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Original Article

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ABSTRACT

Over recent years, mounting pressure has been placed on countries to assess their role in the ivory trade, with a view to tackling the rapidly declining numbers of elephants, due to poaching. The United Kingdom has been identified as a large re-exporter of ivory. Despite much of this trade being reported as legal or antique ivory, such provision of ivory to meet demand is known to fuel illegal markets and provide trade routes for modern ivory sales. Aside from ivory species and age, further analysis to evaluate geographic provenance, can inform where an elephant had lived, and so identify a source region or population where poaching occurred. The purpose of this study was to determine the age and species of ivory objects surrendered or seized in the UK and assess their likely geographic provenance through comparison of results from mitochondrial DNA and stable isotope analysis to publicly accessible georeferenced African elephant databases. The results demonstrated that the objects tested from an airport seizure were modern and matched existing haplotypes allowing for regional geographic inferences (supported by both techniques) to be obtained for most of these objects. In contrast, antique and modern ivory was detected amongst the amnesty objects, and several new mtDNA haplotypes were identified. Regional geographic inferences were achieved for some but not all of the objects tested. Our findings show this combination of methods provides a wealth of information which, could provide insight into targeted elephant populations and assist in disrupting international wildlife trade networks.

1. Introduction

The continued exploitation of elephants by poachers to fuel the international demand for ivory has drastically reduced the number and distribution of these iconic mammals [1,2]. Increasingly, pressure has mounted on governments to address the role their country plays in facilitating the ivory trade. The United Kingdom has been identified as a large re-exporter of ivory, considered in part to be due to the prevalence of antique ivory in the country [3,4].

UK legislation currently allows elephant ivory carved or worked prior to 1947 (considered to be 'antique'), to be traded legally; however, the commercial use of raw ivory is prohibited regardless of age [3,5]. In order to address and reduce the role played by the UK in the illegal ivory trade, the UK government has drafted legislation that will prohibit the commercial trade of all elephant ivory regardless of age, unless the objects meet very specific exemptions [3,6]. It is expected that this "Ivory Act (2018)" will be implemented in the very near future.

Ivory is derived from the tusks or teeth of several species, with differing levels of protection and differing laws relating to their trade. Many types of ivory have been observed in UK trade, including whale, hippopotamus and mammoth ivories [3,4,7].

To ascertain whether any ivory seized is illegal, the species of origin must first be determined. The measurement and comparison of morphological features unique to elephants and their ancestors, called Schreger lines, are commonly used to identify elephant ivory. The exact measured angle between these visible lines can distinguish between living and extinct elephant species [7]. However, these angles cannot differentiate among the three extant elephant species; Asian elephants

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(*Elephas maximus*), African savanna elephants (*Loxodonta africana*) and African forest elephants (*Loxodonta cyclotis*) [7]. Morphological identification is typically much easier for raw or part-worked ivory where features can be easily visualised and several measurements taken. However, in many ivory seizures, when objects are in a worked, carved or decorated condition, morphological features are not often visible on the external surface. In these circumstances, other techniques, such as mitochondrial DNA analysis, can assist with species identification [7,8].

It is currently an offence in the UK to sell ivory harvested from an elephant that was alive after 1947 [5]. Radiocarbon dating can be used to age elephant ivory and ascertain whether the elephant was alive during the nuclear era (1947 onwards) or prior to this period [8,9]. The relative ratios of radiocarbon to stable carbon, $^{14}C/^{12}C$, (termed the fraction modern value, $F^{14}C$) present in each sample are calculated and values greater or equal to 1 are indicative of an elephant that was alive after 1955 [10,11]. Where this value falls between 0.97 and 0.98, subsequent calibration produces several discrete calendar age ranges between the mid-1600s through to 1955 [10]. This is due to the shape of the calibration curve during this period. Calculated calendar age ranges may include the period between 1947 and 1955, where it is only possible to say that the object is pre-1955, not pre-1947 as stipulated by UK legislation. However, probabilities associated with the different calibrated age ranges are also provided. Within the period from 1955 to present day, two possible age ranges are calculated due to the shape of the "bomb calibration peak", where these correspond to the same $F^{14}C$ value on the up-slope and down-slope of the peak.

Further information can be gleaned from seized ivory through the use of analytical techniques which could assist the investigation of the wider ivory trade network, such as pinpointing poaching hotspots or identifying possible trade routes. Disruption of the network is often an objective when investigating organised criminal activity, whether it relates to people smuggling, weapons trade, drug smuggling or wildlife crime [12].

Here we examine the potential to geographically assign African elephant ivory (genus: *Loxodonta*) to countries or regions of origin using two alternative methods: DNA and stable isotope analysis.

Geographic assignment using DNA has been made possible through an increased understanding of elephant behaviour and population genetic structures. Elephant herds are philopatric and generally consist of a strongly bonded group of related females led by the matriarch (often the oldest female) [13,14]. This means that maternally inherited mitochondrial DNA, for the most part, remains within a herd and is localised to the region that herd inhabits. Males disperse, leaving their natal groups around puberty and typically live in solitude, or in other, generally smaller groups of males [13,14]. When ready to mate, male elephants compete and seek out females, resulting in male mediated gene flow between populations [14–17]. These behaviours give rise to the differences observed between mitochondrial and nuclear phylogeographic patterns in African elephants [14,16,18]. Despite this, it has been demonstrated that both nuclear and mitochondrial DNA can be used to infer geographic origins of seized ivory objects [18–21].

Wasser *et al.* [19,20] analysed sixteen polymorphic microsatellite loci from georeferenced African elephant samples and calculated allele frequencies for these markers. Alleles at these same markers from a seized ivory object can then be compared by applying a 'smoothed continuous assignment technique' to generate mapped allele frequency data from which possible geographic origins for the tested sample are statistically evaluated to ascertain which is more likely. The reported accuracy of this method is that 50 % of elephant samples are geographically assigned within 499 km of their true origin [19].

