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Development of a targeted forensic test for the identification of Eurasian beaver DNA

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ABSTRACT

Eurasian beaver (*Castor fiber*) has recently been reintroduced in Scotland after more than 400 years of extinction and in 2019 received legal protection; deliberate killing or disturbing beavers without a license is therefore now an offense. We present a validated polymerase chain reaction (PCR)-based Eurasian beaver identification test for use in forensic casework where persecution of Eurasian beaver is suspected. Primers were designed to target a 271 base pair region of the mitochondrial cytochrome b (*Cytb*) gene in Eurasian beavers, and positive amplicons were confirmed by sequence analysis. Validation was carried out across two laboratories in Scotland, and included studies on sensitivity, specificity, reproducibility, and robustness. The developed test reliably detects Eurasian beaver DNA to the lower limit of 0.1 pg DNA input and differentiates *Castor fiber* from other species, including congeners. In conclusion, the developed test was successfully optimized and validated to identify Eurasian beaver DNA and will be a valuable tool in wildlife forensic laboratories in cases of suspected persecution of Eurasian beavers.

1. Introduction

Eurasian beaver (*Castor fiber*) is a large mammal belonging to the family Castoridae. Beavers affect biodiversity by modifying the environment, creating new wetland habitats and changes in the ecosystem [1]. They originally populated most of Europe and Asia. However, by the 16th century, European populations decreased significantly, leading to extinction of beavers in Scotland due to overhunting for fur, meat, and castoreum [2]. Eurasian beavers were reintroduced to Scotland in 2009 when they were released to Knapdale Forest in Argyll as a part of the Scottish Beaver Trial [3]. Furthermore, another population has established in Tayside, due to escapes or deliberate releases [4]. Ten years after the first licensed releases, in May 2019, the Eurasian beaver received legal protection as a European Protected Species under the Conservation (Natural Habitats, &c.) Regulations 1994, meaning that deliberate killing, harming, or disturbing beavers without a license is an offense [5]. Although beavers now have legal protection, they may be at risk of persecution as anglers and land managers have raised concerns regarding blocking the movement of fish and causing damage to crops [6].

The 2020 Beaver Management report by NatureScot states that the Scottish beaver population is expanding, as the number of territories with observed beaver activity has more than doubled since 2017. However, due to damage to agriculture and infrastructure, 115 beavers were killed under license between the start of their protected status in 2019 and the end of 2020 [7]. Currently, the extent of any illegal persecution of beavers is unknown.

Several studies have assessed genetic structure and variation within Eurasian beaver populations [8–10]. According to McEwing et al., the two Scottish beaver populations have different genetic origins. Argyll beavers are known to originate from Norwegian populations, while Tayside beavers exhibit genetic signatures similar to those from Bavaria, Germany [11]. Therefore, any developed test to identify Eurasian beavers in Scotland must work across these divergent populations [10,11]. Currently, there is no published literature on existing forensically validated targeted tests to identify Eurasian beaver DNA.

Species identification of biological samples is an important tool in forensic science to investigate wildlife crime offenses. Wildlife forensic science laboratories often encounter low-quality and challenging samples, lacking morphological features to enable species identification. Therefore, the importance of DNA-based forensic testing to identify protected species has increased. Polymerase chain reaction (PCR)-based

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amplification followed by analyses of sequence differences has been shown to be the most specific and reliable method for species identification in a forensic context [12–14]. Mitochondrial DNA (mtDNA) sequences are often used for species identification as they show low levels of intraspecific variation but sufficient interspecific variability [14]. Moreover, mtDNA is much more abundant in cells, with higher resistance to degradation than nuclear DNA [15,16], which is particularly beneficial when samples contain low quantity, degraded DNA, such as trace evidence recovered from an outdoor crime scene [17].

Forensic species testing is usually based on sequence differences of mtDNA markers, most frequently cytochrome b (*Cytb*) [14,18] and cytochrome oxidase I (COI) [19]. To increase the chances of amplification from trace-level, degraded samples, short fragments of loci are targeted [20]. There is currently no standardized locus for species identification because different loci exhibit different variability levels in different taxonomic groups [21]. However, for mammalian species, Tobe et al. compared *Cytb* and COI sequences to determine which had higher distinguishing power, and *Cytb* demonstrated slightly more correctly identified species than COI [22]. While many studies have used universal primers positioned in conserved regions of genetic loci for species identification [12,14,23], in cases where mixed samples are recovered, this type of test could generate mixed sequences which are impossible to resolve [24–26].

In forensic studies *Cytb* has been extensively used for targeted tests for various species including bears [27], tigers [28,29], rhinoceroses [30,31], elephants [32,33], and antelopes [34].

