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Research Article

Ivory species identification using electrophoresis-based techniques

Despite continuous conservation efforts by national and international organizations, the populations of the three extant elephant species are still dramatically declining due to the illegal trade in ivory leading to the killing of elephants. A requirement to aid investigations and prosecutions is the accurate identification of the elephant species from which the ivory was removed. We report on the development of the first fully validated multiplex PCR-electrophoresis assay for ivory DNA analysis that can be used as a screening or confirmatory test. SNPs from the NADH dehydrogenase 5 and cytochrome b gene loci were identified and used in the development of the assay. The three extant elephant species could be identified based on three peaks/bands. *Elephas maximus* exhibited two distinct PCR fragments at approximate 129 and 381 bp; *Loxodonta cyclotis* showed two PCR fragments at 89 and 129 bp; and *Loxodonta africana* showed a single fragment of 129 bp. The assay correctly identified the elephant species using all 113 ivory and blood samples used in this report. We also report on the high sensitivity and specificity of the assay. All single-blinded samples were correctly classified, which demonstrated the assay's ability to be used for real casework. In addition, the assay could be used in conjunction with the technique of direct amplification. We propose that the test will benefit wildlife forensic laboratories and aid in the transition to the criminal justice system.

Keywords:
Electrophoresis / Elephant species / Identification / Ivory / Multiplex
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1 Introduction

Elephants are the largest extant land mammal and have a long history of association with people in various aspects of religion, culture, and economy. They are recognized currently as three distinct species: the Asian elephant (*Elephas maximus*) distributed around Asia; the African elephant (*Loxodonta africana*) inhabiting savannahs, and the forest dwelling African elephant (*Loxodonta cyclotis*) distributed around Eastern, Southern, and West Africa [1,2]. For the past few decades, wild elephant populations have plummeted dramatically and only approximately 40 000 Asian elephants and 500 000 African elephants remain in the wild [3,4]. The tremendous decline in number of these species is mainly due to extreme poaching and the illegal trade of ivory. Recently, Thailand has become one of the largest and most active countries in the world for illegal ivory trafficking, as trading of domestic elephant ivory is currently authorized in Thailand. Consequently, an increasing number of wild elephants in Thailand are poached for their ivory but a substantial quantity of African ivory is laundered labeled as taken from a dead domestic elephant. To prevent the ultimate extinction of elephants from the wild, all species are listed currently on Appendix I of the Convention on International Trade in Endangered Species of Flora and Fauna (CITES). This results in the prohibition of the international trade of elephants and their products by signatories to CITES. Confusingly *L. africana* is on Appendix II for some countries such as Botswana, Namibia, South Africa, and Zimbabwe. Further, ivory from other mammal species, such as hippopotamus, walrus, and narwhals, have less protection.

Forensic science plays an important role to determine if seized or processed ivory has been taken from a protected species such as elephants. A number of noninvasive methods have been proposed for the accurate identification of the elephant species from which the ivory samples originated. These include macroscopic identification, short-wave near-infrared, and Fourier transform Raman spectroscopy [5, 6]. Even though these methods are nondestructive, they provide both qualitative and quantitative data but have limitations. Reasons include an inability to identify elephant

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Abbreviations: **CITES**, Convention on International Trade in Endangered Species of Wild Fauna and Flora; **Cyt b**, cytochrome b; **ND5**, NADH dehydrogenase subunit 5

Colour Online: See the article online to view Figs. 1–3 in colour.

species from which the ivory originated; necessity of expensive equipment; known false positives and negatives results; and a lack of robustness for use with highly degraded or processed samples commonly found in forensic science. DNA-based approaches have therefore been developed as an alternative to overcome these drawbacks. These traditionally employ universal primers designed from mitochondrial cytochrome *b* (cyt *b*) gene [7, 8] or the mitochondrial control region [9, 10] as a DNA marker, complementing nested [8], or traditional PCR following by sequencing technique via capillary electrophoresis. However, these methods are time-consuming, require moderately long intact DNA, which is rarely obtained from processed or poor quality ivory, and cannot be used to analyze mixed DNA samples. Real-time PCR was also reported for elephant species identification [11]; however this study could not differentiate between the two extant African species and was based on a limited sample size.

