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**REVIEW**

# Short Tandem Repeats Loci in Parentage Testing

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**Abbreviations**
AF(s) = alleged father(s)
CPI = combined paternity index
HLA = human leukocyte antigens
IMGT = international ImMunoGeneTics (project)
IVF = in vitro fertilization
MHC = major histocompatibility complex
PCR = polymerase chain reaction
PE = power of exclusion
PI = paternity index
RFLP = fragment length polymorphism
STRs = short tandem repeats
VNTRs = variable number of tandem repeats
W = probability of paternity

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## ABSTRACT

The need for confirmation or exclusion of biological father and / or biological mother is a social phenomenon, which is imposed by socio-economic and, sometimes, by moral-psychological factors. Modern science has significantly contributed to solving this problem, as many medical methods have been applied for this purpose. Biological markers that have been conducted for distinguishing between individuals were the human ABO blood groups, the Rh, MNS, Duffy, Kidd, and Kell systems, as well as the human leukocyte antigens (HLA) system. For a long time the HLA testing represented the standard testing in forensic genetics, but, due to the linkage disequilibrium and the predominance of certain HLA alleles and as the demand for parentage investigations is rapidly increasing during the recent years, this serological era has been replaced by molecular markers through the introduction of "DNA profiling", which is based on polymorphisms of short tandem repeats (STRs) loci. Nowadays, "DNA profiling" by analysis of STR loci is the method of choice for human identification and parentage investigations. This technique is the most informative, accurate, robust, rapid, cost-effective method of genotyping and has worldwide acceptance in the courts, as the probability of parentage will typically be greater than 99.99999%.

## INTRODUCTION

Nowadays, most parentage investigations are usually conducted in order to establish legal responsibility and support for a child. In addition, several other cases are investigated in order to prevent disputes in adoption, in in vitro fertilization (IVF) cases, in prenatal diagnosis, in bone marrow transplantation (follow ups), as well as cases in which a record for immigration is required.

Historically, the first biological markers used to genetically distinguish between individuals were the human ABO blood groups.[1] The ABO blood group phenotyping was introduced to paternity or maternity assessment very early, but soon it started playing a complementary role due to the fact that it is not very informative. While it is useful for excluding an individual from the paternity or maternity test, it is not useful when an inclusion is required. When for example, conventional ABO blood group typing has a power of exclusion (PE) of only 17%, this value increases to 53% using simultaneously the Rh, MNS, Duffy, Kidd, and Kell systems.[2]

The next methodology that was used to carry out parentage investigations was the major histocompatibility complex (MHC) system. The MHC system, known as the human leukocyte antigens (HLA) system, is the most polymorphic genetic system in human genome and expands to a 3.6 Mb region located on the short arm of human chromosome 6 (6.p21.3). More than 300 different genes and pseudo-genes have been identified in this complex. HLA gene products are expressed as glycoprotein heterodimers on the cell surface, specialized to present antigenic peptides to the T-Cell Receptor (TCR) on T cells.[3] The HLA antigens play a fundamental role in immune function, as they control the immune response through recognition of "self" and "non-self".[4,5] Regarding the way of transmission through generations, the HLA genes are inherited en bloc from each parent due to their close proximity on the short arm of human chromosome 6. The set of HLA alleles inherited from each one parent is referred to as a haplotype and it is transmitted according to Mendelian fashion. HLA genes have multiallelic and stable nature with low recombination events, while mutations have not been recorded in family studies.[6] According to the World Health Organization Nomenclature Committee more than 12.200 HLA alleles have been documented in the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla released 3.18.0, 2014-10-10).[7,8] Due to this variability, the HLA system has proved to be a useful tool in paternity testing. For parentage investigations, incompatibilities in at least one HLA allele between parents and offspring indicate exclusion. Many studies have shown that HLA serological markers can provide a PE value greater than 98%, while the probability of paternity (W) value can reach 99.99% in many cases. However, due to linkage disequilibrium and the predominance of certain HLA alleles in particular ethnic groups, in some cases the HLA loci are not informative enough and do not provide high discrimination power, leading to the need for new markers and new methods.[9]

