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Final Technical Report

Development of a cost effective method to analyze degraded DNA evidence in Sri Lankan case work

Grant No: RG/2011/BT/07

Section 1

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iii. Principal Investigator: Dr. Ruwan J. Illeperuma

iv. Co-Investigator/s : Dr. Neil D. Fernandopulle

v. Institute where research is being carried out: Genetech Reserch Institute, 54, Kitulwatte Road Colombo 08.

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xiii. Publications/Communications arising from the project :

- Evaluation of new Mini STR markers for DNA based human identification; An *in silico* approach. *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J illeperuma* (Proceedings of the Annual Sessions – University of Colombo 2012).

- Application of miniaturized human STRs (Mini-STRs) in DNA based identification of a formalin fixed foetus: A case report *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J Illeperuma.* (proceedings annual research symposium 2013, university of Colombo 2013).

- Successful DNA based identification of a formalin fixed foetus using Human Mini STR markers-A case report *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J Illeperuma.* (Medico-Legal Journal of Sri Lanka, August 2014).

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Section 2

Executive Summary of the project:

DNA based human identification technology is the most powerful tool for accurate identification of perpetrators of crime. In forensic DNA typing, Short Tandem Repeat (STR) DNA markers in human DNA from biological evidence/human remains that is collected from crime scene are selectively amplified in Polymerase Chain Reaction (PCR) PCR to generate DNA profiles that unique to an individual.

However, under tropical environmental conditions of high humidity and high temperature DNA in biological evidence tend to degrade resulting fragmentation of DNA molecules in to smaller pieces that fail to amplification in PCR creating a major obstacle on identifying perpetrators of crime. Approximately 21% of samples in DNA analysis in Sri Lanka suffer from being untypable due to heavy fragmentation due to DNA degradation.

Application of a technology that reduce the size of the PCR products to generate a miniaturized STR fragment (miniSTR), has proven to be highly successful to recover information from degraded DNA samples. However analysis of such smaller STRs, demand highly expensive commercial DNA testing kits. The present project developed a low cost in-house method to analyze human mini-STRs system reducing more than 70% of sample analysis cost against commercial test kits. A detailed population genetic study with novel mini human STR markers was also completed to determining the allelic frequencies and forensically important statistical parameters for Sri Lankan human population.

Section 3

Introduction/background

The prevalence of violent crimes in Sri Lanka has become more frequent over the past few years. In the course of investigating such crime the establishment of the identity of the perpetrators of crime is vital to maintain law and order of a country. Examination of polymorphisms in our genetic material by DNA testing is the most powerful tool to accurate identification of criminals and to confirm familial relationships in criminal investigations. Since 2002 DNA based human identification technology has been successfully applied in more than 3400 criminal cases and 3500 civil cases that have been referred by the Courts of Sri Lanka. In forensic DNA typing, Short Tandem Repeat (STR) DNA markers on the human genome are used extensively in identifying individuals from biological evidence/human remains that were collected from scene of crime. DNA extracted from those biological materials (biological evidence) is then subjected to Polymerase Chain Reaction (PCR) amplification to generate DNA profiles that unique to an individual.

However, under certain conditions, such as in the case of mass graves, mass disasters, war and criminal cases it has been found that the DNA analysis using the conventional methods suffer from being untypable due to degradation of DNA. In such instances this limitation of the conventional technology often affects the accuracy of establishing identity of the offenders creating a major obstacle on bringing justice to the victims.

Due to tropical environmental conditions of high humidity and high temperature, the rate of DNA degradation is higher in Sri Lanka, and therefore the possibility of typing nuclear DNA is rapidly lost under these conditions. This DNA degradation turn out by the activity of bacterial, biochemical or oxidative processes resulting fragmentation of DNA molecules in to smaller pieces. The conventional DNA analyzing technology that is being used in Sri Lanka is based on PCR based typing of nuclear DNA to generate DNA fragments (amplicons) in a size range of 100 to 450 nucleotide bases. In situations where DNA in biological samples has been heavily fragmented due to degradation, the PCR amplification at said lengths could not be achieved. Therefore approximately 21% of samples sent for DNA analysis in Sri Lanka suffer from being untypable due to DNA degradation.

Application of a technology that reduces the size of the PCR products to generate a miniaturized DNA fragment of the conventional STR regions (Mini-STRs) has proven to be highly successful to recover information from degraded DNA samples.

Mini STRs

Mini STRs are nothing but reduced sized amplions of conventional STR locations in the human genome. The amplifiable repeating region of the STR locus is the same with the conventional STRs except the length of the amplicon is significantly reduced in size. The reduced sized amplicons help in the analysis of decomposed DNA evidence that contain fragmented DNA, thereby improving the sensitivity and the amplification efficiency of the DNA typing assay (Butler *et.al 2003*).

DNA degradation and PCR inhibitors typically produce a partial genetic profile with allele or locus dropouts. This problem is further exacerbated when larger multiplex PCR

reactions are performed (Butler *et.al.2003*). That is due to the larger sized DNA amplicons (ranging from 100 bp to 450 bp) produced by the conventional multiplex STR markers (Michael *et.al 2005*). In order to avoid allele and locus dropouts in a degraded DNA template, it is sensible to amplify shorter regions of DNA by PCR resulting reduced sized DNA amplicons with MiniSTR markers) by moving forward and reverse primers towards the repeat region as much as possible (Hill *et.al.*,2008).

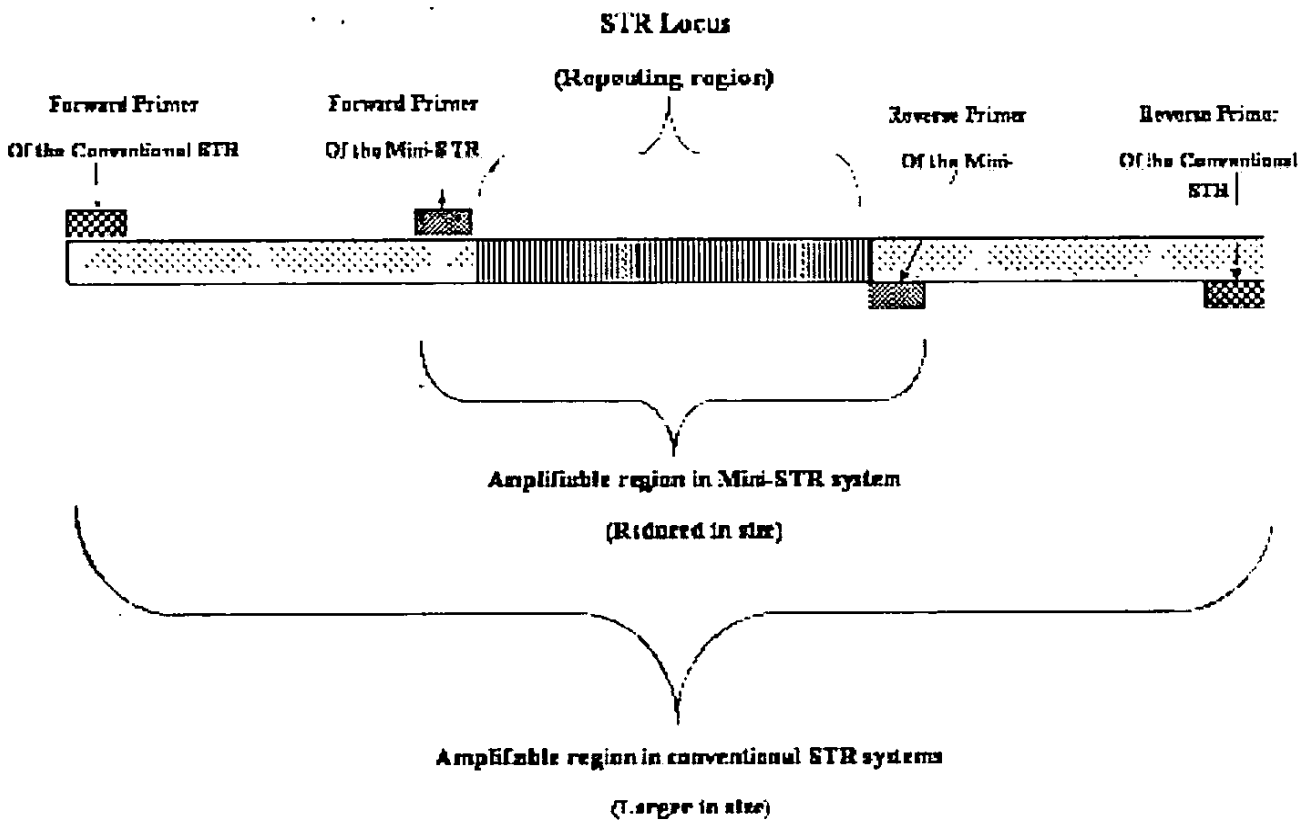

Figure 6.1 PCR amplicon size reduction of a human STR locus by mini-STR analysis

Several studies have been carried out to calculate the allele frequency distribution, forensic parameters and heterozygosity in different populations as well as different ethnicities for the Combined DNA Index System (CODIS) [FBI's program of support for criminal justice DNA databases of USA] and non-CODIS mini STR loci to evaluate their usefulness in forensic casework.