In this study, we aim to develop the first fully functional multiplex PCR assay using elephant (any of the three elephant species) and elephant species-specific SNPs, species-specific primers, and an electrophoresis-based technique. The method proposed is rapid, simple, and can accurately identify authentic elephant ivory to species level. The method developed in this study will also be validated according to forensic standards such that it will be beneficial to analyze ivory casework samples in wildlife forensic laboratories worldwide.

2 Materials and methods

2.1 Sample collection

A total of 113 ivory samples were collected from the Department of National Parks wildlife forensic unit, Wildlife and Plant Conservation, Thailand. These comprised: 52 blood samples from Asian elephant (*Elephas maximus*); 49 ivory samples from African bush elephant (*Loxodonta africana*); and 12 ivory sections and products made from ivory from African forest species (*Loxodonta cyclotis*). Blood from an elephant (*Elephas maximus*) was collected by a veterinarian and kept in a vacutainer tube containing EDTA. Ivory samples were processed by incising the inside of a proximal-hollow ivory pulp cavity (if possible) or the cementum, into small pieces, using a sterile scalpel and bone scissors. The subsamples were then kept separately in a sterile plastic bag until further analysis.

2.2 DNA extraction

Tiny pieces of ivory samples were added to a 1.5 mL microcentrifuge tube, until no more than half full, and 700 μL of 0.5 M EDTA was added. The solution was left for 7 days or until the samples softened. The softened ivory samples were then transferred to a new 1.5 mL microcentrifuge tube and 20 μL of proteinase K (10 mg/mL) was added. The solution was incubated at 56°C for 24 hours after which it was centrifuged at 11 000 rpm for 1 min. The supernatant was then used for DNA extraction using the Favorgen Stool Kit (Favorgen Biotech Corporation, Taiwan). DNA was isolated from the blood sample using the QIAamp DNA Mini Kit (Qiagen, UK) following the manufacturer's protocol for Blood and Tissue. The DNA extracts were stored at –20°C until further analysis.

2.3 Elephant and elephant species-specific SNPs identification

To identify elephant species-specific SNPs, all elephant cyt *b* and NADH dehydrogenase 5 (ND5) DNA sequences available currently on GenBank were downloaded. A total number of 1403 sequences were obtained comprised of 483 from *E. maximus*, 779 from *L. Africana*, and 141 from *L. cyclotis*. These DNA sequences were then aligned using the program Mega 5 [12]. From these sequences, 14 (one from *E. maximus*, 11 from *L. Africana*, and two from *L. cyclotis*) were found to contain several apparent DNA sequence anomalies and were then removed from the analysis. The remaining 1389 elephant sequences were used in this study. The SNPs or nucleotide bases that are specific only to one of the three elephant species were identified manually.

To identify elephant-specific SNPs, cyt *b* gene sequences from the three elephant species and 187 sequences from 124 mammalian species, spanning wide range taxonomic groups, were aligned using the program Genieous. SNPs that were specific to all elephant species and not present in any other mammalian species were identified.

2.4 Primer design

Elephant (all three species) and elephant species-specific primers were designed by incorporating the newly discovered elephant- and elephant species-specific SNPs at the last base of each 3' primer. The candidate primers were checked for their physical parameters such as annealing temperature (Tm), GC content, primer length, and secondary structures using the bioinformatics web-based tool called Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). The two forward primers (IDT, Singapore) were labeled with the dye 6-FAM at the 5/ end. These primers were LC_ND5_F: 6-FAM-TGGAGCCAGAGGAGAGAAATAA; LC_ND5_R: TAGTAACCATCGGCATTAATCAAC; Elephatid_cytb_R: TGGCTRAGAGGTCGGAG; Elephatid_cytb_F: 6-FAM-CTTAC GCCATTCTACGATCTGTA; and EM_cytb_R: CTCTTCCCTGAATACCCTTAGAAAG. Figure 1 shows the positions of the primers and the expected products from the three elephant species.

