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A PATERNITY CASE BASED ON SHORT TANDEM REPEAT (STR) USING DNA FINGER PRINTING TECHNOLOGY

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ABSTRACT

DNA Fingerprinting is the technology which is used to identify each individual on the basis of the molecular characteristics of their DNA. The technologies and applications have continued since 1985. Every individual in the world can be identified at the molecular level on the basis of an extremely greater level of polymorphism in the sequence of DNA which is inherited from biological parents and is identical in every cell of the body. DNA fingerprinting, as this technique of identification is called, can confirm with certainty the parentage of an individual. The method of DNA profiling is carried out currently by the polymerase chain reaction (PCR) based short tandem repeat (STR) profiling. STR technology is used to evaluate specific regions (loci) within the DNA. Variability in STR loci can be used to distinguish one DNA profile from another. The amplification of these microsatellite polymorphisms STR loci has progressed from single locus reactions via multiplex PCR reactions (CTT triplex and Silver triplex), amplifying as many as sixteen loci in one reaction tub. In parallel the detection of STR loci has advanced from polyacrylamide gel electrophoresis (PAGE) and silver stain techniques. This test was carried out by obtaining the dried blood spots from the mother, baby and alleged father. The child and the alleged father were tested. The paternity case analysis was performed on six STR (THO1, TPOX, CSF1PO, D16S539, D7S820 and D13S317) loci. In the DNA profiles tested, when the mother's alleles were excluded in the child's STR profile, the remaining alleles matched with the putative father's profile, thus confirming the paternity of the putative father.

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INTRODUCTION

DNA Finger printing technology

The DNA revolution began in 1984 with the discovery by Alec Jeffreys. The technique is also known as DNA profiling or genetic fingerprinting. Each cell of an organism contains the same genetic material of DNA, the technique used to identify individuals. Many applications are available including forensics genetic for crime investigation, paternity testing, genealogy and genetic research as well as the genetically engineered organisms (Jobling and Gill, 2004). DNA fingerprinting technology is a process to determine genetic identity by comparing DNA sequences which are unique to each individual in the world. It comprises of techniques such as Restriction Fragment Length Polymorphism (RFLP) and Variable Number Tandem Repeat (VNTR) or Short Tandem Repeat (STR) determination. RFLP was the initial approach practiced for DNA fingerprinting (Giardina, 2013). The VNTR and STR methods have since been developed and more constantly applied for DNA fingerprinting due to their less sensitivity and time consuming procedures. Typically VNTR is composed of 17-19 base pair (bp) repeat sequences. As the number of repeats varies from one individual to another, it can be implicated for specific DNA markers. The STR basically consists of 2-4 bp repeat sequences which have been identified as dinucleotide, trinucleotide and tetra-nucleotide polymorphisms (Choi and Ahn, 2008). STR based forensic DNA investigation has achieved worldwide public and ethical acceptance as a reliable means of individual identification. The technology was established for the development of databases such as Federal Bureau investigation (FBI), which comprises 13 STRs with the amelogenin gender determination test. Present approach in forensic casework has now agreed on using commercially developed autosomal STR multiplexes and different sources of genetic variation to find specialized uses

of autosomal Single Nucleotide Polymorphisms (SNPs) and markers on the Y chromosome and mitochondrial DNA (Jobling and Gill, 2004).

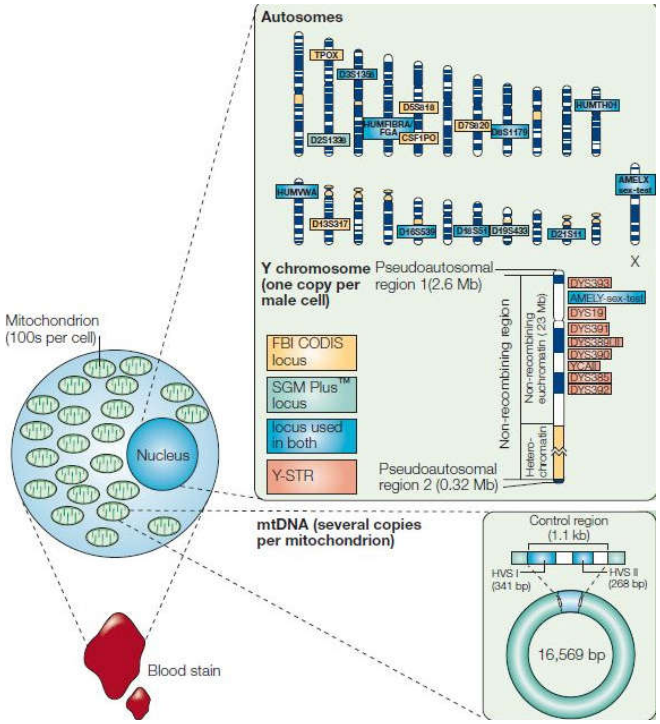

Figure 1. Sources of human genetic variation used in forensics analysis and the different properties of loci (Jobling and Gill, 2004)

