive (see below). Furthermore, the results are definitive and not subject to confidence intervals or probabilistic estimation (Foran et al. 1997). However, our low (24%) DNA extraction success may be cause for concern. The cost is approximately $5.00 [US] per sample for disposable items, enzymes, and the chemicals required for sequencing. Other costs that are not included are for the sequencer, other nondisposable lab equipment such as pipetors, cameras, computers, and salaries of students and technicians.

DNA isolated from fecal material often is of low quantity and quality (Taberlet et al. 1996). In addition, epithelial cells usually are distributed unevenly (Kohn et al. 1995), and our fecal subsamples may not have included the cells required for DNA isolation. Our modest DNA extraction success was attributed to low-quality and low-quantity DNA found on scats (Foran et al. 1997). Furthermore, 203 of the scats we tested were up to 5 years old at the time we conducted DNA analysis. Reed et al. (1997) and Lucchini et al. (2002) reported that fecal sample age affected extraction success and suggested that fresh scats would be more suitable for DNA analysis. We found, as did Foran et al. (1997), that a scat's physical appearance was not a definitive guide to the DNA quality available. We were able to extract DNA from 49 scats that had been stored dry for up to 5 years. Another possibility influencing low extraction success could have been that some of the scats were deposited by non-

target species whose DNA could not be amplified with the canid-specific primers designed for wolf and coyote. We found, however, that the targeted species could be positively identified if a PCR product could be obtained, since DNA too degraded to amplify produced no results as opposed to incorrect results (Foran et al. 1997). Our results were consistent with the findings of Pilgrim et al. (1998) that wolf and coyote mtDNAs were distinct and could be differentiated by a single restriction site and length polymorphism.

With a sample size of 47 scats identified to species using DNA analysis, we suggest that our results be interpreted with caution. Our results demonstrated, however, that identification of Mexican wolf and coyote scats using DNA analysis was more accurate than identification methods previously available. Molecular scatology can facilitate the identification of species, individuals, their gender, food habits, and pathology. This would require an experimental design with extended systematic transects from which fresh fecal samples are obtained, coupled with an appropriate preservation of fecal material and DNA extraction method (Reed et al. 1997, Wasser et al. 1997, Frantzen et al. 1998, Taberlet et al. 1999, Lucchini et al. 2002), and using appropriate species-specific primers (Foran et al. 1997). These data could then be used further to estimate home range, reproductive patterns, kinship structure, and population size (Kohn and Wayne 1997). Molecular scatology also has potential to detect hybridization (Lehman et al. 1991, Wayne et al. 1992, Gottelli et al. 1994, Pilgrim et al. 1998, Vilà and Wayne 1999). Finally, these data could be used for validating presence of wolves in livestock depredation incidents.

Our results suggest that previous diet studies using traditional scat-identification methods may have misrepresented the diets of both the North American gray wolf and the coyote where the 2 species were sympatric. Fecal DNA analysis provides an accurate method for assessing the visual identification of scat samples collected from the field and improves diet analysis (Reed et al. 1997). Molecular scatology appears to have significant potential as a non-invasive sampling technique to monitor and manage free-ranging Mexican wolves where the subspecies is sympatric with other carnivore species.

Acknowledgments. The United States Fish and Wildlife Service funded this research and provided scats from captive Mexican wolves. The University of New Mexico and Arizona Veterinary Diagnostic Lab provided Mexican wolf blood samples. The Museum of Texas Tech University provided coyote and rodent tissue samples. A. Brown, C. C. Perchellet, F. Hoffman, and J. Wickliffe contributed invaluable assistance and dedication in the laboratory. D. Wester provided guidance on statistical analyses. P. Gipson, P. Krausman, and 2 anonymous referees made constructive comments on an early draft. This is a Texas Tech University, College of Agricultural Sciences and Natural Resources technical publication T-9-999.

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