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TECHNICAL NOTE

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A Simple and Inexpensive Molecular Method for Sexing and Identification of the Forensic Samples of Elephant Origin

ABSTRACT: The population of the Asian elephant is being dramatically reduced due to poaching of the ivory from the male. As poaching occurs in remote forests, it often takes weeks or longer for it to be discovered and it is therefore often very difficult to determine the sex of the decomposed body. Data suggest that in the recent past, over 2000 male elephants have been poached in South India. We have developed a technique based on molecular markers to determine that the carcass is an elephant and that it is a male. Using DNA sequence information from Genbank, we have developed two primer pairs: one for the mitochondrial DNA (mtDNA) and the other for the sex-determining region of Y chromosome (SRY) gene of the Indian elephant. After PCR amplification of known elephant DNA, we found that the mtDNA was common in both males and females, whereas the SRY-specific amplicon was observed only in the male.

KEYWORDS: forensic science, elephant, ivory, molecular markers, mitochondrial DNA, SRY, wildlife forensic

The Asian elephant is one of the endangered species, which was due to various reasons. In India, approximately 25,000 Asian elephants reside in various forests, elephant camps, and temples. Unlike the African elephant (the tusk is present in both sexes), the tusk is present only in the male of the Asian elephants. As male Asian elephants are being hunted for their ivory, the male population is drastically decreasing. More than 2000 tusker elephants have been poached in South India in the recent past (1). Hence, there is a grave need to protect male elephants to maintain the sex-ratio of the population, which is required for healthy and proper reproduction. As the poachers make use of the thick forest for their illegal activities, it would take a few weeks to several months before the poaching activity is known. Moreover, the testes of the male elephants are located inside the abdomen; therefore, it is difficult to find out whether the decomposed body is that of a male or a female. Hence, it would be difficult to trace the sex of the elephant in the confiscated cases. Therefore, there is a need for a technique, that would reveal the sex of the elephant unambiguously.

Sexing of human samples is mostly carried out with the amelogenin, which is present on both the X and the Y chromosome, using a single pair of primers spanning a conserved region within the first intron (2) and many other alternative primers (3–8). However, due to the absence of the Y chromosome-specific amelogenin in many fertile men, its use for identifying the sex of an individual is questionable (9–11). As the sex-determining region of the Y chromosome (SRY) is the male sex-determining gene, exclusively present on the Y chromosome and is highly conserved, this is the more appropriate marker for sex identification (10). This is true for almost all the mammals. For species identification, mitochondrial DNA (mtDNA) markers are very frequently used (12–14). In addition, the mtDNA markers were also used to trace the maternal side relatives and our evolutionary past. Among the mitochondrial markers, the D-loop region is being used in many cases, because of its hypervariable features (14). Although majority of the cases utilize the mtDNA D-loop, no studies have been conducted using the mtDNA in Indian/Asian elephants. Therefore, we have undertaken this study among Asian elephants using mitochondrial D-loop and SRY for unambiguous identification of elephants and their sex, which would be more useful in wildlife forensics.

Materials and Methods

A total of 18 elephants, housed in various zoological parks, were included in this study. Fifteen to 30 hair samples from the forehead of each animal (with intact root) were taken. In addition, four forensic case samples (bones and skin) were also included in this study.

Isolation of DNA

Hair roots were cut with a sterile surgical blade, the skin sample was chopped into fine pieces using a surgical blade, and the bone sample was powdered using an indigenous stainless-steel homogenizer. All the samples were washed twice with normal saline (0.89% w/v NaCl in double distilled water) and 500 mL of lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM EDTA (pH 8.0)), 50 mL of 20% (w/v) SDS, and 15 mL of protinase-K (20 mg/mL stock) was added, and incubated at 55°C for 8.0 h with gentle rotation in an hybridization oven. The homogenized solution was processed for DNA isolation using the phenol:chloroform method

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(15). DNA isolated from hairs, skin, and bones were further purified using the GeneClean Kit (Q-Biogene, Morgan Irvine, CA) for the removal of PCR inhibitory substances from DNA solution.

Duplex PCR Amplification

To amplify the D-loop and the SRY gene, we have developed two sets of primers, one of which is specific to an elephant's mitochondrial D-loop region and the primer sequences are as follows (derived from NCBI Accession No. NC_005129): EdlF 5′-GAGGCCCTAACACAGTCAAGCAAC-3′ (position 16026–16049) and EdlR 5′-CGTGTACGCTGGGAATTTAGGTT-3′ (position 16140–16162). Another set of primers was specific to the SRY gene of elephant and the primer sequences are as follows (derived from NCBI Accession No. AF180946): EsryF 5′-GGGATAC CAGTGGAAAATGCTTA-3′ (position 107–129) and EsryR 5′-GTTCGGGTATTTCTCTCGGTGCA-3′ (position 181–203).

Duplex PCR amplification was carried out in a 20 μL reaction volume containing 10–30 ng of template DNA (2 μL directly from the DNA of skin and bone), 150 μM each of dNTPs, 2.5 pmol of EdlF/R primer, 3 pmol of EsryF/R primer, 1.5 mM $MgCl_2$, 0.6 U of Taq Gold (Applied Biosystems, Foster City, CA) and 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl). The PCR cycling conditions of the above primers were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles each of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. The final extension was 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide (0.5 mg/mL), and observed under a UV transilluminator.

DNA Sequencing and Sequence Analyses

In order to sequence the mtDNA D-loop and the SRY gene, the amplicons were treated with exonuclease-1 and shrimp alkaline phosphatase (USB, Cleveland, OH) for 15 min each at 37°C and 80°C, respectively, to dephosphorylate and degrade any residual primer. The PCR amplicons containing mtDNA D-loop and SRY gene were sequenced using their respective primers, and an automated ABI 3700 DNA Analyzer (Applied Biosystems). Sequences were aligned using AutoAssembler software (Applied Biosystems). The sequence information has been deposited in the NCBI gene bank (D-loop—*DQ078275*; SRY—*DQ078276*). The D-loop sequences obtained from this study were compared with a large number of D-loop region sequences of various species using ClustalX (16).

