Biological Conservation 229 (2019) 50–58

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques☆,☆☆,☆☆☆,☆☆☆☆

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ARTICLE INFO

Keywords:
eDNA
qPCR
Monitoring
Carnivores
Noninvasive

ABSTRACT

The management of rare species is a conservation priority worldwide, but this task is made difficult by detection errors in population surveys. Both false positive (misidentification) and false negative (missed detection) errors are prevalent in surveys for rare species and can affect resulting inferences about their population status or distribution. Environmental DNA (eDNA)—DNA shed from an organism in its environment—coupled with quantitative PCR (qPCR) analyses, has become a reliable and extremely sensitive mean for identifying rare species in aquatic systems. Due to the demonstrated effectiveness of these methods, we tested their efficacy in surveys for rare species in terrestrial settings to reduce detection errors for three rare forest carnivores of conservation concern: Canada lynx (*Lynx canadensis*), fisher (*Pekania pennanti*), and wolverine (*Gulo gulo*). We specifically investigated our ability to reliably: 1) identify species directly from snow samples collected within tracks; 2) identify species by collecting snow in locations where an animal had been photographed; and 3) identify species from hair samples collected during the summer after being deployed throughout the winter (i.e., overwinter surveys). Our findings indicated that qPCR assays can effectively detect DNA of all three species, including from snow-track surveys, snow collected at camera stations, and overwinter samples that failed to amplify with conventional PCR techniques. All results indicate that the sources of targeted DNA collection provided adequate quantities of DNA for robust species detection. We suggest that using qPCR methods to detect DNA has the potential to revolutionize winter surveys for rare species in terrestrial settings by reducing or eliminating misidentifications and missed detections.

☆ Declaration of interest: The authors declare no financial or other conflict of interests.

☆☆ Acknowledgements: We would like to thank the field crews from Rocky Mountain Research Station, Idaho Department of Fish and Game, MPG Ranch, Beaverhead-Deerlodge National Forest, and Nez Perce-Clearwater National Forest for bait station support and collection of snow samples.

☆☆☆ Funding: This study was funded by the USDA Forest Service Northern Region and MPG Ranch.

☆☆☆☆ Role of funding: The funding sources had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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https://doi.org/10.1016/j.biocon.2018.11.006
Received 7 August 2018; Received in revised form 20 October 2018; Accepted 3 November 2018

1. Introduction

Wildlife conservationists, federal and state agencies, and non-governmental organizations spend significant amounts of time and money managing rare species (e.g., Miller et al., 2002; McCarthy et al., 2012). Rare carnivores are a group of rare species that often have formal legal protections, garner strong public attention, and drive management and conservation decisions. Reliable detection and monitoring information is therefore an important component of these decisions (Zielinski and Kucera, 1995; Reynolds et al., 2016). However, obtaining reliable information about rare carnivore populations to inform decisions is challenging due to their low densities, which make them difficult to detect (Thompson, 2004). Nevertheless, many efforts to detect or understand the ecology of rare carnivores have been successful in documenting distribution (e.g., Squires et al., 2004) or presence (McKelvey et al., 2006; Ulizio et al., 2006).

Over the past two decades, noninvasive survey methods—defined here as track, camera, or noninvasive genetic sampling—have revolutionized rare carnivore surveys (e.g., Long et al., 2008). Prior to the development of these methods, there was limited information on rare carnivores (Zielinski and Kucera, 1995; Long et al., 2008). In most boreal and temperate forests, noninvasive carnivore survey methods conducted in winter offer many advantages. For example, carnivore snow-tracks are common and easily located in winter. Canada lynx (*Lynx canadensis*) travel 1–9 km per day, leaving large numbers of tracks which can result in a 95% probability of detecting a Canada lynx if it is present given appropriate snow conditions and survey design (Squires et al., 2004, 2012). In addition, most carnivores are reliably attracted to baited stations in winter, resulting in high detection rates (e.g., Mulders et al., 2007). These methods, combined with the advantages of conducting surveys in the winter season, have allowed new insights into carnivore ecology and monitoring (e.g., Squires et al., 2004; McKelvey et al., 2006; Ulizio et al., 2006).

However, winter noninvasive surveys for rare carnivores present many challenges. Noninvasive methods, like all wildlife survey methods, are subject to two major detection errors: false positives (species misidentifications) and false negatives (missed detections). Snow-track surveys and camera traps are particularly vulnerable to misidentifications (e. g. Heinemeyer et al., 2008, see Box 3.1, Clare et al., 2017). Some species, such as fisher (*Pekania pennanti*) and marten (*Martes caurina* or *M. americana*), cannot be reliably separated via snow-tracks (Zielinski et al., 2006; Zielinski and Truex, 1995), leading to high levels of misidentification (Aubry et al., 2017; Aubry and Lewis, 2003; Clare et al., 2017). Other visually similar species, such the Canada lynx and bobcat (*Lynx rufus*), cannot always be reliably distinguished from one another in still or video images captured by cameras (Nielsen and McCollough, 2009). Winter noninvasive methods are also vulnerable to missed detections for a variety of reasons, including the rarity of the species of interest on the landscape, the failure of baits or scent lures to attract the target species, and the failure of detection devices (e.g., cameras, track plates, hair snares) to document species presence (Gompper et al., 2006).

