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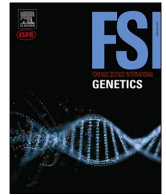

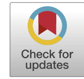

LAMPSEQ: Colorimetric LAMP and nanopore sequencing for rapid species identification in the illegal wildlife trade

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ABSTRACT

The illegal ivory trade in animal parts such as ivory, horn, and bone, threatens the survival of many protected species including elephants, rhinoceros, hippopotamus, and walrus. Accurate and reliable species identification is critical for enforcement under CITES, yet traditional DNA-based methods are time-consuming, resource-intensive, and are often inaccessible in regions most affected by poaching. This proof-of-concept study presents a rapid, portable, two-step workflow combining colorimetric loop-mediated isothermal amplification (LAMP) with nanopore sequencing for the authentication of elephant ivory and its legal and illegal substitutes. Novel LAMP assays were developed for elephants, mammoths, rhinoceros, hippopotamus, walrus, water buffalo, and common domestic species, targeting conserved mitochondrial DNA regions to enable both presumptive detection and sequencing-based confirmation. Using synthetic DNA, all assays demonstrated high sensitivity, with limits of detection down to 1–100 fg, with good consistency between replicates. A multiplexing strategy was designed to group closely related species into a single assay. Separate assays were designed for all elephant species, all mammoths, and the two closely related rhinoceros species (*R. sondaicus* and *R. unicornis*), enabling rapid presumptive screening followed by species-level confirmation via sequencing. To address challenges associated with the tandem repeats present in LAMP products, LAMPSEQ, a custom bioinformatics pipeline, was developed to extract, align, and accurately identify species from LAMP sequencing data. This sensitive, scalable, and field-deployable workflow expands the molecular toolkit, delivering innovative solutions to wildlife crime investigations and supports the protection of the world's most vulnerable and treasured species from extinction.

1. Introduction

The illegal trade in animal parts such as ivory, horn, and bone remains a major driver of population decline among some of the world's most iconic and ecologically significant species. Elephants are heavily targeted for their ivory [1–3], which has led to all elephant species (*Elephas maximus* and *Loxodonta spp.*) being listed under Appendix I of the Convention on International Trade in Endangered Species of Fauna and Flora (CITES), enforcing the strictest international trade regulations [4]. As global restrictions on elephants have tightened, demand for alternative substitutes like rhinoceros horn [5–7], hippopotamus (*Hippopotamus amphibius*) canines [8–11], and walrus (*Odobenus rosmarus*) tusks [10,11] have grown, with these species now listed under CITES Appendices I, II, and III. Legal substitutes, like mammoth (*Mammuthus spp.*) ivory [3,10–12], water buffalo horn or bone [10], and bone from domestic species (e.g., cow, cat, or dog) [11,13,14] have also been commonly used to imitate elephant ivory. The widespread use of these materials in wildlife trafficking demonstrates the need for accurate species identification to determine the legal status of seized materials and to support conservation efforts.

Morphological analysis of ivory and substitutes has been well established as a rapid, cost-effective species identification method [15]. Despite being able to determine elephant from mammoth, these methods cannot reliably distinguish between elephant species [15], heavily rely on specialist expertise, and are often unreliable for processed or degraded specimens [16]. Chemical techniques, including x-ray fluorescence [17–19], Fourier-transform infrared spectroscopy [20–22], and trace element analysis [23,24], identify items based on

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elemental composition. DNA-based approaches such as species-specific PCR [25–28], and DNA barcoding using Sanger or massively parallel sequencing (MPS) [29], offer species-level identification by targeting unique genetic markers. Contamination introduced during illicit handling and processing from poachers or importers can introduce exogenous human DNA that may preferentially amplify, masking target DNA and limiting its detectability [14]. Both chemical and DNA-based methods are complex, costly, time-consuming, and reliant on specialised laboratory infrastructure, making them impractical for in field or low-resource settings.

Loop-mediated isothermal amplification (LAMP) presents a promising alternative to conventional PCR. LAMP rapidly amplifies DNA at a constant temperature using 4–6 species-specific primers and a simple heat source [30,31], with results detected visually [16]. The use of multiple primers provides high specificity [32], critical for distinguishing closely related or legally distinct species such as protected elephants versus unprotected mammoths and has demonstrated utility in various CITES-related applications [16], including traditional medicines [32,33], sharks [34,35], and white rhinoceros [36]. When paired with nanopore sequencing, LAMP products can be confirmed [37,38], enabling accurate and reliable species identification in the field. Despite these advantages, LAMPs use of multiple primers increases the risk of non-specific amplification and false positives [39,40]. Its exponential amplification can generate aerosols, posing a contamination risk when opening tubes post-amplification [16]. These issues can be mitigated through careful primer design and reaction optimisation [41,42]. Multiplexing is also challenging, though some studies have implemented different gene targets and detection dyes [43,44], or performed multiple singleplex reactions in parallel using microfluidic devices [45,46].

In wildlife crime cases, where species identification underpins legal prosecution, these limitations warrant a conservative, evidence-driven approach [16]. This study supports a two-step strategy: LAMP-based visual detection for rapid presumptive screening followed by confirmatory nanopore sequencing. This ensures a rapid, portable, and cost-effective workflow, facilitating robust species identification. However, sequencing LAMP products introduces additional complexity. Unlike PCR, LAMP generates multiple tandem repeats of the target sequence [30,31], complicating downstream sequence alignments and interpretation. Tools such as BLAST [47], while commonly used, rely on local alignments and can yield inaccurate or ambiguous results which are difficult to interpret. These issues are further compounded by the limited availability of high-quality reference data for endangered species, which are often underrepresented in public databases [16,48]. When species are underrepresented, similarity scores are lower and misidentifications are more likely [48], potentially undermining prosecution. These analytical and interpretation issues highlight the need for a robust, purpose-built analysis workflow tailored to LAMP sequencing data and the legal requirements of wildlife crime cases.

This study addresses these challenges by combining presumptive colorimetric LAMP with confirmation nanopore sequencing to develop a rapid, portable, and low-resource method for the authentication of elephant ivory and common substitutes. Novel LAMP assays were developed to identify elephants, mammoths, rhinoceros, hippopotamus, walrus, water buffalo, as well as domestic species (cow, cat, dog, human). As a proof-of-concept study, these assays were evaluated using synthetic DNA to assess sensitivity. This study also developed LAMPSEQ, an optimised analysis workflow for interpreting LAMP nanopore sequencing data, enabling high-confidence species identifications for some of the most heavily trafficked species in the illegal wildlife trade.

