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Estimating gray wolf pack size and family relationships using noninvasive genetic sampling at rendezvous sites

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Studying the ecology and behavior of pack animals often requires that most, or all, of the pack members are sampled. A unique opportunity to sample all gray wolf (*Canis lupus*) pack members arises during the summer months when reproductive packs localize in rendezvous sites. We collected 155–296 scat and hair samples from each of 5 wolf rendezvous sites in central Idaho to evaluate intrapack relationships and determine the efficacy of noninvasive genetic sampling (NGS) for estimating pack size and family relationships. We detected 65 wolves (5–20 wolves per pack) with NGS, and the pack counts from NGS were the same or higher for adults and the same or slightly lower for pups compared with the counts from observation and telemetry. The wolves in each pack were closely related to one another, and all packs included at least 2 years of offspring from the current breeding pair. Three of the packs had additional breeding adults present. In 1 pack pups were produced by a parent–offspring pair and a pair of their inbred full siblings, indicating multiple cases of inbreeding. This targeted NGS approach shows great promise for studying pack size and wolf social structure without the use of radiotelemetry or direct observations.

Key words: *Canis lupus*, fecal genotyping, inbreeding, kinship, multiple litters, noninvasive genetic sampling, pack structure, pedigree, wolves

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Studying the ecology and behavior of animals with a pack social structure requires detailed knowledge of the number of individuals in a pack and the relatedness of those individuals. For example, gray wolf (*Canis lupus*) pack size is correlated with hunting efficiency, prey type, and territory size (Mech and Boitani 2003). In addition, the family relationships in a pack dictate breeding status and competition for food (Packard 2003). Wild pedigrees are fundamental tools for examining biological questions (Liberg et al. 2005; Pemberton 2008; Visser 2008), but a key challenge when inferring genealogical relationships among pack animals is collecting data from all, or nearly all, animals in the pack. Pedigree reconstruction from wild species typically requires observational and genetic data (e.g., red wolves [*Canis rufus*—Adams 2006], brown bears [*Ursus arctos*—DeBarba et al. 2010], and African wild dogs [*Lycaon pictus*—Girman et al. 1997]), but some studies have reconstructed genealogies using only genetic data collected from noninvasive sources (e.g., western gorilla *Gorilla gorilla*—Bradley et al. 2004] and southern hairy-nosed wombat [*Lasiorhinus latifrons*—Walker et al. 2008]). Similarly, studies examining wolf pack genealogy typically use a combined data source approach (i.e., radiotelemetry, field observation, and genetic sampling), which requires handling and direct observation of individuals (Adams 2006; Liberg et al. 2005; Rutledge et al. 2010; vonHoldt et al. 2008). Alternative methods for assessing pack social structure and relatedness would be particularly useful for wolf packs in areas where radiotelemetry and direct observation are less feasible.

The use of noninvasive genetic data to describe pack structure in gray wolves is still largely unexplored, but substantial benefits are associated with this method. First, it provides the potential to sample all wolves in a pack over a short time period. Comprehensive sampling of family groups

allows for increased accuracy in parentage, pedigree, and kinship analyses (Jones and Ardren 2003). In a single day genetic samples potentially could be collected from every individual in the pack because scat and hair samples remain after wolves have moved. In the summer, 8–20 weeks after wolf pups are born, reproductive packs localize to areas called rendezvous sites, where the pups are kept while they are too young to travel with the pack (Packard 2003). This is an ideal time to sample reproductive packs because it is likely that all pack members have visited the site and left some source of DNA.

Wolf packs typically consist of a mated pair with their offspring and also can include siblings of the breeders (Mech and Nelson 1990) and unrelated wolves (Jędrzejewski et al. 2005; Lehman et al. 1992). The proportion of unrelated wolves in a pack (''adoptees'') varies among studies, with some studies showing no cases of unrelated wolves (vonHoldt et al. 2008) but others finding adoptees in 80% of packs (Grewel et al. 2004; Rutledge et al. 2010). The establishment of adoptees into packs can increase with intense harvest (Jędrzejewski et al. 2005; Rutledge et al. 2010), during the time when similarly aged pack members disperse (Meier et al. 1995), or when maturing females are present (Mech and Boitani 2003). In addition, multiple litters of pups born to different mothers in the same year have been documented (Meier et al. 1995; Rutledge et al. 2010; Van Ballenberghe 1983; vonHoldt et al. 2008), but the frequency and mechanisms for multiple litters in a pack are unknown (Meier et al. 1995; Packard and Mech 1980). Researchers hypothesize that multiple breeding occurs when food is plentiful (Mech et al. 1998) or in heavily exploited packs (Ballard et al. 1987). Finally, although wolves have many opportunities for incestuous mating, inbreeding typically is avoided in wild wolf populations (Smith et al. 1997; vonHoldt et al. 2008).

The purposes of this study were to estimate wolf pack size and determine pack pedigrees with noninvasive genetic sampling (NGS) for a reintroduced, unharvested wolf population in central Idaho and assess the efficacy of NGS to investigate questions related to the ecology and social structure of packs. We used a targeted NGS survey focused on collecting hair and fecal samples from a single sweep of predicted gray wolf rendezvous sites. Because previous studies have not used NGS to investigate pack size and pedigrees, it was important to test working hypotheses that would reveal limitations of NGS and highlight the potential for NGS to answer questions of wolf ecology and social structure. First, we evaluated whether NGS at rendezvous sites would detect all pack members because pack members, including the breeding male, sometimes leave the rendezvous site for an undetermined amount of time (Mech et al. 1998), and packs can occupy 2 rendezvous sites simultaneously (Harrington and Mech 1979, 1982). We hypothesized that every wolf in a pack visited the rendezvous site and would be detectable via NGS. Second, because pack pedigrees can diverge from the simple pack structure of an unrelated breeding pair with their offspring (Mech and Boitani 2003), we assessed whether NGS could reveal the prevalence of multiple breeding and inbreeding. We hypothesized that each pack would have 1 breeding pair comprised of 2 unrelated individuals because this is the most typical pack structure (Mech and Boitani 2003). To evaluate the efficiency of NGS as a tool for testing the previous hypotheses we ran subsampling analyses to provide recommendations for the design and implementation of future studies sampling hair and scat at rendezvous sites to study wolf pack size and structure.