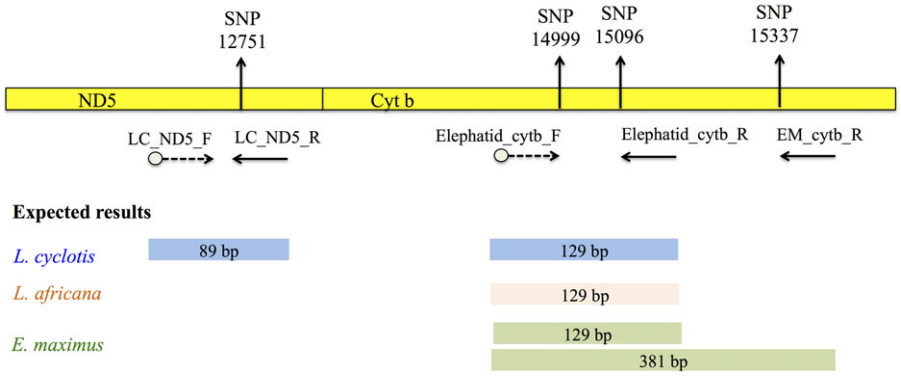

Figure 1. A schematic diagram showing the positions of the five primers and four SNPs used in this study. Two forward primers (dashed lines) are labeled with 6-FAM dye (o). In combination with three reverse primers (solid lines), three PCR products are generated: elephantid-specific product (129 bp), *L. cyclotis* specific product (89 bp), and *E. maximus*-specific product (381 bp).

2.5 PCR amplification

Both singleplex and multiplex PCRs were performed in a total volume of 20 μL, containing 10 μL of Phire® Animal Tissue PCR Buffer (includes deoxynucleoside triphosphates and $MgCl_2$), 1.0 unit Phire® Hot Start II DNA Polymerase, three primer pairs at the optimal concentrations (LC_ND5_F: 0.385 μM, LC_ND5_R: 0.385 μM, Elephatid_cytb_R: 0.1 μM, Elephatid_cytb_F: 0.7 μM, and EM_cytb_R: 0.7 μM), and 1 μL DNA template (ranging from 2.0 to 0.0625 ng/μL). The PCRs were conducted using a T100 Bio-Rad thermal cycler. Amplification conditions were as follows: initial denaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 5 s, annealing and extension at 66°C for 25 s; and a final extension at 72°C for 1 min. The reactions were stored at 4°C until further analysis.

2.6 Direct PCR

The process of direct PCR was as Kitpipit [13]. Essentially, a scraping or fragment of ivory no more than 1 mm^2 was submerged in a 1.5 mL microcentrifuge tube with 20 μL of PBS and heated. The PCRs were conducted as described in Section 2.5.

2.7 DNA separation and detection

All PCR products were separated and detected using both gel and capillary electrophoresis. A 2% agarose gel was prepared on which the products plus a 100 bp DNA ladder (Bio-Rad, California, USA) were separated. Electrophoresis was performed at 100 V for 30 min. The gel was visualized using Gel Doc™ EZ System (Biorad, USA). For capillary electrophoresis, mixture of 0.1 μL PCR product, 13 μL Hi-Di™ Formamide, and 0.2 μL of GeneScan-500 LIZ size standard (Applied Biosystems) was prepared and separated on a 310 Genetic Analyzer (Applied Biosystems) using a 36-cm capillary and POP-4-polymer. The mixture was heated at 95° for 5 min and then cooled on ice for 5 min before it was loaded to the instrument. The parameters used for separation on the 310 included: oven temperature at 60°C, filter set F, injection time of 5 s, injection voltage of 15 kV and a run time of 28 min.

All raw data were analyzed by Genemapper version 3.2. Alleles were scored based on the size marker with the number in base pairs (bp) based on the nearest whole base. The height of each allele was recorded based on its relative fluorescent unit.

