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RESOURCE ARTICLE

Individual genotypes from environmental DNA: Fingerprinting snow tracks of three large carnivore species

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Abstract

Continued advancements in environmental DNA (eDNA) research have made it possible to access intraspecific variation from eDNA samples, opening new opportunities to expand non-invasive genetic studies of wildlife populations. However, the use of eDNA samples for individual genotyping, as typically performed in non-invasive genetics, still remains elusive. We present successful individual genotyping of eDNA obtained from snow tracks of three large carnivores: brown bear (*Ursus arctos*), European lynx (*Lynx lynx*) and wolf (*Canis lupus*). DNA was extracted using a protocol for isolating water eDNA and genotyped using amplicon sequencing of short tandem repeats (STR), and for brown bear a sex marker, on a high-throughput sequencing platform. Individual genotypes were obtained for all species, but genotyping performance differed among samples and species. The proportion of samples genotyped to individuals was higher for brown bear (5/7) and wolf (7/10) than for lynx (4/9), and locus genotyping success was greater for brown bear (0.88). The sex marker was typed in six out of seven brown bear samples. Results for three species show that reliable individual genotyping, including sex identification, is now possible from eDNA in snow tracks, underlining its vast potential to complement the non-invasive genetic methods used for wildlife. To fully leverage the application of snow track eDNA, improved understanding of the ideal species- and site-specific sampling conditions, as well as laboratory methods promoting genotyping success, is needed. This will also inform efforts to retrieve and type nuclear DNA from other eDNA samples, thereby advancing eDNA-based individual and population-level studies.

KEYWORDS
eDNA-based population studies, high-throughput STR genotyping by sequencing, individual identification, large carnivores, non-invasive genetics, snow track sampling

1 | INTRODUCTION

Environmental DNA (eDNA) sampling and analysis, using organismal DNA extracted from environmental samples (Taberlet et al., 2012), are revolutionizing the way we assess biodiversity, enhancing the scope of ecological investigations and conservation studies (Beng & Corlett, 2020; Cristescu & Hebert, 2018; Deiner et al., 2017, 2021; Taberlet et al., 2018). So far, eDNA applications have primarily focused on species detection and ecosystem-level diversity (Beng & Corlett, 2020), but continued advancements within eDNA research have resulted in increased effectiveness of approaches for recovering eDNA potentially suitable also for addressing intraspecific diversity and population-level questions (Adams et al., 2019; Sigsgaard et al., 2020).

In the context of wildlife studies of macroorganisms, the ability to access intraspecific genetic variation from various eDNA sources represents an advancement in non-invasive genetic methods typically based on the collection of scats, hair, feathers, urine, etc. (Andrews et al., 2018; Waits & Paetkau, 2005). DNA traces in the environment, in fact, offer new opportunities to non-invasively genetically sample animals in their natural setting, without handling or even observing them (Adams et al., 2019). One main challenge is that environmental samples comprised DNA of several species and individuals of the same species, all diluted in the sample matrix and contributing unequal amounts of DNA to the eDNA mixture (Barnes & Turner, 2016; Sigsgaard et al., 2020). However, through targeted eDNA sampling aimed at maximizing DNA retrieval of the target species and sometimes individuals, researchers have been able to assess mitochondrial DNA (mtDNA) haplotype diversity, frequency and distribution and even compile mitogenomes (Dugal et al., 2022; Farrell et al., 2022; Parsons et al., 2018; Sigsgaard et al., 2016; Székely et al., 2021). Further, studies are now showing real potential for calling nuclear variants in eDNA samples for use in a population genetic framework (e.g. Andres et al., 2021; Jensen et al., 2021).

Reliable analysis of nuclear DNA (nDNA) is key in enabling eDNA-based population studies because of the higher information content and resolution of nDNA compared to mtDNA (Adams et al., 2019; Sigsgaard et al., 2020). Typing of nDNA will also allow for individual identification, which is the basis of wildlife non-invasive genetic surveys, genetic monitoring programs and forensics (Kelly et al., 2012; Ogden et al., 2009; Schwartz et al., 2007). However, individual genetic profiling from eDNA sources remains elusive. Retrieving nDNA of a target species from an environmental mixture in sufficient quantity and quality is more difficult compared to mtDNA because nDNA is present in significantly lower copy number (except for the multi-copy regions) and it degrades faster due to the absence of organellar membranes protection (Parsons et al., 2018; Sigsgaard et al., 2020).

Snow tracks, that is, footprints left by animals while walking in the snow, are an ideal setting for targeted eDNA sampling in population-level wildlife studies. eDNA from animal tracks originates from cells present on the animal paw and deposited on the snow surface due to friction against the ground. Therefore, a first advantage of snow track eDNA sampling in terrestrial ecosystems is that an animal's DNA is found in a well-delimited area as opposed to samples from aquatic environments where eDNA dilution and mixing from multiple sources is greater (Dalén et al., 2007; Franklin et al., 2019; Howell et al., 2021). This feature also increases the chances of collecting DNA from single individuals of the target species. Secondly, snow limits DNA degradation by acting as a natural freezer (Dalén et al., 2007; Howell et al., 2021) and hence facilitates the preservation of nDNA. Finally, snow tracks of terrestrial animals are commonly found in winter in snowy ecosystems (Kinoshita et al., 2019), potentially allowing for adequate sample sizes in population studies.

Snow track eDNA has already been used for species detection of several predators through mtDNA analysis (Barber-Meyer et al., 2020; Dalén et al., 2007; Franklin et al., 2019; Kinoshita et al., 2019). A number of published studies have attempted to analyse nDNA with varying results for lynx (Hellström et al., 2019), wolf (Barber-Meyer et al., 2020, 2022) and polar bear (Von Duyke et al., 2023) with only this latter recent study being successful in achieving reliable multilocus genotyping for individual identification in a single species. However, individual genotyping from snow track eDNA as a wildlife non-invasive genetic method still remains elusive. Several reasons have been called into play for the earlier failures, spanning from field conditions and collection methods to laboratory protocols. All these previous works evaluated the amplification and genotyping performance of existing microsatellite loci (i.e. short tandem repeats – STR) either on agarose gel or by capillary electrophoresis.

