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CRISPR-Dx and Metabarcoding Perform Similarly for Monitoring Mammals With eDNA on the Catchment Level in High-Alpine Ecosystems

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ABSTRACT

Biodiversity monitoring in difficult-to-access areas, such as rugged mountain ranges, is currently challenging and thus often absent. Environmental DNA (eDNA) offers new opportunities to monitor remote or strictly protected areas, as rivers integrate the biodiversity information of entire catchments. Environmental samples can be analyzed either with metabarcoding or using species-specific assays. Species-specific assays like quantitative polymerase chain reaction assays do not require a fully-equipped laboratory and thus can be used in settings with limited resources and are especially suited to monitoring elusive or threatened species of management concern. Recently developed molecular tools, such as CRISPR-based diagnostic systems (CRISPR-Dx), provide new avenues to facilitate eDNA analysis through species-specific assays. Here, we combine multispecies primers with CRISPR-Dx to detect terrestrial mammal species in parallel with one amplification to detect multiple species with CRISPR-Dx. Given the short length of metabarcoding amplicons, designing species-specific assays within them can be challenging. We designed species-specific CRISPR-Dx for eight terrestrial mammals within a commonly used metabarcoding amplicon ~59 base pairs in length and tested the assays on eDNA samples collected in high-alpine catchments. Additionally, we compared the detections from CRISPR-Dx with metabarcoding results of the same samples and with catchment-based species inventories obtained through traditional monitoring. First, we show that designing species-specific CRISPR-Dx within a short amplicon allows terrestrial mammal detection in eDNA. Second, we demonstrate that CRISPR-Dx assays combined with multispecies primers are comparable in sensitivity to metabarcoding and thus can bridge a gap between species-specific assays and community analysis without requiring fully equipped laboratories. Third, we highlight that catchment-based eDNA sampling can be used to monitor terrestrial mammals in remote or protected areas. Overall, we demonstrate that eDNA and particularly CRISPR-Dx are a promising tool to monitor inaccessible and/or protected areas and to detect rare species across large spatiotemporal scales, thereby promoting biodiversity conservation.

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1 | Introduction

With increasing anthropogenic pressure on earth's biodiversity, vertebrate populations across the globe are declining (Capdevila et al. 2022; Collen et al. 2009), increasing the demand for high-quality biodiversity monitoring data to reliably detect trends (Marconi et al. 2021) and remaining populations (Miranda-Chumacero et al. 2020). The location and subsequent protection of remnant populations is a key task in preventing (local) extinctions (Bolam et al. 2021; Turvey et al. 2008). Several species extinctions were prevented in the last decades through conservation measures, including the formation of protected areas (Bolam et al. 2021). However, detecting rare and elusive species is challenging even for large vertebrates, such as ungulates, given their evasive behavior and scarcity in the landscape (Kindberg et al. 2009). Vertebrates were initially monitored via audio or direct visual observations. In the last decades, passive recorders, such as camera traps, have increased the range of monitored species and areas (Cordier et al. 2022). However, these traditional methods—including camera trapping—can only survey a small part of the landscape at once. Hence, scaling up monitoring to include forested or rugged areas involves substantial additional effort and accompanying costs, making covering these areas unsustainable at large spatial scales (Beaudrot et al. 2016; Joshi et al. 2020; Kavčić et al. 2021). Most biodiversity data therefore stems from easily accessible areas (Jetz et al. 2019) even though threatened species typically inhabit remote areas (Polaina et al. 2019), hindering effective conservation measures (Scheele et al. 2019; Titley et al. 2017). Thus, monitoring methods which require less personnel and can integrate information over larger spatial scales are needed to complement the existing traditional monitoring schemes (Schmeller et al. 2017).

Environmental DNA (eDNA) has emerged as an alternative for detecting populations of vertebrates in inaccessible areas or in protected areas where a noninvasive monitoring method is required (Leempoel et al. 2020; Reinhardt et al. 2019; Thomsen and Willerslev 2015). Originally applied to aquatic vertebrates (Ficetola et al. 2008), most eDNA studies since then have focused on aquatic organisms such as fish and amphibians (Takahashi et al. 2023). Several studies have showcased the successful detection of rare species with eDNA (Lopes et al. 2021; Miranda-Chumacero et al. 2020; Reinhardt et al. 2019; Valsecchi et al. 2023). For example, a frog species thought to be regionally extinct was rediscovered with eDNA (Lopes et al. 2021). DNA fragments (e.g., from shed hairs or skin cells) in river water can be transported several kilometers downstream, and eDNA samples of river water thus integrate information on the species community upstream (Carraro et al. 2018, 2021; Deiner and Altermatt 2014; Lyet et al. 2021; Pont et al. 2018). Hence, species can be detected over a large spatial scale with only a few eDNA samples (Carraro et al. 2021). Filtering a large volume of river water increases the chances of detecting rare species occurring in the upstream area of a catchment, which are rarely observed otherwise (Cantera et al. 2019; Coutant et al. 2021; Lyet et al. 2021). Sampling at multiple elevation levels within one catchment can increase the number of species detected (Reji Chacko et al. 2023), but sampling at the bottom of a catchment is recommended to maximize the number of species detected when only one sample can be taken (Carraro et al. 2021). Despite the promising results of eDNA for biodiversity monitoring, its application to large spatiotemporal monitoring programs is currently limited by the requirement for well-equipped laboratories and the time-consuming analysis protocols (Holdaway et al. 2017).

eDNA analyses are mainly performed either through metabarcoding, to sequence the DNA of entire species communities using universal primers, or through species-specific assays including quantitative polymerase chain reaction (qPCR) (Takahashi et al. 2023). Compared with other species groups, such as amphibians, there are relatively few qPCR assays available for mammals (Takahashi et al. 2023), and most studies rely on metabarcoding if more than one species is of interest. Metabarcoding offers a list of all taxa of the target group, that is, mammals, detected within a catchment (Lyet et al. 2021) and thus additionally provides estimates of species distributions and richness. However, this approach requires sequencing of the amplified DNA and relies on well-equipped and costly laboratories, both of which slow down the analysis process and restrict the use of eDNA in large-scale biodiversity monitoring programs. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based diagnostic systems (Dx) were initially developed for health care diagnostics (Kellner et al. 2019; Pardee et al. 2016) and have more recently been leveraged for eDNA analyses (Williams et al. 2019, 2021). CRISPR-Dx assays are highly specific and can discriminate sequences with two nucleotide differences (Cox et al. 2017; Kellner et al. 2019). As CRISPR-Dx can be applied with limited resources, this approach does not require well-equipped laboratories and thus could be used across large spatial scales and in many different regions of the globe (Blasko and Phelps 2024; Nagarajan et al. 2024; Yang et al. 2024). CRISPR-Dx assays have the potential to be applied directly in the field to successfully detect rare and elusive species (Leugger et al. 2024, 2025; Nagarajan et al. 2024). However, most CRISPR-Dx assays are performed with species-specific primers (Nagarajan et al. 2024; Williams et al. 2019, 2021; Yang et al. 2024), preventing their application for monitoring multiple species of the same taxonomic group simultaneously.

A new approach, termed ampliscanning, combines multispecies primers, amplifying the DNA of a broad taxonomic group, with CRISPR-Dx for species detections. In this way, ampliscanning leverages the benefits of metabarcoding with those from taxa-specific assays (Leugger et al. 2024). The assays are highly sensitive, due to the amplification step, and specific, due to the CRISPR-Dx assay step (Leugger et al. 2024). The ampliscanning concept was developed to detect rare amphibians (Leugger et al. 2024) but has the potential to be adapted to other species groups as well. Given the short length of amplicons within multispecies primers (usually ~50–80 nucleotides) and the fact that less-specific CRISPR-Dx reactions have been reported (Li et al. 2022; Metsky et al. 2022), designing species-specific assays within the short amplicon is challenging. Thus, it is yet unknown to what extent the concept of ampliscanning can be applied to other organism groups/primers, that is, to mammals. Compared with amphibians, the diversity of mammal species is generally higher and more species co-occur in an area (Jenkins et al. 2013), making the design of species-specific CRISPR-Dx assays within a short amplicon

more challenging. Additionally, it remains poorly understood how the performances of CRISPR-Dx and metabarcoding readouts of eDNA compare.