2. Methods

2.1. LAMP primer design

Mitochondrial DNA sequences for all target species were obtained from GenBank (Supplementary Table S1) [49], selected based on the following criteria to ensure the use of accurate and reputable reference sequences: (1) full mitochondrial genome, (2) published in peer-reviewed journal, (3) RefSeq accession for higher quality control if possible, (4) BLAST search consistency with target species, (5) alignment of multiple sequences per species to confirm accuracy. Target species included elephant (*Elephas maximus, Loxodonta africana, Loxodonta cyclotis*), rhinoceros (*Ceratotherium simum simum, Diceros bicornis, Dicerorhinus sumantrensis, Rhinoceros unicornis, Rhinoceros sondaicus*), hippopotamus (*Hippopotamus amphibius*), walrus (*Odobenus rosmarus*), mammoth (*Mammuthus primigenius, Mammuthus columbi*), cow (*Bos taurus*), cat (*Felis catus*), dog (*Canis lupus familiaris*), water buffalo (*Bubulus bubalis*) and human (*Homo sapiens*).

Species-specific SNPs in target genes (*16S rRNA, 12S rRNA,* and *CytB*) were identified by aligning sequences using the multiple align function in Geneious Prime (v2024.0.7) [50]. Target genes and candidate SNPs were selected based on: (1) general acceptance of the gene as a marker for species identification; (2) presence of species-specific SNPs sufficient to differentiate the target species from other closely related species; (3) ability to design all six LAMP primers; and (4) amplicon length of less than 250 bp to ensure efficient amplification from degraded DNA.

LAMP assays were designed using New England Biolab's LAMP Primer Design Tool 1.4.1 (https://lamp.neb.com/#!/), as summarised in Table 1. Full primer sequences are provided in Supplementary Table S2. Each LAMP assay included two inner primers (FIP/BIP), two outer primers (F3/B3) and two loop primers (LF/LB). LAMP primers were synthesised by Integrated DNA Technologies, IA, USA, reconstituted to 100 μM with nuclease-free water and pooled for a final concentration in the amplification reaction of 0.2 μM (F3/B3), 1.6 μM (FIP/BIP), and 0.4 μM (LF/LB).

For closely related species such as elephants, mammoths, and rhinoceros species *R. unicornis* and *R. sondaicus*, it was possible to design one LAMP assay to capture all species within the species group (Table 1). In these cases, conserved regions within *CytB* were selected that allowed amplification across species, while containing sufficient SNPs for species-level resolution in downstream nanopore sequencing. For more

Table 1
LAMP assay names, their target species and gene, and CITES protection for assays developed in this study.

LAMP Name	Species Group	Species captured with this assay	Target	CITES Protection
ELE	Elephant	All elephant species including *E. maximus, L. africana,* and *L. cyclotis*	*CytB*	Appendix I
MAM	Mammoth	Mammoth species including *M. primigenius* and *M. columbi*	*CytB*	Unprotected
RHI_Rhi	Rhinoceros	Closely related rhinoceros' species, *R. unicornis* and *R. sondaicus*	*CytB*	Appendix I
RHI_Csim		*C. simum* only	*CytB*	Appendix I
RHI_Dbic		*D. bicornis* only	*CytB*	Appendix I
RHI_Dsum		*D. sumantrensis* only	*CytB*	Appendix I
HIPPO	Hippopotamus	*H. amphibius* only	*16S rRNA*	Appendix II
WAL	Walrus	*O. rosmarus* only	*12S rRNA*	Appendix III
COW	Cow	*B. taurus* only	*16S rRNA*	Unprotected
CAT	Cat	*F. catus* only	*12S rRNA*	Unprotected
DOG	Dog	*C. lupus familiaris* only	*12S rRNA*	Unprotected
WBUF	Water buffalo	*B. bubalis* only	*CytB*	Unprotected
HUM	Human	*H. sapiens* only	*16S rRNA*	Unprotected

genetically diverse rhinoceros species like *C. simum, D. bicornis* and *D. sumantrensis*, and groups with one species (hippopotamus, walrus, cow, cat, dog, water buffalo and human), one LAMP assay was designed for each species.

2.2. Synthetic oligonucleotides

Mitochondrial target regions for each species included in this study were purchased as lyophilized gBlock Gene Fragments (Integrated DNA Technologies, IA, USA). Each target region corresponded to the *CytB, 12S rRNA* or *16S rRNA* locus used for LAMP primer design, as outlined in Table 1. The GenBank accession numbers for each of these sequences are provided in Supplementary Table S1. All synthetic oligonucleotides were reconstituted and diluted to the desired concentration using nuclease-free water.

2.3. Colorimetric LAMP reaction

Sensitivity of each LAMP assay was tested in duplicate with input amounts of 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg. Each LAMP reaction was prepared as per the manufacturer's instructions with a total reaction volume of 25 μL including: 12.5 μL WarmStart Colorimetric LAMP 2X Mastermix with UDG (New England Biolabs), 2.5 μL 10X LAMP primer pool, 8 μL nuclease-free water and 2 μL target synthetic oligonucleotide. The LAMP reaction was then incubated at 65°C for 30 min, followed by denaturation at 95°C for 5 min using a thermal cycler and results were determined by visual inspection of a colour change from pink (negative), orange (weakly positive) to yellow (positive). An amplification negative control (ANEG) of nuclease-free water was used in each batch for each LAMP assay tested.

2.4. LAMP sequencing

LAMP products were purified using 1X AMPure XP beads (Beckman Coulter), quantified using Qubit 1X dsDNA HS assay (Thermofisher Scientific) and normalised to 200 fmol per sample. Libraries were prepared using the manufacturers protocol (Oxford Nanopore Technologies) for ligation with amplicons using the Native Barcoding Kit (SQK-NBD112.96). The final library was loaded onto a R9.4.1 flow cell and sequenced for 48 h.

2.5. LAMPSEQ analysis

Raw *FAST5* files were basecalled and demultiplexed using standalone Guppy-GPU (v6.5.7) [51]. The demultiplexed *FASTQ* files were used as input data for the custom LAMPSEQ bioinformatics pipeline, which is available on GitHub (https://github.com/oliviayugovich/LAMPSEQ/).

