Rwanda Medical Journal RMJ

ORIGINAL ARTICLE Open Access

Paternity testing using 21 STR Loci in a biotechnology approach: case of Rwandan Population

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ABSTRACT

INTRODUCTION: We focused on a sample size of 141 unrelated Rwandan persons to genotype 21 STR loci that were relied up in establishing allele frequencies, heterozygosity and power of exclusion. This study aims at exploring allele frequencies on a representative sample from Rwandan population to determine probability of paternity for sampled families basing on polymorphic STRs loci, using 21 autosomal-STR loci by Genetic Analyzer 3500X.
METHODS: This was an experimental study and global filer TM Express PCR Amplification kit was used to amplify 21 autosomal STR loci.
RESULTS: The total number of observed alleles was 270; the largest number of different alleles was seen in SE33 and D18S51 loci. The locus with the highest heterozygosity was SE33, while locus TH01 had the lowest heterozygosity. The heterozygosity of the 21 STR loci ranged from 71.3% (TH01) to 91.6% (SE33) with an average of 81.1% a good indicator of high genetic variability. For all microsatellites analyzed the power of exclusion ranged from 43.4% (TH01) to 78.1% (SE33) with an average of 58.2%. For seven of eight cases examined in paternity test alleged father was not excluded as biological father of child. The results found in examination of case 8 indicated that the alleged father was not the biological father of the child.
CONCLUSION: Based on calculated statistical parameters, the population of Rwanda may use these 21 STR loci as a vital tool for forensic identification and paternity testing.

Keywords: Humans, Autosomal, Forensic, DNA Sequence, Heterozygosity, Paternity Testing

INTRODUCTION

DNA paternity testing is the use of DNA profiling to determine whether two individuals are biologically parent and child. A paternity test establishes genetic proof whether a man is the biological father of child and a maternity test establishes whether a woman is the biological mother of child [1]. DNA testing is currently the most advanced and accurate technology to determine parentage. As demonstrated in DNA parentage test, the result called the probability of parentage is 0% when the alleged parent is not biologically related to the child and the probability of parentage is typically 99.99% when the alleged parent is biologically related to the child [1].

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Received: 13nd April 2023; **Initial decision given:** 20th February 2023; **Revised manuscript received:** 05th May 2023; **Accepted:** 28th May 2023.
 Publisher: Rwanda Biomedical Centre (RBC)/Rwanda Health Communication Center, P. O. Box 4586, Kigali. ISSN: 2079-097X (print); 2410-8626 (online)

Citation for this article: A. Ndungutse; F. Karege; C. Nsanzabaganwa et al. Paternity testing using 21 STR Loci in a biotechnology approach: Case of Rwanda Population. Rwanda Medical Journal, Vol. 80, no. 2, p. 60-68, 2023. https://dx.doi.org/10.4314/rmj.v80i2.9

Paternity can be determined by highly accurate tests conducted on cheek cells, blood or tissue samples from the father, mother and child [2]. Such tests can exclude a man who is not the biological father, and can also show the likelihood of paternity if he is not excluded. If the father could be one of several men, each may be required to take a genetic test. There are several different ways to establish whether an alleged father is the natural and legal father of the child [3], including blood typing, human leukocyte antigen (HLA), restriction fragment length polymorphism (RFLP) and short tandem repeats (STRs). Each of these techniques targets hereditary information or genes inherited from parent to child. If the alleged father does not possess targeted genes, then he cannot be the biological father of child.

Worldwide, DNA paternity test is used for different purposes. As explained by Barata[4], immigration authorities in various countries, such as U.S, Canada, France, and others have been requesting immigration petitioners and beneficiaries in a family-based immigration case to voluntarily take the DNA parentage test when primary documents such as birth certificate to prove biological relationship are missing or inadequate. According to EasyDNA in South Africa, DNA paternity testing is used for court cases as a court admissible proof of a biological relationship between the alleged father and the child [5]. In Rwanda, DNA technology including paternity test is rapidly emerging and applied in legal and civil affairs [6].

This study aims at making a prospective study on paternity tests using 21 autosomal-STR loci by Genetic Analyzer 3500X, establish allele frequencies on a representative sample from Rwandan population hence determining probability of paternity for sampled families basing on polymorphic STRs loci.