For a long time the HLA testing represented the standard procedure in forensic genetics. However, very early in the 1990's, as the demand for higher statistical significance increased, this serological era was replaced by molecular markers through the introduction of "DNA profiling" based on polymorphisms located in the microsatellite regions (details of published STR studies http://www.cstl.nist.gov/div831/strbase). The human genome is full of repeated sequences which show widespread allocation more or less throughout the human genome, accounting for about 3% of the entire genome.[10] These tandem repeated sequences are classified, according to the length of the core repeat unit and the number of repeat unit, into two groups, 1) Variable number of tandem repeats-VNTRs (also called minisatellites), and 2) Short tandem repeats-STRs (also called microsatellites). In 1985 the English geneticist Alec Jeffreys studied the minisatellites regions and developed the "DNA fingerprinting" methodology based on the restriction fragment length polymorphism (RFLP) analysis. There are loci with long motifs, ranging from 8 to 80 bp, and the number of repeat units varies significantly between individuals. But, as the demand for identity testing increased rapidly, and as they are not amenable to automation because of their large size, minisatellites were abolished and finally replaced by the STR loci analysis (http://www.cstl.nist.gov/biotech/strbase/seq_ref.htm).[11]

## PARENTAGE TESTING BASED ON "DNA PROFILING"

Historically, STRs were identified in the early 1980s and were first described as an important tool for human identity testing in the early 1990s by Alec Jeffreys. STRs loci have been widely used in forensic casework since 1993 and, nowadays, they are the method of choice for human identification and parentage investigations. Microsatellites or STRs or simple sequence repeats (SSRs), are non-coding tandem repeat units composed of small motifs of 2 to 6 nucleotides (bp) repeated typically from 5 to 30 times.[12] The length of repeat regions is shorter than 100 bp. Most STRs loci are located in the non-coding regions, while only about 8% are found in intergenic regions. They are believed to have no biological uses and regarded as non-functional "junk DNA". However, studies have shown possible correlation between some human neurodegenerative disorders, such as Huntington's disease. According to the length of the repeat unit, STRs are classified into mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats, and the most common STRs in the human genome are dinucleotide repeats.[13] One typical example of a microsatellite is the 5'-3' $(TCAT)_n$ marker (named as THO1 with chromosomal location 11p15.5, tyrosine hydroxylase 1st intron), where n varies between alleles. These hypervariable regions have a large number of alleles, depending on the number of repeats, showing sufficient variability among individuals in a population with high degree of heterozygosity, frequently greater than 0.7.[14] Thus, the probability of two random individuals, except identical twins, having exactly the same alleles at each STR loci is extremely rare.[15] Scientists can use these variable regions to create a genotyping of an individual referred to us as a "DNA profile", using samples of blood, bone, hair, saliva, semen and other kinds of body tissue and products.[16] Although the human genome contains thousands of STR markers (~500.000 loci), only a small core set of loci has been selected for use in human identity testing. Their exact chromosomal location only recently became available with the completion of the Human Genome Project[17] (Table 1).

The inheritance of STRs markers follows the basic Mendelian principles.[18] Every individual has a pair of STR alleles, one inherited from the mother (maternal origin), and the other from the father (paternal origin). There are two possibilities.

**TABLE 1.** Locus details and genomic coordinates of commercial markers (17 core loci) routinely employed in paternity/maternity assessment.

| Locus designation | Chromosome location | Repeat Sequence 5'-3' | Alleles included in AmpFlSTR® NGM SElect™ Allelic Ladder[†] |
|---|---|---|---|
| D10S1248 | 10q26.3 | GGAA | 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 |
| vWA | 12p13.31 | TCTA complex | 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 |
| D16S539 | 16q24.1 | GATA | 5, 8, 9, 10, 11, 12, 13, 14, 15 |
| D2S1338 | 2q35 | TGCC complex | 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 |
| Amelogenin* | X:p22.1-22.3<br>Y:p11.2 | NA | X, Y |
| D8S1179 | 8q24.13 | TCTA complex | 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 |
| D21S11 | 21q11.2-q21 | TCTA complex | 24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38 |
| D18S51 | 18q21.33 | AGAA | 7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 |
| D22S1045 | 22q12.3 | ATT | 8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 |
| D19S433 | 19q12 | AAGG complex | 9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2 |
| THO1 | 11p15.5 | AATG | 4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3 |
| FGA | 4q28 | TTTC complex | 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2 |
| D2S441 | 2p14 | TCTA | 9, 10, 11, 11.3, 12, 13, 14, 15, 16 |
| D3S1358 | 3p21.31 | TCTA complex | 12, 13, 14, 15, 16, 17, 18, 19 |
| D1S1656 | 1q42.2 | TAGA complex | 9, 10, 11, 12, 13, 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18.3, 19.3, 20.3 |
| D12S391 | 12p13.2 | AGAT complex | 14, 15, 16, 17, 18, 19, 19.3, 20, 21, 22, 23, 24, 25, 26, 27 |
| SE33 | 6q14 | AAAG complex | 4.2, 6.3, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20.2, 21, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37 |

*Amelogenin is not an STR but displays a 106-base, X-specific band and a 112-base, Y-specific band.