Materials and Methods

Sampling strategy.—We surveyed for wolves at predicted rendezvous sites using a habitat model developed from 300 known wolf pack rendezvous sites in Idaho, 1996–2006, based on the habitat predictors of green leaf biomass, surface roughness, and profile curvature (Ausband et al. 2010). Focusing on these predicted rendezvous sites decreased the sampling area and precluded the need for radiotelemetry. In late June to August 2008 we surveyed 79% of predicted rendezvous sites in 2 study areas in central Idaho—Sawtooth (~44°20′31.8″N, 115°30′14.8″W) and Salmon (~45°4′12.6″N, 114°12′51.4″—Stenglein et al. 2010b; Fig. 1)—supporting high densities of wolves (5–7 packs/~3,500 km^2—Nadeau et al. 2009).

A rendezvous site was considered occupied when multiple wolves were heard howling, wolf trails and heavy use areas were found, or pups were sighted or their sign was present (i.e., pup play areas—Joslin 1967). The day after we located an occupied rendezvous site, a 6-person crew collected all scat and hair samples they could find, with concentrated effort in the areas of heavy use. We used multiple observers to minimize the time spent at an occupied site. Additionally, sampling always occurred after mid-June when pups were more mobile and we expected packs to be less sensitive to disturbance (Thiel et al. 1998).

We collected a small sample of the side portion of each scat (Stenglein et al. 2010a) with sterilized tweezers and placed each sample in 2.0 ml of dimethylsulfoxide–ethylenediaminetetraacetic acid–Tris–salt solution (Frantzen et al. 1998). Scat samples $>$ 2.5 cm in diameter were considered adult wolf (Weaver and Fritts 1979) and those $<$ 2.5 cm were considered wolf pup. Hair samples were distinguished as either from daybeds (hereafter, daybed hair) or other hair found in clumps on the ground or snagged on trees (hereafter, other hair) and were collected in individual envelopes and stored in silica until DNA extraction. Sampling within the rendezvous site took 3–5 h in 1 day, but we collected additional samples outside the rendezvous site while we visited other probable rendezvous sites in the study area (Stenglein et al. 2010b).

From July to September reproductive packs in the study area were monitored by a separate crew using radiotelemetry to locate packs and count the adults and pups visually. This crew operated independently from the field technicians surveying predicted rendezvous sites and conducting NGS sampling. The telemetered wolves were part of the United

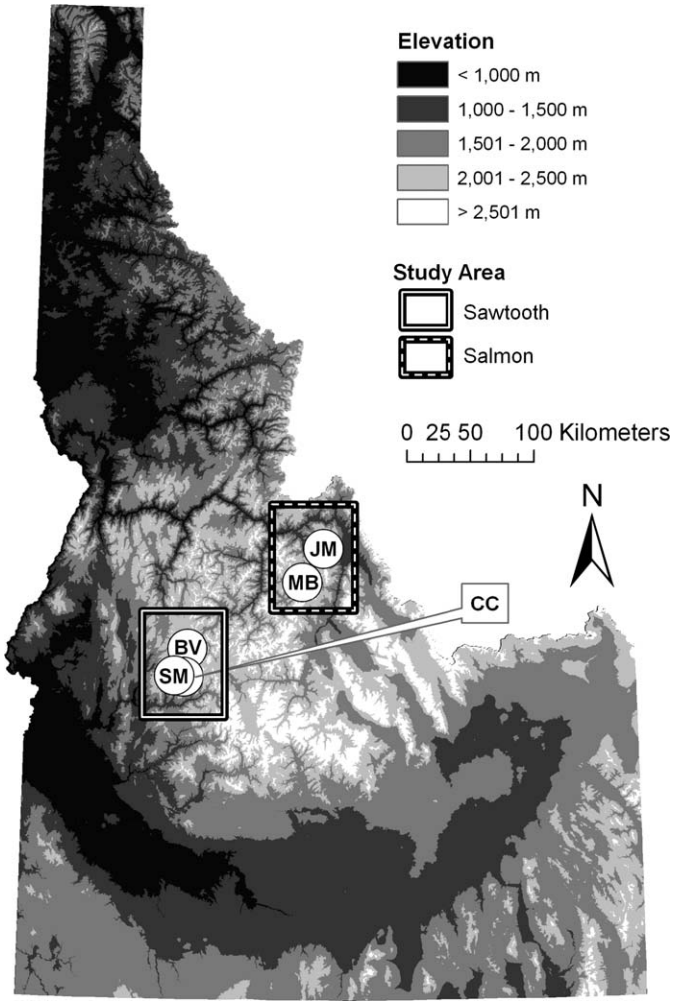

Fig. 1.—Approximate locations of Jureano Mountain (JM), Moyer Basin (MB), Bear Valley (BV), Scott Mountain (SM), and Casner Creek (CC) gray wolf packs in the Sawtooth and Salmon study areas of central Idaho. Locations are drawn centered on their rendezvous sites.

States Fish and Wildlife Service Northern Rocky Mountain Distinct Population Segment monitoring program implemented by Idaho Department of Fish and Game and the Nez Perce Tribe (United States Fish and Wildlife Service et al. 2006; Wolf Oversight Committee 2002). We obtained genetic samples for the 11 radiocollared wolves in our study packs (1–3 radiocollared wolves per pack) from the United States Fish and Wildlife Service Forensics Laboratory (Ashland, Oregon).