Short tandem repeat based DNA profiling

DNA regions with repeat units that are 1-6 bp in length are known as microsatellites or STRs. The number of repeats in STR markers are highly variable among individuals, collocated these markers effective for human identification purposes. Polymorphic STR loci can be copied simultaneously using multiplex PCR, comfortably by adding more than one primer set to the mixture. As the technology has evolved quickly, the number of STRs that can be simultaneously amplified has augmented from 3 or 4 with silver stained systems to over 15 STRs (El-Alfy and Abd El-Haafez, 2012).

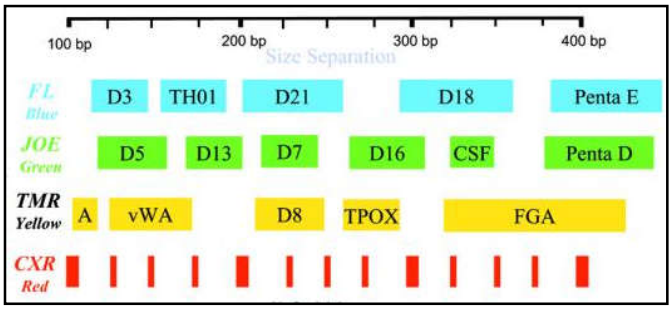

Figure 2. Schematic representation of the STR loci shown use three different fluorescent dyes (M. Ruitberg, M. Butler and Reeder, 2001)

STRs are widespread through the human genome and are capable sources of highly polymorphic markers which can be detected by PCR. Multiplex STR typing was used to study the 15 STR loci (D8S1179, D7S820, D21S11.CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA) in addition to a gender identification marker amelogenin (Shrivastava *et al*., 2012). STR DNA sequences are that involve a repetitive region of 1-6 bps repeated typically 5-30 times, casting series with lengths of up to 100 nucleotides. STRs are widely found in prokaryotes and eukaryotes, including humans. They appear scattered abundantly or less evenly throughout the human genome, accounting for about 3% of the entire genome (Tamaki and Jeffreys, 2005). Their distribution within chromosomes is not quite uniform; they come in to existence less frequently in subtelomeric regions. Most STRs are located in non-coding regions, while only about 8% found in the coding regions. In the extreme, their densities vary slightly among chromosomes. In humans, chromosome 19 has the greatest density of STRs. On average one STR occurs per 2000 bp in the human genome. The abundance common STRs in humans are A rich units including A, AC, AAAN, AAN and AG. The STR locus is named as, for example, D3S1266, where D represents DNA, 3 means chromosome 3 on which the STR locus locates, S stands for STR and 1266 is the unique identifier (Lareu, 2013).

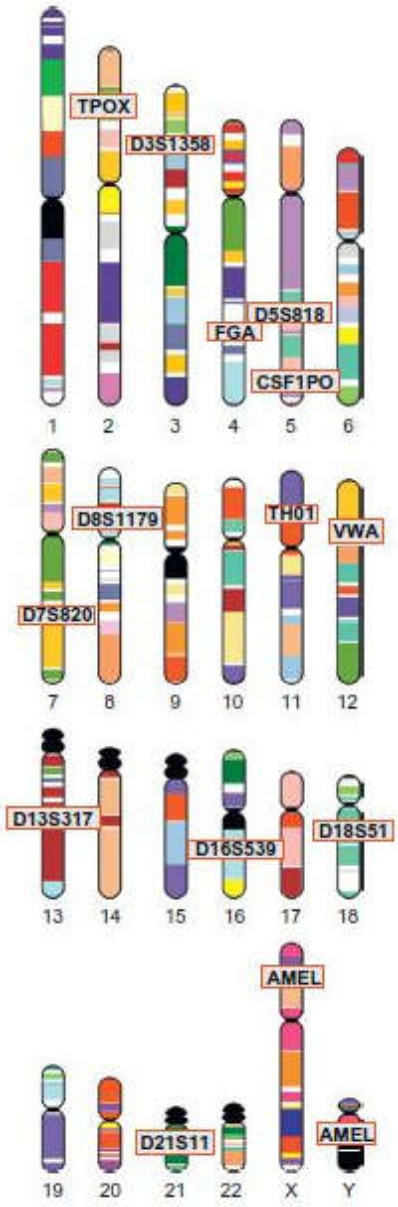

Figure 3. Names and the relative positions of the 13 core Combined DNA Index System (CODIS) STR loci on human chromosomes (Godbey, 2014)

