



Full Length Research Paper

Varian in D-Loop of mitochondrial DNA from hair fibers of some Indonesian people

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Abstract

Mitochondrial DNA is widely used as a biomarker in the relationship analysis between individuals, because of its number copies and its level of polymorphism is high. The biomarker of mtDNA is unusual embedded in hypervariable regions HV1 and HV2. This study reported the nucleotide diversities of (HV1) and HV2 in human mtDNA D-loop from some Indonesian people that live in Surabaya. The research was developed by amplification of mtDNA d-loop from human hair samples then sequencing of HV2 and HV1 regions. The amplification of mtDNA d-loop was successfully performed by primers MH and HV2R at 55 °C as annealing temperature and result the d-loop fragment of mtDNA at 1 kb. The sequencing and alignment analysis of mtDNA d-loop showed that the region of HV1/HV2 of Indonesian mtDNAs are distinct, having the highest level of polymorphism, and showing specific each individual. The genetic diversity value for the combined HV1 and HV2 of human Indonesian mtDNA was 0.97 following the nucleotide diversity was 0.098.

Keywords: D-loop mtDNA, HVS1, HVS2, Indonesian people.

INTRODUCTION

The mitochondrion is one of two cellular organelles that has self genome which different with the nucleus genome. Mitochondrial DNA (MtDNA) shows uniqueness, ie relatively no effect on the phenotype and has a high mutation rate than nuclear DNA, which is about 10-17 times from the nuclear DNA (Wallace et al., 1997) so that the impact on the high level of polymorphism. This can occur due to the process of mtDNA replication that has no a system repair. Mitochondrial DNA polymerase has no proofreading activity exonuclease 5' → 3' and 3' → 5' (Wallace et al., 1997; Bellwood et al., 2003). Moreover the mtDNA is inherited only through the maternal lineage with no recombination. This phenomenon led to that the child inherits the mother's mtDNA (maternally inherited). This nature, likely to take advantage of mtDNA as a tool to determine the identity of an individual or population through the maternal lineage (Wallace et al, 1997). Furthermore, the mtDNA genetic code system is different from the genetic code of the nucleus genome (Anderson et al., 1981). The mtDNA copy number per cell is high (over 1000 copies) in each

cell, whereas nuclear DNA amounted to only two. This gives technical advantages which specimens in poor condition (or conditions of sample burning fossil) it is still possible to be analyzed.

The nucleotide sequences of the human mitochondrial DNA was first published by Anderson et al (1981) and revised by Andrews et al (1999). The DNA sequence called rCRS (revised Cambridge Reference Sequence). RCRS sequence (16569 bp) has been widely used as a standard reference in the study of diversity (polymorphisms) of human mtDNA sequences.

mtDNA comprises coding and noncoding regions. The coding region contains 37 genes for 2 rRNAs, 22 tRNAs, and 13 polypeptides for subunits of enzyme complexes that involved in oxidative phosphorylation, namely: subunits 1, 2, 3, 4, 4L, 5, and 6 of complex I, subunit b of cytochrome b, subunit I, II, and III of complex IV (cytochrome oxidase) and subunits VI and IIX of complex V (cytochrome oxidase). Most of these genes are transcribed from the H strand, namely 2 rRNA, 14 of the 22 tRNAs and 12 polypeptides. MtDNA has no introns

Table 1. The nucleotide of primer

No	Primer Name	Nukleotides sequence of primers (5'→ 3')
1.	HV2R	CTG TTA AAA GTG CAT ACC GCC
2.	HV2F	GGT CTA TCA CCC TAT TAA CCA C
3.	MH	CAC CAT TAG CAC CCA AAG CT
4.	ML	TGA TTT CAC GGA GGA TGG TG

and all genes located adjacent in coding region (Anderson et al., 1981; Wallace et al., 1997). While other proteins that also function in oxidative phosphorylation such as metabolic enzymes, DNA and RNA polymerase, ribosomal proteins and mtDNA regulatory factors are all encoded by nuclear genes, synthesized in the cytosol and then imported into the organelle (Wallace et al., 1997; Melton et al., 2001).