Species, individual, and sex identification.—DNA was extracted in a laboratory for genetic analysis of noninvasive genetic samples using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, California) for scat samples and the Qiagen DNeasy tissue kit for hair samples. We included 1 extraction negative in each group of extractions to check for contamination. We extracted hair samples with at least 3 guard hairs or underfur because preliminary analyses demonstrated poor amplification success (15%) for samples with 1 or 2 guard hairs. We 1st conducted a test of species identification using the control region of mitochondrial DNA (Onorato et al. 2006) to cull all coyote (*Canis latrans*) and low-quality DNA samples (i.e., samples that failed to amplify).

Nine microsatellite loci (FH2001, FH2054, FH2088, FH2137, FH2611, FH2670, FH3725, C09.173, and Cxx.119—Breen et al. 2001; Guyon et al. 2003; Holmes et al. 1994) were combined into a polymerase chain reaction (PCR) multiplex and amplified using conditions described in Stenglein et al. (2010b). All PCR primers were labelled with fluorescent dyes, and all loci have a PCR product size of <250 base pairs (bp). We included a negative extraction and PCR control in each group of reactions to test for contamination. Initially, we amplified and ran each wolf sample twice using the 9-locus multiplex, and alleles were sized using an Applied Biosystems 3130*xl* ABI capillary machine (Applied Biosystems, Inc., Foster City, California) and viewed with GeneMapper version 3.7 (Applied Biosystems).

Samples that amplified at 5–9 loci were rerun in 1–3 additional PCRs to finalize the consensus genotype. An allele was accepted in a heterozygous consensus genotype after it was seen in $\geq$2 independent PCRs, and we required $\geq$3 independent PCR replicates to accept a homozygous consensus genotype. Once we confirmed 8 or 9 loci for a sample we compared the genotype to all consensus genotypes using GIMLET version 1.3.3 (Valière 2002) to check for matches. RELIOTYPE (Miller et al. 2002) was used to determine if further repetitions were needed to obtain 95% certainty in the accuracy of genotypes observed in only a single sample. The more rigorous cumulative probability of identity based on siblings ($P_{ID(sibs)}$) was calculated in GenAlEx version 6.2 (Peakall and Smouse 2006) for 8 or 9 loci to ensure we were using enough loci to distinguish siblings (Waits et al. 2001). We calculated rates of allelic dropout and false alleles from the 2 initial PCRs and the probability that we retained 1 of these errors in our final genotypes using methods described in Broquet and Petit (2004).

Consensus genotypes were analyzed for sex identification with canid-specific primers (Seddon 2005), and an additional 10 loci were added using the sample for each individual with the best initial microsatellite amplification. We initially ran the sex-identification PCR 3 times for each sample; a female was not confirmed until the X chromosome alone was seen 3 times, and a male was not confirmed until the Y was seen at least twice. We combined the 10 loci (AHT103, AHT109, AHT121, AHTk200, C05.377, C37.172, Cxx.250, FH2004, FH2010, and FH2145—Breen et al. 2001; Holmes et al. 1995; Ostrander et al. 1993) into a PCR multiplex. The 7-μl PCR consisted of 0.03 μM C37.172, C05.377, and FH2010; 0.05 μM AHT121 and Cxx.250; 0.08 μM AHTk200 and FH2145; 0.1 μM AHT109 and FH2004; 0.35 μM AHT103, 1× concentrated Qiagen Master Mix; 0.5× concentrated Qiagen Q Solution; and 1 μl DNA extract. The PCR profile had an initial denaturation step of 15 min at 94°C followed by a touchdown with 15 cycles of 30 s at 94°C, 90 s at 63°C with a decrease in annealing temperature by 0.5°C each cycle, and 1 min at 72°C, followed by 20 cycles of 30 s at 94°C, 90 s at

55°C, and 1 min at 72°C. Acceptance of the genotype followed the same criteria as described above, with at least 17 of 19 loci required for a consensus genotype.

We used GENEPOP version 3.4 (Raymond and Rousset 1995) to test for deviations from Hardy–Weinberg and linkage equilibrium using a larger population of wolves in the study areas that we sampled with NGS in 2007 and 2008 (122 wolves—Stenglein et al. 2010b). Because many of these wolves were known to be closely related, we retested for deviations from Hardy-Weinberg and linkage equilibrium with a data set where all known offspring ($N = 66$, based on genetic data) were removed. Tests were considered significant at a Bonferroni-corrected $\alpha = 0.05$ level (Rice 1989).

We assessed the power of our marker set in distinguishing between candidate relationships using the analytical method in program KinInfor version 1 (Wang 2006). We used the data set with all known offspring removed, a significance level of 0.05, relative precision value of 0.01, and a 1% genotyping error rate per locus. We assessed power for the 19-locus set and the 17 least informative loci, as determined by the rank order of their information content for the power analysis, to ensure conservative estimates of power. Statistical assessments of the power in our marker set to assess parent–offspring, full-sibling, and half-sibling relationships were foundational for correct interpretation of our results.

Pack size.—To test the hypothesis of whether all wolves in a pack were detectable at a rendezvous site, we compared the minimum counts derived from NGS to independent, visual counts using radiotelemetry data combined with observation (hereafter, telemetry/observation). For NGS counts we distinguished wolf pups from adults by scat size in the field (see ''*Sampling strategy*'' above). Adult or pup status could not be determined for hair samples, but 98% of the individuals detected from hairs also were detected with scat samples.

Pack pedigrees, genetic diversity, and breeding pairs.—We found potential parent–offspring trios in each pack with the exclusion method (Jones and Ardren 2003) in program GIMLET, with a maximum of 1 mismatch allowed. All potential parent–offspring trios were then run in CERVUS with a likelihood approach; delta values were considered significant at the strict (95%) and relaxed (80%) levels. If a parent–offspring trio was unresolved at the relaxed level, we considered a single-parent duo. To assess confidence we computed delta values using allelic frequencies from the larger population with known offspring removed (56 wolves). We simulated 10,000 offspring with 25 parent-pairs, allowing for 17% of the genotypes to be incomplete, a 1% genotyping error rate as determined in our data set, and 20% of the population remaining unsampled.

