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Haplogroups determination from RNR1 and RNR2 regions of mitochondrial DNA

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***Abstract*---**Mitochondrial DNA (mtDNA) has two regions, i.e. coding and control regions which are also known as hypervariation (HV) regions. Analysis of hypervariation regions or the entire human mtDNA genome is an important element that could be used during human evolutionary, forensic or phylogenetic studies. Although most human phylogenetics studies often use HV regions; the lack of information from those regions can complicate the process of haplogroup determination. Therefore, in this study, the human mtDNA coding regions such as MT-RNR1 and MT-RNR2 genes were used as an alternative to determine the haplogroups. A total of four

primer pairs were designed based on MT-RNR1 and MT-RNR2 genes using the Primer Designing Tool software. The human mtDNA sequence from the National Center for Biotechnology Information (NCBI) database (accession number NC_012920.1) was used as a reference. These primers were used in the PCR amplification approach using DNA genomic samples isolated from human peripheral blood. Polymorphism analysis of MT-RNR1 and MT-RNR2 regions using the Mitomaster database was performed to determine haplogroups. The results indicated that three haplogroups were identified which are N11a, H2a and H1. In conclusion, the primers designed from mitochondrial RNR1 and RNR2 genes can be used for human haplogroup identification.

Keywords---Haplogroup determination; hypervariation regions; mitochondrial DNA.

Introduction

Determination of haplogroups using mtDNA is an important technique in forensic research and archaeological studies to determine a person's identity and race (Shahrul Hisham et al. 2007). The variations that occurred in the mtDNA genome may discriminate against individual populations (Sahidan et al. 2014). This widespread use is due to the unique characteristics of mtDNA that make it highly suitable for evolutionary studies. A single cells has many copies of mitochondrion DNA as compared to nuclear DNA (nDNA). (Amorim, Fernandes & Taveira 2019; Duan, Tu & Lu 2018; Pakendorf & Stoneking 2005; Willerslev & Cooper 2005). Therefore, the chances of obtaining mtDNA from a sample in a limited quantity are higher as compared to nDNA. Moreover, its circular shape and small size (16569 bp) make it difficult to be degraded as compared to nDNA which has a linear shape and large size.

MtDNA is inherited maternally where only the mother could transfer it to all her children. The mtDNA maternal inheritance pattern also shows a haploid genome inheritance pattern rather than through fusion. Therefore, the variation in mtDNA is not due to recombination but more to mutations (Amorim, Fernandes & Taveira 2019; Drobná & Celec 2018; James T. Case 1981; Liu et al. 2020). In addition, the mutation rate of mtDNA is higher than nDNA (Amorim, Fernandes & Taveira 2019) due to severely lacking repair mechanisms as well as accuracy during DNA synthesis by a polymerase enzyme. This will increase the mitochondrial DNA mutation rate, especially in some polymorphism areas where a higher rate of mutation occurs (Karki et al. 2015).

Most human phylogenetic studies often use HV regions, however, the lack of information from this region can complicate the process of haplogroup determination. On the other hand, polymorphism analysis of coding regions such as the MT-RNR1 and MT-RNR2 could be used as an alternative region to determine individual haplogroups. The objectives of this study were to amplify MT-RNR1 and MT-RNR2 genes from human mitochondrial DNA and determined the haplogroup for potential application in human phylogenetic studies.

Materials and Method

Primer design and DNA isolation

The determination of human population haplogroups involved a total of four primers with each pair designed specifically for MT-RNR1 and MT-RNR2 regions using the Primer Designing Tool software from the National Center for Biotechnology Information (NCBI) database with accession number NC_012920.1 (Table 1). A total of 3 mL of blood samples were taken and placed in an EDTA vacutainer tube (BD Bioscience, USA). Blood sample extraction, followed by mtDNA isolation was performed on the same day. Mitochondrial DNA isolation was performed using the InnuPrep Blood DNA Mini Kit (Analytik Jena, Germany) based on the recommendation by the manufacturer's manual. The purity of the isolated DNA was determined by spectrometry O.D (optical density) readings and DNA size using 1.7% (w/v) gel electrophoresis analysis.

Table 1. Primers were designed based on MT-RNR1 and MT-RNR2 genes.

