MUTATION ANALYSIS OF MITOCHONDRIAL DNA CONTROL REGIONS: A STUDY ON ECTODERM TISSUES

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ABSTRACT

Mitochondrial DNA nucleotide sequence data are known and examined until the mutations level. However, comparison of mutation that occurred in the D-loop and non-D loop in the mtG on the origin of various different tissues on certain individuals who have not yet reported. D-loop is a non-coding region, containing some important sequences such as the promoter for heavy chain replication. On this research analyzed the diversity of D-loop region mtG on the various tissues that different origin on a particular individual. The aim of the research is to see if there is diversity of mtG that come from tissues that originated from the ectoderm tissue and how the mutation pattern nucleotide D-loop mtG the original came from tissue that is different. mtG analysis is done on some parts of the human body cell, such as brain and skin cells. This research begins with the individual samples in the different body tissue through an autopsy and to follow procedures / ethics applicable. Lysis cells to get the template mtDNA using QIAamp DNA Mini Kit. Then, amplified with the PCR technique using REPLI-g Mitochondrial DNA. PCR fragments of DNA D-loop region sequenced with the Sanger Method. Comparative nucleotide sequence analysis shows the diversity of mutation that occurred in the D-loop mtG on certain individuals. Nucleotide sequence data on the tissues in the region D-loop has been successfully

authenticated and published in the International Nucleotide Sequence Database Collaboration (INSDC). Research determining the sequence of mtG human nucleotide of ectoderm in various tissues is involved and to contribute to frontier of knowledge. The result is expected to give benefit the development of biotechnology. Research is expected to generate future process of cooperation in the fields of biochemistry, molecular biology, bioinformatics, and forensic medicine.

Keyword: mtDNA, Control Region, and Ectoderm

INTRODUCTION

DNA is one object of research in the field biotechnologi important today. Analysis of DNA sequence can be of great benefit in various fields, including health. Various studies conducted by experts have revealed a link between mutation of DNA with a number of diseases. In addition, analysis of DNA can be utilized in the field of forensics.

Inside the cell, there are two types of DNA, ie nuclear DNA and mitochondrial DNA (mitochondrial DNA, mtDNA). Nuclear DNA analysis performed by isolating DNA from the cell nucleus, whereas mitochondrial DNA analysis conducted by collecting DNA contained in mitochondria. In this study, reported the results of a study of nucleotide sequence of the D-loop region of mitochondrial DNA. Mitochondrial DNA is unique compared to nuclear DNA contained in the nucleus. The uniqueness in question include the mitochondrial DNA of a specific pattern of inheritance through maternal lineages, a high polymorphism and a system of genetic code that differ from the standard genetic code system in the process of translation of codons into amino acids (Anderson et al, 1981.)

Analysis of mitochondrial DNA can provide solutions for scientists in overcoming the problems encountered when researching nuclear DNA. Samples that have been long, have no cell nucleus material again, thus not allowing nuclear DNA analysis of these samples. However, mitochondrial DNA can still be obtained from these samples. Mitochondrial DNA analysis was also conducted in the field of forensics. Maternal mitochondrial DNA is inherited, so it can be used to determine a person's identity. Human mitochondrial DNA sequence has been determined in 1981, through research conducted by Anderson et. al.

Mitochondrial genome consists of 16,569 base pairs, and is composed of 37 genes involved in energy production and storage in the form of ATP, which takes place in mitochondria. Although most of the mitochondrial genome consists of genes, there are areas noncoding region.

At first, people came from single cells that are the result of fertilization process. This single cell has multiple copies of mitochondrial DNA with the same nucleotide sequence. Furthermore, there has been growth in the number of cells become more numerous, through the process of embryogenesis. When the number of cells that have reached a certain level, these cells had differentiated into the three embryonic layers, namely layers ectoderm, mesoderm, and endoderm. To date not yet known whether the mutation occurred during embryogenesis in the mitochondrial DNA. To know this, should be an analysis of mitochondrial DNA sequences in cells derived from human embryos that layer. The cells in the lining of embryos experiencing differentiation and produce a variety of tissues and organs. Research on the differentiation of tissues or organs of each layer of these embryos can be done to determine if during the process of embryogenesis, ongoing mutation of mitochondrial DNA. In this study reported the results of analysis of mitochondrial DNA in skin and oral mucosal tissue obtained from the same individual. Skin and oral mucosal tissue is the result of differentiation of human embryonic ectoderm layer.

