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Allele Frequencies of STRs F13A01, FES/FPS and vWA in Random Temiar Population of Gua Musang, Malaysia

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Forensic

ALLIA BINTI SHAHRIL

School of Health Sciences
University Sains Malaysia
Health Campus
16150, Kubang Kerian, Kelantan
Malaysia

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2. INTRODUCTION

Forensic scientists are searching for biological traits in human which are very unique to an individual to accomplish identity and individualization with certainty. Fingerprints have such characteristic as they are never the same in two individuals. However, the circumstances under which fingerprints are left and recovered in good condition are limited. Blood and hair are usually found in heinous crime. By determining blood type and hair form does not provide unique characteristics. The analysis based on blood type can narrow down the identification process to a very large group. With the advancement in DNA technology, it is proved that DNA sequence is unique to an individual. DNA profiling based identification is widely used for human identification from biological specimen.

DNA analysis is perhaps the most significant advance in technology in modern crime solving and law enforcement. It enables identification of individual present in the scene of crime based on biological evidence, such as, blood stain, semen stain, or even a single hair.

DNA which is an acronym for deoxyribonucleic acid is the genetic material of all living organisms. It is found in the nucleus of all living cells. In human, the number of chromosomes are 46; which half of it is contributed from the mother and another half from the father. One of the pair comprises the sex chromosomes, either XX or XY.

A single DNA unit is made up of three components; a sugar, a nitrogenous base, and a phosphate. The sugar and phosphate groups are involved in the backbone structure of the DNA molecule and the sequence of bases carry the genetic information. The nitrogenous bases can be categorized into two groups, which are purine; Adenine (A), Guanine (G), and pyrimidine; Thymine (T) and Cytosine (C). According to Watson and Crick (1953), DNA consists of a long linear chain of bases. DNA is double stranded and the two strands are

linked by hydrogen bonds. These two strands are arranged parallel to one another in opposite orientation (Rudin *et al.*, 2002).

Human genome consists of approximately 3 billion base pairs of nucleotides, in which 5% to 10% are coding regions and 90% to 95% are non-coding regions. Some regions of non-coding DNA sequences are highly polymorphic, so they vary from person to person in terms of the length of the repeated sequence and the number of times the sequence is repeated. This polymorphic spots are referred to as minisatellites or microsatellite characterized by repeated blocks of DNA (Edward *et al*, 1991).

DNA typing is based on the variability of several non-coding DNA stretches in the human genome. In 1985, the first DNA typing technique for identification was introduced by Sir Alec Jeffrey (Jeffrey *et al.*,1985). This classical DNA fingerprinting target on loci up to 10kb long. The core units of these repeats are composed of hundreds of nucleotides which are repeated many times. This type of repetitive DNA is called minisatellite and is also known as Variable Number of Tandem Repeat DNA (VNTR). The method of DNA fingerprinting introduced by Sir Alec Jeffrey (Jeffrey *et al.*,1985) is called Restriction Fragment Length Polymorphism (RFLP). However, the use of this classical fingerprinting is limited to high quality and quantity of DNA. Degraded DNA which results to DNA being broken down into pieces is not suitable to be analyzed using RFLP. Therefore, forensic DNA typing requires the use of techniques that allow detection of short but informative repetitive loci.

After the introduction of DNA fingerprinting, a new technique for identification was discovered (Nakamura *et al.*, 1987). DNA fingerprinting is replaced by DNA profiling. This DNA typing make use of short and highly repetitive DNA loci called Short Tandem Repeat (STR) which is another type of VNTR used in identification. STRs are also known as

microsatellites consist of 2 to 7 base pair core repeat unit and are repeated to a limited length of 80 to 400 base pairs. STRs are useful for genetic mapping, linkage analysis, and identity testing since they show a high degree of variability among individuals in a population. Analysis of polymorphic STR loci forms today's method of choice for human identification (Edward *et al*, 1991).

With the invention of Polymerase Chain Reaction (PCR) in 1986 by Kary Mullis (Mullis *et al.*,1986), minute amount of DNA can be amplified at a specific sequence. This technique is implemented in the process of DNA typing where STR loci are amplified using PCR. The total length of most of the STRs is less than 400bp and it is more suitable to be run in PCR compared to minisatellites. Although PCR-based STR typing is less discriminating than RFLP, the power of discrimination can be greatly increased by using several markers. PCR-based STR enables several loci to be amplified simultaneously in one reaction by using STR multiplex system. The advantages of using multiplex system are; small quantity of DNA sample is required, fewer reagents are consumed, and the time is greatly reduced. However, if two or more loci are to be amplified together, the amplification parameters of the STR loci must be compatible.