These errors can significantly affect resulting inferences about population status (MacKenzie et al., 2005; McKelvey et al., 2008) and even delay conservation actions. When the target species is rare, even low rates of misidentification can lead to erroneous conclusions (McKelvey et al., 2008). As a result, misidentification of the relatively common Pacific marten (*M. caurina*) as the rarer fisher in the Pacific states masked a precipitous contraction in geographic range for the fisher, delaying appropriate conservation actions (Aubry and Lewis, 2003; McKelvey et al., 2008). Misidentifications not only inflate range estimates, but also lead to inaccurate niche models (Aubry et al., 2017). Surveyors can account for missed detections with field methods including distance sampling, multiple observer methods, repeat visits, or time removal methods (Golding and Dreitz, 2016, see Table 1) and modern statistical modeling tools such as Bayesian hierarchical models (e.g., Royle and Nichols, 2003; Royle and Dorazio, 2008; Kéry and Schaub, 2012; Iknayan et al., 2014; Guillera-Arroita, 2017, see Table 4) that can explicitly account for missed detections. However, high rates of missed detections diminish the reliability of inferences derived from a sampling effort, particularly for small populations (Menkens and Anderson, 1988). As a result, there has been a tremendous amount of research to address these errors and improve the reliability of surveys for rare species (Gu and Swihart, 2004; Kéry and Schmidt, 2008; Zipkin et al., 2010).

Surveyors employ a variety of techniques to reduce these negative consequences and diminish these sources of error in winter noninvasive surveys (Table 1). To reduce track survey misidentification, surveyors use backtracking to locate genetic samples (i.e., scats or daybeds) (McKelvey et al., 2006; Ulizio et al., 2006). This can often require snowshoeing many kilometers off trail in uncompacted snow (e.g., Squires et al., 2012). As a potential alternative to backtracking, Dalén et al. (2007) extracted DNA from arctic fox (*Alopex lagopus*) tracks using conventional PCR techniques typically applied to scat and hair samples, but had limited success (16.7%, 1/6). Misidentifications from photographs can be reduced by using hair-snaring devices in conjunction with camera sets to collect genetic samples (Kendall and McKelvey, 2008; Moriarty et al., 2009). Unfortunately, this requires behavioral responses by the target organism (e.g., climbing a tree to access bait) and not all animals respond reliably (e.g., Robinson et al., 2017). Misidentifications can also be accounted for with modeling techniques (e.g., Miller et al., 2013), but this requires a data stream of known species identifications, which is not always available. To reduce missed detections and quantify detection probability, most survey designs require multiple visits (e.g., MacKenzie et al., 2005). But because researchers often have difficulty accessing terrain in winter surveys, their ability to implement multi-visit protocols varies greatly across space and time, which can leave large roadless areas unsurveyed due to inaccessibility. In addition the utility of genetic samples derived in all noninvasive surveys is not certain due to degradation over time or amount of DNA in the sample (e.g., Taberlet et al., 1999; McKelvey et al., 2006). Degradation can be partially overcome with a 2-week sampling interval, which also may limit the number of individuals that visit the station and provide a more accurate population assessment (Clare et al., 2017). However, this effectively limits DNA-based winter surveys to areas that can be accessed relatively easily and safely, potentially resulting in a landscape sampling bias. All of these techniques to reduce error are costly or time intensive and therefore not always possible to accomplish.

Environmental DNA (eDNA), a rapidly developing research tool, has transformed the detection of rare species in aquatic systems, and may provide a sensitive and reliable technique for detecting rare carnivores in terrestrial environments. While eDNA is usually of insufficient quality to provide individual or sex identification, the genetic material is sufficient for reliable species detection and identification via quantitative PCR (qPCR), which minimizes misidentification and missed detection errors associated with traditional sampling. Species-specific eDNA assays analyzed with qPCR methods are extremely sensitive, and can detect even a few copies of DNA with high reliability (Dysthe et al., 2018; Wilcox et al., 2013), making this an effective method for addressing missed detections. For example, Jane et al. (2015) placed five small fish in cages and sampled the water at 50 m intervals, with a maximum downstream limit of 240 m. The analysis of eDNA via qPCR detected the caged fish in all 162 eDNA samples. As a result of this sensitivity, eDNA sampling has proved effective for delineating distributions of rare species (McKelvey et al., 2016; Spear et al., 2015), detecting invasive species (Franklin et al., 2018; Thomsen et al., 2012), and detecting species that are difficult to sample using traditional approaches (Taberlet et al., 2012). Environmental DNA assays reduce missed detections resulting from samples dominated by non-target DNA, because properly designed assays are species-specific (Wilcox et al., 2013). In one study, eDNA methods successfully detected

Table 1
Rare carnivore species survey error types, rates, and ways errors have been addressed. All survey methods currently employed to study rare species have high false negative detection rates. Additionally, snow-track surveys have high false positive detection rates.

Survey method	Species misidentification (false positive)			Missed species detection (false negative)			Bias (sampling errors)		
	Rate	Cause	How it is addressed	Rate	Cause	How it is addressed	Rate	Cause	How it is addressed
Camera	Varies	- Unclear picture of species (indistinguishable from closely related species – i.e., lynx/bobcat)	- Add noninvasive genetic component to survey	High	- Species not present during sampling period - Camera malfunction (ex. fogging in cold weather)	- Statistical methods in study design and data analysis - Unable to address	High	- Inability to access sites in winter - Improper camera setup/ camera failure	- Overwinter scent station - Frequent visits to inspect photos
Noninvasive genetic sampling	Very low	- Contamination/error	- Negative control	High	- Species not present during sampling period - DNA obtained but too poor quality to amplify to species - Mixture of hair samples fails to detect all species	- Statistical methods in study design and data analysis - Re-extract DNA or unable to address - Unable to address	High	- Inability to access sites in winter	- Not developed
Snow-track	High	- Misidentification of track	- Add noninvasive genetic component to survey	High	- Species did not walk in survey area - Tracks not identifiable	- Statistical methods in study design and data analysis	High	- Inability to access sites in winter - Lack of appropriate weather to allow track identification	- None