2.8 Assay validation

The accuracy of the test was determined by analyzing 113 samples from the three elephant species to confirm correct identification.

The LOD, being the sensitivity of the assay, used serial dilutions of DNA. Samples of DNA from the three elephant species were quantified by Nanadrop 2000c (Thermoscientific, USA) from which six DNA concentrations (2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 ng/μL) were prepared using a twofold serial dilution. Measurements were taken using 260 nM.

Twenty-seven other mammalian species commonly encountered in wildlife forensic investigations were analyzed as part of the specificity test. These DNA samples were from tiger (*Panthera* tigris), white rhino (*Ceratotherium simum*), cow (*Bos taurus*), human (*Homo sapiens*), bison (*Bison bison*), horse (*Equus caballus*), banteng (*Bos javanicus*), camel (*Camelus sp.*), Eld's deer (*Rucervus eldii*), large indian civet (*Viverra zibetha*), Pig-tailed macaque (*Macaca nemestrina*), leopard (*Panthera pardus*), slow loris (*Nycticebus coucang*), Malayan sun bear (*Helarctos malayanus*), lion (*Panthera leo*), rabbit (*Lepus peguensis*), white-handed gibbon (*Hylobates lar*), chimpanzee (*Pan Troglodytes*), Asiantic black bear (*Ursus thibetanus*), Dusky leaf langur (*Tachypithecus obscura*), hog deer (*Axis porcinus*), orang utan (*Pongo pygmaeus*), malayan tapir (*Tapirus indicus*), sambar deer (*Cervus unicolor*), Indian hog deer (*Axis porcinus*), wild boar (*Sus scrofa*), and bitorong (*Arctictis binturong*).

Ten out of 140 DNA extracts from the elephant and other mammalian biological samples were selected at random to perform single blind testing.

Evaluation of the possibility of using direct amplification was tested using 20 confiscated ivory samples were analyzed with the developed assay using a conventional DNA extraction

Table 1. Elephant species and Elephatid-specific SNPs used for primer design and assay development in this study

Species	SNP 12751		SNP 15337		SNP 14999		SNP 15096	
E. maximus	C	(201)	**C**	(249)	A	(19)	C	(18)
L. africana	C	(551)	T	(643)	A	(682)	C	(683)
L. cyclotis	**T**	(96)	T	(113)	A	(135)	C	(135)
Other 147 mammalian species					**C/T**	(99/37)	**T**	(136)

The number in brackets is the number of the GenBank sequences shared for each species at this base. The position was determined by aligning the sequences with the *E. maximus* sequences accession number DQ316068.

and direct amplification following protocol of Kitpipit et al. [13].

3 Results

3.1 The informative SNPs for elephant and elephant species identification

Four informative SNPs were identified from cyt *b* and ND5 gene sequences and used for primer design and multiplex assay development. A SNP at the position 12751 was identified as specific to *L. cyclotis* and a SNP at 15337 was identified as specific to *E. maximus*; both of which are transition. SNPs at the position 14 999 and 15 096 were specific to all the three elephant species but different from other 124 mammalian species. None of SNP was found to specific to *L. africana*. Details of the SNPs used in this study are shown in Table 1.

3.2 Assay development and analysis result

A multiplex PCR-electrophoresis based assays for elephant species identification was successfully developed in this study. The assay was compatible with both simple agarose gel and high-resolution capillary electrophoresis so it can be used as a screening and confirmatory test. Using 1.0 ng DNA input, both analyses showed that a DNA sample from all three elephant species (*Elephas maximus*, *Loxodonta africana*, and *Loxodonta cyclotis*) was clearly identified. Peak/band patterns observed in the electropherogram and agarose gel are concordant (see Fig. 2), in which *Elephas maximus* showed