Mitochondrial DNA (mtDNA) sequences from large numbers of georeferenced African elephants have been analysed by many research groups as detailed by Zhao *et al.* [21]. These research groups targeted sections of the mitochondrial control region and published their sequences. Zhao *et al.*[21] compiled an open access, searchable database (Loxodonta Localizer; www.loxodontalocalizer.org) using 316 bp segments of these georeferenced sequences. Currently, reference data spans 24 countries, with over 1900 individual sequences divided into 125 haplotypes. Sixty two percent of these haplotypes are country specific and useful for inferring ivory provenance. Country specific or regional geographic inferences combined with knowledge of elephant population ranges, patterns of movement (including physical barriers) and geographic boundaries (such as borders) can aid investigations. Identifying an overlap in provenance from samples within a seizure or from different seizures can also allow for the identification of poaching hotspots or highlight recently targeted populations [20,21]. It follows that increased protection, monitoring and law enforcement could be focused on these areas.

Other haplotypes have been identified with a widespread distribution and as such are less informative [21]. The simplicity of the method and open access web platform of the Loxodonta Localizer database makes it accessible to wildlife forensic laboratories internationally.

In addition, Debruyne [22] demonstrated the presence of two highly divergent groups of mtDNA sequences amongst African elephants which could be separated into 'F' (forest) and 'S' (savanna) clades. These clades do not completely align to species identification amongst African elephants due to historic hybridisation events. Through the analysis of longer mtDNA sequences, Ishida *et al.* [23] further divided these clades into eight subclades, and geographically determined the distribution of each subclade across Africa. It follows that regional geographic provenance can be inferred for mtDNA sequences which can be assigned to these subclades. Within the 316 bp region there are 42 polymorphic sites and sequences can be sorted into these subclades using an identification key generated by Ishida *et al.* [23].

Stable isotope analysis is an alternative method for inferring the geographic origin of a biological specimen. Elemental isotopes are assimilated into tissues, including ivory, whilst an animal is alive. Measurement of carbon, nitrogen and sulphur isotope ratios in ivory (in the form of; $^{13}C/^{12}C = \delta^{13}C$, $^{15}N/^{14}N = \delta^{15}N$ and $^{34}S/^{32}S = \delta^{34}S$) can provide information on the vegetation, geology, habitat and environment occupied by the elephant at the time of its growth [8,24].

Carbon fixation pathways utilised by plants during photosynthesis affect their $\delta^{13}C$ values, and thus influence the amounts of each carbon isotope assimilated into ivory. Plants which use the Calvin cycle (C3 plants), are mostly found in temperate climates and have more negative $\delta^{13}C$ values, compared to plants which use the Hatch-slack method (C4 plants) or Crassulacean acid metabolism (CAM), which are mostly found in drier, more arid environments and exhibit less negative $\delta^{13}C$ values [25,26,28]. It follows that $\delta^{13}C$ values of African elephants reflect the prevalence and selection of plants in their habitat. In general, $\delta^{13}C$ values from African elephants fall between -15‰ and -28‰ [24,26,27]. However, individuals feeding in drier, more arid, environments have provided more extreme $\delta^{13}C$ values at the higher end of this scale, between -10‰ and -16‰ [26,28]. Whereas, African elephants feeding in moderate climates with more water availability, for example, in forests, have shown $\delta^{13}C$ values between -22‰ and -29‰ [27,28].

Nitrogen isotope ratios ($\delta^{15}N$) in African elephants reflect the humidity of an environment and subsequent nitrogen fixation in soils [24, 26,29]. The range of $\delta^{15}N$ values in African elephants has been reported as between 4.2‰ and 17.2‰, with those inhabiting humid environments returning values at the lower end of this range [24].

Values for sulphur isotope ratios ($\delta^{34}S$) in African elephants were found to correlate latitudinally across the continent, linked to the geology of an area, providing a range between 0.6‰ and 18.5‰, where enriched $\delta^{34}S$ values were recorded for elephants inhabiting countries in the south of Africa [24].

The calculation of isotopic ratios for specific elements or combinations of elements from African elephant ivory can be used to assign geographic provenance. Ziegler *et al.* [24,29] compiled results of isotopic analyses from georeferenced African elephant samples into an open access, searchable database (IvoryID; www.ivoryid.org). The IvoryID database was used to geographically assign 50 % of samples

analysed to within 381 km of their true origin, allowing for the regional assignment of unknown samples [24]. Ziegler [29] also reports a geographic assignment accuracy of 88 % using $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in combination. Currently, data present in the IvoryID database spans 29 African countries, with isotopic measurements from 694 African elephant samples. However, fewer than five reference samples are included in the database for 13 of these countries, the majority of which are part of the forest or mixed habitat zones, and therefore accurate isotopic profiles from these regions may be under represented [29].

We present the results of a study that examined thirty-nine ivory objects from two sources: surrendered voluntarily within the UK, or seized by UK Border Force at Heathrow Airport. Using data from both mtDNA and stable isotope analysis, we interrogated both the Loxodonta Localizer and IvoryID databases to investigate the origins of these objects. We also conducted radiocarbon dating to determine the likely age of these ivory objects. Finally, we evaluate our findings within the context of the ivory seizure and discuss the potential ramifications of the results.

2. Method

2.1. Sample collection

Thirty-nine suspected elephant ivory objects were provided by UK Border Force. All ivory was either part-worked or carved and received in tamper-proof evidence bags with full chain of custody. All ivory objects were assigned a unique identifier with the prefix IV.

The 39 ivory objects originated from two sources;

- 24 ivory objects were from UK residents who had surrendered suspected ivory to an amnesty held by the International Fund for Animal Welfare (IFAW). These had subsequently been transferred to UK Border Force for destruction, no further information was known about these ivory objects.
- 15 ivory objects were part of a UK seizure at Heathrow Airport in 2017, whose country of origin was thought to be Angola/Democratic Republic of the Congo (DRC), and had been routed via Nairobi.

2.2. Sample preparation

The outer surface of each ivory object was removed and discarded by drilling or using a Dremel ® sanding tool. Decontamination of drill bits, saw blades and other sampling equipment used was carried out using 1 % Chemgene™ solution and exposure to UV light for five minutes before and after each ivory object.

For DNA analysis the newly exposed surfaces were then drilled at a low speed to collect between 0.25 mg and 0.5 mg of powdered ivory.

For stable isotope and radiocarbon dating analysis small sections of ivory were cut using a saw and approximately 50 mg was required for further analysis.

2.3. Mitochondrial DNA analysis

2.3.1. Decalcification

Decalcification was carried out using 1 ml of EDTA (0.5 M, pH 8.0). All samples were incubated on a Thermomixer Comfort (Eppendorf, Hamburg) at 900 rpm and 4 °C for 24 h. Samples were pelleted by centrifugation (10,000 rpm for 10 min) and the supernatant was discarded and replaced with fresh EDTA for the process to be repeated. Resulting pellets were washed twice using 1 ml of nuclease free water.