In this study, we present the first successful individual genotyping from eDNA in snow tracks of three large carnivore species in temperate ecosystems: brown bear (*Ursus arctos*), wolf (*Canis lupus*) and Eurasian lynx (*Lynx lynx*). We sampled snow tracks in the field and used an extraction protocol for water eDNA samples and a genotyping approach based on amplicon sequencing of STRs and a sex marker on a high-throughput sequencing (HTS) platform (Figure 1a). We discuss genotyping success in relation to field conditions, the ecology of eDNA (Barnes & Turner, 2016) of the three species and laboratory protocols with implications for advancing the use of eDNA approaches for population-level wildlife studies (Wilcox & Jensen, 2022).

2 | MATERIALS AND METHODS

2.1 | Snow track eDNA sampling

Snow tracks were collected opportunistically during winter in 2019, 2020 and 2022 in the Slovenian Alps and Dinaric Mountains (seven brown bear samples and nine lynx samples) and in the French Alps (10 wolf samples) (Table 1). Field personnel including volunteers, field biologists and park/forest rangers performed the sampling in areas known for the stable presence of the species.

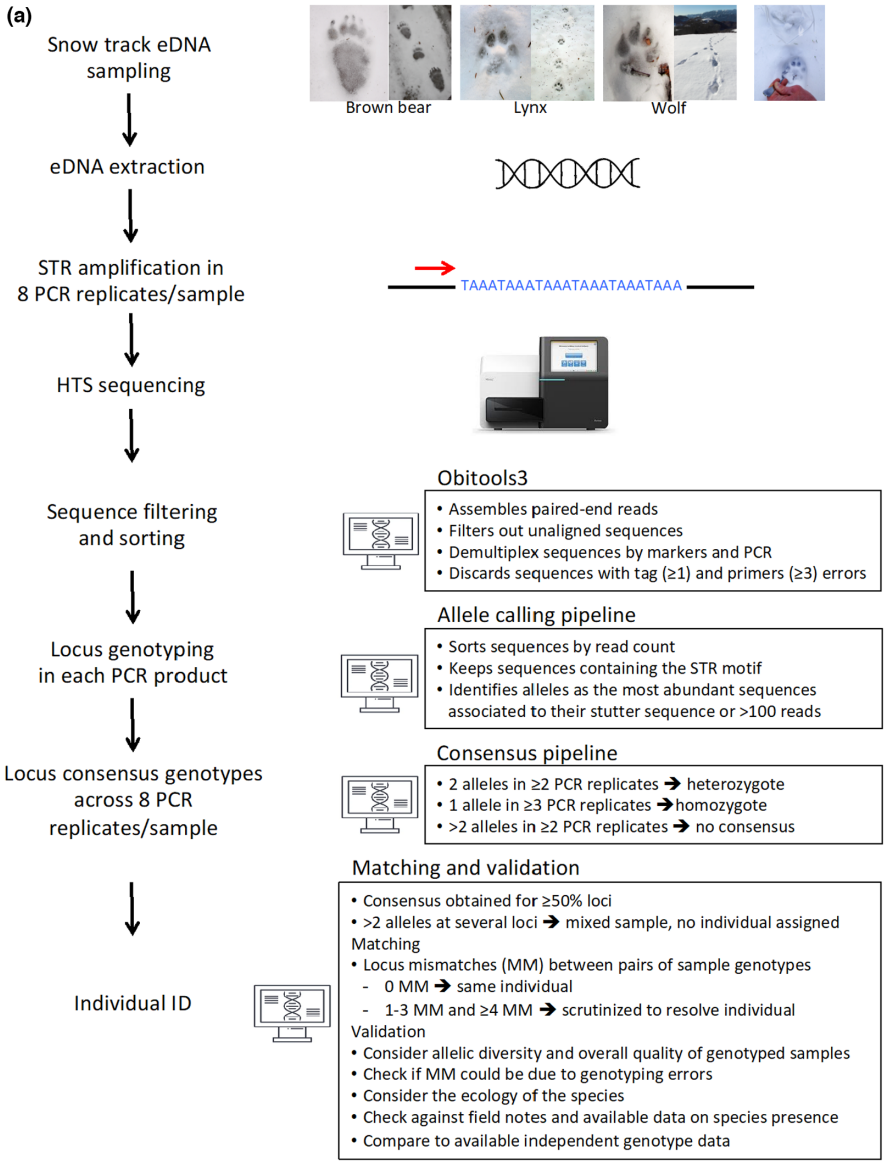

FIGURE 1 Workflow of snow track eDNA genotyping. (a) Components of the workflow from eDNA sampling to individual identification, with main steps of the data analysis outlined. (b) Flowchart of the matching and validation of individual assignment process for pairs of sample genotypes, detailing how brown bear, lynx and wolf snow track samples were assigned to individuals. In (b) blue text indicates the sample genotypes for each specific case described, while grey dashed arrows indicate cases not represented in the sample genotypes analysed. ADO, allelic dropout; FA, false allele; MM, mismatches; QI, quality index. Snow tracks photo credits: Miha Krofel.

Samples were collected upon discovery of trails of snow tracks visually attributed to the target species. Brown bear is the only ursid in southern Europe and it occurs at high density in the study area. Footprints of adult brown bears are readily distinguishable from other wildlife. Lynx and wolves are closely monitored as part of ongoing projects. Therefore, to locate trails on snow for these species, we took advantage of available fine-scale information on presence and movement from GPS-telemetry and camera trapping for individual lynx and wolves within previously identified packs. A sterilized spoon was used to scrape the surface of a snow track and place the snow in a sterile plastic bag (Fisherbrand Sterile Polyethylene Sampling Bags, 10″×12″). Multiple bags were used when larger volumes of snow were collected for a sample. The number of tracks collected for a given sample ranged from 1 to 17. Sampling location, sample characteristics and environmental conditions at the sampling site were recorded by field operators (Table 1, Table S1). Plastic bags containing the snow were labelled and transported frozen to the genetic laboratory, where they were kept at −20°C until DNA extraction.