The non coding region of mtDNA has 1122 bp size, starting from 16024 to 576 nucleotides and is located between the tRNA^{phe} gene and tRNA^{pro} gene. This area contains regions with high variation called the displacement loop (D-loop). D-loop is a three-stranded region (triple stranded). The third strand is known as 7S DNA (Hill et al., 2007; Krause et al., 2010). D-loop has two regions with a high rate of polymorphism so the sequence is highly variable between individuals, the hypervariable I (HVS1) and hypervariable II (HVS2). Non coding region also contains a control region because it has the origin of replication for the H strand (OH) and promoter transcription to H and L strands (PL and PH) (Anderson et al., 1981; Gill et al., 1994). In addition, the noncoding region also contains three areas of conserved sequence called conserved sequence block (CSB) I, II, III. Conserved region is thought to have an important role in mtDNA replication (Anderson et al., 1981; Gill et al., 1994; Bellwood et al., 2003). We have investigated the nucleotide diversity of HVS2 and HVS1 in the mtDNA d-loop from some Indonesian people using hairs as samples.

MATERIALS AND METHODS

Samples

Samples in the research were hair cells that collected from five unrelated volunteers. All volunteers are Indonesian people who live in Surabaya. The number of sampled hairs each individuals are seven.

Extraction of Mitochondrial DNA

Mitochondrial DNA was prepared by lysis of hair cells each individuals in buffer (5 mM, pH 8.5 TrisCl; 0.1 mM EDTA pH 8.5, and 0.5% Tween-20 (b/v)) containing 0.2 mg/mL proteinase K. The mixture was incubated at 50 °C for one hour and then final incubation at 95 °C for 3 min. The cellular debris was removed by centrifugation at

11,000 g for 10 min. The supernatant obtained was used as DNA template for PCR.

Primer design

The primer design has been done with the Clone Manager program using the primary mtDNA Cambridge Reference Sequence (CRS) as template. Within the human mitochondrial genome, the nucleotides sequence of HV1 is located in the region of approximately between base numbers 16024–16365, while HV2 is approximately between base numbers 73 and 340 (Anderson et al., 1981). We have created primer sequences MH and HV2R for amplification of mtDNA d-loop region for a 1-kb product that containing the HV1 and HV2 collectively. The nucleotide primers were presented at Table 1.

Polymerase chain reaction

MtDNA D loop amplification performed by PCR using the template results hair cell lysis and primer pair design results. PCR reaction mixtures will be done in a volume of 25 mL containing 12.5 pmol forward and reverse primer, 5 µL solution of template containing around 50 ng DNA, 1.25 units of Taq-DNA polymerase (Amersham, New Jersey, USA), 1x PCR buffer (10 mM Tris HCl pH 9, 1.5 mM MgCl₂, 50 mM KCl), and 200 µM dNTPs. Process will be carried out by PCR-DNA Thermal Cycler machine with the following steps: pre-denaturation at 94 °C for 4 min, 35 cycles of denaturation step at 94 °C for 1 min; annealing at the optimum temperature of 50-60 °C for 1 min, and polymerization step at 72 °C for 1 minute. Post-elongation was conducted at 72 °C for 7 min. The PCR products were analyzed in agarose gel electrophoresis and purified by GFX purification kit (Amersham, New Jersey, USA).

DNA sequencing

The d-loop of mitochondrial DNA was sequenced by an automatic nucleotide sequencer (ABI PRISM, Macrogen, Seoul-Korea). All oligonucleotide primers used in the sequencing of *mtDNA d-loop* are presented in Table 1.

Alignment analysis

The *mtDNA d-loop* from each sample was analyzed *in silico* by aligning the sequences of their nucleotide to be compared to CRS sequences. Alignment of the

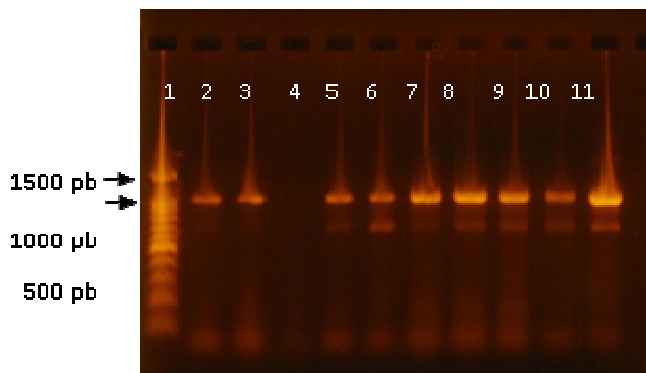


Figure 1. The product of mtDNA d-loop amplification that run at 58 °C for annealing temperature. The DNA fragment of mtDNA d-loop has a measurement of 1 kb. Lane 1, Marker 100 bp leader; lanes 2&3, the PCR product using a mtDNA template from sample 4; lane 4 from sample 12; lane 5&6 from sample 13; lane 7, 8&9 from sample 29; and lane 10&11 from sampe 34