In this study, we aimed to expand the ampliscanning concept to mammals and to compare the newly developed ampliscanning assays to metabarcoding using eDNA samples from a strictly protected area in the European Alps, in which proximity around 35 mammal species occur (infofauna.ch). Additionally, we used a species inventory obtained from the Swiss National Park as validation of the catchment-based eDNA sampling and subsequent analyses (Figure S1). We designed CRISPR-Dx assays for eight species occurring in the Swiss National Park based on the ampliscanning approach (Leugger et al. 2024) within the ~59-nucleotide Mamm01 amplicon (Lyet et al. 2021; Taberlet et al. 2018). We intended to answer the following questions: (1) Can we design species-specific CRISPR-Dx assays within the short Mamm01 amplicon for multiple mammals living in high-alpine catchments? (2) Do the species found in eDNA samples with ampliscanning match the species detected through metabarcoding? (3) Does sampling river water at the bottom of a catchment enable the detection of species known to occur in the catchment? By comparing ampliscanning with metabarcoding and with inventories of species known to occur in the catchments, our study allows us to show the benefits and uncertainties of using eDNA with metabarcoding or ampliscanning for large-scale biodiversity monitoring in inaccessible areas.

2 | Methods

2.1 | Study Area

We conducted our study in the Swiss National Park, which is located in the canton of Grisons in the European Alps, ranging from 1400 to 3174 m a.s.l. The park was founded in 1914 and covers an area of 170 km^2 that is strictly protected (International Union for Conservation of Nature category Ia: strict nature reserve). The area of the Swiss National Park is rigorously monitored (Baur 2014) and several alpine mammal species inhabit the park, including four native ungulate species. An emblematic species of the park is the alpine ibex (*Capra ibex*), a high-alpine ungulate species that nearly went extinct 100 years ago and inhabits the steepest slopes often above the treeline (Graf et al. 2021), at around 2200 m a.s.l. in the Swiss National Park. We sampled eDNA from river water in the Swiss National Park, with the aim to compare detections of terrestrial mammals based on eDNA and traditional monitoring.

2.2 | eDNA Sampling & Extractions

We collected eDNA samples at six sites (catchments) within the Swiss National Park from 26 to 29 September 2022 (see Table S1 for precise date at each location). We sampled close to the park border or official hiking trails to minimize the impact on the park's flora and fauna, which is an important advantage of eDNA for biodiversity monitoring compared with traditional methods (Leempoel et al. 2020). The sampling sites ranged from 1507 m a.s.l. (Val Cluozza) to 2613 m a.s.l. (Macun) (Table 1, Figure 1). We calculated the catchment area with the rivnet package v0.5 (Carraro 2023) in R v4.3.3 (R Core Team 2024) and estimated the catchment length with an online tool available from SwissTopo (map.geo.admin.ch). All catchments (above the sampling location) lie entirely in the Swiss National Park. At each sampling site, we filtered water for 2 h with a peristaltic pump (Athena; Pegasus, Nottingham, UK) through a single-use filtration capsule with 0.45 μm pore size (VigiDNA, SPYGEN, Le Bourget du Lac, France), which corresponds to approximately 80 L of filtrated water. We simultaneously took two filter replicates at each site, as a compromise between costs and the ability to detect more species. We added 80 mL of CL1 conservation buffer (SPYGEN) to the filter capsules, shook them thoroughly for 2 min immediately after sampling, and stored them at room temperature until extraction.

We followed the protocol of Pont et al. (2018), with the modifications detailed in Leugger et al. (2024), for the eDNA extractions in a clean laboratory environment. In brief, we performed an ethanol precipitation followed by multiple cleanup steps with columns. We extracted the DNA over 2 days and included a negative extraction control for each day to test for contamination during the laboratory processes. We measured the DNA concentration with the Qubit high-sensitivity dsDNA kit (Thermo Fisher Scientific, Waltham, MA, USA) (Table S1) and stored DNA extracts at −20°C until the amplification.

2.3 | Metabarcoding

For library preparation, we used a two-step PCR protocol. We performed the first amplification step using Mamm01

TABLE 1 | Catchment names and characteristics (elevation range, size) of the sampled catchments within the Swiss National Park.

Catchment name	Elevation of sampling point [m a.s.l.]	Maximum elevation [m a.s.l.]	Catchment length [km]	Catchment area [km^2]
Macun (Lai d'Immez)	2613	3045	1.9	2.4
Val Cluozza	1506	3164	10.2	26.7
Val dal Botsch	1885	3012	3.9	4.1
Val Foraz	1760	3092	4.4	4.7
Val Tantermozza	1744	3164	5.3	9.2
Val Trupchun	1871	3070	4.5	10.8

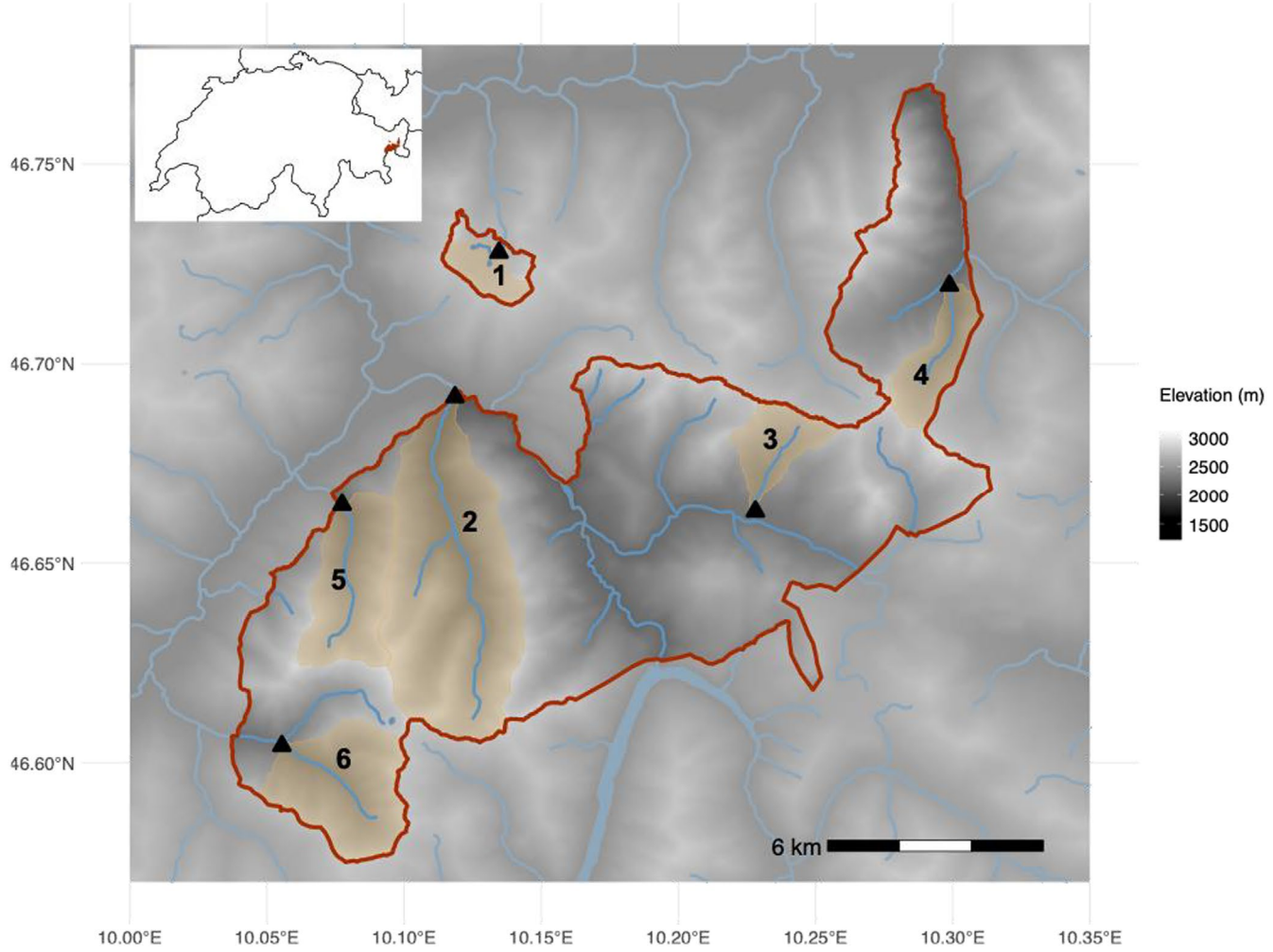

FIGURE 1 | Sampling sites and catchment names within the Swiss National Park. Black triangles indicate the sampling locations. (1) Macun (Lai d'Immez), (2) Val Cluozza, (3) Val dal Botsch, (4) Val Foraz, (5) Val Tantermozza and (6) Val Trupchun. The park boundary is shown with a thick red line. The inset map in the top left shows Switzerland and the location of the Swiss National Park (in red).