In forensic work, STRs with 4 repeat unit or tetranucleotide are the most commonly used (Edward *et al.*,1991). They can be amplified by PCR with higher fidelity than dinucleotide repeats that very often show intensive slippage, or stutter bands, leading to patterns that are rather difficult to interpret (Hohoff *et al*, 1999).

The detection of polymorphisms of STR loci is primarily based on the analysis of length polymorphisms by means of electrophoresis in a polyacrylamide gel. This is to achieve high resolution necessary for separation of single strands STR alleles. With this method,

samples are electrophoresed in polyacrylamide gel supported between glass plates with high voltage. The charged DNA molecules migrate through the gel matrix at rates determined by their size. The electrophoretic migration of the STR fragments is later visualized by silver staining.

To determine the probability that a particular genotype might occur at random in a population, population data must be compiled to estimate the frequency of each possible allele and genotype (Gill *et al.*,1995). Allele frequencies differ according to ethnic and racial group and therefore, population database are compiled based on ethnic and racial group (Chakraborty R., 1992).

Population DNA databases can be used to obtain information regarding the probable ethnicity of an unknown offender. This can assist investigators by narrowing their search for the true perpetrator. The approach generally taken is to examine alleles present in the evidentiary profile and compare them with the allele frequencies found in various population data sets. Likelihood ratios can then be created based on hypotheses that the profile could have come from one population or another population (Butler, 2006).

In this study, population database for three STR systems (F13A01, FESFPS, and vWA) for random individuals of Temiar population in Gua Musang, Malaysia was compiled. The multiplex system was chosen because the STRs are validated STRs for forensic analysis. Moreover, the STRs contain simple repeat motifs to aid interpretations, high discriminating power, high sensitivity across all of the loci and low intensity of stutter peaks.

The allele frequencies were statistically analyzed to ascertain it conforming to the Hardy Weinberg expectation using chi square test. Heterozygosity of three STR alleles in Temiar population is obtained. If the observed heterozygosity is low, there might be

inbreeding in the population. The allele frequencies for the three STRs in Temiar population in Gua Musang, Malaysia were then compared with other populations.

3. LITERATURE REVIEW

The use of DNA in personal identification commenced in the mid 80's with the introduction of RFLP based DNA fingerprinting (Jeffrey *et al.*,1985). Since then, DNA typing of biological material has become one of the most powerful tools for personal identification in criminal investigation (Benecke, 1997).

All living organisms are composed of cells, which are the basic units of biological activity (Tijo *et al.*,1956). Each somatic cell contains two sets of chromosomes inherited from the parents. Humans have 22 sets of autosomes and 2 sex chromosomes, making a total of 46 chromosomes (Tijo *et al.*,1956). Chromosomes within the nucleus of the cell include proteins, and DNA carrying gene. In 1944, Oswald Avery defined the role of the cellular component known as DNA as the vehicle of generational transference of heritable traits (Rudin *et al.*,2002).

Watson *et al.* (1953) elucidated the structure of DNA molecule as double helix. DNA is a polymer of four nucleotides or bases, (A) adenine, (G) guanine, (T) thymine, and (C) cytosine arranged in a varying sequence (Rudin *et al.*,2002). According to Chargaff (1949), each nucleotide consists of one of the four nitrogen-containing bases, together with a sugar (deoxyribose) and a phosphate group (Rudin *et al.*, 2002). The phosphates and sugars of adjacent nucleotides are linked together to form the backbone of the single stranded DNA chain (Rudin *et al.*,2002).

Only 1 to 2% of the DNA making up the 3.3×10^9 base pairs of the human haploid genome codes for genes and about 90% of human DNA is noncoding and consists of repetitive DNA sequences (Trent, 2005).

