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Identification with mitochondrial DNA typing from one sperm cell isolated by micromanipulation

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Abstract
Due to the limited or high degree degradation of nuclear DNA (Deoxyribonucleic acid) content in some cases of sexual assault at forensic cases, successful results in standard genetic profiling techniques are not achieved. The aim of this study is with swab to characterize the male mitotype by mtDNA (mitochondrial DNA) typing from individually collected sperm cells together with physically differentiation of the perpetrator spermatozoa among the victim epithelium cells using micromanipulation technique. In this study, 10 samples of oral swabs were collected for the first step (from 5 volunteer pairs), and vaginal swabs for the second step after the sexual intercourse. In the first step, intra-oral swab specimens were analysed and the mtDNA profiles of the male and female were determined. These profiles were compared to the Revised Cambridge Reference Sequence. For each couple, SNP regions not seen in women present in men were detected. Based on these detected SNP (Single Nucleotide Polymorphism) regions, each couple's male-specific SSPs (Sequence Specific Priming) were designed. In the second step, DNA isolation was performed by using micromanipulation technique from vaginal swab samples taken from women after sexual intercourse and sperm cells were individually collected. PCR (Polymerase Chain Reaction) was performed using SSPs designed in the first step. Then male mtDNA profiles were obtained. According to the results, the overlapping regions in relatives from the maternal side since SSP is not designed, and not able to be used in cases which relative men are involved are the disadvantages of the method. However, thanks to the micromanipulation technique used in the study, the risk of contamination is reduced and individual samples can be isolated with sperm isolation and sequenced with SSPs designed for the individual, indicating that it is a method that can be prefered in sensitive situations such as pack rape crimes involving more than one person from the same male lineage.

Keywords: Forensic science, sexual assault crimes, mtDNA, SSP, single sperm cell, micromanipulation

Introduction

Sperms belonging to aggressor/aggressors in cases of sexual assault taken from victim and crime scene are among the most important biological evidence that can be used to illuminate the event. Traces of the aggressor can be found on the objects at the scene, while the traces of the aggressor can be found in the underwear of the victim, between the hair, on the body surface, in the mouth, between the nail, in the vagina and in the anus [1]. In cases of sexual assault, in some cases it may be necessary to identify the suspect from other body fluids, mixed semen and vaginal cells. In such cases, it is very important to isolate sperm DNA from the DNA of other cellular sources or vaginal cells [2].

The vast majority of vaginal swab samples collected after sexual assault contain 167 times more female cells than sperm cells, which is why the preferential amplification of female nuclear DNA is the most important problem that poses a risk to the analysis of vaginal swab samples. Another problem is that the DNA of the female originating from the cells that break down with time after sexual assault sticks to the sperm membranes.

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Again, this risk the analysis and specific amplification of the male STR (short tandem repeat) fragments [1-3]. The Y-STR (Y-Chromosome Short Tandem Repeat) assay used in the analysis of male DNA is successfully applied in a variety of critical situations [3-5]. However, in some instances of judicial investigation, the amount of nuclear DNA may be limited or may be at a high level of degradation. In addition, this method does not allow the identification of a single cell [1,6]. However, the Y chromosome is transferred from the father to the son, so it is the same in the relatives men on the paternal side. For this reason, the actual alleged criminal with Y-STR analysis of sexual assault incidents involving more than one man from the same male lineage can not be determined precisely [7].

In such sexual assault cases, the presence of multiple copies of mitochondrial DNA (mtDNA) in a single cell, where there are fewer cells present, or situations where DNA is degraded, protection of their ring structure against exonuclear reactions, and mtDNA inheritance from the mother make these analyses valuable [4,8]. The aim of this study is with swab to characterize the male mitotipine by mtDNA typing from individual collected sperm cells together with physically differentiation of the guilty spermatozoa among the victim epithelium cells using micromanipulation technique.

Materials and Method

Oral and vaginal swab samples from 10 volunteers (5 couples) were used in the study. Experimental investigations were carried out at Istanbul University, Institute of Forensic Sciences student laboratory and Istanbul In Vitro Fertilization Center, Embryology Laboratory. This study was approved by Cerrahpasa Medical Faculty, Ethics Committee (Ethical number: 6594).