Primer Name		Primer Sequence	Gene	Annealing Temperature (°C)	Expected Size (bp)
P4	Forward	5' ATCCCCGTTCCAGTGAGTTC 3'	MT-RNR1	59.6	265
	Reverse	5' TTGGGGAGGGGGTGATCTAA 3'			
P5	Forward	5' TAGATCACCCCCTCCCCAATA 3'	MT-RNR1	61.7	211
	Reverse	5' TAAGCTGTGGCTCGTAGTGT 3'			
P16	Forward	5' AGGGATAACAGCGCAATCCT 3'	MT-RNR2	62.8	252
	Reverse	5' TATCATTTACGGGGGAAGGCG 3'			
P19	Forward	5' CGCCTTCCCCCGTAAATGA 3'	MT-RNR2	65.0	258
	Reverse	5' TTGGGGCCTTTGCGTAGTT 3'			

PCR amplification

The purified mitochondrial DNA was amplified using the PCR approach. The PCR mixture contained DNA templates, buffer, $MgCl_2$, forward and reverse primers, dNTP, and DNA polymerase. PCR amplification was initiated with a pre - denaturation process at 95 °C for 2 min followed by 35 cycles of denaturation process at 94 ° C, annealing at 59.0 °C - 65.0 °C depending on the primers (Table 1) and elongation at 72 ° C. Finally the elongation process occurs at 72 °C for 5 min. The amplified PCR products will undergo automated sequencing. The obtained sequences were analyzed bioinformatically using BLAST software for comparison with the database sequences at NCBI (www.ncbi.nlm.nih.gov). On the other hand, mutation positions on the sequence were identified and analyzed using Mitomap software to determine haplogroups (www.mitomap.org).

Results and Discussion

The DNA genome consisting of mitochondrial DNA was isolated, however, only the nucleus DNA genome with a size of more than 10kb was able to be visualized during agarose gel electrophoresis analysis (Fig. 1A). This could be due to the low amount of mitochondrial DNA in the samples. A concentration of DNA that had successfully isolated was 47.0 ng/µL. The optical density (OD) reading at the absorption ratio of A260/280 was 1.8 and A260/230 was -182.20. However, a total of four types of DNA fragments were successfully amplified using PCR methods specific to the constructed primers for mitochondrial DNA namely P4 and P5 for the MT-RNR1 gene region (Fig 1B and C), while P16 and P19 for the MT-RNR2 gene region (Fig 1D and E) respectively.

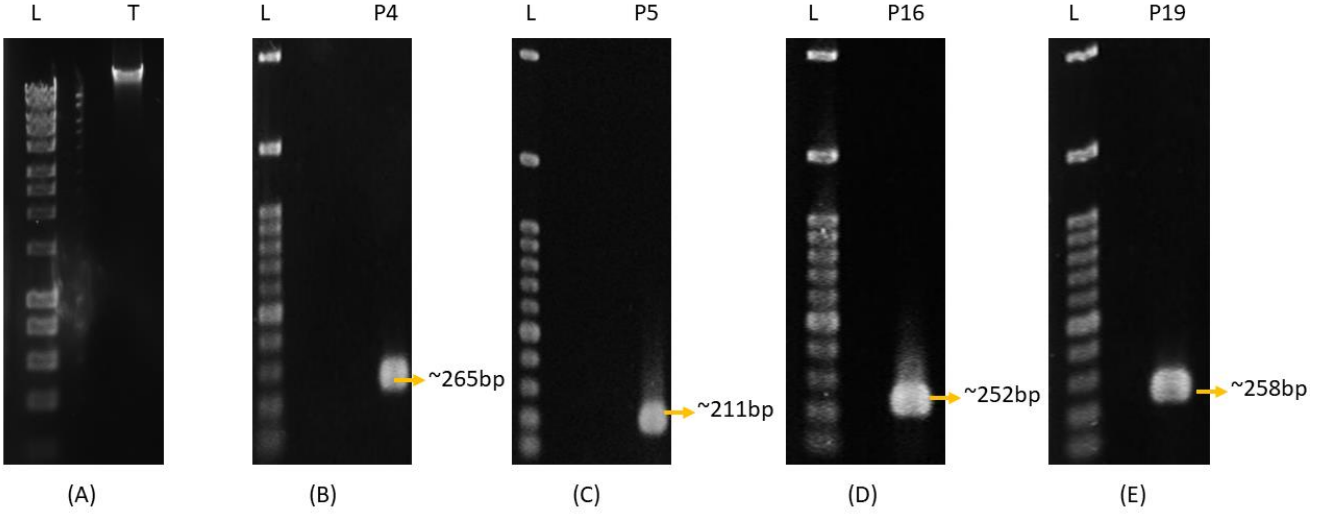

Figure 1. DNA isolation (A) showed a DNA size > 10 kb, where L1 was a 1 kb marker (Promega, USA). The amplification results were analyzed using 1.7 % (w/v) agarose gel together with DNA marker H3 RTU 100 bp; L1 (GeneDirex, Taiwan). The mtDNA fragment P4 showed the band sizes of ~265 bp (B), P5 (~211 bp; C), P16 (~252 bp; D) and P19 (~258 bp; E).