The existence of multiple alleles of a genetic marker at a single locus is called polymorphism. Polymorphisms can occur either in the sequence of bases at a particular locus or in the length of a DNA fragment between two defined endpoints (Rudin *et al.*, 2002). Sequence polymorphism is defined as the variation in specific base pairs at a particular locus while length polymorphism is defined as the locus that exhibits variations in length when cut with restriction enzymes or amplified with PCR primers (Edward A. *et al.*,1991). Tandemly repeated DNA sequences are widespread throughout the human genome and they exhibit length polymorphism and they are important genetic markers for mapping studies, disease diagnosis, and human identity testing (Ruitberg *et al.*,2000). Tandem repeat DNA is defined as repeating units of an identical DNA sequence arranged in direct succession in a particular region of a chromosome (Rudin *et al.*,2002)

VNTRs can be of two types, minisatellites and microsatellites based on the size of the repeated blocks and they are highly variable between unrelated individuals (Jeffrey *et al.*,1985; Nakamura *et al.*,1987).

Minisatellites consist of repeat units of 9 to 100bp (Hohoff *et al.*,1999) with total repeat length up to kilobases (Tamaki *et al.*,2005). DNA revolution in forensic investigation began in 1984 with the discovery of hypervariable minisatellite loci detected with multi locus probe which later switched onto single locus probe to circumvent limitations of multi locus probe (Tamaki *et al.*,2005). These minisatellites were detected by hybridization of probes to Southern blots of restriction-enzyme-digested genomic DNA, to reveal restriction fragment length polymorphism (RFLPs). The pattern produced by this RFLP method is known as DNA Fingerprint (Tamaki *et al.*,2005). RFLP method requires high quality and quantity DNA making it often unsuitable to be used to type DNA evidence from crime scene which usually

comes in degraded form and in minute quantity. Therefore, RFLP method of DNA fingerprinting was replaced with DNA profiling of microsatellites or short tandem repeat (STR) (Benecke *et al.*,1997).

Microsatellite, also known as STR consists of tandemly repeated simple nucleotide units of about 2 to 7 base pairs and total repeat length is less than 400bp (Nakamura *et al.*,1987). Microsatellite can show substantial polymorphism although they are far less variable than the most variable minisatellite, and are abundant throughout the human genome (Tamaki *et al.*, 2005). Microsatellites are particularly suitable for analyzing forensic specimens containing degraded and limited amount of DNA (Nakamura *et al.*, 1987).

In forensic science, analysis of polymorphic STR loci forms today's method of choice for human identification (Schneider, 2007). Highly specific, rapid, and discriminative systems have been developed that allows for working with degraded DNA because fragment length usually range from 100 to 300bp (Hohoff *et al.*,1999). Because the total length of STR is short, it is suitable for PCR (Nakamura *et al.*, 1987).

According to Hohoff *et al.* (1999), for application in forensic caseworks STRs is able to be amplified robustly by PCR, have alleles that are distinguishable, and highly heterozygous. STR loci are also feasible to become co-amplified with other loci in multiplex PCR. Small PCR products can be sized with precision by polyacrylamide gel electrophoresis (PAGE) and automated capillary electrophoresis.

There are several types of STRs being classified by Urquhart *et al.* (1994). They are simple STRs consisting of one repeating sequence (HUMFESFPS,CD4), simple STRs with additional nonconsensus alleles (F13A01 and HUMTH01), compound STRs consisting of two or more different repeated sequences (HUMGABRB15), compound STRs with nonconsensus

alleles (HUMvWA), complex repeats (D21S11) and complex hypervariable repeats (HUMACTBP2) (Hohoff *et al*, 1999).

The objective of DNA profiling is to determine the genotype of a person at several STR loci. Inheritance of STRs follows basic Mendelian inheritance (Demerec M,1933). This means that individual receives one allele from the mother and the other allele from the father (Edwards *et al.*,1991). Same DNA sequence of two paired chromosomes in an individual at a specific locus is termed homozygous and if different, the chromosome are heterozygous at that locus (Rudin *et al.*,2002). If a person is heterozygous at a particular STR locus, two bands will be visible at the lane of polyacrylamide gel. One band will be observed if the person is homozygous at that particular STR locus (Rudin *et al.*,2002).