primers (Lyet et al. 2021; Taberlet et al. 2018; Integrated DNA Technologies [IDT], Coralville, IA, USA) targeting a region of the 12S mitochondrial locus. These primers provide a high taxonomic resolution to discriminate among mammalian species (Taberlet et al. 2018) and are often used in eDNA metabarcoding studies to detect terrestrial mammals (Holm et al. 2023; Juhel et al. 2021; Lyet et al. 2021; Mena et al. 2021). We used both forward and reverse primers in an equimolar mix of four primers, including three frameshift primers. We performed eight PCR replicates for each DNA extract and included a negative control with molecular-grade water. The final PCR reaction volume was 25 μL and included: 12 μL of AmpliTaq Gold 360 master mix (Applied Biosystems, Waltham, MA, USA), 0.3 μM of each primer mix, 4 μM of human blocking primer (Lyet et al. 2021; IDT), 0.2 μg/μL of BSA (Thermo Fisher Scientific), 5% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific), 3.25 μL of molecular-grade water, and 3 μL of DNA extract. The PCR protocol started with an enzymatic activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 30 s. We performed a final elongation step at 72°C for 5 min. For each sample, i.e., one filter replicate, we pooled the PCR replicates and analyzed them using agarose gel electrophoresis. We performed a cleanup step with AMPure XP beads (Beckman Coulter, Brea, CA, USA) both before and after the indexation PCR. We applied the indexation PCR following the manufacturer's protocol (Illumina, San Diego, CA, USA), with 10 cycles of amplification using Nextera XT adapters (Illumina). Afterwards, we quantified the samples in duplicate using the fluorescent dsDNA BR Qubit assay (Thermo Fisher Scientific) and the Spark M10 plate reader (Tecan, Männedorf, Switzerland). We then normalized and pooled the samples into a single library. We analyzed the library by capillary electrophoresis and quantified it by qPCR using the Kapa quantification kit (Kapa Biosystems, Wilmington, MA, USA) and the LightCycler 480 Real Time PCR system (Roche, Basel, Switzerland). We performed paired-end sequencing using the MiSeq Reagent Kit v3 (2 × 75 base pairs) (Illumina) at a theoretical sequencing depth of 200,000 reads per sample.

For the bioinformatics analysis, we first trimmed the primer sequences with the default parameters in cutadapt v4.6 (Martin 2011), accepting a maximum error rate (−e) of 0.1 and a minimum length (−m) of 20 to remove empty reads. Subsequently, we used the dada2 R-package v1.22 (Callahan et al. 2016) to filter the reads before merging them. We did not allow ambiguous bases (N, default), we set the maximum

expected error rate at 2 (maxEE, default), and we truncated reads at the first instance of a quality score of 11 or lower (truncQ). After learning the error rates and denoising the reads with default parameters, we merged the forward and reverse reads with a minimum overlap of 20 (minOverlap) and removed chimeras. Finally, we assigned species using the assignTaxonomy and addSpecies functions in dada2, with default settings and a custom reference database built with CRABS (Jeunen et al. 2023). The custom reference database included amplified sequences with the Mamm01 primers from the NCBI GenBank (Benson et al. 2017), accessed in November 2024, and the European Molecular Biology Laboratory (EMBL) database (Kanz et al. 2005), accessed in June 2023, using default parameters. We applied a minimum threshold of 10 reads per taxa per filter for downstream analyses and manually corrected genus-level identifications when only one species of this genus was known to be present in the study area. We removed sequences with a frequency below 0.01%. Additionally, we removed species found in the negative extraction control. In cases where a single sequence was assigned to multiple species but only one of these species occurs in the proximity of the study area, we selected this species. We performed all downstream analyses on the species level if not otherwise indicated, and we combined (taxon) data from the two filter replicates for comparisons on the site/catchment level.

2.4 | CRISPR-Dx Assay Design

We used the ampliscanning concept to combine taxa-specific CRISPR-Dx assays with multispecies primers to detect several target species with the same amplification step (Figure 2; Leugger et al. 2024). For the PCR-based amplification, we used the Mamm01 primers (Mamm01 forward primer 5′-CCGCCCGTCAC YCTCCT-3′; reverse primer 5′-GTAYRCTTACCWTGTTACGA C-3′) (Lyet et al. 2021; Taberlet et al. 2018). We followed the procedure outlined in Leugger et al. (2024) to design CRISPR-Dx assays for a subset of species occurring in the Swiss National Park, ranging from locally rare to very abundant. The CRISPR-Dx guide sequence is 28 nucleotides long and must lie within the amplified sequence. In brief, we applied an in silico PCR to obtain the Mamm01 amplicon of the target species' DNA sequences from NCBI Genbank (Benson et al. 2017) to design the guide sequences.

As the specificity of CRISPR-Dx is not yet fully understood and likely context dependent (Molina Vargas et al. 2024), creating reliable 28-nucleotide species-specific guides requires both in silico and in vitro testing (Gootenberg et al. 2017; Williams et al. 2019). Following best practices for validating eDNA assays (Thalinger et al. 2021), we first in silico filtered target sequences for potential off-target hits having four or fewer mismatches (Leski et al. 2023; Leugger et al. 2024; Metsky et al. 2022) (see Figure S2 for the positions of the guide

