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Research Article

Evaluation of Noninvasive Genetic Sampling Methods for Cougars in Yellowstone National Park

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ABSTRACT Conventional methods for monitoring cougar, *Puma concolor*, populations involve capture, tagging, and radio-collaring, but these methods are time-consuming, expensive, and logistically challenging. For difficult-to-study species such as cougars, noninvasive genetic sampling (NGS) may be a useful alternative. The ability to identify individuals from samples collected through NGS methods provides many opportunities for developing population-monitoring tools, but the utility of these survey methods is dependent upon collection of samples and accurate genotyping of those samples. In January 2003, we initiated a 3-yr evaluation of NGS methods for cougars using a radio-collared population in Yellowstone National Park (YNP), USA. Our goals were to: 1) determine which DNA collection method, hair snares or snow tracking, provided a better method for obtaining samples for genetic analysis, 2) evaluate reliability of the genetic data derived from hair samples collected in the field, and 3) evaluate the potential of NGS for demographic monitoring of cougar populations. Snow tracking yielded more hair samples and was more cost effective than snagging hair with rub pads. Samples collected from bed sites and natural hair snags (e.g., branch tips, thorn bushes) while snow tracking accurately identified and sexed 22 individuals (9 F, 13 M). The ratio of the count from snow tracking to the count from radio-telemetry was 15:24 in winter 2004, 13:12 in 2005, and 22:29 for both years combined. Annual capture probabilities for obtaining DNA from snow tracking varied considerably between years for females (0.42 in 2004 and 0.88 in 2005) but were more consistent for males (0.77 in 2004 and 0.88 in 2005). Our results indicate that snow tracking can be an efficient, reliable NGS method for cougars in YNP and has potential for estimating demographic and genetic parameters of other carnivore populations in similar climates. © 2011 The Wildlife Society.

KEY WORDS cougars, genotyping errors, hair snares, individual identification, noninvasive genetic sampling, *Puma concolor*, radio-collar, snow tracking, Yellowstone National Park.

Reliable information on populations is essential for successful conservation and management of many wildlife species. Carnivores such as cougars (*Puma concolor*) are particularly difficult to study due to their large home ranges, low densities, and secretive nature (Logan and Sweanor 2001). Conventional methods for monitoring cougar populations involve capture, tagging, and radio-collaring, but these methods are time-consuming, expensive, and logistically challenging. For difficult-to-study species such as cougars, noninvasive genetic sampling (NGS) may be a useful alternative. Extracted DNA from hair or scat can be used to identify and sex individuals, estimate abundance, distribution, and population growth rates, and examine patterns of genetic population structure (Foran et al. 1997, Woods et al. 1999, Mowat and Paetkau 2002, Palomares et al. 2002, Frantz et al. 2004, Long et al. 2008, Kendall et al. 2009). The ability to identify individuals from genetic samples provides many opportunities for developing population-monitoring tools, but the utility of these survey methods is dependent upon the collection of a representative sample from the population and the accurate genotyping of those samples.

Although NGS methods are gaining popularity, problems with sample collection and genotyping errors may be difficult to overcome (Taberlet et al. 1999, Mills et al. 2000). Scat sampling has been proven to be a useful method for identifying individual cougars (Ernest et al. 2000), but the merits of hair sampling have not been fully evaluated. Hair samples are difficult to obtain from some wild felid species (Downey

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et al. 2007, Ruell and Crooks 2007), but 2 collection methods show promise: hair snares and snow tracking (McDaniel et al. 2000, McKelvey et al. 2006, Ulizio et al. 2006). Neither hair snares nor snow tracking has been adequately tested for obtaining hair samples from cougars and little is known about the effectiveness and reliability of these methods.

Genotyping error is another problem that can exist with noninvasively collected samples. Noninvasive samples such as hair and scat contain low concentrations of DNA, which can lead to errors during polymerase chain reaction (PCR) and genotyping, particularly allelic dropout and false alleles (Taberlet et al. 1999). These errors can be difficult to track and quantify (Bonin et al. 2004, Broquet and Petit 2004). As individuals are usually identified on the basis of unique, multi-locus genotypes, genotyping errors can lead to generation of false DNA profiles and overestimation of population size (Waits and Leberg 2000, Creel et al. 2003). Numerous methods have been proposed to minimize and correct genotyping errors in population studies (e.g., Taberlet et al. 1996, Paetkau 2003, McKelvey and Schwartz 2004, Miquel et al. 2006, Scandura et al. 2006), but few wildlife studies have examined the reliability of genetic data by comparing genotypes from hair or scat samples collected in the field with genotypes from the same individuals produced from blood or tissue samples, which have much higher concentrations of DNA and therefore lower rates of genotyping errors (Bayes et al. 2000, Lathuilliere et al. 2001). These comparisons of NGS to independent field data are necessary to evaluate and validate the information derived from noninvasive sampling studies (Arrendal et al. 2007).

Long-term population research on cougars in Yellowstone National Park (YNP) provided an opportunity to evaluate NGS methods using a radio-marked population (Ruth et al. 2003). Our goals were to: 1) determine which DNA collection method, hair snares or snow tracking, provided a better method for obtaining hair samples for genetic analysis, 2) evaluate the reliability of genetic data derived from hair samples collected in the field, and 3) evaluate the potential of NGS for demographic and genetic monitoring of cougar populations.