Forensic DNA analysis is based on the frequency of every DNA fragment at the tandemly repeated regions being known and forensic biologists are able to calculate how often an allele combination appear in a population by knowing the frequency of certain STR allele fragment length in a population (Benecke, 1997). Population databases are compiled based on ethnic or racial groups (Benecke, 1997) as the distribution of allele in different population differ (Edward *et al.*, 1991; Lewontin *et al.*, 1991; Benecke, 1997; Ruitberg *et al.*, 2000; Rudin *et al.*, 2002; Panneerchelvam *et al.*, 2003; Trent *et al.*, 2005; Butler, 2006; Shneider, 2006; Conrad *et al.*,2007). It is important to know the frequency in population of various markers that make up the genotype in order to know the probability of them being present in another individual or in other words to demonstrate that the genotype are likely to be unique to an individual (Trent, 2005).

Besides autosomal STR typing and RFLP, there are many other methods that can be used for DNA typing (Rudin *et al.*,2002). Those methods are; Amplified Fragment Length

Analysis (AFLP), Random Amplified Polymorphic DNA (RAPD), Y- STR typing, X-STR typing, mitochondria DNA (mtDNA) analysis and single nucleotide polymorphism (SNP) (Rudin *et al.,* 2002). In the future, SNPs might provide an alternative typing platform that might replace STR as the most widely use procedure for DNA typing (Tamaki *et al.,*2005).

There are published data for STRs for three major ethnic population groups of Malaysia which are Malay, Chinese and Indians (Seah *et al.*,2003). Typing of other populations of Malaysia is being done (Pannerchelvam *et al.,*2003; Seah et al.,2003). In the present study, allelic distribution for three validated STRs (F13A01, FESFPS and vWA) for random Temiar population of Gua Musang, Malaysia was studied.

4. OBJECTIVES

The aim of this research is to:

1. To compile a DNA database on 3 types of STRs, F13A01, FESFPS and vWA STRs in random Temiar population of Gua Musang, Malaysia that can be referred throughout the world.

2. To compare the allelic distribution pattern of STR of Temiar population in Gua Musang, Malaysia with other population.

5. MATERIALS AND METHODS

5.1. Materials

Steriled reagents and materials were used throughout this research. The short tandem repeat (STR) typing procedure was followed according to the guidelines of manufacturer of STR kits (GenePrint STR System Technical Manual D004, Promega, USA).

5.1.1. Sample Source

Buccal swabs were collected from 100 unrelated Temiar individuals of Malaysia. Two steriled cotton buds were used for each individual. They were then asked to swipe the cotton buds inside their mouth at both cheeks for at least 10 seconds. The cotton buds were air dried and put inside plastic envelopes separately. The plastic envelopes were labeled with subject's full name and gender. The samples were stored in the refrigerator at -20°C and kept away from direct sunlight.

5.1.2. Reagents

Digestion buffer (1M Tris HCl pH 7.5, 0.5M EDTA, 20% SDS, 5M NaCl), proteinase K (20µg/µl) (Promega, USA), chloroform-isoamyl alcohol (24:1), 3M sodium acetate anhydrate (Fluka Garante), 2M sodium acetate, 70% ethanol, Tris-EDTA buffer, 10% NaOH, 0.5% acetic acid in ethanol, acetic acid, bind saline, Rain X (Blue Coral-Slick 50, USA), Q421A, MSDS (Promega, USA), 10% ammonium persulphate, 40% acrylamide, 10× TBE buffer, 0.5× TBE buffer, distilled water, E-pure water, FFv Multiplex kit consisting of STR 10× buffer (500mM KCl, 100mM Tris-HCl pH 9, 15mM $MgCl_2$, 1% Triton X-100, 2mM each dNTP), multiplex 10× FFv primer pair mix, Taq DNA polymerase (5µ/µl), STR 2× Loading

Solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF), FFv Allelic Ladder Mix (Promega, USA), Silver staining solution, Fix/Stop solution and developer solution.

5.1.3. Chemicals

Tris base (Promega, USA), concentrated HCl, Na_2EDTA (Promega, USA), NaOH (Merck, Germany), NaCl (Merck, Germany), sodium dodecyl sulphate (SDS) (Bio-Rad Lab), chloroform (Merck, Germany), isoamyl alcohol (Merck, Germany), sodium acetate (Merck, Germany), glacial acetic acid (Merck, Germany), absolute ethanol (Merck, Germany), EDTA (Promega, USA), ammonium persulphate (Promega, USA), bisacrylamide (Promega, USA), boric acid (Promega, USA), urea (Promega, USA), silver nitrate (Promega, USA), 37% formaldehyde (Promega, USA), sodium thiosulphate (Promega, USA), sodium carbonate (Promega, USA), TEMED (N,N N'N' tetramethylethylene diamine) (Promega, USA), phenol (PIERCE, USA), ethidium bromide (Sigma, USA), Agarose (Promega, USA), and Orange-G dye (Sigma, USA).