LAMPSEQ first performs quality control on each *FASTQ* file by running FastQC (v0.12.1) [52], with results compiled using MultiQC (v1.28) [53]. A custom Python script was then used to identify and extract full-length LAMP repeats from each read. Reads were scanned for the presence of both forward and reverse flanking sequences, 20 bp regions located just inside the outer LAMP primers (F3 and B3), that are specific to each assay. Reads are retained if both flanks are detected in any orientation and if the sequence between them is within ±20 bp of the expected target amplicon length. These sequences are extracted into an output *FASTA* file for downstream alignments.

Reads passing this filter were aligned to a combined reference file containing all expected species using Bowtie2 (v2.5.4) [54,55] (-I 0 -X 800 -p 12 –very-fast -f). Alignment *SAM* outputs were converted to *BAM* format using SAMtools (v1.21) [56]. Species assignment was then performed using R (v4.4.3) [57] and the Rsamtools package (v2.22.0) [58] based on the alignment position in the reference file. LAMPSEQ generates a final *CSV* file containing barcode identifiers, species calls, and read counts, along with a summary *PDF* report. An analytical threshold of 5 % of total read count was applied to define off-target alignments and positive species identifications were identified as having > 95 % mapped reads.

3. Results

3.1. Presumptive LAMP

To evaluate the sensitivity of the LAMP assays for both CITES-protected and unprotected species, synthetic DNA was serially diluted from 5 ng to 1 fg and amplified in duplicate, with presumptive results recorded after 30 min (Fig. 1). For elephants (ELE), mammoths (MAM), and the closely related rhinoceros species *R. sondaicus* and *R. unicornis* (RHI_Rhi), a single LAMP assay was designed to detect all species within each group. The presumptive LAMP results (Fig. 1) observed for the elephant (ELE) and mammoth (MAM) assays demonstrated limits of detection (LOD) of 100 fg and 10 fg, respectively, with consistent results across species and replicates. For RHI_Rhi, slight preferential amplification of *R. unicornis* (LOD = 10 fg) over *R. sondaicus* (LOD = 1 pg) was observed, likely reflecting greater diversity between these species compared to elephant and mammoth.

Due to the higher diversity among the other rhinoceros species, *C. simum, D. bicornis,* and *D. sumatrensis*, detecting multiple species with a single LAMP assay was not feasible (data not shown), and individual assays were designed for each rhinoceros species. The observed LODs for these species were 10 fg (RHI_Csim), 1 fg (RHI_Dbic), 100 fg (RHI_Dsum). For the remaining species, single-species assays were sufficient, simplifying primer design with LODs for these assays being 100 fg for hippopotamus, 10 fg for walrus, cat, dog, water buffalo, and human, and 1 fg for cow. For water buffalo, one 1 fg replicate was positive (yellow), but the other was weakly positive (orange). In the human assay, one replicate at 1 fg was positive (yellow) and the other negative (pink), suggesting that 10 fg was a more reliable detection threshold for both assays. All other assays showed good consistency between replicates. These results demonstrate that the developed LAMP assays are highly

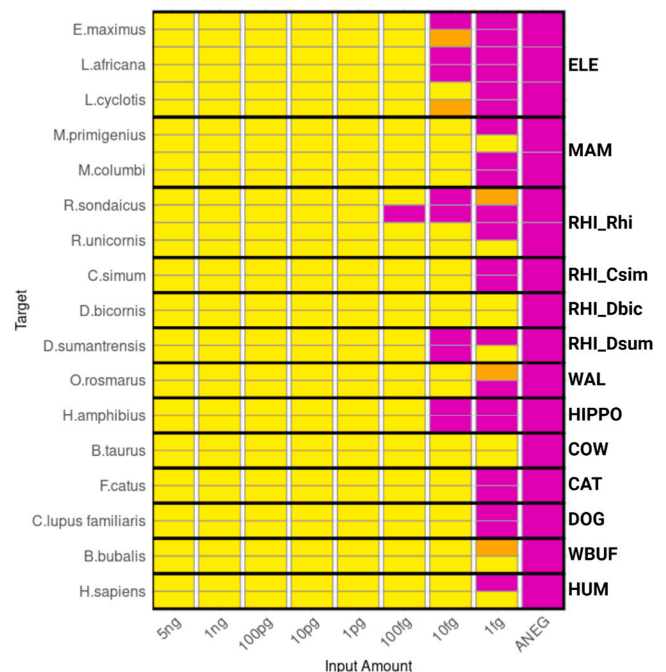

Fig. 1. Presumptive results for all LAMP assays and species using synthetic DNA (target) diluted from 5 ng to 1 fg (input amount) and amplified in duplicate. Results are indicated by a colour change from pink (negative), orange (weakly positive), to yellow (positive). The corresponding LAMP assay is labelled to the right, ANEG detonates the amplification negative control.

sensitive, reliably detecting target DNA in the femtogram range. Good consistency across species and replicates was observed, showing good promise for presumptive detection of both protected and unprotected species.

3.2. Building a bioinformatics pipeline for confirmation LAMP

Following presumptive LAMP, sequencing was performed to confirm species identification and to resolve species-level assignments for each assay. This was particularly important for the ELE, MAM, and RHI_Rhi assays, where co-amplification of multiple species was intentional, with species resolution being possible from the sequencing data.

To support this, a custom bioinformatics pipeline was designed with the primary goal of enabling high-confidence species identification while accounting for the increased noise associated with LAMP amplicons and nanopore sequencing data. Unlike PCR, which produces a single copy of a target sequence, LAMP generates concatemers (tandem repeats of the target amplicon) resulting in sequencing reads with variable lengths (Fig. 2A). In Fig. 2B, peaks below 150 bp represent excess primers and primer dimers (10.9 %), while the larger peaks correspond to LAMP products with one or more amplicon repeats. Most reads contained one (31.3 %), two (27.1 %), or three repeats (10.1 %), with more than 3 repeats found in < 6.9 % of the reads.

These concatemers introduce challenges for downstream bioinformatic analyses as commonly used aligners like Bowtie2 [54,55] and BLAST [47] are optimised for single-copy PCR amplicons and will discard or misalign reads with multiple repeats. To address this, a custom amplicon extraction script was designed prior to aligning sequences to identify a single, full-length repeat from each read, regardless of how many repeats were present.

The script relies on identifying consistent flanking regions, 20 bp sequences located just inside the outer LAMP primers (F3 and B3), which were found to be well-sequenced and reliably located at the ends of each repeat (Supplementary Figure S1). These flanking sequences were chosen after visual inspection of the sequence alignment in Geneious Prime (v2024.0.7) [50], where the actual primer sites (F3/B3) fell within poorly sequenced regions and were not consistently detected. The script scans each read for the presence of both the forward and reverse flanks being present in any direction and is within the expected target amplicon length (±20 bp). Reads meeting these criteria are extracted into an output *FASTA* file, and any sequence outside the flanks including partial repeats are removed. If a read contains multiple repeats, only the first instance that matches both flanks and the expected length is retained.