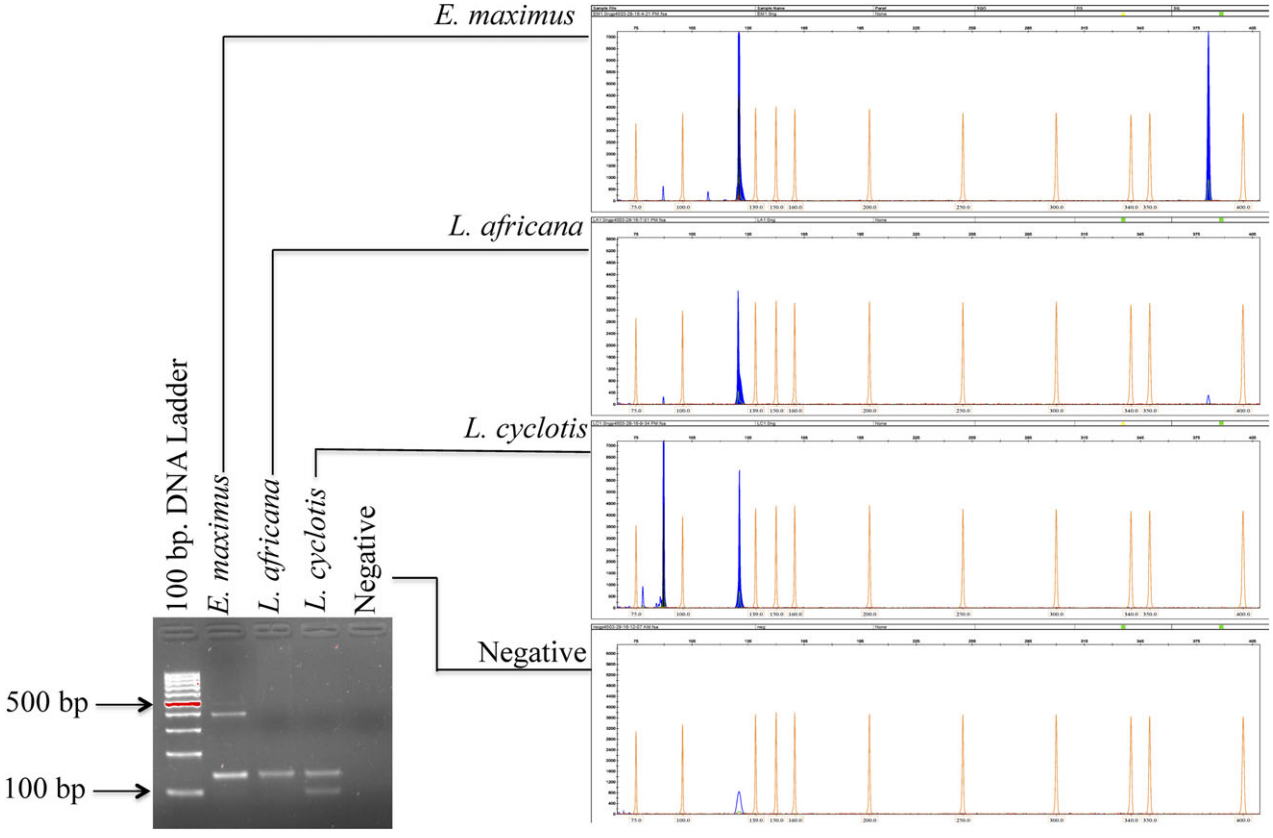

Figure 2. Agarose gel and CE analysis result of the DNA from three elephant species using the developed assay.