[†] Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: www.cstl.nist.gov/div831/strbase/

An individual is either homozygous (having the same number of repeats) or heterozygous (having different number of repeats) at a particular locus.

However, autosomal microsatellite analysis has certain drawbacks. It is known that in vivo the mutation rate of microsatellites is much higher, ranging from $10^{-2}$ to $10^{-6}$ nucleotides per locus per generation, than that of other parts of the human genome (approximately $10^{-9}$ nt per generation). The range differs among different loci, and depends on repeat number, repeat unit and repeat structure, the composition of the STR, recombination, gender and age.[19,20] Several mechanisms have been proposed to explain the high mutation rate. Among others the strand-slippage replication appears to be as the main cause of STR mutation, which has also been called DNA slippage, polymerase slippage or slipped strand mispairing.[21] In parentage investigation, as well as in human identification, the possibility of mutation should be taken into consideration when a genetic mismatch is observed, which normally can lead to non-inclusion. Nowadays, for the exclusion of an alleged father, the "paternal obligate alleles – POA" should not be found in the offspring in three or more testing STRs loci.[22]

In disputed parentage investigations, genetic markers of the child, the alleged mother and/or the alleged father are compared to determine exclusion or non-exclusion. It should be emphasized that medical methods lead to the exclusion of the alleged father and/or mother at a certainty of 100% or non-exclusion probability at a rate that depends on the number of controlled genetic markers and the frequency of the alleles encountered in the controlled population. On the other hand, proof of paternity or maternity is not possible with a 100% certainty because there is always a chance for another random individual to have the same genetic blueprint with the alleged father (AF) or alleged mother.[23] In cases where an alleged father is not excluded, mathematical formulas were developed for the analysis of parentage using allele frequency databases.[24] More specifically, the combined paternity index

(CPI) and the probability of paternity (W) values are calculated using the assigned paternity index (PI) for each tested STR marker from a reference population database. PI is a statistical measure of how powerfully a match at a particular marker indicates paternity. The CPI indicates the overall probability of an individual being the biological father of the tested child as related to any random man from the entire population of the same race. The CPI is converted into the W value, showing the degree of relatedness between the AF and the child.[25] Finally, using the STRs procedure in parentage testing, the W value can reach the probability factor of 99.999999% or 1 in 10 billion of the population.[26] It should be underlined that "DNA profile" cannot distinguish the true biological father only in cases where the two alleged fathers are monozygotic twins. On the other hand, in mutation events, where there are only one or two mismatches, the PI value at a single or double mismatching genetic locus is not documented as zero, but shall be modified using equations that incorporate the mutation rate at the specified genetic loci.[27]

To date, several countries have legislated to implement national DNA databases based on STRs.[28] The National Institute of Standards and Technology (NIST) of the USA has compiled and maintained an STR DNA internet database available at http://www.cstl.nist.gov/biotech/strbase/ commonly referred to as STRBase. On the other hand, statistical analyses were conducted by several programs such as STATCEL (OMSPublishing Inc.), Powerstats version 1.2 (Promega Corp.) and OmniPop (http://www.cstl.nist.gov/biotech/strbase/population/OmniPop). Additionally, there are several programs for creating pedigrees and calculating the W value, such as the Familias program (download free at http://www.nr.no/familias).

## LEGAL ISSUES IN PARENTAGE TESTING

DNA testing is a necessary tool in family law. Apart from cases that might relate to people concerned, there are many cases, which fall within the field of civil law (e.g. cases of recognition of illegitimate children, waivering paternity of a child born in wedlock) or criminal law (e.g. rape, incest, abandonment and abuse of pregnant woman), where the test is performed after a court order and the judge appoints a special expert.

A lot of countries have restrictions/regulations on human DNA testing regarding the principles of blood sampling, processing, testing and the statistical analysis of the data. For judicial recognition of paternity, the experts appointed by the court must use the most appropriate medical tests and methods which must be internationally admissible and not at the stage of experimentation and they must follow the rules of the procedure strictly. A report of the statistical significance of a match must be submitted by the expert.

