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T he Development of Multiplex PCR-RFLP of Fat Mass and Obesity-Associated (FTO) Genetics Variants

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Abstract

Background: Major health concern throughout the world is the increasing rate of obesity, either in elder group or younger group of multiracial ethnics that are believed to be associated with genetic factor that plays important role in the pathogenesis of obesity. The very first gene to be associated with obesity is fat mass and obesity associated (FTO) gene with the involvement from many single nucleotide polymorphisms (SNPs) in the first intron of FTO. Early detection of obesity-related SNPs is very crucial in the effort of reducing obesity rate which is expected to increase by 2025.This has led to the urge of developing a method to detect individual with predisposing factors towards obesity, with the hope that this method will eventually be helpful in assisting healthcare authorities to rapidly detect individual who are at high risk of being obese. Multiplex PCR-RFLP is a method for rapid detection of obesity-related SNPs using gene sequence amplifying technique and optimization done using gradient PCR technique. Thus, this study was conducted to evaluate the potential of multiplex PCR-RFLP to amplify FTO genetic variants. Materials and methods: A total of 14 UniSZA students were recruited to obtain 2 ml of peripheral whole blood for DNA extraction. Single gradient PCR and multiplex PCR were employed to determine the optimum annealing temperature for all assigned FTO SNPs as rs9939609, rs1421085 and rs17817288. The PCR products were run on 1% of agarose gel electrophoresis and visualized under UV Gel doc Image Analyzer. These PCR products were introduced to restriction enzymes for the cutting site Bfal, HpyCH4V, HpyCH4IV for rs9939609, rs1421085 and rs17817288, respectively. Next, the PCR fragments were identified on 2% agarose gel electrophoresis under UV Gel Doc Image Analyzer. Results: The optimum annealing temperature to amplify all FTO SNPs obtained through gradient PCR was 60°C. The amplification of FTO rs9939609, rs17817288 and rs1421085 regions, showed specific size product of 151 bp, 140 bp, and 131 bp respectively. In RFLP, the fragments after restriction enzyme digestion produced homozygous wild type, heterozygous and homozygous mutant type for genotype characterization based on allele cut site by specific restriction enzyme. Conclusion: This study may become preliminary data on the potential of multiplex PCR-RFLP in provide rapid diagnosis to amplify multiple FTO genetic variants.

Keywords:
Obesity, PCR-RFLP, rs9939609, rs17817288, rs1421085, Restriction Enzyme

Introduction

Fat mass and obesity-associated gene (FTO) is one of many genes that is associated with susceptibility towards obesity (da Fonseca et al., 2020). Genome-wide association studies (GWAS) found single-nucleotide polymorphisms (SNPs) are associated with obesity which is mainly located in the first intron of FTO (Todendi et al., 2020). FTO SNPs seem to be associated with obesity-related traits such as body mass index (BMI) and others. Several FTO SNPs found to be related with obesity are rs9939609, rs9930506 (Loos & Yeo, 2014), rs17817288 (Apalasamy et al., 2012), rs1421085 (Hebbar et al., 2020) and many more. Rs9939609 is frequently mentioned in literature as a polymorphism commonly found to be associated with obesity in various populations. Frayling et al. (2007) first reported its association in European white individuals then followed by other populations such as in Caucasian Romanian, western Spain, and Portuguese (Ursu et al., 2015; Gonzalez et al., 2011; Albuquerque et al., 2013). As for rs1421085 polymorphism, a study in Portuguese children found a significant association with weight, BMI, BMI Z-score, waist circumference, and hip circumference (Albuquerque et al., 2013). rs17817288 is rarely mentioned in literature. To date, there is only one article by Apalasamy et al. (2012) that found its association with low-density lipoprotein cholesterol in Malaysian Malays. Hence, the study suggested that rs17817288 may be involved in adipogenesis or lipid metabolism in the population. Therefore, due to the significant effects of these polymorphisms towards obesity, there is a need for the study to be done in the local population looking at these specific SNPs of FTO gene.

Obesity is defined as an excess accumulation of fat that may predispose an individual to various chronic illnesses (Fruh, 2017). Obesity can be due to genetics and environmental factors with genetics contributing towards as much as 50% to 80% risk of obesity (Sheikh et al., 2017). There are several of genetic factor associated with obesity that has been identified and continuous research are still on going to identify more genetic variants that may become a predisposing factor for an individual to become obese (da Fonseca et al., 2020). These genes include leptin (LEP) gene, leptin receptor (LEPR) gene, melanocortin 4 receptor (MC4R) gene, as well as fat mass and obesity associated (FTO) gene (Frayling et al, 2007).