With new legal protection in place and known conflict between this species and human land users, there is a need for a validated species identification test which can be applied to evidential items from a beaver persecution investigation. Items such as traps, tools, and clothing may be recovered, potentially some time after any beaver DNA is deposited, and also potentially present as a mixture with human DNA or other wildlife species. Here we present a DNA-based species identification test using a targeted primer pair, which amplifies a short (271 bp) *Cytb* region from Eurasian beaver. The developed test was optimized, validated to forensic testing standards [26,35], and applied to casework-type samples to demonstrate its utility.

2. Material and methods

2.1. Primer design

Primers targeting Eurasian beaver DNA were designed using NCBI Primer-BLAST to amplify a 271 bp region of the mitochondrial *Cytb* gene (Table 1) using a reference Eurasian beaver *Cytb* sequence downloaded from GenBank (FR691687.1 [8]) as a template. Multiple full *Cytb* sequences from Eurasian beaver were aligned using Geneious R11 software (https://www.geneious.com/): a reference sequence produced at SASA, and four other sequences published by Horn et al. [8]. Primers were compared against these sequences to assess whether they would amplify among different Eurasian beaver lineages.

2.2. In silico primer testing

Primers were tested *in silico* using NCBI Primer-BLAST software to assess specificity and identify any non-targeted sequences primers may bind to and amplify when conducting PCR.

Table 1
Targeted primers used for amplification of Eurasian beaver DNA.

Primer pair	Primers	Coordinates (FR691687.1)	Sequence (5'→3')	Tm
Cfib4	Forward	14931–14950	CAGCCAACCCCCTAAACACA	60.18
	Reverse	15201–15182	ATGAATGGGGGTTCAACGGG	60.32

2.3. Samples and DNA extraction

One North American beaver (*C. canadensis*) sample and eight Eurasian beaver (*C. fiber*) samples were used in this study (Table 2). The *C. fiber* samples included representatives from the two distinct lineages that established the Tayside and Argyll populations in Scotland [11]. A human DNA extract (provided by a volunteer with consent) and DNA extracts from multiple other species (Section 2.6.2) were used for specificity testing from collections at SASA.

DNA extraction was carried out using the QIAamp DNA Investigator kit (QIAGEN) following appropriate protocols for different sample types. DNA quantity and quality were assessed by measuring DNA absorbance at 260 nm and 280 nm, using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). As Nanodrop® measures the concentration of total DNA including both nuclear DNA and mtDNA, the measurements were used as an estimate for the true amount of targeted DNA (mtDNA) in this study.

2.4. PCR amplification

Annealing temperature for the test was initially optimized using gradient PCR. Amplification of beaver DNA was performed at 12 annealing temperatures, ranging from 50 °C to 65 °C.

The optimized PCR method was carried out in a total volume of 20 μL, containing 10 μL 1× TypeIT Microsatellite Master Mix (QIAGEN), 1 μL of each forward and reverse primer at 10 μM concentration, 6 μL nuclease-free water, and 2 μL DNA extract. Amplification at the Centre for Forensic Science was conducted in a SureCycler 8800 thermocycler (Agilent Technologies), while at SASA the cycling was performed in a ProFlex™ PCR system (Applied Biosystems). Cycling conditions under which amplification was carried out were as follows: polymerase activation (95 °C, 5 min), followed by 35 cycles of denaturation at 95 °C, annealing at 65 °C and extension at 72 °C, for 30 s each step, with a final extension at 72 °C for 10 min and a final hold at 10 °C.

After PCR amplification, 5 μL of each product was mixed with 1 μL loading dye (PCR Biosystems) and run on a 1% agarose gel (Sigma-Aldrich) stained with SYBR Safe (Thermo Fisher) to check for amplification.

2.5. Sequencing of amplicons

Sanger sequencing was used to determine whether the test amplified the targeted sequence of Eurasian beaver. Amplicons were purified using Exo-Pro Star (GE Healthcare Bio-Sciences). Sequencing reactions were performed in forward and reverse directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), sequenced using a 3500 series Genetic Analyzer (Applied Biosystems), and edited in Geneious by trimming off poor-quality regions and primers. Forward and reverse sequences generated from the same PCR product were aligned to produce consensus sequences, and BLASTn used to compare sequences against NCBI's GenBank database [36] and an in-house SASA reference database. Genetic distances between query and reference

Table 2
Beaver samples used in this study. All samples were provided from existing collections.