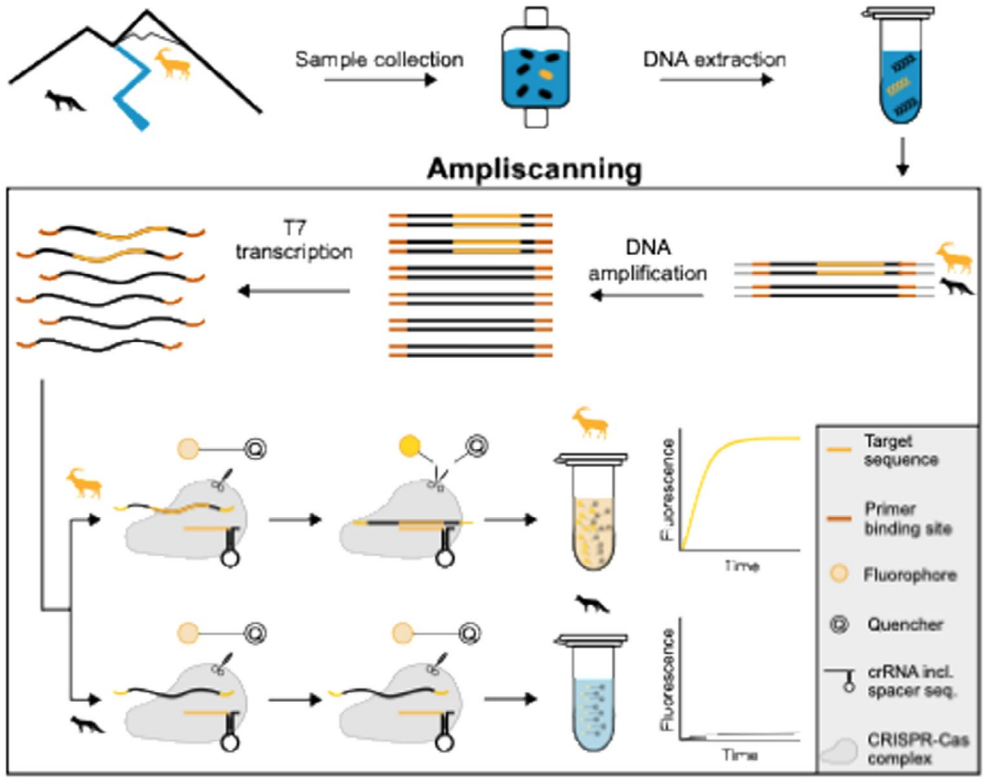

FIGURE 2 | Ampliscanning concept for eDNA samples, exemplified for the alpine ibex as target species. First, eDNA water samples are collected and DNA is extracted. Ampliscanning consists of amplification (using a multispecies primer pair) to amplify the eDNA at low concentrations. The amplified product is then used as input for the CRISPR-Dx assays to detect the target species. For this study, we used the Mamm01 primers (Lyet et al. 2021; Taberlet et al. 2018), the same primers used for metabarcoding. During the incubation of the CRISPR-Dx, the DNA is transcribed to RNA, as Cas13a recognizes RNA. The CRISPR-Cas complex with the crRNA including the spacer sequence then scans the RNA strands. Upon target recognition (RNA complementary to the spacer sequence), collateral cleavage is activated and the quencher is cut from the fluorophore, which then emits a signal. The signal can be measured over time and indicates detection of the target DNA.

sequences within the amplicon). Similarly to previously published CRISPR-Dx or qPCR assays and recommendations, we further challenged the selected guides' specificity against potentially co-occurring species in vitro (Baerwald et al. 2023; Thalinger et al. 2021; Yang et al. 2024). As domestic animals are forbidden in the Swiss National Park, we only included wild mammals in these specificity tests. We tested the specificity of the CRISPR-Dx assays using synthetic DNA (gBlocks; IDT; sequences provided in Table S2). Synthetic DNA has been shown to be an adequate replacement for tissue extracts for testing the specificity of individual assays (Leugger et al. 2024) and has been used successfully to test CRISPR-Dx specificity for eDNA studies (Blasko and Phelps 2024; Leugger et al. 2024; Yang et al. 2024). As the concentration of the target DNA affects CRISPR-Dx specificity (Molina Vargas et al. 2024), pure synthetic DNA input might underestimate the specificity of CRISPR-Dx compared with highly complex eDNA samples characterized by very low target DNA concentrations. We used a concentration of 1 pg/μL as input for the PCR (see details below). Additionally, we aligned the CRISPR-Dx guide sequences to the metabarcoding sequences to investigate if there could be any unexpected off-target hits. On the other hand, to assess the detection limit, that is, sensitivity, of the ampliscanning (amplification & CRISPR-Dx), we applied the alpine marmot (*Marmota marmota*) CRISPR-Dx assay with synthetic DNA as PCR input. We used a serial dilution down to 0.6 copies per reaction (pre PCR) and used the same PCR and CRISPR-Dx setup as for the eDNA samples. Additionally, we compared the sensitivity of using pooled PCR products with that of using unpooled PCR products to examine if such pooling prior to the CRISPR-Dx reduces the sensitivity of ampliscanning.

2.5 | CRISPR-Dx Assays

We attached the T7 promoter sequence to the 5′ end of the Mamm01 forward primer (T7-mamm01-fwd: 5′-GAAAT TAATACGACTCACTATAGGGCCGCCCGTCACYCTCC T-3′) for the transcription to RNA in the CRISPR-Dx assay. Additionally, we added the 'GAAAT' on the 5′ end of the T7 promoter to increase binding affinity (Baerwald et al. 2020; Kellner et al. 2019). We set up each PCR at a final volume of 25 μL, with 1 μL of (pooled) DNA extract, 1.25 μL of forward and reverse primers (IDT; final molarity: 0.5 μM), respectively, 9 μL of molecular grade water, 12.5 μL of Q5 High Fidelity Master Mix (New England BioLabs, Ipswich, MA, USA), and 0.035 μL of bovine serum albumin (BSA) (New England BioLabs; final concentration: 0.02 ng/μL). The settings for the PCR were as follows: initial denaturation at 98°C for 30 s, followed by 35 cycles of 5 s at 98°C, 15 s at 57°C, 5 s at 72°C, and a final elongation step at 72°C for 120 s.

For each extract, we performed eight individual PCR replicates, which we then combined into three pools to reduce costs for the subsequent CRISPR-Dx assays (PCR replicates 1–3 in pool 1, reps 4–6 in pool 2, and reps 7–8 in pool 3). We performed one CRISPR-Dx assay per target species for each pool and followed the protocol of Leugger et al. (2024) to set up the CRISPR-Dx reactions. We used fluorescence measurements as a readout. We visually verified the classifications of a machine learning algorithm developed in Leugger et al. (2024) to classify a CRISPR-Dx assay as positive or negative. Extracts were classified as positive if at least one of the CRISPR-Dx assays was positive and a site/catchment was classified as positive if at least one extract was positive for a target species (Leugger et al. 2024; Yang et al. 2024), following the guidelines of the eDNA Society (Minamoto et al. 2021).

2.6 | Catchment-Based Species Inventories

We received catchment-based mammal species inventories from the Swiss National Park. Rangers and scientists perform traditional monitoring at the Swiss National Park through visual censuses and camera trapping. Opportunistic recordings from hikes by park staff are included as well. The data was combined at the catchment level from standardized censuses and the opportunistic records. It included expert knowledge to estimate if a species could be present at the time of eDNA sampling or not, as some records were from previous years. We considered all species that were classified as known to be present in the catchment as "known occurrence" (detected with traditional monitoring). We classified species that might be present but whose presence was not confirmed as "maybe present" (this only concerned the water shrew, *Neomys fodiens*) and all others as "likely absent".

2.7 | Methods Comparison

To investigate how well ampliscanning results matched the metabarcoding results, we performed a McNemar test, as implemented in R (Biggs et al. 2015; McNemar 1947). Additionally, we tested the correlation between the number of positive CRISPR-Dx assays per site and metabarcoding reads using a Pearson correlation test and a generalized linear model, both in R. We also compared the species detected with ampliscanning with data from a catchment-wise species inventory. We considered species classified as "detected with traditional monitoring" for the comparison between the results from the eDNA analysis methods and from traditional monitoring. As in the comparison between the eDNA methods, we applied a McNemar test to assess differences between detections made with ampliscanning and traditional monitoring.

3 | Results

3.1 | Metabarcoding

From the eDNA metabarcoding that we performed to assess the terrestrial mammal community on the catchment level, we obtained 2,806,789 reads in total before filtering (Table S3), which corresponded to 187,119 reads per extract, including the negative controls (standard deviation 38,177 reads). The negative controls included reads from the wild boar (*Sus scrofa*) and great apes (family Hominidae). After removing low-abundance reads and sequences present in the negative extraction control, 1,359,822 reads were left (Table S4). We were able to assign 1,025,060 reads (75.3% of filtered reads) to the species level. If only a single species of a genus was

present in the region, we manually assigned the reads to the species level (this concerned the red deer [*Cervus elaphus*], red fox [*Vulpes vulpes*], and brown rat [*Rattus norvegicus*]). We used only species-level assignations for all further analyses. The most reads were obtained for ungulate species (Figure 3 and Table S5), with the red deer having the most reads in several sites (Table S5). Between 3 and 16 species per site were detected with metabarcoding. The most commonly detected species of our subset of eight species for which we designed CRISPR-Dx was the northern chamois (*Rupicapra rupicapra*), which was detected at all six sites. The red deer and the red fox were the second most common species, each with detections at five out of six (83%) sites. We detected the alpine ibex at four sites, the roe deer (*Capreolus capreolus*) at two sites, and the water shrew at one site. We never detected the alpine marmot or mountain hare (*Lepus timidus*) with metabarcoding.