Genotyping of the FTO SNPs can be performed via multiplex polymerase chain reaction (PCR) with Restriction fragment length polymorphisms (RFLPs) using extracted deoxyribonucleic acid (DNA) from blood samples. Multiplex PCR-RFLP allows simultaneous amplification of more than two loci using more than one pairs of primer in a single reaction as compared to a traditional PCR-RFLP. Instead of traditional PCR that amplify only one specific target gene. This multiplex PCR greatly saves time and money by amplifying more than two loci in a single reaction (Han,Z et al, 2018). However, multiplex PCR also has its own disadvantages. In multiplex PCR, there will be less flexibility in primer selection because simultaneous amplification of more than one target sequence requires designated primers to be specifically amplified the specific target sequence (World Health Organization, 2011). All primers must be within close range of melting temperature to allow amplification process to be carried out at the selected temperature, to avoid the possibility of primers anneal to the DNA template in a cycle that another primer is at different stage. Therefore, the primers used in multiplex PCR should be carefully selected so that all primers have similar annealing temperature, and the primers must not be complementary to each other to avoid interference. The size of the amplicon should be different for each fragment to produce a distinctive band when visualized with gel electrophoresis (Shen, 2019a; Sint et al., 2012).

The present study was an experimental study with a total of 14 participants, aimed to develop multiplex PCR-RFLP for FTO genetic variants for rapid detection of obesity-related SNPs using gene sequence amplifying technique.

Materials and Methods

Study design

This study involved a total of 14 students from UniSZA, Terengganu. During recruitment, both male and female subjects aged 18 to 25 years old were enlisted in this study. However, we excluded the subjects who were active smoking, having chronic diseases and pregnant. A signed informed consent was obtained from subjects to draw whole peripheral blood.

Ethical statement

This study was approved by UniSZA Human Research & Ethics Committee (UniSZA/UHREC/2022/453) of Universiti Sultan Zainal Abidin (UniSZA).

DNA extraction

Blood (2 mL) was withdrawn from the subjects and stored in EDTA tubes. Genomic DNA was extracted from whole blood (200 µl) using the GeneAll®ExgeneTM Blood SV (Korea) with a Spin Protocol before genotyping. The remaining blood samples were stored in -20°C with no longer than three months from the day it was collected. Any unused blood sample will be discarded after the storage period expired.

Gradient PCR

A total PCR mixture of 25 µl was prepared. The mixture consists of 3.5 µl of extracted genomic DNA and 21.5 µl PCR reaction as described in Table 1. The temperature range for gradient PCR was set between 50°C to 65°C with increment of 2.5°C which are 50°C, 52.5°C, 55°C, 57.5°C, 60°C and 62.5°C for each specific thermal block in the thermal cycler were shown in Table 2.

Table 1: Specific volume of gradient PCR mixture used in a single reaction for amplification of FTO SNPs rs9939609, rs17817288 and rs1421085

PCR reagents	Concentration	Volume (µL)
ddH2O		7
PCR Master Mix	1X	12.5
Forward primer	10 µM	1
Reverse primer	10 µM	1
DNA template	3.5 ng/µL	3.5
Total		25

Table 2: Thermocycler program for gradient PCR amplification

Step	Program	Temperature	Time
1	Pre-incubation	95°C	2 minutes
2	Denaturing	95°C	30 seconds
3	Annealing	50°C - 65°C (±2.5°C)	45 seconds
4	Extension	72°C	45 seconds
5	Repeat step 2 & 3		X 35
6	Extension	72°C	5 minutes
7	End		

Multiplex PCR-RFLP

For multiplex PCR-RFLP protocol, a total of PCR reaction mixture of 25 µl was prepared. The mixture consisted of double distilled water, PCR master mix (BiotechRabbit, Berlin, Germany), forward and reverse primer for each FTO SNPs (Integrated DNA Technologies, Singapore) and DNA template. A negative control containing double distilled water instead of genomic DNA was also prepared. Multiplex PCR parameters consisted of pre-denaturation step for 2 min at 95°C, denaturation step for 30 sec at 95°C, annealing step for 60˚C, Extension step for 45 sec at 72˚C, followed by 35 cycles and final extension step for 5 min at 72°C.

Then, the multiplex PCR product was digested at restriction cut site with enzyme (refer Table 3). A total of RFLP mixture of 50 µl was prepared. The mixture consisted of PCR product of each sample, 10X NEBuffer, restriction enzyme (New England BioLabs Inc., U.K.) and nuclease free water. The mixture was gently mixed and incubated at 37˚C for 15 min and heat inactivated at 65˚C for 20 min in water bath.

