Journal of Applied Sciences

ISSN 1812-5654

science alert

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Journal of Applied Sciences 14 (2): 197-200, 2014
ISSN 1812-5654 / DOI: 10.3923/jas.2014.197.200

Haplogroup Determination Using Hypervariable Region 1 and 2 of Human Mitochondrial DNA

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Abstract: Analysis of human mitochondrial DNA (mtDNA) is commonly used in ancestry determination due to variation in its Hypervariable (HV) regions. The aim of this study is to amplify HV 1 and 2 regions of human mtDNA to determine individual geographic ancestry using human peripheral blood sample. Twelve pairs of primers were designed using the Primer Premiere 5 Software based on the HV region in human mtDNA. Six human peripheral blood samples (1, 1a, 2, 2a, 3 and 3a) were used in this study. DNA samples were amplified using Polymerase Chain Reaction (PCR) followed by bioinformatics sequence analyses using BLASTn in NCBI. Sequence mutations were analysed using Mitomap to determine the possible haplogroup. Twelve fragments of six DNA samples were successfully amplified and sequenced. Mitomap analysis showed that sample 1 and 1a, 2 and 2a, 3 and 3a come from the same maternal lineage as they share the same sets of mutations in the HV region. Sample 1 and 1a were estimated belongs to haplogroup M10b, sample 2 and 2a belongs to haplogroup F1a and sample 3 and 3a belongs to haplogroup D5b. In conclusion, the twelve primers designed can be used as haplogroup determination hence geographical ancestry.

Key words: Human mtDNA, haplogroup determination, hypervariable region, mutation analysis, ancestral determination

INTRODUCTION

Mitochondrial DNA (mtDNA) is commonly used for ancestral determination in archaeological specimens, evolutionary studies, human migration and forensic identification (Pakendorf and Stoneking, 2005; Ariffin *et al*., 2007). It contains 16, 569 bp sequences and can be the only source of surviving DNA in highly degraded specimens or samples with low quantity of DNA such as human bones or hair shafts (Graffy and Foran, 2005; Adler *et al*., 2011). The invention of PCR made it possible to amplify the targeted DNA to millions of copy number (Yang *et al*., 2003). The ability of mtDNA to survive over time is due to its high copy number per cell as compared to nuclear DNA (nDNA) which has one copy per cell. In addition, mtDNA is closed and circular in shaped while nDNA is linear shaped with 3 billion of base pairs making nDNA prone to degradation due to strand break (Alonso *et al*., 2004). Furthermore, mtDNA is maternally inherited, meaning that the offspring will inherit the same mtDNA (Giles *et al*., 1980). mtDNA consist of coding and non-coding regions. Non-coding region also known as Hypervariable (HV) or Displacement (D) loop region. The region of interest in human mtDNA that was used in ancestral determination is HV region which contained HVI and HV2 regions (Meyer *et al*., 1999). This region has high rate of mutation which can be used as a marker for human ancestry identification (Horai and Hayasaka, 1990). Several studies showed that each population has their own sets of mutation or haplotype in their mtDNA (Zainuddin and Goodwin, 2004; Hoong and Lek, 2005; Chen *et al*., 2008). A cluster of haplotypes classify a haplogroup that shares common variable characters which defined them as having similar ancestry (Budowle *et al*., 2003). Therefore, the objective of this research is to develop a set of primers which covers the whole HV region on human mtDNA to determine geographical ancestry by analyzing the sets of mutations in each sample.

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MATERIALS AND METHODS

Genome DNA isolation: All blood samples were taken from individuals of Peninsular Malaysia. Six samples of human peripheral blood were used in this study. Three samples labeled as the 1, 2 and 3 samples while another three blood samples were obtained from individual that have the same maternal lineage with the 1, 2 and 3 sample, labeled as 1a, 2a and 3a samples respectively. Approximately 1 mL of peripheral blood was taken by certified medical personnel and collected in EDTA vacutainer tube (BD, USA). The DNA of blood samples were immediately isolated using Invisorb Forensic Kit I (Stratec, Germany). The procedure of DNA isolation followed manufacturer instruction manual. OD reading was obtained to determine the quantity and quality of isolated DNA samples using Biophotometer (Eppendorf, Germany). The size of isolated genome DNA was determined by 1% (w/v) of agarose gel electrophoresis.

