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The Efficacy of Obtaining Genetic-Based Identifications from Putative Wolverine Snow Tracks

TODD J. ULIZIO,[1] *Wildlife Biology Program, University of Montana, Missoula, MT 59812, USA*

JOHN R. SQUIRES, *United States Forest Service, Rocky Mountain Research Station, Forestry Sciences Laboratory, Missoula, MT 59801, USA*

DANIEL H. PLETSCHER, *Wildlife Biology Program, University of Montana, Missoula, MT 59812, USA*

MICHAEL K. SCHWARTZ, *United States Forest Service, Rocky Mountain Research Station, Forestry Sciences Laboratory, Missoula, MT 59801, USA*

JAMES J. CLAAR, *Carnivore Program, Region 1, United States Forest Service, Missoula, MT 59801, USA*

LEONARD F. RUGGIERO, *United States Forest Service, Rocky Mountain Research Station, Forestry Sciences Laboratory, Missoula, MT 59801, USA*

Abstract

*Snow-track surveys to detect rare carnivores require unequivocal species identification because of management and political ramifications associated with the presence of such species. Collecting noninvasive genetic samples from putative wolverine (*Gulo gulo*) snow tracks is an effective method for providing definitive species identification for use in presence–absence surveys. We completed 54 backtracks of approximately 1.4 km each and collected 169 hairs and 58 scats. Amplification rates of mitochondrial DNA (mtDNA) used for species identification were 62% and 24% for scats and hairs, respectively. The average distance traveled to collect a sample containing high-quality mtDNA for species identification was 1,330 m. Genetic analysis confirmed 35 snow tracks (64%) as wolverine. The remaining 19 snow tracks consisted of 8 that did not provide samples and 11 that contained nonamplifiable samples. Collection of both hairs and scats provided 28% more track verifications than would have occurred using only one type of sample. Collecting noninvasive samples from snow tracks also may provide individual wolverine identification that may provide a basis for obtaining minimum population estimates, relatedness tests, or mark–recapture population estimates given sufficient sample sizes. To that end, we analyzed nuclear DNA (nDNA) from the same samples to produce individual genotypes. Amplification rates of nDNA from scats and hairs ranged from 25% to 52% and 13% to 16%, respectively, and produced individual genotypes for 23 of the 54 snow tracks (43%).* (WILDLIFE SOCIETY BULLETIN 34(5):1326–1332; 2006)

Key words

detection, Gulo gulo, *mtDNA, nDNA, occurrence, snow tracks, survey method, wolverine.*

Snow-track surveys have been a common management and research method for determining species presence and distribution (Thompson et al. 1981, Beauvais and Buskirk 1999, D'Eon 2001). However, the subjective nature of making track identifications for sympatric species with similar track characteristics, such as midsized carnivores, is a major weakness of this method (Halfpenny et al. 1995, Prugh and Ritland 2005). Evidence to substantiate species identification has historically been limited to a suite of track measurements, photos, and track casts (Halfpenny et al. 1995), none of which are completely reliable. Potential for error is increased by the fact that field biologists often are faced with identifying tracks under a broad array of snow, track, and weather conditions. Sources of error are difficult to control, may produce ambiguous results, and ultimately, call into question the results of such surveys. Halfpenny et al. (1995) stressed that survey methods must provide unequivocal evidence that will hold up to scrutiny by the professional community and the court system because of potential economic and management ramifications associated with the reporting of rare species presence.

Noninvasive genetic sampling, which is the collection of genetic samples without capturing or handling animals, has provided new opportunities to study rare and elusive species (Taberlet et al. 1997, Sloane et al. 2000, Palomares et al. 2002, Eggert et al. 2003) and might provide opportunities to improve snow-track methods. However, we did not know if wolverines (*Gulo gulo*) naturally deposit scat and shed hair of suitable quality along their tracks at rates that would allow collection of these genetic samples to provide definitive identification. In addition to improving surveys for presence and distribution, genetic samples from snow tracks may be of adequate quality to allow identification of individuals that could provide a framework for monitoring populations. We applied noninvasive genetic sampling to snow-track surveys for wolverine in an effort to improve reliability of wolverine detection and monitoring data.