5.1.4. Apparatus

Desiccators Nucerite (Nalgene/ Syborn Corp), Vortex mixer EVM-6000 (ERLA), Spectrafuge 16M (National Labnet Co., USA), Gilson varying volume pipette (France) (1000µl, 200µl, 100µl, 20µl, and 10µl), parafilm "M" (Pechiney Plastic Packaging, Chicago), MJ Research PTC-200 Peltier Thermal Cycler (Gradient cycler) used for 0.2µl tubes, MJ Research PTC-100^{TM} Peltier Thermal Cycler used for 0.5µl tubes, monopan balance sensitive for smaller weights (DRAGON 204 Mettler Toledo), Fisher & Pagkal N308 Freezer, Hitachi refrigerator,

Mammart waterbath, SA 32 Electrophoresis apparatus (GIBCO BRL Sequencing System), High voltage powerpack EC 3000-90 (E-C Apparatus Co., USA), fumehood (Model: RICO), plastic trays for staining, stopwatch, Greiner bio-one filter tip 100, Microwave (Sanyo), Elite 300 Plus (Wealtec), Photo printer P91D UVP (Mitsubishi), Software on Chemi System, UVP (Bioimaging System), UV Transilluminator, EpiChemi Darkroom, UVP (Bioimaging system), pH Cyberscan 1000, Model RS232 Meter (Eutech Instruments, Singapore), Hot plate and magnetic stirrer, EMS-HP-7000 (ERLA), Disposable latex/vinyl examination gloves, MF 22 (Fiocchetti, Italy), DuranFavorit WHL (Genristo Ltd, England), E-pure machine (Barnstead), Diamond aluminium foil (Renaults Consumer Products, USA), Sharps collector (Dispo-Med, Malaysia), 3M Comply^{TM} Indicator Tape, C-fold Handtowels (Scott), Penguin Double Clips No.0222, B51 (China), Multipurpose assorted buds (Wang Zheng Co., Malaysia), Eve-Tape, 50ml Syringe W12875 (B-D, Singapore), Pharmacia Gel Electrophoresis Apparatus GNA-100 (Pharmacia Biotech, Sweden), short and long glass plates, Spacers, Combs, Beakers, Conical flask (Asahi Techno Glass), WTF Binder 7200 (Tuttlingen, Germany) and Autoclave: Steam Sterilizer Model MC-30LDP (ALP Co., Japan).

5.2. Methods

5.2.1. Reagent Preparations

1M Tris HCl pH 7.5

121.1g Tris base is dissolved in 800ml deionized water and the pH is adjusted to 7.5 with concentrated HCl. Solution is made up to 1000ml and autoclaved.

0.5M EDTA

186.1g Na_2EDTA is dissolved in 800ml deionized water and the pH is adjusted to 8.0 with NaOH pellets. The solution is made up to 1000ml and autoclaved.

5M NaCl

292.2g NaCl is added to 800ml deionized water and made up to 1000ml.

20% SDS

100g Sodium dodoecyl sulphate is added to 400ml deionized water. Solution is stirred with heat using magnetic stirrer. The solution was made up to 500ml and stored in an autoclaved bottle.

Digestion buffer

1ml of 1M Tris HCl pH7.5, 2ml of 0.5M EDTA, 10ml of 20% SDS, 1ml of 5M NaCl and 86ml of deionized water are mixed together using magnetic stirrer. The solution was then autoclaved.

Proteinase K

20mg of Proteinase K is mixed with 1ml of deionized water,

Chloroform-isoamyl alcohol (24:1)

240ml of chloroform is added to 10ml of isoamyl alcohol.

3M sodium acetate

102.025g of sodium acetate are added to 200ml of deionized water. The pH is adjusted to 5.2 with glacial acetic acid. Solution is made up to 250ml and autoclaved.

2M sodium acetate

16ml of 3M sodium acetate is dissolved in 8ml to deionized water.

70% ethanol

350ml of absolute ethanol is mixed with 150ml deionized water.

TE buffer

10ml of 1M Tris HCl is mixed with 0.2ml 0.5M EDTA. 989.8ml of deionized water is added and autoclaved.

