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Correlation of Plant Species from Botanical Evidences in Criminal Cases Using DNA Technique- A Case Study

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Abstract: *Botanical evidences play a significant role in criminal investigations. These evidences help in linking a victim or a suspect to the crime scene and eventually help in correlating them. In present study, it was correlated that the botanical evidences found at the crime scene with the victim or suspect, whether the place of murder and the place where the dead body was found are same or not, using RAPD-PCR technique. The DNA extracted from the botanical evidences using CTAB method and the RAPD-PCR profile of the standard and the suspected samples were then matched by using TotalLab softwares, TotalLab Quant and Phoretix 1D, data analysis report. Out of three cases, in two cases, separated RAPD-PCR products were matched with the standard and the suspected sample, while in one case RAPD-PCR profile of both samples did not matched.*
Keywords: *Botanical evidences, CTAB, RAPD-PCR, TotalLab.*

I. Introduction

Forensic Science is a field in which we never know what type of evidence will be encountered at the crime scene. Each case has its own uniqueness and individuality and so thus is true with the evidences. The commonly found evidences at the crime scenes are fingerprints, footprints, blood, semen, saliva etc. In most of the outdoor crime scenes such as murder, drug related cases etc, botanical evidences play an important role. These evidences are sometimes considered as trace evidence because these are transferred from the crime scene to the victim's clothes, hair, shoes, socks or any other part of the body. These evidences are mostly go unnoticeable or neglected while collecting evidences at the crime scenes because of lack of awareness. So, a thorough search should be done at the crime scene because we may never know which evidence will help in solving a crime and apprehending the criminal.

Botanical evidences include pollens, leaves, roots, fruits, flower, petiole, seeds, stems etc. As the Court of Law relay on the evidences, trace evidences or more precisely botanical evidences prove to be good evidence which sometimes support the statements of the eye-witnesses. These evidences also help because of lack of eye-witnesses and in some cases where there are eye-witnesses they either biased or unreliable or become hostile.

As human's blood group cannot be used as a tool for individualization, the same goes true for the plants also. Many plants look like the same and when we get botanical evidences at the crime scenes we surely cannot tell which plant it is by looking at it. Even if we examine its anatomy, then again we cannot determine the plant. By isolating the DNA of the botanical evidence and by using an appropriate DNA technique we can distinguish between two or more plants.

The DNA fingerprinting is based on the fact that there is a variation in DNA of every individual except for monozygotic identical twins. This variation helps in differentiating two dissimilar samples. The technique which Alec Jeffreys used was Restriction Fragment Length Polymorphism (RFLP). RFLP uses restriction enzymes which cut the DNA from a particular site resulting in small DNA fragments. These DNA fragments are then electrophoresed and then Southern blotting is done. But RFLP requires a pure, non-degraded, intact DNA. In Forensic Science, we come across samples which are not in proper condition and the isolated DNA also comes out to be in degraded form or not in pure form. For such cases another DNA technique called Random Amplified Polymorphic DNA (RAPD) can be used.

RAPD is a Polymerase Chain Reaction (PCR) based technique in which ten bases long (oligonucleotides) random primers are used which amplify the DNA at low annealing temperatures. The amplified products are separated by agarose gel electrophoresis. These primers are commercially available from various sources such as Operon Tech Inc. Welsh and McClelland independently developed a similar methodology using about 15 nucleotides long and different amplification and electrophoretic conditions from RAPD and called it the Arbitrarily Primered Polymerase Chain Reaction (AP-PCR) technique. PCR amplification with primers shorter than 10 nucleotides (DNA Amplification Fingerprinting (DAF)) has been used producing more complex DNA fingerprinting profiles. [1] In RAPD-PCR, the difference from normal PCR is that only single oligonucleotide of random sequence is used and there is no need of knowing the whole genome of the sample. If the sample has the sequence which is complementary to the sequence of the random

primer, only then the amplification is positive. As in Forensics, we do not know which plant it is, so RAPD-PCR is best suited for such cases. This technique is cost efficient and faster than other techniques.

II. Materials And Methods

1.1. Botanical evidences

Six botanical evidences from three cases (one standard and one suspected sample) were received from the police station.

