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Short Communication
Optimization of Allele Specific PCR for the Development of Human Mitochondrial DNA Typing Method

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Abstract
Background and Objective: Human DNA is categorized into nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Mutation occurred in these two types of human DNA can be utilized for the purpose of medical reports, molecular genetic analysis, haplogroup variations, forensic crime search and many others. The polymorphisms were selected from mtDNA in both coding and control regions. Main objective of this project was to produce a simple, cost effective yet robust typing method for human identification using allele specific PCR (asPCR) specifically designed for southeast Asian population. **Materials and Methods:** A total of 60 subjects with ethical approval were randomly collected with 30, 20 and 10 of them represented Malay, Chinese and Indian population. Polymerase Chain Reaction (PCR) was carried out using 25 sets of primers, that amplify the fragments bearing the selected SNPs. In the second round PCR, two types of allele specific primers labelled as wild type allele specific primer (wtASP) and variant type ASP (vtASP) were used to amplify both Cambridge Reference Sequence (CRS) and variant sequence. The PCR was used for first round and for the second round, the as PCR was applied. Finally, the amplified products were directly viewed using UV light. **Results:** Overall, out of 30 selected SNPs, the designed ASP managed to amplify only 20 SNPs. The selected SNPs in this study were SNP 146, 195, 1709,1719, 1872, 3705, 3027, 3552, 4491, 7684, 9080, 8440, 13626, 16108, 16291, 16274, 16355, 16093, 16335 and 16148 that reported to belong to macrohaplo group M, B, F, E and N. All the amplified products of selected mtSNPs were observed in wild type lane except for SNP 195 that the amplified product was observed in variant lane. **Conclusion:** The variant allele managed to be amplified with simple technique yet robust to be brought on site. Generally, mutation found using this technique may narrow down the individual and also population hence it is beneficial in cases such as forensic crime and mass disaster.

Key words: Variant allele, human population, polymorphisms, molecular genetic analysis, mutation, mitochondrial DNA, haplogroup variations

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Deoxyribonucleic Acid (DNA) is unique to each individual and genetically inherited. The two types of DNA are nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). The nDNA is inherited from both parents while mtDNA is inherited only from mother. DNA has been used in molecular genetics and medical diagnosis and has contributed great findings in forensic research[1]. Compared to mtDNA, nDNA has given more interest in forensic research due its strong ability for individual identification[2]. Regardless of its limitation, nowadays mtDNA is preferred by researchers. Having circular double helical shape, mtDNA is more stable compared to nDNA and able to survive in extreme conditions[3]. Due to this advantage, it is useful in forensic investigation as forensic cases always deals with degraded, small quantity and contaminated samples[4]. Furthermore, variations that occurred in mtDNA genome may discriminate individual population[5].

Variation of mtDNA sequence occurs due to several inherited events, such as point mutation. Human evolution and geographical area are reported factors that influence human mtDNA variations[6]. According to previous studies, the Last Glacial Maximum (LGM) has caused the migration of people out of Africa. People were believed to first originate from Africa and later migrated out, formed populations and expanded in every new place they live. The mtDNA phylogenetic tree was developed where L group was stated as root of the tree. This L group was divided into L0 till L7 sub-haplo groups where L3 was specifically allocated for people that migrated out of Africa towards Asia[7]. This L3 group has branched out and formed macrohaplo group M and N. Subclades of M group including group D, G, Q, C, Z and E are extension to these two groups, under M macrohaplo group. Under N macrohaplo group, there were group A, X, O, S, I, W and Y and this N group has also branched out into superhaplo group R with subhaplo group[8] of P, H, V, J, T, F and K.

Haplo group is a group of populations that shared mutation which can be linked back to their ancestors[9]. These mutations are inherited throughout generations that can help in population discrimination. Mitochondrial DNA Single Nucleotide Polymorphisms (mtSNPs) are the variants found in mitochondrial genome that is useful in matrilineal study[10].