According to the available literature, the Mini-STR loci have demonstrated to be an effective approach in recovering genetic information from degraded biological evidences which can be often found from a scene of a crime (John M. Butler, 2003, Denise *et al.*, 2004). The polymorphism studies that have been carried out for CODIS and Non-CODIS STR loci so far had indicated the applicability of these systems in forensic identification purposes. There are a vast number of readymade mini-STR kits which are commercially available under brand names ABI AmpFSTRMiniFiler and Promega PowerPlex® S5 System of which use automated genotyping technology as their analysis method. These kits are priced in a range of US$3000 – US$3500 for the US market (~ Sri Lankan Rs 360,000- 400,000) (ABI AmpF'STRMiniFiler Cat No: 4343872, Promega PowerPlex® S5 Cat No: DC 6951). These highly priced reagent and chemical kits to be purchased from the instrument manufacturer as long as the testing is being carried out. Despite affording highly priced kits, extensive amount of initial capital should be invested on

automated genotyping instrumentation and also for periodic purchases of chemical replenishments for the instruments.

Therefore a low cost method for analysis of "mini STRs" was developed with an intention to make the technology more affordable and sustainable in Sri Lanka. During the study a low cost in-house method was developed to analyze human mini-STRs systems by integrating it with current methods of analysis using conventional STR systems in Sri Lanka. The novel method was validated against commercially available kits in order to maintain the concordance of results. The cost reduction of analysis of a sample is 70% per sample (Cost per sample ~Rs. 1,100/=).

According to the available literature, Mini-STR systems have not been studied thoroughly for the South Asian populations. Therefore a population study was conducted in order to determine the frequency distribution of mini-STR alleles among Sri Lankan population. Three mini- STR allelic frequency databases were created at ethnic group level along with forensically important statistical parameters of each ethnic population of Sri Lankans.

General Objective/s

1. Development of a low cost detection method for the following nine (09) Mini STR markers (detailed in Table: 1 above)

   Loci: CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A

2. Development of methods to analyze three (03) novel MiniSTR markers that have not been included in CODIS system and to perform a population study.

   This population study includes determination of allelic frequencies, and vital forensically important parameters of these three novel MiniSTR markers in the Sri Lankan human population.

Specific Objective/s

1) Optimization of PCR conditions to perform multiplexing (simultaneous analysis of multiple STR loci in a single PCR reaction) of the nine mini- STR loci; CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A.

2) Integration of allele detection method (genotyping) into a low cost polyacrylamide gel based silver staining detection method.

3) Establishment of the concordance of results of the new system by comparing the results against a commercially available mini STR kit (AmpFℓSTR® MiniFiler).

4) Development of methodologies to analyze three novel human MiniSTR markers and to determine their heterozygosity, polymorphism and allele ranges.

5) A population study was carried out by analyzing at least 100 randomly selected blood samples from each of the three ethnic groups (Sinhalese, Tamils and Moors)

for these 03 new MiniSTR markers to determine the following forensically vital statistics of each marker;

Forensically vital statistics-

i. Allele Frequencies
ii. Matching Probability (MP)
iii. Power of Discrimination (PD)
iv. Polymorphism Information Content (PIC)
v. Power of Exclusion (PE)
vi. Typical Paternity Index (TPI)
vii. percentage homozygotes and percentage heterozygotes

Materials and methods

Preparation of buffers, stock solutions and reagents

Buffers, stock solutions and reagents were prepared from chemicals purchased from Promega USA, unless otherwise stated below. All the chemicals used to prepare the following solutions were of either molecular biology grade or analytical grade, and except those stated, were prepared according to Sambrook *et al.,* (1989). These solutions were stored at room temperature, except where otherwise stated. The composition and the method of preparation of solutions were given in Appendix 1.

Collection, handling and storage of blood samples from donors.

Collection of blood samples was done on the voluntary informed consent of the donors. A sample of 1ml of venous blood was collected from each donor.

Blood samples were collected from a total of 444 healthy and unrelated individuals belonging to the three major ethnic groups of Sri Lanka; Sinhalese (n=228), Tamils (n=93), Sri Lankan Muslim (n=123), after having their informed written consent. Ethical clearance has been obtained for the project from the Ethical Review Committee of the University of Sri Jayawardhanapura.

Any donor whose close blood-relative had also donated blood for this study was not included in the study.

DNA extraction

Chelex-100 extraction of DNA from blood samples.

25µl of whole blood was used for DNA extraction (Promega Technical Manual 1998,Walsh *et.al* 1998). For each sample of blood was put in to a 1.5 ml micofuge tube and 1 ml distilled water was added. The sample then agitated gently and incubated at room temperature for about 30 minutes to 45 minutes , mixing occasionally by inverting. Tube was then centrifuged at 13000 rpm for 3 minutes and the supernatant was discarded followed by addition of 1 ml sterile distilled water. The solution was mixed gently. The

step one was repeated for two more times and after the third washing the supernatant was discarded by pipetting leaving about 30 µl in the pellet. Then 200 µl of 5% chelex 100 solution was added to the remaining pellet of the 1.5 microfuge tube and incubated at 56 °C for 20 minutes. Samples were vortexed at high speed for 7 seconds and incubated at 100 °C for 8 minutes. Samples were vortexed for 7 seconds at high speed and were centrifuged at 12,000 rpm for 2 minutes. 5 µl-10 µl from the upper most layer of the supernatant were used for the PCR immediately after the incubation step at 100 °C and the final centrifugation.

PCR amplification of STR Loci

PCR amplification was done for each sample using conventional STR primers (Promega Geneprint STR Kit) as well as newly designed Mini STR primers.

Designing of Mini STR primers

Optimization of PCR conditions to perform multiplexing (simultaneous analysis of multiple STR loci in a single PCR reaction) of the nine mini- STR loci; CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A.

Mini STR primer designing for Loci CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A

Mini STR Primers for the existing loci were designed using the web based primer designing tool 'primer 3'. The reference sequence for each STR marker was obtained from http://www.cstl.nist.gov/strbase/seq_ref.htm. Nine primers were designed using the reference sequences and were analyzed for the specificity using the NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/#).

PCR amplification of novel Mini STR primers

Designed Mini STR primers were optimized with adjusting the primer concentrations, Mg^{2+} ion concentrations and dNTPs concentrations in order to obtain a successful amplification of the DNA template. Conditions were optimized for concentrations of PCR component, optimal PCR reaction volume, and PCR thermal cycling parameters.

The master mix was prepared by adding 10X PCR buffer without $MgCl_2$, forward and reverse Mini STR primer mix, dNTP mix and sterile distilled water and Taq DNA polymerase. The ability to make those primers work into multiplexes was also verified by performing PCR with primer cocktails. PCR products were subjected to agarose gel electrophoresis followed by polyacrylamide gel electrophoresis.

Amplification of allelic ladders for Mini STR primers
Allelic ladders which are needed for manual Silver Staining were generated for CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A loci by using Promega GenePrint kit templates (Baechtel *et.al.* 1993) along with the newly designed MiniSTR primers. The amplification step was as follows.

For 25 µl volume	
10X PCR buffer (without MgCl2)	2.5 µl
25mM MgCl2	1.5 µl
2.5mM dNTP mix	2.5µl
2.5µM Primer (F+R)	2.5µl
DNA template	1.0 µl
Sterile distilled water	up to total of 20 µl
Taq DNA polymerase	0.2µl
Total	25 µl

The PCR product obtained after the amplification was run on 2 % Agarose gel to analyse the intensity. Then it was run on 6% polyacrylamide gel along with the standard positive control (K562 DNA) amplified with relevant Mini STR primer sets. Alleles of the positive controls were checked for confirmation and then sample alleles were assigned according to the standard nomenclature (Bar 1997).

Amplification of allelic ladders for the three novel Mini STR markers.

Development of allelic ladders for the novel STR loci D4S2632, D6S2436 and D19S589 were achieved by amplifying DNA isolated from fifty blood samples obtained from fifty unrelated individuals belonging to three major ethnicity (Sinhalese, Tamil and Moors). The PCR products obtained for each STR loci analysis were subjected to polyacrylamide gel electrophoresis followed by silver staining method for the visualization of DNA bands. Subsequently, three alleles from each STR loci were selected and re-amplified in order to subject them to DNA sequencing. Through DNA sequencing, it was found the corresponding number of repeat motifs in each allele amplified in each three novel STR loci. Finally, using the generated sequencing data, Allelic ladders were designed for D4S2632, D6S2436 and D19S589 loci.

PCR amplification with Promega GenePrint Kit Primers (Conventional PCR)
When using Promega GenePrint kit primers, The PCR buffers as well as other PCR reagents were used according to the instructions given by Promega corporation. Since monoplex PCR was needed in the study it was achieved by just adding forward and reverse primers (Concentration :2.5 µM) to the PCR given below.

For 25µl reaction volumes;

	For multiplex Kit	For monoplex
10x PCR Buffer	2.5 µl	2.5 µl
Primer Mix	2.5 µl	2.5 µl
DNA Template	variable volumes	variable volumes
Sterile D.W	up to total of 20 µl	up to total of 20 µl
075 Taq DNA polymerase Diluted in 1 X STR Buffer	5.0 µl	5.0 µl
Total	25 µl	25 µl

Master mix was prepared by adding 10X STR buffer, forward and reverse primer mix, sterile distilled water and Taq DNA polymerase which was diluted in 1X STR buffer. The volumes of these reagents were calculated according to the number of samples that were to be amplified. An extra sample was also counted to above calculation to compensate the pipetting errors.

Agarose gel electrophoresis

After the PCR amplification of the samples the, PCR products were checked for the their amplification by loading 7.5 µl of the PCR product in to 2% agarose gels containing 1X Tris-borate(TBE) buffer (pH8) followed by subjecting electrophoresis under 100V for about an hour to separate the PCR products of different STR loci that have amplified.

Integration of allele detection method (genotyping) into a low cost polyacrylamide gel based silver staining detection method.