We built pedigrees from the parentage analysis and tested all pairwise relationships in terms of their expected inbreeding coefficient (F), probabilities of identity states ($\Delta 7$ and $\Delta 8$—Weir et al. 2006), and relatedness (r) based on the pedigree against the F, $\Delta 7$ and $\Delta 8$, and r genetic marker calculations without pedigree information (Wang 2011). As an example, a noninbred parent–offspring pair in the pedigree would have F $= 0$, $\Delta 7 = 0$ and $\Delta 8 = 1$, and r $= 0.5$ (Weir et al. 2006) from the pedigree configuration, but this might not match the F, $\Delta 7$ and $\Delta 8$, and r calculated from their genetic marker data. We derived the F, $\Delta 7$ and $\Delta 8$, and r values based on the pedigree from identical-by-descent (IBD) probabilities calculated in program R version 2.9.2 (R Development Core Team 2009) with package cic (Cheng 2010). We assumed that the best pedigree configuration would be the one that had the fewest discrepancies between the pedigree calculation and marker-based calculation of F, $\Delta 7$ and $\Delta 8$, and r. We used the Triadic IBD method (Wang 2007) to calculate $\Delta 7$ and $\Delta 8$ and r (for inbred packs), the Queller and Goodnight (1989) moment estimator to calculate r (for noninbred packs), and the Lynch and Ritland (1999) moment estimator to calculate F in program COANCESTRY version 1 (Wang 2011). For these calculations we used allelic frequencies from the larger population with known offspring removed, a 1% rate of genotyping error, potential inbreeding, 100 referenced individuals, and 100 bootstrapped simulations to create 95% confidence intervals (*CI*s) on the estimates. The Triadic IBD method incorporates a reference individual to obtain a dyad's relatedness estimate; the relatedness estimates are improved for dyads of closely related individuals because genes that are identical-in-state are less likely to be assumed as IBD for the dyad (Wang 2007). The best pedigree configuration for each pack was reconstructed with Pedigraph version 2.4 (Garbe and Da 2008). We located the radiocollared wolves that were known breeders on the pedigree to assess whether their status determined from telemetry-related observations matched the pedigree.

We calculated the following statistics as pack averages: allelic richness in FSTAT version 2.9.3 (Goudet 1995), expected heterozygosity in GENEPOP, and relatedness in KINSHIP (Goodnight and Queller 1999). Our test for inbreeding was whether the 95% *CI* for each breeding pair's r was different than 0.25 (half sibling) and 0.50 (full siblings or parent–offspring) and which relationship was best supported by $\Delta 7$ and $\Delta 8$. Both were calculated in program COANCESTRY with the Triadic IBD method described above.

Sampling considerations.—We evaluated the type of samples collected (i.e., scat or hair), spatial location of samples, and the number of samples collected for each pack to recommend an efficient sampling method at a rendezvous site. We calculated the distance from the rendezvous site center to each sample in ArcMap version 9.2 (ESRI, Redlands, California). The rendezvous site center was determined as the centermost sample in the rendezvous site using the central feature tool in ArcMap. Because this was always within the area of high pup activity, the rendezvous site center could be determined approximately in the field by denoting the center as the concentration of pup sign, which we found to be a consistent characteristic of the occupied rendezvous sites. In addition, we built accumulation curves of the unique genotypes to assess the number of samples we needed to analyze for the curve to reach an asymptote. All of the samples collected at a site, regardless of finalized genotyping success,

TABLE 1.—Scat and hair samples collected from 5 gray wolf rendezvous sites in central Idaho, with the number of consensus genotypes and wolves confirmed for each pack. The allelic richness, average expected heterozygosity (H_E), and average relatedness (r) are shown for each pack.

Pack[a]	Adult scat	Pup scat	Other hairs	Daybed hairs	Total samples	Consensus genotypes	No. wolves	Allelic richness	Average H_E	Average r
BV	44	54	39	18	155	70	20	2.77	0.72	0.50
CC	54	116	76	14	260	150	8	2.78	0.76	0.51
JM	84	80	109	23	296	160	16	2.50	0.64	0.49
MB	61	80	27	45	213	118	16	3.10	0.74	0.43
SM	30	28	62	46	166	99	5	2.79	0.78	0.44
Total	273	358	313	146	1,090	597	65	2.79[b]	0.73[b]	0.47[b]

[a] BV = Bear Valley, CC = Casner Creek, JM = Jureano Mountain, MB = Moyer Basin, SM = Scott Mountain.
[b] Averaged over all packs.

were used, and the data set was resampled 100 times for each number of samples collected (1 to the total number of samples collected) in program R.

RESULTS

Sampling strategy.—We collected a total of 1,090 samples, 155–296 from each of 5 occupied rendezvous sites (Table 1). The precautions to minimize disturbance of the wolf packs appeared effective because telemetry data indicated that no packs moved rendezvous sites in response to sampling. An additional 36 scat samples obtained outside occupied rendezvous sites were from wolves detected at rendezvous sites, and we used these samples to infer individual space use for sampling recommendations.

Species, individual, and sex identification.—We successfully amplified DNA from 89% of the samples for species identification, and 7 coyotes subsequently were removed. In the larger population 9 loci (47%) deviated from expectations of Hardy–Weinberg equilibrium, and 98 (57%) pairs of loci deviated from linkage equilibrium. However, when we removed the known offspring and recalculated the tests, no loci deviated from expectations of Hardy–Weinberg equilibrium and no pairs of loci deviated from linkage equilibrium. These results indicate that deviations from equilibrium in the larger data set likely were due to sampling of related individuals. The estimated allelic dropout (0.13) and false allele (0.03) error rates led to a low probability of retaining a false homozygote (0.002) or false allele (0.009) error, given our multiple replication protocol. The set of microsatellite loci had enough power to distinguish closely related individuals ($P_{ID(sibs)} < 0.001$), and alleles per locus averaged 6.4. High power (range: 0.72–1.00) characterized distinctions between 2 candidate relationships with 19 loci and only slightly lower power (0.63–1.00) for 17 loci (Table 2).