2.3.2. Extraction, amplification, and sequencing

DNA extraction was performed using the DNeasy Blood and Tissue Kit extraction (Qiagen) protocol. DNA was eluted from the columns with 50 µl warmed AE buffer. To minimise analysis time, samples were assumed to be from elephant and PCR amplification was initially attempted, targeting the mitochondrial control region used in the Loxodonta Localizer database. If unsuccessful, species identification was subsequently performed via sequencing of the mtDNA cytochrome b gene. An extraction control was also included for each extraction batch.

A 522 bp fragment of the mitochondrial DNA (mtDNA) control region was amplified using primers CR-F1 (5′-TGGTCTTGTAAGCCATAAATGAAA-3′) and CR-R2 (5′-TGGTCCTGAAGAAAGAACCAG-3′) [30]. PCR was performed in a 20 µL reaction volume containing 2 x TypeIt master mix (Qiagen), 10 µM of both the forward primer and the reverse primer, and 2 µL genomic DNA of varying concentration.

PCR was carried out with the following program: 95 °C for 5 min; followed by,

- 3 cycles of: 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s,
- Four sets of 5 cycles of: 95 °C for 15 s, 'X' °C for 30 s, and 72 °C for 30 s, where 'X' is 58 °C for the first set and 56 °C, 54 °C, 52 °C for each set thereafter
- 22 cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s
- 72 °C for 7 min and held at 4 °C

PCR products were visualised on a 1 % agarose gel. Where no amplification was observed in the first PCR, suggesting the ivory was not from African elephant, a 471 bp fragment of the mtDNA cytochrome b gene was amplified using primers MCB398 (5′-TACCATGAGGACAAATATCATTCTG-3′) and MCB869 (5′-CCTCCTAGTTTGTTAGGGATTGATCG-3′) [31], using the same reaction mix and primer concentrations described above.

PCR was carried out with the following program: 95 °C for 5 min, followed by 35 cycles of; 95 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min 30 s, followed by 72 °C for 10 min and held at 15 °C.

Appropriate extraction and PCR negative controls and PCR positive controls were included with each amplification.

All visible PCR amplicons, plus all control reactions, were cleaned up and sequenced in both directions using BigDye® Terminator v3.1 (Life Technologies) chemistry on a Genetic Analyzer 3500xL (Applied Biosystems).

Sequences were trimmed and edited using Geneious v9.0 (https://www.geneious.com), generating a consensus from forward and reverse sequences. All control region consensus sequences were edited to 316 bp, assigned to a mtDNA subclade using an identification key [23], and queried against the Loxodonta Localizer database (https://www.loxodontalocalizer.org/), as described below. Cytochrome b sequences were queried using the BLAST algorithm against the GenBank database [32].

2.4. Loxodonta Localizer database analysis

The 316 bp sequences were grouped into haplotypes and queried against the georeferenced sequences held on the Loxodonta Localizer database. Where a matching sequence exists in the database, details of that sample, including accession number and mapped location are provided as outputs (mapped output shown in Fig. 1). Where no exact sequence match is identified (a novel sequence), closest matching sequences are suggested in ascending order of nucleotide mismatches. An increased number of mismatches between the queried sample and the closest sequence in the database decreases the reliability of the suggested geographic origins.

2.5. Radiocarbon dating and stable isotope analysis

Objects assigned to each haplotype were selected for further analysis, where multiple objects were assigned to a haplotype at least two objects from each haplotype were analysed further (11 objects in total). As were all objects that yielded novel mtDNA sequences (10 in total).

Collagen was extracted from these samples using the modified Longin method [33,34]. Collagen samples were combusted in an

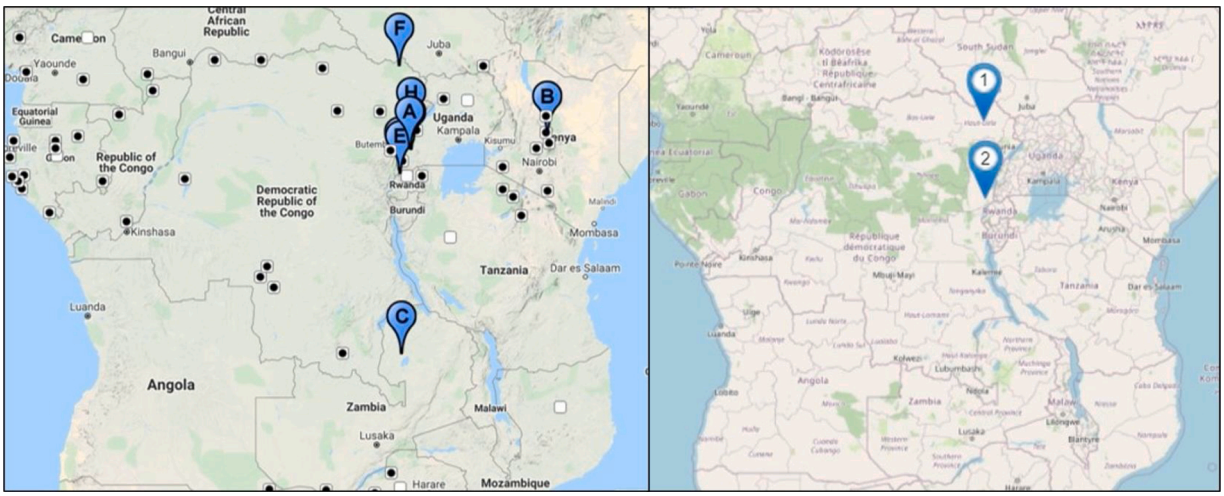

Fig. 1. Mapped outputs returned by the Loxodonta Localizer database (left) and the IvoryID database (right). The Loxodonta Localizer output shows the geographic locations of samples in the database grouped within haplotype LL068. The IvoryID output shows the geographic suggestions (both with good fit) for the isotope ratio combinations ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of two of the ivory objects in this study (IV002 and IV015) which matched haplotype LL068, plotted onto a single map.

evacuated combustion tube, in the presence of copper oxide and silver foil, by furnacing overnight at 850 °C. Following combustion, 3 ml subsamples of carbon dioxide (CO_2) were reduced to graphite using the zinc-iron reduction method described by Slota *et al.* [35]. The graphite was then pressed into aluminium cathodes and the radiocarbon content determined by accelerator mass spectrometry. Calculation of ^{14}C was performed by SUERC using the method set out in Brown and Southon [36]. Radiocarbon ages were calibrated to the calendar timescale using the Oxford Radiocarbon Accelerator Unit calibration program OxCal v4.4 [37], and the post-bomb atmospheric NH3 curve [38] for modern samples and IntCal20 atmospheric curve [39] for antique samples. Calibrated date ranges (with 95 % confidence) are reported.