Unlike the genetic system of the cell nucleus consisting of chromosomal DNA, mtDNA has no proofreading activity. Thus, replication errors can not be eliminated in this manner and easy mutation occurs. A high mutation rate in mtDNA cause mtDNA sequence differences between individuals can be easily observed. D-loop region is an area that has the highest level of polymorphism in mtDNA. (Wallace, 1989).

In this study, carried out an analysis of the fragment D-loop and ATPase in mtDNA. D-loop is a non-coding regions, while the ATPase is an area that mengode ATPase enzyme in mitochondria. D-loops serve as controls for the process of mtDNA replication and transcription. Therefore this is not a local area pengode, mutations in this region has no effect on the function of any protein. Mutations in the gene ATPase would affect the function of proteins (enzymes) are produced. Impaired function of this protein can cause illness and even death. Research carried out on a network that is the result of differentiation of human embryonic ectoderm layer.

MATERIALS AND METHODS

Sample Preparation

This study aims to analyze mitochondrial DNA (mtDNA) in tissues that contained in the ectoderm layer in humans. Therefore, in this study, the sample is obtained by taking tissue samples of skin and oral mucosa. Both samples of skin and oral mucosal tissue were obtained from a single individual. The second network is the result of differentiation of human embryonic ectoderm layer. Tissue is taken using a surgical scissors that have been sterilized with ethanol.

Cell lysis

Samples of tissue were prepared and then lysis to obtain mtDNA template. Lysis process took place with the addition of lysis buffer, consisting of 0.5 M Tris-HCl pH 8.5, EDTA 0.01 M pH 8.0, Tween-20 5%. In addition, also added proteinase K and ddH2O. Sample at this stage of lysis, 20 µL been prepared, put into Eppendorf tubes 1.5 µl, then, add 20 µL proteinase K and ddH2O, and 10 µL lysis buffer 10×, 170 µl ddH_2O, this mixture was incubated in a waterbath at 50 ^oC for 1 hour. Furthermore, deactivation of the enzyme performed by incubation at a temperature of 95 ^oC for 3 minutes. The mixture then extracted using the tool sentrifugation 5415 type C with a speed 12.000 rpm for 3 minutes. Supernatant obtained was used as a source of template DNA in the PCR process.

MtDNA PCR Amplification Method

Amplification carried out on the D-loop fragment located at position 16024 -576 in mtDNA. PCR reaction for fragment D-loop performed in 200 µL of reaction mixture consisting of 25 µL tube containing 2.5 µL PCR buffer 2,5 mM $MgCl_2$, 0.5 10 mM dNTP mixture (AmershamµPCR buffer, 2.5 Pharmacia 0,5 µl of each forward and reverse primers), 0.5 µL of enzyme Taq DNA polymerase 5µ l, 1 µconcentration of 20 pmol /µL and 0, 5 µ l, plus ddH2O up to volume 25 µunits / the lysis supernatant (source of template DNA). Amplification primers used in the D Loop is the M1 and HV2R. Fragments are expected to have usuran 0.9 kb. ddH_2O reagent mixture is added to the total volume to 25 µl

PCR process is done by using GeneAmp PCR system 2400 machine (Perkin Elmer) by 30 cycles consisting of several stages. The first stage is initial denaturation at 94 °C (4 minutes), then go to program PCR cycles, with each cycle consisting of three stages: denaturation step at 94 ° C (1 min), annealing phase, and phase extension or initial polymerization at 72 ^{o}C. At the end of every cycle, the polymerization process was extended by heating at 72 °C for 4 minutes to complete the polymerization reaction is not perfect. In the process of amplification of the D-loop, use annealing temperature of 50 oC for 1 minute with initial polymerization for 1 minute. DNA PCR product is then stored at -20 °C before being processed further.

Agarose gel electrophoresis

Amplification results were analyzed by electrophoresis method, using 1% agarose gel. Gel was prepared by mixing 0.3 g agarose, then added 30 ml solution of 1 x TAE (tris-acetate 0.04M, 0001 M EDTA pH 8). This mixture is heated until all the agarose dissolves, then left at room temperature. After the agarose solution temperature reaches 50-60 °C, added 2 µl into EtBr 10µg/mL. This solution was then homogenized by turning the erlenmeyer flask slowly. Solution which is homogeneous, poured into the gel mold (tray) which has been installed insulation at the tip and "comb" as forming the well (well).