Analysis of mitochondrial DNA (mtDNA) from noninvasive hair and scat samples has been used to identify species (Foran et al. 1997*a*,*b*, Reed et al. 1997, Ernest et al. 2000, Mowat and Strobeck 2000). Noninvasive sampling also has been used to identify individuals within a species through analysis of nuclear DNA (nDNA), allowing for population estimates and population genetic analysis (Woods et al. 1999, Sloane et al. 2000, Palomares et al. 2002, Hedmark et al. 2004). Many analytical problems associated with low-quality or low-quantity DNA typical of noninvasively collected samples have been identified (Kohn and Wayne

[1] *E-mail: Todd_Ulizio@yahoo.com*

1997, Frantzen et al. 1998, Taberlet et al 1999, Mills et al. 2000*a*,*b*) and mitigated through laboratory and statistical techniques (Navidi et al. 1992, Taberlet et al. 1996, Alpers et al. 2003, McKelvey and Schwartz 2004).

Collecting genetic samples from snow tracks to identify species of forest carnivores has been reported for lynx (*Lynx canadensis*; Schwartz et al. 2004, Squires et al. 2004, McKelvey et al. 2006), wolverines (Flagstad et al. 2004), and coyotes (*Canis latrans*; Prugh and Ritland 2005). Flagstad et al. (2004) used scat samples collected along wolverine snow tracks in Scandinavia to determine individual genotypes and reported that nDNA quality (i.e., genotype success rate) was higher than reported for other species. Despite growing use of noninvasive sampling of snow tracks, we do not yet understand the distribution or relative quality of hairs and scats along wolverine tracks (i.e., frequency and location), nor the ability of field technicians to detect samples when they are present. Such understanding is necessary before sampling protocols can be developed for monitoring and management purposes.

Our goal was to evaluate the feasibility of using genetic samples collected from snow tracks (i.e., effort involved to collect samples and DNA amplification rates) to improve species identification for traditional snow-track surveys for wolverines. If so, this sampling method may provide individual identification that could be used to monitor populations. Our specific objectives were to 1) determine if hair and scat samples can be consistently collected from snow tracks, 2) quantify the required snow-tracking distance, 3) determine mtDNA amplification rates from samples to assign reliable species identifications of putative wolverine snow tracks, and 4) determine nDNA amplification rates to determine if samples from snow tracks can identify individual wolverines.

Study Area

The study area was located on the Beaverhead-Deerlodge National Forest and included the Pioneer, Beaverhead, Anaconda-Pintler, and Flint Creek mountain ranges in southwest Montana, USA (Fig. 1). Elevations ranged from approximately 1,830 to 3,500 m. The dominant forest cover was lodgepole pine (*Pinus contorta*). At lower elevations, Douglas fir (*Pseudotsuga menziesii*) and sagebrush (*Artemisia* spp.) steppe dominated south-facing slopes. Mixed Engelmann spruce (*Picea engelmanni*)–subalpine fir (*Abies lasiocarpa*) forests were found on wet aspects at higher elevations. Whitebark pine (*Pinus albicaulis*) occurred at the highest elevations near timberline. Riparian communities were dominated by willow (*Salix* spp.) that often transitioned into sagebrush-dominated meadows.

Methods

Backtracking and Sample Collection

We conducted ground-based surveys to detect wolverine snow tracks during the winters of 2003 and 2004 following protocols presented by Squires et al. (2004). We divided all potential wolverine habitats into a grid of 76 survey units,