3.2 | Ampliscanning Specificity & Sensitivity Tests

Our in silico and in vitro specificity analyses showed that 75% (6/8) of the designed CRISPR-Dx assays were specific to the target species in our study region (Table 2). The in silico analyses revealed that none of the CRISPR-Dx guide sequences had any potential off-target hits with fewer than four mismatches, except for the alpine ibex, which had two to three mismatches to the sequence of the domestic goat (*Capra hircus*) and four to the domestic sheep (*Ovies aries*) (Table S6). Additionally, the CRISPR-Dx assay of the mountain hare had four mismatches to the brown hare (*Lepus europaeus*). Our in vitro analyses (Figure 4, Table S7) using synthetic DNA (gBlocks) showed that the CRISPR-Dx assays of the mountain hare could detect the brown hare despite having four mismatches. The next lowest and tested number of mismatches between a CRISPR-Dx assay guide sequence and a DNA target sequence was six mismatches, which did not show increased fluorescence values (Figure 4, Table S7). We subsequently aligned the CRISPR-Dx guide sequences to the sequences obtained at the species level from metabarcoding to test for potential additional off-target hits. The only species that had four or fewer mismatches to the target species' CRISPR-Dx guide sequence was the domestic goat, with two mismatches to the alpine ibex guide sequence. Metabarcoding detected the domestic goat only at one site (Val Tantermozza), where it detected the alpine ibex as well. Therefore, we expect that the other detections of alpine ibex with the CRISPR-Dx assays represented true positive detections of this emblematic species. We hence used the CRISPR-Dx assays for the alpine ibex at the species level for downstream analyses.

The sensitivity tests of ampliscanning (amplification with multispecies primer pair and species-specific CRISPR-Dx) using the CRISPR-Dx assays for the alpine marmot to detect synthetic DNA targets (gBlocks) revealed that pooling the PCR products did not reduce the sensitivity, as all three pools were positive down to 2.23 DNA copies per reaction, whereas only six out of eight (75%) unpooled PCR replicates were positive and two out of three (67%) pools were positive for 1.1 DNA copies per reaction (Table S8).

3.3 | Ampliscanning on eDNA Samples

We observed no signs of contamination in the 48 negative controls that we ran in parallel with the 288 CRISPR-Dx assays. Of the 288 CRISPR-Dx assays for eDNA samples, 73 (25%) were positive. The red deer was detected most often, with 27 positive assays (out of 36, 75%; Table S9), followed by the northern chamois and alpine ibex, with 21 (58%) and 14 (39%) positive assays, respectively. Three species were not detected with ampliscanning: the

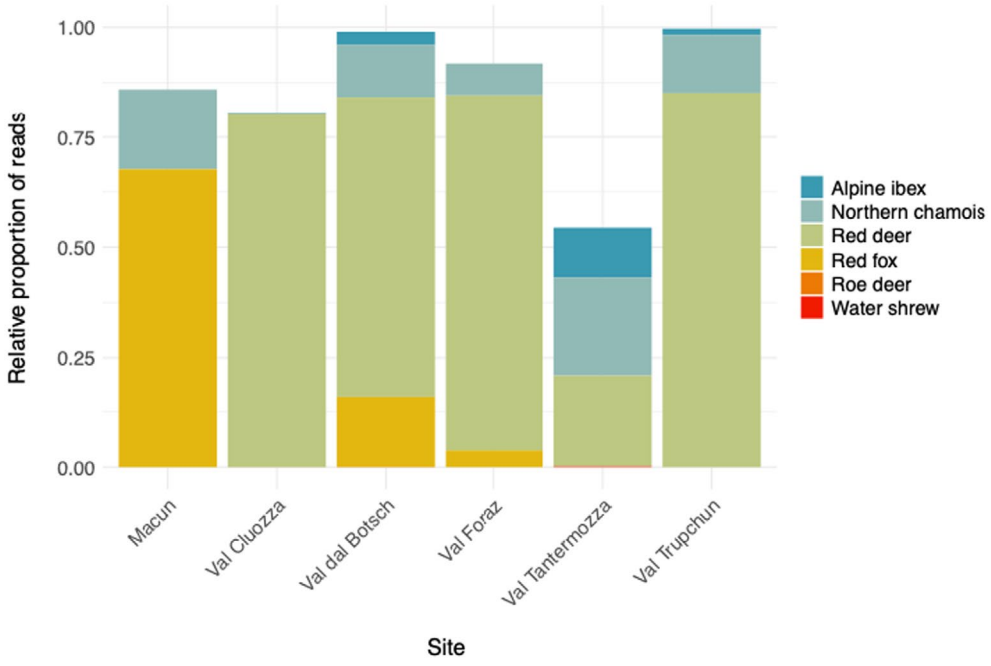

FIGURE 3 | Relative proportion of reads per species detected with metabarcoding across the six catchments in the Swiss National Park (one sampling site per catchment). Only species for which we designed CRISPR-Dx are shown. See Table S5 for detailed read numbers per species and site.

TABLE 2 | Sequences of the CRISPR-Dx assays for the species included in this study.

Species	Scientific name	CRISPR-Dx sequences (5′–3′)	Red list status
Alpine ibex	*Capra ibex*	TAAATATAATGCACTCAAGCCTATTTACGTTTTAGTCCC CTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTG AGTCGTATTAATTTC	LC
Alpine marmot	*Marmota marmota*	ATACTTTAATACAACTCTATTAATAATTGTTTTAGTCC CCTTCGTTTTTGGGGTAGTCTAAATCCCCTATA GTGAGTCGTATTAATTTC	LC
Mountain hare	*Lepus timidus*	ACTGAATATTAATTATAAATAATTCTTAGTTTTAGT CCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGT GAGTCGTATTAATTTC	NT
Northern chamois	*Rupicapra rupicapra*	CAGGACACTCAAAACCTATTTAAACACAGTTTTAGTCCC CTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGT GAGTCGTATTAATTTC	LC
Red deer	*Cervus elaphus*	CACTCAAATTTATTTGCACGTATTAATCGTTTTAGTCCC CTTCGTTTTTGGG GTAGTCTAAATCCCCTATAGTGAGTCGTATTAATTTC	LC
Red fox	*Vulpes vulpes*	AGGCCATAAACATATTAACTCACATCAAGTTTTAGTCCCC TTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGA GTCGTATTAATTTC	LC
Roe deer	*Capreolus capreolus*	ACATTTAAATTTATTTATACGTATAAACGTTTTAGT CCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTG AGTCGTATTAATTTC	LC
Water shrew	*Neomys fodiens*	AACTAAAATATTCCTAATTTAATGGTAAGTTTTAGT CCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATA GTGAGTCGTATTAATTTC	VU

Note: The spacer sequence is underlined, followed by the direct repeat sequence. The sequences have to be converted to RNA. The Red List status is from Capt (2022): LC, least concern; NT, near threatened; VU, vulnerable.

alpine marmot, the mountain hare, and the water shrew. Thus, the assays of the mountain hare, which were previously shown to not be specific at the species level, did not show any positive detections. We detected two to five species per site with ampliscanning, including multiple ungulate species, except at Macun, where we detected only the northern chamois and red fox.

3.4 | Comparison Between Ampliscanning and Metabarcoding

Comparing ampliscanning and metabarcoding revealed that for the subset of species for which we created CRISPR-Dx assays, detections with metabarcoding and ampliscanning largely overlapped (21 out of 23 cases [91%]; Figure 5). We did not detect species with ampliscanning at sites where there was no detection with metabarcoding, indicating that ampliscanning did not lead to false positives, that is, due to unspecific CRISPR-Dx assays. Compared with the detections with ampliscanning, we observed an additional detection of the roe deer in Val Cluozza and the water shrew in Val Tantermozza with metabarcoding, which might have been due to amplification stochasticity. Thus, the difference between CRISPR-Dx assays and metabarcoding was marginal (McNemar test: p value = 0.48, $\chi^2 = 0.5$, df = 1).