STUDY AREA

We conducted NGS surveys on the Northern Range of Yellowstone National Park (NRYNP) during winter of 2003–2005. The NRYNP is characterized by steep, rocky slopes with primarily south and north facing aspects along the Yellowstone River corridor. Elevations ranged from 1,500 to 3,000 m although most surveys were limited to elevations below 2,400 m due to snow accumulation. Vegetation consisted primarily of grasslands interspersed with patches of Douglass fir (*Pseudotsuga menziessi*) and juniper (*Juniperus occidentalis*; Despain 1990). This region experienced cold, dry winters and provided habitat for many of the Park's ungulates, particularly elk (*Cervus elaphus*) and mule deer (*Odocoileus hemionus*; Frank and McNaughton 1992, Singer et al. 1994). This area supported a variety of large carnivores, which fed on the seasonal abundance of ungulates, including cougars, grizzly bears (*Ursus arctos*), black bears (*U. americanus*), wolves (*Canis lupus*), and coyotes (*C. latrans*). The study area was bordered by the Absoraka-Beartooth Wilderness Area and the Gallatin National Forest to the north and 1 paved road along the south.

METHODS

Cougar Capture and Radio-Collaring

In 1998, Ruth (2004*a*) initiated a study to determine the effects of wolf reintroduction on the population of cougars on the NRYNP. Ruth et al. (2008, 2010) captured, tagged, and radio-collared 83 cougars to determine survival rates, habitat use, prey selection, and predation rates following wolf reintroduction. Cougar capture, handling, collaring, and sexing procedures are described in Ruth et al. (2010). December through March, 1998 through 2005, T. Ruth (Hornocker Wildlife Institute/Wildlife Conservation Society, unpublished data) estimated the minimum number of cougars present each winter by surveying for cougar tracks in snow while traversing nonoverlapping transects (approx. 1,500 km each winter) and through intensive efforts to capture cougars (average = 226 person days/4–5 months of winter) following Murphy (1998). Ruth et al. (2010) permanently marked cougars with numbered colored ear tags and tattooed them with the same number in the opposite ear (Logan and Sweanor 2001). They pre-punched a hole in the center of an ear using sterile procedures prior to affixing ear tags. They macerated the removed tissue and placed it into a tube of lysis buffer and stored it at room temperature for future genetic analyses. Ruth also collected blood samples for use in pathologic and genetic studies (see Biek et al. 2006*a*,*b*). When time allowed Ruth also plucked hair from individuals captured during the 3-yr DNA study to aid with optimization of DNA extraction and PCR protocols. Ruth dried hair samples and stored them in silica desiccant to prevent bacterial degradation (Roon et al. 2003). Ruth et al. (2010) fitted each cougar with a very high frequency (VHF) collar (Telonics, Inc., Mesa, Arizona) or Global Positioning System (GPS) collar (Televilt/TVP Positioning AB, Lindesberg, Sweden) to obtain location and mortality data (Ruth et al. 2010). In 2006 Ruth ceased monitoring and removed any remaining collars.

Collection Methods

We synchronized the timing of hair snaring and snow tracking so that we could make direct comparisons of the 2 hair collection methods. We accessed survey sites primarily by foot and snowshoe travel from the south and west due to the limited road access. We allocated effort differently between the 2 collection methods across years in response to poor performance of hair snares in year 1.

Hair snares.– We conducted hair snaring from January through March of 2003 and 2004. We sampled in winter to allow direct comparisons with snow tracking and because cougar home ranges were more concentrated during this time. We delineated a NGS study area boundary of

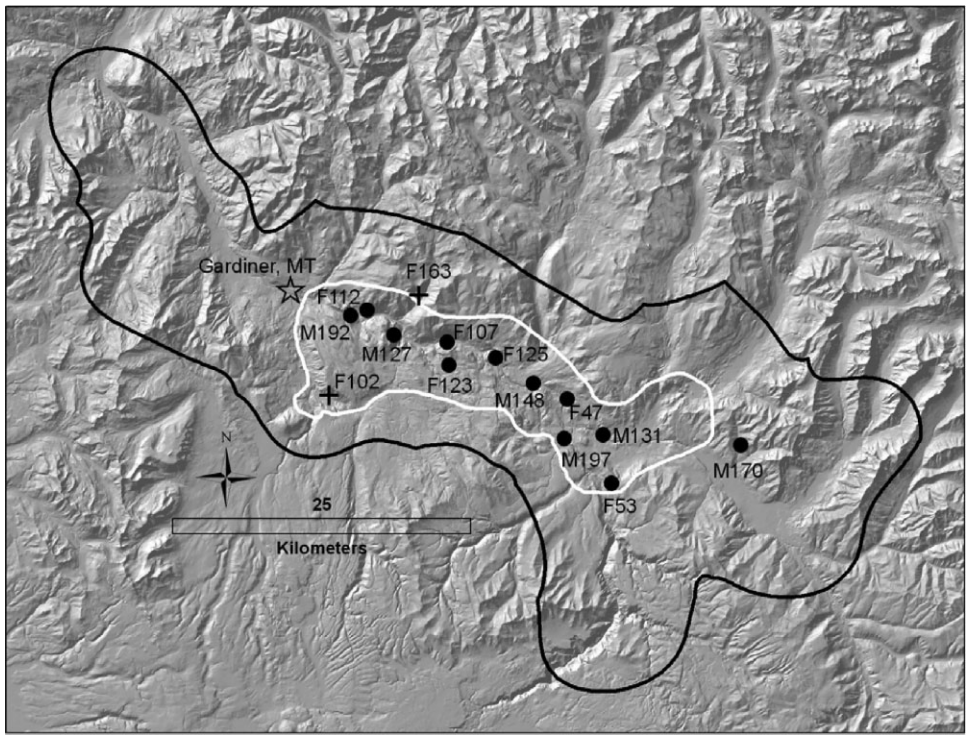

Figure 1. Map showing noninvasive genetic sampling (NGS) study area (white polygon) encompassed by the Yellowstone Cougar Project (YCP) winter study area (black polygon). Centroids for all adult cougars ($n = 14$) radio-located within the NGS study area during winter 2004 or 2005 are shown: closed circles represent adult cougars we detected from hair samples collected while snow tracking ($n = 12$), crosses represent adult cougars we did not detect with snow tracking ($n = 2$). Centroids are labeled with the animal's individual identity (ID) for the YCP and denoted F for females and M for males. We did not include on the map radio-collared cats not detected within the NGS study area during winter 2004 or 2005 ($n = 5$).