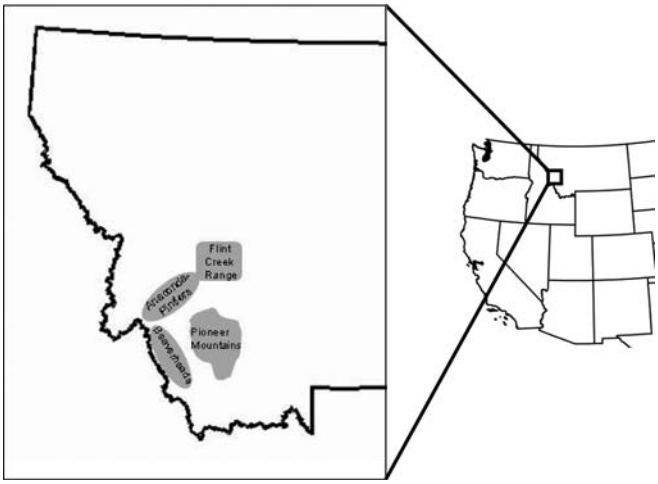

Figure 1. Wolverine study area in southwest Montana, USA, 2003–2004.

each measuring 8 × 8 km, and surveyed 10 km within each unit. We randomly sampled units without replacement until a census of units was completed. We repeated this census 2 times during each winter. We followed all putative wolverine tracks detected during surveys for approximately 2 km using snowshoes or backcountry skis depending on snow conditions, topography, and vegetation types. We collected samples (hairs and scats) from footprints, daybeds, foraging areas, tree boles, and coarse woody debris along the path of the animal. Technicians walked slowly alongside each track while visually scanning each footprint and tree bole along the path and sifted through the top layer of snow at daybed and foraging sites. We stored hair and scat samples in 50-ml vials (Fisherbrand, Houston, Texas) filled with silica gel desiccant (Reagent A.C.S. 10–18 mesh; Fisherbrand). We considered all hairs from a single location a single sample that may have consisted of a hair fragment, a single hair, or many hairs. We used the vial itself to scoop the sample from surrounding snow to prevent contamination. This technique prevented hair follicles frozen into the snow from being stripped off during collection, as well as contamination or the loss of the sample during transfer to the vial.

We stored vials containing hair samples in a dark area at room temperature prior to analysis. In 2003 we dried all scat samples in a paper bag over a lamp for 4–6 hours and stored them in fresh desiccant vials. In 2004 we preserved scats by soaking them in a 95% ethanol bath for 24 hours, draining, and leaving the cap vented until evaporation was complete. We delivered all samples to the genetics lab within 4 months of initial collection.

Mitochondrial DNA Analysis

We used genetic analysis of mtDNA to identify species for samples collected along snow tracks. We extracted genomic DNA from hair and scat samples according to manufacturer protocols (Qiagen Inc., Valencia, California) and determined species identification using restriction digest of a 442-bp segment of the *cytochrome b* region diagnostic for

wolverines (Riddle et al. 2003). All samples not producing wolverine identification using restriction digest were subsequently sequenced. We sequenced and analyzed both strands on a Li-Cor 4300 DNA imager (Li-Cor Biosciences, Lincoln, Nebraska) using standard protocols and compared sequences to reference samples collected by the lab or to sequences located in the National Institutes of Health GenBank.

Microsatellite Analysis

We screened genetic samples for individual identification by using only samples that had adequate mtDNA for species identification. Given the higher copy numbers per cell for mtDNA, this screening procedure reduced the cost and effort associated with analyzing poor-quality samples. We analyzed 15 wolverine tissue samples at 29 microsatellite markers to determine a panel of markers with adequate power to differentiate between individuals (P[id]; Waits et al. 2001) and minimize the shadow effect (Mills et al. 2000*a*), which is the probability that 2 different individuals are considered the same animal because they share identical genotypes at the markers examined. We selected Ggu216 and Ggu238 (Duffy et al. 1998), and Gg4, Gg7, Ma2, Ma8, and Tt4 (Davis and Strobeck 1998), and analyzed these 7 loci using a multitube approach (Navidi et al. 1992, Taberlet et al. 1996) with 3 repeats. Per locus genotypes were screened for consistency (Mowat and Paetkau 2002) and all samples that amplified at <4 loci were discarded. For samples with scoreable products at 4–6 loci, lab personnel re-amplified any missing or inconsistent loci 3 additional times. Samples were then rescreened and any samples not containing consistent scores at 6 or more loci were discarded. Per locus genotypes were accepted when analysis produced 3 consistent homozygote or 2 consistent heterozygote scores (Flagstad et al. 2004). Genotypes were compared to field records to determine the proportion of samples and backtracks that provided individual identifications. We also compared number of individuals detected and identified by snow tracking to those known to exist from our trapping and telemetry activities (Squires et al. 2006) during the same period and in the same study area to estimate how well the method detected wolverines.