Polyacrylamide gel electrophoresis

Poyacrylamide gel electrophoresis was carried out according to Promega technical manual (2006) with several minor changes.

2.5 ul of each mini-STR PCR amplicon (samples and standard sixe markers) was loaded in the 6% Polyacrylamide gels. Electrophoresis power was set at 50W, with a voltage of 1250 V and a current of 50mA. Samples were prepared by adding 2.5 µl of PCR product to 2.5 µl of STR 2X loading solution prior loading the gel. Standard sixe markers (Allelic ladders) were run after every two samples, so that every sample was adjacent to an allelic ladder. Negative controls consisting of PCR reactants in which no template DNA was added were run. Positive controls consisting of amplified products of K562 DNA were also run, and allele assignments were confirmed for both conventional and mini STR systems.

Silver staining

Amplified products were detected using a DNA sliver staining procedure described by Bassam et.al. (1991) this is a very sensitive method that allows rapid evolution of amplified STR fragments. After electrophoresis the glass plates were separated and the shorter plate, on to which the gel has bound was placed in a shallow tray and treated as follows according to Bassam et.al (1991) and promega manual 1998, with reagent supplied by the Promega corporation; Fix/stop solution (20 min) distilled water (2 min) repeated this step twice, staining solution (30 min), distilled left for 5 min. Placed in distilled water for 2 minutes, air dried overnight.

Stained gels were labeled, photographed and the alleles were converted in to numeric DNA profiles.

Establishment of the concordance of results of the new system by comparing the results against a commercially available mini STR kit (AmpFℓSTR® MiniFiler).

DNA profiles of four DNA based paternity tests each comprising of mother, child and father trios were tested using conventional STR methods, with newly designed primers in Mini STR system and with the commercially available mini STR kit (AmpFℓSTR® MiniFiler).
For each batch of STR loci tested, universal human DNA control (K562 DNA) was also amplified using PCR reagents of conventional STR methods, with newly designed Mini STR system and with the reagents of commercially available mini STR kit (AmpFℓSTR® MiniFiler) respectively.
For AmpFℓSTR® MiniFiler assay, kit instructions provided in the instructions manual by Applied biosystems USA were adhered to.

Results obtained from each method were compared to verify the concordance of results of the new system.

Electrophoresis and genotyping

PCR amplicons from conventional STR methods and newly designed Mini STR system were genotyped by Poyacrylamide gel based silver staining procedure.

PCR amplicons from AmpFℓSTR® MiniFiler assay were genotyped by automated capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems) employing ABI Fragment Analysis Data collection software. Sizing and genotyping of the products were performed using Genemapper ID V3.2 software from Applied Biosystems.

Development of methodologies to analyze three novel human MiniSTR markers and to determine their heterozygosity, polymorphism and allele ranges.

Ten STR loci were selected initially from a panel of 700 STR markers from the Mammalian Genotyping services database of Marshfield clinic web facility. These markers were selected based on the level of polymorphism (PIC values) observed, size of the repetitive region and the loci that has clean flanking regions around their repetitive region. Out of ten STR loci selected, three STR loci were finalized after analyzing them for intrinsic structural properties such as Nucleosome Forming Potentials (NFP's) using the algorithm of Nucleosome eXclusion Sensor (NXSensorversion 1.3.1) (http://www.sfu.ca/_ibajic/NXSensor/) web based software.

The population study for 03 new MiniSTR markers and to determine forensically informative parameters.

A population study was carried out by analyzing at least 100 randomly selected blood samples from each of the three ethnic groups (Sinhalese, Tamils and Moors) for these 03 new MiniSTR markers to determine the following forensically vital statistics of each marker.
Samples were collected from volunteers after obtaining their written informed consent and the donors were requested to give some personal details voluntarily. The ethical clearance for sampling of human subjects has been obtained from). The personal details included ethnicity, age, sex, place of birth.

The samples collected from volunteers were venous blood (0.5 ml- 1ml). Proper sample collection, labeling, recording and storage methods will be adhered to.

Statistical analysis

Determine their heterozygosity, polymorphism, allele ranges and other forensically vital statistics in the population study;

The genotypic data of each sample that were recorded as numerical data, and were subjected to various statistical analysis.

Determination of forensically important statistical parameters; Autosomal STR Allele frequencies, Polymorphism information content (PIC), power of discrimination (PD), power of exclusion (PE), and typical paternity index (TPI) were calculated for each locus using Powerstats v1.2 software (Tereba, 1999).

Results

General objective:

Development of a low cost detection method for the following nine (09) Mini STR markers (detailed in Table: 1 above)

Loci: CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A

Designing of mini-STR primers

Mini STR primers which were designed for loci CSF 1PO, TPOX, THO1, D7S820, D13S317, D16S539, D8S1179, F13A and vWA are listed in the table 1. The melting temperature and the distance from the repeat region are also listed.

Table: 1 Primer sequences designed to generate Mini-STR amplifications in PCR

Locus		mini STR primer sequence 5'-3'	Tm (°C)
CSF 1PO	F	AGATATTAACAGTAACTGCCTTCA	52.1
	R	GTGTCAGACCCTGTTCTAAGTA	51.2
TPOX	F	TAGGGAACCCTCACTGAATG	51.8
	R	GGTCCTTGTCAGCGTTTATTTG	51.8
THO1	F	CCTGTTCCTCCCTTATTTCC	50.7
	R	GAGGGAACACAGACTCCATG	51.7
D7S820	F	GCAAACAAAGCAGATCCCAAG	54.3
	R	GGTGCATCTGTAAGCATGTATCTATC	54.5
D13S317	F	ACTTGTCATAGTTTAGAACGAAC	51.1
	R	GTCCACATTTATCCTCATTGACAG	52.6
D16S539	F	TGACCCATCTAACGCCTATC	52.1
	R	GCAGAAAGATAGATAGATGATTGATTG	50.9
D8S1179	F	GTGTACATTCGTATCTATCTGTCTA	52.4
	R	GTTCACTGTGGGGAATAGATAGA	51.7
F13A	F	GCAACAGAGCAAGACTTCATC	53.5
	R	GCTTTAATAATGCCATGCAGATTAG	51.1
vWA	F	GTATGTGACTTGGATTGATCTATC	51.5
	R	GGATAGAGATAGGACAGATGATAAATAC	51.7

Specific Objective

Optimization of PCR conditions to perform multiplexing (simultaneous analysis of multiple STR loci in a single PCR reaction)

Following reaction components and volumes were found to be optimum for novel Mini STR primers which can deliver successful typing results from both non degraded and degraded DNA samples analysed in this study.

For 25µl reaction volumes;

	For monoplex PCR
10x PCR Buffer without MgCl2	2.5 µl
3.5mM dNTP mix	2.5 µl
Primer Mix (F+R)	2.5 µl
BSA	0.2 µl
DNA Template	5.0 µl
Sterile D.W	up to total of 20 µl
5u/ µl DNA polymerase	0.2 µl
Total	25 µl

Optimized PCR protocol for Mini STR system

Table: 2 Optimized PCR conditions for nine mini- STR loci; CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A each to be amplified in single PCR reactions (monoplexes).

	Initial Incubation	Programmed Ramp time	Cycling for first 10 cycles	Programmed Ramp time	Cycling for first 10 cycles	Extension Step	Hold step
1	94°C for 2 minutes	Default ramp to 94°C 30 sec Default ramp to 54°C 45 sec Default ramp 70 °C 1.5 min		Default ramp to 94°C 30 sec Default ramp to 54°C 45 sec Default ramp to 70 °C 1.5 min		60°C for 45 min	4°C

The newly designed primers (Table: 1) were used to amplify the universal human DNA standard K562 DNA to check for the compatibility. Results were compaired with the results for K562 DNA results derived from conventional STR amplification system of GenePrint STR-Promega, USA). The results (Table: 3) confirmed that the designed primers do not deviate from the results obtained from the conventional STR. The standard DNA (K562) gave the desired allelic readings against the amplified Mini STR ladder confirming the compatibility of the newly designed mini STR system with the conventional STR system.

Table: 3 Validating the primers with universal human DNA standard (+ve control)

STR Locus	NCBI GenBank Accession	Universal human DNA standard (K562 DNA) results	
		Conventional STR (GenePrint STR Systems-Promega)	Mini STR results in the present study
CSF1PO	X14720	9,10	9,10
TPOX	M68651	8,9	8,9
THO1	D00269	9.3,9.3	9.3,9.3
D16S	NT_010498.15	11,12	11,12
D7S80	AC004848	8,8	8,8
D13S317	AL353628	9,11	9,11
D8S1179	AF216671	12,12	12,12
F13A	ACM21986	4,5	4,5
vWA	M25858	16,16	16,16

Results obtained from Mini-STR system were evaluated and determined the number of alleles observed and allelic range for each STR locus. Minimum PCR amplicon length size of each mini-STR loci was compared with that of the conventional STR amplification system of GenePrint STR-Promega, USA). Results are presented in Table: 4.

Table: 4 The size reduction achieved by novel mini STR primers

STR Locus	NCBI GenBank Accssion	Number of Alleles observed in the given sequence in Mini STR	Allele Range in Mini STR	PCR Product Size		Size Reduction by Mini-STR system (In base paires)	Percentage Size Reduction by Mini-STR system
				Conventional STR (GenePrint STR Systems-Promega)	Mini STR		
CSF1PO	X14720	12	7-15	295-327	90-122	205	65.9%
TPOX	M68651	11	06-13	224-252	67-95	157	65.9%
THO1	D00269	09	05-11	179-203	56-80	123	64.4%
D16S539	NT_010498.15	11	05-15	264-304	135-175	129	65.4%
D7S820	AC004848	13	06-14	215-247	158-190	57	24.6%
D13S317	AL353628	11	07-15	165-197	90-122	75	41.4%
D8S1179	AF216671	13	8-16	127-159	59-91	68	47.5%
F13A	ACM21986	07	4-16	283-331	171-219	112	36.5%
vWA	M25858	20	13-20	139-167	111-139	28	18.3%

a. Optimization of PCR conditions to perform multiplexing of the nine mini- STR loci; CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A to be amplified in three or four PCR reactions simultaneously (multiplexing).