2.2 | Snow track eDNA extraction

We extracted DNA from snow track samples using the DNeasy PowerWater Sterivex Kit (Qiagen, Germany) following manufacturer's instructions (DNeasy PowerWater Sterivex Kit Handbook

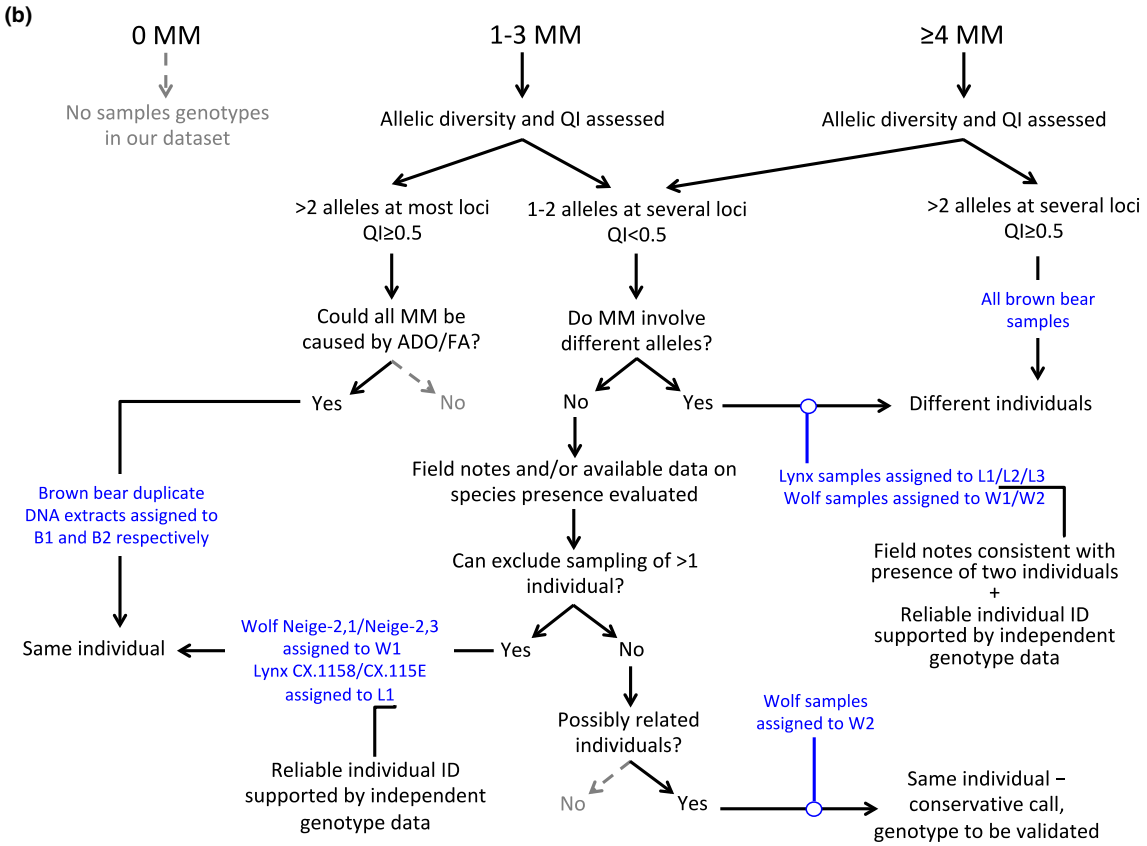

FIGURE 1 (Continued)

05/2019) with slight modifications as described below. We processed 10–12 samples at a time, with each set of extractions taking 3 working days: the first day for snow melting, the second day for water filtering and the third day to complete the DNA extraction.

Snow samples in plastic bags were completely thawed at room temperature (this took up to 24 h, depending on the amount of snow). The following day, melted snow was left to settle until large forest debris was deposited on the bottom of the bag. For each sample, the resulting water was filtered through a Sterivex filter (Millipore cat. no. SVGPL10RC) using a 60-mL volume syringe (Omnifix Luer Lock Solo 50 mL). We measured the amount of water filtered by collecting it in a graduated container. For two brown bear samples, we performed two extractions for each sample using two filters because the first filter clogged before filtering all the available water (this resulted in a total of nine DNA extractions analysed for the brown bear) (Table 1). Once all samples were filtered (this step took up to a full working day), filters were stored in a freezer at −20°C until the next morning. DNA extraction was completed following the kit protocol, omitting the incubation step at 90°C and the steps with the PowerBead Tubes as recommended for samples containing easy-to-lyse organisms or where less DNA shearing is desired. The centrifuge was used instead of the vacuum manifold with kit handbook settings and collection tubes provided with the kit. DNA was eluted in 100-µL volume. An extraction negative control was included with all sets of extractions to monitor contamination and was processed with the snow samples in all subsequent stages of the analysis. DNA extraction and the following PCR set-up were carried out in a room dedicated to low-quantity/quality DNA samples.

2.3 | STR amplicon sequencing

We performed individual profiling using genotyping by HTS of STR amplicons (De Barba et al., 2017; Fordyce et al., 2011). For each species, we used a set of STR markers designed for optimal multiplex amplification and HTS genotyping. The brown bear set includes 13 STR recently described and used for individual profiling from faecal DNA (De Barba et al., 2017), with the addition of a sex-specific marker (Pagès et al., 2009). For wolf and lynx, we used 13 new STRs (Table S2) developed following criteria outlined in De Barba et al. (2017).

For each species, STRs (and a sex-specific marker for brown bears) were co-amplified in a single multiplex PCR. Reactions were carried out in a 20-µL volume and contained 1× concentrated Platinum Multiplex PCR Master Mix, 1% GC enhancer (brown bear) or 0.0032 mg of BSA (lynx, wolf), 0.035–0.1 µM of each primer (Table S2) and 2-µL DNA template. The thermocycling profile had an initial denaturation step of 2 min at 95°C, followed by 50 cycles of 30 s at 95°C, 90 s at 57°C (brown bear)/60 s at 55°C (wolf, lynx), 60 s at 72°C and a final elongation step of 10 min at 72°C. Amplifications were performed in eight replicates per

TABLE 1 Sample information and genotyping performance for the snow tracks analysed in this study.