The comparison between the metabarcoding read numbers and the positive detections with ampliscanning highlights the sensitivity of the latter, as very few (< 40) metabarcoding reads were successfully detected with ampliscanning (due to the amplification step). For example, the red fox had 32 and 37 reads in Val Trupchun and Val Tantermozza, respectively, and the roe deer had 40 reads in Val dal Botsch that we also successfully detected with ampliscanning (Figure 5 and Tables S5 and S9). Generally, the number of positive CRISPR-Dx assays of a species per site and the number of metabarcoding reads of the same species at the respective site showed a slight correlation ($r = 0.63$, $p < 0.01$; Figure 5 and Figure S3; see Table S5 for a comparison between read numbers and numbers of positive CRISPR-Dx assays). Specifically, only 1 of 6 CRISPR-Dx pools was positive for the metabarcoding detections with 32–40 reads, indicating that it is likely that only one PCR replicate amplified the respective sequences. Two detections from metabarcoding that were missed with ampliscanning had relatively few reads, with 66 for the roe deer and 318 for the water shrew. This implies that the amplification for rare sequences can be stochastic and that the sample/community composition might affect amplification success.

3.5 | Comparison With Catchment-Based Species Inventory

From our subset of eight species, five are known to occur in all sampled catchments within the Swiss National Park: the alpine

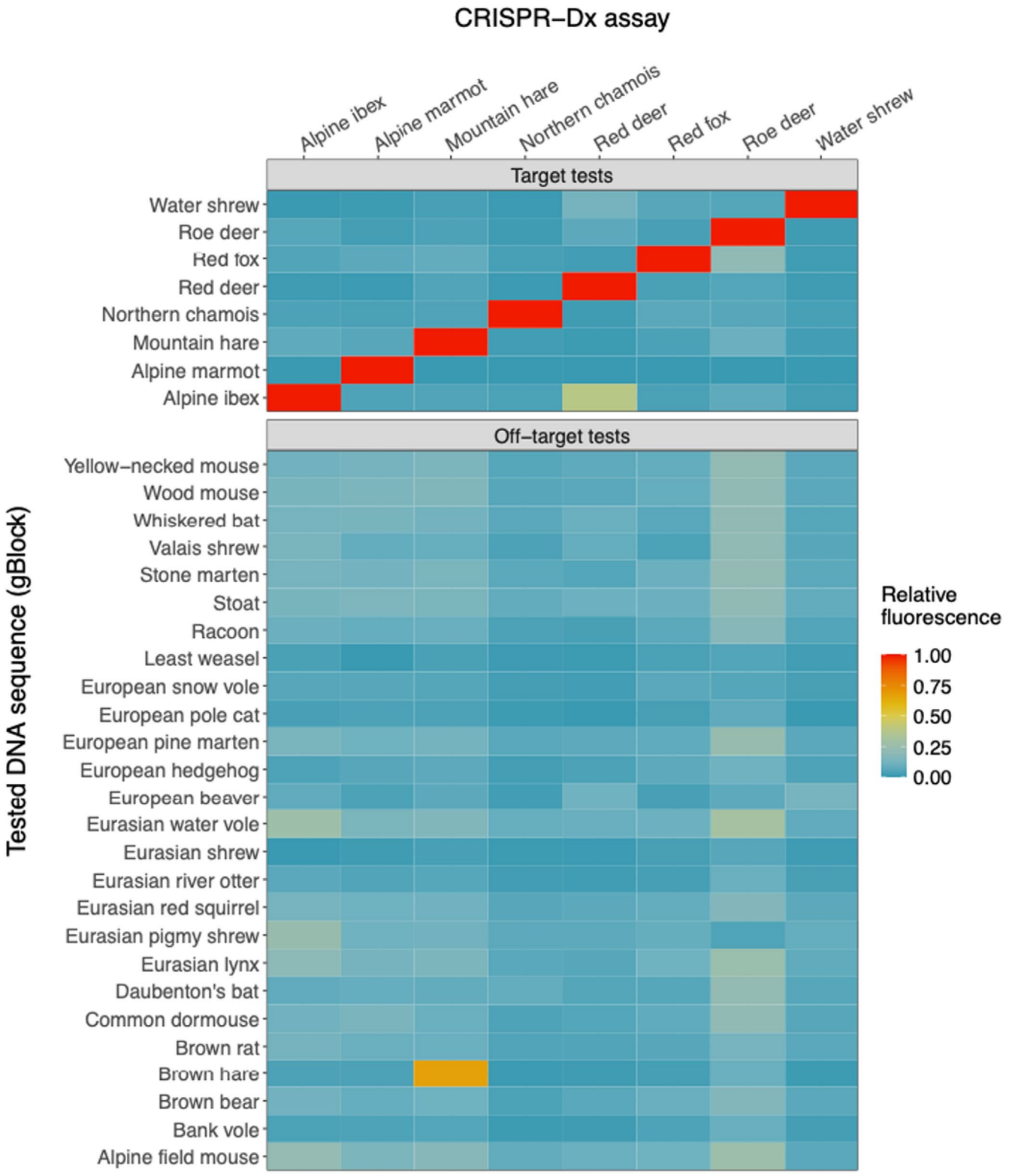

FIGURE 4 | Specificity tests of the CRISPR-Dx assays against synthetic DNA sequences (gBlocks) of potentially co-occurring species. The CRISPR-Dx of the mountain hare also produces a detectable signal for the brown hare.

marmot, mountain hare, northern chamois, red deer, and red fox. The alpine ibex has been documented to inhabit four catchments and the roe deer two. There was no confirmed detection of the water shrew in the catchment-based species inventory (Table S10).

The comparison of detections from ampliscanning with catchment-based species inventories obtained from traditional monitoring indicated significantly fewer detections with ampliscanning (21 vs. 37; McNemar test: p value <0.01, $I^2=14.1$, df $=1$). The difference was mainly caused by the inability of the

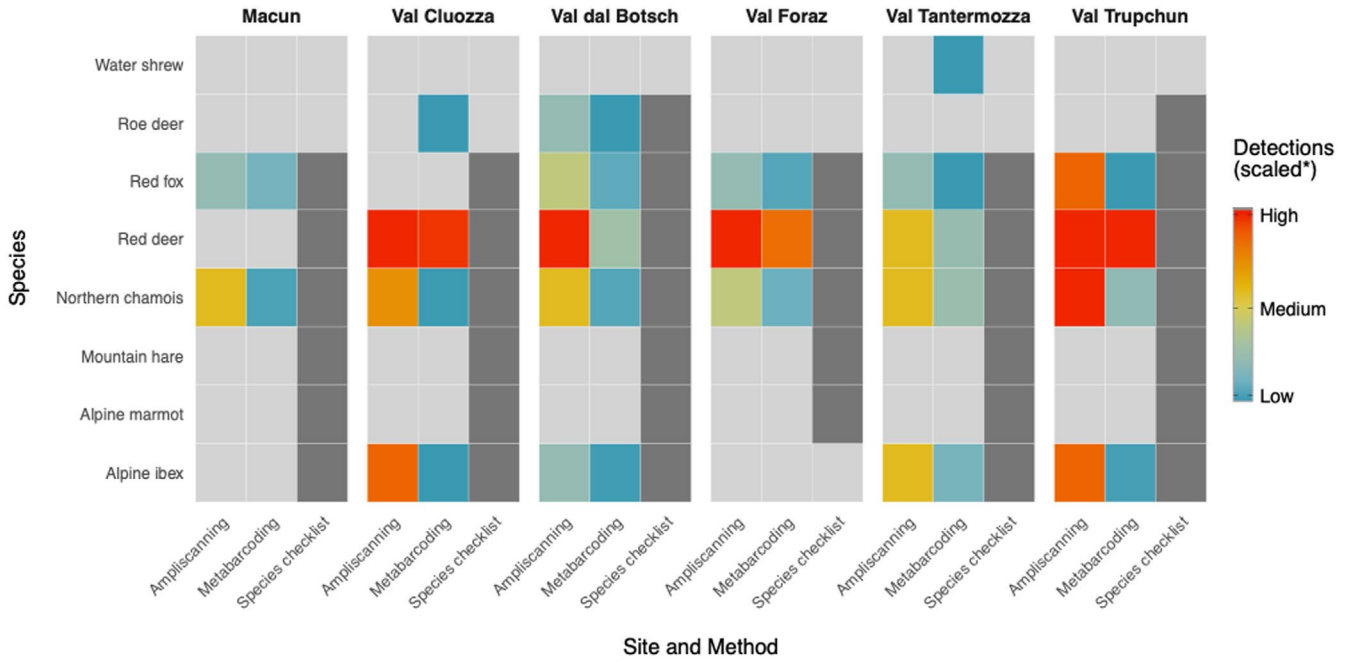

FIGURE 5 | Species detected per catchment with the different methods. Ampliscanning and metabarcoding are based on the same eDNA extracts, whereas the catchment-based species inventories combine traditional monitoring and expert knowledge from the Swiss National Park. The number of positive ampliscanning pools (maximum of six per species and site) and the number of metabarcoding reads per species and site are scaled and log10 + 1 transformed. Light gray cells indicate no detections, and dark gray cells indicate detections with traditional monitoring where no scaling was possible. Only species for which we designed CRISPR-Dx assays are shown.

