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TECHNICAL NOTE

The selection of a standard STR panel for DNA profiling of the African elephant (*Loxodonta africana*) in Kenya

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Abstract The African elephant (*Loxodonta africana*) population in Kenya is declining at an alarming rate due to habitat destruction, human-elephant conflicts and the current escalation of poaching for ivory. This study established a standard protocol for forensic analysis of *L. africana* and their products such as ivory. Three multiplex PCR panels consisting of 17 Short Tandem Repeat markers were selected from 40 markers using bioinformatics tools, amplification and polymorphism. Genotyping was successful and reproducible. This method is efficient, accurate and cost effective and has potential for application in conservation of *L. africana*.

Keywords Microsatellites · Standard panel · Forensic analysis · African elephants

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Currently there are around 0.5 million vulnerable African elephants with Kenya accounting for about 28,000 (IUCN 2013a, b). In Africa, approximately 25,000 and 22,000 elephants were illegally killed in 2011 and 2012 respectively (CITES 2013; Vira and Ewing 2014). Highly polymorphic microsatellite markers can provide a useful tool for traceability of elephant products to geographic origin (Wasser et al. 2006).

This study evaluated the suitability of 40 published STR markers (Comstock et al. 2000; Archie et al. 2003; Eggert et al. 2000; Nyakaana and Arctander 1998; Nyakaana et al. 2005) for forensic analysis. Initial selection criteria included the removal of 15 imperfect STR markers: FH102, LaT05, LaT07, LaT16, LaT17, LaT26, LA1, LA2, LA4, LafMS07, LafMS08, LafMS09, LafMS10, LafMS11 and LafMS01, to avoid the formation of variant alleles that have no equivalency between fragment length and amplicon sequence (Guichoux et al. 2011). In forensics, perfect motifs are preferred (Gusmao et al. 2006).

The primers were then assessed for hairpin formation and self-annealing using oligocalc® (Kibbe 2007). Primers from seven markers: FH1, FH65, FH40, LAT13, LAT18, LAT25 and LafMSO3 formed hairpins. Four markers were modified using oligocalc® while three markers: FH65, LAT18 and LafMSO3 were excluded because they were difficult to modify. Hairpin loops, if present can reduce the efficiency of the reaction by limiting primer ability to bind to the complementary fragment (Vinay et al. 2000). Primers from three markers: Lat 06, LafMSO4 and LafMSO5 had potential for self-annealing. Two were modified while one, LafMSO4, was excluded due to the position of the complementary base pairs. Details on modification of primers are provided as supplementary material, S1.

The primers' annealing temperature (Ta) was determined using oligocalc®. Primers with Ta higher than 60 °C

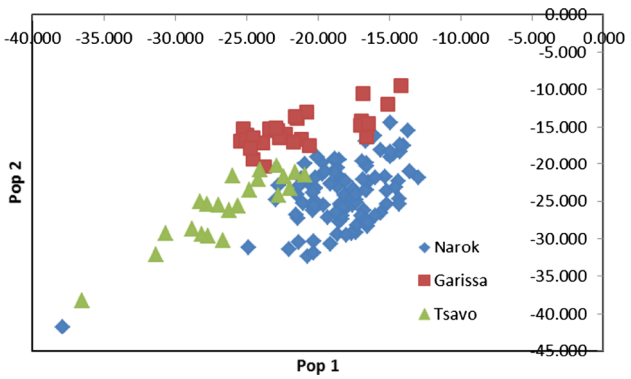

Fig. 1 Showing population assignments into self, closest or distant population, with log-likelihoods as negative values, the highest (least negative) value indicates the most likely population

were modified to standardize the Ta within a range of 5 °C. Three multiplex PCR panels of six, seven and seven primers pairs were designed based on Ta and expected fragment length. The primers were tested for cross annealing using vector NTI 11.1 (Invitrogen, CA, USA) and no cross-annealing occurred. GenBank BLAST searches revealed no alignment with other parts of the elephant genome.

Genomic DNA was extracted from 147 Kenyan elephant samples from Narok (98), Garissa (27) and Tsavo (22) using Qiagen kits. Amplification was first tested in single-plex gradient PCR and later in multiplex PCR. Two Ta of 55 and 58 °C were chosen as most primers amplified at those temperatures. Biometra PCR calculator (2007) software was used to calculate the quantities of primers, water, Taq DNA polymerase (Thermoscientific, 1U), reaction buffer (Thermoscientific, 1X), magnesium chloride (Thermoscientific, 2.0 mM) and dNTPs mixes (Thermoscientific, 0.2 mM) in each multiplex PCR reaction. The cycling parameters were: one cycle of enzyme activation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 or 55 °C (Table 1) for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 20 min. The success of the multiplex PCR was confirmed by comparing the results with the single-plex and pseudo multiplexed PCR products. Primer modification enhanced co-amplification of primers in the same reaction.