284 km^2 within the NRYNP study area based on detections of cougars from previous winters track surveys (Fig. 1). We deployed hair snares following the National Forest Lynx Detection Protocol (McKelvey et al. 1999). Hair snare sites consisted of carpet pads baited with a mixture of beaver castorium, catnip oil, and dried catnip along with an aluminum pie plate attached to a tree branch with wire and swivel. The 10-cm × 10-cm carpet pads had roofing nails driven through them to maximize collection of hair; we nailed pads to the base of a tree at a height of 30 cm. We used pie plates as visual attractants and carpet pads and bait to entice animals to rub their cheeks and deposit hair on the barbed pads. We established 5 of these stations per line transect oriented uphill to the point of origin. We overlaid a 3.2-km × 3.2-km point grid randomly over our study area to determine the point of origin for transects. We used the grid design to distribute effort across the study area and minimize capture variation. We chose grid size based on the Lynx Detection Protocol and the minimum winter home range size for female cougars in our study area (McKelvey et al. 1999).

In 2003, we deployed 73 hair snare transects consisting of 5 stations per transect. We set and maintained 365 stations from 9 January to 19 April 2003. We re-baited all stations at least once 10–14 days after installation and left them out for ≥20 trap nights. Station set-up required approximately 90 min/transect, re-baiting took 60 min/transect, and removal took 45 min/transect. During this first trial winter, we spent more time and money but collected fewer samples with hair snaring than with snow tracking (Fig. 2). Hair pad results prompted us to allocate more time to snow tracking during winters 2004 and 2005. In 2004, we deployed 40 hair pad transects consisting of 5 stations per transect. We placed snares in 10 locations frequented by cougars based on >10 yr of radio-telemetry data (Murphy 1998, Ruth 2003). We

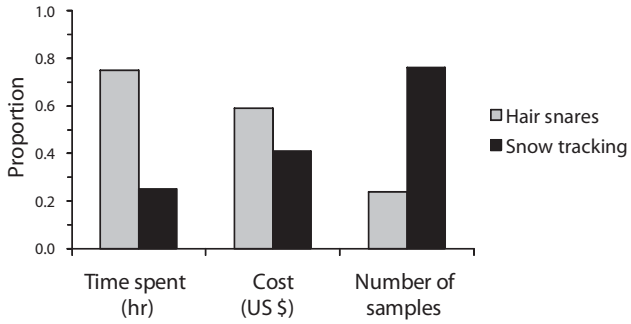

Figure 2. Hair snares versus snow tracking DNA collection methods: comparing proportions of time, cost, and sample yield for data collected during winter of 2003 in Yellowstone National Park, USA. We estimated time spent and costs due to the impossibility of differentiating between overlapping costs and time spent on the 2 methods. Expenses of $13,971.14 included hair pad station materials and costs that overlapped between hair snares and snow tracking such as collection materials, fuel for vehicles, stipends for 2 volunteers, and 1 graduate student salary. During the winter 2002–2003 sampling period, we placed more effort on hair snaring and less effort on snow tracking, although we also spent more money to conduct hair snaring. Snow tracking also yielded many more DNA samples. We compared the estimated time spent, estimated cost, and sample yield of the 2 methods and decided to allocate most of our effort to snow tracking for the following 2 field seasons, as it took less time, was cheaper, and yielded more samples.

checked and re-baited hair pads once a week during 15 January to 17 February, then every other week from 17 February to 25 March. We removed hair pads from the field by 25 March 2004. We deployed no hair snares in 2005 due to 2 yr of poor DNA collection success.

Snow tracking.– We conducted snow-tracking surveys to locate hair samples from January to March of 2003 and 2004 and from December to March 2005. We forward or back-tracked cougar tracks until we located a hair sample, tracking snow degraded to a level where tracks were no longer visible, or until time limited following the track further. Initially we found hair samples at only bed and kill sites, but during the second sampling season we discovered that careful trackers often found cougar hair within tracks and on natural hair snags such as thorn bushes, branch tips, and rock edges. During the period from January to March 2003 we looked for cougar tracks opportunistically while hair snaring. In winters 2004 and 2005 we used the hair snaring grid to guide our survey efforts but we focused effort on snow tracking. We converted the 3.2-km × 3.2-km point grid to cells by connecting the points.

We created 16 survey routes that we could walk in one day based on logistical considerations such as coverage of cells, travel efficiency, and vehicle access. We designed these routes to maximize probability of encountering a cougar track so we targeted rocky outcrops and creek bottoms. We used track surveys from capture and monitoring efforts in previous years to determine likely places to encounter cougar tracks; we used this information to build the NGS survey routes. We walked $\geq$1 km within each grid cell every 2 weeks and surveyed every route $\geq$6 times during the 2004 and 2005 field seasons. We determined Universal Transverse Mercator (UTM) coordinates of track locations and hair samples using a hand-held Garmin 12 Global Positioning System (Olathe, KS). We recorded all locations, survey routes, and backtracking segments on 1:25,000 scale maps. We used a Scalex PlanWheel (Carlsbad, CA) to determine the distance of the survey route and to measure tracking distances.