MATERIALS

Population and sample collection

Buccal swabs were collected from 15 healthy individuals (including 8 children and 7 parents) corresponding to 8 cases. For both first and second case samples were collected from a father, mother, and a child. For the third case samples were collected from a father and a child. For the fourth and fifth case samples were collected from a father, mother, and a child. For the sixth and seventh samples were collected from a father and a child. For eighth case, samples were collected from three individuals including father, mother and a child.

DNA amplification

Polymerase Chain Reaction (PCR) is the process used to amplify a specific region of DNA. It is possible to create multiple copies from a small amount of template DNA using this process. The PCR amplification was carried out in ProFlex PCR system (Applied Biosystems) according to the manufacturer's recommendations [6]. After buccal rubbing, the head of each swab were detached from swab shaft and placed immediately in 1.5-ml tube. All tubes were brought in DNA extraction room where 400µL of Prep-n-GoTM buffer were added to each tube and let stand for 20 minutes at room temperature to lyse the sample. PCR reagents Master Mix and Primer mix were prepared as well as volume of each component (master mix and primer mix) needed to add to PCR reactions. Six µl of master mix and 6µl of primer mix (12 µl) were required per one reaction and multiplied 16 reactions (15 reactions of samples and one for positive DNA control 007) to sum up to 192µl (96 µl of master mix plus 96 µl of primer mix) into an appropriately sized polypropylene tube to make reaction mix. The reaction mix was vortexed for 3 seconds. 12µl of reaction mix were dispensed into each PCR tube. 3µl of DNA extract from sample lysate tube were added to each PCR tube to make total volume of 15 µl (12 µl of reaction mix plus 3 µl of DNA test sample). The negative control tube of 3 µl Prep-n-GoTM Buffer was required to be put in thermal cycler as well as PCR reaction tubes. Thermal cycling conditions were programmed. At the first, machine was incubated at 95oc for one minute; optimum cycle number was set where denaturation temperature was adjusted at 94oc for three seconds and annealing/extension temperature was 60oc for 30 seconds; and the final extension step at 60oc for 8 minutes. The final hold of thermal cycler was maintained at 4oc for more than 24 hours. After settings, PCR reaction tubes and negative control were loaded into thermal cycler in order to start running.

Electrophoresis of the amplification products

In the time of waiting for readiness of PCR products, we were preparing the third major step of forensic DNA test called capillary electrophoresis after extraction and amplification. In performing this

task, 0.5 µl of GeneScanTM 600 LIZ® Size Standard v2.0 and 9.5µl of Hi-DiTM Formamide per one reaction were pippeted into an appropriately size polypropylene tube. Total volume required for all reactions was 180 µl (including 9 µl of GeneScanTM 600 LIZ® Size Standard v2.0 and 171 µl of Hi-DiTM Formamide). The tube containing mixture was then vortexed and centrifuged briefly. Into each well of a MicroAmp® Optical 96-well Reaction Plate, 10 µl of the formamide/size standard mixture and 1 µl of PCR product or Allelic ladder were added. To respect injection requirements (8 samples per injection), 10 µl of Hi-DiTM Formamide were added into blank wells. Then reactions' plate was sealed with appropriate adhesive film to prevent evaporation and heated in thermal cycler for 3 minutes at 95oC. Immediately, plate was placed on ice for 3 minutes. Finally, the remaining work was to prepare the plate assembly on auto sampler to start the electrophoresis using Genetic Analyzer 3500X (applied Biosystems) [7]. To obtain the sample genotype, the allelic ladder is the standard to which STR alleles are compared. By comparison of the size of a sample's alleles to the size of alleles in allelic ladders for the same loci being tested in the sample, the STR genotyping was conducted.

Allele frequency determination

In the present study, 141 DNAs of unrelated individuals from Rwandan population were used. We converted their genotypes for 21 STRs into allele frequencies by counting the number of times each allele was observed in all giving samples. Since there are some alleles which were not sampled sufficiently and that an estimate of an allele frequency is uncertain if the allele is so rare that it is represented only once or a few times in a dataset, it is recommended that each allele be observed at least five times to be used in forensic calculations. As explained by Hameed et al. [8], the minimum allele frequency is 5/ (2n) where n is the number of individuals sampled and 2n is the number of chromosomes (as autosomal are in pairs due to inheritance of one chromosome from each parent). STRs loci studied are D3S1358, Vwa, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, and D2S1338.