Table 3: Primers and restriction enzymes used for genotyping

Variants	Primers (5' to 3')	Restriction enzyme	PCR product (bp)	PCR-RFLP (bp)
rs9939609	For: TAGGTTCCTTGCGACTGCTGTGAATCT Rev: AAATACAAAAAATTAGCCAGGTGTGGTG	BfaI	151	C=390
rs17817288	For: AACTCATAGAATGACAGAAAAATAAATGG Rev: TTTAATTCTTTGTGGTTTTTACAAGCCCT	HpyCH4V	140	A=225
rs1421085	For: GGCAGACTTGTAAGGAACAA Rev: GTTGATTAAGTGTCTGATGAGAATTTGTA	HpyCH4IV	131	C=131

For: forward direction; Rev: reverse direction

Data analysis

The PCR and PCR-RFLP product was preceded with 1%-2% of agarose gel electrophoresis, stained with Florosafe DNA stain (1st BASE, Apical Scientific Sdn. Bhd., Malaysia) and viewed with the Sygene G:BOX Chemi XRQ (New England BioGroupTM)

Results

Amplification of FTO SNPs rs9939609

Figure 1 showed presence of amplicons in all Lane A, B, C, D, E, and F, reflecting the success of PCR reaction for amplification of FTO rs9939609 region. Lane G served as negative control, showing no presence of amplicon as no DNA was amplified. Lane B and F, circled with red line in Figure 4.3 which had been set to run on annealing temperature of 52.5˚C and 62.5˚C, respectively, showed distinctively thick, intense bands compared to gradient PCR products on other lanes. 52.5˚C and 62.5˚C were chosen as the possible optimum annealing temperature for amplification of FTO rs9939609 region and these two different temperatures were considered as an option when choosing for the optimum annealing temperature for simultaneous amplification of all FTO genetic variants.

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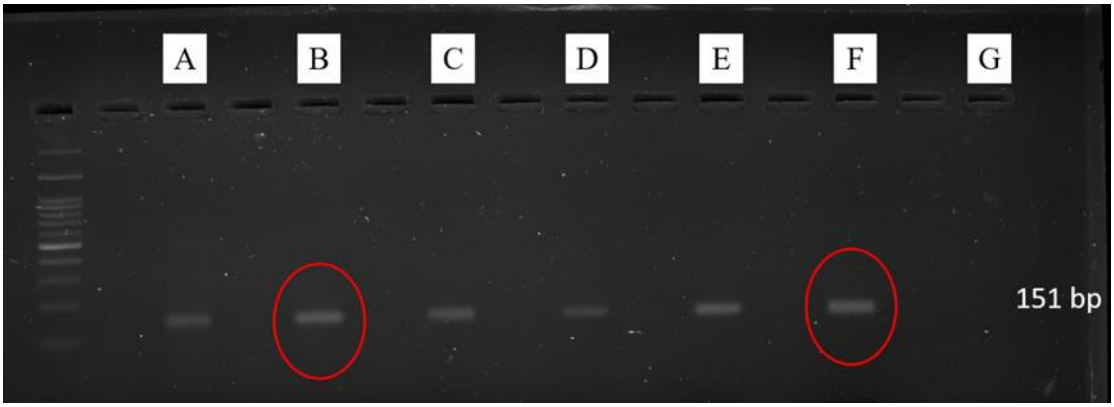

Figure 1: Electrophoretic analysis of gradient PCR products for the amplification of FTO SNPs rs9939609. Lane B and F (circled with red line) showed intense bands. 100 bp DNA marker was used.

Amplification of FTO SNPs rs17817288

Figure 2 shows presence of amplicons in all Lane A, B, C, D, and E reflecting the success of PCR reaction for amplification of FTO rs17817288 region. Lane F served as negative control, showing no presence of amplicon as no DNA was amplified. Lane E, circled with red line in Figure 4.4 which had been set to run on annealing temperature of 60˚C showed distinctively thick band compared to gradient PCR products on other lanes. 60˚C was chosen as the possible optimum annealing temperature for amplification of FTO rs17817288 region.