Species	Sample ID	Collection date	Country	Number of tracks collected	Estimated track age	Track size at collection	Water filtered (mL)	AS	ADO	FA	GS	QI
Ursus arctos	CX.1138[a]	07-Mar-20	Slovenia	8	1 day	cca 16 cm	330	0.923	0.283	0.067	0.923	0.644
	CX.113A[a]	07-Mar-20	Slovenia	8	1 day	cca 16 cm	420	0.894	0.324	0.054	1	0.673
	CX.113C	07-Mar-20	Slovenia	10	2 days	cca 16 cm	100	0.99	0.08	0	1	0.923
	CX.113E[b]	07-Mar-20	Slovenia	15	1 day	cca 8 cm	320	0.75	NA	NA	NA	NA
	CX.115H	28-Mar-20	Slovenia	1	8 h	14 cm	660	0.663	0.313	0.011	0.692	0.462
	CX.1175	29-Feb-20	Slovenia	1	0.5 days	11 cm	600	0.962	0.063	0.058	1	0.913
	CX.1176[c]	28-Mar-20	Slovenia	1	6 h	14 cm	500	0.962	0.231	0.011	1	0.779
	CX.1176.2[c]	28-Mar-20	Slovenia	1	6 h	14 cm	200	0.99	0.014	0	1	0.981
	CX.1177	27-Feb-20	Slovenia	1	4–8 h	14 cm	200	0.413	0.153	0	0.462	0.317
	Average							0.893	0.183	0.025	0.885	0.712
Lynx lynx	CX.1156	15-Dec-19	Slovenia	15	1 day	8 cm	600	0.683	0.597	0.112	0.923	0.346
	CX.1157	22-Feb-22	Slovenia	8	1 day	Kitten	240	0	NA	NA	0	NA
	CX.1158	28-Mar-20	Slovenia	1	8 h	8 cm	540	0.317	NA	0.059	0.692	0.269
	CX.115A	28-Mar-20	Slovenia	1	12 h	8 cm	450	0.048	NA	0	0.077	0.019
	CX.115C	14-Jan-22	Slovenia	17	2–3 days		450	0	NA	NA	0	NA
	CX.115E	28-Mar-20	Slovenia	1	6 h	8 cm	540	0.74	0.308	0.042	0.923	0.606
	CX.115F	22-Feb-22	Slovenia	8	1 day	Adult	350	0	NA	NA	0	NA
	CX.115J	15-Dec-19	Slovenia	15	1 day	8 cm	660	0.837	0.547	0.013	0.923	0.442
	CX.115K	22-Feb-22	Slovenia	10	1 day	Young lynx	240	0.01	NA	NA	0	NA
	Average							0.293	0.484	0.045	0.393	0.336
Canis lupus	Neige-1	1-Feb-19	France	1	>24 h		75	0.019	NA	NA	0	NA
	Neige-2,1	29-Feb-20	France	1			187	0.394	0.36	0.094	0.846	0.26
	Neige-2,2	29-Feb-20	France	1			220	0.538	0.621	0.075	0.923	0.365
	Neige-2,3	29-Feb-20	France	1			80	0.731	0.218	0.021	0.923	0.635
	Neige-2,4	29-Feb-20	France	1			100	0.452	0.7	0.041	0.846	0.346
	Neige-3,1	7-Mar-20	France	1	<24 h		80	0.587	0.483	0.073	1	0.423
	Neige-3,2	7-Mar-20	France	1	<24 h		55	0.567	0.229	0	1	0.5
	Neige-4	8-Feb-20	France	1	>24 h		27	0.423	0.5	0.076	0.846	0.346
	Neige-5	24-Mar-19	France	1	>24 h		275	0.067	NA	0	0.077	0.019
	Neige-6	9-Feb-20	France	1	>24 h		75	0.01	NA	NA	0	NA
	Average							0.379	0.444	0.048	0.646	0.362

Abbreviations: ADO, allelic dropout; AS, amplification success; FA, false allele; GS, genotyping success; NA, not analysed; QI, quality index (See full Table S1 for additional information).

[a]First set of duplicate DNA extractions.

[b]Possible mixed sample.

[c]Second set of duplicate DNA extractions.

sample, following a full multitube approach (Taberlet et al., 1996). Tagged primers, modified by the addition of molecular identifiers on the 5′ end, were used in each PCR to uniquely label any given PCR product for retrieving the respective sequence data in post-sequencing bioinformatic analysis. Tags consisted of eight nucleotides enabling a minimum of five mismatches between any pair of tags (Coissac, 2012). An additional 1–2 specified nucleotides were added to the tags 5′ end to increase complexity for cluster detection on the flow cell. PCR negative (water) and positive (a non-invasive DNA sample previously successfully genotyped) controls and "tagging system" controls (corresponding to unused tag combinations) were included in the PCR set-up to facilitate the detection of potential contamination, false positive caused by tag-jumps (Schnell et al., 2015), and monitor the performance of the amplification and the sequencing process (De Barba et al., 2014; Zinger et al., 2019).

For each species, PCR products were pooled equivolume, purified using the MinElute PCR purification kit (QIAGEN, Germany) (all samples) and Spribeads kit (SPRIselect, Beckman Coulter, Indianapolis) (lynx and wolf samples only) and then quantified with Qubit v03 Fluorometer (Life Technologies). Separate sequencing libraries were constructed for each pool targeting approximately 500–2000 reads/marker/PCR. As samples were processed in different laboratories, different library preparation protocols and sequencing platforms were used. Brown bear samples analysed at the University of Ljubljana were sent to a commercial service (www.eurofinsgenomics.eu) for library preparation and sequencing on a NovaSeq platform (2×150 bp) (Illumina Inc.). In contrast, at the University of Lausanne, the Tagsteady protocol, a procedure for library preparation that significantly reduces the impact of tag-jumps (Carøe & Bohmann, 2020), was implemented with lynx and wolf snow tracks, and samples were sequenced on a Miniseq platform (2×150 bp) (Illumina Inc.).