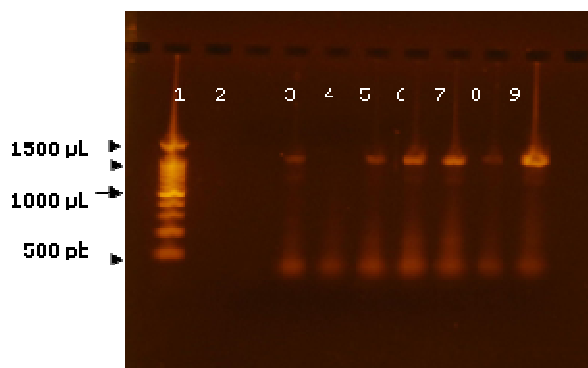


Figure 2. Results of d-loop mtDNA amplification that run at 50 °C for annealing temperature. Lane 1, Marker 100 bp leader; lanes 2, negative control; lane 3&4, the PCR product using a mtDNA template from sample 4; lane 5 from sample 12; lane 6, from sample 13; lane 8, from sample 29; and lane 9 from sampe 34

nucleotide sequences was performed by the DNA star program for SeqManTMII instruction (Lasergene 1997).

RESULTS

PCR primer design

Amplification of mtDNA d-loop requires primer pair. The primer pair was designed directly from the nucleotide sequence of human mtDNA Revised Cambridge Reference Sequence (rCRS) (GI: 251 831 106). The primer design was done in silico with DNA Star program and Clone Manager and resulted in primer pair namely MH and HV2R (Table 1).

The PCR Product of mtDNA D-loop

The mtDNA d-loop was successfully amplified by PCR using primers MH and HV2R and generate DNA

fragments that have a measurement of 1 kb (Figure 1). This result indicated that all steps of the PCR process is going well. Optimization of annealing temperature in the PCR process is performed at a temperature of 50-60 °C. By using of annealing temperature at 58 °C, the PCR produced a clear band for target DNA fragment of mtDNA d-loop (1 kb), but non target band also appeared sharp (Fig 1).

The PCR that was run at 50 °C for annealing temperature could generate a 1 kb DNA fragment corresponding to mtDNA d-loop, but the band of the fragments were slight (Figure 2). When annealing was carried out at 55 °C, the PCR generate a sharp band at 1 kb DNA fragment for the mtDNA d-loop target and followed by appearing of very slight non targeted DNA fragment (Figure 3). The target DNA that had a measurement at 1 kb was isolated from the gel and purified with GFX purification kit for nucleotides sequencing.

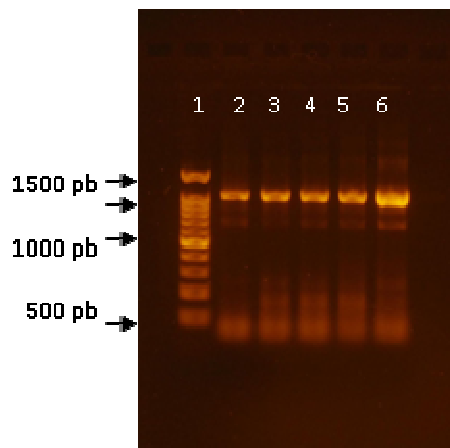


Figure 3. Results of d-loop mtDNA amplification at 55 ° C annealing temperature. Lane 1, Marker 100 bp leader; lane 2-5, the PCR product using a mtDNA template from sample 4. 12, 13, 29 and 34 in a series.



Figure 4. Sequencing scheme for mtDNA D-loop

The Variant of HVS1 and HVS2 in each individuals mtDNA d-loop.