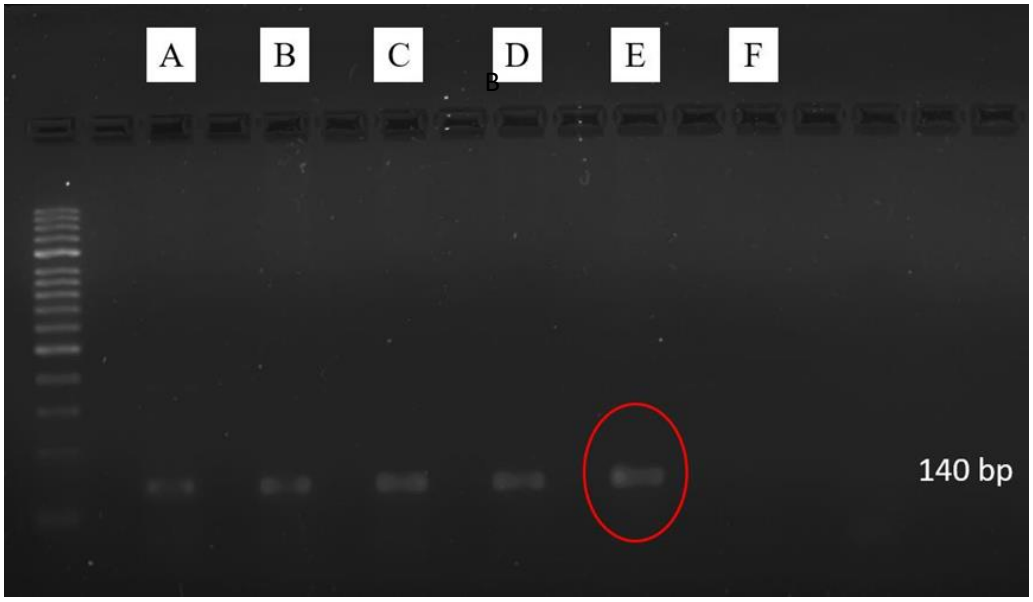

Figure 2: Electrophoretic analysis of gradient PCR products for the amplification of FTO SNPs rs17817288. Lane E (circled with red line) showed intense band. 100 bp DNA marker was used.

Amplification of FTO SNPs rs1421085

Figure 3 shows presence of amplicons in all Lane A, B, C, D, E, and F, reflecting the success of PCR reaction for amplification of FTO rs1421085 region. Lane G served as negative control, showing no presence of amplicon as no DNA was amplified. Lane E, circled with red line in Figure 4.5 which had been set to run on annealing temperature of 60˚C showed distinctively intense band compared to gradient PCR products on other lanes. 60˚C was chosen as the possible optimum annealing temperature for amplification of FTO rs1421085 region.

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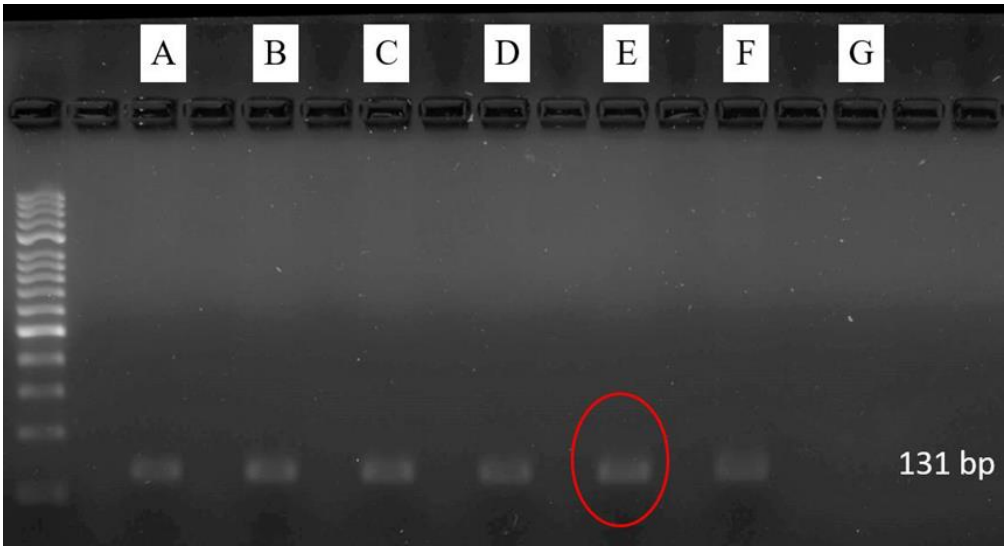

Figure 3: Electrophoretic analysis of gradient PCR products for the amplification of FTO SNPs rs1421085. Lane E (circled with red line) showed intense band. 100 bp DNA marker was used.

Integrity visualization of Multiplex PCR product

Figure 4-5 shows the presence of all three bands in all Lanes indicating that the multiplex PCR reaction for simultaneous amplification of FTO rs9939609 (150bp), rs17817288 (140bp), and rs1421085 (131bp) areas was successful. Last lane acted as a negative control, displaying no amplicon because no DNA was amplified. All FTO SNPs were effectively amplified at an optimum annealing temperature of 60°C, and all three bands of FTO SNPs in each lane showed different bands based on their positions.