Materials

Instrumentation	Reagents	Consumables
Micropipette (0.5-10µl)	Distilled water	Blood impregnated filter paper
Micropipette (2-20µl)	95.5% Ethanol	Lancet kit
Micropipette (20-200µl)	70% Alcohol	Tourniquet
Micropipette (200-1000µl)	20mg Saponin	Forcep
Magnetic stirrer	Phosphate buffered saline	Scissor
Refrigerator	Chelex 100 resin	Hole puncher
Vortex Machine	5X Buffer	Falcon tube (50ml)
Microcentrifuge machine	25 mM $MgCl_2$	Beakers
Incubator	2.5 mM dNTP	Conical flask
Heat block	5U/mM Taq polymerase	1.5 Microcentrifuge tube
Digital weight scale	CTT primers (F/R)	500µl Microcentrifuge tube
Refrigerator	Silver III primers (F/R)	pH paper
Vacuum machine	1X Buffer and	Pasteur pipette
Thermometer	K562 DNA	Sterilized blades
Thermal cycler (PCR)	Fluorescent dye (Hoechst bisbenzimidazole dye)	Temperature sensitive strips
Power supply	0.5% acetic acid	Tube rack
Timer	Gel repellent solution	Tube container
Fluorometer	Bind silane	Bunsen burner
White light box	99.5% Urea	Aerosol free pipette tips
Incubator	10X TBE buffer	Crushed ice
Fume hood	38.0g Acrylamide	Polyacrylamide gel electrophoresis apparatus
	2.0g Bis acrylamide	Glass plates
	Tetramethylethylenediamine (TEMED)	Side spacers
	1% ammonium persulfate	14 cm vinyl double fine sharkstooth comb(s)
	0.5X TBE buffer	Clamps
	Bromophenol dye	Cotton wool
	STR 2X Loading Solution	Micron filter
	DNA Markers	Squeeze bottle
	Xylene cyanol	50cc syringe
	10% Acetic acid	19-Gauge needle
	0.5% Silver nitrate	Wash tubs
	40% Formaldehyde	
	Pure Sodium carbonate	
	99.5% Sodium thiosulfate	

On the basis of different repeat units, STRs can be classified into different types. According to the length of the major repeat unit STRs are classified into mono, di, tri, tetra, panta and hexa nucleotide repeats. The total number of each type decreases as the size of the repeat unit increases. Di nucleotides are the most common STRs in the human genome. According to the repeat structure STRs are classified into perfect repeats (simple repeats), containing only 1 repetitive unit, and imperfect repeat units (compound repeats) containing of different composition repeats (Fan and Chu, 2007).

Allele inheritance and Paternity testing

A paternity testing is to determine paternity whether a man is the biological father of another person. Paternity testing can be done by older methods including ABO blood group typing, analysis of various other proteins and enzymes or using human leukocyte antigen (Mishra, S and SK, 2015). The paternity index (PI) comments the probability of paternity. The PI depends on the pattern of inheritance, which alleles were contributed by the mother and which by the alleged father. If multiple loci are applied to determine paternity then the product of all the individual PI for each locus is the combined paternity index (CPI). The CPI also conclude a probability of paternity, the probability that the alleged father is in fact the biological father. Due to the vast genetic diversity in the world, knowledge of the gene frequency of individual systems in studied populations is very important in paternity testing. The CPI is converted into a probability of paternity describing the degree of relatedness between the alleged father and child. Presently confirmation of paternity necessitates a probability of 0.999999 (99.9999%) (Stephenson, 2010). The DNA of a person is almost exactly same in each somatic cell.

Sexual reproduction provides the DNA of both parents together randomly to create an inimitable combination of genetic material in a new cell. The genetic material is located in the nucleus except some genes that are located in the mitochondrion from mother's egg. Half the genes of a person received from the father are located in 23 chromosomes and the other half genes of the person received from the mother are located in the other 23 chromosomes. Comparing the DNA sequence of a person to that of another person can show if one of them are derived from the other or not. Usually specific sequences are looked at to see if they are copied verbatim from one of the individuals' genome to the other. Establishing a relationship based on comparison of mitochondrial genome is much easier than that based on the nuclear genome (Ma *et al*., 2006).