In 2003, we conducted snow track surveys opportunistically while en route to hair snare transects. We surveyed >948 km from 9 January to 19 April 2003. In 2004 and 2005, we used the 3.2-km × 3.2-km hair snare grid to guide our snow-tracking surveys. We surveyed >1,285 km from 27 December 2003 to 25 March 2004 and >1,140 km from 27 December 2003 to 20 March 2004.

Reliability of Noninvasive Genetic Sampling Data

We performed DNA extraction and PCR set up in a room isolated from amplified DNA with minimal movement of people and materials between facilities to prevent contamination of genomic DNA with amplified DNA (Paetkau 2003). We extracted DNA from blood using the Qiagen Blood Kit (Qiagen, Valencia, CA). We extracted DNA from tissue using the Qiagen DNeasy Tissue Kit. Before DNA extraction, we rinsed hair follicles with distilled water and placed them in microcentrifuge tubes. We used 5–10 follicles when possible but we also extracted DNA from single hair follicles when necessary. We finished the extraction using the user-developed protocol posted on the Qiagen website (Isolation of genomic DNA from nails and hair using the DNeasy Tissue Kit, unpublished protocol at http://www1.qiagen.com).

We evaluated 19 microsatellite loci developed for the domestic cat by Menott-Raymond et al. (1999). We chose a subset of 7 loci based on their heterozygosity and performance using blood or tissue samples taken from 42 individual cougars during capture. We genotyped all 42 individuals twice at all 7 loci to establish accurate genotypes for each individual. We also looked for Mendelian inheritance between mothers and litters as another way to validate genotypes. We used Program MM-dist (Kalinowski et al. 2006) to calculate allele frequencies and heterozygosities from blood and tissue samples. We used HW-QUICKCHECK to check for Hardy–Weinberg equilibrium (HWE, Kalinowski 2006). We chose loci Fca008, Fca057, Fca083, Fca096, Fca132, Fca205, and Fca293 for individual identification and sample quality screening based on their ability to amplify, heterozygosities, number of alleles, and fragment lengths (Buchan et al. 2005, Broquet et al. 2007). We used GENECAP (Wilberg and Dreher 2004) to detect genotyping errors in our capture-mark-recapture (CMR) data set and to calculate probability of identity *P*(ID) and probability of identity siblings (*P*(ID)sib; Waits et al. 2001) for the 7 loci. We then used an error-checking and removal procedure developed by Paetkau (2003) for use on poor-quality samples. We removed hair samples from further analysis if they failed to amplify at $\geq$4 loci. We also removed samples from analysis if they produced multiple alleles at $\geq$1 loci, an indication that the sample is mixed or contains hair from >1 individual (Roon et al. 2005). Two independent observers determined consensus genotypes based on strength and confidence in the results. Our approach differed from Paetkau (2003) in that we amplified each locus at least twice and only accepted consensus genotypes if 2 heterozygotes and 3 homozygotes were replicated and confirmed through visual inspection by both observers. We used Amelogenin and Zn-finger regions to determine genders for all blood, tissue, and hair samples (Pilgrim et al. 2005).

We used GENECAP to identify samples that produced multi-locus genotypes that differed by only 1 or 2 loci, a potential warning sign that a genotyping error occurred (Paetkau 2003). Once we had consensus genotypes for all 7 microsatellite loci, we ran the multi locus data set through GENECAP. If multi locus genotypes differed by 1–2 alleles, we either reran the loci that mismatched or scrutinized the electropheragrams from previous runs. Once we were satisfied that we had accurate genotypes, we ran the hair samples again along with the tissue samples through GENECAP to see if any of our hair samples produced multi-locus genotypes that differed from the capture samples by only 1–2 loci. This last step provided a robust test of the reliability of the noninvasive genetic data, as it directly compared genotypes from hair samples to those from blood and tissue samples.

We used 2 tests in Program DROPOUT to identify problem loci and evaluate the reliability of our final set of con-

sensus genotypes (McKelvey and Schwartz 2004, 2005). We used the Examining Bimodality test to detect if our CMR data set contained genotyping errors. This test assumes that in a population sampled in a manner that generates recaptures, some of the samples will be genetically identical, whereas the rest will differ (McKelvey and Schwartz 2004). An ideal sample free of errors produces a unimodal distribution with individuals being less likely to differ at many loci or few loci. Multi locus genotypes that differ at only 1 or 2 loci are an indication that those samples came from the same individual and that ≥1 genotyping errors occurred (Mowat and Paetkau 2002, Paetkau 2003). We identified problem loci by examining the effect of including and excluding loci on the number of individuals using the Difference in Capture History test (McKelvey and Schwartz 2005).

Comparison of Snow-Tracking and Radio-Telemetry Data

We produced 2 abundance measures for winters of 2004 and 2005: 1 count from hair samples collected while snow tracking (snow-tracking count) and 1 count from radio-telemetry data (radio-telemetry count). We defined the snow-tracking count as the total number of males and females detected via DNA analysis during a winter sampling period. We defined the radio-telemetry count as the total number of radio-collared males and females located within the study area at least once during a winter sampling period. We evaluated the efficacy of using snow tracking as an index of cougar population abundance by examining the ratio of the snow-tracking count to the radio-telemetry count. We also estimated annual capture probabilities (proportion of the population sampled in a given year) by dividing the snow-tracking count by the total count. We calculated total counts as the sum total of all unique individuals detected on the study area from radio-telemetry and DNA sampling adjusted by adding the number of unmarked individuals known to be on the study area from snow track surveys used to estimate percent accuracy (PA) following Logan and Sweanor (2001).