Sixty-eight (6%) of our samples appeared to be mixed because they had genotypes from >1 individual (i.e., >2 alleles at multiple loci) and were removed from the data set. These mixed samples represented 1% of the total scat samples, 22% of the daybed samples, and 10% of the other hair samples. We obtained consensus genotypes for 597 (55%) samples, and we detected 65 wolves (31 males and 34 females; Table 1). Individuals were detected 1–10 times in Bear Valley, 9–33 times in Casner Creek, 3–16 times in Jureano Mountain, 1–15 times in Moyer Basin, and 9–44 times in Scott Mountain, and 94% of the wolves were detected more than once. We averaged 7.9 detections per adult and 12.4 detections per pup. We detected DNA from all 11 radiocollared wolves with a scat or hair sample.

Pack size.—More wolves were detected with NGS data than were detected with telemetry/observation in Bear Valley, Jureano Mountain, and Moyer Basin packs (Table 3). In Bear Valley and Jureano Mountain packs telemetry/observation and NGS pup counts were identical, but we detected more adults with NGS. In Moyer Basin pack more adults and 1 fewer pup were detected with NGS than were detected with telemetry/observation counts. For the other 2 packs, Casner Creek and

TABLE 2.—The multilocus power to detect one relationship over another from 19 microsatellite loci and the 17 least-informative loci in an Idaho wolf population with a 1% rate of error per locus.

Relationship 1	Relationship 2	19 loci	17 loci
Parent–offspring	Full sibling	0.855	0.767
Parent–offspring	Half sibling	0.972	0.919
Parent–offspring	Unrelated	1	1
Full sibling	Half sibling	0.770	0.694
Full sibling	Unrelated	1	1
Half sibling	Unrelated	0.720	0.632

TABLE 3.—The number of gray wolf adults and pups counted in 5 packs in central Idaho. The pack counts from noninvasive genetic sampling (NGS) are compared to counts from radiotelemetry and associated observation (RT) data.

	Adult counts		Pup counts	
Pack[a]	NGS	RT[b]	NGS	RT[b]
BV	16	8–12	4	≥3
CC	5	5	3	4
JM	10	4 or 5	6	6
MB	12	9	4	5
SM	4	3 or 4	1	2 or 3

[a] BV = Bear Valley, CC = Casner Creek, JM = Jureano Mountain, MB = Moyer Basin, SM = Scott Mountain.
[b] A range of values in the adult and pup counts or a minimum number in the pup counts are shown when radiotelemetry counts were uncertain.

Scott Mountain, NGS total pack counts were lower than telemetry/observation counts by 1 or 2 pups (Table 3).

Pack pedigrees, genetic diversity, and breeding pairs.—Genetic parentage analysis resolved parent–offspring relationships for 51 (78%) wolves with 47 parent–offspring trio assignments and 4 single-parent assignments. Forty-two (82%) of the assignments were resolved at the 95% confidence level, and 9 (18%) were resolved at the 80% level. Twenty-eight percent of the dyads from the pedigrees incorporating only the parentage resolved in CERVUS were rejected because they were not within the 95% *CI* of both the Δ7 and Δ8 coefficients calculated from the genetic marker data. However, slight modification of Bear Valley, Jureano Mountain, and Moyer Basin relationships improved the pedigrees so that only 18% of the dyads had discrepancies with Δ7 and Δ8 95% *CI.* Of the dyads with discrepancies, 87% had an r from the pedigree that fell within the 95% *CI* for r from the genetic marker data, indicating a close match between the pedigree and marker-based calculations. The full-sibling dyad in the Bear Valley pack of female F53 and male M55 was resolved, and the 2 siblings were determined to be the offspring of F16 (Fig. 2). In the Jureano Mountain pack we resolved that the breeding pair M118/F32 was parent–offspring. Finally, in the Moyer Basin pack we determined F54, F94, F105, and M102 to be full siblings with no parents present (Fig. 2). Jureano Mountain was the only pack with inbreeding, and 81% of the individuals' F from the pedigree fell within the 95% *CI* for F calculated from genetic marker data. The 3 known radiocollared wolves that were identified as alphas through telemetry-related observation were determined to be breeders in the current and previous year(s) with the pedigree analysis; wolf B145 (Mack and Holyan 2004), wolf B106 (Nadeau et al. 2007), and wolf B375 (Nadeau et al. 2009) correspond to F105, M118, and M75, respectively, in Fig. 2.

The mean intrapack allelic richness was 2.79 alleles/locus (range: 2.50–3.10 alleles/locus), the mean intrapack expected heterozygosity was 0.73 (range: 0.64–0.78), and the mean relatedness between all pairs of individuals within packs was 0.47 (range: 0.43–0.51; Table 1). Jureano Mountain pack had the lowest heterozygosity and allelic richness of any pack, and the highest relatedness was in Casner Creek pack (Table 1).

At least 1 breeding pair was present in each pack, and additional breeders were detected in 3 packs (Fig. 2). In the Moyer Basin pack the breeding male had produced offspring with 3 sisters in previous years (i.e., offspring of these pairings were sampled as adults), but the pups of the year came from only 1 pairing (Fig. 2). Alternatively, multiple litters were detected in the current year from 2 different breeding pairs in Jureano Mountain pack (Fig. 2). We obtained complete 19-locus genotypes for all individuals in Jureano Mountain pack and, based on the exclusion method, found 11 individuals as offspring of the M118/F32 parent–offspring breeding pair. We excluded 3 pups as offspring of the main Jureano Mountain breeding pair M118/F32 because of mismatches at 2–5 loci (11–26% of the loci) per pup, but these pups had no mismatches with M84/F31, and all were supported as offspring of M84/F31 and full siblings with each other based on their Δ7 and Δ8 coefficients. The breeding pair relatedness ranged from 0.16 to 0.08, except for both Jureano Mountain breeding pairs, which were related at the r = 0.50 level, suggesting 2 cases of inbreeding. The Δ7 and Δ8 coefficients supported a parent–offspring relationship for M118/F32 and a full-sibling relationship for M84/F31 (Fig. 2).