After agarose gel freezes, comb and sealing the end of the tray is removed, then the tray containing agarose gel is placed on the *Mini sub^{TM} DNA Electrophoresis Cell* (Biorad) which was filled with TAE 1x buffer solution until the surface of the submerged agarose gel. Into each well included 7 µl loading buffer of PCR product and 2 µl mixture consisting of 5 µL (50% sucrose, 0.1 M EDTA pH 8.0, 0.1% blue bromfenol pH 8.0) . Electrophoresis process underway with 1x TAE as conductive medium flows, the voltage of 80 volts for 45 minutes. Edctrophoresis results of amplfication performed in conjunction with a marker or markers. Marker or DNA size marker used was pUC19/HinfI that has 5 bands (each measuring 1419 bp, 517 bp, 396 bp, 214 bp and 75 bp).

Visualization of DNA with Ultraviolet Rays

Visualizing the results of electrophoresis performed using a series 9814-312 nm UV lamp (Cole Parmer). Prediction of the size and concentration of DNA PCR can be done by

comparing the position and intensity of PCR bands with ribbon marker whose concentration had been determined previously (Sambrook et al, 1989).

Nucleotide Sequence Determination and Analysis of Sequencing Results

Determination of the nucleotide sequence or sequencing performed by Macrogen Inc., Seoul, South Korea. The method used is dideoxy Sanger. For a one-time sequencing reaction, put 800 to 1200 ng samples of PCR product and 2.5 µl. Primer concentration of 10/3pmol /µl. Primer sample and placed in a micro tube and wrapped with parafilm. The data obtained are in the form of electropherogram *abi.file*. In the electropherogram, alkaline nucleotide shown with different colors. Base A, indicated by the green color, base G base with black, bases T are shown in red, and the base C is shown in blue.

Analysis of nucleotide sequence by using SeqMan program, which is one of the software package DNASTAR program version 4.0.0. by using this program, conducted comparing the sample sequence with the nucleotide sequence of nucleotides standard Cambridge Reference Sequence (CRS). The program will mark the bases at certain positions that are different bases on the standard CRS. In addition, also used EditSeq.

RESULTS AND DISCUSSION

DNA Template Preparation

Tissue samples of skin and oral mucosa obtained from the Forensic Laboratory, Hasan Sadikin Hospital, Bandung. Both samples came from a single individual. Skin and oral mucosa is the tissue lining the differentiation of human embryonic ectoderm. Preparation of template was done by lysis of cells found in tissue samples. At this stage, 0.5 mg samples of each tissue sample dilisis, by using lysis buffer, Proteinase K and Tween 20. Tween 20 is non inonik detergents that form micelles in solution. Tween-20 structure consists of the hydrophilic compound composed of esters and alcohols and the hydrophobic compound is a hydrocarbon. Interaction of hydrophobic micelles of Tween-20 with a compound of membrane phospholipids resulting in compounds soluble membrane phospholipids to form mixed micelles with Tween-20 (Noer et al., 1994).

Mixed tissue samples, lysis buffer, Proteinase K and Tween-20 was heated at 55 oC. This heating causes the structure of cell membranes become damaged. Proteinase K enzyme

contained in this mixture can catalyze the degradation process DNAse enzymes and other proteins. Enzyme activity was deactivated by heating at a temperature of 95 oC for 5 minutes. The results of this analysis be used as a template for PCR reaction (Noer et al., 1994).

Amplification of Mitochondrial DNA D-loop

DNA amplification performed using PCR method. In the PCR process, takes place by performing nucleotide polymerization temperature regulation. Amplification took place in a specific region bounded by a pair of primers on the DNA strand. The process of PCR to obtain fragments of the D-loop performed using M1 and HV2R primers. Expected fragment size of 0.9 kb. The success of the PCR process is known to perform electrophoresis of PCR results. Sample electrophoresis of PCR performed using agarose gel 1% (w / v). In this agarose gel, add ethidium bromide (EtBr). EtBr is a flat molecule which can insert between the DNA chain. When viewed under UV light, then EtBr which inserts the DNA chain will produce luminescence (Dale, 2004). State is utilized to detect the presence of DNA fragments after electrophoresed. The position of the electrophoresis sample band compared with marker to evaluate the suitability of the results obtained with the expected. Based on the electrophoresis is done, the D-loop fragment in the two samples produced bands whose positions lie between the first and second bands of the marker pUC / HinfI. Both these bands each measuring 1419 bp and 517 bp. Ribbon amplified DNA fragments against the D-loop region in between, measuring about 0.9 kb as expected.