RESULTS

Collection Methods

Hair snaring proved ineffective for collecting cougar hair. During winter 2003, we collected 24 hair samples from rub pads, 5 of which appeared through visual examination of the hair to be from cougars. We also collected samples from coyotes, wolves, bears, and possibly bobcat (*Lynx rufus*). In 2 instances we located cougar tracks within 1 m of hair pads without the cat investigating or rubbing on the pad. In 2 other instances, cougars made kills within 30 m and 40 m of hair pads but did not rub on them. During winter 2004, we collected 15 hair samples from hair pads, only 1 of which appeared to have come from a cougar. We also collected samples from coyotes, wolves, bears, and possibly bobcat. None of the 14 samples collected from the hair snares in 2004 amplified at any locus indicating those hairs were not from cougars.

Table 1. Snow tracking effort for cougars in Yellowstone National Park, USA (2004–2005). We defined individual identity (ID) as a hair sample that amplified at enough loci to produce a multi-locus genotype and unique DNA profile.

Effort	*n*	$\bar{x}$ (km)	SD
Length of survey	231	10.5	4.38
Distance backtracked	67	1.09	1.44
Distance to DNA sample	64	0.32	0.42
Distance to individual ID	33	0.81	2.24

Snow tracking was an efficient and cost-effective way to sample the study population and obtain individual identities (ID's) from free-ranging cougars in YNP. During winter 2003, we did not conduct any surveys specifically to locate cougar tracks. Although we were only looking for tracks opportunistically while hair snaring, we were able to collect 12 cougar hair samples from bed or kill sites. We determined species identification of hair samples through both visual inspection and track identification of species; therefore all samples collected had a high probability of coming from a cougar. We were able to successfully backtrack to a hair sample 80% of the time when tracking conditions were favorable. During winter, 2004, we collected 81 cougar hair samples; 79% of hair samples came from bed sites and we collected 21% from natural hair snags, mostly broken branch tips and thorn bushes. We determined species identification of the samples through accurate track identification of species then confirmed these identifications by microsatellite analysis. During winter 2005, we collected 128 cougar hair samples; we collected 38% of hair samples from bed sites and 62% of hair samples came from natural hair snags.

During the 2 yr we concentrated on backtracking, 2004 and 2005, average distance backtracked from the start of a track to a sample was 1.09 km (Table 1). Average distance to a sample that produced an individual ID was 0.81 km. We spent an average of 2.25 hr backtracking each time we were able to follow a track. We spent an average of 1.09 hr following a track to a hair sample that resulted in an individual ID (Table 1). Average survey length was 10.5 km and average survey duration was 6.10 hr. We encountered cougar tracks on 34% of survey days, were able to follow 92% of tracks we encountered, and collected DNA from 88% of those backtracks (Table 2). Although our overall success rate for obtaining an individual ID from a survey was only 15%, we were able to obtain an individual ID from 49% of the tracks that we followed (Table 2).

Table 2. Snow tracking success for cougars in Yellowstone National Park, USA (2004–2005). We defined individual identity (ID) as a hair sample that amplified at enough loci to produce a multi-locus genotype and unique DNA profile.

Success	*n*1	*n*2	Proportion
Survey routes with cougar tracks	85	253	0.34
Tracks able to backtrack	78	85	0.92
Survey routes with ≥1 DNA sample	69	253	0.27
Backtracks with ≥1 DNA sample	69	78	0.88
Backtracks with ≥1 individual ID	38	78	0.49
Survey routes with ≥1 individual ID	38	253	0.15

Reliability of Noninvasive Genetic Sampling Data

To evaluate the reliability of genetic data generated from hair samples, we first assessed accuracy of our blood and tissue genotypes. Running HWQuickCheck (Kalinowski 2006) with blood and tissue genotypes indicated that none of the 7 loci departed significantly from HWE. We calculated *P*(ID) as 0.000003 and *P*(ID)Sibs as 0.0041, which were low enough for individual identification (Mills et al. 2000). Once we optimized PCR conditions, we detected no genotyping errors in the blood and tissue sample data set using GENECAP and DROPOUT. Results from these analyses were encouraging and validated our lab techniques. Additional evidence for accurate genotypes came from a test for Mendelian inheritance; all of the offspring from 11 marked litters shared $\geq$1 allele with their respective mothers. Both gender tests matched the genders we determined during capture of all 42 individuals.

After optimizing our protocols with blood and tissue samples, we genotyped 21 hair samples plucked from captured cougars. Nineteen (90%) of these samples produced IDs and 2 (10%) of them failed to amplify. Multi-locus genotypes from these hair samples matched the genotypes from blood or tissue samples from the same individual further validating our methods.

Of 112 samples collected from bed sites in 2004 and 2005, 112 (100%) were extracted, 44 (39.2%) produced IDs, 53 (47.3%) failed to amplify, and 15 (13.3%) were mixed (Fig. 3). Of 104 samples collected from natural hair snags, 97 (93.3%) were extracted and 7 (0.07%) contained inadequate material for extraction. Of the 97 samples that were extracted, 24 (25%) produced IDs, 68 (70.1%) failed to amplify, and 5 (0.05%) were mixed (Fig. 3). Of the 22 individuals identified from genetic samples obtained from snow tracking, we identified 13 from samples collected at bed sites, 2 from natural hair snags, and 7 from both bed sites and natural hair snags.