Specificity of the primers was tested by comparative analysis using samples from other species: common zebra, gnu, cow, grant gazelle, dik dik, giraffe, sheep, kongoni, thomson gazelle, goat, impala, lesser kudu, warthog and donkey and no amplification products were obtained indicating species specificity. Fidelity was high as the markers were found to amplify low quality DNA from dung and ivory although better amplification was observed from the biopsy tissue samples. Reproducibility was tested by repeat amplification and by use of different thermocyclers: GeneAmp® 9,700, GeneAmp® 2,720, veriti® (life technologies), Gene Pro (BIOER Technology) and results were similar for all these instruments.

Genotyping was done using a 3,730 Genetic Analyser ™ (Life Technologies) capillary electrophoresis system with three fluorescent labels (FAM, NED and VIC) and Genescan™ 500 LIZ™ standard. The data was analyzed using GeneMapper® version 4.1 (Life Technologies). The number of alleles per locus for the 147 samples ranged from 1 to 20. The markers: FH 94, LA3 and LafMSO5, had one or two alleles and were excluded from the test, leaving a final list of 17 STR markers: FH1, LA5, FH103, FH60, LaT24, FH39, LafMSO6, LA6, FH19, FH40, FH67, LafMSO2, FH48, LaT08, LaT06, LaT25 and LaT13 (S1). To test the ability of the proposed 17 STR markers in assigning confiscated ivory to source population, the method of Paetkau et al. (1995, 2004) was used. The results showed that 142 individuals (97 %) were correctly assigned into their populations, 4 individuals (2.7 %) were assigned to the closest neighboring population (Fig. 1) and one individual (0.7 %) assigned to a more distant population. Three test ivory samples, one from each population were correctly assigned into their source populations. The 17 STR markers could therefore reliably assign confiscated ivory to the source population in Kenya. We recommend the establishment of a DNA database of *L. africana,* with control DNA samples, to provide an opportunity for match determination of confiscated ivory.

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Supplementary material

Supplementary Table 1: Three PCR multiplex panels including the 17 microsatellite markers selected for the final test with dye label, primer sequence, concentration, Ta and length of primer and that of expected fragment.

Primer	Primer Sequence 5'-3'	Dye	Conc' (uM)	Fragment length	Ta °C	Na
Panel 1*						
FH1-F	GATCAGACCATGGCATGAG	VIC	0.2	80-90	58	6
FH1-R	ACAGTCTCCCTTGGGAAGA				58	
LA5-F	GGGCAGCCTCCTTGTTTT	NED	0.1	139-155	58	8
LA5-R	CTGCTTCTTTCATGCCAATG				58	
FH103-F	TGTGCTGCCACTTCCTACAC	6-FAM	0.2	147-157	58	6
FH103-R	GATGTTGAGACAGTTCTGTAAG				58	
FH60-F	CAAGAAGCTTTGGGATTGGG	VIC	0.05	143-163	58	7
FH60-R	CCTGCAGCTCAGAACACCTG				58	
LaT24-F	AAGTTGAGAGATCAGCAAAGCA	VIC	0.1	124-264	58	14
LaT24-R	GATGTTCAGTCCTTCCTTAGCA				58	
FH39-F	GTATTCCTGGGCATTCCATG	6-FAM	0.05	235-253	58	10
FH39-R	CTTGGAATATGACCCTGTTTG				58	
Panel 2*						
LafMS06-F	AGCTGTCCTAAGTCATAAATACACA	6-FAM	0.1	138-158	58	8
LafMS06-R	ACAGCCACTGAAACCCCATG				58	
LA6-F	AAAATTGACCCAACGGCTC	VIC	0.4	145-177	58	10
LA6-R	TCACGTAACCACTGCGCTA				58	
FH19-F	GAAGCTCATGGTCAAGGTCAC	6-FAM	0.2	185-207	58	9
FH19-R	CTGCATACTCATCGAAGTCACC				58	
FH40-F	GCTTTCTAGCCACCTCCTTC	VIC	0.2	209-263	58	14
FH40-R	GCTCACATTCACTTGCTGACC				58	
Panel 3*						
FH67-F	GCTTCTCTAGAAATGTGTATGC	NED	0.4	88-110	55	11
FH67-R	GGCGTATAGGATAGTTCCAC				55	
LafMS02-F	GAAACCACAACTTGAAGGG	NED	0.3	136-168	55	11
LafMS02-R	TCGCTTGTAAGAAGGCGTG				55	
FH48-F	GAGTCTCCATAATCAAGAGCG	NED	0.3	166-178	55	7
FH48-R	CCTCCCTGGAATCTGTACAG				55	
LaT08-F	ATGGACAGGCAGAAAGATTT	6-FAM	0.1	177-225	55	13
LaT08-R	TCCCAATAACAGGATAGCATT-				55	
LaT06-F	AGCCAGGCACATTAAGTGT	6-FAM	O.2	270-394	55	19
LaT06-R	CTCCTAGAAAAGGTTACCAC				55	
LaT25-F	GACCGTCTTCATGAGATG	VIC	0.2	291-331	55	10
LaT25-R	ATGCAAGCTTACAATGGCAG				55	
LaT13-F	AGCTTCTGTAGGCTCTGA	NED	0.3	216-272	55	9
LaT13-R	ACTCGATAAACAGTGTTGA				55	

*The number of multiplexed markers can be increased to end up with one or two panels by using four instead of the three fluorescent dyes used in this study. Na-Number of alleles per locus.