Per Unit Effort Analysis

To quantify the snow-tracking distance required to collect genetic samples, we recorded the path of the animal and distance between samples using a Global Positioning System (GPS) unit (Trimble Geoexplorer 3). We collected GPS points every 7 seconds to create a line feature delineating the animal's track. In addition, we recorded all sample locations, daybeds, and foraging areas. Backtrack routes were delineated by hundreds or thousands of points with GPS error that overstated distances (D'Eon et al. 2002, Frair et al. 2004, DeCesare et al. 2005). Therefore, we smoothed the track to remove measurement bias without losing the true tortuosity of the track (DeCesare et al. 2005). We then used the "Surface Length" extension (Jenness 2004; Jenness Enterprises, Flagstaff, Arizona) for ArcGIS 8.3 to calculate 3-dimensional track distances to reflect the influence of slope on distance traveled in mountainous terrain.

Table 1. Qualitative categories for assigning wolverine snow tracks a relative condition class based on amount of snow in the track and degradation of the track due to environmental exposure in southwest Montana, USA, 2003–2004.

Condition category	Description
Good	Tracks appear fresh and are clearly visible Less than 10 cm of snow in track No melting or drifting No debris in footprints
Fair	10–20 cm of snow in track Track has some melting or drifting, but track outline is still intact Debris is present in footprints
Poor	More than 20 cm of snow in track Track is heavily degraded by melting or drifting of snow Track outline is obscured, making track measurements difficult

We calculated number of tracks that produced at least one sample and average distance between subsequent samples. We then factored in genetic results by using only amplified samples to calculate the frequency of samples capable of providing species and individual identifications.

We compared amplification rates for scats and hairs across collection locations to determine if the collection method should be refined to focus on a specific sample type or locations. Gagneux et al. (1997) and Goossens et al. (2004) suggested multiple hairs with follicles should be used for analysis to reduce error rates. We used logistic regression to determine if amplification rates for single hairs with and without follicles differed compared to multiple hairs with and without follicles.

We hypothesized that track condition could affect the observer's ability to detect a sample that was present, as well as actual hair-deposition rates by the wolverine. We tested this hypothesis by categorizing each track into 1 of 3 qualitative "track condition" groups (good, fair, and poor) based on track age, depth of snow in the track, and amount of debris in the track (Table 1). We used multivariate response permutation procedures (MRPP; Mielke et al. 1981, Zimmerman et al. 1985) to evaluate relationships between track condition and distance required to collect a hair sample.

Results

Sample Collection and Distribution Along Snow Tracks

We completed 54 backtracks during winters 2003 and 2004 that were 77 km in total length and averaged 1,430 m (range = 200–3,200 m). Variation in backtrack length was due to amount of time available to follow tracks, our ability to follow a track due to track condition or topography, and technician effort. We collected 169 hair samples and 58 scat samples from snow tracks and found 46 of 54 tracks (85%)

Table 2. Amplification rates of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) for all hair samples collected from snow tracks in southwest Montana, USA, 2003–2004.

Sample type	*n*	Amplification rates	
		mtDNA	nDNA
Single hair without follicle	70	11% (8/70)	3% (2/70)
Single hair with follicle	39	26% (10/39)	10% (4/39)
Multiple hairs without follicles	24	29% (7/24)	21% (5/24)
Multiple hairs with follicles	30	57% (17/30)	33% (10/30)

Table 3. Frequency and amplification rates of hair samples in each of 4 snow-track location categories from wolverine snow tracking conducted in southwest Montana, USA, 2003–2004.[a]

Location	Total no. of hairs	% hairs with wolverine mtDNA	% hairs with wolverine nDNA
Footprints	112	19	10
Daybeds	23	39	30
Tree boles	18	44	11
Foraging sites	16	13	6