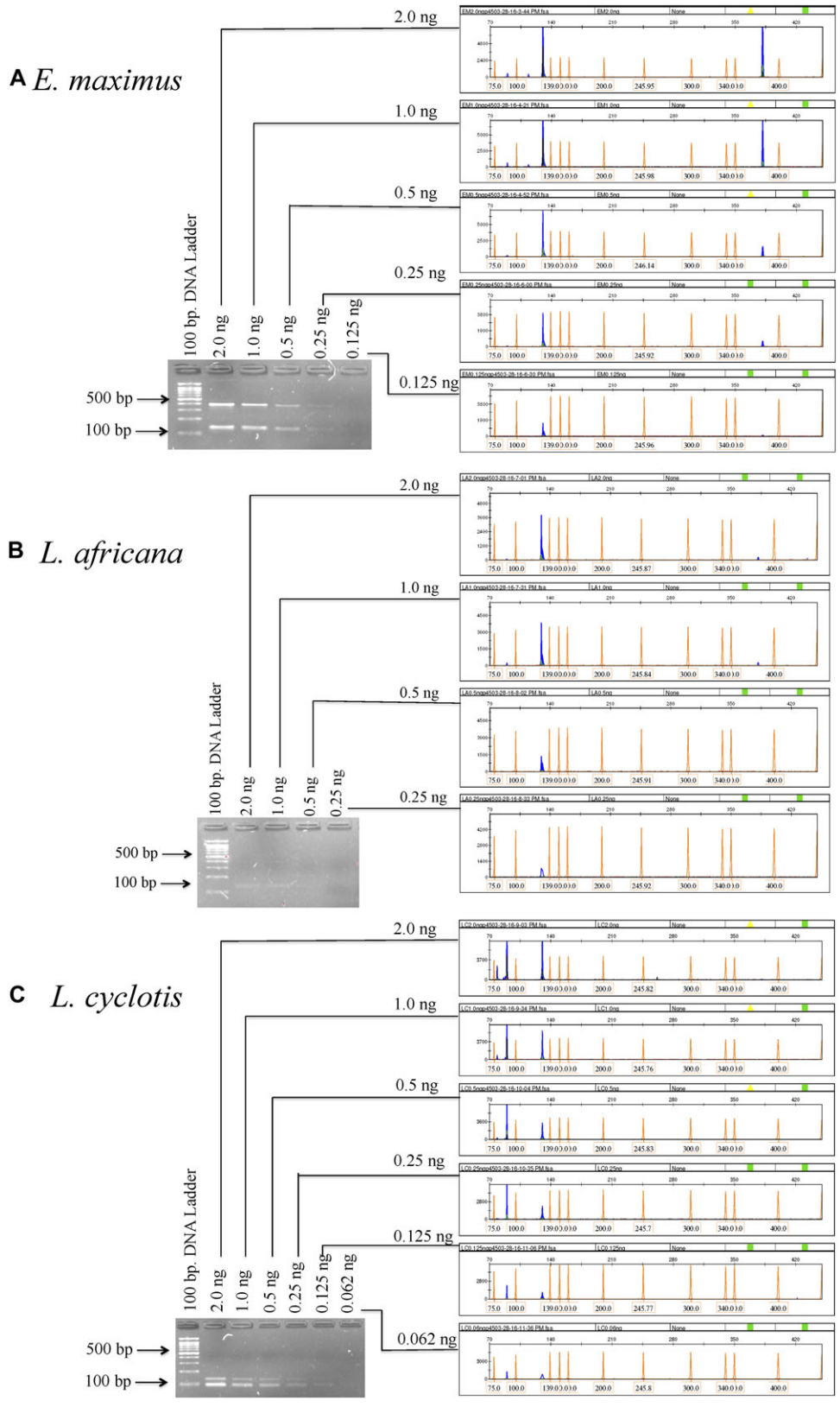

Figure 3. Agarose and CE results showing sensitivity of the test using six different amounts of DNA (2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 ng) from the three elephant species.

two distinct PCR fragments at approximate 129 (Elephatid-specific allele) and 381 bp (*E. maximus*-specific allele) in size; *Loxodonta cyclotis* showed two PCR fragments at 89 (*L. cyclotis*-specific allele) and 129 bp (Elephatid-specific allele) in size; and *Loxodonta africana* showed a single fragment of 129 bp (Elephatid-specific allele) in size. No PCR fragment was observed from any negative control, indicating no DNA contamination was noted. Dye artefacts at an approximate size of 128 bp were observed in all samples, corresponding to previously reported for 6-FAM dye blob [14].