To evaluate whether the script could be adapted to extract multiple repeats from a single read, LAMP amplicons were visualised (Fig. 3) using dot plot comparisons in Geneious Prime (v2024.0.7) [50]. This was done to assess whether repeat boundaries could be reliably identified and used to split concatemers into individual full-length amplicons. Fig. 3A represents two identical target sequences that match perfectly and are in the forward direction. In contrast, when the same target sequence is compared to an untrimmed single-repeat read (Fig. 3B), there are unmatched bases at the start, a small internal deletion, and short non-specific repeats towards the end. In the untrimmed two-repeat read (Fig. 3C), the first repeat aligns well with the target sequence (denoted by the red line in the dot plot), but the rest of the read consists of fragmented regions, including incomplete forward and reverse repeats (denoted by the purple, blue and green lines). In the six-repeat read (Fig. 3D), the sequence is highly fragmented, showing a mixture of forward and reverse fragments with no intact full-length amplicon present.

These comparisons demonstrate that multiple repeat reads often contain incomplete, low-quality fragments with inconsistent boundaries, making it unsuitable for reliable amplicon sequence extraction. As a result, the custom script could not be adapted to extract multiple full-length repeats from individual reads as these were not present in the data. Instead, multiple repeat reads were composed of noisy sequences with internal deletions and varying strand orientations, which would likely reduce alignment accuracy and compromise reliable species identification. For these reasons, only high-quality, full-length amplicons were retained for downstream analysis.

Given that our sequence extraction approach strictly retains only reads that both match the flanking sequences exactly and the expected

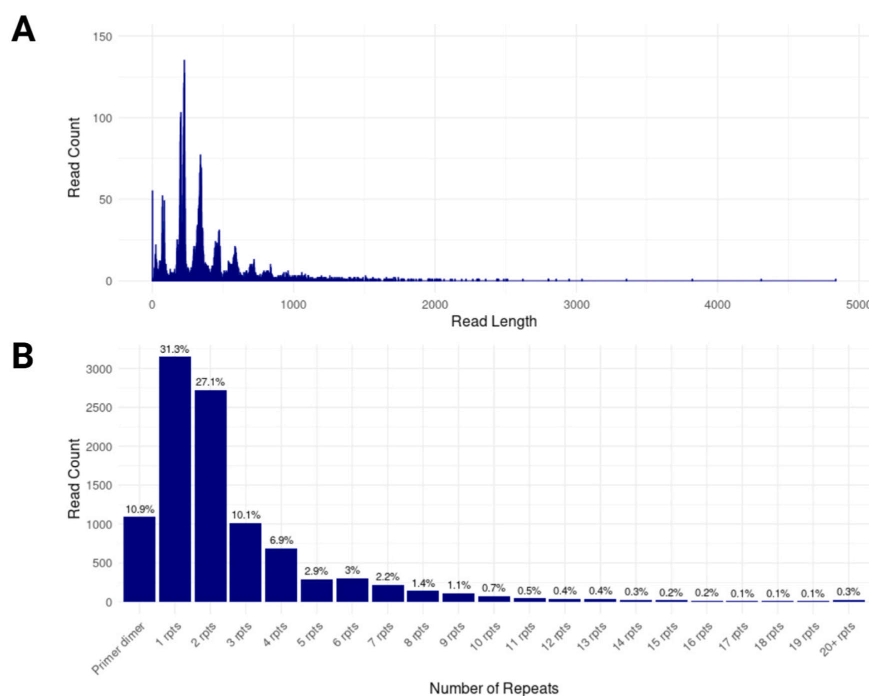

Fig. 2. Composition of LAMP amplicons generated from amplifying 1 ng of synthetic *E. maximus* DNA using the ELE assay. A similar trend was observed across all species and samples. (A) Distribution of read lengths and total read counts, showing the presence of LAMP products with varying concatemer lengths. (B) Distribution of repeat counts (rpts) based on read length and expected size of the target amplicon, with the proportion of total reads (%) shown for each repeat number.

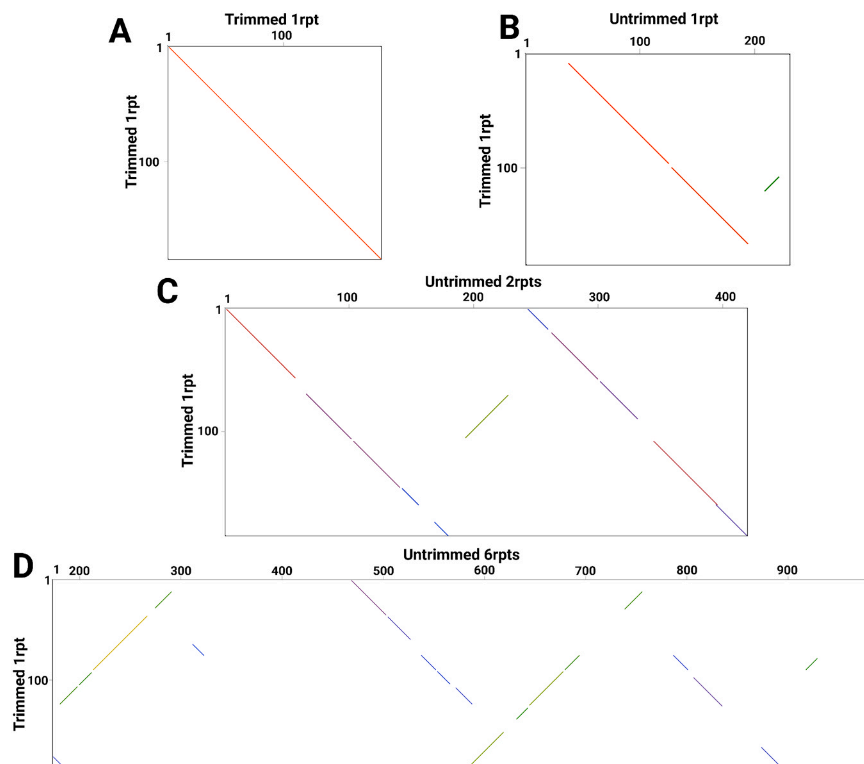

Fig. 3. Dot plot visualisation of LAMP in Geneious Prime v2024.0.7 (EMBOSS v6.5.7 dottup tool, low sensitivity/fast mode, word size = 12), generated from 1 ng of synthetic *E. maximus* DNA using the ELE assay. Similar results were observed across all species and samples. (A) Target sequence (trimmed 1 rpt) matching perfectly; (B) target sequence compared to an untrimmed 1 repeat (rpt) read; (C) target sequence compared to an untrimmed 2 rpt read; and (D) target sequence compared to an untrimmed 6 rpt read. Each chart is scaled according to the length of the reads (in bp). Solid lines indicate matching base pairs between the compared sequences. Line direction reflects strand orientation (forward or reverse), and line colour corresponds to specific sequence matches.