[a] mtDNA = mitochondrial DNA; nDNA = nuclear DNA.

contained at least one genetic sample. We found hair samples in footprints ($n = 112$; 66%), daybeds ($n = 23$; 14%), on tree boles or other woody debris ($n = 18$; 11%), and in snow at foraging sites ($n = 16$; 9%). We collected scat samples from footprints ($n = 48$; 83%), foraging sites ($n = 8$; 14%), and daybeds ($n = 2$; 3%). The average distance between hair samples was 490 m (SE = 70), while average distance between scat samples was 1,435 m (SE = 341). When combined, the average distance between all genetic samples was 370 m (SE = 45).

Species Identification

The overall mtDNA amplification rate for all scat samples, regardless of species, was 74% (43 of 58). Of 43 scats that amplified, 22 belonged to wolverines (51%), while the others belonged to red fox (*Vulpes vulpes*; $n = 13$), coyotes (*Canis latrans*; $n = 5$), ungulates ($n = 2$), and marten (*Martes americana*; $n = 1$). The overall mtDNA amplification rate for hair samples, regardless of species, was 28% (47 of 169). Of 47 that amplified, 40 (85%) produced wolverine species identifications, with ungulates ($n = 3$), coyotes ($n = 2$), and squirrel (Sciuridae; $n = 2$) making up the difference. The presence of nontarget species in our sample confounded our ability to calculate exact mtDNA amplification rates for wolverine scats and hairs. We therefore calculated a possible range of values using 2 approaches. The first approach removed 20 scats and 7 hairs known to be from other species and assumed the remaining 200 samples were wolverine. The second, more conservative, approach removed 77 samples collected on tracks where multiple species were present and assumed the remaining 150 samples were wolverine. Our amplification rate for mtDNA from wolverine scats ranged from 59–65%, whereas hair ranged from 22–25%. Given the similarity in rates between methods, we averaged results together to produce amplification rates of 62% for scats and 24% for hair samples.

Thirty-five of 54 snow tracks (65% of all tracks, 76% of tracks with at least one sample) followed could be identified as wolverine based on genetic analysis of samples collected along tracks. Remaining tracks either lacked samples ($n = 8$) or samples contained inadequate mtDNA to provide species identification ($n = 11$). Multiple species often were present along the same tracks ($n = 18$ tracks), with all but one of these tracks containing at least one wolverine sample. Other species represented along tracks were red fox ($n = 8$ tracks), ungulates ($n = 5$ tracks), coyotes ($n = 4$ tracks), and marten ($n = 1$ track). We removed nonamplified samples and recalculated the per unit effort required to collect a sample capable of providing species identification. Average distance between amplified hairs was 2,025 m (SE = 454), whereas amplified scats were 3,990 m (SE = 981) apart. For scats and hairs combined, average distance between any amplified samples decreased to 1,330 m (SE = 212).

Samples containing multiple hairs had a higher amplification rate ($P = 0.001$, df = 2, $n = 160$) than single-hair samples, whereas hair samples with at least one follicle had higher amplification rates (39% amplification rate, $P = 0.021$) than hair samples or fragments without follicles (16% amplification rate; Table 2). Similar distances were required to collect a hair sample for good (293 m) and fair tracks (299 m), but differed moderately ($P = 0.59$) for poor tracks (585 m) due to decreased visibility of samples along the track. Stronger differences existed between amplification rates of hair samples in each track condition ($P = 0.002$), increasing the average distance to an amplifiable hair sample to 813 m for good tracks, 1,145 m for fair tracks, and 3,500 m for poor tracks. The proportion of good, fair, and poor tracks that provided samples was 100%, 95%, and 61%, respectively. Amplification rates of mtDNA from hair samples varied by location along the track in which they were collected, with hairs from tree boles and daybeds producing the highest rates (Table 3).