Table: 5

STR Loci		Forward and Reverse Primers	Size Range (bp)	Tm	GC%	Size reduction (bp)	Multiplexing
CSF1PO	F	AGATATTAACAGTAACTGCCTTCA	90-122	52.1	33.3	205	Set 1
	R	GTGTCAGACCCTGTTCTAAGTA		51.2	42.9		
THO 1	F	CCTGTTCCTCCCTTATTTCC	56-80	50.7	50	57	
	R	GAGGGAACACAGACTCCATG		51.7	52.6		
D7S820	F	ACTTGTCATAGTTTAGAACGAAC	158-190	51.1	34.8	123	
	R	GTCCACATTTATCCTCATTGACAG		52.6	39.1		
F13A	F	GCAACAGAGCAAGACTTCATC	171-219	53.5	47.6	112	Set 2
	R	GCTTTAATAATGCCATGCAGATTAG		51.1	33.3		
TPOX	F	TAGGGAACCCTCACTGAATG	67-95	51.8	50	157	
	R	GGTCCTTGTCAGCGTTTATTTG		51.8	42.9		
vWA	F	GTATGTGACTTGGATTGATCTATC	111-139	51.5	37.5	28	
	R	GGATAGAGATAGGACAGATGATAAATAC		51.7	33.3		
D16S 539	F	GCAAACAAAGCAGATCCCAAG	135-175	54.3	47.6	129	Set 3
	R	GGTGCATCTGTAAGCATGTATCTATC		54.5	40		
D13 S 317	F	TGACCCATCTAACGCCTATC	90-122	52.1	50	75	
	R	GCAGAAAGATAGATAGATGATTGATTG		50.9	30.8		
D8S1179	F	GTGTACATTCGTATCTATCTGTCTA	59-91	52.4	36	68	Set 4
	R	GTTCACTGTGGGGAATAGATAGA		51.7	40.9		

Optimized PCR conditions for all four sets of PCR reactions.

I. 94 °C 5min ,
II. 30 cycles - 94 °C 1 min, 50.0 °C- 55.0 °C 1 min, 72 °C 1 min
III. 60 °C 45 min

Specific objective: Integration of allele detection method (genotyping) into a low cost polyacrylamide gel based silver staining detection method.

1) Allele detection of the new mini-STR PCR amplicons (genotyping) was integrated into in to a low cost polyacrylamide gel based silver staining detection method.

   Allelic ladders (standard size markers) for each loci (Table: 1) were constructed by performing PCR amplification with respective mini-STR primers designed in Table: 1 using Promega GenePrint kit ladders (Baechtel *et.al.* 1993) as PCR templates.
   Each PCR amplicon was subjected to polyacrylamide gel electrophoresis along with PCR products of K562 DNA and samples which were amplified by the same mini-STRs primers. The alleles were visualized by staining the gels with Silver staining procedure.

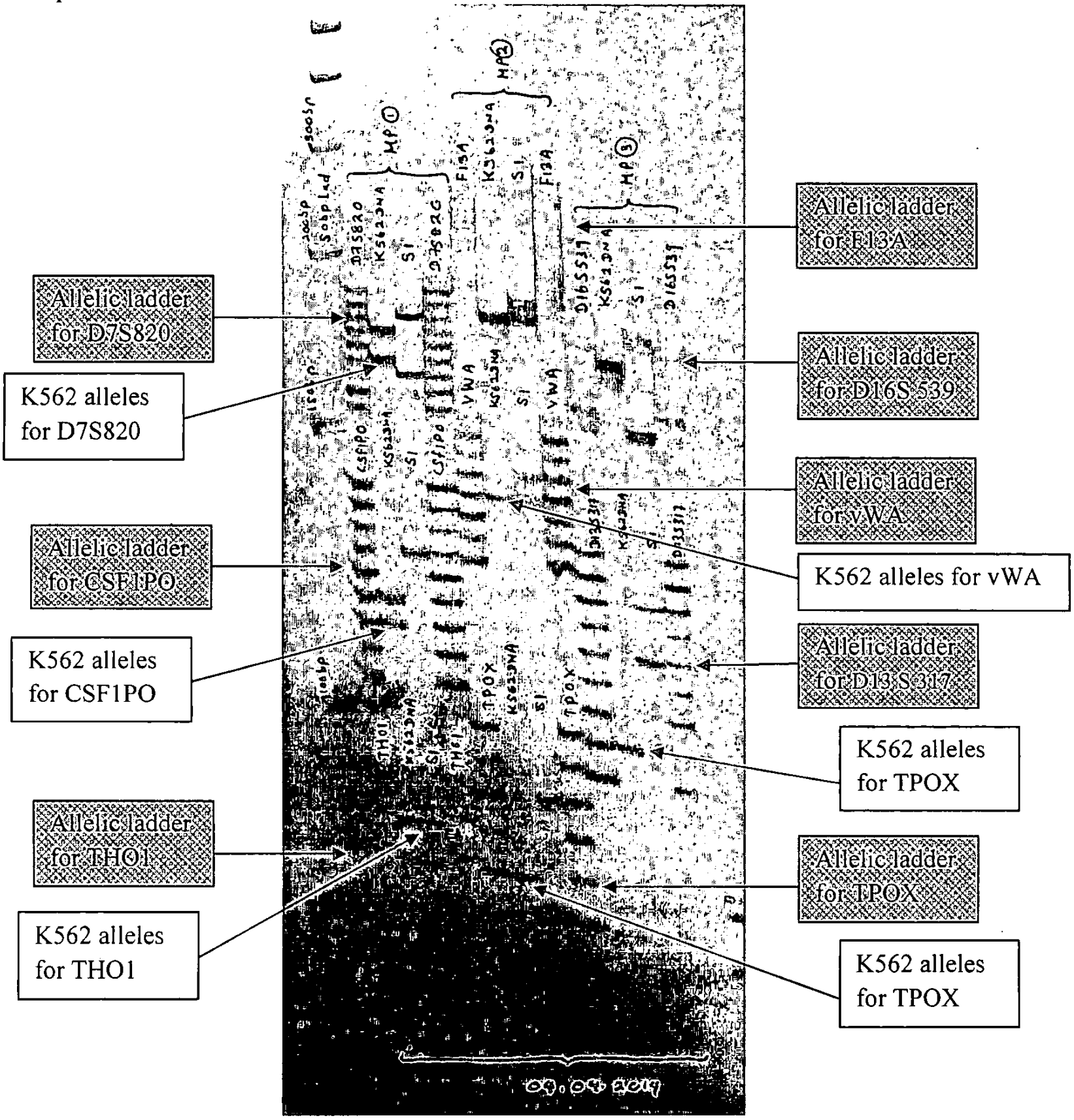

Figure: 1 Silver stained polyacrylamide gel with amplified products and size makers (ladders) for each mini- STR loci.

Table: 4 Verifying concordances of mini-STR results by comparing against the commercially available AmpFℓSTR® MiniFiler kit. Four mother, child and father trios of positive DNA based paternity tests were used for comparison.

Sample ID	CSF1PO	TPOX	THO1	D16S	D7S	D13S	F13A	vWA
Case 1-M	11, 11	9,11	8,9	8,13	8,8	8,11	3.2,14	17,18
Case 1-1C	11, 11	9,11	8,9.3	8,11	8,11	11,14	3.2,3.2	16,18
Case 1-1F	10,11	11,11	8,9.3	11,13	10,11	11,14	3.2,5	16,18
Case 2-M	12,12	8,11	6,9	10,11	10,13	10,12	5,6	17,18
Case 2-1C	10,12	8,11	6,7	10,10	11,13	8,10	5,6	16,17
Case 2-1F	10,10	10,11	7,9.3	9,10	11,12	8,10	5,5	16,19
Case 3-M	11,11	9,11	8,9	9,10	8,10	12,13	5,6	15,17
Case 3-1C	11,11	11,11	9,9	9,11	10,10	11,13	5,14	15,18
Case 3-1F	10,11	11,11	8,9	11,11	10,10	8,11	3.2,14	17,18
Case 4-M	11,12	8,8	7,7	9,11	8,10	12,12	4,4	15,16
Case 4-1C	10,11	8,9	7,9.3	9,13	8,10	12,12	4,5	14,16
Case 4-1F	10,11	9,11	6,9.3	10,13	8,10	9,12	3.2,14	17,18
K562	9,10	8,9	9.3,9.3	11,12	8,8	9,11	4,5	16,16

General objective 2: Development of methods to analyze three (03) novel MiniSTR markers that have not been included in CODIS system and to perform a population study.

Out of ten STR loci analyzed from Mammalian Genotyping services database of Marshfield clinic web facility, three STR loci were finalized after analyzing them for intrinsic structural properties such as Nucleosome Forming Potentials (NFP's) using the algorithm of Nucleosome eXclusion Sensor (NXSensorversion 1.3.1)

Table: 5 Three novel human MiniSTR markers developed by the present study.