full amplicon length, we evaluated whether the extracted high-quality reads would provide sufficient coverage for accurate species identification. As shown in Fig. 4, although less than 10 % of the total reads per sample passed the filtering criteria, this typically still represented 500–1000 reads for each sample which is more than sufficient read coverage for species identification. For samples with fewer than 100 reads, the results correlated with the presumptive LAMP results of being weakly positive (orange) or negative (pink), so were consistent with what was expected. These findings demonstrate that if the LAMP amplification step is successful and produces a strong signal (as indicated by a complete colour change to yellow), sufficient high-quality reads can be obtained for species identification, despite the stringent filtering used. Importantly, this trend was observed across input amounts, with comparable filtered reads generated from these samples.

Following the read extraction step, we evaluated different alignment strategies to determine the most appropriate approach for accurate species identification in a field-deployable workflow suitable for wildlife forensic casework. Although BLAST [47] is commonly used for species identification, it was deemed unsuitable for our pipeline due to both scientific concerns about the interpretability of results and practical limitations related to computational efficiency.

BLAST [47] is designed to answer the question of 'what is this sequence similar to' rather than 'what is this sequence'. It does this by maximising sequence similarity by aligning only the best matching region of a read, often truncating sequences. While this tends to improve alignment scores, it can also obscure low-quality or noisy sequences rather than identifying them as poorly sequenced reads, often leading to misleading results. This is particularly problematic in wildlife forensic casework where high confidence in species identification results is critical to provide scientifically robust evidence. In this study, species-specific LAMP primers were designed to amplify only the intended target species from single-source samples and provides a

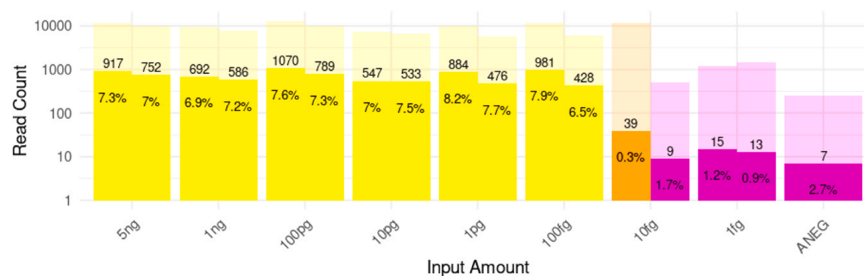

Fig. 4. Total and filtered read counts from synthetic *E. maximus* DNA diluted across a range of input amounts (5 ng to 1 fg) and amplified in duplicate using the ELE assay. A similar trend was observed across all species and assays. Each bar shows the total reads for each sample, with the number and percentage of filtered reads labelled. Bar colours correspond to presumptive LAMP assay results: yellow (positive), orange (weakly positive) and pink (negative). A logarithmic scale is used for the y-axis. ANEG refers to the amplification negative control.

presumptive species identification result prior to sequencing. Therefore, we are not sequencing completely unknown or multi-species samples, rather these are targeted detections that require confirmation and species-level resolution using sequencing. This means we expect the sequencing data to produce a precise match to the species we are expecting, and a mapping to reference approach was considered more appropriate.

Due to the alignment approach used by BLAST [47], outputs can also be difficult to interpret especially with closely related species like elephants and mammoths where sequence similarity is already high. To visualise this, we performed BLAST analysis on one trimmed and one untrimmed read (Supplementary Figure S2) from amplifying 1 ng synthetic *E. maximus* DNA. For the trimmed read, BLAST returned top hits not only to the correct species (95.9 % identity), but also to off-target African elephants, mammoths, and the extinct *Palaeoloxodon* elephant species, with identity scores above 94 %. The presence of these off-target species in the BLAST output complicates the interpretation of these results. It is difficult to say with high confidence that the sample originated from the target species and not the off-target species due to high sequence similarity and this could be easily refutable in court. Similar species identification results were observed for the untrimmed read, although due to these reads containing known noise sequences at either end, the top identity score dropped to 86.7 %. This also justified why the read extraction step was necessary, with untrimmed reads decreasing specificity and increasing misleading alignments.

The computational demand of BLAST [47] was another reason why this method was deemed impractical for a rapid, portable analysis workflow. Running BLAST on thousands of reads per sample is both time consuming and resource-intensive which conflicts with analysis being easily performed on a standalone laptop at or near the point of sample collection. For these reasons, we concluded that BLAST was not suitable for our data and analysis needs. Instead, we evaluated a mapping-based alignment approach using the reference sequences that were used for LAMP primer design to identify each species using Bowtie2 [54,55].

Bowtie2 [54,55] is a widely used aligner optimised for short-read, high-throughput sequencing data, typically from PCR amplicons. By using the read extraction filtering, we have adapted the LAMP sequencing data to be compatible with Bowtie2's alignment model. We assessed multiple alignment strategies, including mapping individual species references versus a combined reference sequence containing all species, and using trimmed or untrimmed data (Fig. 5). Alignments to the combined reference (Figs. 5A and 5C) consistently outperformed individual species references (Figs. 5B and 5D) in both specificity and sensitivity.