1.2. Solutions

A CTAB buffer (pH-8.0) consisting 100 mM Tris buffer, 25 mM EDTA, 1.5 M NaCl, 2% CTAB was prepared. In addition, Chloroform : Isoamyl alcohol (CIA) (24:1), 70% ethanol, a TE buffer consisting of 10 mM Tris buffer, 1 mM EDTA were also prepared. For electrophoresis, a TAE buffer containing 2 M Tris buffer, glacial acetic acid and 0.5 M EDTA and a TBE buffer containing 2 M Tris buffer, boric acid and 0.5 M EDTA were prepared. For loading DNA, 6 X DNA loading dye consisting 10 mM Tris HCl, 0.03% Bromophenol blue, 60% glycerol and 60 mM EDTA was prepared.

1.3. Isolation of DNA

Same procedure was followed for all the samples, i.e., for the standard and the suspected samples except for one sample (C-3 Std). This sample was first kept in 70% ethanol for overnight and then DNA was isolated by same procedure.

Each botanical sample or evidence was first washed with tap water and then with 70% ethanol and lastly with distilled water. The washed sample was dried at room temperature. The sample was then taken in the mortar and was crushed by using pestle with warm CTAB buffer till a fine slurry obtained. About 1 ml fine slurry was transferred into a 1.5 ml centrifuge tube and 5 µl β-mercaptoethanol was added. The tube was subjected to invert mixing and vortex. The tube was incubated at 60^{o}C for 1 hour to increase the DNA binding capacity and at this temperature RNA becomes inactive. Two-three times invert mixing and vortexing was done in between incubation. After incubation, the tube was cooled down at room temperature and centrifuged at 10,000 rpm for 10 minutes to spin down cell debris. The supernatant was transferred into a new centrifuge tube and equal volume of CIA (24:1) was added. Invert mixing was done. It was again centrifuged at 10,000 rpm for 10 minutes. After centrifugation, three layers were formed, the top layer of nucleic acid, middle layer of proteins and bottom layer of chloroform, lipids, carbohydrate, fats etc. The top layer was collected into a new tube. This CIA (24:1) step was repeated twice. Equal volume of chilled Isopropanol was added and invert mixing was done. Isopropanol precipitates DNA. It was centrifuged at 10,000 rpm for 10 minutes. A white coloured tiny pellet was formed at the bottom of the centrifuge tube. The remaining solution was thrown. If the colour of the pellet is not white or the size of the pellet is large enough then only the pellet is washed with 70% ethanol and again centrifuged at 10,000 rpm for 10 minutes. The pellet was air dried and 50 µl TE buffer was added to the tube. The DNA sample was stored at 4^{o}C till further use.

1.4. Qualitative and Quantitative Analysis of DNA

The quality of DNA was checked on 0.8% TAE agarose gel. The electrophoresis conditions were 70 Volts for 45 minutes to 1 hour. The quantitative analysis of DNA samples were done by using α-Helios Spectrophotometer by measuring absorbance at 260 nm and 280 nm. The purity of DNA was determined by calculating ratio of absorbance at 260 nm to that of 280 nm. Yield and concentration of the DNA samples were also calculated using the following formulas:

$$\textbf{Concentration} = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}\ \mu g/\mu l \quad (1)$$

$$\textbf{Dilution factor} = \frac{\text{Volume of TE buffer used for measuring absorbance}}{\text{Volume of DNA sample taken for measuring absorbance}} \quad (2)$$

$$\textbf{DNA yield (µg)} = \textbf{DNA concentration} \times \textbf{total volume of DNA sample (µl)} \quad (3)$$

1.5. PCR reactions and electrophoresis

Nine Operon Technology Inc. Oligonucleotides were used. The primers used were OPA-16, OPA-18, OPB-04, OPB- 07, OPD-11, OPD-15, OPD-18, OPD-20 and OPN-09.