Source_ ID	Species	Sample type	Geographic origin
NMS_R216.00	*Castor canadensis*	Tissue	Captive collection
NMS_GH133.15	*Castor fiber*	Tissue	Tayside, Scotland
NMS_PH22.96	*Castor fiber*	Tissue	Norway
NMS_R36.01	*Castor fiber*	Tissue	Sweden
NMS_R95.03	*Castor fiber*	Tissue	Germany
RZSS_BEV754	*Castor fiber*	Blood	Argyll, Scotland
RZSS_BEV312	*Castor fiber*	Blood	Argyll, Scotland
SASA_RES0070	*Castor fiber*	Tissue	Tayside, Scotland
SASA_V0324	*Castor fiber*	Tissue	Tayside, Scotland

sequences were analysed to determine intraspecific and interspecific sequence similarities.

2.6. Validation

The validation parameters to be assessed followed recognized recommendations and guidelines [26,35] including: (1) sensitivity, (2) specificity, (3) reproducibility, (4) robustness and (5) casework-type samples. These validation components were undertaken across two laboratories, with the majority being carried out at the Centre for Forensic Science, University of Strathclyde, and the remainder at SASA.

2.6.1. Sensitivity

The sensitivity of the developed test was determined by performing PCR with samples of different DNA concentrations. Four beaver DNA samples were used to prepare 10x serial dilutions ranging from 5 ng/μL to 0.5 fg/μL using nuclease-free water. Each dilution was mixed at 900 rpm and room temperature for an hour before generating the next serial dilution.

For each sample, the lowest input of DNA giving a positive agarose gel electrophoresis result and at least one more concentrated sample were sequenced.

2.6.2. Specificity

To determine the specificity of the developed test, PCRs were conducted in the presence of DNA from closely related species, potential contaminant species, and other species often encountered in UK wildlife forensic casework: North American beaver (*Castor canadensis*), human (*Homo sapiens*), red deer (*Cervus elaphus*), sheep (*Ovis aries*), rabbit (*Oryctolagus cuniculus*), hare (*Lepus europeus*), dog (*Canis lupus familiaris*), otter (*Lutra lutra*), Atlantic salmon (*Salmo salar*), chicken (*Gallus gallus domesticus*), and common buzzard (*Buteo buteo*). DNA input was between 2 ng and 20 ng. Eurasian beaver DNA was included as a positive control. All positive amplicons were sequenced.

Following the results of the specificity study, a mixture study was conducted to determine whether the presence of more than one DNA source that could amplify with this primer set would interfere with PCR amplification of targeted Eurasian beaver DNA. DNA extracts from human and Eurasian beaver were mixed at different concentrations with the non-target always at least 1000 times more concentrated than the target DNA. The total volume of DNA added was 4 μL (2 μL of each species) and water was adjusted to 4 μL per reaction. DNA input was 2 ng of human DNA and either 2 pg, 0.2 pg or 20 fg of beaver DNA. The experiment was replicated with two beaver samples and all PCR products were sequenced.

2.6.3. Robustness

A robustness study was conducted, in which the temperature of each PCR step was lowered or increased by 1.5 °C to ensure the test is robust enough to give positive results when the thermal cycler is out of calibration. The standard protocol was carried out as a positive control. Amplification was conducted with six different Eurasian beaver individuals using two DNA inputs, 0.1 pg and 0.1 ng, to ensure the temperature change does not affect amplification in the presence of low concentrations of DNA. PCR products for one Eurasian beaver sample for each DNA input for both deviations from the standard protocol were sequenced.

Additionally, PCRs were performed with JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma) using two Eurasian beaver samples, and input DNA at 0.1 pg to examine the robustness of the developed test with different reagents. Volumes and concentrations of reagents, primers, water, and template were the same as for TypeIT Microsatellite Master Mix.

2.6.4. Reproducibility

To demonstrate the developed test's reproducibility, PCRs were also conducted at SASA's laboratory by a second analyst, using a different thermal cycler, with two Eurasian beaver DNA extracts (SASA_RES0070 and SASA_V0324). Reaction DNA inputs ranged from 1 ng to 10 fg. For each sample, PCR products from the two lowest concentrations that produced an observable band on the agarose gel were sequenced. Additional PCRs were performed to produce amplicons from all Eurasian beaver samples used in this study (Table 2) for sequencing.

2.7. Casework-type samples

Casework-type samples were prepared and tested to demonstrate the utility of the developed test in a casework setting using two blood samples (Table 2). Casework-type samples were prepared in triplicate by pipetting 10 μL of blood onto a cleaned metal surface (samples M_1-M_3) or washed piece of fabric (samples F_1-F_3) and smearing the drops with sterile swabs. Samples were placed on an indoor or outdoor windowsill for one week. Samples were exposed to similar light conditions, with outdoor samples additionally exposed to changes in temperature (5–23 °C) and wind, while being protected from rain.