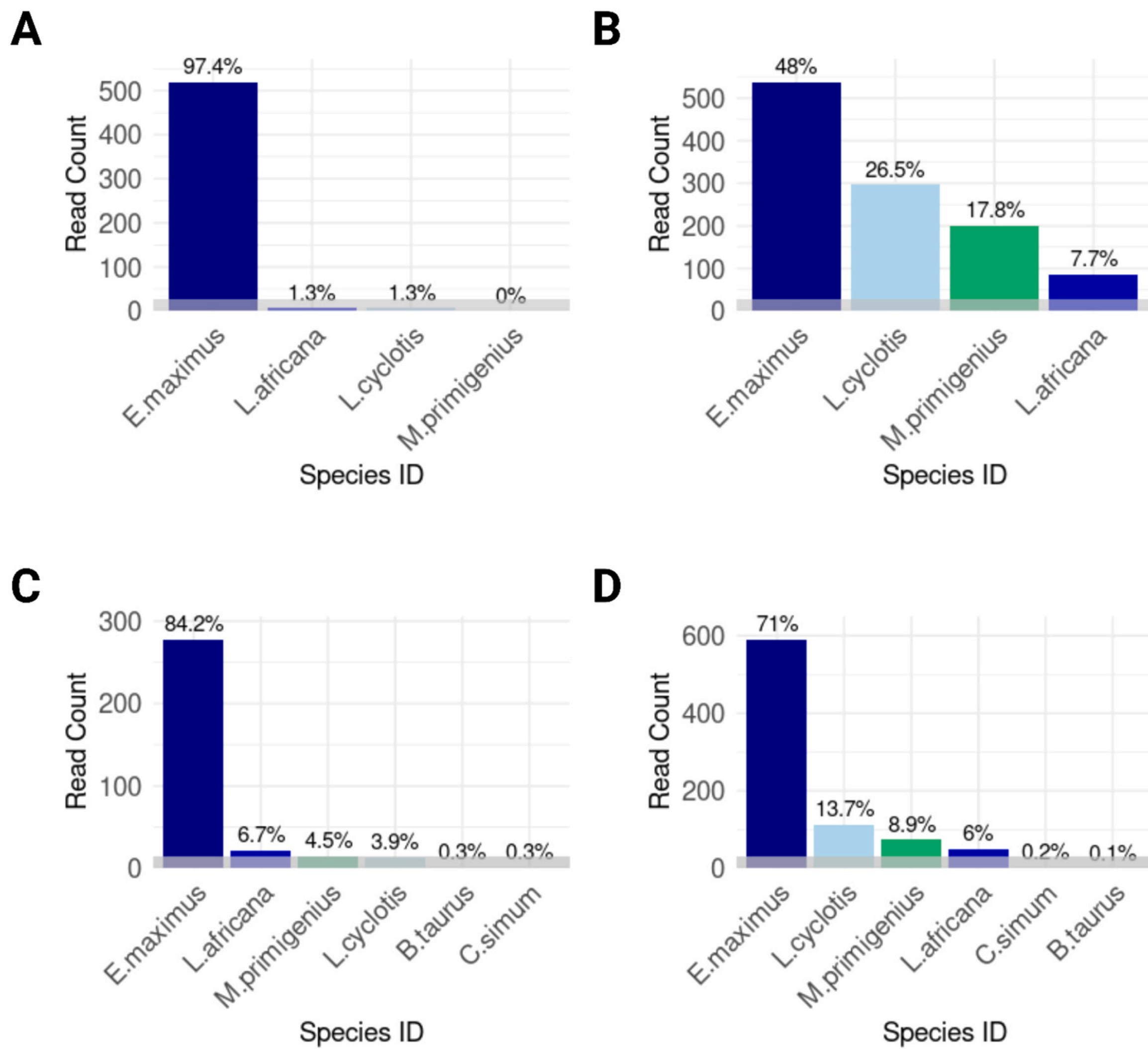

Fig. 5. Read counts and proportion of mapped reads (%) for alignments using Bowtie2 generated from 1 ng synthetic *E. maximus* DNA amplified using the ELE assay. A similar trend was observed across all species and assays. Trimmed reads (A and B) aligned using a combined reference (A) and each species individually (B); untrimmed reads (C and D) aligned using a combined reference (C) and each species individually (D). The analytical threshold (<5 %) is shown as a transparent grey bar.

Trimmed reads aligned to the combined reference (Fig. 5A) yielded the highest specificity for on-target *E. maximus* with 97.4 % reads mapping, only 1.3 % reads mapping to off-target elephant species, and no reads mapping to mammoth (*M. primigenius*). Untrimmed reads (Fig. 5C) resulted in 84.2 % on-target reads, with increased off-target mapping (<6.7 %) to species not seen when using the trimmed data including *M. primigenius*, *C. simum*, and *B. taurus*, and off-target elephant (*L. africana*) present above the analytical threshold. Manual inspection of off-target alignments from untrimmed reads showed that these were primarily poorly sequenced primer sequences that had spuriously matched to these off-target species highlighting increased noise in untrimmed data. Trimmed reads produced higher read counts for *E. maximus* (520 reads) compared to untrimmed reads (278 reads), indicating improved sensitivity with trimming.

When aligning to individual species references (Figs. 5B and 5D), both trimmed and untrimmed reads showed increased off-target mapping exceeding the analytical threshold. This resulted in reduced on-target mapping to 48 % (trimmed) and 71 % (untrimmed). This observation reflects the inherent behaviour of Bowtie2's alignment model. When given reference sequences individually, the tool evaluates each alignment in isolation and without the context of other possible matches, this can produce high mapping scores in regions of high sequence similarity. When Bowtie2 is given all possible reference sequences, the aligner can compare all possible options simultaneously and can more easily determine the best matching reference. This is especially important when distinguishing between closely related species, like elephants and mammoths, where conserved regions increase the risk of misalignment in the absence of competing reference sequences.

These results demonstrate that aligning trimmed reads to a combined multi-species reference provides the most sensitive and specific species identifications for LAMP sequencing data. This optimised strategy was incorporated into the LAMPSEQ bioinformatics pipeline and applied to all assays.

3.3. Sensitivity using LAMPSEQ

The finalised LAMPSEQ workflow was used to evaluate both the sensitivity of sequencing-based species identifications and concordance with presumptive LAMP results. Species identification results for all assays and input amounts are summarised in Fig. 6. All LAMP assays demonstrated high sensitivity and species-level specificity across input amounts ranging from 5 ng to 1 fg. Correct species identifications were consistently achieved down to 100 fg, with > 98 % of reads mapping to the expected species. The dog assay showed some variability in the percentage of mapped reads, although these remained > 95 % across input amounts. High amplification efficiency was found in the MAM and RHI_Rhi assays, consistently generating > 2000 reads regardless of input amount. As reflected in the presumptive results, preferential amplification of *R. sondaicus* over *R. unicornis* was observed in the RHI_Rhi assay, leading to dropout of one *R. sondaicus* replicate at 100 fg, consistent with its negative presumptive result.