Isotopic compositions were derived from the collagen samples as described by Sayle *et al.* [40]. Values were determined as the difference in parts per thousand (‰) between the isotopic ratios in the samples to the ratios in the appropriate international standards and expressed using the delta (δ) notation [41]. For carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and sulphur ($\delta^{34}S$) the standards were Vienna Pee Dee Belemnite (VPDB), Atmospheric Nitrogen (AIR) and Vienna Canyon Diablo Troilite (VCDT), respectively [40]. Laboratory uncertainty values were calculated as ±0.1‰ for $\delta^{13}C$, ±0.2‰ for $\delta^{15}N$ and ±0.3‰ for $\delta^{34}S$.

2.6. IvoryID database analysis

Isotope analysis for the IvoryID database, conducted directly on powdered ivory, includes isotopes from both bioapatite and collagen fractions. In order for our results from collagen to be comparable to, and compatible with, the IvoryID database records, offsets calculated by Ziegler *et al.* [24] were applied (0.3‰ for $\delta^{13}C$ and 0.4‰ for $\delta^{15}N$). No specific offset was required for sulphur as the majority of sulphur detected in a powdered sample is likely to have originated from the collagen component [24,29]. The corrected isotope ratios ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of each object were searched in combination against the IvoryID database (https://www.ivoryid.org/) and geographic assignments were provided as mapped outputs (Fig. 1) along with a strength of assignment.

The IvoryID database works by assessing the similarity of isotopic combinations recorded in the database to those measured in the sample, and assigns a weight to them (using the weighted k-Nearest Neighbour (NN) Classifier method). The Euclidian distance to each of these 'nearest neighbours' is calculated and ranked using the Wilcoxon signed rank test. If the p-value calculated is more than 0.05, the test concludes that the unknown sample and database sample (neighbour) are similar, and that the origin could be the same [29]. The strength of geographic assignment is assessed by algorithms within the IvoryID database according to the number of statistically similar isotopic fingerprints identified as 'neighbours'. If at least two similar neighbours were identified, the suggested geographic origin would be deemed as a 'good fit'. Where only one similar reference was identified, the suggested geographic origin would be deemed as a 'moderate fit' and where no statistically similar neighbours were identified, an 'uncertain fit' [29].

3. Results

3.1. Mitochondrial DNA analysis – overview

Robust 316 bp control region sequences were generated for thirty-two of the thirty-nine objects, and sixteen unique sequences were obtained, seven of which had been published previously (Supplementary Materials, Table 1). Five objects (all from the amnesty) produced mixed DNA sequences, where either minor DNA types could be seen in several parts of the sequence generated or where nucleotide calls within the sequence could not be accurately resolved to allow for robust comparison to other sequences. The remaining two objects (IV009 and IV021) failed to amplify with the CR-F1 and CR-R2 primer pair, so further analysis of the mtDNA cytochrome b gene was conducted to assist with species identification. The resulting sequences were BLAST searched against GenBank, and matched sequences described as *Hippopotamus amphibious* (Supplementary Materials, Table 1). No further analysis will be reported on these seven ivory objects.

3.2. Radiocarbon and stable isotope analysis - overview

Twenty of twenty-one objects provided enough material following combustion to undergo radiocarbon dating (IV040 was unsuccessful). $F^{14}C$ values and calibrated calendar ages are reported in Table 1. Isotope analysis was successful for nineteen of these objects, with one unsuccessful object (IV031) yielding insufficient collagen for analysis. For all other objects the stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) fell within previously reported ranges for African elephants (Table 2).

3.3. Results – surrendered ivory (twenty-four objects)

Calibrated calendar ages revealed the presence of both modern and antique ivory amongst the surrendered objects confirmed as elephant ivory. One was antique, dating to no later than 1930. Three were likely

Table 1
^{14}C results for twenty ivory objects presented as ^{14}C fraction modern values with calibrated calendar age ranges and their associated probabilities in parentheses at overall 95.4 % confidence.

Sample numbers	Ivory Set	^{14}C fraction modern ($F^{14}C \pm 1\ \sigma$)	Radiocarbon dating - Calibrated calendar age estimations in calAD (95.4 % confidence)	
			Early age range	Late age range
IV024 SUERC-85740 (GU50828)	Airport Seizure	1.0463 ± 0.0027	1957 (20.9 %)	2007–2009 (74.6 %)
IV023 SUERC-85739 (GU50827)	Airport Seizure	1.0328 ± 0.0024	1956–1957 (73.6 %)	2015- (21.8 %)
IV025 SUERC-85744 (GU50829)	Airport Seizure	1.0398 ± 0.0031	1955–1957 (67.4 %)	2007–2009 (28.1 %)
IV028 SUERC-85745 (GU50830)	Airport Seizure	1.0630 ± 0.0031	1957–1958 (3.7 %)	2004- (91.8 %)
IV029 SUERC-85746 (GU50831)	Airport Seizure	1.0384 ± 0.0027	1955–1957 (80.4 %)	2008–2009 (15.1 %)
IV030 SUERC-85747 (GU50832)	Airport Seizure	1.0562 ± 0.0031	1957 (3.4 %)	2006- (92.1 %)
IV015 SUERC-85737 (GU50825)	Amnesty	1.0461 ± 0.0031	1957 (21.0 %)	2007–2009 (74.4 %)
IV002 SUERC-85728 (GU50818)	Amnesty	0.9876 ± 0.0029	1690–1730 (25.4 %)	1800–1930** (70.1 %)
IV004 SUERC-85729 (GU50819)	Amnesty	1.1712 ± 0.0035	1958–1959 (8.2 %)	1987–1990 (87.2 %)
IV010 SUERC-85736 (GU50823)	Amnesty	1.4241 ± 0.0036	1962–1963 (6.5 %)	1973–1974 (89.0 %)
IV034 SUERC-86214 (GU50836)	Amnesty	1.5019 ± 0.0058	1963 (8.6 %)	1969–1972 (86.9 %)
IV019 SUERC-85738 (GU50826)	Amnesty	1.2563 ± 0.0032	1961–1962 (21.1 %)	1980–1982 (74.3 %)
IV003 SUERC-85757 (GU50938)	Amnesty	1.2801 ± 0.0033	1962 (6.3 %)	1979–1981 (89.2 %)
IV006 SUERC-85730 (GU50820)	Amnesty	0.9773 ± 0.0023	1660–1810 (78.1 %)	1920–1955* (17.3 %)
IV001 SUERC-85727 (GU50817)	Amnesty	1.4896 ± 0.0044	1962–1963 (17.8 %)	1969–1972 (77.6 %)
IV008 SUERC-85735 (GU50822)	Amnesty	1.4946 ± 0.0044	1962–1963 (15.3 %)	1969–1972 (80.2 %)
IV011 SUERC-86047 (GU50824)	Amnesty	0.9777 ± 0.0026	1650–1810 (76.4 %)	1910–1955* (19.0 %)
IV033 SUERC-85750 (GU50835)	Amnesty	1.0115 ± 0.0030	1955–1957 (95.4 %)	–
IV031 SUERC-85748 (GU50833)	Amnesty	1.3761 ± 0.0036	1962–1963 (9.5 %)	1974–1976 (86.0 %)
IV037 SUERC-85755 (GU50838)	Amnesty	0.9751 ± 0.0029	1650–1810 (84.2 %)	1920–1955* (11.3 %)

