A new D-Loop mitochondrial nucleotide variation from individual at Naga and Kuta traditional village

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Abstract. The D-Loop region of human mitochondrial DNA (mtDNA) has a higher level of polymorphism compared to nuclear DNA. Therefore, the mtDNA D-Loop area can determine a particular individual or ethnic identity. This study aims to determine the variation of the D-Loop region nucleotide sequence, namely the Hipervariable I (HV1) region in normal tribal individuals in Kampung Naga and Kampung Kuta. The sample used is the hair root. In determining the variation of nucleotides has been carried out a series of stages of research, namely the isolation of mitochondrial DNA from hair root samples using buffer lysis, mtDNA sample amplification using PCR techniques with M1 primers and HV2R primers, detection of D-Loop region mtDNA fragments by agarose gel electrophoresis, and sequencing of PCR products by the Sanger Dideoxy method and nucleotide sequence analysis of the sequencing results. The results of DNA amplification by PCR showed a band in the area of 1.0 kb. Homology analysis was carried out by comparing the nucleotide sequences of samples with the Cambridge nucleotide sequence, which shows that there are 10 variations. The new variation (morph) is determined by comparing the results of the variations of the two samples with variations from Homo Sapiens Indonesia including Baduy, Sundanese, Javanese (Sangiran), and Madura. Homology results showed 4 new variations, namely c(16184)A, t(16209)C, a(16272)G, t(16519)C. Sapiens Kuta village and Sundanese (general) have a close genetic relationship. The ancestors of the two Homo Sapiens were closely related to Homo Sapiens Naga, Homo Sapiens Naga are closely related to the Homo Sapiens of the Baduy. The ancestors of Homo sapiens, Kuta, Sundanese, Naga and Baduy, are closely related to Homo sapiens Sangiran (Java). Homo Sapiens Madura is most likely the oldest Homo sapiens seen from phylogenetic trees. The results of this study are expected to be able to add Indonesia's normal human database.

1. Introduction
Phenotype human were different from others. It is come from difference genetical information and their environment. Moreover, every human has a different either phenotype or genotype even they have the same ancestor. The environment where they live seem to be a major factor of the difference. Genetical information were coding in deoxyribonucleic acid (DNA). It played a role in inheritance of genetical information. DNA were place in nucleus and mitochondrial of human body [1].
Mitochondrial DNA is unique. It only inherit to a daughter from her mother. Genome of mitochondria consist of 13 gene which coding sub unit protein respiration enzyme, 12s rRNA gene, 16S rRNA gene, 22 tRNA encoding gene, and non-coding area namely D-Loop. D-Loop is a special region in mitochondria that has a control of gene transcription, and it has a high rate of polymorphism [2]. Therefore, D-Loop could be used as human identity, detection of hereditary disease, evolution study, and modern human global migration [3].

D-loop nucleotide sequence divides into two regions, namely hypervariabel 1 (HV1) and hypervariabel 2 (HV2). To date, Hypervariabel characteristic was connected to comparison sequence, and human nucleotide mtDNA mutation, age, and tribe [4].

The objection of this research is to contribute data base mtDNA of native people in West Java, Indonesia. The research was specifically analyse variation pattern D-Loop nucleotide sequence from Sunda native tribe which live in Naga and Kuta village, Tasikmalaya.

Naga and Kuta village have strict regulation according to ancestral culture. There are prohibit for filling the house with chair, desk, and bed. Also having two doors from opposite direction is prohibited. They only do Endogamy marriage [5].

Unfortunately, Sunda tribe still have unclear information of their history yet. This is happened because it lacks of its historical heritage and native tribe research. In year 2001 a research gain information about genetic relationship from ethnic group in Indonesia except Sunda tribe based o Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) [5].

2. Materials and methods

2.1. Materials

Materials that were used in this research are mtDNA template from follicel cell oh human hair. Lysis buffer (500 µL Tris - HCl pH 8; 20 µL EDTA pH 8; 5% Tween - 20), Proteinase - K (Thermo Scientific), ddH₂O, 100 pmol / µL M1 primer (MacroGen), 100 pmol / µL HV2R primer (MacroGen) (Table 1). DreamTaq Green Master Mix (2X) (Thermo Scientific) (buffer PCR, MgCl₂, dNTPs), ddH₂O, agarose gel (Thermo Scientific), TAE buffer (Tris - asetat 0,05 M; EDTA 0,001 M pH 8), 1 µL loading dye (Thermo Scientific), 0,5 µL Red gel (Thermo Scientific), and GeneRuler 1 Kb DNA Ladder (Thermo Scientific).

| Primer | Sequence | Position | Size |
|--------|----------|----------|------|
| M1     | -CACCATTAGCACCCAAAGCT- | 15.978-15.997 | 20 Nucleotide |
| HV2R   | -CTGTAAAAGTGCAATACGC- | 409-429 | 21 Nucleotide |

2.2. Methods

2.2.1. DNA extraction from follicel cell

As much as 12 hair root were placed in microtube. The DNA extraction was done by using lysis buffer method. The name of all samples were list in Table 2.

| Sample code      | Age (year) | Sex   | Native kampong   |
|------------------|------------|-------|------------------|
| KT-KIMFST-UINSGD | 75         | Female| Kuta Kampong     |
| NG-KIMFST-UINSGD | 45         | Female| Naga Kampong     |

2.2.2. Amplification of mtDNA fragment. Amplification were done by using Polymerase Chain Reaction (PCR) technique. A pair of primer M1 and HV2R is used to amplificate D-loop fragment of mtDNA. This primer was designed to amplificate HV1 and HV2 region. PCR condition were described in Table 3.
Table 3. PCR mastermix composition.