STR Locus	Chromosomal location	Chromosomal pb Position	Repeat Motif	GenBank accession	GenBank allele	PCR amplified fragment range (bp)
D4S2632	4p15-p14	35704165	(GATA)n	G08391.1	13	105
D6S2436	6q24.1	154136091	(GATA)n	G27284.1	9	91
D19S589	19q13.42	58498394	(GATA)n	G08026.1	13	98

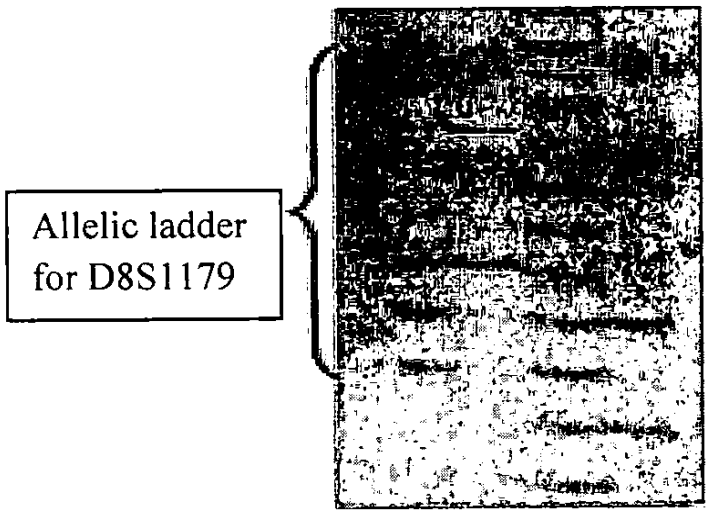

Figure: 2 Silver stained polyacrylamide gel with amplified size maker (ladder) for mini- STR loci D8S1179.

Specific Objective 3: Establishment of the concordance of results of the new system by comparing results against a commercially available mini STR kit (AmpFℓSTR® MiniFiler).

Table: 3 Verifying concordances of mini-STR results by comparing with results of conventional STR analysis method. Four mother, child and father trios of positive DNA based paternity tests were used for comparison.

		Confirmation of Concordance of Mini STR Vs Conventional STR															
	CSF1PO		TPOX		THO1		D16S		D7S		D13S		F13A		vWA	
Sample ID	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR	MINI STR	CONV.STR
Case 1 -M	11,11	11,11	9,11	9,11	8,9	8,9	8,13	8,13	8,8	8,8	8,11	8,11	3.2,14	3.2,14	17,18	17,18
Case 1 -1C	11,11	11,11	9,11	9,11	8,9.3	8,9.3	8,11	8,11	8,11	8,11	11,14	11,14	3.2,3.2	3.2,3.2	16,18	16,18
Case 1 -1F	10,11	10,11	11,11	11,11	8,9.3	8,9.3	11,13	11,13	10,11	10,11	11,14	11,14	3.2,5	3.2,5	16,18	16,18
Case 2 -M	12,12	12,12	8,11	8,11	6,9	6,9	10,11	10,11	10,13	10,13	10,12	10,12	5,6	5,6	17,18	17,18
Case 2 -1C	10,12	10,12	8,11	8,11	6,7	6,7	10,10	10,10	11,13	11,13	8,10	8,10	5,6	5,6	16,17	16,17
Case 2 -1F	10,10	10,10	10,11	10,11	7,9.3	7,9.3	9,10	9,10	11,12	11,12	8,10	8,10	5,5	5,5	16,19	16,19
Case 3 -M	11,11	11,11	9,11	9,11	8,9	8,9	9,10	9,10	8,10	8,10	12,13	12,13	5,6	5,6	15,17	15,17
Case 3 -1C	11,11	11,11	11,11	11,11	9,9	9,9	9,11	9,11	10,10	10,10	11,13	11,13	5,14	5,14	15,18	15,18
Case 3 -1F	10,11	10,11	11,11	11,11	8,9	8,9	11,11	11,11	10,10	10,10	8,11	8,11	3.2,14	3.2,14	17,18	17,18
Case 4 -M	11,12	11,12	8,8	8,8	7,7	7,7	9,11	9,11	8,10	8,10	12,12	12,12	4,4	4,4	15,16	15,16
Case 4-1C	10,11	10,11	8,9	8,9	7,9.3	7,9.3	9,13	9,13	8,10	8,10	12,12	12,12	4,5	4,5	14,16	14,16
Case 4 -1F	10,11	10,11	9,11	9,11	6,9.3	6,9.3	10,13	10,13	8,10	8,10	9,12	9,12	3.2,14	3.2,14	17,18	17,18
K562 DNA	9,10	9,10	8,9	8,9	9.3,9.3	9.3,9.3	11,12	11,12	8,8	8,8	9,11	9,11	4,5	4,5	16,16	16,16

Upon developing the primers for the above three STR loci as accordance with the methods mentioned in the "Designing of Mini STR primers"; PCRs were performed and the amplicons were subjected to sequencing.

Sequences were subjected to BLAST searches and the results were as follows; The target repeating regions of each loci are highlighted

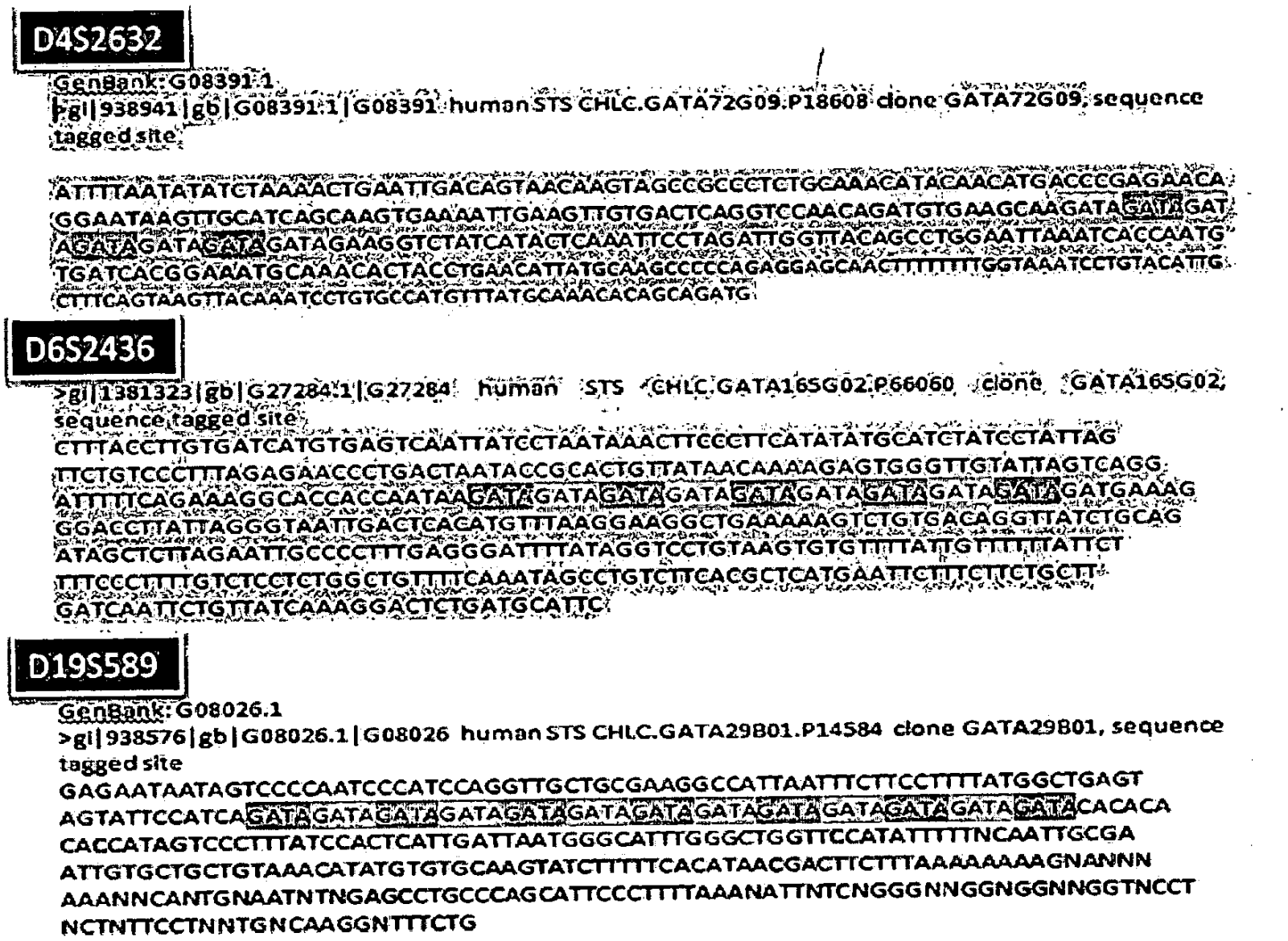

Figure: 3 Sequencing results of the novel Mini-STR loci. Tetra nucleotide tandem repeats are highlighted.

Specific objective 4: Development of methodologies to analyze three novel human MiniSTR markers and to determine their heterozygosity, polymorphism and allele ranges

1) A population study was carried out by analyzing a total of 441 randomly selected individuals from each of the three ethnic groups (Sinhalese, Tamils and Moors) for these 03 new MiniSTR markers. Following forensically vital statistics of each marker were also determined;

   Forensically vital statistics-

Table: 6 Determination of the alleleci frequency distributions and other forensically vital statistics of the three novel Mini-STR loci D4S2632, D6S2436and D19S589.