Samples were taken from the metal surface using sterile swabs wetted with a drop of sterile nuclease-free water, while stains were cut from the fabric. A swab and piece of fabric with fresh beaver blood were included as positive controls, while a sterile swab and piece of fabric with no beaver blood were included as negative controls. DNA extracted from a tissue sample was used as a PCR positive control with 0.1 ng input DNA.

After DNA extraction, all casework-type samples were amplified, results checked by agarose gel electrophoresis and one of each of the replicates sequenced.

3. Results

3.1. In silico testing

When testing primers *in silico* using Primer-BLAST, no species that might contaminate Eurasian beaver samples were identified as potentially being amplified. The search returned only sequences originating from Eurasian beaver.

3.2. Interspecific and intraspecific variation

Using pairwise comparisons of sequence identity, intraspecific variation among all eight Eurasian beaver samples was 99.5–100% for the 271 bp fragment of *Cytb* amplified (sequences produced during this study have been submitted to GenBank under accession numbers ON017616-ON017624). Extending this dataset to include twelve further *C. fiber* sequences mined from GenBank increased intraspecific variation to 98.59–100% sequence identity. Interspecific differences among sequences produced in this study and congeneric species *C. canadensis* were 86.85–87.88%, demonstrating this locus is suitable for identification of Eurasian beaver.

3.3. Validation

3.3.1. Sensitivity

The sensitivity study results showed successful amplification down to at least 0.1 pg of DNA input for all Eurasian beaver samples. Dilutions from one sample (SASA_RES0070) amplified at 0.1 pg, two samples (SASA_V0324, NMS_PH22.96) produced positive results at 10 fg, and one sample (NMS_GH133.15) successfully amplified at 1 fg (Fig. 1). Sequencing of PCR products was successful down to 10 fg of DNA input; although these weaker amplicons produced lower quality sequence, BLAST results still returned the highest similarity score for *C. fiber*.

3.3.2. Specificity

Strong amplification was observed with the congeneric North

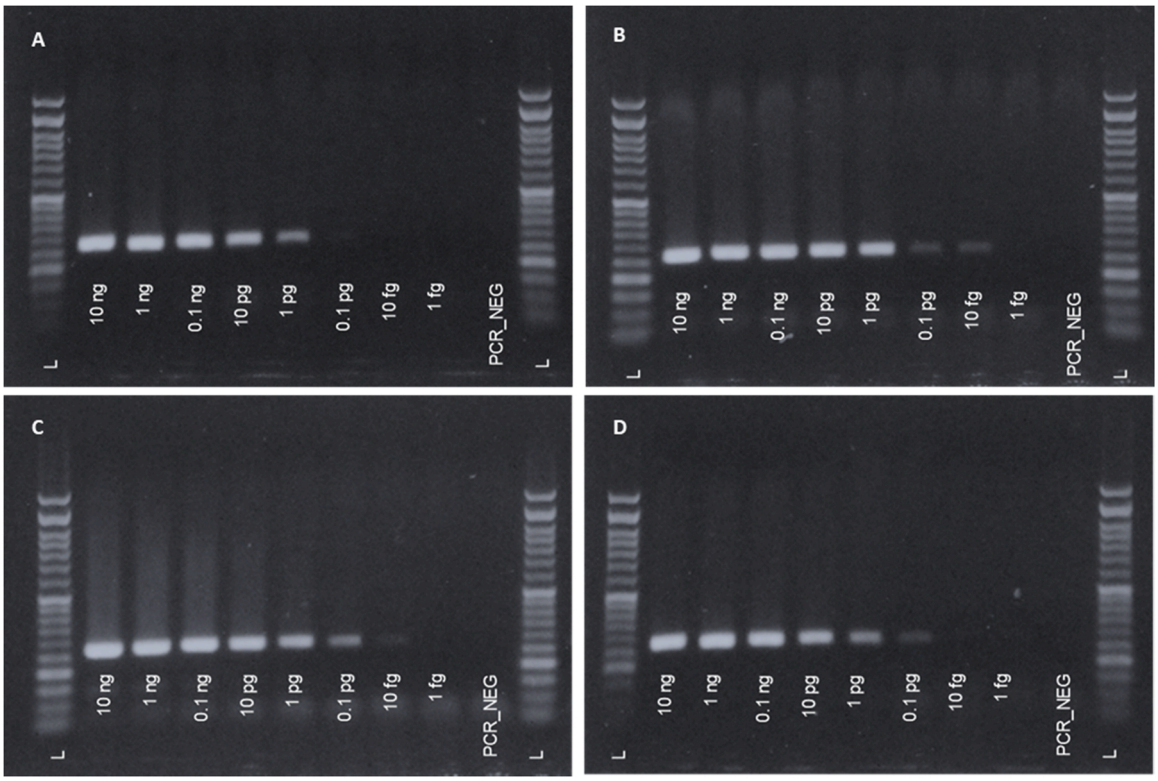

Fig. 1. Agarose gel images showing results of the sensitivity study for four Eurasian beaver DNA samples: (A) SASA_RES0070, (B) SASA_V0324, (C) NMS_PH22.96, and (D) NMS_GH133.15. L = PCRBIO ladder III 50–1500 bp (PCR Biosystems), PCR_NEG = PCR negative control without DNA template.