Analysis of oral swab samples

To perform mtDNA analysis from oral swab samples, DNA isolation was performed using QIAamp® DNA Investigator kit from Qiagen, and the DNA amount was determined fluorometrically. Quant-iT dsDNA HS Assay kit was used for quantification of samples and measurements were made using Qubit® fluorometer. PCR analysis was performed using the SolisBioDyne kit. The amounts of distilled water added to the stock solutions and primer sequences used in this step are shown in Table 1. After the PCR step, purification of the products was performed using InvitrogenPureLink PCR Purification Kit and typing for the electrophoresis step using the BIGDye®Terminator v3.1 CycleSequencing kit. Electrophoresis results were analyzed using SeqScapiverite 2.6 software.

The mtDNA profiles obtained from the samples were compared with the reference sequence, and the single nucleotide polymorphism regions used in the study of Pereira et al. [1]. These SNP regions are shown in Table 2. In all couples, SNP regions are compared within themselves. SNP regions not seen in female present in male were detected and sequence specific primers were designed for the male of each couple. SSPs designed for couples are shown in Table 3.

Table 1. Quantities of added distilled water to prepare primer sequences and 20 µM stock solutions

Primer region	Name of primer	Sequence of primer	Quantity (nmol)	Added water (µL)
HVI	L15996-F	5´-CCA CCA TTA GCA CCC AAA GC-3´	21,1	211
	H16401-R	5´-TGA TTT CAC GGA GGA TGG TG-3´	20,9	209
HVII	L27-F	5´-CAG GTC TAT CAC CCT ATT AAC-3´	22,8	228
	H411-R	5´-ACT GTT AAA AGT GCA TAC CG-3´	20,5	205

Table 2. SNP points used during within couples comparisons [1].

mtDNA Region		HVI																					HVII									
rCRS Position	15904	16080	16093	16162	16166	16183	16189	16192	16193	16249	16256	16264	16270	16278	16293	16298	16304	16311	16356	16398	16465	16519	16526	73	146	150	152	195	263	285	309	315	320
rCRS Base	C	A	T	A	A	A	T	C	C	T	C	C	C	C	A	T	T	T	T	G	C	T	G	A	T	C	T	T	A	C	C	C	C

Table 3. SSPs designed specifically for men in couples.

	Amplification region	Forward	Sequence
1st Pair	HVII	SSP73G:	5´-TTCGTCTGGGGGGTG -3´
2nd Pair	HVII	SSP150T:	5´-TTTGATTCCTGCCTCATT -3´
3rd Pair	HVI	SSP16093C:	5´-CCCATCAACAACCGCTATGTATC -3´

Analysis of vaginal swab samples

Vaginal swab samples were taken rapidly from female participants immediately after sexual intercourse from volunteer couples.

Dried in air, placed in sterile tubes and stored at +4°C. Vaginal swab samples were waited in 1.5 ml ependorf tubes containing 200 µl medium solution for one hour. For the isolation of sperm by micromanipulation, the swab was pressed against the wall of the tube to facilitate release of the sperm cells from the swab. Drops from these liquids were placed on separate dishes. Sperm cells were collected using a DMIRB/E inverted microscope and transferred to PCR tubes containing 1.5 [mu] l of ATL buffer. Qiagen's QIAamp (R) DNA Investigator kit was used for DNA isolation from sperm cells. The primer sequences of the designed SSPs used in this step and the added distilled water quantities for the stock solution are shown in Table 4.

Table 4. Quantities of added distilled water to prepare primer sequences and 20 µM stock solutions

Pairs	Name of primer	Sequence of Primer	Quantity	Added water (uL)
1st Pair	H411-R	5´-ACT GTT AAA AGT GCA TAC CG-3´	20,5	205
	SSP73G-F	5´-TTCGTCTGGGGGGTG-3´	4,1	141
2nd Pair	H411-R	5´-ACT GTT AAA AGT GCA TAC CG-3´	20,5	205
	SSP150T-F	5´-TTTGATTCCTGCCTCATT-3´	14,9	149
3rd Pair	H16401-R	5´-TGA TTT CAC GGA GGA TGG TG-3´	20,9	209
	SSP16093C-F	5´-CCCATCAACAACCGCTATGTATC-3´	13,1	131

At the subsequent step with PCR, an agarose gel electrophoresis and imaging by the Biometra Gel Imaging system were performed in order to determine whether DNA was replicated. After the observation of the bands indicating the replication of DNA by the agarose gel electrophoresis, DNA purification process was performed using Invitrogen's purification kit in order to purify the PCR products from the remnants. Following the purification of the initial PCR products, 'BigDye®Terminator v3.1 Cycle Sequencing' Kit was used for sequencing. The amounts of the instant mix, primer, and the purified PCR product, which were used to replicate the samples, were determined by modifying the amounts recommended in the kit procedure. In order to perform sequencing after the second PCR process, the residual deoxy stains in the products were removed using Qiagen's DyeEx 2.0 Spin kit. The second PCR products, which were purified, were dried in the incubator for an hour at 60˚C. After the drying process, the products were then transferred to the plate adding 12 µl formamide and the sequence analysis was performed by the ABI 3130 capillary electrophoresis.