* Potentially antique ivory. Probability favours pre-1947.
** Antique ivory pre-dating 1947.

Table 2
Stable isotope ratios obtained from nineteen ivory objects. Analysis was carried out on collagen and therefore offsets (0.3‰ for $\delta^{13}C$ and 0.4‰ for $\delta^{15}N$) were added to the listed ratios before searching against the IvoryID database as described by Ziegler *et al.* [24].

Sample numbers and Laboratory Codes	Ivory Set	Isotope Ratios ‰		
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
IV024 SUERC-85740 (GU50828)	Airport Seizure	−22.0	8.3	8.5
IV023 SUERC-85739 (GU50827)	Airport Seizure	−21.9	8.7	7.9
IV025 SUERC-85744 (GU50829)	Airport Seizure	−22.4	9.4	7.9
IV028 SUERC-85745 (GU50830)	Airport Seizure	−20.7	9.2	8.3
IV029 SUERC-85746 (GU50831)	Airport Seizure	−22.5	8.0	6.9
IV030 SUERC-85747 (GU50832)	Airport Seizure	−22.6	8.0	6.7
IV015 SUERC-85737 (GU50825)	Amnesty	−19.4	8.2	4.4
IV002 SUERC-85728 (GU50818)	Amnesty	−22.7	7.4	5.7
IV004 SUERC-85729 (GU50819)	Amnesty	−20.2	7.8	6.0
IV010 SUERC-85736 (GU50823)	Amnesty	−21.5	6.2	4.4
IV034 SUERC-86214 (GU50836)	Amnesty	−21.5	7.0	4.7
IV019 SUERC-85738 (GU50826)	Amnesty	−24.6	11.0	9.5
IV003 SUERC-85757 (GU50938)	Amnesty	−24.1	8.9	5.0
IV006 SUERC-85730 (GU50820)	Amnesty	−20.6	6.6	9.3
IV001 SUERC-85727 (GU50817)	Amnesty	−26.2	10.9	3.6
IV008 SUERC-85735 (GU50822)	Amnesty	−21.7	6.8	6.1
IV011 SUERC-86047 (GU50824)	Amnesty	−15.9	7.2	3.1
IV033 SUERC-85750 (GU50835)	Amnesty	−24.0	12.4	5.8
IV037 SUERC-85755 (GU50838)	Amnesty	−20.6	6.0	7.3

to be antique as the calibrations from their calculated ^{14}C fraction modern values fell in an area of the calibration curve that potentially dates them as post-1947 and also as early as the mid-1600s (Table 1). However, probabilities provided for the date calibrations of these objects favour the earlier (antique) date.

Seventeen ivory objects yielded robust mtDNA control region sequences; representing thirteen different haplotypes. Three sequences matched haplotypes on the Loxodonta Localizer database and are reported with their respective IvoryID outputs in Table 3. Maps produced for objects which had both a haplotype match and stable isotope results can be found in the supplementary materials (Supplementary Materials, Fig. 1). The haplotypes found in this study were:

- **LL068** (objects IV002 and IV015) found in Uganda, DRC-Uganda border, Rwanda, DRC, Kenya and Zambia, and was part of the east central mtDNA subclade. The IvoryID database search of the isotopic combinations from both objects suggested the Democratic Republic of Congo, in locations close to the Ugandan border (with a good fit) as the likely geographic origins (Fig. 1). These findings correlate with the locations suggested by Loxodonta Localizer and the geographic distribution of the mtDNA subclade.
- **LL071** (object IV004) found in Uganda and Kenya and was part of the northern savanna mtDNA subclade. The IvoryID output for this object suggested geographic provenance of Southern Zambia (with a good fit), which does not correlate with the Loxodonta Localizer findings however Zambia has a limited representation on this database. It also does not currently fall within the mtDNA subclade range.
- **LL062** (objects IV010, IV020, IV022 and IV034) has a widespread distribution and was part of the savanna-wide mtDNA subclade. Two of the four objects (IV010 and IV034) were tested further and IvoryID suggested possible provenance as Malawi and Mozambique (both with a moderate fit). Although both fall within the mtDNA subclade range, due to the moderate fits, these assignments may not be particularly informative.

Of the ten remaining haplotypes not represented on the Loxodonta Localizer database, one (IV040) contained a single deletion within the trimmed target fragment, and therefore was only 315 bp in length. This sequence was not compatible with the Loxodonta Localizer search functionality, as sequences containing deletions or ambiguous bases are not currently accepted as a query input. However, it did match a

Table 3
Results of molecular and isotopic tests conducted on the surrendered ivory objects (amnesty). Mitochondrial DNA sequence search results from the Loxodonta Localizer database are provided alongside associated geographic assignments and isotope ratio ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) geographic assignments from the IvoryID database (including strength of fit). Also included is the mitochondrial subclade assignment based on the identification key produced by Ishida *et al.* [23] and the estimated age following radiocarbon dating.