common carp (*Cyprinus carpio*) even though this species' DNA represented ≤0.0004% of the total DNA present in the sampling area (Turner et al., 2014). The specificity of this method also minimizes misidentification errors (Wilcox et al., 2013). Other sources of misidentification, specific to eDNA, exist (see Bohmann et al., 2014; Box 2), but the degree to which these sources are problematic is highly dependent on sampling circumstances and study design. For example, eDNA may be associated with dead organisms (Kamoroff and Goldberg, 2018), but the terrestrial methods we are evaluating (e.g., snow-tracks) are closely associated with animals known to have recently been alive. When thinking about terrestrial applications of eDNA methodologies, it is important to note that the sensitivity and specificity are associated with the application of qPCR assays that can be applied to DNA samples whether formally associated with eDNA or from other methods.

In this paper, we determine the potential of applying state-of the-art eDNA and qPCR methods to the detection of rare carnivores in winter noninvasive survey methods. Specifically, we investigated our ability to reliably identify species: 1) from snow-tracks (thereby reducing the rates of misidentification in track surveys); 2) detected via camera traps by collecting snow samples from the location where the animal was photographed (thus reducing rates of misidentification from camera detections); and 3) from genetic samples considered too poor in quality to achieve this goal using methods relying on conventional PCR methods. To address these questions, we selected three rare carnivore species of conservation concern: Canada lynx, protected under the Endangered Species Act since 2000 (USFWS, 2000); fishers, petitioned for listing in 2009 and 2013 (USFWS, 2010, 2016a); and wolverines, petitioned for listing in 1994 and 2013 and currently proposed as threatened (USFWS, 2016b).

2. Methods & materials

2.1. Snow-track testing

Snow-tracks were found opportunistically, primarily by crews involved in other winter carnivore surveys and trained to identify the tracks of our target species from 31 January 2018 to 6 April 2018. Snow samples associated with all putative lynx tracks and most putative wolverine tracks were collected by crews hired to capture and collar Canada lynx in the Clearwater River drainage approximately 6–18 km from Seeley Lake, Montana (Fig. A1). Lynx snow-tracks were collected during the exploratory phase of the lynx survey and, to minimize the risk of contamination, the collection of snow-tracks ended once lynx trapping began. Wolverine snow-tracks were collected opportunistically throughout the survey, but collection ceased after a wolverine visited one of the lynx traps, again to minimize potential contamination. Snow-track collection ceased once target animals were handled because of the risk of transferring the animal's DNA to clothing and equipment, and from clothing and equipment to snow-track samples during collection. Two wolverine snow-tracks were collected near a bait station in the Warm Springs Creek drainage approximately 22 km from Anaconda, Montana (Fig. A1; see Snow-Column Sampling, below) where a wolverine had been captured on camera earlier that day. Putative fisher snow-tracks were collected in areas where field crews were monitoring radio-collared fishers in the South Fork of the Clearwater River drainage approximately 14–16 km from Elk City, Idaho, (Fig. A1).

Sampling kits for collecting snow-tracks consisted of a clean 2-l Nalgene bottle, two 1-gallon plastic bags to serve as gloves, and a plastic scoop (Fig. 1); prior to use, all materials were stored inside the Nalgene bottle. A blank datasheet printed on waterproof paper was taped to the outside of the bottle, and completed during sample collection. Plastic bags were used to isolate samples from the collector's hands, so that the field crews could wear thick winter gloves during the collection process. On later samples, 46-cm poly food service gloves, which extend to or above the elbow, were used. Crews were directed to restrict their sampling to the snow under and near the snow-track. If

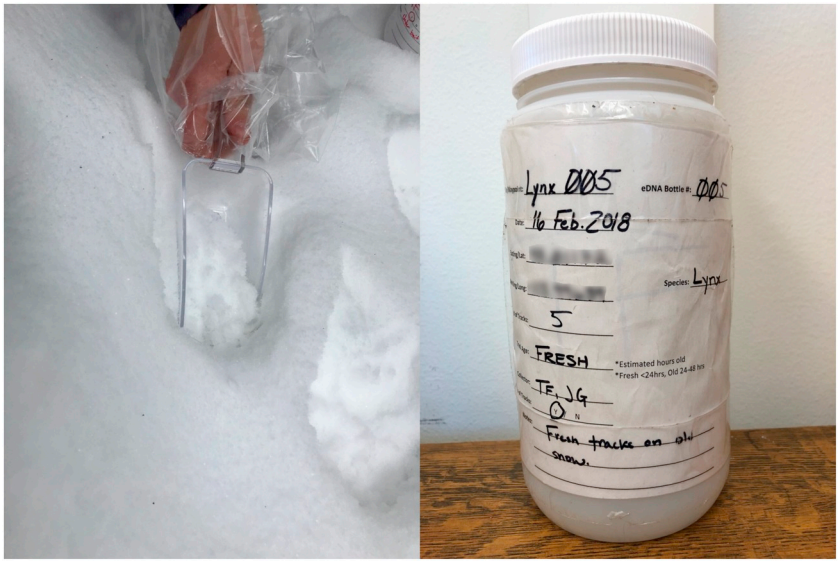

Fig. 1. Snow associated with tracks was collected with a scoop and deposited in a 2-l bottle for transportation. The scoop and plastic gloves (or bags) were stored in the 2-l bottle prior to use to ensure that they remained uncontaminated.