The RHI_Dbic and RHI_Dsum assays showed good sensitivity down to 10 fg, but one replicate at 100 pg, 10 pg, 10 fg, and 1 fg produced lower mapped reads (<300) for each assay. In these cases, sequencing reads were split between *D. bicornis* and *D. sumantrensis,* although the correct species always retained a higher percentage of mapped reads than the off-target rhinoceros species. This shows a difficulty in species-level resolution in these two closely related rhinoceros species, reducing mapping efficiency and lowering read counts in some replicates. For these cases, it would be appropriate to report the result conservatively at the genus-level. For the replicates with high amplification efficiency and high sequencing reads, the assays worked as expected with 99–100 % reads mapping to the target species.

At 10 fg, most assays still produced positive species identifications, although dropout occurred in the ELE, RHI_Rhi, and HIPPO assays. For elephants, the presumptive LAMP results (Fig. 1) showed one weakly positive and one negative replicate for *E. maximus*, while both *L. africana* replicates were presumptively negative. In the sequencing results (Fig. 6), no sample across these two elephant species produced sufficient

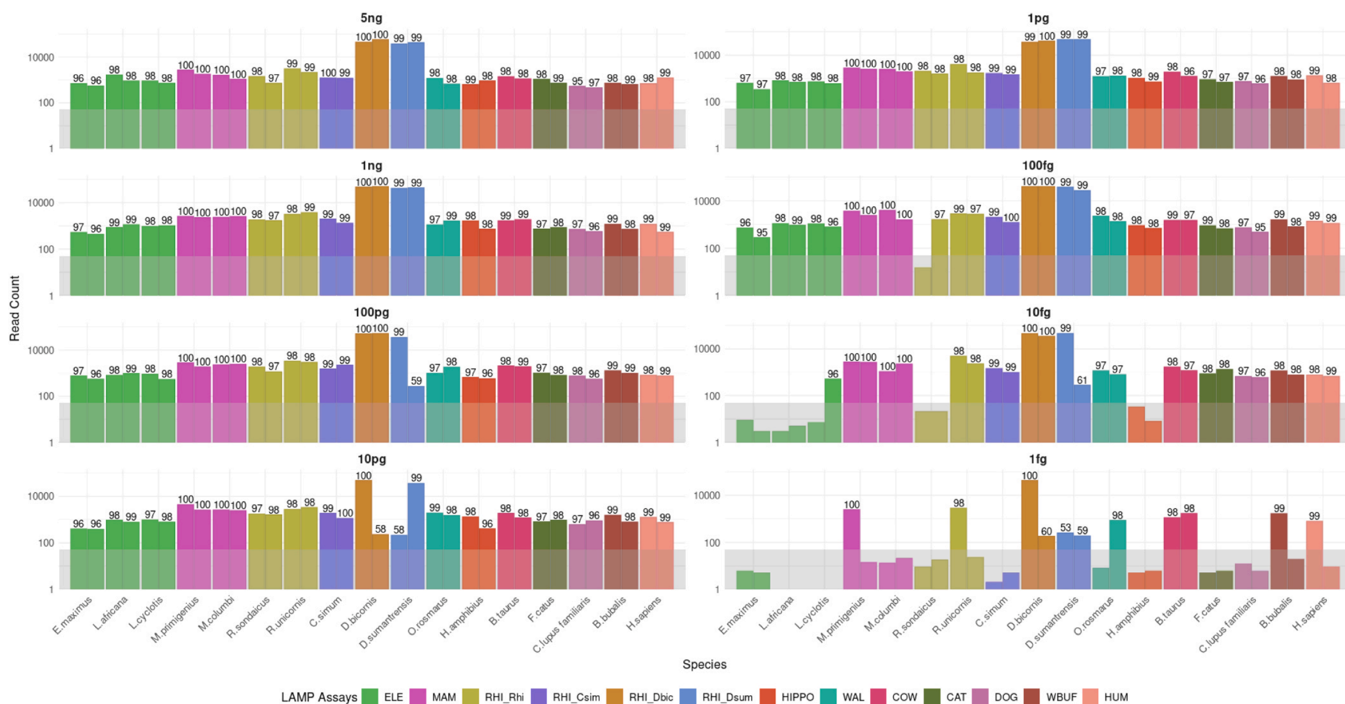

Fig. 6. Species identification from confirmatory sequencing across all LAMP assays and input amounts. Synthetic DNA from each target species was serially diluted (5 ng to 1 fg) and amplified in duplicate using the corresponding LAMP assay. All presumptive LAMP results were sequenced and analysed using the LAMPSEQ workflow. Bars represent read counts for each species and coloured by assay. The grey transparent bar shows on-target reads < 50 to demonstrate poorly sequenced samples. Panels are ordered by input amount to illustrate assay sensitivity. Numeric labels indicate the percentage of reads mapping to the identified species.

reads for species identification at this input amount. For *L. cyclotis,* one replicate was presumptively positive and the other weakly positive, with only the presumptively positive sample generating enough sequencing reads to confirm the species. At this input amount, complete dropout of *R. sondaicus* was observed using the RHI_Rhi assay, while *R. unicornis* remained detectable with high read counts. The dropout observed in the HIPPO assay was corroborated by a presumptive negative result for this replicate, with fewer than 35 sequencing reads being insufficient for reliable detection.

At 1 fg species dropout became more extensive across assays. Consistent with presumptive results, only *M. primigenius, R. unicornis, D. bicornis, O. rosmarus, B. taurus, B. bubalis,* and *H. sapiens* were detected, with *B. taurus* being the only species successfully identified in both replicates.

The MAM assay consistently failed to differentiate closely related *M. primigenius* and *M. columbi* species (Supplementary Figure S3), with reads split approximately evenly between the two species. The splitting of reads across both mammoth species lowered the mapping accuracy and consistently caused misidentifications across input amounts and replicates. Across all 16 replicates amplified with *M. primigenius*, exactly half (8/16) were identified as *M. primigenius*, while the remaining half was misidentified as *M. columbi*. While distinguishing between these two mammoth species would be ideal, the assay's primary goal is to reliably detect mammoth DNA distinctly from elephant. Consequently, results were conservatively reported at the genus-level. Using this approach, 100 % mapped reads consistently identified mammoth with no off-target elephant detection (Fig. 6). Given that *M. primigenius* is considered to be the predominant source of mammoth ivory in the trade [3], this assay remains appropriate and robust for detecting mammoth.