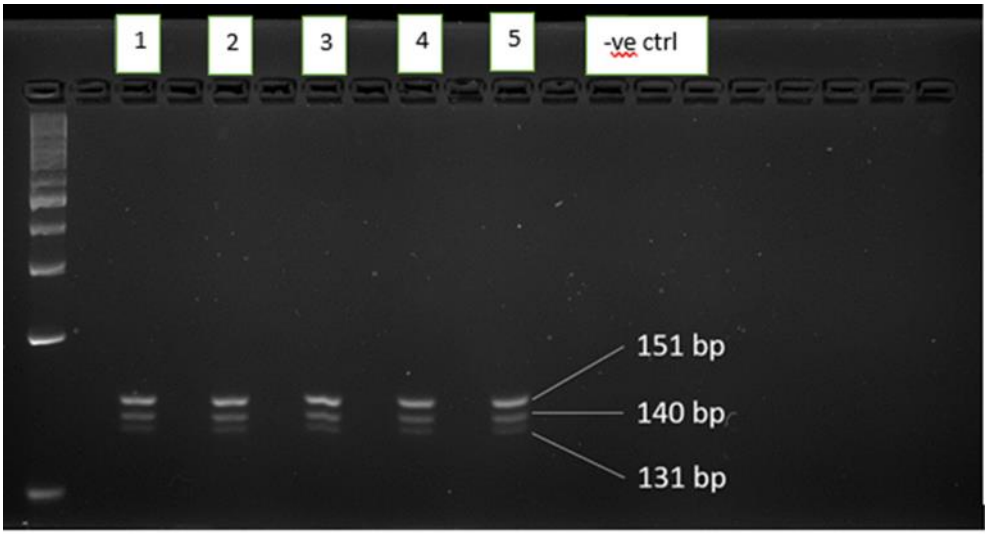

Figure 4: Electrophoretic analysis of multiplex PCR for FTO SNPs from DNA product from sample 1-5. Lane 1-5 contains amplicon FTO SNPs and negative control. 100bp DNA marker was used. The 100 bp DNA Ladder serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

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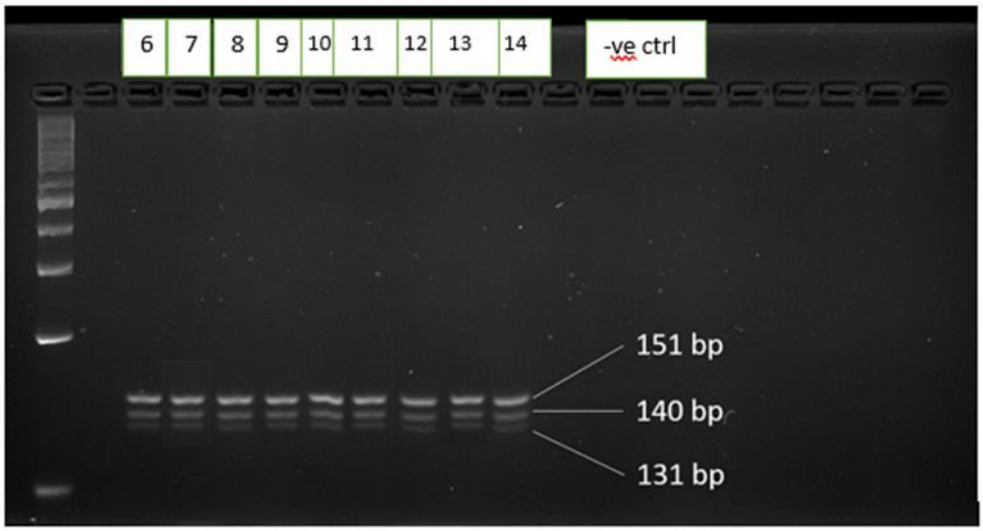

Figure 5: Electrophoretic analysis of multiplex PCR for FTO SNPs from DNA product of sample 6-14. Lane 6-14 contains amplicon FTO SNPs and negative control.100bp DNA marker was used. The 100 bp DNA Ladder serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

Integrity visualization of restriction enzyme for Bfal product for the amplification of FTO rs9939609 of 14 samples

Based on Figure 6-7 for RE Bfal of FTO SNPs rs9939609 were successfully amplified. All two distinctive dark (forward and reverse), thick intense bands were present with specific size product according to the restriction enzyme used. All samples exhibited homozygous wild type.