Differences in sample quality were apparent between bed site samples and natural hair snag samples. The number of follicles per sample, an indirect measure of sample quality (Goossens et al. 1998), was higher for bed site samples than for samples collected from natural hair snags. We placed samples into 3 subjective categories of low quality (0), fair to moderate quality (1–5), and good to high quality (6–10). Bed site samples had a much higher proportion of samples in the good to high category than did the natural hair snag samples (Fig. 4). Error-checking procedures aided in identifying and removing genotyping errors before they were incorporated into multi-locus genotypes (DNA profiles). Program DROPOUT did not identify any suspicious multi-locus genotypes. The Examining Bimodality test produced a plot of the differences in genotypes between hair samples collected through snow tracking, which showed a relationship indicating the sample was error-free, with no 1—locus mismatches and few 2—locus mismatches (McKelvey and Schwartz 2004). The Difference in Capture History test produced another plot with a flat horizontal line, which indicated that none of the loci were particularly problematic or more prone to genotyping errors. In other words, no individuals would be added or removed if a locus was included or excluded from our multi-locus data set.

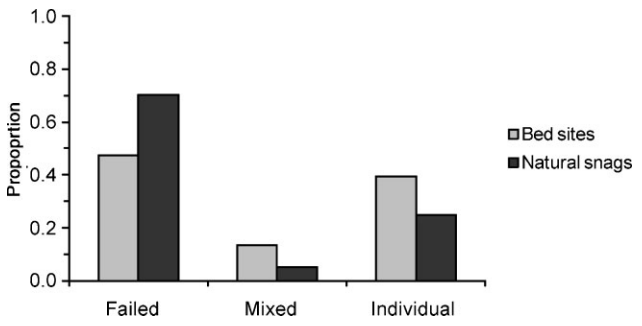

Figure 3. Results of DNA amplification of 209 hair samples collected during winters of 2004 and 2005 while snow tracking to bed sites or natural hair snags in Yellowstone National Park, USA. We defined individual identity (ID) as a hair sample that amplified at enough loci to produce a multi-locus genotype and unique DNA profile.

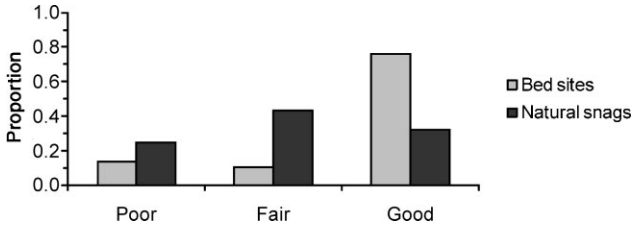

Figure 4. Proportion of cougar hair samples that were of poor, fair, or good condition collected from beds or natural snags in Yellowstone National Park, USA, during winters of 2004 and 2005.

Once we removed genotyping errors, we compared our final hair genotypes to blood and tissue genotypes. We identified 22 cougars (9 F, 13 M) from hair samples, 20 of which matched genotypes from blood and tissue samples. Genders from all 20 of these individuals matched the genders we determined from live capture. Two individuals genotyped from hair that did not have matching blood or tissue genotypes were identified as kittens from separate litters that we observed but never captured. We collected one of the hair samples from the kittens as a mortality while backtracking. The kittens had multi-locus genotypes that were unique and differed by $\geq$2 loci with any other individual. Both the Amelogenin and Zn-finger tests produced results indicating these 2 individuals were female. A test of Mendelian inheritance confirmed their putative mothers.

Comparison of Snow-Tracking and Radio-Telemetry Data

We detected 22 individuals through genotyping hair samples collected from bed sites and natural hair snags while snow tracking during winters of 2004 and 2005 (Table 3). We detected 15 individuals in 2004 (5 F, 10 M), 13 individuals (6 F, 7 M) in 2005, and 22 individuals (9 F, 13 M) for both years combined. The number of individuals we detected on the study area with radio-telemetry was 24 (12 F, 12 M) in 2004, 12 (6 F, 6 M) in 2005, and 29 (15 F, 14 M) for both years combined.

Table 3. Counts from snow tracking and radio-telemetry sampling and annual capture probabilities, *P*, for snow tracking. We calculated snow-tracking count as the total number of unique DNA profiles generated from hair samples collected while following cougar tracks in snow. We calculated the radio-telemetry count as the total number of individuals radio-located on the DNA sampling area during a winter. We calculated total counts by summing the total number of individuals known to have been on the study area from snow tracking and radio-telemetry at least once during a winter. We adjusted total counts by adding the number of unmarked individuals estimated to be on the study area using PA following Logan and Sweanor (2001); we added 1 unmarked male individual to the count in 2004 and zero unmarked individuals in 2005. We calculated annual capture probabilities for snow tracking by dividing the snow-tracking count by the total count.

	2004			2005			2004–2005		
Parameter	**F**	**M**	**Total**	**F**	**M**	**Total**	**F**	**M**	**Total**
Snow-tracking count	5	10	15	6	7	13	9	13	22
Radio-telemetry count	12	12	24	6	6	12	14	15	29
Total count	12	13	25	8	7	15			
Annual *P*	0.42	0.77	0.60	0.88	0.88	0.87			