Supplementary material on modification of the primers

In Loci FH1, the reverse primer had a potential to form a hairpin,

5' CAGTCTCCCTTGGGAAGAC 3,' and this was corrected by removing the last base C resulting into ACAGTCTCCCTTGGGAAGA.

In Loci FH65, the forward primer had a potential to form hairpin,
5' GGCTGTAGCATTTTACACTCCC 3', but this was difficult to correct due to its position and the primer was then dropped.

In Loci FH40, the forward primer had a potential to form hairpin,
5' GGCTTTCTAGCCACCTCCTTC 3'. This was corrected by removing the first base G resulting into GCTTTCTAGCCACCTCCTTC.

In Loci LaT13, the forward primer had a potential to form hairpin,
5' TGAGCTTCTGTAGGCTCTGA 3', which was corrected by removing the first two bases TG resulting into AGCTTCTGTAGGCTCTGA. The reverse primer also had a potential to form hairpin, 5' GCACTCGATAAACAGTGTTGA 3', which was corrected by removing the first two bases GC resulting into ACTCGATAAACAGTGTTGA.

In Loci LaT18, the forward primer had potential of forming two hairpins,
5' AATCCAAGATTGGGCAACAC 3' and 5' AATCCAAGATTGGGCAACAC 3', which were difficult to correct hence it was dropped.

In Loci LaT25, the forward primer had potential of forming a hairpin,
5' TGAGACCGTCTTCATGAGATG 3,'which was corrected by removing the first three bases TGA resulting into GAC CGT CTT CAT GAG ATG

In Loci LafMS03 the forward and reverse primers both had potential of forming hairpins, 5' CATATGAACATACCGGAAC 3’ and 5'AAACTCCTCGAGTAGTAGAA 3,' which were difficult to correct and hence dropped from the marker set.

In locus Lat06, reverse primer, TCTCCTAGAAAAGGTTACCACA, had potential for self-annealing as shown below but it was corrected by removing the first base T and the last one, A, resulting into CTCCTAGAAAAGGTTACCAC

5' TCTCCTAGAAAAGGTTACCACA 3'

3' ACACCATTGGAAAAGATCCTCT 5'

5' TCTCCTAGAAAAGGTTACCACA 3

3' ACACCATTGGAAAAGATCCTCT 5’

In the locus LafMS04, both forward and reverse primers had potential for self-annealing as shown below hence dropped

5' GGGACACATGTGTGCATAA 3'

3' AATACGTGTGTACACAGGG 5'

5' GGGACACATGTGTGCATAA 3'

3' AATACGTGTGTACACAGGG 5'

5' TTATGTCTGCATAGACAGGTTGG 3'

3' GGTTGGACAGATACGTCTGTATT 5'

5' TTATGTCTGCATAGACAGGTTGG 3'

3' GGTTGGACAGATACGTCTGTATT 5'

5' TTATGTCTGCATAGACAGGTTGG 3'

3' GGTTGGACAGATACGTCTGTATT 5'

The reverse primer of Locus LafMS05, AATGGACTTGGGACTTGCCAAAATGT had a potential for self-annealing as shown below and this was corrected by removing the first three bases AAT and the last five bases AATGT resulting into GGACTTGGGACTTGCCAA.

5' AATGGACTTGGGACTTGCCAAAATGT 3'

3' TGTAAAACCGTTCAGGGTTCAGGTAA 5'

5' AATGGACTTGGGACTTGCCAAAATGT 3'

3' TGTAAAACCGTTCAGGGTTCAGGTAA 5'

The forward primer of microsatellite marker LA3, TACTCTGCTCCTCTGCCTATCC, had annealing temperature of 64.2^{o}C. This was reduced to 58.4^{o}C by modifying the primer by removing the last two bases CC. The reverse primer of microsatellite marker LafMS06, ACAGCCACTGAAACCCCATGGA, had annealing temperature of 64.2 ^{o}C. This was reduced to 60.5 ^{o}C by modifying the primer by removing the last two bases GA. After

modification of the primers, the forward and reverse primers of each primer pair had annealing temperatures within 5ºC of each other to avoid inefficient amplification.