METHODOLOGY

Sample collection: A fresh blood was spotted on blood impregnated filter paper discs from each individual of mother, baby and alleged father by finger prick method following aseptic techniques using sterile needle with lancet kit. The dry blood spot was partitioned and placed it into a 1.5ml microcentrifuge using hole puncher and samples were labeled according to their personal details of name, date of birth, address and contact numbers for quality control purposes.

Chelex based DNA extraction

- The dry blood spot paper was portioned in to 3mm x 3mm pieces from each individual of mother, baby and alleged father using sterile blade and placed into a labeled 1.5ml microcentrifuge tubes.

- The 1% of saponin 1ml was added into the each microcentrifuge tube contained the dried blood spot and the samples were incubated at room temperature (20^0C) for 30 minutes.
- The samples were centrifuged for 2 minutes at 13000rpm to remove the drops from the inner lid and aspirated the saponin from each tube using non barrier pipette tip attached to a Pasteur pipette on a vacuum assembly machine.
- Then PBS of 1ml was added into the each microcentrifuge tube with centrifuged for 1 minute at 13000rpm to remove the PBS and furthermore repeated the step twice.
- Then 20% Chelex of 50µl was added by using modified pipette tip attached to 1-10µl micropipette and added 150µl of distilled water into the each microcentrifuge tube containing the spot.
- Then the tubes were heated at 98°C for 8 minutes using heat block to elute DNA, vortexed for 20 seconds and centrifuged at 30000rpm for 1 minute.
- The supernatant was transferred into a new microcentrifuge tube without disturbing the pellet to measure the DNA concentration.

Fluorometric assay

- A volume of 2.0µl of each respective individual's extracted DNA was added into the separate 500µl microcentrifuge tubes each containing 198µl of fluorescent mix.
- The prepared samples were subjected to fluorescent based fluorometric assay separately to obtain DNA yield and the DNA was kept at 44°C to be used within a few hours or at -20°C for long time storage.

Multiplex PCR amplification

- The PCR master mix components and volumes were equally used separately for two multiplex PCR methods of CTT triplex and Silver III triplex.
- The 5X buffer, 25mM $MgCl_2$, 2.5mM dNTP mix, distilled water and 2.5mM primer mix (Forward / Reverse) were thawed and placed on ice and the reagents were vortexed for 15 seconds before use, Further, 5U/mM Taq polymerase of 0.25 µl was thawed by tapping several times.
- Five 500µl eppendorf tubes were placed for each reaction into a rack, and labeled appropriately to set up the number of reactions with positive and negative control reaction.
- To prepare the PCR master mix, 5.0µl of 5X buffer, 25mM $MgCl_2$ 3.0µl, 2.5mM dNTP mix 2.5µl, distilled water 9.25µl, 2.5mM primer mix (Forward / Reverse) and 5U/mM Taq polymerase 0.25 µl were added in to the microcentrifuge tube for each sample.
- The required amount was calculated for each component of the PCR master mix. The volume per sample was multiplied by the total number of reactions to obtain the final volume.

From the PCR master mix, 22.5µl was added into each 500µl tubes labeled as sample1, sample 2, sample 3, negative and positive and placed on ice to inhibit any produced imbalanced amplification of multiplexed loci.

- For the positive amplification control, 2.5µl of K562 DNA was added and for the negative amplification control, 2.5µl of sterile water was added into a tube containing 22.5µl of PCR master mix.
- Finally the samples were centrifuged briefly to bring the contents to the bottom of the tube and the PCR tubes were placed in the PCR machine.
- The CTT triplex was optimized to be compatible with the Perkin-Elmer model 480 thermal cycler reactions and SILVER III triplex was optimized to be used with the GeneAmp® PCR system 9600 thermal cycler reactions.
- The CTT triplex reactions were subjected to initial denaturation at 96°C for 2 minute ; 30 cycles of denaturation at 94°C for 30 seconds; primer annealing at 66°C for 30 seconds ; and primer extension at 72°C for 1.30 minutes with finally 2 cycles of holds at 72°C for 10 minutes and 4°C for last.