Sampling considerations.—Individual wolves were sampled from 1 to 44 times. On average, scat samples provided 6.2 detections per wolf compared to an average of 3.0 hair samples per wolf, and 64 of the 65 wolves were detected using fecal sampling compared to 44 of the 65 wolves with a hair sample. We could have omitted daybed hair samples from the analysis without an effect on the number of wolves sampled. All wolves were detected within 1 km of the rendezvous site center, 95% of the wolves were detected within 250 m of the center, and 80% of the wolves were detected within 100 m of the center. Thorough sampling close to the rendezvous site center (i.e., within a 250-m radius) was an efficient method for detecting most wolves. The accumulation curves for each rendezvous site reached an asymptote in 3 of the 5 packs (Fig. 3). For those packs whose curves reached an asymptote, the number of samples required to sample all individuals varied with the total number of individuals in the pack. It ranged from ~70 samples for Scott Mountain to ~180 samples for Jureano Mountain (Fig. 3). At 50 samples collected we detected 65–100% (median of 100 resampled data sets) of the members in each pack, at 100 samples we detected 90–100% of the members in each pack, and at 150 samples we detected all members in each pack (Fig. 3).

Discussion

We detected multiple litters and inbreeding, which was inconsistent with our hypothesis that each pack would be composed of a single, unrelated breeding pair. The configuration of the extra breeding pairs found in this study varied by pack, and both cases were in the Salmon study area. Multiple breeders in a season, producing multiple litters, have been documented in areas of high exploitation (Ballard et al. 1987) and highly productive and unexploited populations such as Yellowstone National Park (Mech et al. 1998; vonHoldt et al. 2008). Although the mechanism for multiple litters in this study is unclear, multiple litters in wolf packs might be higher than expected and typically remain undetected unless 2 distinct den sites are observed (Ballard et al. 1987) or genetic data are used (vonHoldt et al. 2008; this study). The 2 litters in 2008 in Jureano Mountain pack resulted in 6 pups, which is within the range of average litter size (Mech and Boitani 2003), so without genetic confirmation 2 litters would not have been suspected.

We detected inbreeding in multiple years and between a parent–offspring pair and a full-sibling breeding pair in the Jureano Mountain pack. The Jureano Mountain pack was one of the 1st packs established after the reintroduction (Mack et

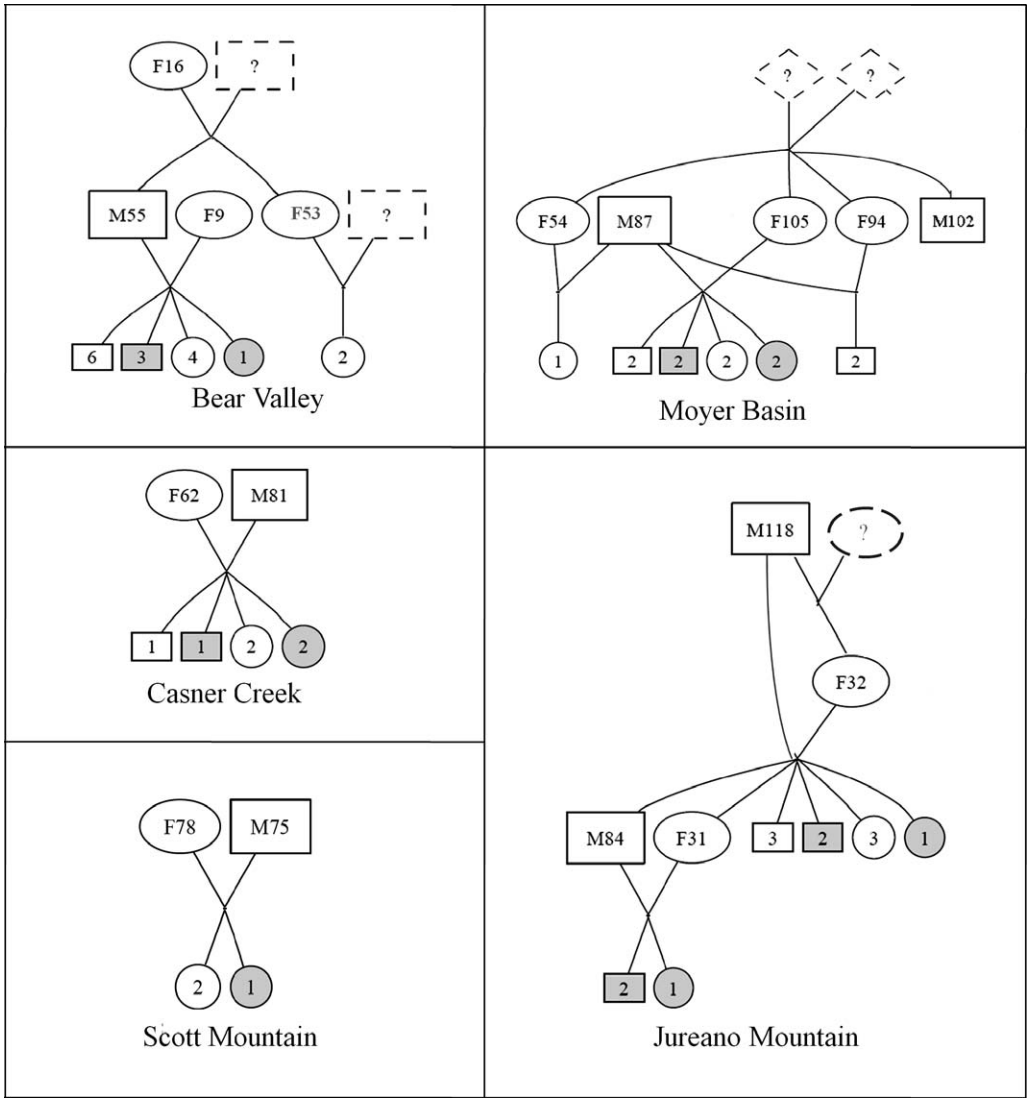

Fig. 2.—Pedigrees for 5 gray wolf packs in central Idaho. Female wolves are shown with circles or ovals, males are shown with rectangles, and unknown sex is shown with diamonds. Dashed shapes with question marks are unsampled parents. All breeders and individuals discussed in the text are labelled with their sex and field identification. Other numbers inside small circles and rectangles are used to indicate the number of offspring per sex, with the shaded shapes showing pups of the year.