Analysis and Computation

Gene Mapper® ID-X Software was used to perform sizing and allele calling, to make the final genotype or support reviews, editing and further processing of the sample. Microsoft Office Excel 2007 was used to calculate various parameters relevant to this study. fx COUNTIF was used to determine alleles count; fx QUOTIENT was used to calculate allele frequencies; fx PRODUCT was used to determine Homozygosity, Power of exclusion and Combined paternity index. In addition, FX-82MS (Scientific calculator) Display type DOT/LCD and Algebraic input logic S-V.P.A.M was also used for simple calculations [9]. The formula used to compute various parameters for population data analyses were described in chapter of literature review.

RESULTS

We illustrated the findings of the experiments in Appendices 1 to 6. Appendix 1 shows allele frequencies, while others (Appendix 2 to 6) illustrate paternity testing results.

Allele frequency results

In the Appendix 1 each allele has two values presented in the same row. The first value which follows a given allele in a row is allele count standing for number of times the allele was counted on a given locus within a population of 141 individuals. The second value is allele frequency standing for a ratio between allele count and total chromosomes (2n).

Paternity testing results

This section shows the results obtained by typing autosomal STRs of the family members. In some families, DNA profile of mother was available but in others not. GlobalFilerTM Express PCR amplification system was used for typing 21 autosomal STRs loci in alleged fathers, mothers (for some cases) and children.

The table 1 and 4 illustrates a column of 22 STR loci genotyped for the present member of the family; columns of a child and parent (alleged father and mother for some cases); a column of an obligate allele that a father should has donated to a child, while remaining column(s) show(s) calculated paternity index. At the bottom, there is a combined paternity index (CPI) with corresponding paternal affiliation probability (POP).

Combined paternity index (CPI) obtained is 15550738.19 and probability of paternity (POP) is

Table 1: Forensic efficiency parameters of the autosomal 21 genetic loci

Locus	Homozygosity	Heterozygosity	Power of Exclusion
D3S1358	0.26273	0.73727	0.465939
TH01	0.286922	0.713078	0.434296
D21S11	0.157137	0.842863	0.631112
D18S51	0.140189	0.859811	0.662658
D5S818	0.213163	0.786837	0.537407
D13S317	0.228001	0.771999	0.514998
D7S820	0.215057	0.784943	0.534495
D16S539	0.209065	0.790935	0.543761
CSF1PO	0.212677	0.787323	0.538157
VWA	0.158478	0.841522	0.628684
D8S1179	0.194563	0.805437	0.566847
TPOX	0.222427	0.777573	0.523308
FGA	0.136136	0.863864	0.670446
D19S433	0.191785	0.808215	0.57138
D2S1338	0.094042	0.905958	0.757409
D22S1045	0.193117	0.806883	0.569202
D1S1656	0.156755	0.843245	0.631805
D10S1248	0.207887	0.792113	0.545601
D2S441	0.227703	0.772297	0.515439
D12S391	0.174689	0.825311	0.600091
SE33	0.083349	0.916651	0.781403

99.9999967% for the first case; CPI is 18061812.21 and POP is 99.999999972% for second case; CPI is 463469608.6 and POP is 99.9999998% for third case; 3588893.003 and POP is 99.999986% for fourth case; 15902241.36 and POP is 99.9999968% for fifth case; 20965051.99 and POP is 99.9999976% for sixth case, while it was 3843092.86 with POP of 99.9999869% for seventh case. Table 4 for case eight does not illustrate paternal affiliation probability since the results exclude alleged father who is not the biological father of a child.

DISCUSSION

The relation of our results to other works having experiments related to this study and their conclusions are here discussed. It involves a comparison of results and an analysis of their significance.