The sequencing of mtDNA D-loop nucleotides from PCR product were performed with four primers (Figure 4). By using of these primers, sequencing could read sample for 1000 nucleotides, in which there are HVS1 and HVS2 sequence.

Variant in HVS1 and HVS2 of samples was determined by comparing of their nucleotides to CRS. Each individual has a different sequence of HVS2 and HVS1 from another and some nucleotides variant were met in both HVS2 and HVS1 (Table 2).

DISCUSSION

It is unusual for individuals to match over the entire mtGenome, even when they are identical in HV1/HV2. Currently the forensic testing in mtDNA targets involves sequence analysis of the most often targeting hypervariable regions one and two (HV1/HV2) for a total of 610 base pairs (Coble et al., 2004; Wallace et al., 1997). The ability to discriminate between HV1/HV2 sequences has many important applications. Among those are distinguishing among multiple matching suspects, distinguishing between victim and suspect, and distinguishing among multiple matching families in mass disasters or missing persons projects (Wallace et al., 1999; Coble et al., 2004). In this paper, HV1/HV2 refers to the following sequence ranges: HV1=16024–16365

and HV2=73–340 (Anderson et al., 1981; Andrews et al., 1999). In determining the HV1/HV2 sequences to target for the research, we chose human hair as samples since the hairs have been used in forensic investigations for over a century. Hairs are readily available for transfer, easily transferred, and resilient.

To characterize better Indonesian mtDNA variation, we have determined the HV1/HV2 sequence types by the analysis of 5 samples from individuals that live at Surabaya. The result in Table 2 shown that in the five individual samples from Indonesian people, there were 31 different mtDNA sequences for HV1, and 29 different mtDNA sequences for HV2. The variation of mtDNA HV1 and HV2 regions was confined to 61 positions, of which 31 was observed in the HV1 and 29 in the HV2. Sequence variation consisted of 30 transitions and 1 insertion for HV1, and 27 transitions and 2 insertions for HV2. The genetic diversity value for combined HV1 and HV2 regions was 0.97 in this Indonesian population sample, and the nucleotide diversity was 0.098. For HVS1, the minimum variant is met in sample PRK2 that has 2 variants, and the biggest is 11 that was in sample PRK34, while for HVS2, the lowest variant is 3 in sample PRK12 and the biggest is 10 in sample PRK 4. Variant that was found in samples PRK4, namely C16223T variant has been found by other researchers in human Indonesia from different tribes or regions. The C16223T variant was found in individuals Sundanese, southern Borneo (Banjarmasin), North Sulawesi (Gorontalo) and the Moluccas (Ternate) (Ratnayani et al., 2007). The

Table 2. Variant of HVS1 and HVS2 d-loop mtDNA samples compared with CRS

Nomor Sample	HVS1 (CRS→sampil)	Number of variants	HVS2 (CRS→sampil)	Number of variant
PRK4	T16024G C16030A C16223T C16261T T16362C	5	T142G C144G C145G T152A T180A A198T A201T A202T A263G G329A	10
PRK12	C16129A A16162G	2	T172A T179A C309T	3
PRK13	C16223T T16311C T16315A T16318A T16322A	5	A278T A286G C299A A302C	4
PRK29	T16094C G16129A C16223T T16263C G16274A T16311A A16343G T16357C	8	G185C C198T A263G G316C	4
PRK34	A16183C T16189C Insertion C at 16194 C16224T A16259C A16270C T16272C T16312C C16314A T16316A C16340A	11	G100T G101T C110G C118A T209A A263G Insertion C at 309 Insertion C at 311	8

C16223T variant is likely unique from population of Indonesian people.

Our results show that the region of HV1/HV2 of Indonesian mtDNAs are distinct, having the highest level of polymorphism, and showing specific each individual, therefore is of great utility in profiling a marker and searching kinship system as well as providing additional forensic resolution.

CONCLUSION

Amplification of DNA fragments containing d-loop sequence of mtDNA HVS2 and HVS1 from each individual hair cells sample have been successfully performed using primers MH and HV2R at optimum temperature 55 °C. The amplification obtained a DNA fragment at 1 kb in size. The sequencing results and alignment analysis of nucleotide sequences indicates that

each individual has the nucleotide sequences of HVS2 and HVS1 that distinct from one another.

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