We backlogged cougars into the winter population and estimated proportion of adults marked each year using the PA method of Logan and Sweanor (2001). An estimated 68% and 88% of adult cougars present in our study were radio-marked by winters 2000–2001 and 2001–2002, respectively, with 88–93% radio-marked in all subsequent years until the final year of study (Ruth et al., Hornocker Wildlife Institute/Wildlife Conservation Society, unpublished data). The ratio of snow-tracking count to radio-telemetry count was 15:24 in winter 2004, 13:12 in 2005, and 22:29 for both years combined (Table 3). Total counts were 25 in 2004 (12 F, 13 M) and 15 in 2005 (8 F, 7 M; Table 3). Using total count as population size, estimates of annual capture probabilities for females with snow tracking ranged from 0.42 in 2004 to 0.88 in 2005 (Table 3). Annual capture probabilities for males were 0.77 in 2004 and 0.88 in 2005 (Table 3). Although DNA analysis cannot determine age, an examination of our radio-telemetry data revealed that our hair samples detected 6 adult females, 6 adult males, 2 juvenile females, and 7 juvenile males (we pooled kittens and independent subadults together as juveniles). Over the 2-yr sampling period, we detected 12 of 14 (86%) adult individuals that were radio-located on the study area. One of the adult females that were not detected, F163, had a home range centered on the north side of the Yellowstone River, which is primarily south-facing and snow-free for much of the winter (Fig. 1). The only other adult that was radio-located on the DNA study area but not detected with DNA sampling was F102, who was only alive for the first season of sampling and had a home range that had little overlap with the DNA sampling area (Fig. 1). Radio-collar data also revealed that we had collected samples from 2 individuals, F53 and M148, after they died. In the case of adult female F53, we lost a set of cougar tracks due to poor tracking conditions and collected a sample from a nearby snow-free bed site. This hair sample, collected on 14 March 2004, produced a multi-locus genotype which matched the genotype from F53's blood sample even though she had been killed by wolves on 2 February 2004. In the case of M148, we lost a set of cougar tracks due to lack of snow and collected a hair sample from a nearby bed site located under a protected rock outcrop. We collected that hair sample on 18 February 2005 and it produced a genotype that matched M148's tissue sample taken during capture even though he died on 16 January 2004 due to injuries sustained during a confrontation with another male cougar.

DISCUSSION

Our evaluation supports previous research (Boulanger et al. 2004, Kendall et al. 2008, Ruell et al. 2009, Williams et al. 2009, Stetz et al. 2010) indicating NGS can provide a low-cost, long-term, population-monitoring tool for YNP and other management agencies, thus reducing the need for capturing and collaring cougars depending on management needs and objectives. Methods to identify individual cougars from their tracks (Smallwood and Fitzhugh 1993, Grigione et al. 1999, Lewison et al. 2001) and using counts of cougar tracks as a measure of abundance (Van Dyke et al. 1986, Van Sickle and Lindzey 1991, Beier and Cunningham 1996, Anderson and Lindzey 2005, Choate et al. 2006) have limited application for monitoring cougars, as those methods typically cannot accurately identify and sex individuals. Ours is the first study to identify and sex individuals using non-invasive hair sampling methods and evaluate the genetic data by comparing genotypes from hair with genotypes from blood and tissue and relating the number of detections to the number of cougars known to be on the study area from radio-telemetry.

Collection Methods

Although hair snares have the potential to be an effective hair collection method (e.g., lynx [*Lynx Canadensis*], gray fox [*Urocyon cinereoargenteus*]), they were not cost effective and yielded few samples ($n = 5$) using our sampling design (Fig. 2). Other studies have reported a similar lack of success with hair snares targeting felids (Downey et al. 2007). We limited hair snaring to winter, when cougars were restricted to lower elevation winter range, the population was assumed to be closed, and bears were primarily nonactive while in winter dens. However, colder air temperatures during winter may inhibit dispersion of scent lures, which could lower capture probability. Testing multiple visual and scent lures may be necessary to optimize this procedure for cougars. Research on whether seasonal variation affects behavioral response of cougars to scent lures could also better determine the usefulness of hair snare stations to obtain DNA from cougars. Further, cougars may avoid hair pads that have been

visited by wolves or bears, species that are dominant competitors (Murphy 1998, Ruth 2004*b*).

Snow tracking was an efficient and cost-effective way to sample the study population and obtain IDs from free-ranging cougars in YNP. The effort required to follow tracks in snow to hair samples was reasonable (Table 1). Average distance and time spent backtracking to a sample and to obtain a sample yielding an individual ID was small compared to the total time spent on a backtracking survey route. We encountered cougar tracks on 1 out of every 3 days and were able to follow most tracks that we encountered. Although our overall success rate for obtaining an individual ID from a snow-tracking survey was low, we were able to obtain an individual ID for many of the tracks that we followed. Forty-one surveys produced an individual identification and 7 of 41 surveys identified >1 individual. These data demonstrate snow tracking can be an efficient and productive NGS method.

Advantages and disadvantages exist for both hair snares and snow tracking. Advantages of hair snaring include applicability to other felines, success is unrelated to snow availability and snow conditions, and hair samples may be of higher quality. Disadvantages of hair snares include tendency towards nontarget species detections (Downey et al. 2007), equipment costs, and dependency on a specific behavioral response (rubbing face on carpet pad with nails).

The advantages of snow tracking include greater sample yield during winter, high detection probability along with auxiliary information about age (track measurements), and locating kills where bed sites are easily located with large quantities of hair. Another benefit of snow tracking is that >1 sample type can be collected; individuals have been successfully identified from blood, urine, and scat samples found while following tracks in snow (Valiere and Taberlet 2000, Flagstad et al. 2004, Scandura 2005). A disadvantage of snow tracking is that sampling is strongly influenced by snow conditions to the extent that individuals may be missed or, conversely, DNA from dead individuals may be collected. These omissions and inclusions may limit the accuracy of the method, yet even in light of the limitations, snow tracking was a better, more efficient method than hair snares at obtaining hair samples for genetic analysis in our study area.