Results

The concentrations of the DNAs isolated from the study participants were measured by the Qubit™ fluorometer. The measured concentrations were 3.10-8.39 ng/µl, 1.86-2.01 ng/µl, and 0.05-0.09 ng/µl in the swab samples from the mouth, vagina, and sperm respectively. Because the number of cells studied at the second step of the study was quite low, in order to observe the replication of DNA, an agarose gel electrophoresis was performed at the first PCR step following the isolation process. The outcomes of this process are presented in Figure 1.

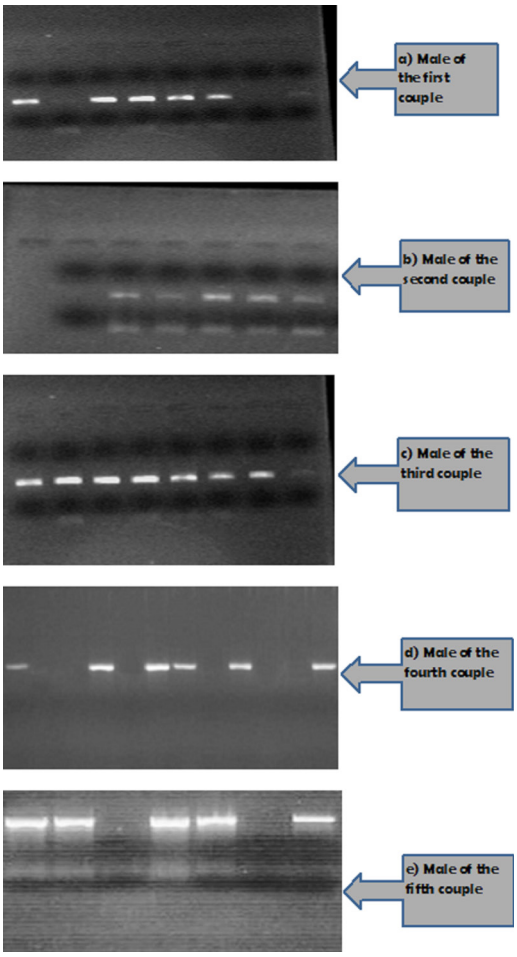

Figure 1. The results of the agarose gel electrophoresis at the second phase of the study. The agarose gel electrophoresis was performed at the first PCR step using the isolated DNAs in order to observe the replication of DNA (a, b, c, d and e).

After visualization of the agarose gel process, the consequent steps and electrophoresis were performed to sort the products out. Electrophoresis results were analyzed using SeqScapiverite 2.6 software. SNP regions were determined by comparing the samples to the Revised Cambridge Reference Sequence for analysis of mtDNA profiles. In all pairs, SNP regions were compared within themselves. During the comparison, SNP regions not seen in the female present in the male were detected. Based on these detected SNP points, the SSPs of the male of each couple were designed. In the studied specimens from 2 couples, no variations were observed at the SSP points of the male and female samples, therefore, the samples from 3 couples were analyzed. Figure 2 shows the electropherogram of the female of the first couple, figure 3 shows the results of the electropherogram comparison of the first couple, figure 4 shows the electropherogram of the female of the second couple, figure 5 shows the results of the third electropherogram comparison of the second couple, figure 6 shows the electropherogram of the female of the second couple, and figure 7 shows the results of the electropherogram comparison.