This study was aimed to develop an optional method for South East Asia (SEA) human mtDNA typing hence selection of SNPs was based on southeast Asia haplo groups. A total of 30 SNPs were selected from both mtDNA control and coding regions. These SNPs were reported in previous studies to belong to southeast Asia haplo groups that includes SNPs from haplo group[11-14] E, M9, B4, N21, R21 and F1a1a. The 30 selected SNPs are 153, 146, 195, 479, 1709, 1719, 1872, 3027, 3394, 3552, 3705, 4491, 7684, 8440, 9080, 9512, 13362, 13626, 16093,16108,16287, 16390, 16309, 16274, 16148, 16291, 16335, 16355, 16261 and 16266.

The mtDNA typing method was designed to discriminate individuals based on population using the mtDNA sequence variation. Successful amplification of variant allele may narrow down the individual and also population and therefore the technique offers in this mtDNA typing method with simple, cost effective yet robust is reliable[15]. Hence, allele specific PCR was proposed with the aid of internal PCR control to amplify the CRS and variant sequence. The developed typing method may aid human identification in mass disaster and can be used in population studies.

MATERIALS AND METHODS

Samples collection and first round PCR: For first round PCR, 30 sets of primers for SNP 16390, 16261, 16266, 16355, 16291, 16093, 16108, 16274, 13626, 153, 146, 3552, 16148, 16309, 16287, 1709, 1719, 195, 9080, 3394, 13362, 4491, 3027, 3705, 9512, 7684, 479, 1872 and 8440 were designed to amplify one of the collected DNA sample. A total of 60 buccal swabs were collected from Malay, Chinese and Indian population with ethical approval from Universiti Sains Malaysia. PCR were successful using all the designed primers[16].

Purification of the amplified products: The PCR products from the first round PCR were purified using purification kit from Qiagen following the protocol provided in the kit. The purified PCR products were divided into two portions. Half of the purified products was sent for sequencing and the other half was used for second round PCR. The sequencing was carried out to confirm the sequence of each amplified fragment.

Second round PCR: A total of 60 allele specific primers (ASP) were designed (30 for wild type ASP and 30 for variant) using available online tool (http://bioinfo.biotec.or.th/WASP). For PCR reaction, the PCR reagents from Bioline were used as follows: Buffer (2.5 µL), dNTPs (0.5 µL), $MgCl_2$ (0.75 µL), ddH_2O (19.5 µL), forward and reverse primers (0.5 µL each) and DNA template (0.5 µL). The PCR program was as follows: 95°C for initial denaturation, 95°C for denaturation, 50 and 60°C for annealing, 72°C for extension, 72°C for final extension and the samples were kept at 4°C for final step. The optimum annealing temperature for all ASP is listed in Table 1.

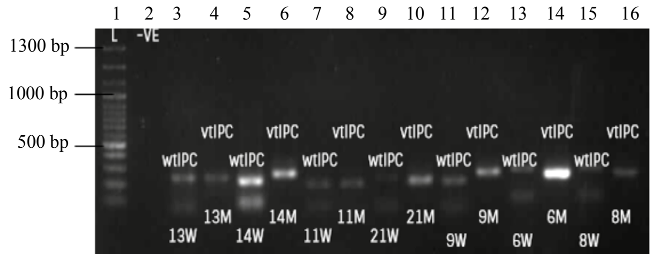
Table 1: Optimized annealing temperature for wild type ASP and variant ASP

ASP	Annealing temperature (°C)
Wild type for SNP 16 148	56
Variant for SNP 16 148	
Wild type for SNP 3552	52
Variant for 3552	
Wild type for SNP 16 335	50
Variant for SNP 16 335	
Wild type for SNP 1872	52
Variant for SNP 1872	
Wild type for SNP 1709	53
Variant for SNP 1709	
Wild type for SNP 16 093	50
Variant for SNP 16 093	
Wild type for SNP 16 355	52
Variant for SNP 16 355	
Wild type for SNP 195	52
Variant for SNP 195	
Wild type for SNP 16 274	50
Variant for SNP 16 274	
Wild type for SNP 8440	52
Variant for SNP 8440	
Wild type for SNP 13 626	50
Variant for SNP 13 626	
Wild type for SNP 16 291	52
Variant for SNP 16 291	
Wild type for SNP 9080	52
Variant for SNP 9080	
Wild type for SNP 3705	52
Variant for SNP 3705	
Wild type for SNP 4491	52
Variant for SNP 4491	
Wild type for SNP 146	50
Variant for SNP 146	
Wild type for SNP 1719	53
Variant for SNP 1719	
Wild type for SNP 16 108	50
Variant for SNP 16 108	
Wild type for SNP 3027	52
Variant for SNP 3027	
Wild type for SNP 7684	52
Variant for SNP 7684	

Gel electrophoresis: The products from second round PCR were separated through electrophoresis using 1% agarose gel and visualized under UV light. The amplified products would appear either in wild type lane or variantlane[17].