2.4 | Bioinformatics analysis of the sequence data

DNA sequence data analysis was performed using a modified version of the pipeline published in De Barba et al. (2017) (Figure 1a), implemented using in-house Python and R scripts, on a standard desktop computer running Linux or MacOSX (pipeline description available at https://github.com/PazhenkovaEA/ngs_pipelines.py). Initially, Illumina reads were processed using the OBITools3 (Boyer et al., 2016) to assemble paired-end reads, filter out unaligned sequences, demultiplex sequences by markers and samples discarding sequences without a perfect tag match and at least three primer mismatches. STR alleles were inferred from the observed sequences and relative read counts in each PCR product following the process already described in De Barba et al. (2017). In summary, alleles were defined as the most abundant sequences containing the STR motif of the locus and associated with their relative stutter sequence. If a sequence had no stutter and a lower number of reads than the user-defined threshold (default 100 reads), it was discarded. Consensus genotypes at each locus for a sample were determined based on STR sequence alleles observed across the eight PCR replicates, requiring that an allele be observed at least twice for heterozygotes and three times for homozygotes. Similarly, with the sex marker, males were scored by the detection of the homologous X and Y sexual chromosome sequences in at least two replicate PCRs, while females were scored by the detection of the X chromosome sequence in at least three replicate PCRs.

2.5 | Genotyping performance and individual identification

For each sample, we estimated i. amplification success (AS), as the proportion of positive PCR replicates at each STR locus, that is, replicates yielding reads assigned to at least one allele sequence, averaged across loci; ii. rate of allelic dropout (ADO) and iii. rate of false alleles (FA) averaged across loci following formulas in Broquet and Petit (2004) using data for each PCR replicate compared to the consensus; iv. locus genotyping success (GS), as the proportion of loci analysed for which a consensus genotype was obtained and vi. the quality index (QI), as the proportion of PCR replicates at each locus in which the consensus genotype was observed, averaged across loci (Miquel et al., 2006).

For each species, we calculated overall multilocus genotyping success (MGS), as the proportion of samples that were identified to individual. Sample individual assignment was a multistep process that considered all genotypic, field and ecological information available for the analysed samples and the species in the study area (Figure 1a,b). We first required that samples had a consensus genotype obtained at >50% of the STR loci analysed and excluded samples with more than two alleles detected at several loci. Then, to reliably assign samples to different individuals, we evaluated sample genotype similarity by calculating the number of locus mismatches between pairs of sample genotypes (Paetkau, 2003) using a custom R script (provided in Supplementary Information). With moderate/high allelic diversity (i.e. >2 alleles at most loci for the genotypes compared) and sample QI ≥0.5, sample genotypes with ≥4 mismatches (4 MM) were considered as originating from different individuals. Pairs of similar genotypes presenting 1–3 locus mismatches (1–3 MM) were scrutinized to determine whether mismatches could have been caused by genotyping errors, assuming that samples with no mismatches (0 MM) were left by the same individual. However, with fewer alleles observed (i.e. 1–2 at several loci) and error-prone samples (i.e. QI <0.5), we adopted more stringent criteria for individual assignment, as, under these premises, genotyping errors could be difficult to distinguish from true genotypic differences. In these cases, we specifically checked if mismatches between pairs of genotypes involved different alleles at some of the loci (i.e. MM not compatible with ADO/FA) before assigning samples to different individuals. In addition, we used field notes (Table S1) and available monitoring data, that is, about the presence or transit of single/multiple individuals at the sampling site, to ascertain dubious individual

assignment and the ability for accurate individual genotyping. For lynx, we also disposed of genotyping data previously obtained at the same markers from samples collected from collared animals that were compared with snow track genotypes.

Genotypes were organized in a custom Microsoft Access database. All calculations were performed in R (v.4.2.1) and Microsoft Excel.

3 | RESULTS

Sequencing of the snow track samples generated 4,818,564 reads assigned to markers and samples, 3,667,315 for brown bear, 224,061 for wolf and 927,188 for lynx, with an average of 1529 (bear), 228 (wolf), 876 (lynx) reads/marker/PCR that were used for genotyping. The average proportion of reads cumulatively attributed to alleles for all loci multiplexed in an amplification reaction was 59% (14%–82%) across all samples. Remaining sequences included stutter sequences and a variable number of less abundant sequences originating from PCR and sequencing errors. The level of reads observed in the negative and tagging system controls was very low in general, and negligible in the samples prepared with the Tagsteady protocol (Appendix S1).

Short tandem repeats genotyping performance differed among samples analysed and for the three species (Tables 1 and 2). Brown bear samples showed generally higher genotyping success, resulting in a consensus genotype for 6–13 of 13 loci (GS = 0.46–1). However, among the five lynx samples that had non-zero GS, three samples had 12 out of 13 loci genotyped (GS ≥0.92), and among the eight wolf samples that had non-zero GS, seven samples had at least 11 out of 13 loci genotyped (GS ≥0.85). Number of alleles per locus in the samples analysed was 2–5 for brown bear, and 1–3 for both lynx and wolf (Tables S2 and S3).

A consensus genotype at ≥7 loci was reached for eight of the brown bear DNA extracts corresponding to six snow track samples (Table 2). One sample genotype (CX.113E) had >2 alleles at three loci (UA06, UA16 and UA51) indicating a possible mixed sample containing DNA from multiple individuals. Each of the genotypes of the remaining five samples had at least four-locus mismatches with genotypes of other samples and was assigned to an individual, resulting in MGS = 71.4% (5/7 samples) for individual identification. The genotype identified from the brown bear tracks extracted using two filters matched between duplicate extractions, except for one allele difference at one locus, due to ADO or FA (locus UA14 and UA64, respectively, in each of the duplicate extraction sets). Sex was successfully identified from all five (one female and four males) of the six brown bear samples for which an individual genotype was obtained, and was concordant among duplicate DNA extracts (Table 2). The sex marker was typed also for the mixed sample, but sex ID could not be ascertained in this case (Table 2).