PCR was carried out in 25 µl volume reaction mixture. The reaction mixture contained 15.5 µl double distilled water, 3.0 µl 10X buffer, 1.0 µl dNTP (0.3 mM), 2.0 µl $MgCl_2$ (0.25 mM), 1.0 µl random primer (0.3 µM), 2.0 µl DNA sample (10-100 ng), 0.5 µl Taq polymerase (0.5 U). The PCR conditions were initial denaturation at 95^{o}C for 5 minutes, followed by 36 cycles, denaturation at 95^{o}C for 20 seconds, annealing at

32°C for 20 seconds and extension at 72°C for 40 seconds and final extension at 72°C for 5 minutes and stored at 4°C for 1 hour.

The RAPD-PCR products were fractioned by electrophoresis using 1.5% TBE agarose gel at 80 Volts for 1 hour and were photographed. The RAPD-PCR amplified product sizes were evaluated by TotalLab software (TotalLab Quant).

III. Result And Discussion

There were three suspected and three standard samples. The three cases were named as follows-

1. Case-1: C-1 Std (for standard sample) and C-1 Susp or Sus (for suspected sample)
2. Case-2: C-2 Std (for standard sample) and C-2 Susp or Sus (for suspected sample)
3. Case-3: C-3 Std (for standard sample) and C-3 Susp or Sus (for suspected sample)

1.1 Physical appearance of the samples

1. C-1 Std: Green grass like sample
2. C-1 Susp: Green grass like sample
3. C-2 Std: Light green long leaf like structure with little roughness on one side
4. C-2 Susp: Dark green leaf with roughness on one side.
5. C-3 Std: Seeds of Datura
6. C-3 Susp: Brown coloured slurry

The DNA was isolated from each sample and was checked by 0.8% agarose gel electrophoresis at 75 Volt for 30 minutes.

The absorbance, ratio of absorbance, concentration and yield of isolated DNA samples were calculated as given in (1), (2) and (3) and are given in Table No.-1.

Table No.-1: Absorbance, Concentration and yield of DNA samples

Samples	Absorbance (nm)			Concentration (µg/µl)	Yield (µg)
	A_{260}	A_{280}	$\frac{A_{260}}{A_{280}}$		
C-1 Std	0.200	0.084	2.3	2.00	200
C-1 Susp	0.137	0.046	2.9	1.37	137
C-2 Std	0.058	0.042	1.38	0.58	58
C-2 Susp	0.135	0.068	1.98	1.35	135
C-3 Std	0.609	0.551	1.10	6.09	609
C-3 Susp	0.982	0.876	1.12	9.82	982

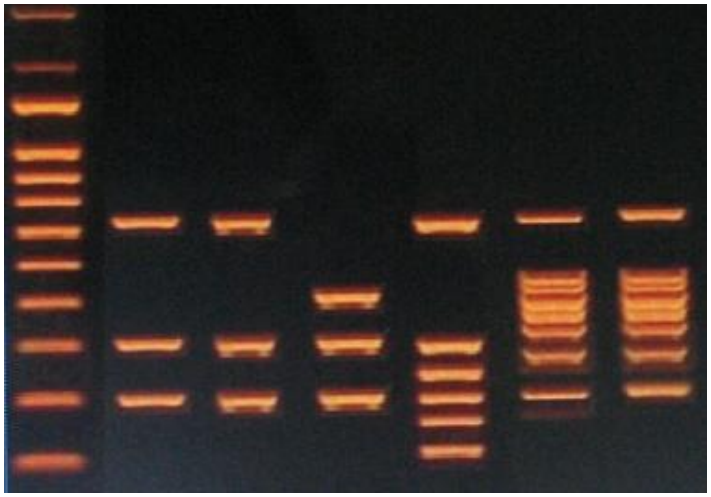

Figure-1: Result produced by OPD-18 with 100 bp ladder

Table No.-3: Lane number assigned to sample number

Lane No. of above image	Sample No.
Lane 1	100 bp ladder
Lane 2	C-1 Standard sample
Lane 3	C-1 Suspected sample
Lane 4	C-2 Standard sample
Lane 5	C-2 Suspected sample
Lane 6	C-3 Standard sample
Lane 7	C-3 Suspected sample