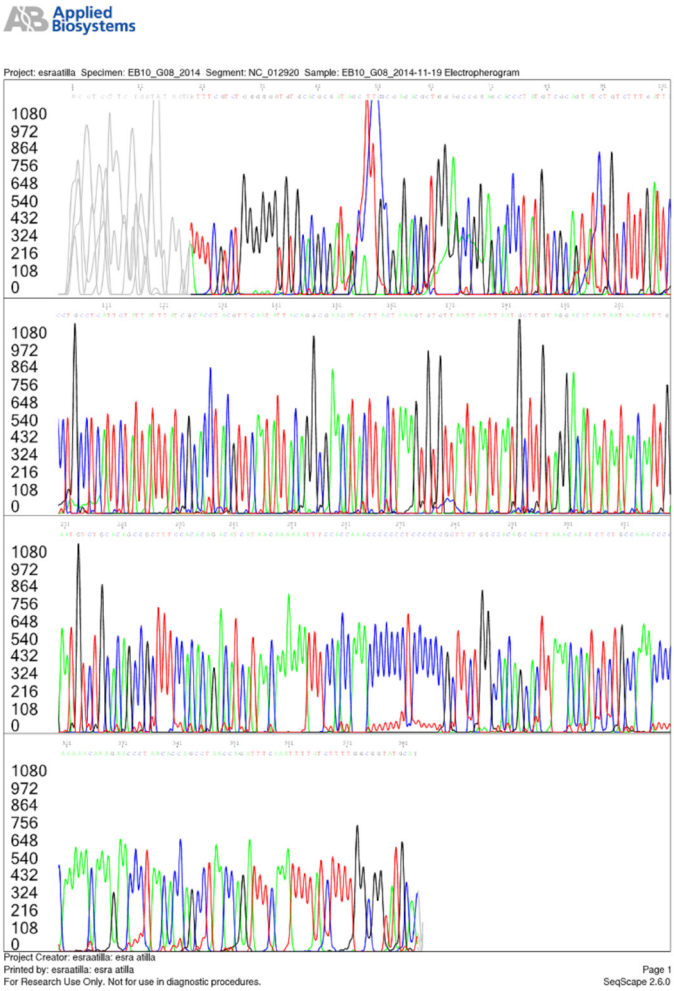

Figure 2. The electropherogram of the female of the first couple

Physical identification of the spermatozoa from the males was performed in the swab samples, however, each sample from the couples was examined separately and the SNP points differentiating the male and the female was identified by mtDNA typing of the individually collected sperm cells. Then, male-specific SSPs were designed on these identified SNP points and the characterization of the male mitotype was determined (Figure 2,3,4,5,6,7). The variations of the SNP points designed to be specific for each of the males individually were observed as follows: In the first couple,

they were observed in the G-C couples of the SSP73G primer of the male. In the second couple, they were observed in the T-C couples of the SSP150T primer of the male. And, in the third couple, they were observed in the C-T couples of the SSP16093 primer of the male.

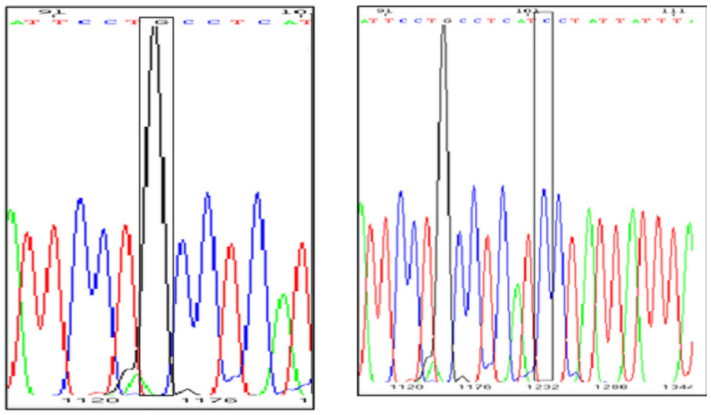

Figure 3. Comparison of the electropherogram of the first couple [image on the left belongs (SSP73G) man, image on the right belongs woman)