snow-tracks were found in deep, recently fallen snow, crews followed the tracks until they passed under the forest canopy and collected snow from a shallower location. Snow-track collection continued by following a single set of tracks and scooping individual snow-tracks until the 2-l bottle was full of packed snow. Bottles were then sealed, and researchers recorded the track's geographic coordinates, the collector's putative identification of the species that created the track, and other details on the datasheet (Fig. 1). Once collected, snow was kept frozen until it reached the National Genomics Center for Wildlife and Fish Conservation (NGCWFC) in Missoula, Montana. Bottles containing snow samples were thawed at room temperature in a separate building having no previous contact with laboratory equipment or the target species to prevent contamination. We filtered the resulting water, closely following the protocol outlined in Carim et al. (2016). Filtered field samples were paired with filtered distilled water blanks to test for potential environmental contamination associated with the thawing and filtration facility. We performed eDNA extractions using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), and scissors and forceps were cleaned with 50% bleach. Unless otherwise noted, all spins were carried out for 2 min at 20,000 ×*g*. First, we halved each sample filter and placed each half in a 1.7 ml tube. One half was used for DNA extraction and the other was stored at −20 °C for future analysis. We then added 360 µl of ATL and 40 µl of proteinase K to the filters, and incubated them for 40–48 h at 56 °C. Next, we added 400 µl AL and 400 µl ethanol, then incubated for 10 min at 70 °C. To acquire solution from the filters, we centrifuged them in QIAShredder columns (Qiagen, Valencia, CA, USA). All solutions were loaded and spun through into a single DNeasy spin column, using as many spins as necessary. Samples were washed with 500 µl each of AW1, ethanol, and AW2, with the last spin increased to 4 min. Lastly, we eluted the DNA in 100 µl of warm IDTE buffer (pH 8.0, 10 mM Tris, 0.1 mM EDTA; Integrated DNA Technologies) for 2 min at 9600 ×*g* after a 10 min incubation at room temperature.

We designed species-specific qPCR assays for detecting Canada lynx, fisher, and wolverine DNA (see Appendix A1.1) and tested the efficacy of each qPCR assay when applied in vivo to environmental samples collected from the snow-tracks putatively left by Canada lynx, fisher, or wolverine. The eDNA extracts were analyzed using the optimized assays (Table A2) in triplicate, 15-µl reactions containing 7.5 µl of 2× Environmental Master Mix 2.0 (Life Technologies), 0.75 µl of 20× assay, 4 µl of eDNA extract, a TaqMan Exogenous Internal Positive Control (IPC; Life Technologies) including 1.5 µl of 10× IPC assay and 0.30 µl of 50× IPC DNA, and 0.95 µl of PCR-grade distilled water. The thermocycling profile remained the same as described in the methods for assay design and testing (see Appendix A1.1). As with the in vitro tests, in vivo analyses were prepared inside a hood where pipettes, tube racks, and consumables were exposed to UV light for a minimum of 1 h prior to set-up. Further, for every analysis, we included qPCR positive controls containing approximately 0.4 ng DNA extracted from the Canada lynx, fisher, and wolverine tissues, and negative controls containing distilled water in the place of DNA template. We used IPC to evaluate if inhibitors were impacting the PCR analyses, and the mean cycle number value (Ct) of the IPC for each sample was compared with the mean Ct of the negative control. A sample was considered inhibited if the mean Ct of the IPC in the sample was more than one Ct later than the mean Ct of IPC in the negative control.

2.2. Species identification from snow collected at camera stations

In the Anaconda-Pintler Mountains, a camera trap had recorded a putative Canada lynx twice, on 2 November 2017 and again on 29 January 2018. However, the pictures were not diagnostic and the persistent presence of lynx in this area was novel. We therefore used this occurrence to investigate question two. We located the area where the putative Canada lynx had walked based on the camera images. We used data from a nearby (< 7 km distance and < 90 m elevation difference) SNOTEL recording station (Schaefer and Paetzold, 2001; Station 930) to approximate the depth in the snow column associated with the two visits. We visited the camera-trap site on 6 April 2018, and excavated three snow pits: one at each area identified from the photos as having lynx tracks in November and January, and one at the base of the tree containing the lure; the camera did not photograph the base of the tree, but we presumed that the lynx visited it. We collected snow samples from the layers associated with both November and late-January snow conditions. Samples were collected by shaving snow from the edge of the pit from those regions of the snow column associated with snow that was present at the time of the recorded visits and filling large (60.6-l) plastic containers with snow. Because it was difficult to prevent contamination during this process, we ensured that the shovels and plastic containers were new, and that neither the crew nor the snowmobiles had previous contact with either lynx or lynx DNA. Additionally, at this location we collected a field blank consisting of a snow sample from an untracked area. Pictures from the camera set and fresh tracks indicated that a wolverine had visited the survey station

Table 2
Data from the lynx (n = 11), fisher (n = 3), and wolverine (n = 17) snow-track samples. Snow-tracks are labeled based on the putative morphology-based species identification. "Fresh" tracks were created no > 24 h prior to sampling, "Old" refers to all tracks older than 24 h. The respective DNA extracts were analyzed with the Canada lynx (Lynx), Fisher, and Wolverine qPCR assays. Detection information, including the number of replicate reactions (# of reps) with positive detection and DNA concentration estimates (Copies per reaction), is included. Location information is also displayed in Fig. 1A.