al. 2005), and the breeders in the pack have produced pups every year (Mack et al. 2005; Nadeau et al. 2007, 2008, 2009; United States Fish and Wildlife Service et al. 2006), including litters in past years from a parent–offspring pair (this study). At least 2 interpretations can be proposed for the observed inbreeding, and because only 5 packs were sampled, it is difficult to make conclusive statements about the reason for the inbreeding and the extent to which it is abnormal. First, past management actions (Nadeau et al. 2007, 2008, 2009) might have removed breeders from this pack and left only related individuals. However, this pack's territory abuts 4 wolf packs (Nadeau et al. 2009), and therefore it is unclear why unrelated wolves have not joined the pack as breeders to avert inbreeding (Smith et al. 1997; vonHoldt et al. 2008). The parent–offspring breeding pair likely has produced offspring for at least 3 years (8 adults and 3 pups sampled as offspring in 2008), which implies that even if this pairing was initiated by management action that removed breeders or unrelated wolves in the pack, time was sufficient for an unrelated breeder to join the pack. Second, inbreeding might be more common than previously noted (Smith et al. 1997). Although inbreeding typically was averted for 17 eastern wolf (*Canis lycaon*) packs in Algonquin Provincial Park, Canada, 2 (12%) of the packs had breeding pairs related at the half- or full-sibling level (Rutledge et al. 2010). Additional studies that sample all pack members might find additional cases of inbreeding in healthy and growing wolf populations, such as Idaho (this study) and Canada (Rutledge et al. 2010).

Similar to wolf packs in Yellowstone National Park (vonHoldt et al. 2008), we did not detect any unrelated adoptees, even though many more individuals were identified in some packs than were thought to be present from telemetry/observation. The high intrapack kinship that we detected in Idaho's unharvested wolf population (the population was not

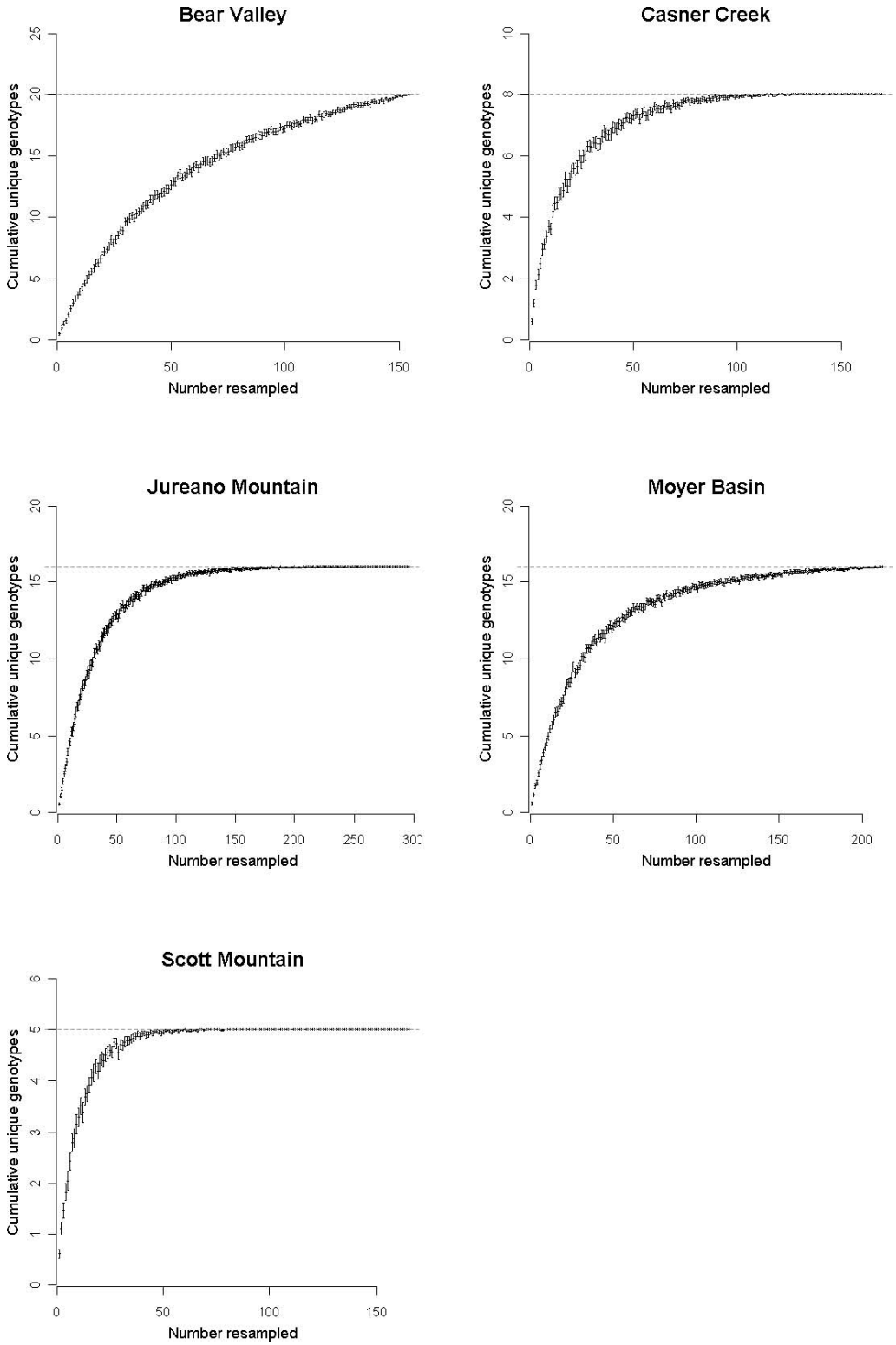

Fig. 3.—Accumulation of individual gray wolves as all scat and hair samples collected at each of 5 rendezvous site in central Idaho were added. The 95% confidence intervals show the variation of 100 iterations for each number of samples from 1 to the actual number of samples collected. The horizontal dashed lines show the number of wolves detected with noninvasive genetic sampling from analyzing all scat and hair samples.