American beaver (data not shown), but the sequence produced gives clear separation between these two beaver species. Some very weak amplification was observed with human, hare, chicken, buzzard, and Atlantic salmon DNA (Fig. 2). Only human and Atlantic salmon gave readable sequences. The human sequence was mitochondrial in origin although not from the *Cytb* region and the Atlantic salmon sequence was a genomic fragment. Comparison with Eurasian beaver sequence confirmed that the bands on the gel were not due to contamination with beaver DNA.

Beaver DNA was successfully detected in all mixtures of human and Eurasian beaver DNA, based on sequence results, even when beaver DNA input was much lower (down to 20 fg input) than human DNA (2 ng).

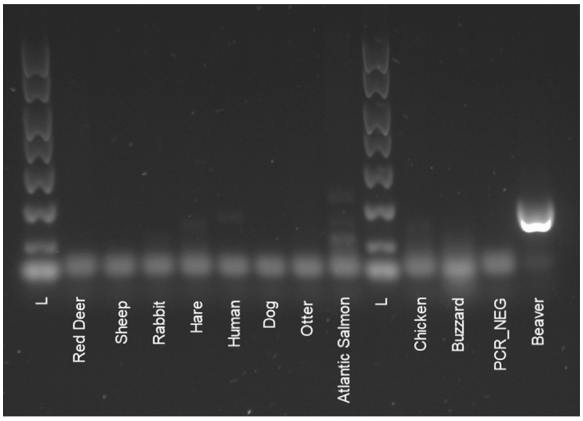

Fig. 2. Agarose gel image showing results of the specificity study. L = Sigma Aldrich 50–2000 bp ladder, PCR_NEG = PCR negative control without DNA template.

3.3.3. *Robustness*

Samples in the robustness study were successfully amplified and sequenced when PCR temperatures were altered by + /− 1.5 °C (Fig. 3). Two beaver samples were also successfully amplified using a different PCR master mix (data not shown).

3.3.4. *Reproducibility*

The primers successfully amplified Eurasian beaver DNA from two individuals down to 10 fg DNA input when performed at SASA (Fig. 4). All sequences successfully identified Eurasian beaver down to 10 fg of DNA input, similar to results obtained in the original sensitivity study performed at the Centre for Forensic Science, University of Strathclyde.

3.4. *Casework-type samples testing*

Samples extracted from blood smears on different substrates exposed to indoor and outdoor conditions were successfully amplified and sequenced, confirming the presence of Eurasian beaver DNA (Fig. 5). Qualitatively the amplification appears similar across replicates and substrate type.

4. Discussion

This paper presents a validated targeted DNA test for Eurasian beaver, based on amplification and sequencing of a 271 bp mitochondrial *Cytb* region.

Validation studies involving protected species can encounter difficulties obtaining samples [37] so we utilized external reference collections to improve the number of samples from identified specimens [38]. The developed test successfully amplified and sequenced samples from eight Eurasian beaver individuals, including representatives from the two main genetic lineages of this species in Europe (Table 2) [10,11]. Having these data from voucher specimens prevents misinterpretations when comparing data with GenBank where some erroneous sequences