Allele	D4S2632				D6S2436				D192589			
	Moors	Tamils	Sinhalese	Sri Lanakn Pop	Moors	Tamils	Sinhalese	Sri Lanakn Pop	Moors	Tamils	Sinhalese	Sri Lanakn Pop
	N=122	*N=93*	*N=228*	*N=443*	*N=117*	*N=84*	*N=204*	*N=405*	*N=123*	*N=92*	*N=226*	*N=441*
6						0.173	0.007	0.009				
7					0.179	0.024	0.132	0.154				
8	0.082	0.118	0.066	0.081	0.026	0.19	0.025	0.025				
9	0.004	0.011	0.002	0.005	0.124	0.071	0.174	0.163				
10					0.09	0.077	0.098	0.090				
11	0.004			0.001	0.111	0.31	0.083	0.090	0.049	0.027	0.004	0.002
12	0.02	0.027	0.009	0.016	0.308	0.137	0.343	0.326	0.093	0.158	0.022	0.031
13	0.102	0.07	0.101	0.095	0.12	0.018	0.115	0.121	0.439	0.429	0.082	0.101
14	0.205	0.247	0.206	0.214	0.026		0.02	0.021	0.293	0.255	0.407	0.421
15	0.234	0.258	0.228	0.236			0.002	0.001	0.093	0.109	0.299	0.288
16	0.115	0.102	0.112	0.111					0.028	0.016	0.126	0.113
17	0.053	0.075	0.123	0.094					0.004	0.005	0.053	0.039
18	0.078	0.038	0.072	0.067							0.007	0.006
19	0.033	0.005	0.013	0.017								
20	0.004		0.004	0.003								
21	0.004		0.009	0.006								
22	0.008	0.005	0.011	0.009								
23	0.029	0.016	0.022	0.023								
24	0.008	0.005	0.009	0.008								
25	0.012	0.011	0.011	0.011								
26	0.004	0.011	0.002	0.005								
Forensic												
Matching Probability	0.039	0.059	0.04	0.038	0.055	0.078	0.063	0.059	0.157	0.128	0.118	0.127
Expressed as 1 in ...	25.5	16.9	24.7	26.5	18.2	12.8	15.8	16.9	6.4	7.8	8.5	7.9
Power of Discrimination	0.961	0.941	0.96	0.962	0.945	0.922	0.937	0.941	0.843	0.872	0.882	0.873
PIC	0.85	0.82	0.84	0.84	0.8	0.78	0.78	0.79	0.66	0.67	0.68	0.67
Paternity												
Power of Exclusion	0.517	0.652	0.714	0.644	0.621	0.639	0.579	0.604	0.493	0.456	0.427	0.451
Typical Paternity Index	2.03	2.91	3.56	2.84	2.66	2.8	2.37	2.53	1.92	1.77	1.66	1.75
Allele Frequencies												
Homozygotes	24.60%	17.20%	14.00%	17.60%	18.80%	17.90%	21.10%	19.80%	26.00%	28.30%	30.10%	28.60%
Heterozygotes	75.40%	82.80%	86.00%	82.40%	81.20%	82.10%	78.90%	80.20%	74.00%	71.70%	69.90%	71.40%
Total Alleles	244	186	456	886	234	168	408	810	246	184	452	882

Discussion

The emphasis of designing mini-STR primers was to obtain the smallest possible PCR amplicon for each STR marker of interest without affecting the primer binding ability to the flanking regions in PCR. When designing primers attempts were made to bring the forward and reverse primers as close as possible to the STR repeat region.

The nine mini-STR primer pairs were developed (Table:1) to accommodate an annealing temperature ranging from 50.7 to 54.5°C. However each mono plex PCR generated amplifications having minimal amount of non specific primer binding at an annealing temperature of 54°C (Table: 2) with 30ng of DNA template. Newly designed Primers were validated at initial mono-plex PCRs using Universal human DNA standard (K562 DNA) [Table: 3]. There were no abnormalities of the results obtained from the new primers when compared with the results of the commercially available conventional STR analysis kit of GenePrint STR Systems-Promega, USA.

The PCR amplicon size reduction achieved by the newly designed system with compared to the conventional STR analysis was between 18% to 65.9%. In STR loci CSF1PO, TOPX, THO1 and D16S539 size reduction was more than 60% each having a mean length of 106, 81, 68 and 155 base pairs respectively indicating the effectiveness of these loci in analyzing highly degraded biological samples which contained fragmented DNA. Therefore new mini STR system of the present study will increase the level of sensitivity in amplifying degraded biological samples (having DNA molecules less than 219bp in length) which could not be amplified in most of the Loci in conventional PCR system that demand lager template DNA fragments (224-327 bp).

Due to the small amplicon sizes (Ranging from 26 bp-219 bp) of each amplified loci, it limits the incorporation of all the nine loci into a single multiplex PCR amplification. However, the nine loci were incorporated in to a system which can be co-amplified in four sets of PCR reactions (Table: 5). This four reaction PCR system that comprise of two triplex reactions, one duplex and a monopelx has reduced the cost of PCR reagents nearly by 40% than they are amplified as in individual PCR reactions (Mono-plexes).

Since STR loci D8S1179 could not be made in to a multiplex due to its overlapping amplified fragment lengths (PCR product sizes ranging from 59- 91) and annealing temperature, it is amplified as monoplex in order to genotype it along with other eight loci in low cost polyacrylamide gels.

All the nine mini-STRs were integrated into a low cost polyacrylamide gel followed by Silver Staining genotyping method. The size of each allele in the allelic ladders (standard size markers) constructed for each mini- STR were validated by using universal human DNA K562 (Figure: 1 and Figure: 2) . All the amplicons that were subjected to polyacrylamide gel electrophoresis were successfully visualized by low cost Silver staining procedure without the need of expensive automated genotyping. Allele sizs of each sample were determined by comparing them with validated ladders.

Allele sizes of the samples that were amplified using the new system were validated using a comparative concordance study with two commercially available kits (GenePrint STR Systems-Promega, USA -conventional STR kit and AmpFℓSTR® MiniFiler kit, Applied Bio systems, USA- Commercial mini-STR kit). Allelic results of four positive paternity testes that were obtained using conventional STR analysis of (Table: 3) and commercially available AmpFℓSTR® MiniFiler were found to be consistent with the results obtained by the mini-STR system of the present study. No non-specific primer annealing or allele drop-outs in PCR were detected at any loci in the newly established system.

Three primer pairs were developed for three novel STR loci D4S2632, D6S2436 and D19S589 that generate PCR amplicons having lengths of 105, 91 and 98 base pairs respectively (Table: 5). All the three STR loci comprise of having tetra nucleotide repeats. The repeating region and the flanking regions of each PCR amplicon was explored by sequencing.

Allelic frequencies and forensically vital statistics were determined for D4S2632, D6S2436and D19S589 upon performing the population study. The feasibility of using each maker for human identification purposes were assessed using the statistic "Power of Discrimination" (PD). PD were high in D4S2632 and D6S2436 (more than 0.92) and D19S589 recorded a value of 0.843. The Polymorphism information Content (PIC) of each of the three mini-STR loci among the ethnic populations was more than 0.6 indicating a high level of polymorphism across these STRs among them. This further justifies that these three loci are polymorphic enough to discriminating individuals at DNA level.

The most heterozygote loci was D4S2632 (82%) while D19S589 report as having the least heterozygosity (71.4%). The PIC values for 11 conventional STR loci that are presently being used for forensic human identity testing is between 0.57 to 0.80 (Illepruma et al., 2008).

Cost effectiveness of the new mini-STR system was evaluated by comparing the cost per sample with that of a commercially available test kit. The price quoted by the Sri Lankan agent of Applied Biosystems, USA for the commercially available mini-STR system AmpFℓSTR® MiniFiler kit is Rs. 508,690/-. The kit contains reagents to 100 individual PCR reactions each of which will accommodate a total of 09 mini-STR loci to be co-amplified in a single PCR reaction. After performing PCR, detection of alleles of each system (genotyping) has to be completed using an automated genotyping instrumentation manufactured by the same company which makes reaction kits. The cost to complete one PCR reaction (one sample) would be Rs. 5086/-. For genotyping, an additional amount of Rs. 2000/- has to be spent for the chemicals used for the procedure that would increase the total expenditure for reagents alone up to Rs. 7086/- per sample. Nonetheless an additional initial capital of 7.5 million rupees need to be invested on a automated genotyping instrument (Applied Biosystems® 3130/3130xl Genetic Analyzer).

However the cost per PCR reaction in the newly enveloped method is Rs. 300/-. Therefore a total cost of Rs. 1200/- would be placed to cover all the nine STRs of a sample. Since the genotyping procedure is a polyacrylamide based system, the cost per sample to be genotyped is only Rs. 400/-. Therefore the total expenditure to complete the analysis of a sample by the new system is Rs.1600/.

Allele frequencies, Polymorphism information content (PIC), power of discrimination (PD), power of exclusion (PE), typical paternity index (TPI), homozygosities and heterozygocities were determined as forensically vital statistics for Human STR loci D4S2632, D6S2436and D19S589 and has presented in Table: 6 of the final report. The feasibility of a STR locus to be used for DNA based human identity testing is evaluated by these forensically vital statistics. These statistics should be determined by a population study that has to be carried out by analyzing a representative sample of randomly selected individuals from the respective population. A total of 441 randomly selected individuals from each of the three ethnic groups of Sri Lanka (Sinhalese, Tamils and Moors) were tested for the population study for these 03 new Mini-STRs.

Allelic frequencies of the tested DNA loci are determine in order to use them when calculating the significance of a DNA profile match in DNA based paternity and identity testing. An allele with more than 0.5 allelic frequency is not very useful in paternity testing (Weir, 1996) which will result in lowering the Power of discrimination between individuals in DNA based human identity testing.