Sample number	Loxodonta Localizer Haplotype match	Geographic assignments from Loxodonta Localizer (Haplotype locations)	mtDNA subclade	Geographic assignments from IvoryID database (Good fit, † Moderate fit, ° Uncertain fit)	Age
IV015	LL068	Uganda, DRC-Uganda border, Rwanda, DRC, Kenya, Zambia	East Central	DRC, Rumangabo	Modern
IV002	LL068	Uganda, DRC-Uganda border, Rwanda, DRC, Kenya, Zambia	East Central	DRC, Gangara na Bodio	Antique
IV004	LL071	Uganda, Kenya	Northern Savanna	Southern Zambia	Modern
IV010	LL062	Widespread	Savanna-Wide	Malawi, Rhumpi, Vwasa Marsh Game Reserve †	Modern
IV034	LL062	Widespread	Savanna-Wide	Mozambique, Chipupa / Rovuma †	Modern
IV020	LL062	Widespread	Savanna-Wide	Not tested	Not tested
IV022	LL062	Widespread	Savanna-Wide	Not tested	Not tested
IV019	mismatch (2 bp)	No exact match obtained	West Central	DRC, Lukolela	Modern
IV003	mismatch (2 bp)	No exact match obtained	Northern Savanna	DRC, Loyo	Modern
IV006	mismatch (5 bp)	No exact match obtained	North Central	Southern Zambia	Likely Antique
IV001	mismatch (6 bp)	No exact match obtained	South Central	DRC, Mongende †	Modern
IV008	mismatch (1 bp)	No exact match obtained	Savanna-Wide	East Zambia, east to North Luangwa National Park †	Modern
IV011	mismatch (2 bp)	No exact match obtained	South Central	DRC, Garamba park °	Likely Antique
IV033	mismatch (6 bp)	No exact match obtained	South Central	DRC, Medje °	Modern
IV037	mismatch (1 bp)	No exact match obtained	East Central	Northeastern Zambia, near Chilonga °	Likely Antique
IV031	mismatch (2 bp)	No exact match obtained	South Central	Sample fail	Modern
IV040	315 bp sequence (deletion)	Not searchable	Southeast Savanna	Sample fail	Sample fail

previously published sequence on GenBank (Supplementary Materials, Table 1). This object also failed to yield enough material for radiocarbon dating or isotope analysis. The final nine haplotypes had between 1 and 6 bp differences (mismatches) to other recorded haplotypes on the Loxodonta Localizer database (Table 3), and were unique to this study. One of these objects (IV031) failed to produce enough collagen for isotope analysis but the remaining eight were successful. The IvoryID database suggested geographic origins for these eight objects with varying degrees of confidence (Table 3). These geographic assignments could be compared to the regional distributions of the mtDNA subclades they were assigned to, but not to Loxodonta Localizer results.

IV003 was assigned genetically to the northern savanna mtDNA subclade, and IV019 to the west central mtDNA subclade. Both objects were assigned to the Democratic Republic of Congo using the IvoryID database (with good fits). Both of these assignments fall within the respective ranges of their subclades.

The objects grouped in the south central mtDNA subclade (IV001, IV011, IV033) all had isotopic assignments as locations within the Democratic Republic of the Congo. The isotopic assignments of object IV008 (grouped in the savanna-wide subclade), and object IV037 (grouped in the east central subclade) suggested a geographic assignment of Zambia. Despite these geographic assignments correlating with the range of their respective mtDNA subclades, they were all considered as moderate or uncertain fits by the IvoryID database, due to a deficiency in similar isotopic fingerprints present. It follows that caution must be applied when using these assignments in isolation.

IV006 was assigned genetically to the north central mtDNA subclade and the IvoryID database suggested Zambia as the geographic provenance of this object (with good fit). This assignment does not fall within the range of the north central subclade.

3.4. Results - seized ivory (fifteen objects)

Calibrated calendar ages of the six ivory objects tested from the seizure generated modern dates (post-1955) (Table 1).

Mitochondrial DNA sequences were obtained from all fifteen ivory objects, producing three different haplotypes which were all present on the Loxodonta Localizer database (Table 4). Maps produced for objects which had both a haplotype match and stable isotope results can be found in the supplementary materials (Supplementary Materials, Fig. 1). The haplotypes found in this study were:

- **LL003** (objects IV005, IV012, IV014, IV016-IV018, IV023, IV025-IV028) found in Botswana, Zimbabwe and Namibia and is part of the south central mtDNA subclade. Three of these objects (IV023, IV025 and IV028) were selected for isotope analysis, and when queried on the IvoryID database, each provided a location of Southern Zambia (with a good fit). However, the geographic assignments cannot completely correspond due to the lack of sample representation from Zambia on the Loxodonta Localizer database. Regionally, these findings correlate with the geographic distribution of the mtDNA subclade.
- **LL067** (object IV024) found in Tanzania, Kenya, South Africa, Uganda and West Zimbabwe, and assigned to the savanna-wide mtDNA subclade. The IvoryID output for this object suggested a geographic provenance of Southern Zambia (with a moderate fit). Although this location falls within the widespread haplotype and mtDNA subclade range, due to the moderate strength of fit, this assignment has limited informative value.
- **LL085** (objects IV013, IV029 and IV030) found in Northern Zimbabwe and Botswana and assigned to the south central mtDNA subclade. Two of these objects (IV029 and IV030) were tested further and the IvoryID output suggested Mozambique (with good fits) as the geographic origin for both objects. Although neither the samples assigned to this haplotype nor the geographic distribution of the south central mtDNA subclade overlap with the isotope findings, all

Table 4
Results of molecular and isotopic tests conducted on the seized ivory objects (airport seizure). Mitochondrial DNA sequence search results from the Loxodonta Localizer database are provided alongside associated geographic assignments and isotope ratio ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) geographic assignments from the IvoryID database (including strength of fit). Also included is the mitochondrial subclade assignment based on the identification key produced by Ishida *et al.* [23] and the estimated age following radiocarbon dating.