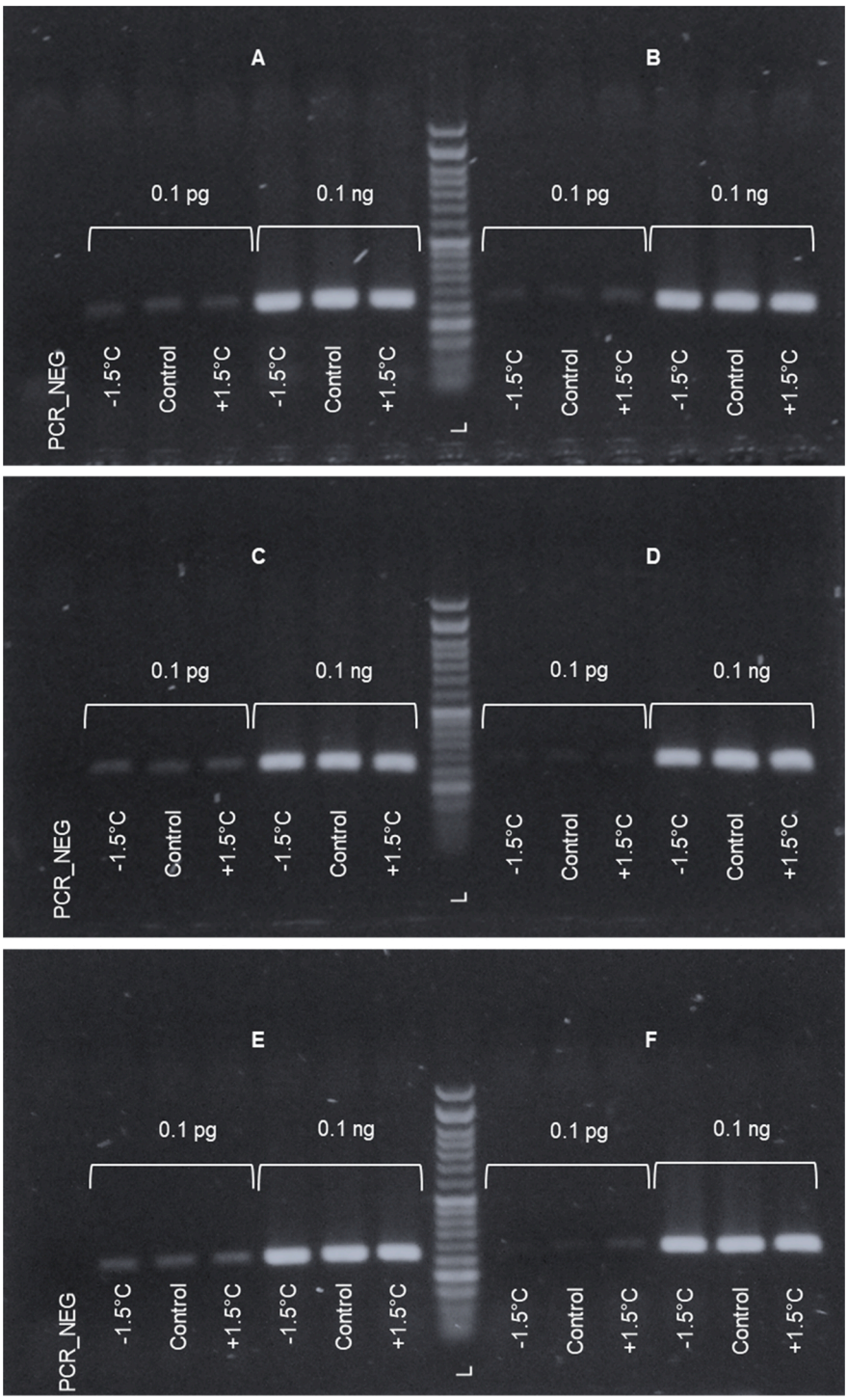

Fig. 3. Agarose gel images showing results of the robustness study. DNA from four Eurasian beaver individuals ((A) SASA_V0324, (B) SASA_RES0070, (C) NMS_PH22.96, (D) NMS_R36.01, (E) NMS_R95.03, (F) NMS_GH133.15), input at either 0.1 ng or 0.1 pg, amplified using the standard PCR program (Control) or a program with temperature deviated by + /− 1.5 °C. L = PCRBIO ladder III 50–1500 bp (PCR Biosystems), PCR_NEG = PCR negative control without DNA template.

may be present [39,40]. Relying only on reference sequences in GenBank could therefore lead to inaccurate identifications. However, Pentinsaari et al. showed that errors in sequence preparation and interpretation were the main causes of misidentification [41]. The quality controls applied when undertaking species identification by DNA sequencing is therefore crucial for accurate results. Validated reference databases are recommended for DNA-based species identification by both the International Society for Forensic Genetics (ISFG) [26] and the Society for Wildlife Forensic Science (SWFS) [35].

Genetic variability has been observed between and within *C. fiber* populations across Eurasia [10,11]. Our results showed low intraspecific variation within the amplified *Cytb* gene region (98.59–100%) and the closest interspecific comparison was with the North American beaver, *C. canadensis* (~87% sequence identity in pairwise comparisons). This gap of over 10% in identity from intraspecific to interspecific comparisons makes this short amplicon suitable to differentiate *C. fiber* from other species.