Reliability of Noninvasive Genetic Sampling Data

Our results show that reliable genetic data can be derived from a population of free-ranging cougars using NGS if care is taken to remove problematic samples and genotyping errors. Schwartz et al. (2006) highlight the importance of removing genotyping errors from data sets to avoid an artificial increase in abundance estimates. The results of the Examining Bimodality and Difference in Capture History tests indicate that our final data set is free of genotyping errors and hence reliable for CMR purposes. Our comparison of the data derived from the hair samples with the data from the blood and tissue samples was a robust test of whether we had genotyping errors and false DNA profiles in our data set. Our use of 2 independent sex ID tests validated our methods since the results of the 2 tests agreed with each other for all samples. We also compared these results to the known genders from capture and those genders matched for every individual for which we had a blood or tissue sample ($n = 20$) further validating our methods. Both bed sites and natural hair snag samples detected unique individuals but bed sites appeared to be more productive, as they produced larger samples and had better amplification rates (Fig. 3). Samples collected from bed sites were higher quality than those taken from natural hair snags (Fig. 4) because when we located a bed site, there was usually ample hair for collection and extraction. The number of hairs found on a natural hair snag was limited by the short duration of exposure to the cougar, whereas cougars will often spend hours in one bed site and 3–6 days bedded near kills in between feeding bouts (Murphy 1998, Murphy and Ruth 2010). Even though bed site samples had higher amplification rates, they also had a higher proportion of mixed samples than did natural hair snag samples (Fig. 3). We speculate that this is due to the tendency of family groups to bed together and the affinity for certain bed sites by multiple individuals. Regardless of the limitations, our research has shown that snow tracking can be used to obtain cougar hair samples for genetic analysis and that those hair samples can produce reliable genetic data for individual identification and gender determination. Our results also suggest that hair samples collected from snow tracking could even be used to examine genetic structure in cougar populations similar to studies done with tissue such as Sinclair et al. (2001), Anderson et al. (2004), and McRae et al. (2005).

Comparison of Snow-Tracking and Radio-Telemetry Data

Snow tracking was an effective and reliable method for collecting genetic information on the minimum abundance and sex ratio of the population of cougars on the NRYNP. When designing the study, we focused our efforts on a small area that we intensively studied rather than a larger area with animals that we did not know as well but with a sufficient number of individuals for a CMR estimate. Because we did not design our study for abundance estimation, we correctly predicted that our data would be too sparse to reliably estimate abundance and capture probabilities using CMR methods. However, if our methods of collection are subsequently applied to a larger area, we expect that CMR estimates will be more precise than what would have been achievable previously.

We accurately identified 86% of the total count of male and female cougars from hair samples collected while snow tracking (Table 3). Yet, we also identified some limitations, in that we missed an individual overlapping the DNA study area, F163, due to lack of snow for tracking (Fig. 1). We included other individuals in the snow-tracking count after they had died. Optimizing sampling after heavy snows, at times when snow persists on all areas to be sampled, and only collecting hair in beds associated with fresh tracks should help minimize omission and inclusion. Yet, bed sites still have the potential to yield hair samples from multiple indi-

viduals, including those that may have died or emigrated prior to sampling.

We also supplemented our radio-telemetry data with DNA data by identifying and sexing 2 female kittens, 1 of which we removed from our counts as we collected it as a mortality. Although we had 2 false detections of cougars that were dead, we are confident that these situations could be avoided with careful sample collection and meticulous record keeping. Regardless, it is both remarkable and concerning that we were able to genotype a sample from M148 that was in the field for >13 months.

We documented greater heterogeneity in annual capture probabilities for female than male cougars, which could possibly be explained by improvements to our methods over time. However, we had surprisingly little variation for males, possibly a function of lower male densities and greater male movements, which are typical in a primarily nonhunted cougar social system (Logan and Sweanor 2001). Although our data were too sparse to use CMR modeling, the high capture probabilities we obtained from these methods suggest that a larger study area would yield adequate detections to provide precise abundance estimates. Further, these data are well suited for estimating population growth rates using open models such as those developed by Pradel (1996) and recently explored for grizzly bears in the northern Rocky Mountains of Montana (Stetz et al. 2010). Combining snow tracking with other methods such as biopsy darts, which were successfully used in Northwest Washington, USA to obtain tissue samples from cougars for individual identification and population enumeration (Beausoleil et al. 2008), may result in greater sampling coverage yielding more precise and less biased abundance estimates (Boulanger et al. 2008).

MANAGEMENT IMPLICATIONS

Successful conservation and management of cougars, and many other carnivore species, depends on the ability to reliably estimate demographic and genetic parameters for populations of interest. The results of our research have global applicability for managers interested in using NGS to monitor elusive carnivore species, though our findings may not be directly applicable everywhere and results may vary for different tracking substrates and conditions. We have shown that important parameters such as population size and sex ratio could be estimated by analyzing the DNA found in hair samples collected while following tracks in snow. We make the following recommendations to wildlife managers considering the use of snow tracking to monitor cougar or other carnivore populations: 1) conduct preliminary track surveys to determine the most likely locations and optimum snow conditions for finding tracks in a study area, 2) be sure to match a track with a corresponding DNA sample to avoid false detections of dead individuals, 3) take detailed measurements of tracks so that individuals identified from hair samples can be put into age classes, 4) follow tracks to bed sites whenever possible to maximize hair sample collection, but try to avoid collecting mixed samples from family groups, and 5) collect samples from natural hair snags opportunistically along the way to bed sites to increase sample yield and the number of individual identifications.

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