alpine marmot and mountain hare to be detected through eDNA (which together correspond to 12 traditional monitoring detections). Ampliscanning for those species successfully detected pure synthetic DNA targets of the respective species, which might indicate that the sensitivity is reduced in complex eDNA samples. If these two species were left out of the comparison, the difference in detections between eDNA with ampliscanning and traditional monitoring was much smaller (21 detections with ampliscanning vs. 25 with traditional monitoring) and no longer significant (McNemar test: p value < 0.01, $\chi^2 = 2.25$, df = 1). In the Macun catchment, we observed the lowest detection rate with eDNA compared with the species inventory, and two species that were detected in most of the other catchments with eDNA were not detected here. The results for metabarcoding were very similar to the comparison between ampliscanning and traditional monitoring, due to the large overlap in eDNA detections, as both the alpine marmot and mountain hare were also missing in the metabarcoding results. To summarize, detections made with ampliscanning mainly overlapped with metabarcoding and traditional monitoring, except for two species that were missed with both eDNA analysis methods.

4 | Discussion

Here, we demonstrate that sampling large volumes of river water (i.e., 80 L) at the bottom of a catchment offers valuable insights into (terrestrial) species diversity on the landscape scale. We generally detected similar mammal species by analyzing just two filter replicates at the bottom of the rugged and remote catchments as with extensive traditional monitoring, highlighting the benefits of eDNA to efficiently monitor areas that are difficult to access (Reinhardt et al. 2019). For example, we successfully detected ibex populations that were at least 2 km away from the sampling location, adding evidence that eDNA can be transported over several kilometers in running waters (Deiner and Altermatt 2014; Pont et al. 2018) and that sampling at the bottom of small to medium catchments offers a good overview of the biodiversity on a catchment level (Carraro et al. 2021). Furthermore, we show that ampliscanning using a CRISPR-Dx readout performs similarly to metabarcoding and thus can be used to quickly detect multiple species simultaneously. As CRISPR-Dx assays omit eDNA sequencing, this method can be used in settings where resources are limited or when obtaining results rapidly is crucial (Budd et al. 2023; Nagarajan et al. 2024; Yang et al. 2024). Furthermore, combining CRISPR-Dx with multispecies primers, as in this study with ampliscanning, could foster biodiversity monitoring of landscapes with low accessibility.

We successfully expanded the ampliscanning concept to mammals by designing species-specific CRISPR-Dx assays for multiple species within the short Mamm01 amplicon. In this way, we could circumvent the limitations of most previously published CRISPR-Dx relying on species-specific primers (Baerwald et al. 2020, 2023; Nagarajan et al. 2024; Wei et al. 2023; Williams et al. 2019, 2021, 2023; Yang et al. 2024) for multispecies detections. Our in vitro tests using synthetic DNA revealed that CRISPR-Dx could cross react with sequences having up to four mismatches, contrasting earlier reports (Gootenberg et al. 2018; Kellner et al. 2019). It remains unclear, however, why sometimes several mismatches are tolerated (Li et al. 2022). The location within the guide sequence and the type of mismatch affect the CRISPR-Dx activity differently, as the formation of a guide–target

duplex may be less efficient (Cox et al. 2017; Metsky et al. 2022). For example, the seed region (positions 13–24 for *Lwa*Cas13 were used in this study) has been reported to be hypersensitive to mismatches (Cox et al. 2017), as this region is crucial for initial target binding and for activating collateral RNase cleavage (Tambe et al. 2018). Cox et al. (2017) showed that the seed region is enlarged for double mismatches, indicating that consecutive mismatches strongly reduce binding affinity and thus cleavage. Interestingly, we received a false positive with the mountain hare CRISPR-Dx guide and the target of the European hare, with two consecutive mismatches (positions 17 and 18) in the seed region and additional mismatches at positions 25 and 27. This guide–target combination is unlikely to produce a signal given the number, position, and distribution of the mismatches in the machine learning model of Leski et al. (2023). Advanced machine learning models, such as BADGERS, which are based on deep neural networks and trained on several thousand CRISPR-Dx and target combinations, could be used to further improve CRISPR-Dx guide design, with the aim to improve specificity (Mantena et al. 2024). However, such models have usually been used to identify CRISPR-Dx guides across larger regions of (viral) genomes and not within a short (i.e., ~59 base pair) amplicon, where multiple closely related species potentially differ by only a few nucleotides (Arizti-Sanz et al. 2022; Mantena et al. 2024; Metsky et al. 2022). The utility of such models for complex eDNA samples with many closely related species and—in the case of ampliscanning—predefined short amplicons requires further training and testing. Improved machine learning models trained on large eDNA data sets should increase our understanding of the pairing and cleavage activity and help us to better predict the CRISPR-Dx activity and design better CRISPR-Dx.

Despite the short targeting space within the Mamm01 amplicon, we were able to design species-specific CRISPR-Dx assays for six out of eight species. The additional two species could only be distinguished at the genus level. Adding artificial mismatches to the spacer sequence can increase the ratio of on to off target activity in highly similar sequences (Baerwald et al. 2020; Gootenberg et al. 2017; Mantena et al. 2024; Wessels et al. 2024). Such a strategy could be explored to separate closely related species, such as the mountain hare and brown hare, further increasing the versatility of CRISPR-Dx. Although testing the specificity of CRISPR-Dx through synthetic DNA (gBlocks) may allow CRISPR-Dx assay activity to be more accurately predicted (Leski et al. 2023; Mantena et al. 2024; Metsky et al. 2022), gBlocks cover only the amplicon—a short DNA fragment amplified with the primer pair—and not the entire genome. Using genomic DNA could be advantageous in detecting rather unlikely unspecific amplifications in other parts of the genome. In a previous study, no difference in on- and off-target detections was observed when synthetic DNA was compared with genomic DNA (Leugger et al. 2024). Furthermore, other studies have used synthetic DNA for testing the specificity of CRISPR-Dx tests (Blasko and Phelps 2024; Yang et al. 2024). Using genomic DNA could lead to false negatives if samples from a region are used that include local variants that are different from the variants in the actual study region and therefore not detected through the CRISPR-Dx assays (Elbrecht et al. 2018). We have circumvented this problem by comparing detections obtained with ampliscanning with those obtained through metabarcoding (using the same primers) to identify obvious cases of false negatives or false positives (off target hits) and thus dysfunctional CRISPR-Dx assays.

The sensitivity of ampliscanning for the CRISPR-Dx of the alpine marmot was in a similar range as reported in other studies using CRISPR-Dx for species detection (Baerwald et al. 2023; Leugger et al. 2024; Nagarajan et al. 2024), supporting the high sensitivity of CRISPR-Dx capable of detecting very few DNA copies when CRISPR-Dx are combined with a preamplification step. Overall, we highlight that, with appropriate design, testing, and validation, CRISPR-Dx on a common short metabarcoding amplicon can detect multiple species in parallel, with high sensitivity.