harvested until 2009, after this study was completed) might correspond to relatively low human-caused mortality because intense harvest that compensates for natural causes of mortality can increase the incidence of adoption of unrelated wolves into packs (Rutledge et al. 2010). Our approach of sampling entire packs at rendezvous sites could provide valuable data about how recently enacted hunting seasons might impact the social structure of wolf packs in the northern Rocky Mountains.

It is important to consider sources of error that could have affected our results. First, contamination and genotyping errors can confound results when using NGS. To avoid contamination we processed all samples in a room dedicated to low-quantity DNA sources and included negative controls in all DNA extractions and PCRs. The probability of observing a false homozygote or false allele error using our multiple replication protocol was $\leq$0.009/locus, and we required that genotypes of all single-capture individuals ($<$10% of all individuals) met $\geq$95% reliability criteria (Miller et al. 2002). Estimating family relationships for samples collected by NGS can be challenging because the age of individuals is unknown and many loci are needed for accurate classification of relationships. To address this challenge we chose highly variable loci and required a minimum of 17 loci to include an individual in the analysis. We also used multiple methods to assess and verify the genetic relationships. We used a conservative 1% rate of genotyping error when calculating the multilocus power to distinguish between relationships and analyzing pairwise relatedness. Overall, we had high power to distinguish parent–offspring relationships from other relationships and parent–offspring and full siblings from unrelated individuals, but we acknowledge lower power to discriminate half siblings from full siblings or unrelated individuals. Power to discriminate relationships could be increased by using more loci in future analyses.

We assumed that any successfully genotyped sample collected from a rendezvous site originated from a member of the pack currently occupying the rendezvous site. This appears to be a reasonable assumption for at least 3 reasons. First, PCR amplification of DNA from wolf scat samples decreases 1.14% per day (Santini et al. 2007), and therefore very old samples ($>$1 month) would not likely result in a consensus genotype. Second, because all genotypes from samples collected at a rendezvous site were from closely related individuals and we did not detect any unrelated individuals, the samples met the expectations of pack membership. Finally, we detected $>$90% of the individuals multiple times, which indicates fidelity to the rendezvous site, a typical behavior of pack members (Mech and Boitani 2003).

It is possible that NGS could overestimate or underestimate the number of individuals in a pack. We suggest that undersampling of wolves is not a large problem when using NGS because we detected all 11 wolves known to be in the study area, our estimate of adult wolves met or exceeded telemetry/observation counts, and previous work showed high agreement between NGS and telemetry-based population estimates (Stenglein et al. 2010b). However, in 3 packs we detected fewer pups with NGS than were counted with telemetry/observation methods, which was unexpected because pup activity was highly concentrated in the rendezvous site center, with pups detected an average of 12 times. Also, we calculated high probabilities of distinguishing siblings as unique individuals. Possible explanations for lower pup counts include identical twins (Carmichael et al. 2008), coprophagy of scat by canids or other animals (Livingston et al. 2005), pup mortality between telemetry observations and NGS surveys, and simultaneous use of multiple rendezvous sites, which was observed during monitoring by radiotelemetry (Harrington and Mech 1979, 1982).

Noninvasive genetic sampling of an entire pack can be completed in 3 h by 5 or 6 technicians once the rendezvous site is found. The efficiency of field collection can be increased by restricting sampling to 250 m around the pup activity center, which researchers can identify by the large concentration of pups scats. Because almost all wolves were detected from scat samples only, additional time and cost savings could be achieved by omitting the collection and analysis of hair samples. However, hair samples are important for mark–recapture population estimation (Stenglein et al. 2010b). If hair samples are collected, we recommend excluding daybed samples because many were mixed samples and they were time-consuming to collect.

Because it is not known a priori how many wolves are in the pack, we cannot recommend precisely how many samples to collect, although 100 samples detected 90–100% of the pack members. Accumulation curves are useful in the laboratory analysis stage. Researchers can collect all samples, then extract and analyze DNA from them in a random order while plotting the accumulated unique genotypes. After the accumulation curve reaches an asymptote, researchers could discontinue genetic analysis with high confidence of sampling all individuals, thus saving time and money. Using this approach, we could have stopped analyzing samples for 3 of the packs and saved the time and money associated with $\sim$330 samples ($>$30% of the collected samples).

An NGS approach for studies of wolf pack relatedness is a promising development because all individuals in a pack can be sampled in a single visit to a rendezvous site. If biologists sample more wolf packs in this manner, long-standing questions about the prevalence and mechanism for multiple litters in the same year, multiple breeders, and inbreeding could be investigated further.

Conservation implications.—Several studies suggest that wolf populations can sustain 29–50% human-caused mortality without population-level reduction (Adams et al. 2008; Fuller et al. 2003), but substantial impacts on their social structure could result from these high levels of human-caused mortality (Haber 1996; Rutledge et al. 2010). We conducted our study before the wolf harvest commenced in Idaho; therefore, our evidence of very high intrapack relatedness will be a useful baseline to compare to the future social structure of these

packs subject to human-caused mortality from harvest. The addition of the 2009 Idaho wolf harvest to the other sources of mortality was sufficient to reduce the population by 2%, which was the 1st reduction in population size since wolves were reintroduced (Mack et al. 2010). Based on the findings of Rutledge et al. (2010), we suspect that harvest will decrease the kinship within packs. A better understanding of how pack structure contributes to population viability and fitness of wolves will be important to guide future wolf conservation and management.

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