For the lynx, four samples were genotyped at ≥7 loci (Table 2). Despite low QI values for most samples (Table 1) and low allelic diversity (Tables S2 and S3), three could be reliably assigned to three different individuals L1, L2 and L3 (≥4 MM, including differing alleles). A fourth sample (CX.1158), collected in the same area and day of one of the unique genotypes (L1), was considered having originated from the same individual after accounting for possible ADO at three loci (LL0043, LL0044 and LL0125) and given differing alleles at two loci from the other unique genotypes. This resulted in MGS = 44.4% (4/9 samples genotyped to individual). L1 genotype matched that of a lynx sampled the same day from a hair tuft collected in the area (lynx monitoring data not shown). The other two unique genotypes (L2 and L3) were identified from samples that, based on field notes (Table S1), were left by an adult lynx and a younger individual possibly stepping on the same tracks, initially raising concerns on the ability of distinguishing their genotypes. However, these two genotypes matched those previously determined from buccal swabs collected from a GPS-collared female lynx monitored in the area and from her kitten, supporting reliable individual identification.

For the wolf, seven samples were genotyped at ≥7 loci (Table 2). These samples were collected from the area occupied by a single pack (Table S1) and presented low allelic diversity, that is, 1–2 alleles at most loci (Tables S2 and S3). In addition, they had low QI values (Table 1). After accounting for genotyping errors and consulting field notes, sample genotypes could be assigned to at least two individuals detected in two and five samples, respectively, resulting in 70% MGS (7/10 samples). Specifically, the two sample genotypes assigned to one individual (W1) matched at all genotyped loci except two (CI285 and CI291), with allelic differences compatible with ADO/FA. In addition, they had, respectively, 3–6 (sample Neige-2,1) and 5–7 (sample Neige-2,3) locus mismatches with sample genotypes assigned to the other individual, with mismatches involving different alleles. The other five samples were all conservatively assigned to a second individual (W2). Their sample genotypes differed at six loci (1–6 mismatches between pairs of sample genotypes) with mismatches compatible with ADO (loci CI233, CI285, CI291, CI308, CI527) and FA (locus CI375). However, field notes reported the possible presence of two individuals in some of the samples (Table S1). Therefore, we could not exclude that mismatches are actually true genotypic differences or that the DNA profile obtained from some samples resulted from DNA mixing within a track of related individuals with highly similar genotypes. Consequently, W2 genotype remains to be validated and the wolf snow tracks analysed can only indicate the detection of at least two individuals.

Figure 1b provides a schematic illustration of the subsequent decision-making steps described above for assigning sample genotypes to individuals.

4 | DISCUSSION

In this study, we successfully performed individual genotyping of STRs for three large carnivore species, and of a sex marker for one of these species, using snow track eDNA. Multilocus genotyping success rates for individual identification were in the range of those reported for the species using non-invasive genetic sampling

TABLE 2 Snow track genotypes and individual and sex identification at the STR loci analysed for brown bear (a), lynx (b) and wolf (c) and a sex marker for brown bear (a).

(a) Brown bear

Sample ID	UA03	UA06	UA14	UA16	UA17	UA25	UA51	UA57	UA63	UA64	UA65	UA67	UA68	Sex marker[a]	N. loci genotyped	Individual
CX.1138[b]	59 67	67 75	93 98	59 75	93 93	69 69	80 80	74 78	77 80		89 93	89 93	74 74	104 104_2	12	B1
CX.113A[b]	59 67	67 75	93 93	59 75	93 93	69 69	80 80	74 78	77 80	67 71	89 93	89 93	74 74	104 104_2	13	B1
CX.113C	59 67	67 71	98 98	67 75	93 97	69 69	68 80_2	74 78	77 84	63 67	89 93	85 89	74 78	104 104_2	13	B5
CX.113E[c]	59 59	63 71 83	98 98	71 75 79	89 93	69 77	72 76 80	74 74	77 84	67 67				104 104_2	10	/
CX.115H		71 71	93 98			69 69	72 76	74 78	80 80	63 63	89 93	93 93		104 104_2	9	B3
CX.1175	59 63	71 79	93 98	67 71	97 97	65 69	72 76	70 74	80 80	63 67	89 89	85 89	66 98	104 104	13	B4
CX.1176[d]	59 67	71 71	93 93	75 79	89 93	69 69	68 76	74 78	77 80	63 67	85 93	89 93	66 74	104 104_2	13	B2
CX.1176.2[d]	59 67	71 71	93 93	75 79	89 93	69 69	68 76	74 78	77 80	63 63	85 93	89 93	66 74	104 104_2	13	B2
CX.1177	67 67			67 67	93 93		72 76	74 78		63 67					6	/

(b) Lynx

Sample ID	LL0033	LL0043	LL0044	LL0045	LL0047	LL0051	LL0077	LL0105	LL0116	LL0125	LL0136	LL0140	LL0216	N. loci genotyped	Individual
CX.1156		75 79	64 64	66 70	45 61	92 92	62 62	88 92	55 59	69 69	59 59	69 77	59 59	12	L2
CX.1157														0	/
CX.1158	105 105	79 79	72 72	74 74				92 92	59 59	65 65	59 59	73 73		9	L1
CX.115A								92 92						1	/
CX.115C														0	/
CX.115E	105 105	75 79	68 72	74 74	45 61	92 96	54 62	92 92		65 73	59 59	73 73	59 59	12	L1
CX.115F														0	/
CX.115J	97 105	75 79		66 70	45 61	92 96	62 62	88 92	59 63	69 73	59 59	73 77	59 59	12	L3
CX.115K														0	/

(c) Wolf

Sample ID	CI147	CI233	CI274	CI285	CI291	CI308	CI330	CI366	CI370	CI375	CI507	CI527	Lup23	N. loci genotyped	Individual
Neige-1														0	/
Neige-2,1	65 77	55 55		63 67	76 76	64 68	64 64	76 76		57 57	62_2 62_3	54 58	63 63	11	W1
Neige-2,2	65 65		61 61	67 71	72_2 76	68 68	64 64	76 76	58 58	57 57	62_2 62_3	50 58	63 63	12	W2
Neige-2,3	65 77	55 55	57 61	67 67	72_2 76	64 68	64 64	76 76	58 62	57 57		54 58	63 63	12	W1
Neige-2,4		55 55	61 61	71 71	72_2 72_2	64 68	64 64	76 76	58 58	57 57	62_2 62_3	50 50		11	W2
Neige-3,1	65 65	63 63	61 61	67 71	72_2 76	64 68	64 64	76 76	58 58	57 57	62_2 62_3	50 58	63 63	13	W2
Neige-3,2	65 65	55 63	61 61	67 71	72_2 76	64 68	64 64	76 76	58 58	57 57_3	62_2 62_3	50 58	63 63	13	W2