The LAMPSEQ workflow developed in this study demonstrated high sensitivity and species-level specificity, with strong concordance between presumptive LAMP and confirmatory sequencing identification results across all species targeted. The ability to generate accurate species calls from input amounts as low as 1–100 fg demonstrates the robustness of both assay design and downstream bioinformatics analysis. These features make LAMPSEQ particularly suited for wildlife forensic applications involving processed, low-quality or degraded samples, such as ivory, horn, tusk, and bone, while also supporting rapid, real-time results.

4. Discussion and conclusion

This study presents a rapid, low-resource, two-step workflow integrating colorimetric LAMP with nanopore sequencing for the identification of elephant ivory and common legal and illegal substitutes. The assays developed here demonstrate high sensitivity and species specificity using synthetic DNA, reliably detecting target species at femtogram levels with strong concordance between presumptive and confirmatory results. The LAMPSEQ bioinformatics pipeline designed in this study effectively resolves the analytical challenges posed by LAMP's tandem repeats, enabling robust and reliable species-level identification.

While promising, the laboratory methodology and bioinformatic analyses developed in this study require further work prior to implementation into wildlife forensic casework. As a proof-of-concept, this study used synthetic DNA derived from target species as clean, inhibitor-free template to evaluate assay performance. While appropriate for initial assay development, this does not capture the complexities associated with difficult sample types such as ivory, bone, teeth, or horn, to which this workflow is intended to be applied. These materials are widely recognised as poor sources of DNA, yielding low concentrations (often in the sub-nanogram range), are prone to degradation and contain inhibitors, all of which contribute to reduced amplification efficiency. DNA is embedded within dense structural matrices, including calcium-rich complexes (ivory, bone, teeth), or keratinised fibres (horn), making DNA extraction challenging. For calcified tissues, decalcification with EDTA is necessary, but EDTA carryover into the amplification reaction is a known inhibitor of amplification.

Although limited, some LAMP-based studies suggest that LAMP may be more tolerant to inhibitors than PCR [59–61], but these findings have been largely restricted to clinical diagnostic contexts. Unlike PCR, where inhibitor effects are well characterised across a wide range of applications, equivalent research for LAMP is insufficient and at times, contradictory. Most recently, Nwe *et al.,* [61] observed that LAMP was more tolerant to inhibitors including bile salts, immunoglobulin G (IgG), and urea, but it is less tolerant to calcium chloride compared to PCR. This is of particular relevance for calcified tissues proposed to be used in this study. Further work is required to better understand the potential carryover of Ca^{2+} and EDTA in extracts and how this may affect the ability of the assays developed in this study to efficiently amplify DNA.

The complex nature of these difficult sample types poses significant constraints on developing genuinely field-deployable workflows for ivory, bone, teeth, or horn. Extracting sufficient DNA from these materials still requires laboratory-based protocols, regardless of how rapid, low resource, or cost-effective downstream methods may be. However, even if full field-deployment is not yet feasible for these sample types, workflows such as those presented in this study can still provide substantial benefits by reducing processing delays, lowering costs, and alleviating the burden placed on centralised laboratories. These benefits may be even greater in other wildlife forensic applications where higher quality DNA is more accessible. Tissue, blood, or epithelial swabs encountered in investigations involving the exotic pet trade, bushmeat identification, or illegal fishing, are more compatible with simple or crude extraction protocols. For these samples, combining colorimetric LAMP and nanopore sequencing can support true field-deployable workflows.

As demonstrated in this study, LAMP can achieve high sensitivity, which is critical for successful amplification from low-template samples, but this also introduces additional considerations for anti-contamination procedures. A notable concern specific to LAMP is the generation of aerosols post-amplification which can lead to false positive results. As highlighted in our recent review [16], previous work has shown that this can be mitigated through careful primer design, reaction condition optimisation, and anti-contamination measures such as the use of dual filter tips and closed-tube detection for presumptive testing, and a standard operating procedure already exists for the use of LAMP for herbal medicine authentication [41]. Commercial LAMP mastermixes, including those used in this study from New England Biolabs, incorporate uracil-DNA glycosylase (UDG) to prevent carryover contamination from previous reactions. Finding a suitable balance between robust contamination control and operational feasibility will be essential for low cost and resource-limited settings. This is where well-developed, workflow specific standard operating procedures tailored to LAMP-based wildlife forensic workflows are crucial.

As discussed in our previous work [16], wildlife forensic science lacks the level of standardisation of methods observed in human forensic science, largely due to underfunding. There is a persistent need for more comprehensive developmental validation, quality assurance frameworks, and proficiency testing across laboratories and jurisdictions providing this service. More effort in this space is essential to ensure consistency, credibility, and defensibility in court, and must include assay development, laboratory methodologies, and bioinformatics analysis.

This study demonstrates that a mapping-based alignment approach provided clearer and more defensible species identification results than the more ambiguous similarity-based outputs produced by tools such as BLAST. This strategy was enabled by the workflow design, in which presumptive LAMP results provide an indication of the species present, and nanopore sequencing then gives corroborating evidence and confirms the result with sub-species identification. This intentional design supports a more transparent, reproducible, and court-defensible interpretation of evidence, which is essential in forensic science. DNA barcoding approaches using universal primers cannot rely on similar

mapping-based approaches in the same way. This does not necessarily mean results are not appropriate for court proceedings but demonstrates how different analytical choices have different interpretation constraints and this needs to be well communicated to the court system. Where possible, selecting analytical strategies that have fewer constraints and provide more robust, unambiguous evidence should be prioritised to ensure that bioinformatic choices are aligned with the expectations of legal proceedings.

Similarly to human forensic biology approaches, integrating statistical modelling into this analytical workflow would also provide an additional layer of assurance that evidence is robust and reliable. This study was limited to small sample sizes and only using synthetic DNA so was not possible. Further work to better understand false positive and false negative rates of presumptive LAMP and combining this with cross-validation of sequencing data would enable the application of appropriate statistical models. With more comprehensive, well-tailored standards and the incorporation of statistical modelling, this workflow is strongly positioned as a reliable and robust means of providing evidence in wildlife crime investigations.

Although substantial work is still required before the methods introduced in this study are fully fit-for-purpose in wildlife forensic casework, once achieved, the workflow presented here has strong potential to provide a rapid, low resource molecular approach to support CITES enforcement and conservation efforts aimed at combating the illegal trade in threatened species.

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CRediT authorship contribution statement

Olivia Yugovich: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Shane Sturrock:** Methodology, Writing – review & editing. **Vanessa Cave:** Writing – review & editing, Supervision. **SallyAnn Harbison:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2025.103412.

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