The laboratory validation covered the recommended parameters and

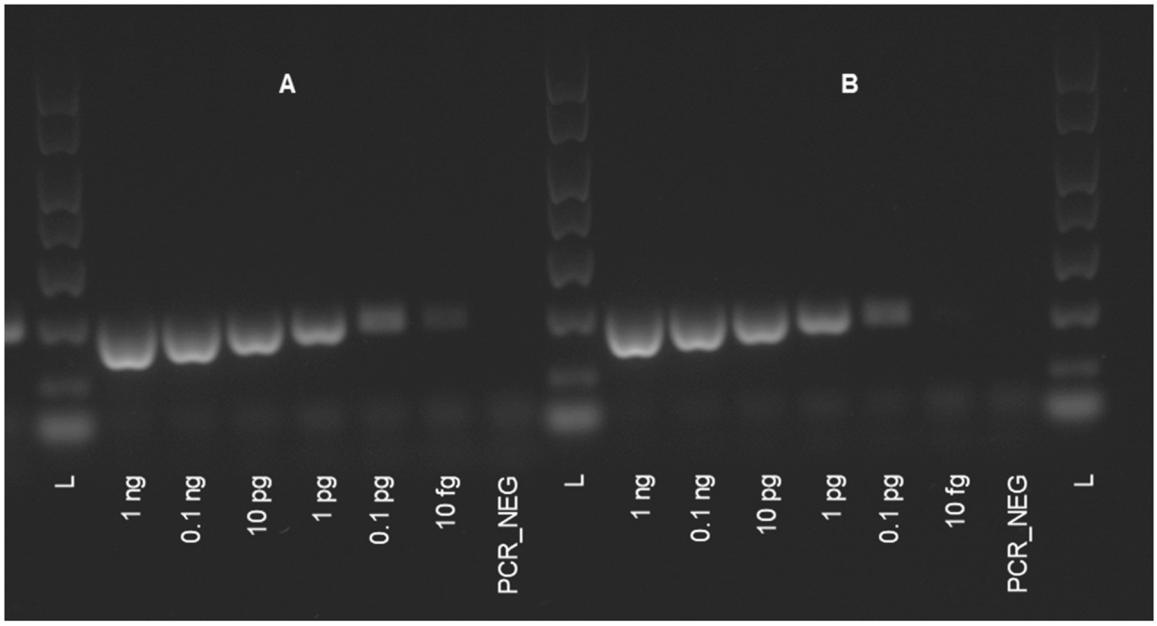

Fig. 4. Agarose gel images showing results of the sensitivity study performed at a second laboratory to demonstrate reproducibility, for two Eurasian beaver individuals: (A) SASA_RES0070 and (B) SASA_V0324. L = Sigma Aldrich 50–2000 bp ladder, PCR_NEG = PCR negative control without DNA template.

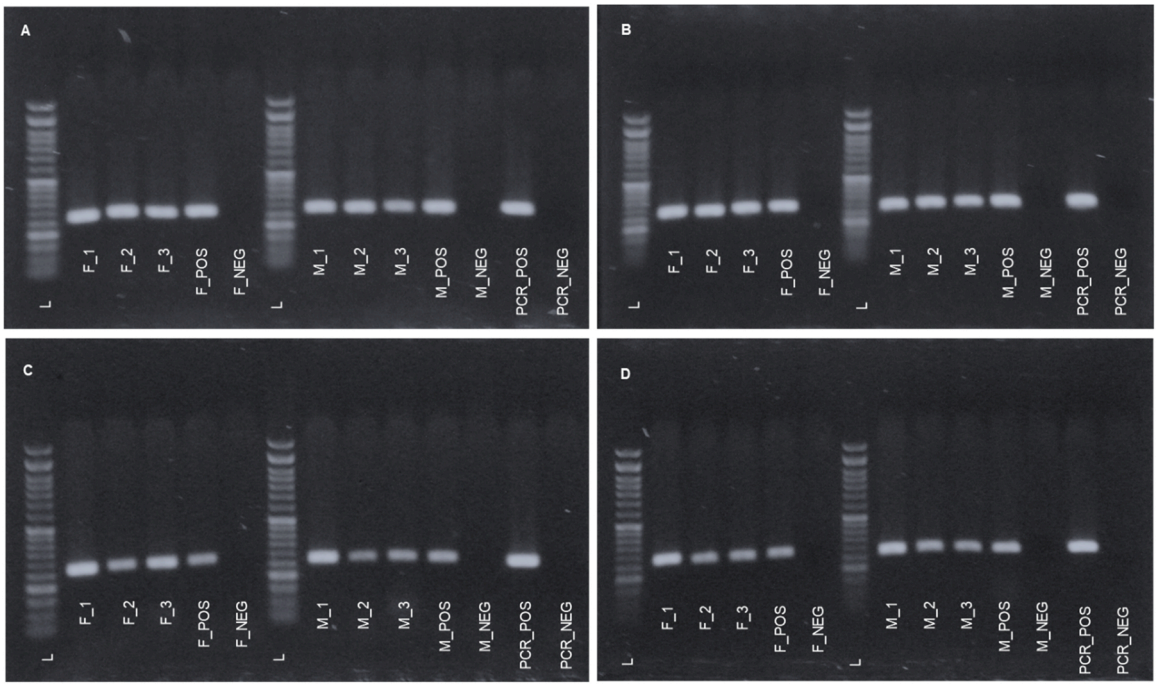

Fig. 5. Agarose gel images showing results from casework testing on fabric and metal surfaces performed in triplicate. (A) RZSS_BEV754 – Outdoor, (B) RZSS_BEV754 – Indoor, (C) RZSS_BEV312 – Outdoor, (D) RZSS_BEV312 – Indoor. L = PCRBIO ladder III 50–1500 bp (PCR Biosystems), F = fabric, M = metal, POS = positive control, NEG = negative control.