Date collected	Location	Number of tracks per sample	Track age	Species tested	Detected	# of reps	Copies per reaction
3-Mar-18	Elk City, ID	?	Fresh	Fisher	Yes	3	45.48
6-Mar-18	Elk City, ID	?	Fresh	Fisher	Yes	3	44.74
10-Mar-18	Elk City, ID	?	Old	Fisher	Yes	3	18.79
31-Jan-18	Seeley Lake, MT	5	Fresh	Lynx	Yes	2	2.65
1-Feb-18	Seeley Lake, MT	5	Fresh	Lynx	Yes	3	13.36
8-Feb-18	Seeley Lake, MT	4	Fresh	Lynx	Yes	1	0.33
9-Feb-18	Seeley Lake, MT	8	Fresh	Lynx	Yes	3	3.34
13-Feb-18	Seeley Lake, MT	6	Fresh	Lynx	Yes	2	0.61
14-Feb-18	Seeley Lake, MT	11	Fresh	Lynx	Yes	3	10.26
21-Feb-18	Seeley Lake, MT	2	Fresh	Lynx	Yes	3	1.52
30-Jan-18	Seeley Lake, MT	6	Old	Lynx	Yes	3	11.57
7-Feb-18	Seeley Lake, MT	2	Old	Lynx	Yes	2	0.87
12-Feb-18	Seeley Lake, MT	6	Old	Lynx	Yes	3	5.06
13-Feb-18	Seeley Lake, MT	7	Old	Lynx	Yes	1	0.38
3-Mar-18	Seeley Lake, MT	4	Fresh	Wolverine	No	0	0.00
10-Mar-18	Seeley Lake, MT	8	Fresh	Wolverine	Yes	3	2.06
11-Mar-18	Seeley Lake, MT	12	Fresh	Wolverine	No	0	0.00
11-Mar-18	Seeley Lake, MT	11	Fresh	Wolverine	Yes	3	43.34
12-Mar-18	Seeley Lake, MT	16	Fresh	Wolverine	Yes	3	49.91
16-Mar-18	Seeley Lake, MT	10	Fresh	Wolverine	Yes	3	1.84
16-Mar-18	Seeley Lake, MT	4	Fresh	Wolverine	No	0	0.00
15-Mar-18	Seeley Lake, MT	10	Fresh	Wolverine	Yes	3	1.96
6-Apr-18[a]	Anaconda, MT	5	Fresh	Wolverine	Yes	3	61.47
6-Apr-18[a,b]	Anaconda, MT	8	Fresh	Wolverine	Yes	3	670.69
13-Feb-18[c]	Seeley Lake, MT	2	Old	Wolverine	Yes	3	8.95
2-Mar-18	Seeley Lake, MT	8	Old	Wolverine	Yes	3	3.55
11-Mar-18	Seeley Lake, MT	4	Old	Wolverine	Yes	3	1.90
17-Mar-18	Seeley Lake, MT	7	Old	Wolverine	Yes	3	97.85
18-Mar-18	Seeley Lake, MT	6	Old	Wolverine	Yes	3	4.84
20-Mar-18	Seeley Lake, MT	4	Old	Wolverine	Yes	3	7.33
21-Mar-18	Seeley Lake, MT	4	Old	Wolverine	No	0	0.00

[a] These tracks were collected opportunistically at a bait station originally visited to confirm lynx (see text).
[b] This track sample, collected at the bait-station, also contained DNA from Canada lynx (# of reps = 3, avg. copies/rxn = 96.8).
[c] This track sample also contained DNA for Canada Lynx (# of reps = 3, avg. copies/rxn = 2.4).

earlier that day, so two wolverine snow-track samples were collected opportunistically. Snow samples were allowed to melt at room temperature in a clean location, and the water was filtered as described previously. These large water samples typically contained forest debris, and the filters clogged after filtering approximately 1-l of water. We therefore exhausted three filters per sample. The filters were extracted as above except the solutions from each filter for a given sample were combined in the spin column during extraction. Environmental DNA extractions followed the same methods as above, except to potentially increase the DNA concentration, we eluted the samples in 50 µl TE after the initial 10-min incubation. We then added an additional 50 µl TE, incubated for 10 min, and eluted again, resulting in a 100 µl total elution volume. Analyses followed the same procedure as for snow-tracks.

2.3. Species identification from noninvasive samples that failed to amplify with conventional PCR techniques

Samples were obtained from remote stations designed to allow automated detection of carnivores throughout the winter in the North Cascade Ecosystem, Washington. To attract carnivores, stations were equipped with a scent disperser designed to attract wolverines. The dispenser consisted of a small pump controlled by an ultra-low power processor and programmed to release 3 ml of liquid scent lure onto a target item (i.e., a partial cow (*Bos taurus*) femur) each day for 6–9 months (full winter season) without maintenance (R. Long, unpublished data). Stations were equipped with both camera traps and hair snares (gun-cleaning brushes; Kendall and McKelvey, 2008) to detect animal visits. We analyzed only samples from stations where researchers obtained photographs of wolverines climbing the tree and were presumed to have made contact with the gun brushes on the tree. Genomic DNA was extracted at the NGCWFC from hair samples using the QIAGEN DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and using modifications for hair samples from Mills et al. (2000). For convention PCR followed by Sanger sequencing, we amplified approximately 360 base pairs (bp) of the 16S rRNA region of mitochondrial DNA (mtDNA) using universal primers (5′-GTGCAAAGGTAGCATAATCA-3′ and 5′-GAATTACGCTGTTATCCCT-3′; Hoelzel and Green, 1992). Reaction volumes of 30 µl contained 50–100 ng DNA, 1× reaction buffer (Life Technologies, NY, USA), 2.5 mM MgCl2, 200 µM each dNTP, 1 µM each primer, 1 U Amplitaq Gold polymerase (Life Technologies, NY, USA). The PCR program was 94 °C/5 min, [94 °C/1 min, 55 °C/1 min, 72 °C/1 min 30 s] × 34 cycles, 72 °C/5 min. The quality and quantity of template DNA were determined by 1.6% agarose gel electrophoresis. PCR products were purified using ExoSap-IT (Affymetrix-USB Corporation, OH, USA) according to manufacturer's instructions. Reactions were sequenced at Eurofins Genomics (Louisville, KY, USA) using standard Sanger sequencing protocols. DNA sequence data were viewed and aligned with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA). All samples that initially failed to sequence were re-extracted and re-analyzed using conventional PCR and, if they failed again, the samples were considered insufficient for sequencing ("poor DNA"). After sequencing these samples, we applied the species-specific qPCR wolverine assay following the same procedures outlined for snow-track samples (above), running each sample in triplicate with a standard curve to allow copy-number quantification. To ensure inhibitors were not impacting the PCR analyses, we analyzed them in triplicate qPCR reactions using IPC as described above for eDNA analyses except we excluded the 20× eDNA assay.