3.3 Validation of the assay–-sensitivity test and impact of DNA input

The minimum and optimum amount of elephant DNA that could be detected by the developed assay was determined using six DNA concentrations from the three elephant species prepared by twofold serial dilution from 2.0–0.625 ng. The effect of reducing the amount DNA template is shown in Fig. 3. A limit of detection was observed with the minimum mass of DNA providing all corresponding or expected bands/peaks was 0.25 ng for *E. maximus*, 0.50 ng for *L. africana*, and 0.125 ng for *L. cyclotis*. Increased DNA input (higher than 0.5 ng DNA for *E. maximus* and 1.0 ng *L. africana*) resulted in one or more nonspecific amplification products but at a very low intensity or peak height. The optimal DNA input recommended for the developed assay was therefore in the range of 0.5–1.0 ng.

3.4 Validation of the assay: Reproducibility

Reproducibility was assessed using 113 known ivory samples (52 from *Elephas maximus*, 49 from *Loxodonta africana*, and 12 *Loxodonta cyclotis*). Both gel and electropherogram results showed that 100 out of 113 sample were successfully amplified and provided the distinct expected fragment(s), representing a success rate of 88.49%. All 100 of these samples generated the expected peaks using an in-put of 1.0 ng DNA. *E. maximus* showed two distinctive fragments at an average peak size of 129.90 ± 0.20 and 381.22 ± 0.18; *L. africana* showed one distinctive fragment at an average peak size of 129.60 ± 0.10; *L. cyclotis* showed two distinctive fragments at an average peak size of 89.76 ± 0.14 and 130.16 ± 0.09. Although nontarget band/peaks for *E. maximus* and *L. cyclotis* were observed in most samples, these were a very small peak and presented less than 10% of the main Elephatid-specific peak height (Table 2). A threshold of 10% could be considered as the cut-off value to exclude nontarget peaks. By using this criterion, all 100 samples were corrected identified and provided a 100% accuracy in identification, indicating that the assay is practical to analyze real-case samples and provide confidential result.

3.5 Validation of the assay: Specificity test

The three elephant and other 27 mammalian species commonly found in the illegal trade of wildlife products were

Table 2. Showing reproducibility of the test based on113 ivory samples

Elephant species	Success rate	*L. cyclotis*-specific peak						Elephantid-specific peak				*E. maximus*-specific peak					
		Size (bp)		Peak height (RFU)		Ratio		Size (bp)		Peak height (RFU)		Size (bp)		Peak height (RFU)		Ratio	
		Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
E. maximus ($n = 52$)	98%	89.45	0.19	371.27	172.66	6%	2%	129.9	0.2	5740.92	2009.37	381.22	0.18	3148.98	2189.15	51%	29%
L. africana ($n = 49$)	84%	89.71	0.14	147.51	125.01	5%	2%	129.6	0.1	2944.15	1833.9	381.25	0.35	205.05	169.15	6%	2%
L. cyclotis ($n = 12$)	83%	89.76	0.14	2763.70	2941.92	146%	41%	130.16	0.09	3643	2939.03	—	—	—	—	—	—

Bp indicates the size in base pairs and RFU is the relative fluorescent unit for each SNP.

tested for determination of assay's specificity. According to both gel and CE results, the expected fragments were generated only from elephant species. None of expected band/peaks was observed in any of the 27 non-Elephatid species, supporting high specificity of the assay to elephant species. Importantly, no products were observed using human DNA as ivory samples will likely be contaminated on the outer surface from humans.

3.6 Validation of the assay using direct amplification

Twenty confiscated ivory samples were analyzed with the developed assay following two methods: conventional DNA extraction and direct amplification. The results showed that the amplification success rate for DNA extraction and direct amplification method was 90 and 85%, respectively. Three samples were amplifiable with conventional PCR while they failed with direct PCR. Conversely, two were amplifiable with direct PCR but failed with conventional PCR. However, samples that were amplifiable with both techniques gave an identical species identification.

3.7 Blind trial testing

Random single blind testing with ten ivory samples was performed to determine the effectiveness of the developed assay. The results showed that all samples were successfully and correctly identified as originating from an elephant species. Also, non-Elephatid samples were excluded using the developed assay.