Homozygosities and heterozygocities values indicate the level of heterozygocity of each allele in the tested STR loci. It is presented in the table as percentages. It is generally accepted that the loci having heterozygocities lower than 50% are not suitable to be used in human identity testing purposes. The most heterozygote loci of the present study was D4S2632 (82%) while D19S589 having the next most heterozygote (71.4%).

The Polymorphism information Content (PIC) expresses the level of polymorphism in each STR loci. Higher PIC indicates the loci is polymorphic and thus have more power in DNA based discrimination between human individual. This power is express in the statistic power of discrimination (PD). Each of the three mini-STR loci among the ethnic populations was reported to be more than 0.6 (Table: 6) indicating a high level of polymorphism across these STRs among them justifying that these three loci are polymorphic enough to discriminating individuals at DNA level in forensic identity testing. The PIC values of the loci in the present study is higher than that of 11 conventional STR loci that are presently being used for forensic human identity testing (between 0.57 to 0.80) as reported by Illepruma et al., 2008.

An alternative way of expressing the feasibility of using each maker for human identification purposes were assessed using the statistic "Power of Discrimination" (PD). PD were high in D4S2632 and D6S2436 (more than 0.92) and in D19S589 it was recorded as 0.843.

The statistic typical paternity index (TPI) is relevant with regard to DNA based parentage testing. If a child and a father are sharing fifty percent of alleles among them that indicate that they are having father child biological relationship. The significance of this conclusion is given by TPI.

Therefore the cost reduction for analysis of a sample by the newly established system is Rs.5486/- (77% net savings in addition to the savings of initial investment for automated instrumentation and its maintenance cost).

Conclusions

1. A validated cost effective method was established for DNA based analysis of human mini STR loci; CSF1PO, TPOX, TH01, D7S820, D8S1179, D13S317, D16S539, vWA and F13A.

2. Analysis methods to test three novel human Mini-STR markers D4S2632, D6S2436and D19S589 were established.

3. The novel human Mini-STR markers D4S2632, D6S2436and D19S589 were polymorphic enough to be used for forensic human identification purposes in Sri Lanka.

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Major findings

1. A net amount of 77% cost reduction could be achieved by the newly developed mini-STR system than analyzing the samples with the commercially available mini-STR kit.

2. Statistics indicate that the three novel human Mini-STR markers D4S2632, D6S2436 and D19S589 could be used for human identity testing in Sri Lanka.

Section 4

Impact of research Results

Relevance of results achieved to scientific advancement

The overall outcome of the project will directly contribute to reduce the cost to analyze a decomposed biological sample. The level of sensitivity of the newly established mini-STR approach was demonstrated on degraded DNA evidence upon completion of the project by Goonawardhana *et al*, 2014. By optimizing the methods for analysing decomposed biological samples and evaluating the feasibility of applying the method to Sri Lanka, it is expected that the success rate of DNA based forensic identification will be increased by 10-15% from the current status.

The research student who has successfully completed the requirements of M.Phil degree by the University of Colombo and has been trained intensively on automated and manual genotyping of mini-STRs in human samples and statistical analysis of forensic genotyping results.

Having successfully completed the project, more junior scientists will have the opportunity to be trained on novel cost effective methods of testing biological evidences.

The methods established for cost effective sample testing at Genetech mark as the establishment of the first validated method to analyze human mini-STRs in Sri Lanka.

Relevance of results achieved to national/socio-economic development

DNA based human identification in criminal casework has been carried out in Sri Lanka since 1999. Up to the present this technology has been applied to resolve nearly 3400 cases that were referred by the Courts. However, as a result of technological limitations in DNA analysis methods, analysis of decomposed biological evidence often remained unresolved. The establishment of the new method of analysing decomposed biological samples and evaluating the feasibility of applying the method in Sri Lanka, it is expected that the success rate of analyzing decomposed biological evidence is increased by 10-15% from the previous status.

This enables the capabilities to analyze degraded DNA evidence at a lower cost, which will eventually provide this service at an affordable cost to the government of Sri Lanka. The payments for testing are settled directly by the Police Department for each case they investigate. Therefore the reduction of cost is directly benefited by the government of Sri Lanka. Maintaining the affordability of the services is a vital factor for sustainability of these novel techniques.

Since the new mini STR system was validated against commercially available kit, it can be assured that this significant cost reduction did not compromise the accuracy of the test results. Therefore, the forensic DNA testing services can be offered in Sri Lanka at a cost which is well affordable while increasing the sensitivity and accuracy of testing.

Application of research project

Genetech is the largest service provider to the government of Sri Lanka for DNA based human identification in criminal and civil case work.

The direct outcome of application of the research findings will be an enhancement of the current detection levels of analyzing biological evidences in DNA based forensic testing at a significantly low cost per sample while maintaining the levels of international standards of accuracy. This will deliver the latest technological services at an affordable cost to the Sri Lankan law enforcement to resolve more crimes.

Section 5

Miscellaneous

List of major equipment acquired during the project period

The present research was carried out at Genetech, which is equipped with all the necessary facilities and instrumentation to perform the proposed research work. Therefore no new equipment were perched for the research project.

List of publications/communications arising from the project

(Copies are attached herewith)

1. Evaluation of new Mini STR markers for DNA based human identification; An *in silico* approach. *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J illeperuma* (Proceedings of the Annual Sessions – University of Colombo 2012).

2. Application of miniaturized human STRs (Mini-STRs) in DNA based identification of a formalin fixed foetus: A case report *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J Illeperuma.* (proceedings annual research symposium 2013, university of Colombo 2013).

3. Successful DNA based identification of a formalin fixed foetus using Human Mini STR markers-A case report *N.D.S. Goonawardhana, N.D. Fernandopulle, Preethi V. Udagama Ruwan J Illeperuma.* (Medico-Legal Journal of Sri Lanka, August 2014).

Section 6

Summary Statement of Expenditure

Expenditure from the institution

Expenditure for sample collection: .....Rs. 33,250.00

Usage of Genetech lab bench space and equipment: .....Rs. 173,650.00

Research student's salary for the study period (2 years): .....Rs. 600,000.00

Total expenditure from Genetech: .....Rs. 806,900.00

Expenditure from funds received from NSF (Grant No. RG/2011/BT/07)

Expenditure to purchase consumables 1st year: .....Rs. 480,000.00

Expenditure to purchase consumables 2nd year: .....Rs. 468,500.00

Post graduate registration fees paid: .....Rs. 11,500.00

Total expenditure: .....Rs. 960,000.00

Section 7

Grantee's signatures

Principal Investigator

Signature: ..... 04/12/2014.

Name: Dr. Ruwan J. Illeperuma

Title: Senior Scientist

Co-investigator – 1

Signature: .....

Name: Dr. Neil D.Fernandopulle

Title: Quality Assurance and Technical Manager, Biology, Centre of Forensic Sciences, Toronto, Canada.

Head of the institution signature

Signature: .....

Name: Mr. D. N. Gunasekera

Title: Head of the Institution

Table 1.1: Buffers, stock solutions and reagents for general purposes

1M Tris	121.1 g of Tris base was dissolved in 800 ml of sterile distilled water. The pH was adjusted as required using concentrated HCl. Sterilized by autoclaving.
0.5M EDTA	186.1 g of disodium ethylenediaminetetra-acetate.2H2O was dissolved in 800 ml of sterile distilled water. The pH was adjusted to 8.0 with NaOH. The volume was made up to 1000 ml with distilled water. Sterilized by autoclaving.
TE (pH 7.6)	10 mM Tris/HCl (pH 7.6), 1 mM EDTA (pH 8.0). Sterilized by autoclaving.
TE (pH 8.0)	10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0). Sterilized by autoclaving.
5x Gel loading buffer for agarose gels	10% w/v Ficoll (MW 400,000), 40% w/v sucrose and 0.25% w/v bromophenol blue in water. Stored at 4 °C.
5x TBE for agarose gels (pH8.0)	0.45 M Tris base, 0.45 M Boric acid and 0.01 M EDTA
Ethidium bromide	10 mg/ml in water, stored in a dark bottle at room temperature.

Table 1.2 Buffers, stock solutions and reagents used in DNA extraction

Proteinase K	20 mg/ml in sterilized water stored at -20 °C.
Protease Buffer	1 M Tris (pH 8.0), 0.5 M EDTA, Sterilized by autoclaving.
Wash Buffer Ispropanol	70% ethanol
Guanidium Lysis buffer	GuSCN, 1 M Tris/HCl (pH 6.4), 0.2 M EDTA (pH8.0), Triton X-100, sterilized by filtration.
Chelex-100 (Bio-*Rad*)	5% Chelex -100 in sterilized water. Stored in room Temperature
TNE Buffer	17mM Tris/HCl (pH 8.0), 50mM NaCl and 7mM EDTA , sterilized by autoclaving.
Lysis solution	10mM Tris–HCl (pH 8.0), 0.5% SDS, 5mM EDTA,sterilized By autoclaving.