Primer design: Twelve pairs of primers were designed based on the HV region of human mtDNA using the Primer Premier 5 software. These primers were designed to produce 12 overlapping sequences which covered the whole mtDNA HV region (HV1: 16024-16569, HV2: 1-576) in order to validate the mutations observed in sequence analysis hence does not require repetition of sequencing using the same primer (Fig. 1). The primers were designed to amplify sequences approximately 262-804 bp in length (Table 1).

PCR amplification procedure: Isolated DNA samples were amplified using PCR which was performed using Eppendorf Mastercycler gradient (Eppendorf, Germany). The PCR mixture consisted of DNA template (10 ng), forward and reverse primers (1μM), GoTaq DNA Polymerase (1.25 U) (Promega, USA), GoTaq buffer (Promega, USA), $MgCl_2$ (2.5 mM), BSA (40 ng L^{-1}) and dNTP mixture (200 μM) (Promega, USA) in a total volume of 20μL. The PCR was conducted using the following program: 94°C for 1 min (denaturation), 50-62°C for 1 min (annealing) and 72°C for 1 min (extension) for a total of 35 cycles. The program was preceded with a pre-denaturation step of 94°C for 3 min and finalized with an extension step at 72°C for 5 min. The PCR products were electrophoresed through 1% (w/v) agarose gel in 1 X TAE buffer and visualized using ethidium bromide staining under UV transillumination. The DNA band of interest were gel purified using MEGAquick-spin Total Fragment DNA Purification Kit (Intron, Korea).

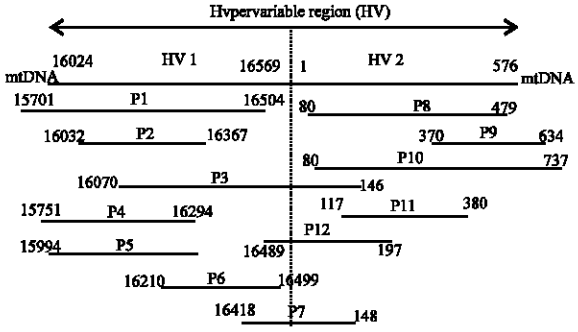

Fig. 1: Twelve overlapping sequences from twelve pairs of designed primers which cover the whole HV region in the human mtDNA

Table 1: Twelve primers were designated as P1 to P12 which produced 12 spcific sequences with the length between 262 to 804 bp. Nucleotide position is based on human mtDNA genome database (Accession No. 012920.1)

Primer	Sequence length (bp)	Nucleotide position
P1	804	15701-16504
P2	336	16032-16367
P3	646	16070-146
P4	546	15751-16294
P5	329	15994-16324
P6	290	16210-16499
P7	300	16418-148
P8	400	80-479
P9	262	370-634
P10	659	80-37
P11	263	116-386
P12	279	16489-197

Cloning and sequencing: The purified genes of interest were ligated into pGEMT Easy Vector System 1 (Promega, USA) as per the manufacturer's instruction. The recombinant plasmid was transformed into TOP10 competent cells through heat-shock. Plasmids were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) and subjected to digestion using EcoR1 to verify the insert size. Samples were subjected to sequencing analyses using T7 forward and SP6 reverse primers separately.

Bioinformatics analysis: The results of DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program at NCBI (http://www .ncbi.nlm.nih.gov/) in order to compare between the sample's sequences with reference sequences from human mtDNA database (accession number NC-012920.1). Mutations of DNA sequences were analyzed using the MITOMAP program (http://www.mitomap.org) to determine the haplogroup of samples and their possible ancestry.