Ampliscanning and metabarcoding performed similarly in detecting terrestrial mammals in eDNA samples, highlighting the potential of CRISPR-Dx to monitor multiple species when sequencing the eDNA is not possible, for example, due to limited infrastructure. Metabarcoding confirmed all ampliscanning detections, whereas two metabarcoding detections with relatively few reads were not detected with ampliscanning, possibly due to stochasticity of the PCR amplification, in particular at low input DNA concentrations (Ellison et al. 2006; Thomsen et al. 2016). The consistency between ampliscanning and an alternative eDNA analysis method in 21 out of 23 cases (91%) is slightly higher than previously reported for CRISPR-Dx and qPCR (Williams et al. 2021). As such, ampliscanning and metabarcoding seem to be comparable regarding their sensitivity. Metabarcoding is preferentially applied to detect a wide range of species, for example, to assess the total diversity of a given taxonomic group, such as mammals (Lyet et al. 2021) or vertebrates in general (Reji Chacko et al. 2023). In contrast, CRISPR-Dx (i.e., with ampliscanning) are ideal to detect just a few taxa, for example, rare, flagship, or invasive species (Blasko and Phelps 2024; Nagarajan et al. 2024; Wei et al. 2023; Yang et al. 2024). Additionally, CRISPR-Dx can—in contrast to metabarcoding—be applied in contexts where resources are limited, as no expensive sequencing and qPCR machines are required (Blasko et al. 2024; Kellner et al. 2019). CRISPR-Dx can be read out using lateral flow tests (Leugger et al. 2025; Nagarajan et al. 2024), and isothermal amplification methods can be applied to circumvent the need for a thermocycler (Kellner et al. 2019; Nagarajan et al. 2024). Given their relatively low price per sample (Hoenig et al. 2023) and their applicability in field-based settings (Nagarajan et al. 2024), CRISPR-Dx furthermore allow many more samples to be collected and thus increase the spatiotemporal resolution or extent required for informed conservation measures (Jetz et al. 2019; Scheele et al. 2019). Together with recent advances in eDNA extraction methods and amplification approaches (Thomas et al. 2020), CRISPR-Dx have the potential to extend the use of eDNA for biodiversity monitoring and conservation by circumventing some of the main limitations of other eDNA analysis methods (Blasko and Phelps 2024; Nagarajan et al. 2024).

eDNA detection rates can be affected by primer bias, dilution of eDNA over distance and time, and patchy distribution and sedimentation of eDNA (Broadhurst et al. 2021; Schenekar et al. 2020; Ushio et al. 2017; Van Driessche et al. 2023), which could explain the detections we did not obtain with eDNA. We did not detect the alpine marmot and mountain hare in any of the eDNA samples, even though they are known to occur in all

sampled catchments. To some extent, eDNA detection rates also depend on the biomass of the targeted taxa (Lamb et al. 2019; Levi et al. 2019; Shelton et al. 2019). As we detected other species of similar biomass, such as the red fox, biomass alone does not explain why we were unable to detect the alpine marmot and mountain hare with eDNA. Species biology and affinity to water bodies can additionally affect the probability of detection with eDNA (Leempoel et al. 2020; Ushio et al. 2017). Both species might interact with streams less than alpine ibex or northern chamois, both of which can more easily cross streams due to their anatomy.

A lack of detection can also be caused by primer bias, which is a known limitation to eDNA metabarcoding (Elbrecht and Leese 2015; Schenekar et al. 2020). A single mismatch in the primer binding region might already be sufficient to reduce primer binding efficiency (Leempoel et al. 2020), in particular if the target DNA is rare. Both the alpine marmot and mountain hare carry one mismatch in the primer binding region of the Mamm01 primers. Ampliscanning detected these species when using pure synthetic DNA of the respective target species, indicating that the amplification in a complex eDNA sample is less likely when a primer mismatch exists. By adding a degenerate nucleotide at the respective location (using a mix of different primers) or using two independent primer pairs, the issue of primer bias could be reduced (Schenekar et al. 2020, 2024). Alternatively, there might be other primers, such as the MiMammal primers, that would be suitable (Ushio et al. 2017). Thus, these results highlight the need for careful primer design and extensive primer testing to benefit most from the versatility of eDNA for biodiversity monitoring (Zhang et al. 2020).

The by far lowest success rate of both eDNA detection methods was at the Macun site, which is the only site where we sampled at the outflow of a lake. Due to the low flow velocity in the lake, eDNA likely sinks to the bottom and thus only very little eDNA can be filtered at the outflow (Turner et al. 2015; Van Driessche et al. 2023). Hence, to increase detection probabilities from catchment-based monitoring with eDNA, the sampling sites should be located at river segments with sufficient mixing and not immediately downstream of ponds or lakes.

The species selected for the CRISPR-Dx assays are all relatively common in the Swiss National Park, except for the water shrew and roe deer. As a result, we could not assess in detail how ampliscanning and eDNA perform in detecting rare species with only a few individuals in the catchment. Given that biomass is related to the detection probability with eDNA, it could be more difficult to find species at very low densities (Crossman et al. 2024; Tetzlaff et al. 2024; Van Driessche et al. 2023). Nonetheless, previous studies show that it is possible, for example, to detect apex predators, such as the mountain lion (*Puma concolor*; Lyet et al. 2021) or the rare Mediterranean monk seal (*Monachus monachus*; Valsecchi et al. 2023) with eDNA. We detected both the roe deer and water shrew with eDNA, but the overlap with the species inventory was poor, indicating that the sampling effort should be increased to detect rare species, for example, by taking more field replicates (Cantera et al. 2019). In our analyses, we used large-volume filtration (up to 80 L); larger volumes can lead to filter clogging, preventing the detection of additional species with the sample. As an alternative, increasing the number of field replicates could enhance detection probability (Cantera et al. 2019). Furthermore, taking temporal replicates at field sites could increase the chances of detecting rare species, and future studies could involve investigating the number of temporal replicates and the collection frequency needed to help optimize eDNA biodiversity monitoring. As we show that species-specific CRISPR-Dx can be combined with multispecies primers for terrestrial mammal detection in this study, this approach makes it possible to reduce the laboratory requirements for eDNA analysis, paving the way for studies with more samples in order to increase our knowledge about eDNA.

5 | Conclusions

In this study, we show how sampling eDNA at the bottom of catchments in a topographically complex landscape integrates information on species in the entire catchment, even for terrestrial mammals, and that the eDNA signal can be detected with both metabarcoding and ampliscanning. Additionally, we showcase the applicability of ampliscanning, which combines multispecies primers and species-specific CRISPR-Dx, to detect multiple species with the same amplification. We demonstrate that ampliscanning performs similarly to metabarcoding on eDNA samples and detects the same species. By circumventing the limitations of metabarcoding, such as access to a fully equipped laboratory, CRISPR-Dx allow eDNA samples to be analyzed in places where it has not previously been possible, revolutionizing eDNA biodiversity analysis (Blasko and Phelps 2024; Hoenig et al. 2023; Leugger et al. 2025; Nagarajan et al. 2024). Given the somewhat patchy and stochastic distribution of eDNA, detecting rare species requires sampling a large water volume and analyzing multiple field and PCR replicates (Ficetola et al. 2008; Seeber and Epp 2022; Ushio et al. 2017). By using CRISPR-Dx, which reduce costs for a single analysis (Arizti-Sanz et al. 2022; Hoenig et al. 2023; Yang et al. 2024), ampliscanning enables more samples to be analyzed for a given price, thereby increasing the spatiotemporal extent that can be monitored. In combination with catchment-based eDNA sampling, this could allow remote and inaccessible areas to be monitored at unprecedented spatiotemporal scales, improving our understanding of biodiversity and ultimately helping to protect it.

Author Contributions

Z.K. and L.P. acquired the funding. F.L., M.L., Z.K., and L.P. designed the research. F.L. and M.S. conducted the field sampling. M.L. and F.L. performed the research with the help of S.T. for laboratory work. F.L. conducted the analyses and wrote the manuscript together with M.L., with contributions from the other authors.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data (including metabarcoding reads, sampling metadata, CRISPR-Dx detections) is available on EnviDat (data repository of the Swiss Federal Institute for Forest, Snow and Landscape Research WSL): https://www.doi.org/10.16904/envidat.661.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.