RESULTS

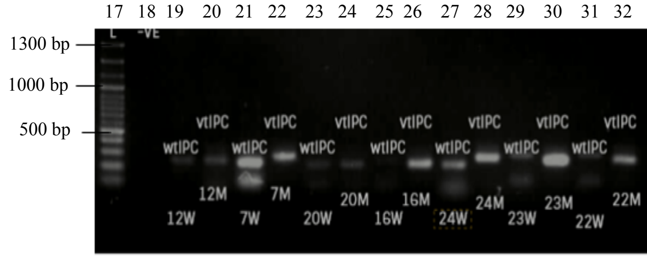
Out of 30 samples, a total of 20 amplified products from first round PCR manage to be amplified using allele specific primers for both wild type and variant in as PCR as shown in Fig. 1-3. From the results obtained, all the amplified products were observed in wild type lane except the SNP 195 (12 M) that the allele has changed from T to C and observed in variant lane. The wild type and variant for all the selected SNPs were



Lane	Sample	Description	Size (bp)
1	L	100 bp ladder	-
2	-VE	Negative control	-
3	wtIPC	Wild type IPC for SNP 16148	288
	13W	Wild SNP 16148	105
4	vtIPC	Variant type IPC for SNP 16148	288
	13M	Variant SNP 16148	-
5	wtIPC	Wild type for SNP 3552	257
	14W	Wild SNP 3552	110
6	vtIPC	Variant type IPC for SNP 3552	257
	14M	Variant SNP 3552	-
7	wtIPC	Wild type IPC for SNP 16355	225
	11W	Wild SNP 16355	115
8	vtIPC	Variant type IPC for SNP 16355	225
	11M	Variant SNP 16355	-
9	wtIPC	Wilt type IPC for 1872	245
	21W	Wild SNP 1872	77
10	vtIPC	Variant type IPC for SNP 1872	245
	21M	Variant SNP 1872	-
11	wtIPC	Wild type IPC for SNP 1709	203
	9W	Wild SNP 1709	89
12	vtIPC	Variant type IPC for SNP 1709	203
	9M	Variant SNP 1709	-
13	wtIPC	Wild type IPC for SNP 16093	238
	6W	Wild SNP 16093	130
14	vtIPC	Variant type IPC for SNP 16093	238
	6M	Variant SNP 16093	-
15	wtIPC	Wild type IPC for SNP 16335	243
	8W	Wild SNP 16335	82
16	vtIPC	Variant type IPC for SNP 16335	243
	8M	Variant SNP 16335	-

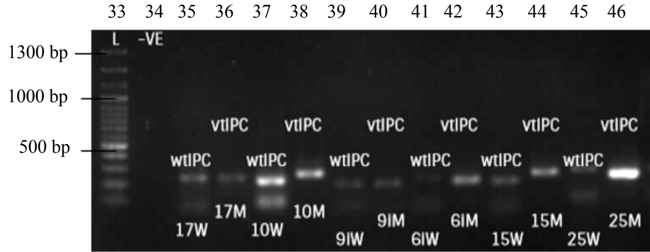
Fig. 1: Agarose gel electrophoresis of as PCR products using wtASP and vtASP for human DNA

listed in Table 2. In as PCR, two types of internal control were designed and labeled as wild type internal PCR control (wtIPC) and variant type internal PCR control (vtIPC). The amplified IPC would be observed on gel electrophoresis together with amplified as PCR products. The function of this IPC was as non-target DNA sequence present in the same sample tube that co-amplified simultaneously with the target sequence to ensure the accuracy of as PCR results. The IPC was designed with the length between 200-300 bp so that it will appear above the as PCR products on gel electrophoresis. The ASP was designed to amplify products less than 200 bp for the purpose of forensic