Nucleotide Sequence Determination and Analysis

Determination of the nucleotide sequence performed by Macrogen Inc.. The data obtained are in the form abi electropherogram files. Electropherogram and sequence of nucleotide bases can be viewed by using the program SeqMan. EditSeq can be used to view the sequence of nucleotide bases only. Sequencing the D-loop fragment in both samples is done through two reactions by using primers HV2R and M1, in order to obtain two data electropherogram for each sample of skin and oral mucosal tissue. Electropherogram obtained help reading the nucleotide sequence of 932 bases. In the electrophoresis results are shown in the example electropherogram data oral mucosal tissue samples, which were sequenced by using primers HV2R. Complete electropherogram of all samples can be seen

in the appendix. This electropherogram data obtained in the form abi file., And can be observed using SeqMan program. Observation of the base sequence of nucleotides, without the electropherogram, it can be done using EditSeq.

In the picture looks the observed nucleotide sequence samples using EditSeq. Furthermore, in silico analysis of this nucleotide sequence. Data complete nucleotide sequence of the D-loop fragment samples of skin and oral mucosal tissue compared with standard nucleotide sequence of D-loop. This analysis is accomplished using the SeqMan. Standard sequence used is the Cambridge Reference Sequence (CRS). In this standard, there is a sequence of 1122 nucleotide base pairs segment of mtDNA D-loop positioned at nucleotide 16 024 to 576.

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   1 tgtgggggggt gtctttgggg tttggttggt tcggggtatg gggttagcag cggtgtgtgt
  61 gtgctgggta ggatgggcgg gggttgtatt gatgagatta gtagtatggg agtgggaggg
 121 gaaaataatg tgttagttgg ggggtgactg ttaaaagtgc ataccgccaa aagataaaat
 181 ttgaaatctg gttaggctgg tgttagggtt ctttgttttt ggggtttggc agagatgtgt
 241 ttaagtgctg tggccagaag cgggggaggg ggggtttggt ggaaattttt tgttatgatg
 301 tctgtgtgga aagtggctgt gcagacattc aattgttatt attatgtcct acaagcatta
 361 attaattaac acactttagt aagtatgttc gcctgtaata ttgaacgtag gtgcgataaa
 421 taataggatg aggcaggaat caaagacaga tactgcgaca tagggtgctc cggctccagc
 481 gtctcgcaat gctatcgcgt gcatacccccc cagacgaaaa taccaaatgc atggagagct
 541 cccgtgagtg gttaataggg tgatagacct gtgatccatc gtgatgtctt atttaagggg
 601 aacgtgtggg ctatttaggc tttatgaccc tgaagtagga accagatgtc ggatacagtt
 661 cactttagct acccccaagt gttatgggcc cggagcgagg agagtagcac tcttgtgcgg
 721 gatattgatt tcacggagga tggtggtcaa gggacccctа tctgaggggg gtcatccatg
 781 gggacgagaa gggatttgac tgtaatgtgc tatgtacggt aaatggcttt atgtactatg
 841 tactgttaag ggtgggtagg tttgttggta tcctagtggg tgaggggtgg ctttggagtt
 901 gcagttgatg tgtgatagtt gagggttgat tgctgtactt gcttgtaagc atggggaggg
 961 ggttttgatg tggattgggt ttttatgtac tacaggtggt caagtattta tggtaccgta
1021 caatattcat ggtggctggc agtaatgtac gaaatacata gcggttgttg atgggtgagt
1081 caatacttgg gtggtaccca aatctgcttc cccatgaaag aa
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Figure 1. Nucleotide sequence of the CRS Standard D-loop region. D-loop region of mitochondrial DNA are at nucleotide position 16024-576. DNA sequence was used as a standard to observe a mutation of DNA in the sample.

Analysis of these data indicate different nucleotides or mutations in skin tissue samples (Ku) with standard sequence of CRS. In tissue samples of oral mucosa (MM) also found differences in the standard sequence of nucleotides with CRS. Followed by examination of nucleotide differences electropherogram of each sample. Data analysis was performed using a sample electropherogram SeqMan program. This program marks automatically if there is a different nucleotide bases between DNA chains being compared.