| PCR Component | Stock | Volume Reagen (µL) |
|---------------|-------|-------------------|
|               |       | 1x                | 2x    | 4x    |
| Master Mx     | 5,625 | 11,25             | 22,5  |
| M1 primer     | 0,45  | 0,9               | 1,8   |
| HV2R primer   | 0,45  | 0,9               | 1,8   |
| NFW           | 11,25 | 22,5              | 45    |
| DNA template  | 5      | 10                | 20    |
| **Total volume** | **22,775** | **45,55** | **91,1** |

Amplification process was done in 30 cycle, each cycle contained 5 stage, pre-denaturation 90°C for 5 minutes, denaturation 94°C for 1 minutes, annealing stage 50°C for 1 minute, polymeration 72°C for 5 minutes, and final polymeration 72°C for 4 minutes. The amplicon was detected by agarose gel electrophoresis. The amplicon concentration was calculated by nanodrop by Nano Quant Plate Tecan 200M Pro.

2.2.3. D-Loop fragment sequence analysis. Nucleotide were sequence by Dideoxy Sanger Method. Software CLUSTALW was using to analyse homological D-loop fragment sequence. To describe relationship between the fragment and others, phylogeny analysis is needed. Phylogeny analysis was explained by phylogenetic tree using Mega 5.2 program Neighbour Joining tree and bootstrap methods with No. Of Bootstrap Replications 1000 software.

3. Results
D-Loop mitochondria fragments were successfully amplified. They were approximately 0.1 kb in size (figure 1). This electroforegram indicated the successfully amplification D-loop fragment using M1 and HV2R primers.

Figure 1. Agarose gel electrophoresis. Line 1 is 1 kb marker. Line 2. NG-KIMFST-UINSGD. Line 3 KT-KIMFST_UINSGD.

3.1. Homology analysis
D-Loop sequence from Cambridge Reference Sequence (CRS) was used as standard sequence. Alignment between all two sample fragment and CRS resulting several variation of DNA. There are five variation found from fragment sample from Naga village (NG-KIMFST-UINSGD), and four variations from Kuta village (KT-KIMFST-UINSGD). All variation occurred in hypevariabel I (HV1) region (Table 4).
A new variation could be discovered by aligning all samples sequence with several ethnic Indonesian sequence and CRS as standard (table 5). We align sample sequence with D-Loop sequence from Baduy tribe, Sunda tribe, Sangiran (Java), and Madura tribe. All sequence was in NCBI database, and have a rule for Indonesian genetic marker.

3.2. Phylogenetic analysis
Genetic relationship of samples sequence was analyzed by building a phylogenetic tree, using Neighbour Joining Bootstrap 1000 (figure 2). We use similar sequence from related tribe as comparison. There are Sunda tribe, Baduy tribe, Madura tribe, and from Sangiran.

There are four new nucleotide variation found in this research. There are c(16184)A, t(16209)C, a(16272)G, and t(16519)C. Interestingly, all new variation located in HV1 region. Also, we found that new variation only occurred in D-Loop fragment of human from Naga and Kuta village.

| Variation Homo Sapiens (Baduy, Sunda, Sangiran, Madura) | Sample variations (Naga dan Kuta village) | New Variant |
|-------------------------------------------------------|------------------------------------------|------------|
| t(16046)C, g(16047)C, g(16048)T, g(16049)T, a(16051)T, c(16052)T, c(16053)T, a(16054)-, c(16056)A c(16057)G, a(16059)C, c(16065)T, c(16052)T a(16080)C, c(16081)T, a(16084)T, a(16091)-, c(16108)T, a(16109)G c(16111)T g(16129)A, t(16131)C, a(16162)G, t(16172)C, t(16183)C, t(16189)C, c(16223)T, c(16278)T, t(16304)C, a(16309)G, t(16325)C. | c(16184)A, c(16223)T, c(16278)T, t(16209)C, a(16272)G, t(16519)C. | c(16184)A, c(16223)T, c(16278)T, t(16209)C, a(16272)G, t(16519)C. |

Figure 2. Phylogenetic analysis of samples.
While, Madura homo sapiens has no correlated with all the sequences. Likely Madura homo sapiens was originated from the same ancestor of all sequence. Therefore, Madura could be the oldest homo sapiens.

4. Discussion

Genetic marker is a difference base nucleotide from people in one species [6]. Analysis new variation generating an assumption that new variation could be used as genetic marker candidate for Sundannese tribe. Another assumption is for some people who have same genetic marker, could be stated that there are relationship between those people. Phylogenetic analysis show that Kuta and Sundanesse tribe has several distances. It is mean a lot of variation that found in one species, it is mean there are a lot of diversion characteristic from that species.

New variation probably as a result of evolution and nature selection. Living creature, including human being is mutan product and product of recombinant. It come from survival. Evolution concept explain that mutan who live at present time and living in suitable space for them will become normal phenotype, or we could mention it a wild type. If, a mutan had a mutation, could adapt with their environment, growing fast and defeating normal phenotype at that time, it will become a new normal phenotype [7].

5. Conclusions

There are nine nucleotide variation found from D-Loop homo sapiens at Naga and Kuta kampong. From nine variation, there four new variation, namely c(16184)A, t(16209)C, a(16272)G, t(16519)C. Homo sapiens from Naga kampong has a close relationship with Sundanese tribe. Whether Naga or Kuta Kampong has the same ancestor. While Madura tribe had a probability as the oldest homo sapiens.

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