Sample number	Loxodonta Localizer Haplotype match	Geographic assignments from Loxodonta Localizer (Haplotype locations)	mtDNA subclade	Geographic assignments from IvoryID database (Good fit, † Moderate fit, º Uncertain fit)	Age
IV024	LL067	Tanzania, Kenya, South Africa, Uganda, West Zimbabwe	Savanna-Wide	Southern Zambia †	Modern
IV023	LL003	Botswana, Zimbabwe, Namibia	South Central	Southern Zambia	Modern
IV025	LL003	Botswana, Zimbabwe, Namibia	South Central	Southern Zambia	Modern
IV028	LL003	Botswana, Zimbabwe, Namibia	South Central	Southern Zambia	Modern
IV005	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV012	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV014	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV016	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV017	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV018	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV026	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV027	LL003	Botswana, Zimbabwe, Namibia	South Central	Not tested	Not tested
IV013	LL085	Northern Zimbabwe, Botswana	South Central	Not tested	Not tested
IV029	LL085	Northern Zimbabwe, Botswana	South Central	Mozambique, Lugenda south bank	Modern
IV030	LL085	Northern Zimbabwe, Botswana	South Central	Mozambique, Mbamba village area - Lugenda	Modern

the suggested geographic origins are neighbouring countries, and Mozambique has poor sample representation on the Loxodonta Localizer database.

4. Discussion

4.1. Geographic inferences for unresolved haplotypes

Despite 62 % of haplotypes on the Loxodonta Localizer database being country specific [21,23], none of these were identified in this study. However, four of the six haplotype matches obtained were considered to be geographically regionally specific. Where matching haplotypes were not found, closest matching haplotype(s) revealed a vast disparity in suggested geographic range. The reliability of geographic inferences by the Loxodonta Localizer database understandably decreases as the number of 'mismatches' increase between queried and reference sequences [21]. We surmise that for the purpose of a forensic investigation, and objects with unknown provenance, inference of origin should only be attempted with the Loxodonta Localizer database where an exact haplotype match is returned from the database. Regional geographic distributions could still be inferred using the mtDNA subclades to which our novel sequences were assigned [23].

Haplotypes LL062 and LL067 belonged to the savanna-wide subclade and showed a widespread distribution (LL062 more so than LL067) [23], and therefore the use of this mtDNA technique alone for inferring geographic provenance for the ivory objects matching these haplotypes was uninformative. Concurrent isotopic analysis of three ivory objects assigned to these haplotypes (IV010, IV034 and IV024) did suggest geographic assignments within their respective haplotype and mtDNA subclade range; however, with only a moderate fit (Tables 3 and 4). The same is true for the geographic origins suggested by isotope analysis of IV008, which generated a novel sequence and was also assigned to the savanna-wide mtDNA subclade. Given the lack of similar isotopic fingerprints represented on the IvoryID database for each of these objects, limited confidence can be placed in these findings, even though there is considered to be sufficient reference representation for the three countries (Mozambique, Malawi and Zambia) in this database [29]. Therefore, geographic inference for these specific ivory objects in this study is not sufficiently robust to inform a criminal investigation.

4.2. Supported geographic assignments

Geographic distributions of both objects (IV002 and IV015) grouped with haplotype LL068 (mtDNA subclade east central) were supported by IvoryID geographic assignments (Democratic Republic of the Congo close to the east and north eastern borders) with good fits such that the outputs of both techniques were corroborated (Table 3). One of the two objects was classified as antique and the other as modern, which could indicate a successful maternal lineage expected of a natural long-lived elephant matriarchal population. However, unknown parts of elephant tusks were used to carve all the objects in this study, and so the estimated age ranges for objects within haplotypes could overlap within an elephant's life span. The latest year of formation for the antique ivory object (IV002) is 1930, compared to 1957 as the earliest formation year for the modern object (IV015) - although probability favours 2007−2009 as the date of formation (Table 1). Interpretation of these findings, along with the proximity of the geographic assignments, mean we are unable to rule out that both ivory objects could have originated from opposing ends of the same tusk. To resolve this, individualisation could be achieved through microsatellite DNA analysis [19] which, if two individuals were identified, could also indicate separate poaching events in this population.

Other considerations must also be taken into account with 'unknown' ivory objects, created from unknown parts of a tusk, as only a narrow location within the elephant's range during its lifetime can be inferred. It is not possible to ascertain a date of death nor assess the intra-specific isotopic variation from part-worked or carved ivory objects, such as those analysed in this study. This would be possible from analysis of whole tusks [24] where multiple samples could indicate dietary changes, thus changing the isotopic fingerprint detected, and

might provide additional supporting provenance information within an individual's range [27]. This would in turn provide additional detail to investigations of seizures involving whole tusks, although these are rare in the UK.

4.3. Implications of georeferenced sample representation on databases

Specific origins suggested for three objects matching LL003 (Botswana/Zimbabwe/Namibia) and two objects matching LL085 (Northern Zimbabwe/Botswana) did not precisely correlate with the isotopic assignments of Southern Zambia and Mozambique respectively. However, these are neighbouring countries and fall regionally close to the distribution of the mtDNA south central subclade, to which they were assigned [23]. Such information may assist an investigation if specific case circumstances or background information was available for the ivory objects or if only regional assignment was required. Zhao *et al.* [21] discuss their examination of four sequences originating from Zambian elephants in a 2018 study [42] using the Loxodonta Localizer. Interestingly one of these sequences matched haplotype LL003 (accession number MF062115 [42]), which if included in the Loxodonta Localizer database would then correlate with the isotopic findings for the objects grouped with LL003. It follows that further testing of elephants in these regions (particularly Zambia and Mozambique), which currently have a single recorded sample each on the Loxodonta Localizer database [21] (compared with 147 and 38 samples on the IvoryID database respectively [29]) may help increase the geographical resolution for these objects.

This is highlighted further through the results from object IV006; assigned to the north central mtDNA subclade (whose distribution includes Cameroon, Central African Republic, Republic of the Congo and the Democratic Republic of the Congo [23]) and was isotopically assigned to Zambia with a good fit. One of the sequences generated from the Zambian elephants in the 2018 study [42] (accession number MF062109) would be assigned to the north central mtDNA subclade using the identification key [23]. This would extend the distribution of this mtDNA subclade to include Zambia and in turn would substantiate the geographic provenance output provided by isotope analysis for IV006.

Recently, results from extensive sampling of African elephants from Tanzania were published, which led to 26 novel mtDNA sequences being obtained [43]. One of these matched the sequence obtained from object IV040 in this study (Supplementary Materials, Table 1). Not only does this provide a potential geographic origin for object IV040 but it was also assigned to the southeast savanna mtDNA subclade, whose geographic distribution includes Tanzania [23]. Further, given this sequence has not previously been seen in the neighbouring countries which have been extensively sampled, it could be that this is a localised haplotype. Additional sampling would assist in determining the geographic distribution of this novel haplotype.