0.5× TBE Buffer

25ml 10× TBE Buffer is diluted in 475ml distilled water.

1% Agarose Gel

1g of agarose is added in 100ml 0.5× TBE Buffer.

10% NaOH

100g of NaOH pellets are dissolved in the 1000ml of deionized water.

0.5% acetic acid in ethanol

0.25ml acetic acid is added to 49.75ml absolute ethanol.

Bind silane

3µl bind silane (silver stain kit) is added to 1ml of 0.5% acetic acid in ethanol.

20% ammonium persulphate

0.2g ammonium persulphate (AP) is dissolved in 1ml deionized water. Solution must be freshly prepared.

40% acrylamide: bisacrylamide (19:1)

190.0g of acrylamide and 10.0g of bisacrylamide are dissolved in 250ml deionized water. Solution is made up to 500ml and kept chilled. Solution is stored in an amber bottle.

10× TBE buffer

107.8g of Tris base and 7.44g of Na_2EDTA are dissolved together in 800ml of deionized water. 46.0g boric acid is added slowly. The pH is adjusted to 8.3 by adding extra 9.0g of boric acid. Solution is made up to 1000ml and autoclaved.

0.5× TBE buffer

50ml 10×TBE buffer is added to 950ml deionized water.

6% Acrylamide gel solution

25.2g of urea, 3.0ml of 10×TBE buffer, 9ml of 40% acrylamide and 29ml of deionized water are mixed together in a 100ml beaker. 40µl of TEMED and 400µl of 20% ammonium persulphate are added just before loading the sample into the well.

Silver Nitrate solution

In a beaker, 600ml of deionized water is added. Then, 0.6g of silver nitrate and 0.9ml of 37% formaldehyde are added in and dissolved. The beaker with contents is wrapped with aluminium foil and stored at 4°C.

Fix/stop solution

120ml of glacial acetic acid is added to 480ml of deionized water.

Gel developer

0.6ml of 37% formaldehyde is added to 120µl of sodium thiosulphate and 18g of sodium carbonate. Solution is made up to 600ml using deionized water.

5.2.2. DNA Extraction

The cotton swabs were cut into small pieces and placed into a 1.5ml microfuge tube. A total of 500µl digestion buffer and 12µl of proteinase K (20mg/ml) was added into the sample and incubated overnight at 56°C. Buffered phenol (120µl) was added and mixed vigorously using vortex. The samples were centrifuged at 10,000 rpm for 3 minutes. By using cut tips, the supernatant of the sample was transferred to a new 1.5ml tube. To this tube, 1 volume

(250µl) of buffered phenol and chloroform-isoamyl was added. The sample was mixed vigorously and again centrifuged at 10,000 rpm for 3 minutes.

Using cut tips, the supernatant was again transferred to a new tube. One volume of chloroform-isoamyl alcohol (same volume as supernatant) was added to the sample and mixed vigorously. Samples were centrifuged at 10 000 rpm for 5 minutes. Supernatant was transferred to a new tube using cut tips. Chilled ethanol (500µl) and 50µl 2M sodium acetate were added to the sample. The tube was inverted slowly to mix the sample. The sample is then centrifuged for 10 minutes at 10 000 rpm. The supernatant was discarded and 0.5ml 70% ethanol is added.

The precipitated DNA pellet was dislodged by tapping the bottom of the tube and again centrifuged at 10 000 rpm for 3 minutes. Supernatant was discarded. The tubes were sealed with parafilm and the pellets were dried using vacuum pump. On drying, 50µl TE Buffer was added and kept overnight at 37°C.The samples were then stored in freezer at -20°C for further use.

5.2.3. Agarose Gel Electrophoresis

The 1% agarose solution is heated in the microwave oven for 3 minutes to dissolve all agarose particles. The bottle was loosely closed with the cap. Meanwhile, 10 well comb was positioned in the gel cast. The agarose gel was then allowed to cool under the pipe water. For 100ml of agarose gel, a total of 10µl of ethidium bromide was added. This solution was then mixed gently. The gel was then poured on the gel cast avoiding any air bubble formation. The gel was left for 30 minutes to set. After the gel had solidified, the comb was removed.