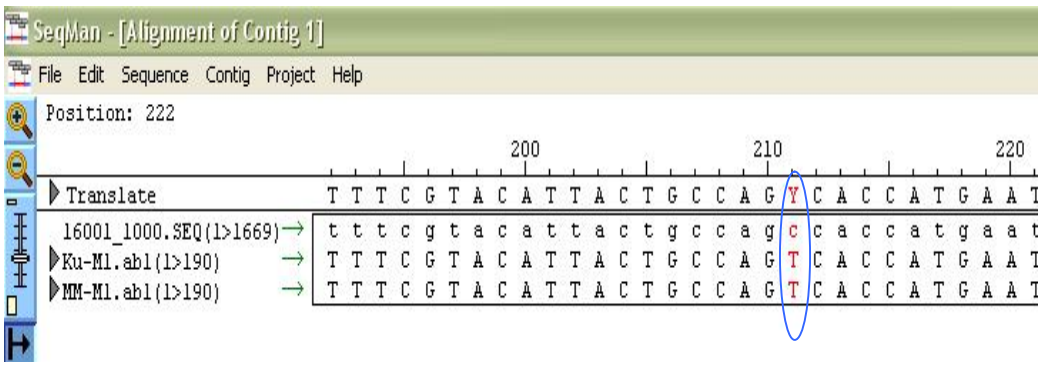

Figure 2. Nucleotide Sequence Comparison Samples of CRS. CRS standard nucleotide sequence contained in the first row. In the second and third rows are the nucleotide sequence of the D-loop fragment of my samples (skin) and MM samples (oral mucosa). SeqMan program marks a different base sequence of nucleotides compared.

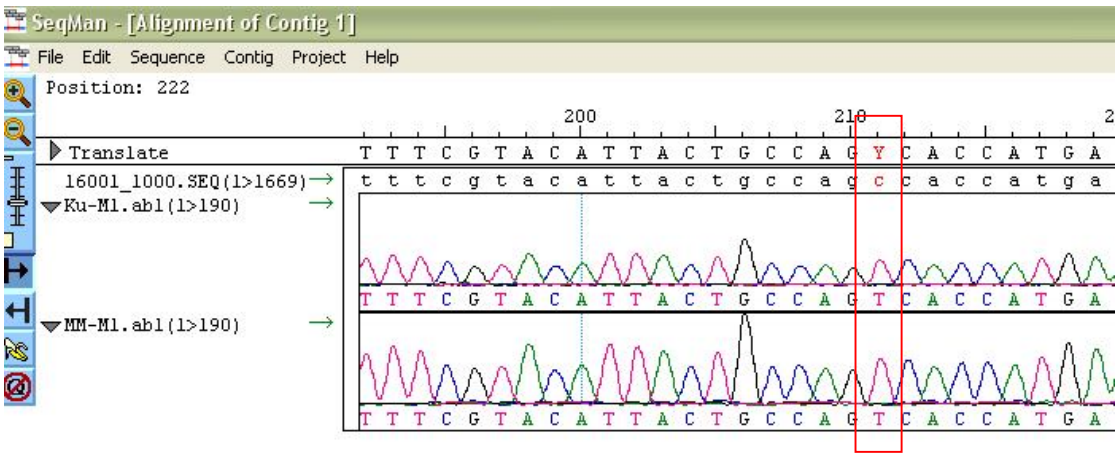

Figure 3. Sample electropherogram Data Analysis Tissues Skin and Oral Mucosal with CRS. Analysis of the data sample electropherogram Ku (Skin) and MM (Mouth mucosa) of images showed a C insertion mutation at a specific position. The program automatically SeqMan mark different nucleotides.

Researchers who had previously conducted research on mitochondrial DNA have reported a number of mutations they found. These mutations have been published in www.mitomap.org. Data published mutation is updated if there are mutations that have not been reported previously.

Among seventeen mutations observed in skin samples, there is a mutation that has not been published, namely C (16 270) G, while sixteen other mutations have been reported.

Table 1. Mutation that is observed in Sample Skin Tissue.