The geographic assignment, following analysis of object IV004 matching LL071 (Uganda and Kenya), does not correlate with the IvoryID suggestion of southern Zambia (with good fit). IV004 falls in the northern savanna mtDNA subclade, within which is a single reference sample ascribed to neighbouring northern Angola [23]. It is therefore feasible that elephants belonging to the LL071 haplotype are also present within Zambia, but a representative reference individual has not yet been analysed. In this case, the use of another technique, for example microsatellite analysis [19,20], could assist in resolving the provenance of this ivory object.

It follows that the geographic disparity between the findings for LL003, LL085, IV006, LL071 is likely to be due to reference sample deficiencies and under representation on the respective databases, rather than genuine discordance. These findings also demonstrate the benefits of using multiple data sources, and analysis techniques for geographic assignments to minimise the effects of any data gaps present in existing databases.

Zhao *et al.* [21], Ishida *et al.* [23] and Ziegler [29] report difficulties obtaining adequate sample numbers from every country. Disproportionate sampling between elephants from more accessible locations, and locations where individuals are harder to detect, such as densely forested regions, was also noted [21,23]. Another explanation could be that there are greatly diminished numbers of individuals carrying a particular haplotype, for example, as a result of historic poaching events [19,44,45]. Okello *et al.* [44] observed a reduced genetic diversity after a large poaching incident, followed by slow reproductive recovery of the herd. A reduced number of individuals carrying a particular haplotype decreases the chance of that haplotype being sampled and subsequently included on a reference database. It is also possible that old mitochondrial haplotypes could have since been lost from current African elephant populations altogether. A recent investigation into ivory recovered from a sixteenth century shipwreck highlighted that only four out of a possible seventeen haplotypes generated had been reported amongst contemporary African elephant populations [45].

For any database, representative numbers of samples, good quality data and reliable results are essential [46–48]. With the Loxodonta Localizer and IvoryID databases specifically, there is an added dimension of sampling from a vast geographic range which, in reality, is difficult to achieve. However, an understanding of these limitations, alongside international data sharing and continued collaboration between researchers [8,48] means that these valuable resources can continue to develop and strengthen.

4.4. Discussion of findings from the ivory surrender and airport seizure

Overall, results obtained from the amnesty objects showed the greatest proportion of unique haplotypes, differences in age estimates and isotope ranges spanning both forest, mixed and savanna habitats. Given the likely random nature of acquisition (such as inheritance or one-off purchases) and the subsequent voluntary surrender of these objects, these findings are not unexpected. This study provides a snapshot of ivory objects currently present in the UK, including both antique elephant ivory and ivory objects from other species and may suggest that some owners are unaware of the true identity of ivory objects in their possession. However, with no background information regarding these objects being available, we are unable to comment on whether this ivory had been marketed fraudulently or how any of the modern ivory objects were procured.

With regard to the ivory objects seized at Heathrow Airport in 2017, all fifteen objects tested were modern. Cerling *et al.* [11] found that across 14 seizures, and the analysis of 231 ivory specimens, only one sample had a lag time (time between estimated death and seizure by law enforcement officials) of more than 6 years. Therefore, given the ivory analysed in this study was seized in 2017, it is unlikely to have been stockpiled from the estimated earlier date ranges (1955–1958). However, consideration must be given to these dates being a period in time during which the individual elephant was alive (rather than an estimated date of death), as the ivory objects were carved from unknown sections of the tusk. Further, isotope ratio ranges obtained were indicative of generally mixed habitats.

All mtDNA sequences generated from the seized ivory objects matched existing haplotypes recorded on the Loxodonta Localizer database, most with regionally restricted distributions which had a degree of overlap. Perhaps of concern is the inclusion of the range states, Botswana, Zimbabwe and Namibia, whose elephant populations were down-listed to CITES Appendix II in 1997, as the inferred geographic provenance for these objects using the Loxodonta Localizer database, as well as their neighbouring countries of Zambia and Mozambique as the suggested geographic origins by the IvoryID database. This regionalisation could indicate that elephant populations in this area are being targeted by poachers as a result of the legislative changes, a concern also raised by others [49,50]. Interestingly, this ivory seizure was suspected to have originated from Angola and/or the Democratic Republic of the

Congo, and the latter does fall within the assigned mtDNA subclade distributions. It is important to note that the Loxodonta Localizer database contains reference data from 202 samples from the Democratic Republic of Congo but is sample deficient for Angola [21]. Similarly, the IvoryID database contains reference data for 83 samples from the Democratic Republic of Congo and only 5 from Angola [29]. Additional georeferenced samples, particularly from Angola, would be required to assess this country as a potential geographic origin for these seized objects. Currently, our results from the Loxodonta Localizer database and stable isotope analysis using the IvoryID database do not support the Democratic Republic of the Congo as the origin of these objects. Increasing database size, as well as the application of additional techniques would assist in resolving their geographic origins with more confidence.

5. Conclusion

This study has demonstrated the utility of a combined approach to scientific testing to maximise the information that can assist in ivory seizure investigations. Validated molecular methods targeting mitochondrial DNA provided reliable and robust species identification which is necessary to determine whether an offence has taken place. Identified African elephant ivory can be subsequently analysed using peer-reviewed open access databases for both mtDNA sequences and stable isotope ratios to provide information on geographic provenance. This study also shows the potential for use of stable isotopes where mitochondrial DNA provides a widespread geographic distribution.

The combined geographic provenance results can provide useful and timely information to investigations, and with background information could assist in identifying probable geographic ranges of targeted elephant populations. These databases highlight the value of continuing collaboration between researchers and international cooperation with regard to data sharing and sample acquisition. This study emphasises the importance of obtaining further georeferenced African elephant samples particularly from countries or populations deemed data deficient for inclusion on both databases.

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Authors' contributions

Catherine Hale: Conceptualisation, Validation, Formal Analysis, Investigation, Writing – Original Draft, Visualisation.

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Lucy M. I. Webster: Conceptualisation, Validation, Investigation, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

Lucy Webster and Rob Ogden are guest editors of this journal special issue, but they did not handle this paper and were not involved in its peer review.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsiae.2021.100027.

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