3. Results

3.1. Snow-track testing

We successfully developed qPCR assays for each species that were efficient and highly sensitive (see Appendix A2.1). The Canada lynx assay detected DNA in all Canada lynx tissue samples (n = 19), the fisher assay detected DNA in all fisher tissue samples (n = 15), and the wolverine assay detected DNA in all wolverine tissue samples (n = 17). The standard curve analyses resulted with the Canada lynx assay efficiency = 94.0% (R^2 = 0.997, y-intercept = 38.74, and slope = −3.47), the fisher assay efficiency = 95.6% (R^2 = 0.996, y-intercept = 38.16, and slope = −3.43), and the wolverine efficiency = 94.0% (R^2 = 0.996, y-intercept = 38.50, and slope = −3.48). In addition, each assay detected target DNA in 6/6 replicates at the 2-copy per reaction dilution level (see Appendix A2.1). In total, 31 snow-track samples were collected. The in vivo analyses using the Canada lynx assay resulted in detection of Canada lynx DNA in 11/11 samples collected from snow-tracks identified as Canada lynx in the field; analyses with the fisher assay resulted in detection of fisher DNA in 3/3 samples collected from snow-tracks identified as fisher in the field; analyses with the wolverine assay resulted in detection of wolverine DNA in 13/17 samples collected from snow-tracks identified as wolverine in the field (Table 2). None of these samples showed signs of inhibition. Two wolverine tracks also contained lynx DNA, one from near Seeley Lake, Montana, and one from a bait station near Anaconda, Montana, where snow-column analyses were conducted. Quantities of DNA associated with snow-track detections ranged widely (0.33–670.69 copies per reaction) and were sufficient for confirming detection in 27/31 samples in total. In triplicate analyses, species were detected in all three wells in 22/27 samples (Table 2). All distilled water blanks were negative for all three species, and none of the samples showed signs of inhibition.

3.2. Species identification from snow collected at camera stations and at known track locations

Two of three snow-column samples collected on 6 April 2018 tested positive for lynx DNA—one from an area where a lynx was observed walking on 2 November 2017, and one from the base of the tree containing the bait. A sample from another area where a lynx had walked on 29 January 2018 tested negative for lynx. For the two positive samples, target DNA quantities were low (0.14 and 0.55 copies per reaction) and, in both cases, amplification only occurred in 1/3 wells. The field blank was negative for all three species, and none of these samples showed signs of inhibition.

3.3. Species identification from noninvasive samples that failed to amplify with conventional PCR techniques

Twenty putative wolverine hair samples were collected during the summers of 2016 and 2017 at 8 stations that had been deployed prior to the previous winter (Table A4). Periods between the last wolverine photographed and the time of hair collection varied from 50 to 256 days. Of the 20 hair samples tested, we detected no wolverines using standard PCR followed by sequencing; one contained Canada lynx DNA. Using the qPCR wolverine assay, we detected wolverine DNA in 17/20 overwinter samples (85%), with average DNA copies per reaction ranging from 0.2–1054.0 copies (Table 3). Three of the samples analyzed failed to detect wolverine DNA. Using the Canada lynx assay and qPCR, we also detected Canada lynx in the sample where it had been detected using standard PCR techniques (Table 3). None of these samples showed signs of inhibition.

4. Discussion

The increased reliability of species identifications provided by qPCR-based DNA analyses creates opportunities for developing more effective methods for sampling rare carnivores. We provided proof-of-concept evaluations of species-specific qPCR assays applied to three potential survey techniques: eDNA from snow-tracks, eDNA from snow in an area where a rare carnivore was captured on camera, and DNA from hair samples collected during summer after being exposed to the elements for at least 1.6 months. For all three techniques tested, species identification rates using qPCR assays were equal to or higher than those obtained using standard PCR techniques. The bobcat (*Lynx rufus*) was the only species closely related to and sympatric with one of our target species. Bobcats have 11 base-pair mismatches in the assay region with Canada lynx, including three mismatches in the probe (Table A1). Thus, it was not difficult to develop species-specific assays for these species (Wilcox et al., 2013). Further, all three assays were highly sensitive and capable of reliably detecting eDNA at concentrations < 10 copies per reaction. Our field tests of these assays indicate detections at estimated copy numbers of < 1 copy per reaction when averaged over the triplicate run (Tables 2 and 3). As such, these assays have the specificity and sensitivity to reduce both misidentifications and missed detections.