Microsatellite Analysis

We used the same approach for calculating overall nDNA amplification rates as we did for mtDNA, resulting in a range of 25% to 52% for scats and 13% to 16% for hairs. To reduce analysis costs, we limited our nDNA analysis to only those samples that had adequate mtDNA for species identification. From this subsample, we produced genotypes for 64% of the 22 scats and 53% of the 40 hairs. Samples providing genotypes were well distributed among backtracks, allowing us to produce a reliable individual identification for 23 of 54 backtracks (43%). The per unit effort required to collect a sample capable of providing individual identification was 2.3 km/sample. Collecting only scats capable of individual identification would have required an average of 7.7 km/sample, while collecting only hairs would have required 3.7 km/sample. In 2003 we used limited snow tracking to detect 4 of 6 wolverines that were known to be present as a result of more intensive trapping efforts, as well as one new individual that had not yet been

captured. In 2004 we detected 6 of 8 wolverines known to be present, while also identifying 3 new wolverines in areas outside our trapping coverage.

Discussion

Using Backtracks to Determine Species Identification

Our ability to verify 65% of all tracks resulted in a high probability of confirming wolverine presence as long as multiple tracks are encountered over the course of the larger survey effort. Although tested on a snowmobile–snowshoe–ski-based survey method (Squires et al. 2004), this verification technique could be used to verify snow tracks detected using any ground or aerially based survey method. Highly degraded tracks that would otherwise be difficult to identify might now be reliably included in surveys. McKelvey et al. (2006) recognized that this would enable biologists to obtain species identification from otherwise ambiguous tracks and greatly expand the conditions under which snow-tracking surveys could be conducted, increasing the efficiency and representativeness of surveys. Even though good- and fair-quality tracks produce more amplifiable samples per kilometer than poor-quality tracks, at least one sample was found on 61% of poor tracks; we suggest these tracks be included in snow-tracking efforts. Samples in footprints and foraging sites on poor tracks were obscured by snow and debris, yet samples in daybeds and on tree boles were more persistent and remained visible even on the worst of tracks.

The average distance to collect an amplifiable genetic sample (1.3 km) from a wolverine track was comparable to the rate of 1.21 km reported by McKelvey et al. (2006) for lynx snow tracks. However, our mtDNA amplification rates of 62% and 24% for scats and hairs, respectively, were lower than the 98% and 81% for lynx scats and hairs. While reasons for differences are unclear, we suspect they may be due, in part, to differences between species, how well microsatellite markers match their DNA, environmental conditions, and collection of higher-quality hairs at daybeds by McKelvey at al. (2006) rather than collection of all hair samples.

Amplification rates for mtDNA collected using snow tracking were much higher for scats than hairs. However, we encountered hairs 3 times more frequently than scats and, thereby, they required less effort to collect. Although many researchers (Foran et al. 1997*a*,*b*, Gagneux et al. 1997, Goossens et al. 1998) limit sampling to scats or multiple hairs with follicles to ensure quality, we believe the increase in overall verification rates from using all available genetic samples, including hairs without follicles, outweighs additional genetic analysis costs when dealing with infrequent tracks for rare carnivores.

We support this line of reasoning from the perspective of collecting only a single sample type versus collecting both sample types. Had we collected only scat, we would have provided species identification for 20 of the 54 tracks (37%). If we collected only hair, 22 tracks (41%) would have produced wolverine species identifications. By collecting both sample types, 35 (65%) tracks produced species identification, which is a 24–28% increase. Sample amplification rates need to be considered in unison with sample collection rates when developing a sampling protocol. Although hair samples had lower DNA quality than scat, we collected 3 hair samples for every 1 scat sample, resulting in the 2 sample types performing almost identically (37% vs. 41%) regarding number of wolverine backtracks confirmed. By collecting both types of samples, we not only confirmed a higher percentage of overall tracks, but we reduced the average distance required to find a high quality sample from 4 km for scats to 1.3 km for both types.