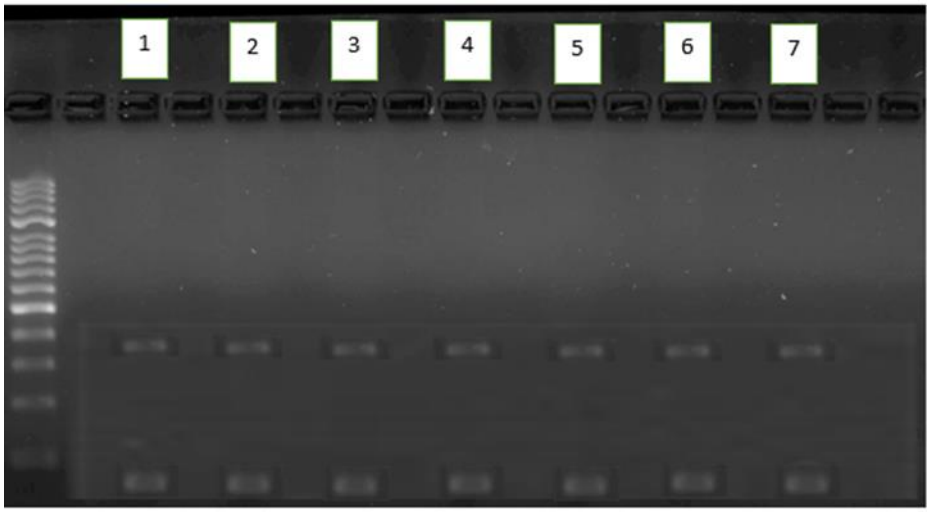

Figure 6: Electrophoretic analysis of RFLP of Bfal for PCR product. Lane 1-7 contains RE. Note forward: 27 bp, reverse: 363bp. 100bp DNA marker was used. The 100 bp DNA Ladder serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

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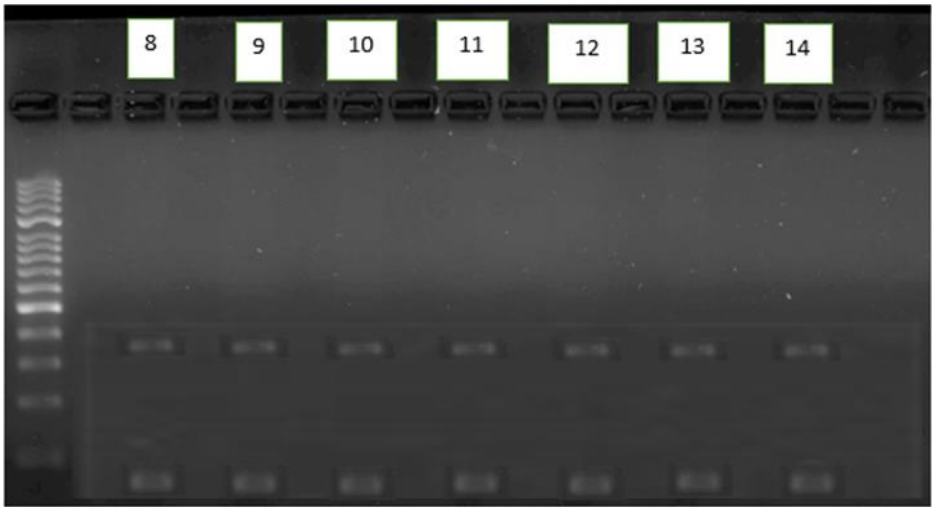

Figure 7: Electrophoretic analysis of RFLP of Bfal for PCR product. Lane 8-14 contains RE. Note forward: 27 bp, reverse: 363bp. 100bp DNA marker was used. The 100 bp DNA Ladder serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

Integrity visualization of restriction enzyme for HpyCH4V product for the amplification of FTO rs17817288 of 14 samples.

Based on Figure 8-9 for RE HpyCH4V were successfully amplified. All two distinctive dark (forward and reverse), thick intense bands were present with specific size product according to the restriction enzyme used. All samples exhibited homozygous wild type.

Figure 8: Electrophoretic analysis of RFLP of HpyCH4V for PCR product. Lane 1-7 contains RE. Note forward: 29 bp, reverse: 196 bp.100bp DNA marker was used. The 100 bp DNA Ladder serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

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Figure 9: Electrophoretic analysis of RFLP of HpyCH4V for PCR product. Lane 8-14 contains RE. Note forward: 29 bp, reverse: 196 bp. 100bp DNA marker was used. which serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

Integrity visualization of restriction enzyme for HpyCH4IV product for the amplification of FTO rs1421085 of 14 samples

Based on Figure 10-11of RE HpyCH4IV of FTO SNPs rs1421085 were successfully amplified. All two distinctive dark (forward and reverse), thick intense bands were present with specific size product according to the restriction enzyme used. All samples exhibited homozygous wild type.