The annealing temperatures for the MT-RNR1 and MT-RNR2 primers were at 59.6 °C (P4) and 61.7 °C (P5) for MT-RNR1 (Figure 1B and C) followed by 62.8 °C (P16) and 65.0 °C for MT-RNR2 (P19) (Figure 1D and E). These amplified fragments were successfully sequenced and analyzed using the Basic Local Alignment Search Tool (BLAST) to compare the sample's sequence with the reference sequences from the human mtDNA genome in NCBI database (accession number NC_012920.1). Each SNP that was found in the sequenced was tabulated and validated through electropherogram using BioEdit software. The analysis of BLAST displayed more than 97% of similarity percentage when the sample's sequence was compared to the mtDNA reference sequence based on the NCBI database (Table 2).

Table 1. Sequencing analysis for mtDNA fragment using BLAST in NCBI database.

mtDNA Fragment	Accession No.	Identities (%)	Percentage
Forward mtDNA fragment P4	NC_012920.1	98.69	
Forward mtDNA fragment P5	NC_012920.1	98.32	
Forward mtDNA fragment P16	NC_012920.1	97.72	
Forward mtDNA fragment P19	NC_012920.1	99.55	

There was a total of 10 polymorphisms found with six polymorphisms at the MT-RNR1 gene region, and two polymorphisms at the MT-RNR2 gene region (Table 3). However, only four polymorphisms were able to identify the haplogroups. The haplogroups were identified using MITOMAP software shows that the the studied DNA samples belongs to the haplogroups N11a, H2a and H1.

The results showed that the SNP present, namely 750G and 813G in the MT-RNR1 region, can be identified as the N11a haplogroup (Kong et al. 2011). N11a is one of the subclades of haplogroup N. Based on the theory of the origin; haplogroup N was originated from Asia although currently can be found throughout the world even with low frequency at Sub-Saharan Africa. The presence of haplogroup N in Africa is most likely due to theresults from the Eurasia migration (Gonder et al. 2007).

Based on SNPs in the MT-RNR2 region which were 1004.1T and 3010A, hence the haplogroups H2a and H1 were identified and these two haplogroups were the subclade of haplogroup H. Haplogroup H is believed to have originated from Southwest Asia which is close to Syria at approximately 20,000 to 25,000 years ago (Achilli et al. 2004). Currently, haplogroup H is also widespread in parts of Africa, Siberia and deep Asia. In addition, haplogroup H is also the mtDNA clade most found in Europe (Ghezzi et al. 2005), which is estimated to represent about 41% of Native Europeans.

Table 3. Location and type of polymorphism in mtDNA of MT-RNR1 and MT-RNR2 genes.

Nucleotide Location	Gene	Nucleotide	Type of Polymorphism
744	MT-RNR1	A → -	Deletion
750	MT-RNR1	A → G	Substitution
813	MT-RNR1	A → G	Substitution
988	MT-RNR1	G → A	Substitution
993	MT-RNR1	A → -	Deletion
1004	MT-RNR1	G → GT	Addition
2968	MT-RNR2	A → -	Deletion
2971	MT-RNR2	A → -	Deletion
2994	MT-RNR2	T → TG	Addition

3010	MT-RNR2	G → A	Addition

Conclusion

Based on mitochondrial mutation polymorphism analysis at the coding region, the haplogroups of studied samples were identified as the N11a, H2a and H1. However, comprehensive molecular human population studies could not focus specifically on the highly hypervariable (HV) region or coding region but should involve the entire mtDNA genome. Complete polymorphism analysis performed on both coding and non-coding regions on mtDNA can create more accurate haplogroups database of mitochondrial DNA genomes that can be further used as a reference in several studies such as human molecular phylogenetic, population studies and forensic science.

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