TABLE 2 (Continued)

(c) Wolf														
Sample ID	CI147	CI233	CI274	CI285	CI291	CI308	CI330	CI366	CI370	CI375	CI507	CI527	Lup23	N. loci genotyped	Individual
Neige-4	65 65	63 63		71 71	72_2 72_2	64 68	64 64		58 58	57 57	62_2 62_2	50 58	63 63	11	W2
Neige-5						64 64								1	/
Neige-6														0	/

Note: Different grey shadings indicate locus data for sets of sample genotypes that were assigned to the same individual but that differed at that locus, with differences compatible with genotyping errors (ADO, FA). Allele sizes followed by underscore indicate allele variants of the same base pair length as another detected allele, but differing by sequence polymorphism (Table S3).

[a]Heterozygote 104 104_2: male; homozygote 104 104: female.

[b]First set of duplicate DNA extractions.

[c]Possible mixed sample with >2 alleles at three loci, no individual assigned.

[d]Second set of duplicate DNA extractions.

in the regions of snow tracks collection (wolf hair, saliva, scat, regurgitate and urine: 22%–60% Dufresnes et al., 2019; lynx hair, scat and urine: 9.4% Sindičić et al., 2013; brown bear scats: 88% Skrbinšek et al., 2019). In addition, the detection of individuals genotyped independently from other DNA sources, multiple observations of a multilocus genotype within the samples analysed and genotyping concordance from duplicate DNA extractions support the ability for accurate profiling. While the current study is a proof of concept, results for three different species show that reliable individual genotyping, including sex determination, from snow track eDNA of wild animals is possible, underlining its great potential for complementing wildlife non-invasive genetic sampling methods, with exciting prospects to expand ecological and conservation studies.

Highly variable per sample AS, GS and QI, as well as genotyping error rates similar to non-invasive genetic studies (e.g. 0.016–0.41 ADO and 0.002–0.08 De Barba & Waits, 2010; Dufresnes et al., 2019; Sindičić et al., 2013; Skrbinšek et al., 2019), suggest that, under certain conditions, snow track eDNA can be preserved and recovered in suitable quantities and quality to allow reliable individual genotyping of nuclear loci. We were able to genotype samples stored in the freezer for over 2 years, including samples taken from a single snow track. Compared to other eDNA sources, targeted sampling at snow tracks is expected to facilitate the detection of individual nDNA, thanks to favourable preservation on the snow substrate and limited DNA mixing (Franklin et al., 2019; Howell et al., 2021). Environmental conditions of the sampling sites and sample characteristics (i.e. number of tracks, track age, size, etc.) varied considerably in our study, and limited sample sizes prevented us from identifying clear patterns and applying statistical testing about factors driving genotyping performance. Still, most samples were collected within 1 day or even a few hours, and all within 3 days since the estimated time of animal passage. Furthermore, collection of several tracks or filtering of larger volumes did not systematically result in higher genotyping success, suggesting that interactions among sample and environmental variables or factors other than those recorded in the field are also key determinants of genotyping success.

We reported differing results between the species considered. Brown bear tracks had higher genotyping performance, and in particular considerably higher overall GS and lower ADO rates, than wolf and lynx tracks. However, most of the samples that were amplified, regardless of the species, resulted in high GS. While we cannot rule out that these differences are due to sampling conditions or laboratory methods in the different laboratories (see below), it may also suggest that the ecology of eDNA of a species could play a role in determining genotyping success. The ecology of eDNA refers to the combination of factors and processes influencing DNA production, state, transport and degradation in a given environment (Barnes & Turner, 2016). For snow track eDNA, this is relevant because the amount and state of DNA shed by each species may differ due to biological and behavioural differences between them, playing a role in eDNA preservation and retrieval. For example, brown bears have larger paws and are heavier, perhaps resulting in more skin cells

being deposited on the snow. In addition, brown bears are known to exhibit pedal marking behaviour, actively twisting their feet on the ground (Sergiel et al., 2017). The amount of DNA left on snow by individuals of a species could be affected by other behaviours, such as animals licking their paws for self-grooming. Beside the amount of DNA, animal behaviour could also affect the accuracy of individual genotyping. For example, it is not unusual for some species, including large carnivores, to step on tracks left by other individuals (Liberg et al., 2011; Sergiel et al., 2017), potentially resulting in eDNA sampling from multiple individuals (mixed samples). If not considered during sample collection or in the sampling design of a study, such instances can imperil individual genotyping efforts and bias results. While using STR genotyping in an outbred, genetically diverse population, mixed DNA profiles would typically be revealed through the presence of >2 alleles at several loci (as in one of the brown bear samples in this study), their detection could be subtler when related individuals are involved. This was a concern with the samples from a parent–offspring lynx pair and from a wolf pack in our study, as high genotype similarity could have resulted in the detection of an erroneous profile resembling that of a single individual. In such cases, field information about track characteristics and knowledge of the study system (i.e. presence of individuals of the target species), as available in our study, could be very important for ascertaining genotyping data and assessing if accuracy can be ensured. Under some circumstances, mixed samples could be resolved at the individual level (e.g. with animals known to be in the area and whose genotype has already been determined). Nonetheless, even if individual identification is prevented, detection and reporting of mixed samples will benefit data accuracy in population studies and support wildlife forensics, management and conservation, for example, indicating the presence of >1 individual at the sampling site, or informing on the efficiency of a sampling method for detecting individuals.