Table 1. Prepared PCR master mix for CTT triplex PCR

PCR Master Mix Component	X1 PCR master mix (volume/ µL)	X6 PCR master mix (volume/ µL)
5 X Buffer	5.00	30.00
$MgCl_2$ (25 mM)	3.00	18.00
dNTP Mix (2.5 mM)	2.5.00	15.00
Primer Mix (F/R) (2.5 mM)	2.5.00	15.00
Distilled H_2O	9.25	55.5
Taq Polymerase (5U/mM)	0.25	1.5
Total	22.5	135.0

Table 2. Prepared PCR master mix for Silver III triplex PCR

PCR Master Mix Component	X1 PCR master mix (volume/ µL)	X6 PCR master mix (volume/ µL)
5 X Buffer	5.00	30.00
$MgCl_2$ (25 mM)	3.00	18.00
dNTP Mix (2.5 mM)	2.5.00	15.00
Primer Mix (F/R) (2.5 mM)	2.5.00	15.00
Distilled H_2O	9.25	55.5
Taq Polymerase (5U/mM)	0.25	1.5
Total	22.5	135.0

Table 3. The cycling conditions of the CTT triplex PCR assay

Initial denaturation		96°C	2 minutes
3 Step : 30 Cycles	Denaturation	94°C	30 seconds
	Primer annealing	66°C	30 seconds
	Primer extension	72°C	90 seconds
2- Holds		72°C	10 minutes

The SILVER III triplex reactions were subjected to initial denaturation at 96°C for 1 minute; 10 cycles of denaturation at 94°C for 30 seconds; primer annealing at 60°C for 30 seconds ; and primer extension at 70°C for 45 seconds. PCR was continued with further 20 cycles of denaturation at 90°C for 30 seconds ; primer annealing at 64°C for 30 seconds; and primer extension at 70°C for 45 seconds with finally 2 cycles of holds at 60°C for 30 seconds and 4°C for finally.

Table 4. The cycling conditions of the CTT triplex PCR assay

Initial denaturation		96°C	60 seconds
3 Step : 10 Cycles	Denaturation	94°C	30 seconds
	Primer annealing	60°C	30 seconds
	Primer extension	70°C	45 seconds
3 Step : 20 Cycles	Denaturation	90°C	30 seconds
	Primer annealing	64°C	30 seconds
	Primer extension	70°C	45 seconds
2- Holds		60°C	30 seconds

Finally the amplified samples were stored at -20 °C for visualization of DNA from polyacrylamide gel electrophoresis.

Polyacrylamide gel preparation: The 40% acrylamide solution was prepared by combining the acrylamide 38g and bis acrylamide 2g with in 100ml distilled water. A 4% polyacrylamide gel solution of 100ml was prepared by combining the components mentioned in the below table and the acrylamide solution was filtered through a 0.2 micron filter.

Table 5. The required components and volumes to prepare the polyacrylamide gel solution

Ingredients	Quantity
99.5% Urea	42.00g
Deionized water	80.00ml
10X TBE buffer	5.00ml
40% acrylamide:bis (19:1)	15.00ml
Total volume	100.00 ml

Finally 82µl of Tetramethylethylenediamine (TEMED) with 800µl of 1% ammonium persulfate was added into the filtered polyacrylamide gel solution and poured between the two glass plates to proceed the polymerization overnight.

Polyacrylamide gel electrophoresis

- PCR products of each individual was prepared by mixing 3.0µl of each sample with 3.0µl of STR 2X Loading Solution.
- To fill out the allelic ladders, 3.0µl of CTT and Silver III DNA markers were added into 3.0µl of STR 2 X Loading solutions for each marker lane.
- The samples were briefly centrifuged and denatured by heating at 98°C for 5 minutes. Then they were immediately placed on crushed ice and 3µl of each sample were loaded into the respective wells.
- Electrophoresis was carried out by applying a constant voltage of 1500 volts (V) at an ambient temperature of 50^0C-60^0C.
- The size ranges were known for each locus and migration characteristics of the dyes stopped electrophoresis any time after the locus of interest passed the midpoint of the gel.

Silver staining

- Initially the gel was placed in a shallow plastic tray (wash tub) which contained 2L of fixative solution and agitated for 20 minutes.
- The gel was kept in a distilled water tray to be cleaned for 2 minutes. This step was repeated twice X2.
- The gel was placed in silver nitrate solution for 30 minutes and immediately cleaned with distilled water to be placed in developing solution within 10 seconds.
- Inside the developing solution the gel agitated until the required band of the locus of interest appeared, and the 2L of fixative/stop solution was added to the same tray to avoid over staining of the gel.
- Finally the same fixative/stop solution was used to avoid the staining of gel for 5 minutes and was placed in distilled water for 2 minutes to clean the gel for visualization.
- Gently agitated during each step, and the steps mentioned below in the table involving solutions containing formaldehyde, was performed in a chemical hood.