Based on the allelic frequencies, some statistical parameters of genetic and forensic efficiency have been estimated. These include the Homozygosity, Heterozygosity, and Power of exclusion as well as Combined paternity indexes for estimating parentage probability in examined cases

The total number of alleles observed across the population was found to be 270 alleles for this particular study (Appendix). It can be concluded that there is a high level of polymorphism of the selected microsatellites. SE33 and D18S51 loci illustrate the largest number of different alleles. The following locations of the most common alleles at the 21 loci were allele 16 for D3S1358 locus, allele 7 for TH01 locus, allele 30 for D21S11locus, allele 16 for D18S51, allele 12 for D5S818, allele 11 for D13S317 locus, allele 10 for D7S820 locus, allele 11 for D16S539 locus, allele 10 for CSF1PO, allele 16 for VWA locus, allele 14 for D8S1179 locus, allele 8 for TPOX locus, allele 22 for FGA locus, allele 13 for D19S433 locus, allele 17 for D2S1338locus, allele 15 for D22S1045locus, allele 14 for D1S1656 locus, allele 13 for D10S1248locus, allele 14 for D2S441locus,

Table 2: DNA profiles of a child, mother and alleged father

DNA Probe/Locus	Child (A2A2)	Mother (A1)	Alleged father (A)	Obligate allele	Paternity index (PI)	Selected paternity index(PI)
D3S1358	16,16	14,16	16,17.1	16	1.42	
VWA	17,18	17,17	16,18	18	2.56	2.56
D16S539	11,13	11,11	11,13	13	2.76	
CSFPO	10,11	10,11	11,13	11	2.47	
TPOX	9,12	9,9	10,12	12	14.28	
D8S1179	14,14	14,15	14,15	14	1.66	
D21S11	28,31	28,31	27,28	28	2.43	2.43
D18S51	15,17	15,19	15,17	17	2.67	2.67
DYS391			10,10			
D2S441	11.3,12	11.3,12	11.3,13	11.3	10.20	
D19S433	13,16.2	13,14.3	14,16.2	16.2	29.41	29.41
TH01	6,7	6,7	6,9	6	2.71	
FGA	22,24	23,24	22,25	22	2.13	2.13
D22S1045	15,17	14,15	17,17	17	6.13	
D5S818	12,12	10,12	8,12	12	1.56	
D13S317	9,12	11,12	9,12	9	23.80	23.80
D7S820	8,10	10,12	8,10	8	2.61	2.61
SE33	13,18	18,18	13,19	13	29.41	29.41
D10S1248	12,15	12,13	14,15	15	2.04	
D1S1656	14,15	12,15	11,14	14	29.41	29.41
D12S391	15,18	15,21	17,18	18	1.62	1.62
D2S1338	20,21	18.3,20	21,25	21	5.05	5.05
Combined paternity index (CPI)	$PI_1*PI_{2.....*}PI_n$ 1, 2……n are obligating alleles at respective loci.				$4.0987*10^{12}$	15,550,738.19
Probability of paternity (POP)	CPI/(CPI+(1-50%))*100				100%	99.999999967%

allele 18 for D12S391locus and allele 19 for SE33locus.

In a population genetics, allele frequencies are used to describe the amount of variation at a particular locus or across multiple loci. According to Chen et al. [10], the occurrence in a population of two or more commonly occurring alleles at a locus usually defined as the frequency of the most common allele lowers variability at locus or across multiple loci, those commonly observed alleles on above loci are associated with low variation in Rwandan population.

As explained by Hameed et al. in 2015, the minimum allele frequency is 5/ (2n) where n is the number of individuals sampled and 2n is the number of chromosomes (as autosomal are in pairs due to inheritance of one chromosome from each parent) [8]. Therefore, for every allele observed less than five times the frequency value assigned is 0.017 obtained from 5/282 (Appendix). The good indicators of the genetic polymorphism within the sample are verified by the number of

Table 3: DNA profiles of a child and alleged father for paternity testing

DNA Probe/Locus	Child (B1)	Alleged father(B)	Obligate allele	Paternity index (PI)	Selected paternity index (PI)
D3S1358	16,18	14,18	18	16.12	
VWA	15,20	14,20	20	32.80	32.80
D16S539	10,10	10,11	10	4.03	
CSFPO	11,11	10,11	11	2.47	
TPOX	8,8	8,11	8	1.62	
D8S1179	12,14	14,14	14	3.32	
D21S11	31,31.2	27,31	31	4.42	4.42
D18S51	15,15	15,17	15	2.94	2.94
DYS391		9,9			
D2S441	12,12	12,14	12	3.01	
D19S433	13,14	13,13	13	3.48	3.48
TH01	8,8	8,8	8	4.71	
FGA	22,26	26,28	26	7.93	7.93
D22S1045	11,17	11,17	11 or 17	2.82	
D5S818	11,12	8,12	12	29.41	
D13S317	11,12	11,12	11 or 12	1.55	1.55
D7S820	8,10	10,10	10	2.84	2.84
SE33	27.2,29.2	26.2,29.2	29.2	23.80	23.80
D10S1248	13,14	12,14	14	2.10	
D1S1656	14,16	16,16	16	4.62	4.62
D12S391	20,23	17,23	23	16.12	16.12
D2S1338	16,24	18,24	24	5.05	5.05
Combined paternity index (CPI)	$PI_1*PI_{2.....*}PI_n$			$9.87625*10^{14}$	463469608.6
	1, 2.....n are obligating alleles at respective loci.				
Probability of paternity (POP)		CPI/(CPI+(1-50%))*100			99.999999998%