We reliably identified all three rare carnivore species from snow-track surveys using qPCR methods and targeted eDNA sampling. Our results suggest that these techniques can be faster in the field than traditional backtracking because no backtracking is required for obtaining snow-track samples. For Canada lynx, backtracking putative tracks for 1 km provides DNA identifiable to species or individual only about 40% of the time (McKelvey et al., 2006). When high quality lynx hair samples are collected through backtracking, amplification success is high (81–98%; McKelvey et al., 2006). However, our success rate at identifying lynx from snow-tracks collected along a travel route from both fresh (< 24 h; n = 7) and older (> 24 h, n = 4) travel routes was 100% successful and did not require backtracking. The combination of ease of collection coupled with high sample success rate suggests a tremendous potential to improve snow-track surveys for rare forest carnivores.

With little additional field time, qPCR analysis has the potential to virtually eliminate track misidentifications. This method expands the conditions under which snow-track surveys can be conducted effectively because track quality is much less critical to achieving a successful identification with qPCR analysis. Current snow-track survey protocols require a specific window of opportunity: a minimum snowfall-free time period (> 24 h) is required to allow tracks to accumulate (e. g. Bayne et al., 2005) and to prevent tracks from being obscured or malformed. These constraints have historically made the planning of snow-tracking problematic. Further, qPCR analysis of snow-tracks eliminates the need to have track-identification experts in the field, enabling citizen scientists to participate in such surveys. More participants mean that more sampling opportunities are possible using qPCR techniques, which will greatly improve the efficacy of survey methods based on locating snow-tracks.

We are confident that our preliminary snow-collection protocol was effective and can be applied for other snow-track collections. One advantage of the protocol is that track collection is methodologically similar regardless of species, and therefore this can be applied to many other species. We had no trouble isolating samples to prevent contamination, the amounts of snow collected appeared to provide sufficient DNA, and, with our current filtering systems, the ~1 l of water that resulted from melting snow approximated the maximum that could be filtered in forested environments without clogging. We were able to control field contamination by limiting the sampling crew to those who had no contact with either lynx or lynx DNA (e. g. hair or scat samples). We recommend that if crews come into close contact with target species DNA, sampling should cease until the contaminated material (clothing, gloves, etc.) are replaced with uncontaminated material. We also recommend that in a formal survey field blanks from recent untracked snow should be collected in addition to the snow-track samples.

Table 3
Results of qPCR analyses for hair samples collected from gun brushes deployed at overwinter camera stations that detected wolverines and, for which, conventional PCR/sequencing failed (i.e., poor DNA). Samples were analyzed using a wolverine-specific qPCR assay (see text) and samples were run in triplicate. Detection information, including the number of replicate reactions (# of reps) with positive detection and DNA concentration estimates (copies per reaction), is included. Additional details on the stations and samples can be found in Table A4.

Station name	Conventional PCR/sequencing	qPCR results			
		Species tested	Detected	# of reps	Copies per reaction
Bowan Meadows	poor DNA	Wolverine	Yes	3	636.29
Bowan Meadows	poor DNA	Wolverine	Yes	3	2.89
Devil's Creek	Lynx[a]	Wolverine	Yes	1	0.25
Devil's Creek	poor DNA	Wolverine	Yes	1	0.32
Devil's Creek	poor DNA	Wolverine	No	0	0.00
Jakita Ridge	poor DNA	Wolverine	Yes	3	56.72
Jakita Ridge	poor DNA	Wolverine	No	0	0.00
Lake View Ridge	poor DNA	Wolverine	Yes	3	1.82
Lake View Ridge	poor DNA	Wolverine	Yes	3	567.94
Lake View Ridge	poor DNA	Wolverine	Yes	1	0.49
Lower Early Winters	poor DNA	Wolverine	Yes	3	82.69
McMillan 1	poor DNA	Wolverine	Yes	1	0.22
McMillan 1	poor DNA	Wolverine	Yes	1	0.23
McMillan 1	poor DNA	Wolverine	No	0	0.00
S. Fork Trout Creek	poor DNA	Wolverine	Yes	2	0.34
S. Fork Trout Creek	poor DNA	Wolverine	Yes	3	21.47
S. Fork Trout Creek	poor DNA	Wolverine	Yes	3	1053.98
Slate Peak	poor DNA	Wolverine	Yes	3	18.09
Slate Peak	poor DNA	Wolverine	Yes	3	156.05
Slate Peak	poor DNA	Wolverine	Yes	3	1.01

[a] Sample was analyzed for Canada lynx using qPCR; lynx presence was confirmed (# of reps = 3, avg. copies/rxn = 22.8).

We reliably detected multiple species within a single track line on two separate occasions (Table 2). Others, such as Murray and Boutin (1991), have documented that in deep-snow environments, animals often take advantage of packed snow and frequently follow the tracks of other animals. In both cases, the tracks were putative wolverine tracks, but Canada lynx DNA was also detected. In Seeley Lake, Montana, it is likely that a wolverine followed the same path previously traveled by a lynx. The putative wolverine tracks at the bait station in Anaconda, Montana, were from a well-used trail leading to the bait station. Photos from the camera at the bait station confirmed that a wolverine was present on the trail the day the tracks were collected and that seven days later a lynx was present following the same trail to access the site. We assume that the lynx visited the station just before our sampling took place, but was not captured on camera. These results support that this sampling technique can be used in an environment where DNA of multiple species is present, such as a well-used track line or packed snow around a bait station. Whether there is a single target species of interest or multiple target species of interest, the extreme specificity of qPCR analysis allows for reliable identification of the desired target. We are continuing to develop eDNA assays for a variety of species active in forested areas during the winter so that we may fully apply the target specificity and multispecies potential of this technology.