Table 6. Basic steps involved in silver staining process

Step	Solution	Time
1	Fix/stop solution - (10% acetic acid)	20 minutes
2	Distilled water	2 minutes
3	Repeat Step 2, twice	2 x 2
4	Staining solution - {2 g/L silver nitrate ($AgNO_3$): 0. 5% formaldehyde (HCOH) (3.0 ml of 40% HCOH/liter)}	30 minutes
5	Distilled water	10 seconds
6	Developer solution - {60 g/L sodium carbonate (Na_2CO_3) 0.056 M formaldehyde (HCOH) (1.5 ml of 37% HCOH/liter) 2 mg/L sodium thiosulfate ($Na_2S_2O_3$ $5H_2O$)}	Up to 5 minutes
7	Fix/stop solution - (10% glacial acetic acid)	5 minutes
8	Distilled water	2 minutes

Finally when the required bands of the locus of interest appeared, the gel plate was placed on the white light box to visualize each locus for interpretation.

RESULTS

Table 7. The measured DNA concentrations in fluorescence based fluorometric assay

Sample	DNA concentration ng/µl
Mother	1.5
alleged father	3.0
Baby	2.0

The DNA quantitation was carried out by fluorescence based fluorometric assay in order to perform the PCR. A sufficient amount of DNA was obtained from each individual of mother, baby and alleged father. A relatively high DNA quantity was shown in the sample of the alleged father. The measured DNA purity in each individual was qualified by avoiding inhibition, to perform PCR reactions.

Table 8. Comparison of the STR alleles after the polyacrylamide gel electrophoresis

Multiplex PCR	Paternity analysis				
	STR allele	+	Mother	Baby	Father
CTT Triplex	THO1	9.3/9.3	9/9	7/9	7/8
	TPOX	8/9	9/11	11/11	10/11
	CSF1PO	9/10	12/12	11/12	11/12
Silver III	D13S317	8/8	8/11	8/9	9/11
Triplex	D7S820	9/11	10/11	11/11	11/11
	D16S639	11/12	10/12	12/14	14/14

The STR allele profiles obtained for 6 loci in each individual (Mother, Baby, Putative father) are listed in table (*Table 8.0*). The amplification products using the CTT (THO1, TPOX, and CSF1PO) multiplex and Silver III (D13S317, 7S820, D16S639) multiplex were separated successfully (Figure 4.0 and Figure 5.0). The lanes containing the positive K562 DNA positive controls were observed. The negative control unit was devoid of amplification products. Numbers to the left of each image indicate the smallest and largest number of repeat units present in corresponding fragments of each allelic ladder.