alleles and the expected heterozygosity (Table 2) found in the Rwandan population. Basically, the number of alleles is highly associated with the size of the sample. This is due to the presence of unique alleles in populations, which occur in low frequencies as indicated in Table 1. Also because of bigger population size, the number of observed alleles tends to increase. The usefulness of the markers (loci) for paternity testing is verified by the number of alleles scored for each marker, meaning that locus SE33, D18S51, D21S11, VWA, FGA, and D1S1656 are most preferred to be used in parentage testing.

The TH01 locus is the least polymorphic marker while SE33 is the most polymorphic marker based on the degree of polymorphism of every marker expressed in heterozygosity and power of exclusion terms (Table 1). According to Casillas S et al. (2017) high heterozygosity means lots of genetic variability and Low heterozygosity means little genetic variability. The expected heterozygosity that was determined along 21 STR loci, ranges from 71.3% (TH01) to 91.6% (SE33) with an average of 81.1% a good indicator of high genetic variability.

Based on Butler et Al. [11], the power of exclusion (PE) can be computed to demonstrate how rare it is to find a random man who could not be excluded

Table 4: Availbale DNA profiles of a child, mother and alleged father

DNA Probe/Locus	Child (C4)	Mother (C1)	Obligate allele	Alleged father (NE1)	Common alleles	Paternity index (PI)
D3S1358	15,15	15,15	15	15,16	15	1.66
VWA	15,16	16,18	15	15,15	15	2.89
D16S539	12,13	9,13	12	11,11	No one	
CSFPO	12,13	10,12	13	7,14	No one	
TPOX	8,9	8,9	9	8,9	9	3.44
D8S1179	14,14	14,14	14	13,14	14	1.66
D21S11	28,30	30,31.2	28	28,28	28	2.43
D18S51	16,16	16,19	16	15,18	No one	
DYS391				10,10		
D2S441	12,12	11,12	12	11,13	No one	
D19S433	13.1,14	12,14	13.1	14,15.2	No one	
TH01	6,9	7,9	6	7,9	No one	
FGA	22,24	21,22	24	17.2,25	No one	
D22S1045	10,17	10,15	17	14,15	No one	
D5S818	12,13	12,12	13	10,12	No one	
D13S317	9,11	11,14	9	13,14	No one	
D7S820	8,10	10,11	8	8,11	8	2.61
SE33	13,19	11.2,13	19	21,21	No one	
D10S1248	15,16	15,16	15	14,14	No one	
D1S1656	15,15	15,16	15	13,14	No one	
D12S391	16,17	15,16	17	15,19	No one	
D2S1338	21,25	21,25	21	17,25	No one	
Combined paternity index (CPI)	$PI_1 * PI_{2\ldots..} * PI_n$ 1, 2……n are obligating alleles at respective loci.					173.74

as the biological father of the child. The PE for every locus in this study was calculated. For all the microsatellites analyzed, the PE ranged from 43.4% (TH01) to 78.1% (SE33) with an average of 58.2%. Based on calculated statistical parameters, the population of Rwanda may use these 21 STR loci as a vital tool for forensic paternity testing.