TBE Buffer (0.5×) was poured into the electrophoresis set until it covers the gel. Before loading the sample, 1µl of orange G loading dye was added to 5µl sample. The samples were electrophoresed at 100V for 1 hour. After the electrophoresis had completed, the bands were observed under UV light. This is to determine the presence of the DNA from the extracted sample and for elimination of their concentration.

5.2.4. Quantification of DNA

The extracted DNA (3µl) from each sample was quantified using spectrophotometer. The optical density (OD) of each samples were done at A_{260} and the DNA samples were diluted appropriately.

5.2.5. PCR Amplification

The amplification of F13A01, FESFPS and vWA were performed according to the manufacturer's recommendations (Promega Corporation, Madison). The STR 10× Buffer and STR 10× FFv Primer pairs were thawed and kept on ice. The number of reactions to be set up was determined. One or two reactions were added to this number to compensate pipetting errors. The required amount for each component of the PCR master mix was calculated as in Table 1. The volume per sample was multiplied by the total number of reactions to obtain the final volume required.

PCR master mix (22.5µl) was added to each tube and placed in ice. DNA sample (2.5µl) was pipetted into the respective tubes containing 22.5µl of the PCR master mix. The samples were then spun briefly to bring the contents to the bottom of the tube. The recommended PCR condition was used to run the samples as in Table 2.

The primers for the STRs that were used are as follows (Pena, *et al.*, 1994)

vWA

3' GCC CTA GTG GAT GAT AAG AAT AAT CAG TAT GTG 5' (forward)

3' GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG 5' (reverse)

F13A01

3' GAG GTT GCA CTC CAG CCT TTG CAA 5' (forward)

3' TTC CTG AAT CAT CCC AGA GCC ACA 5' (reverse)

FESFPS

3' GCT GTT AAT TCA TGT AGG GAA GGC 5' (forward)

3' GTA GTC CCA GCT ACT TGG CTA CTC 5' (reverse)

The tubes were then placed in the thermal cycler for amplification. The thermal cycler is shown in Figure 1.

Table 1. PCR Master Mix Components

PCR Master Mix Component	Volume per sample (µl)
Sterile Water	17.35
STR 10× Buffer	2.50
FFv Multiplex 10× Primer Pair Mix	2.50
Taq DNA Polymerase (5µ/µl)	0.15 (0.75µ)
Total volume	22.50

Table 2. Amplification conditions for FFv multiplex

Initial denaturation	Cycling for first 10 cycles	Cycling for last 20 cycles	Extension Step	Hold Step
96°C for 2 minutes	94°C, 1 minute 60°C, 1 minute 70°C, 1.5 minute	90°C, 1 minute 60°C, 1 minute 70°C, 1.5 minute	60°C for 30 minutes	4°C

Figure 1. Thermal Cycler

5.2.6. Polyacrylamide Fel Electrophoresis (PAGE)

A long and a short glass plates were used to cast the polyacrylamide gel. The short plate was cleaned using 95% ethanol and wiped with paper towel. Rain-X (3ml) water repellant was applied to the longer plate. The solution was spread all over the plate with a dry paper towel using circular motion. Fresh bind silane was prepared and poured. The short plate was wiped with paper towel in circular motion. After 5 minutes, the short glass plate was wiped again with 95% ethanol for three to four times. Long plate was also cleaned using 95% ethanol and wiped with paper towel. Rain-X (3ml) water repellant was applied to the longer plate.

The long plate was later wiped with deionized water. Spacer with thickness of 0.4mm was placed on each side of the long plate. Both the plates were assembled and sealed with sealing tape. A 6% acrylamide gel solution was prepared and filtered using 0.2μ filter. The components of the acylamide gel solution are shown in Table 3.

Then, 40μl TEMED (N, N N'N' tetramethylethylene diamine) and 400μl of 10% ammonium persulphate were added. Using a 50cc disposable syringe with 21-gauge needle, the acylamide solution was poured between the glass plates.

The glass plate with the gel was positioned slant and the straight side of the sharktooth comb was inserted. The gel was left to polymerize for two hours. The sealing tapes were removed and the outside of the glass plates was cleaned. TBE buffer (0.5×) was added to the bottom chamber of the electrophoresis apparatus. The glass plates were placed in the electrophoresis apparatus avoiding air bubbles, long plate facing out. The glass plates were secured by tightening the clamps. TBE buffer (0.5×) was added to the upper buffer chamber.