4 Discussion

Accurate identification of confiscated ivory is a powerful tool combating the illegal trade in ivory. Amplification of a mitochondrial DNA locus followed by DNA sequencing of the amplicon is standard practice in such species testing. This is laborious and leads to uninterpretable data if mixed with human DNA. To overcome these drawbacks we report on a fully functional multiplex PCR-based assay for both screening and confirmation for the presence of elephant DNA from ivory samples. The assay requires five primers, only standard equipment found in forensic laboratory, and does not require sequencing of the DNA. Only a few guidelines are recommended to obtain an accurate identification. Template DNA should be between 0.5 and 1.0 ng DNA and any peak less than 10% of the main Elephatid-specific peak height should be considered as unreliable. Analysis is straightforward as the assay classifies three elephant species by peak/band number and fragment sizes.

Development of the multiplex-electrophoresis based assay relied on accurate sequence alignment leading to high-specific SNPs, primer-specificity, and multiplex optimization with primers of similar melting temperatures. The SNPs were selected from the mitochondrial cyt *b* gene as there is maximum coverage in GenBank at this locus [15–18]. ND5 was also included as this locus is adjacent to cyt *b* and known to contain sequence variants [19, 20]. In addition, all sequences were checked for their accuracy and ambiguous sequences were removed from the analysis. Three (position 12 751, 15 096, and 15337) out of four SNPs used in this study are transitions [21]. The SNP at position 12 751, and 15337 are specific to *L. cyclotis*, and *E. maximus*, respectively. No SNP was specific to *L. africana*, indicating low genetic variation in this species; this result corresponds with the previous reports [8, 22].

Five primers used for assay development were designed based on the above four species-specific SNPs, making them specific to the target species. Both forward and reverse primer for Elephatid identification were designed from Elephatid-specific SNPs (position 14 999 and 15 096), the result of which is high specificity to only elephant species and its ability to separate Elephatid from the other 26 commonly traded wildlife species and humans.

The assay was fully validated for its accuracy, specificity, sensitivity, and blind trail testing, according to recommendation on the use of nonhuman DNA in forensic investigation [23]. The assays were found to be reproducible and provided 100% accuracy in identification of all elephant species and for both ivory and blood samples. The assay required no nested amplification but still provided a success rate as high as 88.49%; this is even higher than that (84.33%) reported by Lee et al. [8] who proposed a nested amplification protocol for ivory identification. The assay can be used to detect a very low amount of unknown elephant DNA; as low as 0.25 ng. This mass is lower than the detection limit of the conventional PCR-sequencing technique.

Direct amplification proved to be efficiently used with the developed assay. The success could be due to several factors such as heating the samples, the use of dilution buffers (i.e. PBS), inhibitor-resistant DNA polymerase, and PCR buffer; these helps to lyse cells, release DNA from ivory, all of which minimize inhibition of the PCR [13]. Only a very small amount of ivory powder is required to obtain a successful amplification (such as a tiny scraping that sits under the 20 μL PBS), being beneficial for processed or degraded ivory and also tiny invasive samples [24]. The process of direct PCR required no DNA extraction step, making this protocol cost-effective and less time consuming, compared to other previous reports [25]. As the process required very little template it is possible to attempt direct-PCR first and, if this is unsuccessful, then the extraction process can be attempted.

In conclusion the multiplex-electrophoresis based assay was successfully developed and validated in this study. It is accurate, reproducible, and highly specific to elephant species. The advantages of our developed assay bring several benefits against the traditional PCR-sequencing technique. Firstly, the assay could selectively generate data from mixed elephant species or human-elephant DNA mixture. Secondly, the assay could be used as both a rapid screening tool and a highly sensitive confirmatory test. Thirdly, presences of ambiguous base, heteroplasmy, or DNA quality will not interfere with any

interpretation. The assay may prove useful not only for forensic purpose but also for ecology and conservation of these species.

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The authors have declared no conflict of interest.

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