According to Cifuentes L et Al. [12], the outcome of a paternity analysis can be one of the following: The results indicate that the alleged father is not the biological father of the child. This conclusion is based on the absence of two or more alleles found in the child that was not donated by the father.
The results indicate that the alleged father cannot be excluded as the biological father of the child. This result is based on statistics calculated for the comparison. Even so, it is not correct to say "you are the father" because that implies 100% probability. Therefore, the results are reported as "the alleged father cannot be excluded as the biological father of the child".
The statistical results derived from the genetic tests fall within the inconclusive range for paternity. This result usually takes place when the sample from the mother is missing or some other difficulties occurred during the analysis.
Basing on Mendel's law of segregation, during the formation of male and female gametes (sperm and ova in humans) alleles are responsible a

particular character separates and are passed into different gametes. Therefore, the relationship between individuals is calculated by the amount of shared genetic material expected between two individuals. The Mendel's first law provides that when individuals with one or more set of alleles are crossed, then the child will have two alleles for each autosomal marker, one from mother and another from biological father [13]. In order to determine who, the father might be, we looked for the obligate paternal allele defined by Cifuentes et al. [12] as the allele that the father must donate to the child (the other allele is donated by the mother). For example, in the Table 3 the mother donated the 15 repeats for the D18S51 STR locus. The alleged father had the ability to donate the obligate paternal allele 17 repeat to the child. The father was the suspect for this STR locus.

The university of Vermont [12] also described Paternity Index (PI) as the likelihood that the alleles the child has come from the man being tested rather than another man at random. In other words, it is how good this DNA profile for determining paternity is. It depends on number of markers genotyped and heterozygosity of markers. Consider the D18S51 STR in table 3, the mother contributed the 15 repeats and the father contributed the 17 repeats. The probability that the alleged father contributed the obligate allele is considered 0.5 or 50% because he was heterozygous for the allele [11,14] and had a 50 % chance of contributing the 17 allele. The probability that a random Rwandan male contributed the allele is 0.187 (as per table 1 of allele frequencies). The PI is then, 0.5 / 0.187 equals 2.67. Then, PI value for D18S51 equals 2.67 the likelihood that allele 17 has come from alleged father A than another man at random. Next, consider the D22S1045 STR; the obligate paternal allele was the 17 repeat, alleged father A was homozygous for the 17 repeats. In this case, the father had a 100% chance of contributing the obligate paternal allele [11,14] the probability that a random Rwandan male contributed the allele is 0.163 (table 1 of allele frequencies). The PI is then, 1 / 0.163= 6.13.

Based on statistics calculated (CPI and POP) for case 1 up to case 7, the results indicate that the alleged father cannot be excluded as the biological father of the child. In addition, the trend of difference in probability of paternity indicates that as CPI increases the POP also increases.

According to Synapse Diagnostics [2], the DNA sample collected from the mother should be typed to help properly deciding which allele was donated by alleged father as well as increasing power of differentiation. It became a hurdle for table 5 of case 3 at locus D22S1045 and D13S317 to decide whether alleged father has donated allele 11 or 17 and 11or 12 respectively, for table 8 of case 6 at locus TPOX and TH01 to decide whether alleged father donated allele 7or 9 and 7or 8 respectively. The results in table 10 of case 8 indicate that the alleged father is not the biological father of the child. This conclusion is based on the presence of more alleles found in the child that were not donated by alleged father. Cifuentes et al. [12] argued that alleged father is excluded as biological father of a child when there is a presence of two or more alleles found in the child that were not donated by the father.

There are some limitations for consideration. This study was done in only one district of the Northern Province of Rwanda (Musanze District), and only 08 paternity cases were performed for the purpose of knowing the real father of the child. Samples were exclusively taken from voluntary families including 08 children and 07 parents. We were also limited on the study of autosomal STR loci. Technically, the size of the population has effect than can be linked to the power of drift and the likelihood of inbreeding within the population. Small populations tend to lose genetic diversity more quickly than large populations due to stochastic sampling error.

CONCLUSION

In total, 270 alleles were observed across the studied population. For seven of eight cases examined in paternity tests, alleged father was not excluded as biological father of child. Using an allele frequency of an obligating allele, a CPI for each of those seven cases was calculated. The calculated paternity indexes were used in Bayesian formula to determine paternal affiliation probability which is approximately 99.99% a maximum value of parentage probability. The results found in examination of case 8 indicate that the alleged father is not the biological father of the child. This conclusion is based on the presence of more alleles in the child that were not donated by alleged father. Based on calculated statistical parameters, the population of Rwanda may

use these 21 STR loci as a vital tool for forensic identification and paternity testing. We strongly recommend that DNA sample from the mother also should be investigated in all paternity test cases to help analysts to decide what allele could have been donated by the father.

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