Many organizations around the world work on a national or international level in order to aid in quality assurance work, such as the Technical Working Group on DNA analysis Methods (TWGDAM), the European Network of Forensic Science Institutes (ENFSI) and the European DNA Profiling Group (EDNAP). Internationally, there are also several organizations responsible for the definition of relevant standards, as well as organizations responsible for the relevant accreditation. Authorized accreditation organizations around the world comprise among others, the College of American Pathology (CAP), the New York State Department of Health-Standards (NYSDOH) for Parentage Testing Laboratories (www.wadsworth.org/labservices.htm), the Standards Council of Canada (SCC), the American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) - International, the Department for Constitutional Affairs (DCA) and the American Association of Blood Bank (AABB).[29]

## BRIEF DESCRIPTION OF THE METHOD

The complete process for STR typing includes:

1. Sample collection and DNA extraction; a testing method requires whole blood in EDTA tubes or blood drops from finger/heel pricks taken onto paper stain cards (bloodstained FTA cards) and/or buccal swabs of saliva. Samples of swab saliva are obtained from the inside of the mouth with cotton or dacron swabs and this can be done immediately after birth. The DNA extraction procedure is either automatic or manual.
2. Polymerase chain reaction (PCR) setup and DNA amplification of multiple STR loci; samples are co-amplified with four fluorescence-labeled primers in a multiplex PCR system. Commercial autosomal STR genotyping kits are commonly used in paternity testing. For instance, the AmpFlSTR NGM SELect™ (Applied Biosystems, Foster City, CA) and the PowerPlex™16 kits (Promega, Madison, WI, Technical Manual PowerPlex®16 HS System www.promega.com.) are able to type 16 and 15 autosomal STR loci respectively with high discrimination power.
3. STR allele separation and DNA sizing; amplified STR fragments are generally separated on the capillary based automated DNA sequencers like ABI PRISM 310, ABI PRISM 3100/3730xl DNA Sequencer systems, MegaBase 1000/500 of Amersham Pharmacia Biotech, USA and Beckman Coultier CEQ 2000XL DNA Sequencer. PCR products of all tested STR loci with overlapping size ranges can be easily distinguished by size and detected with high resolution due to the different characteristic emission spectrum of each fluorescence dye.[30]
4. STR typing and profile interpretation; analysis of the results obtained by the previous step is carried out following

the guidelines and recommendations of the DNA Commission of the International Society of Forensic Genetics (ISFG) and the European DNA profiling (EDNAP) group. To produce the genotyping chromatogram, the GeneMapper® ID V3.2, GeneScan® 3.7 or Genotyper® 3.7 softwares for automatic allele calling (Applied Biosystems, Foster City CA) are used and all alleles are represented as peaks (Fragment Analysis).[31] A sample typing showing two alleles (two peaks in the electropherogram) indicate heterozygosity, whereas one allele (one peak in the electropherogram) indicate homozygosity at a particular locus (Fig. 1).

5. A report of the probability of the respective parenthood is performed by the expert.

The process of recording and archiving of samples as well as the privacy procedure are carried out following the guidelines of the human and ethical research principles.

## OTHER CONTEMPORARY DNA MARKERS IN FORENSIC INVESTIGATIONS

### 1. Y CHROMOSOMAL POLYMORPHISM

In the past 15 years, the use of Y-Chromosome STR loci has seen tremendous increase in forensic as well as in paternity investigations. The Y chromosome has stable nature, is found only in males and it is transmitted from father to son without mutations. For identity testing, although Y chromosome markers have been extensively used in genetic genealogy studies as well as in cases of sexual assaults, they are considered inappropriate to use so as to distinguish men belonging to the same pedigree. For disputed paternity testing, Y-Chromosome STR markers are a useful tool in cases where the alleged father is not available, but another person through paternal lineage is available (Y-STR database http://www.cstl.nist.gov/biotech/strbase/y_strs.htm).[32]

### 2. MITOCHONDRIAL DNA (MTDNA) SEQUENCING

Another system that has been studied for identification purposes is the mitochondrial DNA (mtDNA). MtDNA markers are diallelic polymorphic loci. They are passed en bloc to the next generation and inherited from the mother only and not from the father.[33] Thus, while they are suitable to evaluate maternal relatedness or kinships, they cannot be applied for establishing paternity testing (Human mitochondrial genome database available at http://www.mitomap.org).