Lane	Sample	Description	Size (bp)
17	L	100 bp ladder	-
18	-VE	Negative control	-
19	wtIPC	Wild type IPC for SNP 195	278
	12W	Wild SNP 195	-
20	vtIPC	Variant type IPC for SNP 195	278
	12M	Variant SNP 195	107
21	wtIPC	Wild type IPC for SNP16274	193
	7W	Wild SNP 16274	98
22	vtIPC	Variant type IPC for SNP 16274	193
	7M	Variant SNP 16274	-
23	wtIPC	Wild type IPC for SNP 8440	265
	20W	Wild SNP 8440	108
24	vtIPC	Variant type IPC for SNP 8440	265
	20M	Variant SNP 8440	-
25	wtIPC	Wild type IPC for SNP 13626	241
	16W	Wild SNP 13626	72
26	vtIPC	Variant type IPC for SNP 13626	241
	16M	Variant SNP 13626	-
27	wtIPC	Wil type IPC for SNP 16291	219
	24W	Wild SNP 16291	147
28	vtIPC	Variant type IPC for SNP 16291	219
	24M	Variant SNP 16291	-
29	wtIPC	Wild type IPC for SNP 9080	198
	23W	Wild SNP 9080	83
30	vtIPC	Variant type IPC for SNP 9080	198
	23M	Variant SNP 9080	-
31	wtIPC	Wild type IPC for SNP 3705	176
	22W	Wild SNP 3705	96
32	vtIPC	Variant type IPC for SNP 3705	176
	22M	Variant SNP 3705	-

Fig. 2: Agarose gel electrophoresis of as PCR products using wtASP and vtASP for human DNA



Lane	Sample	Description	Size (bp)
33	L	100 bp ladder	-
34	-VE	Negative control	-
35	wtIPC	Wild type IPC for SNP 4491	308
	17W	Wild SNP 4491	121
36	vtIPC	Variant type IPC for SNP 4491	308
	17M	Variant SNP 4491	-
37	wtIPC	Wild type IPC for SNP 146	285
	10W	Wild SNP 146	58
38	vtIPC	Variant type IPC for SNP 146	285
	10M	Variant SNP 146	-
39	wtIPC	Wild type IPC for SNP 1719	259
	9IW	Wild SNP 1719	72
40	vtIPC	Variant type IPC for SNP 1719	259
	9IM	Variant SNP 1719	-
41	wtIPC	Wild type IPC for SNP 16108	276
	6IW	Wild SNP 16108	145
42	vtIPC	Variant type IPC for SNP 16108	276
	6IM	Variant SNP 16108	-
43	wtIPC	Wild type IPC for SNP 3027	219
	15W	Wild SNP 3027	74
44	vtIPC	Variant type IPC for SNP 3027	219
	15M	Variant SNP 3027	-
45	wtIPC	Wild type IPC for SNP 7684	198
	25W	Wild SNP 7684	70
46	vtIPC	Variant type IPC 7684	198
	25M	Variant SNP 7684	-

Fig. 3: Agarose gel electrophoresis of as PCR products using wtASP and vtASP for human DNA

application. In this project, the longest amplified fragment is 130 bp (SNP 16093) while the smallest amplified fragment is 70 bp (SNP 7684).

DISCUSSION

The nature of as PCR is to capture the targeted SNP mutation in DNA sequence. It is known as a convenient and inexpensive method but accurate in genotyping SNPs and mutations once optimized[18]. The technique requires ASP to bind to the target DNA and detect the mismatch in the sequence[19]. Both ASP are included in different PCR mix but carried out in similar PCR process followed by electrophoresis in 1% gel to separate the amplified products. The concept of ASP is based on extension of primer only when it's 3'-end is a perfect complement to the allele present in the input sample[20]. The as PCR technique has been applied in several previous studies such as sub-typing of multi locus sequence[21] for *E. coli* B2, detection of mutation in the relax circular DNA and the covalently closed circular DNA in hepatitis B virus[18], PCR cloning of low copy nuclear genes[22], determination of IL28B genotype[23], detection of DNA mutation in cancer[24] and many more studies.