Hair samples are more difficult to observe in snow than scat samples. McKelvey et al. (2006) collected hair samples in high-probability locations, namely daybeds and foraging sites, and ignored potential samples from animal footprints. Flagstad et al. (2004) ignored hairs altogether. Even though scanning all footprints to collect hair samples from footprints requires greater attention and a slower observer pace, we found this was worth the effort because footprints provided 66% of all hair samples. Hairs from footprints accounted for 53% of hair samples providing mtDNA species identification and 52% of hair samples providing nDNA-based individual identifications. Samples collected from tree boles and daybeds had higher amplification rates (Table 3) than samples from other locations, which likely is due to plucking of hairs with follicles at these 2 locations rather than sloughing of hair. However, the infrequency of daybeds or tree boles with hair samples would require excessive effort to collect samples only from these locations. Thus, collecting hair from footprints is an important consideration for any snow-track protocol given the substantial improvement in collection and identification rates.

Individual Genotypes

Although no published data exist on nDNA amplification rates for wolverine hair, our rate of 25–52% for scat is lower than the 50–70% reported in Scandinavia (Flagstad et al. 2004; O. Flagstad, Uppsala University Sweden, personal communication). All analysis protocols were consistent with those reported by Flagstad et al. (2004), and differences in sample preservation methods should not have produced differences in amplification rates (Frantzen et al. 1998). Even with these lower rates, we produced genotypes from 43% of snow tracks and were able to identify 8 of 10 (80%) individuals known to be present. Although the effort and coverage of snow tracking was not directly comparable to other research-related trapping activities in the study area, the detection rate of 80% of individuals using snow tracking was higher than anticipated. Based on these findings, we suggest this snow-tracking method is capable of providing useful data for minimum population estimates, individual relatedness, and mark–recapture population estimates (Flagstad et al. 2004, Bellemain et al. 2005) that may ultimately provide a framework for better monitoring and management of wolverines.

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Todd J. Ulizio (photo) works with the United States Forest Service (USFS) Rocky Mountain Research Station (RMRS) in Missoula, Montana, studying wolverine ecology in southwest Montana. He received his B.S. in 1999 and his M.S. in 2005 in wildlife biology at the University of Montana. His work has focused on carnivore ecology and management, with an emphasis on detection and monitoring of wolverine, lynx, and fisher. ***John R. Squires*** is a Research Wildlife Biologist with the USFS Rocky Mountain Research Station located in Missoula, Montana. He studied the effects of oil development on prairie falcons in north central Wyoming for his M.S. degree and trumpeter swan ecology in the greater Yellowstone area for his Ph.D. at the University of Wyoming. From 1991–1997, he was a resident research biologist for the Rocky Mountain Research Station studying seasonal changes in the habitat-use patterns of northern goshawks nesting in Wyoming. He now serves as the team leader for Threatened–Endangered–Sensitive species research in the RMRS Wildlife Unit in Missoula, and is currently studying lynx and wolverine ecology in Montana. ***Daniel H. (Dan) Pletscher*** is a professor and director of the Wildlife Biology Program at the University of Montana. He holds a B.S. in wildlife management from the University of Minnesota, an M.S. in wildlife biology from Kansas State, and a Ph.D. in forestry from Yale University. He has served as President of the Montana Chapter of The Wildlife Society and chaired the steering committee for the 1999 International Wildlife Management Congress in Hungary. His research interests focus on predator–prey dynamics and endangered species. ***Michael K. Schwartz*** is a research ecologist with the RMRS in Missoula, Montana. He obtained a Ph.D. at the University of Montana and is currently the team leader for the conservation genetics team at RMRS. Aside from his genetics program duties, he is finishing several ecological field studies on fisher, lynx, and wolverine. ***James J. Claar*** is the carnivore program leader for the Northern Region, United States Department of Agriculture Forest Service in Missoula, Montana. He has a B.S. in Wildlife Biology from the University of Montana and an M.S. in Wildlife Biology from the University of Idaho. His areas of interest include threatened and endangered species management and in particular, conservation of grizzly bears, gray wolves, canada lynx, wolverine, and fisher. He serves as the national USFS coordinator for Canada lynx and wolverine conservation programs. ***Leonard F. (Len) Ruggiero*** is the Research Unit leader for the Ecology and Management of Wildlife and Habitats at the USFS Rocky Mountain Research Station in Missoula, Montana. He received his B.S from Rutgers University, his M.S. from Virginia Polytechnic Institute, and his Ph.D. from Utah State University.

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