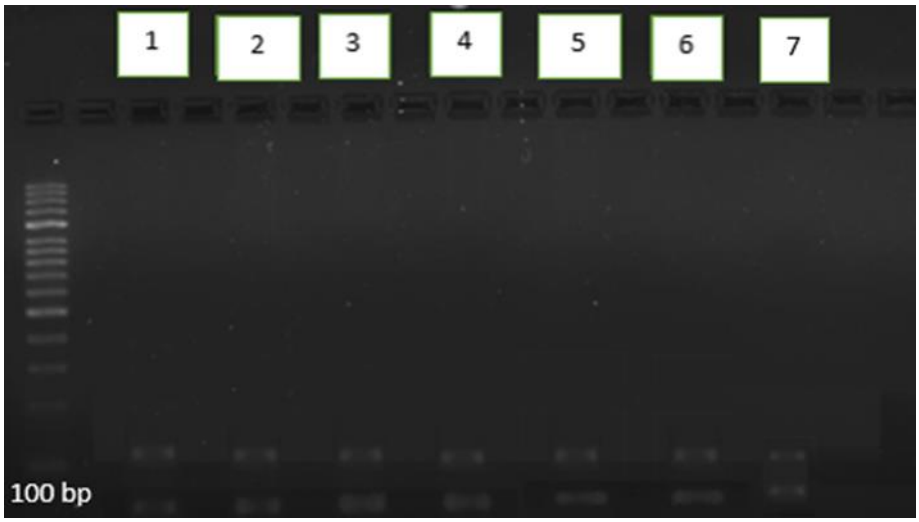

Figure 10: Electrophoretic analysis of RFLP of HpyCH4IV for PCR product. Lane 1-7 contains RE. Note forward: 101 bp, reverse: 30 bp.100bp DNA marker was used, which serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

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Figure 11: Electrophoretic analysis of RFLP of HpyCH4IV for PCR product. Lane 8-14 contains RE. Note forward: 101 bp, reverse: 30 bp. 100bp DNA marker was used, which serves as a visual assistance for tracking the extent of DNA migration during gel electrophoresis.

Discussion

Multiplex PCR is developed to amplify more than one target sequence using more than one primer pair in only one reaction. The development of multiplex PCR requires many parameters that need to be considered. One important parameter that should be given extra highlight is primer. All primer pairs in a single multiplex PCR reaction should be able to amplify their respective target sequence with similar amplification efficiencies. Therefore, it is very important to determine the optimum annealing temperature at which all primer pair would amplify their respective target sequence (Elnifro et al., 2000). In this study, the determination of optimum annealing temperature for simultaneous amplification of all FTO SNPs was done using gradient PCR reaction that allowed a range of annealing temperature to be set in a single PCR reaction, instead of performing multiple traditional PCR reaction using different annealing temperature. Based on this study, gradient PCR product for amplification of FTO rs9939609 region showed annealing temperature of 52.5˚C and 62.5˚C produced distinctively thick, intense bands on the 2 % agarose gel electrophoresis as in Figure 1 (lane B and F). On the other hand, gradient PCR product of amplification of both FTO rs17817288 and rs1421085 regions showed annealing temperature of 60˚C produced intense band on the 2 % agarose gel electrophoresis as in Figure 2 (lane E) and 3 (lane E), respectively. These results made the best possible annealing temperature for amplification of all FTO SNPs region in a multiplex PCR assay ranging from 52.5˚C to 62.5˚C. When choosing one single best optimum annealing temperature, all possible annealing temperature from amplification of all FTO SNPs regions were needed to be considered carefully. Careful consideration of choosing the optimum annealing temperature resulted in using 60˚C as the optimum annealing temperature and used in the multiplex PCR reaction to amplify the FTO genetic variants.

After optimum annealing temperature of 60˚C for amplification of all FTO SNPs was obtained through gradient PCR, the amplification of FTO rs9939609, rs17817288 and rs1421085 region were conducted using multiplex PCR that simultaneously amplify all FTO genetic variants in a single PCR reaction. As shown in Figure 4-5, electrophoretic analysis of multiplex PCR products for amplification of all FTO SNPs assigned as rs9939609, rs17817288 and rs1421085 were successfully amplified in one single reaction of multiplex PCR using optimum annealing temperature of 60˚C. All three distinctive dark, thick intense bands were present with specific size product of 151 bp, 140 bp, and 131 bp for amplification of FTO rs9939609, rs17817288 and rs1421085 regions, respectively.