### 3. SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

Other types of genetic variations applied in parentage investigations are variations of single nucleotide polymorphisms (SNPs). According to recent studies, these diallelic polymorphic loci are considered to be a useful tool in disputed paternity or maternity assessment due to the fact that they have easy application, low cost, but mainly because they have very low mutation rate (100,000 times lower compared to STRs loci).[34] However, in order to achieve the same statistical significance as that of STRs, a panel of 40 short insertion-deletion polymorphisms is required for an efficient human

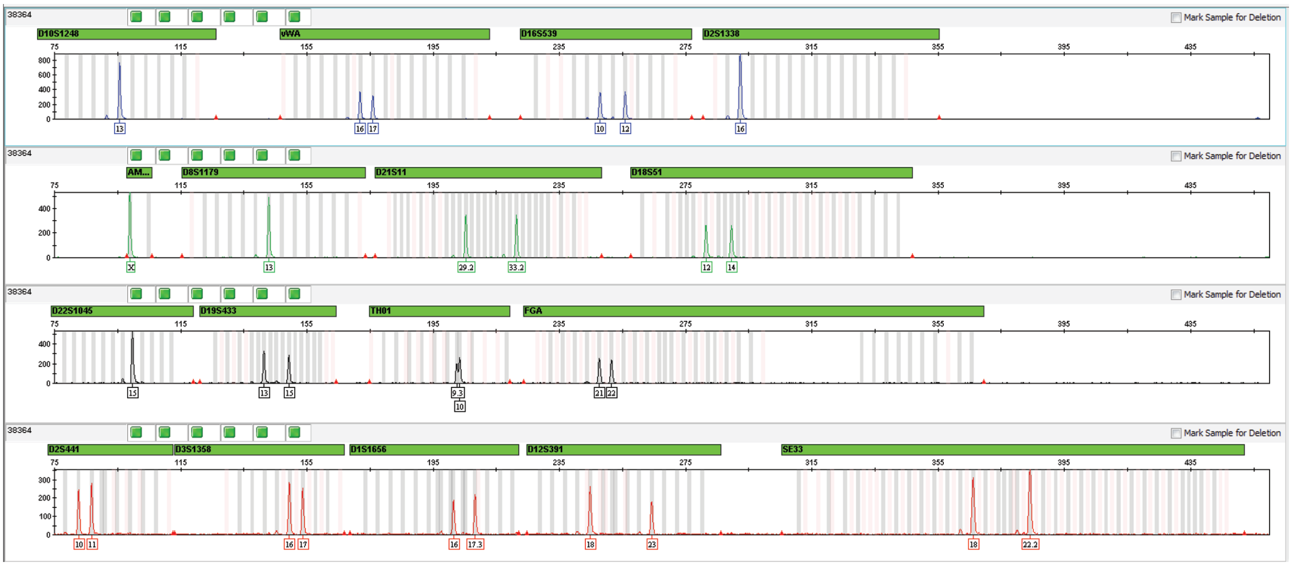

**FIGURE 1.** Map of genotyping of 17 STR loci. Each peak on the electropherogram represents a fluorescently labeled DNA fragment that indicates the maternal or paternal allele. The vWA, D16S539, D21S11, D18S51, D19S433, TH01, FGA, D2S441, D3S1358, D1S1656, D12S391 and SE33 loci are heterozygous, whereas the D10S1248, D2S1338, D8S1179 and D22S1045 loci are homozygous. This sample is from female as indicated by one peak for the amelogenin (ID:38364).

parentage testing. It is considered that in the future it will be a very promising tool for conducting disputed paternity or maternity testing[35] (SNPs database available at http://www.ncbi.nlm.nih.gov/projects/SNP/).

## CONCLUSIONS

The STR genotyping has become an important tool for human identity testing and has increased our ability to solve problems of family relationships and kinships, providing highly sufficient power of discrimination. The high polymorphism, heterozygosity, co-dominance and distribution over the eukaryotic genome have made microsatellites the markers of choice for the construction of genetic maps, population genetics and forensics as well as for parentage investigations. Additionally, the remarkable advances in DNA technologies over the past two decades have had an enormous impact on the development of the “DNA profile” for human identification. Specifically, the availability of capillary sequencers and laser based technology provides a high sample throughput. DNA markers can be easily amplified by a single PCR, even in cases that involve degraded DNA. Commercial STR kits permit reliable amplification of these unlinked loci from small amounts of starting DNA template, using less than 1 ng of genomic DNA and can recover information even at the level of a single cell.