Table 2: Location and type of mutations in mtDNA (a) HV1 region, (b) HV2 region and (c) Haplogroup estimation for each samples

Sample S1	Sample S1a	Sample S2	Sample S2a	Sample S3	Sample S3a
(a) Mutation of HV1					
-	-	16129 G-A	16129 G-A	-	-
16066 A-G	16066 A-G	-	-	-	-
-	-	16172 T-C	16172 T-C	-	-
-	-	-	-	16189 T-C	16189 T-C
-	-	-	-	16223 C-T	16223 C-T
16209 T-C	16209 T-C	-	-	-	-
16304 T-C	16304 T-C	16304 T-C	16304 T-C	-	-
16311 T-C	16311 T-C	16311 T-C	16311 T-C	-	-
-	-	-	-	16362 T-C	16362 T-C
16399 A-G	16399 A-G	-	-	-	-
16519 T-C	16519 T-C	16519 T-C	16519 T-C	16519 T-C	16519 T-C
(b) Mutation of HV2					
73 A-G	73 A-G	73 A-G	73 A-G	73 A-G	73 A-G
89 T-C	89 T-C	-	-	-	-
93 A-G	93 A-G	-	-	-	-
147 C-A	147 C-A	-	-	147 C-A	147 C-A
-	-	-	-	150 C-T	150 C-T
-	-	249-A	249-A	-	-
263 A-G	263 A-G	263 A-G	263 A-G	263 A-G	263 A-G
302 +C	302 +C	-	-	-	-
		309 +C	309 +C		
310 +C	310 +C			310 +C	310 +C
-	-	315 +C	315 +C	-	-
-	-	-	-	456 C-T	456 C-T
-	-	-	-	489 T-C	489 T-C
514-C	514-C	514-C	514-C	-	-
515-A	515-A	515-A	515-A	-	-
(c) Haplogroup					
M10b	M10b	F1a	F1a	D5b	D5b

(A: Adenine, G: Guanine, T: Thymine, C: Cytosine, -C: Deletion of cytosine, -A: Deletion of adenine, +C: Addition of cytosine, C-T: Substitution of cytosine to thymine and vice versa, A-G: Substitution of adenine to guanine and vice versa. accession No. for mitochondrial DNA hypervariable region: NC-012920.1)

RESULTS AND DISCUSSION

DNA of human peripheral blood samples (n = 6) were successfully extracted with high concentration (~352 ng μL^{-1}) and purity (range of 1.8-2.0 for spectrometry absorption 260/280 and 260/230). The results of twelve sequences from both sense and antisense designed primers showed a range of 97-99% identical with the sequence from mtDNA genome database (NC 012920.1) at NCBI using the BLASTn program. There were 15 mutations detected in both the 1 and 1a samples, 12 mutations in the 2 and 2a samples and 11 mutations in both the 3 and 3a samples (Table 2a and 2b).

Mutation analysis showed that the 1, 2 and 3 samples have the same set of mutations with their maternal lineage which were the 1a, 2a and 3a samples in HV region of mtDNA (Table 2a and 2b). This concluded that different maternal lineage carried different set of mutations in their HV region. Types of mutation were mostly nucleotide substitution, deletion and addition. Based on Mitomap analysis of each sample, the 1 and 1a samples were expected belongs to M10b haplogroup, the 2 and 2a samples matched with F1a haplogroup and the 3 and 3a Samples belongs to D5b haplogroup (Table 2c). These haplogroups (F1a, D5b and M10b) are included in several haplogroups that is present in Southeast Asian population (Nur Haslindawaty *et al*., 2010; Hill *et al*., 2006).

CONCLUSION

Based on the findings, two conclusions can be made. The twelve pairs of overlapping primers designed based on the HV region of human mtDNA can be used to determine the haplogroup of individuals and hence, their geographic ancestry. The mutation analysis of hypervariable region of human mtDNA also showed that individual that came from the same maternal lineage share the same set of mutation.

ACKNOWLEDGMENTS

The authors would like to thanks the Ministry of Higher Education (MOHE) for the financial Grants (FRGS/1/2011/SG/UKM/02/13 and ERGS/1/2012/SKK11/UKM/02/5) and Universiti Kebangsaan Malaysia (DPP-2013-024, UKM-DLP-2012-025 and UKM-DLP-2012-001) for supporting this research.

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