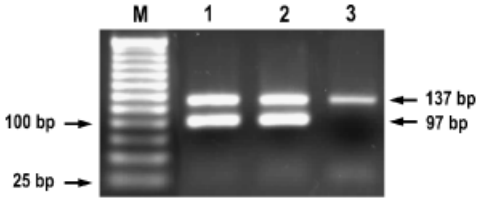

FIG. 1—*PCR amplification of elephant DNA; M: 25 bp DNA marker; lane 1 and 2: male elephant DNA; lane 3: female elephant DNA.*

Results and Discussion

The PCR amplicons generated using the SRY-specific primer pair EsryF/EsryR are 97 bp, and elephant-specific mtDNA D-loop primer pair EdlF/EdlR are 137 bp (Fig. 1). As the SRY is a male-specific DNA, the amplicons obtained from the male elephant yielded both the SRY and the mtDNA D-loop-specific amplicons (Fig. 1, Lanes 1 and 2), whereas the female elephant's DNA, which lack the SRY, gave only one amplicon, specific to the mitochondrial D-loop region (Fig. 1, Lane 3). mtDNA have been used earlier for the species identification of forensic samples (14).

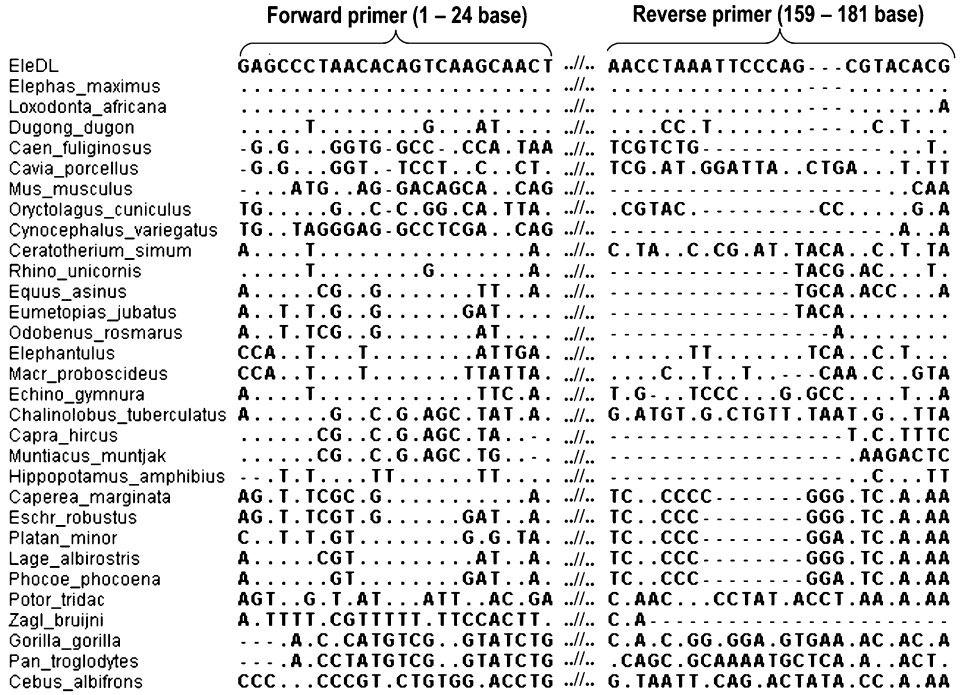

FIG. 2—*Mitochondrial DNA sequence alignment showing the sequence variation at primer site in various species. Note: Dot (·) showing the similarity of sequences; hyphen (-) showing the gaps in alignment.*

In humans, the amelogenin gene is routinely used for sexing of the forensic samples; however, recent findings have questioned the use of amelogenin for this purpose as the normal male also showed the absence of a male-specific amelogenin locus (9–11). Hence, the investigators have suggested the use of SRY for the sex identification (10). Therefore, the use of SRY in the present study for sexing elephants is more appropriate. To validate our result, we have analyzed the DNA of 18 known elephants (12 females and 6 males), which confirms the robustness of this technique. This technique has helped us in resolving some of the wildlife offences where Asian elephants have been involved.

Further, we have tested this technique on the DNA samples of humans, dog, deer, black buck, lion, leopard, tiger, mouse, porcupine, and dolphin to assess whether these markers are unique to the elephant species or whether they also detect the DNA of other species. Interestingly, amplification of the mtDNA D-loop was seen only with the elephant DNA; hence, this set of primers is useful for the unambiguous detection of the elephants. Further, we have carried out *in silico* PCR using the mtDNA sequence, obtained from the NCBI gene bank, of a vast number of animals from various species, which confirms the specificity of the mtDNA primers. In addition, we have aligned the mtDNA sequences of various species with the sequence generated using the primers designed in this study (EdlF/R) to check the sequence variation, if any, within the primer region. This analysis revealed that the primer regions were matching only with the African elephant, but not in other animal species (Fig. 2). Whereas, in the case of SRY, as it is highly conserved across the genera, we have observed a very poor amplification in other mammalian species (humans and dogs).

Conclusion

In conclusion, we have successfully developed two novel primer pairs and demonstrated that they are elephant (mtDNA) and male (SRY) specific. This technique is quick, simple, and inexpensive to identify the decomposed biological remnant of the elephant victim in wildlife crime(s). This technique is also useful for monitoring the ratio of male and female elephants in the forest range.

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