Table 1.3 Buffers, stock solutions and reagents used in PCR

Taq DNA Polymerase	5units/µl. Purchased from Promega Corporation (USA),

	Roche GmbH, Appligene Oncor (France), and Perkin Elmer (USA).
10x PCR buffer	20 mM Tris/HCl (pH8.3), 100 mM KCl, 1mM DTT, 0.1mM EDTA,50%Glycerol, 0.5% NP40, 0.5% TW20 (Sigma, USA)
5 mM dNTP mix (Promega)	50 µl each of 100 mM dATP, dCTP, dGTP and dTTP diluted to 1000µl with sterile distilled water. Stored at -20 °C.
10 mM dNTP mix (Promega)	100 µl each of 100 mM dATP, dCTP, dGTP and dTTP diluted to 1000µl with sterile distilled water. stored at -20 °C.
25 mM MgCl2	
20mg/ml BSA	Diluted with sterile distilled water. Stored at -20 °C

Evaluation of new Mini STR markers for DNA based human identification: An *in silico* approach

N.D.S Goonawardhana[1], Preethi V. Udagama[2], N.D Fernandopulle[1], Ruwan J. Illeperuma[1]

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Introduction

Analysis of Short Tandem Repeat (STR) markers on the human genome is an important tool in the identification of individuals based on their genetic makeup. DNA extracted from materials of biological evidence is subjected to Polymerase Chain Reaction (PCR) to generate DNA profiles, unique to an individual. However, forensic analysts are challenged to obtain a complete DNA profile by the failure in PCR amplification of such biological evidence due to several factors: for example, high humidity and temperature in the tropics (as in Sri Lanka), promote the degradation of such biological materials and rapidly reduce the possibility of typing such nuclear DNA. Conventional PCR technology used in Sri Lanka analyses amplicons generated in the size range of 150 to 450 nucleotide bases. Hence, DNA in a biological sample fragmented into smaller sizes cannot be amplified by PCR. Therefore, we have evaluated the application of three new miniaturized –Short Tandem Repeat markers (mini-STRs) screened for possible application in degraded DNA evidence analysis. These novel STR markers are capable of generating smaller sized PCR amplicons (less than 150 bp) by PCR. No previous studies have been recorded in the application of these three STR loci.

Materials and Methods

Selection of STR loci

The three Mini-STR markers were selected by screening the STR marker sets published in Marshfield Clinic Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/). The selected STRs, namely D4S2632, D6S2436, and D19S589 have not been adapted to human identity testing up to date. The sequences of the STR loci screened were obtained from a BLAST –nucleotide search on www.ncbi.com. The chromosomal positions were determined by BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) and assembly of the human genome version of Feb2009.

Primer designing

The manual primer designing tool of the standalone "Primer premier 3" software was used to effectively locate primers adjacent to the repeat region of each STR locus. The G and C content of the primer were maintained within the range of 40 % to 60 % and the melting temperatures from 53 °C -55 °C. Successfully designing primer sets were evaluated for probable inter-primer interactions using the options in "Primer premier 3" software.

Evaluation of nucleosome forming potentials (NFPs)

Nucleosomes are the basic repeating structural and functional unit of chromatin consisting of nine histone proteins and about 166 bp of DNA (Kogan et al, 2006; Holde, 1988). The algorithm of Nucleosome eXclusion Sensor (NXSensorversion 1.3.1) (http://www.sfu.ca/_ibajic/NXSensor/) was used to determine the nucleosome-free regions of the selected STR marker sequences. The algorithm detects an input sequence for three known nucleosome-free sequences: 10bases of poly-A, 10 bases of poly-T, and a combination of Gs and Cs (A_10, T_10, or [(G/C) 3N2] _3) (Thanakiatkrai et al, 2011). If any of these sequences are detected in the given sequence, the program outputs the sequence in FASTA format and highlights the nucleosome-free region.

The FASTA sequences (~ 100bp up and downstream to the repeat motif) of novel STR markers D4S2632, D6S2436, and D19S589 were entered in to the web based NXSensorversion 1.3.1 and the accessible scores for each STR marker were calculated according to the following formula (Luykx et al, 2006):

$$\text{Accessibility}(OSmin) = \frac{\text{Total length of open contiguous segments } \geq OSmin}{(\text{length of input sequence}) - (\text{total length of ambiguous segments})}$$

Results and Discussion

Screening Candidate Loci

STR Locus	Chromosomal location	Chromosomal pb Position	Repeat Motif	GenBank accession	GenBank allele	Mini-STR amplicon length (bp)	Accessibility (*OSmin*)
D4S2632	4p15-p14	35704165	(GATA)n	G08391.1	13	105	0
D6S2436	6q24.1	154136091	(GATA)n	G27284.1	9	91	0
D19S589	19q13.42	58498394	(GATA)n	G08026.1	13	98	0

Table 1—*Information on three novel STRs evaluated in this study. Chromosomal location and base pair (bp) position of each marker was determined by using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) and the Feb.2009 assembly of the human genome. The miniSTR amplicon length is based on the GeneBank allele observed. The accessibility(OSmin) values for the three STR loci D4S2632, D6S2436 and D19S589*

The potential mini STR markers were screened from previously well characterized STR loci (Ghebranious et al, 2003) including the whole genome screening sets of STRs reported extensively in genetic linkage studies across the entire nuclear genome to determine specific genes that cause or has a linked to human diseases. A subset of the screening markers were also used to characterize the genetic diversity in global populations (Rosenberg et al, 2002 & 2003; Ayub et al, 2003)

When screening the probable STR loci to be used as mini STR markers, we considered several characteristics of each STR locus in order to obtain the best possible loci. Firstly, the STR loci consisting of tetrameric repeat motif were selected, as is strongly recommended in forensic DNA analysis, since these markers are highly polymorphic, with heterozygosity >0.90 and results reduced stutter effect in the PCR reaction (Kimpton et al, 1993). Secondly, the STR loci containing tetrameric repeat motifs were evaluated for "clean flanking regions" upstream and downstream to the repeat motif (approximately

75bp). This approach is very important when the PCR primers are located closer to the repetitive region since a clear flanking region can give rise to good primer hybridization in the PCR reaction. Thirdly, the STR loci that can produce PCR fragments less than 150 bp were selected. Finally, the selected three novel STR loci D4S2632, D6S2436 and D19S589 were evaluated for the nucleosome forming potential (NFPs) by calculating the accessibility (*OSmin*) for each STR marker with the formula (Butler et al, 2003); 1). The accessibility (*OSmin*) values for the STR markers D4S2632, D6S2436 and D19S589 were found to be zero which demonstrates the likelihood of a DNA sequence to be bound by nucleosomes.

Conclusion

This approach outlines the initial efforts to develop mini STR markers that can be used to supplement the loci included in CODIS (Combined DNA Index System) core loci to increase the power of discrimination in analyzing highly degraded DNA evidence in forensic casework. Apart from using these markers for forensic purposes, they can be successfully incorporated in cases where additional markers are needed to conclude the analysis such as human parentage testing or the analysis between closely related individuals.

References:

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Application of miniaturized human STRs (Mini-STRs) in DNA based identification of a formalin fixed foetus: A case report

N.D.S. Goonawardhana[1], N.D. Fernandopulle[1], Preethi V. Udagama[2] and Ruwan J. Illeperuma[1]

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Accurate identification of an individual is fundamental in DNA based forensic investigations. In order to establish identity of a biological sample, Short Tandem Repeat (STR) DNA markers in the human genome are being used extensively considering their high discrimination power over classical methods of human identification. However, biological samples containing environmentally challenged, degraded or damaged and fragmented DNA in low concentration may reduce the chance of obtaining informative results. When unbuffered formaldehyde is added to a biological sample as a preservative, it makes DNA molecules irreversibly fragmented and degraded resulting conventional DNA testing unsuccessful. Further it was reported that the amount of DNA required for a successful PCR amplification from a formalin fixed tissue is dependent on the time of fixation. This limit increase from 10 ng after three hours to 100ng after one week of fixation in unbuffered 10 % formalin. Even though DNA in the sample is fragmented, miniaturized STR DNA markers (Mini-STRs) can still produce successful DNA profiling results due to its ability to generate reduced sized DNA amplicons during Polymerase Chain Reaction (PCR). We have demonstrated a successful genetic identification of a formalin fixed foetus which has been preserved in 10 % formalin for nearly two months, using a newly designed human Mini-STR DNA marker system. This further highlights how a miniaturized PCR amplicon is more suitable over conventional STR amplification using PCR, highlighting the value of Mini-STR approach for analyzing degraded biological samples in DNA based forensics.

Medico - Legal Journal of Sri Lanka - Aug 2014.

Successful DNA based identification of a formalin fixed foetus using mini STR markers-A case report

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Examination of polymorphisms in genetic material, by DNA typing is a powerful tool in the identification of individuals and in the confirmation of familial relationships in criminal investigations. Since 2002, DNA based identification methods has been successfully applied in more than 3300 criminal cases that have been referred by the Courts of Sri Lanka. In forensic DNA typing, Short Tandem Repeat (STR) DNA markers are analyzed from biological evidence with the use of Polymerase Chain Reaction (PCR) amplification to generate DNA profiles that are unique to an individual.

The ability to perform DNA analysis is dependent on the quantity and quality of DNA that is available. The degradation of DNA is caused by microbial activity or environmental conditions. Under certain circumstances, when DNA is degraded, and is found in minute quantities, conventional DNA analysis methods fail. This case report describes the successful identification of a foetus exhumed from a deceased body and preserved in formalin for two months under ambient temperature. The preservation of animal tissues in formalin causes irreversible damage to DNA by depurination, cross-linking between proteins and nucleic acids and fragmentation of DNA.

In the presence of such compromised DNA, targeting larger sized DNA fragments ranging from 200 to 450 base pairs as used in conventional STR analysis has shown to be less successful. The use of reduced sized DNA amplicons by moving forward and reverse PCR primers towards the target STR region enables the analysis of DNA fragmented by preservation in formalin. We have demonstrated successful identification of a foetus using Mini-STR marker system developed in-house for the human STR loci CSF1PO, TPOX, THO1, D7S820, D13S317 and D16S539.

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