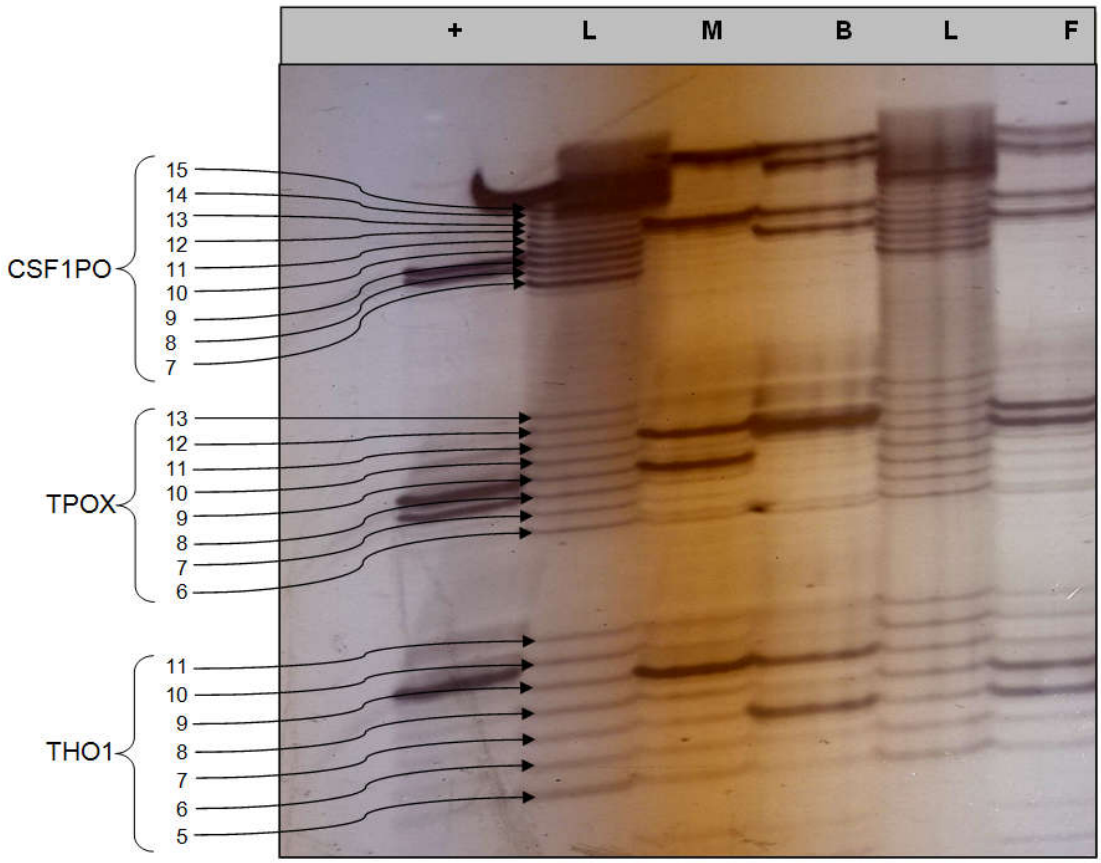

Figure 3.0 DNA profiles in paternity case analysis of CTT (THO1, TPOX, and CSF1PO) triplex. {(L-Ladder), (M-Mother), (B-Baby), (F-Father)}