guidelines from the ISFG [26] and SWFS [35] as well as robustness and casework-type samples as reported by Ewart et al. in the development of a rhinoceros species identification test [31].

Our test is highly sensitive, reliably detecting Eurasian beaver DNA down to 0.1 pg DNA input. Indeed, given the use of the Nanodrop® instrument for quantification, measuring total DNA, the amount of mtDNA will be lower than this estimate so this is a conservative lower limit of detection. This is more sensitive than other similar targeted tests, for example a Pangolin-targeted test with sensitivity to 10 pg [42], a Rhinoceros-targeted test that amplified some species with sensitivity at 20 pg [31], and an elephant-targeted test with sensitivity between 125 and 500 pg [32]. In practice, our test is likely to be applied in situations where only trace evidence is present (e.g., non-visible biological remains on a used trap) rather than larger items recovered in illegal wildlife trade and so this increased sensitivity is beneficial.

As this is a targeted test, specificity studies were important to ensure the test would not amplify a wide range of alternative species – false positive amplification. In addition, the specificity studies considered whether the test would fail to preferentially amplify Eurasian beaver DNA in a mixture with human DNA – a false negative result. Some of the

tested species produced false positive amplification, but readable DNA sequences were only obtained from North American beaver, human and Atlantic salmon. The amplification from the congeneric North American beaver was expected but the sequence produced differed from the Eurasian beaver at more than 10% of bases, making this false-positive easy to identify. Atlantic salmon produced a sequence originating from a genomic fragment, whereas the human amplicon was mitochondrial in origin, of similar size to the target fragment, but from the 16-S ribosomal RNA gene. Despite this, no inhibition of amplification from Eurasian beaver DNA was observed when mixed with human DNA at a range of concentrations. The preference was always for Eurasian beaver DNA even when input was orders of magnitude lower (down to 20 fg input) than the human DNA (2 ng). Primers were designed to match beaver DNA, and there are multiple mismatches when they are aligned against human 16-S ribosomal RNA sequences. Amplification of beaver DNA is therefore preferred over human due to higher binding affinity of primers with beaver *Cytb* sequence compared to human 16-S ribosomal RNA even when substantially more human DNA is present. The observed amplification of human DNA is not a concern as it should not lead to false negative results from potential trace evidence on clothing or weapons where DNA from both beavers and humans may be present. The presence of any non-targeted DNA should not affect the results of this test, as false positives will be identified at the sequence analysis stage.

Robustness checks confirmed successful results when each step of a PCR program was altered by $+/-1.5\,^{\circ}C$, and also when using an alternative, visibly different PCR master mix. Similar validation studies [27,31,42] only adjusted annealing temperature, but our goal was to simulate a thermal cycler being out of calibration, in which case temperatures across the program could be affected. This level of robustness to out-of-calibration type temperature shifts will make this test easier to implement in other wildlife forensic labs. While only two different PCR master mixes were checked, this indicates that the success of the test is not overly dependent on the main PCR master mix used (QIAGEN TypeIT Microsatellite Master Mix). Other master mixes could be checked in other labs to see if they pass a similar internal validation using our standard method which was found to give consistent, reproducible results among the two labs involved in this study.

Successful identification of Eurasian beaver from blood samples on metal and fabric even after exposure to indoor and outdoor environmental conditions demonstrates the utility of the developed test in situations similar to those encountered in casework. Any DNA degradation during the experiment did not affect amplification of DNA, although no formal degradation study was carried out. This was expected as the target is short and located in the mitochondrial genome. The experiment lasted for just one week due to timing constraints, however, these positive results suggest it is worth testing items recovered during an investigation even if the offense occurred some time in the past.

5. Conclusion

We have developed, optimized, and validated a targeted test for the identification of Eurasian beaver from biological remains that may be recovered in criminal casework. The developed test can be used by other laboratories testing samples that potentially contain Eurasian beaver DNA. We demonstrated the utility of the developed test on simulated casework-type samples and ensured that it can reliably identify representative individuals from different genetic lineages of Eurasian beavers in Europe.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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