Beside the sampling conditions discussed above, laboratory protocols, from DNA extraction and amplification to the genotyping approach, differed compared to previous snow track genotyping studies (Barber-Meyer et al., 2020, 2022; Hellström et al., 2019; Von Duyke et al., 2023) and may have contributed to genotyping success. A major difference was the adoption of an HTS approach for amplicon sequencing of STRs. Markers analysed are short (<120 bp) tetranucleotides, selected for optimal multiplexing, to facilitate amplification of degraded DNA and multilocus allele scoring from HTS data. While there is evidence that reliable genotypes can be obtained analysing dinucleotide STRs on capillary electrophoresis (Von Duyke et al., 2023; personal data not shown), HTS sequencing of tetranucleotides, provided appropriate sequencing coverage, allows for greater sensitivity and clearer allele calling that may have enabled genotyping even with limited DNA quantities (Fordyce et al., 2015). In our study, sequencing conditions differed between brown bear samples and wolf/lynx samples, and a higher number of sequence reads was available for genotyping brown bear samples. Libraries for wolf and lynx samples were prepared using a protocol especially developed for minimizing tag-jumps that can form at different steps of the library preparation (Carøe & Bohmann, 2020), while a proprietary protocol was used for preparing the brown bear samples. High incidence of tag-jump reads is a concern because it could reduce allele detection and also lead to inaccurate genotyping. The low level of reads observed in the controls indicates that spurious sequences, including tag-jumps, were not a problem with both protocols and were actually negligible with the Tagsteady protocol used for lynx and wolf samples. Sequencing coverage was, on average, higher for brown than for lynx (almost twice reads/marker/PCR) and wolf (almost seven times reads/marker/PCR). Nonetheless, even with low coverage, individual ID was obtained for a number of samples (up to 70% with wolf that had the lowest read depth). This underlines the high sensitivity of an HTS approach, while also suggesting that increasing sequencing depth to levels similar to the brown bear samples may allow reducing ADO in lynx and wolf samples and increasing genotyping success in marginal samples.

Additional features of an HTS-based method are particularly relevant for snow-track genotyping. The main one is to enable access to the actual allele sequence polymorphism, in addition to length polymorphism, of highly variable STRs and the sex marker. This offers greater discriminating power for distinguishing individuals as well as mixed/contaminated samples due to DNA mixing of individuals of a species or different species (De Barba et al., 2017). Working with sequence data also allows for direct exchange and comparison of genotypes generated by different laboratories and at different times, which will facilitate the use of the collected data in large-scale, transboundary and long-term studies. Another important advantage of the HTS genotyping method is to allow efficient processing of samples requiring high replication levels, such as eDNA samples, that is, through a full multitube approach in a single run, rather than time-consuming screening and selective replication of samples/loci.

The genotyping success reported for the three large carnivores studied indicates that there is a vast potential for the application of eDNA sampling on snow tracks for species inhabiting temperate and polar ecosystems with a snowy season, significantly impacting wildlife research, management and conservation. The species that will benefit the most are those of conservation concern that are extremely elusive and/or difficult to study. Examples include secretive felid species such snow leopard or Siberian tiger (Rodgers & Janečka, 2013) and the polar bear (*Ursus maritimus*) (Von Duyke et al., 2023) among other species for which population data are lacking. In addition, species commonly monitored through non-invasive genetic sampling, such as wolves, brown and black bears and mesocarnivores (Kelly et al., 2012; Mumma et al., 2015), will also profit from genotype data collected through snow tracking. Snow track eDNA can complement other genetic sampling methods, by increasing individual detection and sample sizes, that is, for all age/sex classes or for the winter season, supporting more effective population monitoring and identification of targeted individuals for management purposes (Barber-Meyer et al., 2020). These systems, where ecological information is already available for the study species, are also those that would allow the most robust use of snow track eDNA for reliable individual identification. Here, the genotypes obtained from snow tracks can be used in association with

other available field or genotype data to compensate for possible bias associated with snow track sampling, specifically high ADO rate and eDNA sampling of multiple individuals.

To fully leverage the potential of snow track eDNA genotyping, future studies should work on aspects relating to both sampling in the field and laboratory analysis. In the field, efforts should be directed towards a thorough understanding of the optimal conditions for snow track sampling, investigating factors affecting genotyping success and accuracy related to the sampling site and methods and considering the eDNA ecology of target species. Previous studies have already stressed the importance of understanding the effect of track age, number and conditions of tracks sampled, temperature and UV exposure, equipment utilized for sampling and storage conditions (Barber-Meyer et al., 2020, 2022; Hellström et al., 2019; Howell et al., 2021). We further recommend that these effects be assessed for various target species in their ecosystem in order to evaluate species- and site-specific differences in eDNA deposition and degradation on snow tracks, and ideal sampling conditions for detection of individuals. In the laboratory, we emphasize the importance of DNA extraction protocols maximizing the amount and the quality of DNA retrieved from snow tracks, as well as investigating how sample treatments, e.g. the effect of thawing snow at room temperature for several hours, may affect DNA degradation and observed genotyping performance. We also recommend using highly discriminating individual profiling approaches optimized for accurate detection of low-level allele signals to increase genotyping sensitivity and inform about mixed samples. This includes the employment of library preparation protocols specifically developed for minimizing the occurrence of spurious sequences and therefore the noise-to-allele ratio.

The acquisition of comprehensive knowledge of the multiple factors affecting genotyping success and accuracy is paramount to inform the implementation of cost-effective snow track eDNA sampling efforts for large-scale wildlife surveys, monitoring and population studies in terrestrial ecosystems. Additionally, understanding of the drivers of genotyping success in the simplified snow track system would also inform efforts of nDNA retrieval and typing in more complex eDNA samples, such as water and soil samples.

AUTHOR CONTRIBUTIONS

P.T. conceived the idea. M.D.B., L.F., T.S. and P.T. designed the study. F.B., M.B., M.D.B., M.K., C.M., E.P., N.R., C.S. and T.S. conducted laboratory work and data analyses. M.D.B. wrote the paper with input from all co-authors.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

Sample metadata, marker information, R script used for calculating sample genotype mismatches, read counts in control samples and allele sequences: available in supplementary information. Raw sequence data available from Dryad repository: https://doi.org/10.5061/dryad.9kd51c5q1; ObiTools3 available from: https://git.metabarcoding.org/obitools/obitools3/; Python and R scripts used for bioinformatic analysis of sequence data available from GitHub: https://github.com/PazhenkovaEA/ngs_pipelines.py.

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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