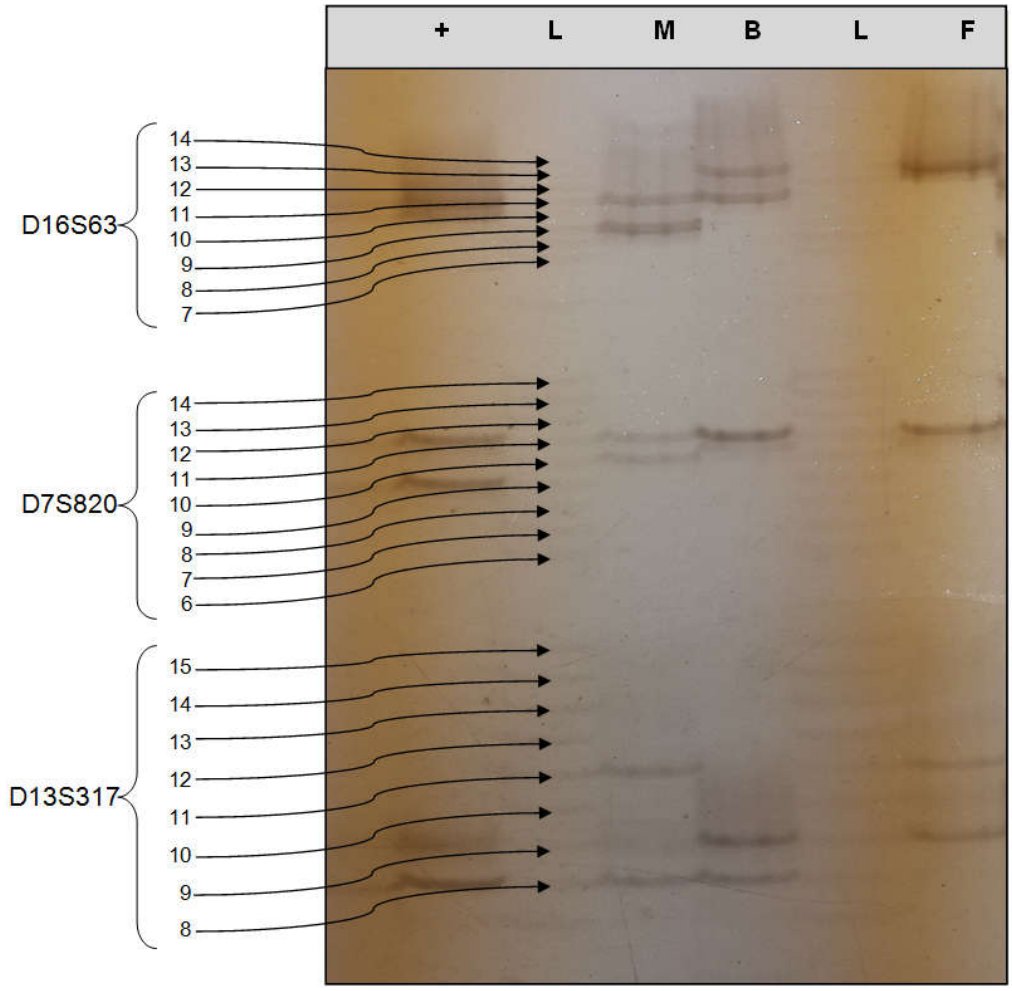

Figure 4.0 DNA profile in paternity case analysis of Silver III (D13S317, 7S820, D16S639) triplex

Table 9. General characteristics of 6 STR loci amplified by CTT triplex and Silver III triplex

Locus	Chromosome Location	Allele Sizes (Bases)	Repeat Sequence 5′→ 3	Alleles Frequency (>0.001)
TH01	11p15.5	179-203	$AATG^2$	5-9, 9.3, 10-11
TPOX	2p25.1-pter	224-252	$AATG^2$	6-13
CSF1PO	5q33.3-34	291-327	$AGAT^2$	6-15
D13S317	13q22-q31	165-197	$AGAT^2$	7-15
D7S820	7q11.21-22	215-247	$AGAT^2$	6-14
D16S539	16q24-qter	264-304	$AGAT^2$	5, 8-15

DISCUSSION

The paternity testing was based on matches of the alleles at the 6 STR loci between the child, the mother and the alleged father (trio cases). To determine the paternity of the child, the allele inheritance was analyzed. The alleles inherited from the mother were excluded from the baby's profile for each locus to be tested, and the remaining alleles were matched with the putative father's respective allele at each locus. The remaining alleles match with the father's profile for that locus. It was established that the paternity was in agreement with that locus. The above results in table (*Table 8.0*) show results of a paternity test and the results indicate that the child and the alleged father's DNA match 100% among these markers. DNA was extracted by cation exchange resin method using Chelex 100. Chelex 100 belongs to the class of weakly acidic ion exchangers. Once the Chelex 100 resin was added to distilled water, it placed immediately on magnetic stirrer to be mixed well prior to the Chelex settle. In order to perform the PCR sufficient amount of DNA was required from forensic sample. The required minimum DNA concentration >0.5ng/µl was extracted by adding sufficient chelating beads in to dry blood spot. Since the Chelex beads were synthesized of a styrene divinylbenzene copolymer containing paired iminodiacetate, the ions serve as chelating groups in binding polyvalent metal ions. The formation of large bead molecules made it unable to be taken in to the pipette tip. Therefore the pipette tips were modified by cutting their edges and were used. Chelex resin works by preventing DNA degradation from degradative enzymes (DNases) and from potential contaminants that might inhibit downstream analyses. The Chelex resin trapped such contaminants leaving behind the DNA in the solution. The Chelex resin arrested cations such as Mg^{2+}, an important co-factor for DNase action, hence protecting the DNA from degradation. Polymorphic short tandem repeat based loci were developed for human identification. The amplification of these STR loci were progressed from single locus reactions to megaplex reactions, amplifying as many as sixteen loci in one reaction tube. Two multiplex systems were used for manual detection using a silver staining procedure thus, advancing the detection of STR loci. The CTT Multiplex contained the loci CSF1PO, TPOX, TH01 and the Silver III Multiplex contained the loci D16S539, D7S820, and D13S317. Both the multiplex systems were used in conjunction to obtain genotype data for 6 loci using a manual detection format. The amplification and detection protocols were provided in the corresponding GenePrint™ STR Systems Technical Manual (Promega, Madison, WI).

Conclusion

The DNA profiles of the child and the alleged father were matched, and the test was considered as a non-excluded paternity case. In this case, no genetic discrepancy for the alleged father–child relationship was observed in any of the 6 STR loci. All alleles of the child at all STR loci monitored were detected in both the mother and the alleged father. The allele inheritance was perfectly matched with putative father and child relationship. DNA Fingerprinting technology which was used to identify the individual on the basis of genetic sequence present on their DNA, was proved to be the powerful tool in the forensic sciences and for the genotypic analysis of various species. DNA analysis provided the ethical background to establish the aristocratic family relationships within the society.

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