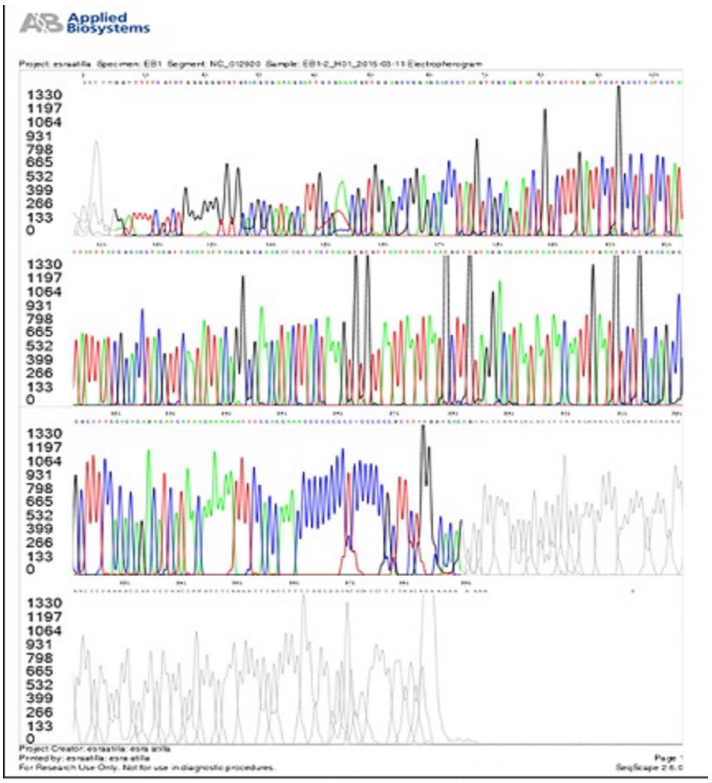

Figure 4. The electropherogram of the female of the second couple

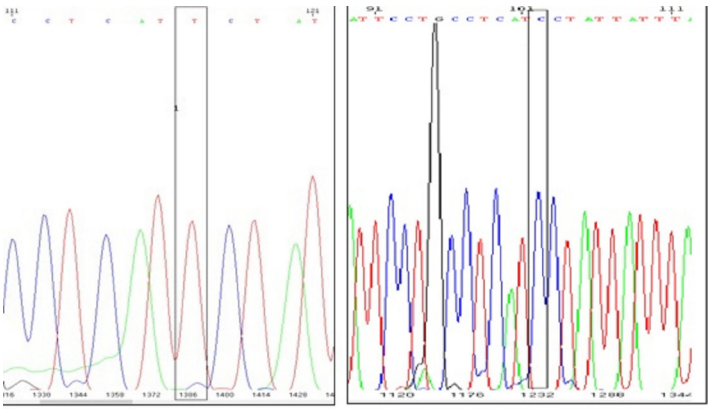

Figure 5. Comparison of the electroforegram of the second couple [image on the left belongs man (SSP150T), image on the right belongs woman]