In the past, identification procedures, such as the ABO blood group antigens, serum proteins and RBC enzymes, had the disadvantage of poor power of discrimination due to low polymorphism and poor sensitivity and carried the risk of giving false paternity inclusions, especially when the mother was not available. On the other hand, VNTR loci have excellent powers of discrimination due to the large number of alleles at each locus but bear limited sensitivity, are time-consuming and cannot be automated. Additionally, due to the predominance of certain HLA alleles and linkage disequilibrium, in some cases HLA genotyping was not informative enough and therefore the statistical power of W was low.[36]

Today, STRs genotyping has completely replaced the HLA typing in paternity investigations. STR profile can overcome the limitations of the HLA system and it is believed that due to high variability, excellent power of discrimination and automated detection, which enable high-throughput sample processing, it will continue to be in use for many years to come. However, the mutation rate of STR loci is higher than that of the HLA or some other genetic markers (Fig. 2). Thus, ac-

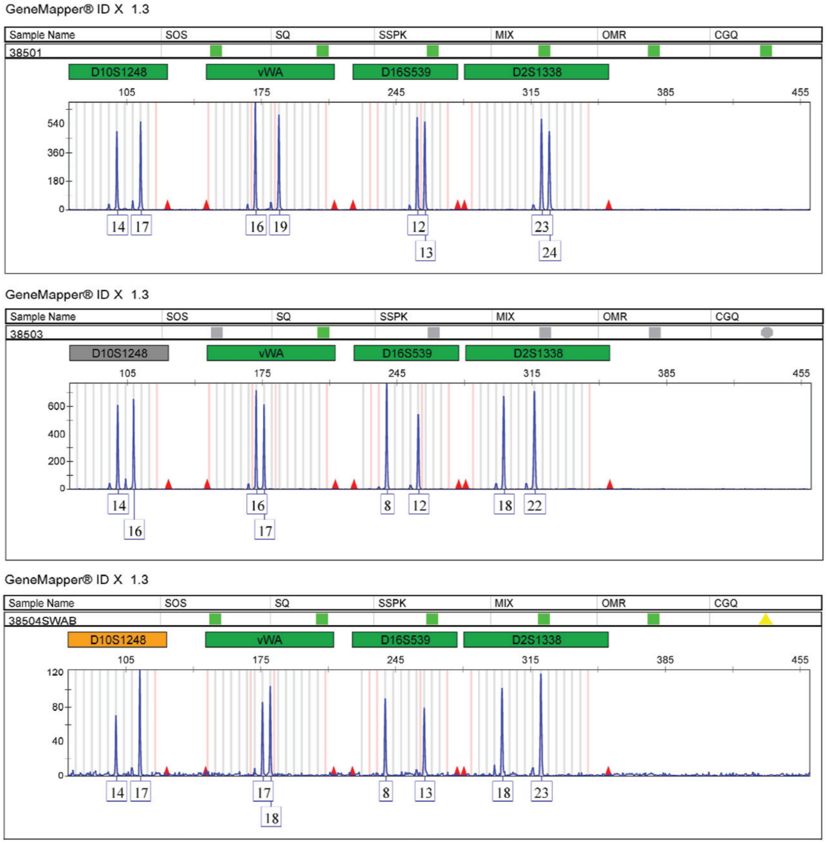

**FIGURE 2.** One genetic discrepancy between the biological father (ID:38501) and the child (ID:38504SWAB) was observed in vWA STR locus (Putative mutation: vWA 19→18 or 16→18). Biological mother (ID:38503). Paternity is not excluded.

cording to global instructions, in order to avoid the risk of falling into a false exclusion of the biological father of the child, the alleged father has to be genetically inconsistent with the child at two or more of the analyzed STR loci. Additionally, in cases when the DNA profile of the mother is not available (motherless cases), the W value is lower than in trio cases (alleged father, mother and child) and the difference gets even more significant when the number of STR markers is limited and the allele frequency is high.[37] For this reason, global guidelines suggest that the number of the tested genetic STRs markers should be increased from 16-17 to 21.[38] On the other hand, in motherless cases with mutation events, more genetic information and more attention to the statistical analyses is required. Thus, it is recommended that the mother should be investigated in all cases.[39] When this is not possible, another genetic system can be used as a supplementary tool, in order to lessen the possibility of false exclusion due to mutation events and reliable results to be achieved.

The use of STRs markers in paternity/maternity assessment has been introduced in the Department of Immunology and Histocompatibility of Evagelismos General Hospital of Athens since 2012 and it is in routine practice for solving problems such cases.

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