We have demonstrated that we can reliably identify species detected on camera via DNA shed at a known location. Thus, our methods can reduce species misidentifications from remote-camera images, particularly for species that are sympatric with closely related species and similar in appearance (e.g., Canada lynx and bobcats, fishers and martens). In addition, we have confirmed that eDNA persists in the snow column throughout the winter season (confirmed from November 2017 to April 2018 via lynx detection in snow-column samples). As Goldberg et al. (2016) note, very weak and unreplicated eDNA results are likely less reliable. Although the detections from the snow-columns were uniformly weak, these results were replicated along with the lynx detection from the snow-track sample collected on the same day the snow-columns were excavated. This demonstrates that detections are replicable at varying temporal scales within a single location in a snow-column, which can help to reduce and quantify missed detections. Importantly, the field blank collected that day was negative for lynx DNA. However, just as we recommend the use of field blanks to control for contamination when performing surveys, we suggest collecting multiple snow samples for confirmation in areas where single track identification is of high interest, such as the presence of an endangered species in areas where they were thought absent.

A snow-column may be a powerful sampling tool because it potentially contains DNA from all of the animals that visited a bait station during the sampling period, and also may provide a record of when the animal visited the site. In the case of snow-tracks and snow-columns, the DNA environment is greatly simplified; the chance of DNA entering the sample due to factors other than direct contact with the species is greatly reduced when compared to aquatic eDNA samples. However, sampling the snow-column must be designed to eliminate contamination because of the sensitivity of qPCR analysis. Digging snow pits provides ample opportunity for DNA to be transferred from one location to another on sampling equipment. We suggest that field collection methodologies for snow-columns should be modified to minimize contamination, such as collecting a snow-column sample within a sterile, DNA-free tube would provide adequate sample isolation to prevent contamination. In addition, given the low number of DNA copies detected in our snow-column samples, we suggest that filtration methods allow more water to be filtered to increase DNA copy number per reaction.

In addition to genetically confirming species identification from snow samples, we were able to reliably identify species in DNA samples from hair that were too poor in quality to yield species identification using standard PCR techniques. These methods create new opportunities for effectively sampling forest carnivores during the winter, especially in areas where limited access could result in sampling bias. We have shown that we can reliably identify species from hair samples collected at overwinter stations established before snowfall and not visited again until after snow melt. This may provide new opportunities for noninvasively sampling carnivores in wilderness areas, where the use of motorized equipment (e.g., snowmobiles, remote cameras) is often restricted. A hair-collection device, a long-lasting attractant, and a qPCR assay for the target species may be all that is needed to reliably detect rare carnivore species in wilderness areas.

While the eDNA and noninvasive sampling methods developed in

this study show great promise, there are still some limitations to overcome. One primary limitation is the need for a broad array of species-specific qPCR assays that will enable positive identification of the animal that was present, rather than simply failing to detect the target species, as we did here. Because of this limitation, the proportion of positive results we report here are conservative. We were unable to verify that wolverines were responsible for all putative wolverine tracks, or that all of the hair samples associated with wolverine photos actually contained wolverine hair. There is a clear need to develop additional assays for more carnivore species, particularly common species whose tracks or appearance might be confused with the target species (bobcats, martens, etc.). This will be an important step in the development of effective and reliable multispecies surveys and may enable future researchers to identify all of the carnivores detected via snow-tracking and camera surveys. As with aquatic eDNA samples, the eDNA samples we collected from snow are open-ended in terms of the quantity of snow that can be analyzed; copy numbers increase as sample size increases (Wilcox et al., 2018). Our primary limitation was water filter clogging prior to analyzing our snow samples. We are currently exploring new approaches to overcome this, including adding a pre-filter step and exploring larger pore sizes. Although the use of qPCR assays to identify species from overwinter hair samples was effective, there may be ways to improve hair traps designed to collect overwintered hair, including better shielding from moisture and ultraviolet light, which can potentially provide higher quantities of DNA.

We believe that qPCR methods for identifying eDNA can revolutionize the sampling of rare terrestrial species by reducing or eliminating key sources of error. While some information is not attainable at this time (such as sex and individual identity), these qPCR analyses are highly sensitive, specific, and reliable for species identifications. Many survey methods such as occupancy modeling (MacKenzie et al., 2017) are designed around species-level data. Thus eDNA snow-track samples can be a source of high quality data that can be used in a variety of current population assessment techniques. Environmental DNA detections have already been used for occupancy estimation in aquatic settings (Schmidt et al., 2013) and can be incorporated into mixed data stream frameworks to estimate abundance, including integrated population models (Zipkin and Saunders, 2018), spatial capture-recapture models with some or no individuals marked (Chandler and Royle, 2013), and partially marked populations (Augustine et al., 2018). The size of snow-track samples is not limited; a great volume of snow could be collected at key locations (e.g., bait stations) and more species reliably detected. This can increase the cost effectiveness of surveys for which identifying the species present is the goal. Snow columns beneath baited or scented stations potentially contain eDNA from every animal that visited the station and, if snow is retained year to year, detections from previous seasons may be possible. Whether or not a camera functions properly and records a diagnostic image of a species, the snow column represents a rich data-source for detecting the presence of carnivores of interest with high confidence. Finally, these techniques are not only applicable to identifying species from eDNA deposited on snow, but can also be used to identify species from a variety of potential DNA sources (e.g., hair, saliva, blood, urine, scats) across any medium, species, or landscape.

Acknowledgements

We would like to thank the USDA U.S. Forest Service's Northern Region as well as MPG Ranch for funding this research. We greatly appreciate the field crews who collected the snow-track samples in Seeley Lake, MT and Elk City, ID. We are also grateful for the useful feedback and comments provided by Karen Lohman, Katherine E. Zarn, and three anonymous reviewers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2018.11.006.

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