Previously, the as PCR technique has been used also in population studies and almost similar to this project. In comparison, the approach of this study, combination of methods, the selection of mtSNPs and the target population

Table 2: List of wild type and variant for selected SNPs

SNP	Wild type	Variant
146	T	C
195	T	C
1709	G	A
1719	G	A
1872	T	C
3027	T	C
3552	T	A
3705	G	A
4491	G	A
7684	C	T
8440	A	G
9080	A	G
13626	C	T
16093	T	A
16108	C	T
16335	A	G
16355	C	T
16274	G	A
16148	C	T
16291	C	T

are different. For instance, in one previous project, probes were attached to the ASP during as PCR to detect the Caucasian group[25]. In the other study, only 16 mtSNPs located in control region were selected to detect the European population[26] while final selection of mtSNPs are specifically belongs to SEA population. In other previous studies, the differences are mostly related to the combination of methods used for the sequence typing.

In this research, the focus was to optimize the designed ASP of selected SNP that specifically belongs to Southeast Asian (SEA) population. The final selected SNPs for as PCR application were SNP 146, 195, 1709, 1719, 1872, 3705, 3027, 3552, 4491, 7684, 9080, 8440, 13626, 16108, 16291, 16274, 16355, 16093, 16335 and 16148. All of the selected SNPs were located in both coding and control region. The role of the ASP at this stage was to bind to either wild type sequence or variant and determine the sequence obtained on the selected SNPs without further sequencing process. Hence, determination of mtDNA sequence able to be carried out on site without further laboratory analysis.

The development of wild type IPC (wtIPC) and variant type IPC (vtIPC) was to make sure that the PCR process work and as indicator the designed ASP functions well[27]. It was developed using in house synthetic oligonucleotides and amplified by a new reverse primer and forward primer from first round PCR. The length of this IPC (250-300 bp) was created with the range that can differentiate between amplified products and IPC. In general, there were two amplification processes in one PCR mix and this was considered as an application of multiplex PCR[28].

Both ASP and ISP were tested using one of the collected DNA samples from subjects with consent. In this case, a sample was used to optimize and provide preliminary results as representative of all the designed ASPs. The proposed concept was if the tested allele is wild type, the amplified product should be observed in wild type lane together with IPC and vice versa. According to Fig. 1-3, all SNPs are wild type except for SNP 195 (12M) that was observed as variant. The results have shown that the designed ASP managed to amplify both wild type and variant SNP. With the results obtained, no further sequencing is required.

However, some difficulties and limitations were found in this project. First, the IPC previewed a bright and sharp band if appeared as a single band in respective lane but quite vague when appeared together with amplified product. A series of optimization has been carried out to overcome this issue. This is because both ASP and IPC competed each other to amplify the target regions and therefore, they shared every PCR components in PCR mix. Second, each of the selected SNPs required duplication of as PCR. One for wild type SNP (wtSNP) and other for variant type SNP (vtSNP). For presentation purpose, only 1 sample was used to present the preliminary result and indicated that ASP can be used for direct determination of mutation.

In future, it is recommended to increase the number of SNPs that belongs to SEA populations and samples quantity in order to increase the power of discrimination. Ability to identify population group in a simple way yet robust may help researchers especially in forensic investigation.

CONCLUSION

It was observed that SNP 146, 195, 1709, 1719, 1872, 3705, 3027, 3552, 4491, 7684, 9080, 8440, 13626, 16108, 16291, 16274, 16355, 16093, 16335 and 16148 that reported to belong to macrohaplo group M, B, F, E and N. Generally, mutation found using this technique may narrow down the individual and also population hence it is helpful in cases such as forensic crime and mass disaster.

SIGNIFICANCE STATEMENT

The study discovered the technique in development of human mtDNA typing method that is simple, with low cost yet robust that can be beneficial for population and also individual identification. This study will help the researcher to uncover the critical areas of human identification and this technique may beneficial especially in forensic cases such as

mass disaster and plane crash that many researchers were not able to explore. In such cases, individual identification may be difficult to be applied but it is possible to narrow down the individual populations. Thus, a new theory on mitochondrial DNA typing method may be arrived at.

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