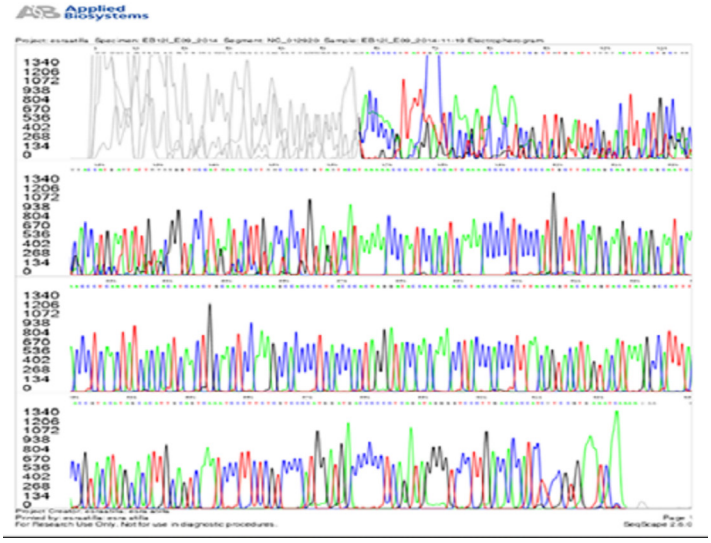

Figure 6. The electropherogram of the female of the third couple

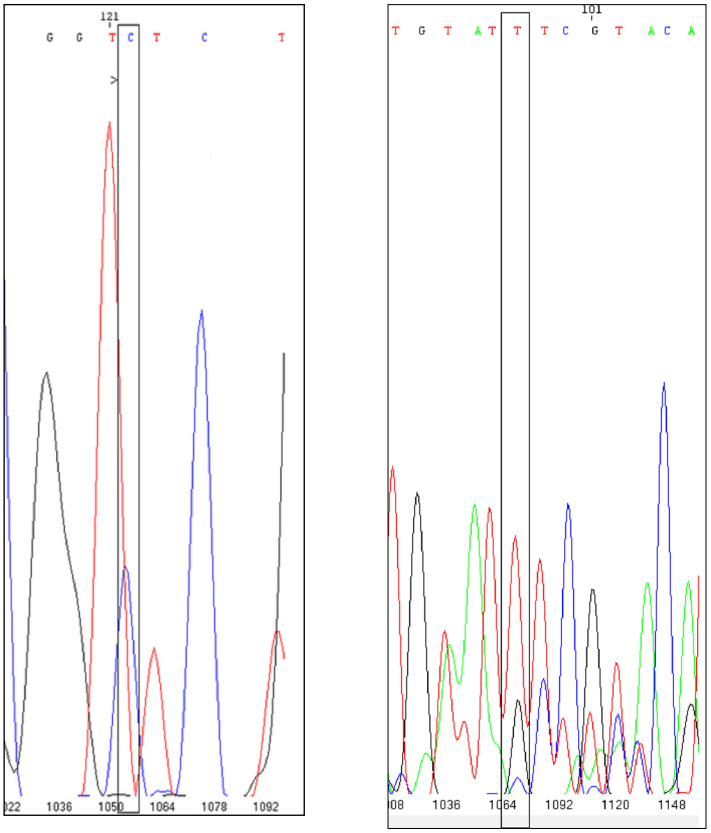

Figure 7. The third electropherogram comparison of the second couple [image on the left belongs man (SSP16093C), image on the right belongs woman]

Discussion

In the case of sexual assaults involving more than one man from the same male lineage, the actual alleged criminal can not be determined with Y-STR analysis. In such sexual assault cases, the presence of multiple copies of mitochondrial DNA (mtDNA) in a single cell, where there are fewer cells present, or situations where DNA is degraded, protection of their ring structure against exonuclear reactions, and mtDNA inheritance from the mother make these analyzes valuable. These advantages that mtDNA provides are rapidly increasing the use of mtDNA typing in forensic sciences [1-3,9]. mtDNA analysis is usually performed by DNA

sequencing of HVI and HVII regions that are highly polymorphic among individuals [10-12]. It is not possible to achieve sufficient discrimination power by analyzing only these regions. Moreover, the fact that sequencing all mtDNA is time-consuming and labor-intensive and not cost-effective also motives forensic scientists to SNPs in mtDNA [13-15]. Mutation rates of SNPs are low. In degraded biological specimens, it is easily amplified. Therefore, SNPs have also begun to be used because of these features [16,17].

In this study, by using the micromanipulation technique, the spermatozoa belonging to the perpetrator were physically distinguished from the epithelial cells of the victim by the assist of the swab, and the male mitotipin was characterized by mtDNA typing from the collected sperm cells. In addition to physical separation by micromanipulation, the couples were examined one by one for characterization of male mitotipine, and the SNP regions separating the male and female were determined, and male specific SSPs were designed on these SNP regions. By this method, the sorting of collected sperm cells separately will enable a solution for gang rape crimes, as well as allowing the exclusion of more than one person from the same male lineage at the time of occurrence, and also ensuring that they come from above the mentioned problems.

For each couple, SNP regions not seen in women present in men were detected. Based on these detected SNP regions, the male specific SSPs of each couple were designed. The study showed that there were similarities in SNP regions belongs to women and men in two volunteer pairs. It was found that one of the couples had all the SNP regions overlapping, and were detected the woman and man have the same SNP regions. When they turned back and were questioned about the kinship of the pair, it was learned that they were relatives (aunt children) by the mother side. When the results of another couple are examined, it was seen that SNPs found at several points in man also existed in woman and while woman was carrying many SNP regions man did not carry. In such cases and in cases involving mother side kinship relations of people, we can state that the discrimination power is limited. In 2012, Pereira and et. al. in Germany were studied with 5 couples in their research. In the evaluation of the couples within themselves, present in man for each couple and SNP regions not seen in the female were detected, and the male specific SSPs of each couple were designed over these points and mtDNA profiles of males were obtained in all pairs using these primers [1]. This coincidence in our study, however, provided a disadvantage of the method, since the relationship of kinship by the mother in this study is not seen in the work of Pereira et al.

According to the results, the time-consuming mtDNA sequencing used in the study, because SSP is not designed, it is disadvantage of the method used by the mother when the relative men are involved due to the overlap of the SNP points in the relatives of the mother. However, thanks to the micromanipulation technique used in the study, the risk of contamination is reduced and individual samples can be isolated with sperm isolation and sequenced with SSPs designed for the individual, indicating that it is a method that can be prefered in sensitive situations such as gang rape crimes involving more than one person from the same male lineage.

Competing interests
The authors declare that they have no competing interest

Financial Disclosure
The financial support for this study was provided by the investigators themselves.

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