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Other factors that determine the success of developing multiplex PCR for amplification for any targeted DNA sequence lies on the primer design. Primer pairs should have amplification efficiencies similar to each other to be able to amplify their respective target sequence. The goal of primer design is to achieve both specificity and efficiency. Amplification of specific target sequence by the primer partly dependent on the length of the primer, GC content of the primer, annealing temperature of the primer and presence of complementary sequences between primer (Bustin et al., 2020). The length of primer is very important because primer needs to correctly identify the designated target complementary regions. The longer the length of the primer, the better it is as it will improve the specificity of the primer to anneal to its targeted complementary regions. Primer usually has the length of 18 to 25 nucleotides (Mubarak et al., 2020) however, increase in the length of primer will cause increased annealing temperature that will cause primer unable to anneal to the DNA template and will result in no amplification of targeted DNA sequence (Bustin et al., 2020; Shahzad et al., 2020).

Primer also has to have optimal GC content between 40% to 60%. GC content is the number of G and C in the primer sequence and presented as percentage of nitrogenous 58 bases of primer sequence that are either G or C (Henegariu et al., 1997; Mubarak et al., 2020). If GC content is too high, it may result in mis-priming as the primer can anneal more stably with nonspecific sequence of DNA template (Kumar & Kaur, 2014). Not only that, the high GC content will also result in higher annealing temperature and primer will poorly anneal to the DNA template and eventually will yield low PCR products (Ehtisham et al., 2016; Kumar & Kaur, 2014; Mubarak et al., 2020). Too high GC content will also result in primer-dimer formation and secondary structure (Kumar & Kaur, 2014). Primers are also recommended to contain one or two G or C within the last five bases of primers at its 3' end, with no more than three G or C, to clamp the primer. This is known as GC clamp (Mubarak et al., 2020). This GC clamp will ensure specific and stable annealing at the 3' end due to the stronger hydrogen bond between G or C residues (Shahzad et al., 2020). GC clamp also helps in improving PCR reaction as it will minimize any "breathing" that may occur (Lorenz, 2012). "Breathing" term means that when primer ends do not stay annealed to the DNA template ends and split apart. Other than that, primers are also recommended to contain the presence of either G or C at the 3'end of the primers but no CG or GC because it may cause the formation of primer-dimers and hairpins (Onodera, 2007). Primer-dimers formed as a result of extra levels of primer over the DNA template concentration, where it may be able to anneal to the other primer, while hairpin loops formed due to the self-annealing primer (Mubarak et al., 2020).

According to NHMS (2022), restriction fragment length polymorphisms (RFLPs), is a variation in the lengths of DNA fragments cut by enzymes. Restriction enzymes are proteins which cut DNA at short, specialized sequences that called as restriction sites. In RFLP analysis, after a segment of DNA has been cut into fragments by one or more restriction enzymes, the resulting restriction fragments are then analysed by separating using gel electrophoresis according to their size. If two individuals have variety in their DNA sequences at particular restriction sites, then the restriction enzymes will cut their DNA into fragments of different lengths. In this study, as the PCR product for 14 samples were successfully obtained, the PCR products were applied with restriction enzyme for identification of cutting site. Through this study, the consistency of the presence of each enzyme restriction site was confirmed by performed RFLP where two distinctive dark, thick intense bands of forward and reverse were present with specific size product, which Bfal (27 bp, 363 bp), HpyCH4V (29bp. 196bp) and HpyCH4IV (101bp, 30bp) respectively. In here 27 bp, 29 bp and 101 bp are forward band and 363bp, 196 bp and 30 bp are reverse band. From all the result for three restriction enzymes, all shows homozygous wild type as restriction enzyme happen to cut on major allele.

Conclusion

In conclusion, this study demonstrated a successful development of Multiplex PCR-RFLP for genetics variants. The findings from the present study suggested the optimum annealing temperature of primer

pairs for efficient amplification of all FTO genetic variants in three FTO SNPs assigned as rs9939609, rs17817288 and rs1421085 was 60°C. This annealing temperature of 60°C then was applied in the multiplex PCR reaction to amplify all FTO SNPs simultaneously. As the PCR product for 14 samples were successfully obtained then the PCR product was applied with restriction enzyme for identification of the cutting site. The integrity of multiplex PCR-RFLP was confirmed by electrophoresis on the agarose gel and visualized under gel doc imager. The presence of distinct dark, thick intense bands on each well with specific size product of 151 bp, 140 bp, and 131 bp, corresponded well with FTO SNPs of rs9939609, rs17817288 and rs1421085 respectively Further analysis with restriction enzymes Bfal, HpyCH4V, and HpyCH4IV for FTO SNPS of rs9939609, rs17817288 and rs1421085 produced specific size product (forward and reverse).

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Conflict of Interest Disclosure
None to declare

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