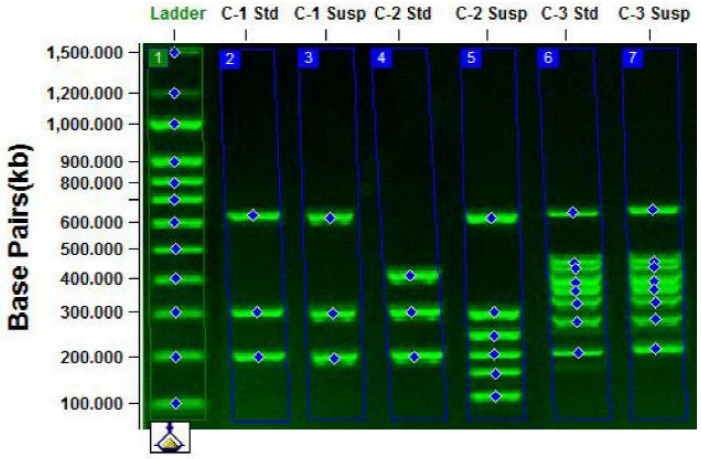

Figure-2: RAPD-PCR profile by TotalLab Quant

The result was obtained with the analysis report by TotalLab softwares. DNA bands of C-1 Std matches with bands of C-1 Susp, means the samples are related to each other. We can say, in this case, that the place where the victim was murdered and the place from where their body was found are same. In Case-2, DNA bands of C-2 Std and C-2 Susp are not matching. We can say that the place of murder and place occurrence of the body is different. In Case-3, DNA bands of both the samples were matching. The content of the mixture and the Datura seeds DNA was matching means in the mixture Datura was present.

The DNA was successfully isolated from each sample by CTAB method and same procedure was followed for every case except for the case (C-3 Std) in which Datura seeds were given as standard sample. As the seeds are building block of a plant, therefore, seeds are protected by a hard covering. The Datura seeds were first washed with tap water, then with 70% ethanol and then with distilled water and the seeds were then kept overnight into 70% ethanol to soften the hard covering. As the seed contains large amount of carbohydrates, proteins, minerals etc, after the isolation of DNA from the Datura seeds when TE buffer was added, there was a white precipitation or turbidity in the solution. This may be due to the contamination or the nutrients present in it. This turbidity also affected the absorbance of the Datura seeds.

DNA isolation from plants have been isolated by various people like Craft *et al.* (2007) [2], Iqbal *et al.* (2013) [3], Ward *et al.* (2005) [4] etc. In our study, we did not use Liquid Nitrogen as described in Deshmukh *et al.* (2007) [5], Zidani *et al* (2005) [6] because it is a cryogenic fluid that can cause rapid freezing on contact with living tissue. If by mistake it falls down on a person's limbs it may cause a serious injury.

In all samples, the DNA was successfully isolated but upon electrophoresis there were smears of DNA instead of clear bands. These smears occurs due to the following reasons-

1. When the DNA is degraded.
2. Too much DNA was loaded on the gel.
3. Improper electrophoresis conditions.
4. There may be too much salt in the DNA.
5. DNA may be contaminated with proteins, carbohydrates, RNA etc.
6. The samples may be over crushed.
7. Incubation of DNA for longer period.
8. Due to excessive vortexing or invert mixing

Although when these DNA were subjected to PCR, there were no such smears as PCR decreases contamination and also amplify the DNA.

In this study, the A_{260}/A_{280} ratio was between 1.10 and 2.9. This ratio should be from 1.6 to 1.9 for DNA. The increase in this ratio in our study may be due to contamination in the sample, improper washing of the sample or equipments or may be carelessness during measurement of the absorbance.

For each sample nine Operon Tech Inc. oligomer primers were used, from which only OPD-18 annealed to each sample and gave good amplification. Out of nine primers, two gave negative amplification in all samples while six primers gave positive but poor amplification.

In the analysis report by TotalLab software, there is some difference in the values of the samples. This may be due to the fact that may be there was some error while detecting band due to which the differences occurred.

IV. Conclusion

The aim of this work was to correlate the botanical evidences based on the DNA profile. It is concluded that the aim has been achieved, showing that, in most of the outdoor crimes, the botanical evidences play an important role and by their DNA those evidences can be correlated with each other.

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