No	Mutations in the sample Ku	CRS	Ku
1	16111	C	T
2	16168	C	T
3	16172	T	C
4	16183	A	--
5	16189	T	C
6	16197	C	T
7	16222	C	T
8	16223	C	T
9	16270	C	G
10	16328	C	T
11	16362	T	C
12	16519	T	C
13	73	A	G
14	152	T	C
15	263	A	G
16	309	C	CC
17	310	T	TC
	Total: 17 mutations		

Table 2. Mutations Observed in Oral Mucosal Samples Tissue.

No	Mutations in the sample MM	CRS	MM
1	16111	C	T
2	16168	C	T
3	16172	T	C
4	16183	A	--
5	16189	T	C

6	16222	C	T
7	16223	C	T
8	16328	C	T
9	16362	T	C
10	16444	C	T
11	16519	T	C
12	73	A	G
13	152	T	C
14	263	A	G
15	309	C	CC
16	310	T	TC
	Jumlah: 17 mutasi		

In this analysis, also carried mutations comparison between the two samples. The comparison showed that among both samples is observed fifteen identical mutation. These mutations occur in the same type and same nucleotide position in both samples. However, the results of this comparison also shows that there are nucleotide differences in the two samples. Mutation C (16 197), T and C (16 270), G is only observed in samples of skin tissue, and not termati in oral mucosa samples. Mutation C (16 444), T is only observed in samples of oral mucosa and absent in samples of skin tissue.

The two samples analyzed showed mutation T to C substitution at nucleotide position 16189 which resulted in a poly-C in both samples. These mutations may disrupt the process of determining the nudeotide sequence through direct sequensing, so that the resulting nucleotide sequence is incomplete. Therefore, bidirectional sequencing electropherogram results using M1 as the primary forward primer and reverse primer HV2R as very helpful reading of the nucleotide sequence of mtDNA samples.

Table 3. Differences Fragments of D-loop mtDNA Samples Skin and Oral Mucosal

No	Mutations in the sample Ku	Mutations in the sample MM
1	C(16111)T	C(16111)T
2	C(16168)T	C(16168)T

3	T(16172)C	T(16172)C
4	delesi A (16183)	delesi A (16183)
5	T(16189)C	T(16189)C
6	C(16197)T	-
7	C(16222)T	C (16222)T
8	C(16223)T	C(16223)T
9	C(16270)G	-
10	C(16328)	C(16328)
11	T(16362)C	T(16362)C
12	-	C(16445)T
13	T(16519)C	T(16519)C
14	A(73)G	A(73)G
15	T(152)C	T(152)C
16	A(263)G	A(263)G
17	C(309)CC	C(309)CC
18	T(310)TC	T(310)TC
	Jumlah: 17 mutasi	jumlah: 16 mutasi

Differences mutation found in both samples, indicating that the tissue or organ which is the result of differentiation of the ectoderm layer, not 100% homologous. At first, it is estimated if the sequence of nucleotides in the two samples showed 100% homology, then the mutation is unlikely to occur during human development from the zygote through embryogenesis process that produces three layers of the embryo, until the formation of tissue or organ differentiation results of the three embryonic layers. However, in the presence of nucleotide differences in the two samples, it is known that a mutation in the tissues derived from this ectoderm layers.

The differences observed in the second mutation affecting the use of mitochondrial DNA samples on homology analysis for forensic analysis. Because both samples have a different mutation, the sequence of nucleotides in the two samples is not 100% homologous. If the results of homology analysis showed differences in mutation like this, it will lead to doubt or even can lead to errors in data interpretation. Based on these reasons, it is suggested the

use of the same network as the source of mtDNA template for homology analysis in forensic analysis. These suggestions are given with the assumption that cells originating from the same network have identical nucleotide sequences of mtDNA.

CONCLUSION

In the D-loop region, the skin samples were observed seventeen mutations, whereas the oral mucosal tissue samples of sixteen mutations were observed when compared with standard nucleotide sequence of the CRS. Among all the mutations that occur in both samples, there are two mutations that have not been reported, that mutations C (1917) T, C (16 270) G, and C (16 444) T. In both samples, there were fourteen identical mutation. In addition, between the two samples also contained different mutations. Thus, it can be concluded that both samples originated from human embryonic ectoderm layer has a degree of similarity or nucleotide sequence homology does not equal 100%. Therefore, the suggested use of the same network as the source of template DNA to examine the homology of the nucleotide sequence of mtDNA in forensic analysis.

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