Determination of Genetic Mutation Profile of *drpr* Gene in *Drosophila melanogaster* using Coupled real time PCR-High Resolution Melting Analysis

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ABSTRACT

Genotype determination of experimental animals is generally conducted using sequencing methods that require expensive cost as well as experience and special equipment. This study aimed to determine the presence of mutation in the drpr gene of Drosophila melanogaster using coupled real time PCR-High Resolution Melting (real time PCR-HRM) *as an alternative method. Two types of fly samples,* w^{1118} *and* $drpr^{\Delta5}$ *were used as wildtype control and mutant genotype, respectively. The DNA from twenty of each* w^{1118} *and mutant* $drpr^{\Delta5}$ *flies were isolated and amplified using real time PCR. The generated amplicons were then further processed by HRM method at the temperature of 60-95°C. This study demonstrated that the real time PCR-HRM method could distinguish wildtype control* w^{1118} *and mutant* $drpr^{\Delta5}$ *based on the HRM data with the confidence level was more than 90%. Therefore, this study provides an evidence that real time PCR-HRM method might be beneficial to screen the mutant genotype from its wildtype counterpart based on differences in the melting temperatures due to changes at nucleotide base level.*

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Introduction

The process of drug discovery begins by identifying the target protein involved in human disease then looking for chemical compounds that can alter the function of disease-causing proteins and optimizing these prospective compounds (Hughes *et al.*, 2011) prior to testing in suitable animal models and human clinical trials (Breyer *et al.*, 2015; Denayer *et al.*, 2014; McGonigle & Ruggeri, 2014). Common approach that usually used was an *in vitro* study and then continued to an *in vivo* study to obtain information about pharmacological effects, pharmacokinetic profiles and toxicity of prospective drugs using wildtype animal models such as rats, mice, and rabbits (Denayer *et al.*, 2014; McGonigle & Ruggeri, 2014). But the process using this approach is very long, complex and expensive. All of those are of concern in relation to a number of biological targets that must be considered for each new drug prior to its approval for clinical use (Denayer *et al.*, 2014; Hughes *et al.*, 2011; McGonigle & Ruggeri, 2014). Currently, several developing studies are trying to solve these problems and every step involved in the process of developing drugs are designed to be more efficient, including the use of alternative model organism to obtain high-throughput results in the *in vivo* context (Breyer *et al.*, 2015).

One of model organisms that have been shown to be useful in the initial stage of drug discovery process is fruit fly *Drosophila melanogaster*. With about 65% homologous genes with humans, this insect can be used as an alternative model organism in several human diseases. (Nainu *et al.*, 2019a; Pandey & Nichols, 2011; Ugur *et al.*, 2016). In addition, the use of fruit fly as model organism has other advantages such as lower maintenance costs, very fast life cycle, the availability of diverse transgenic and mutant genotypes, and can be used in large quantities (Nainu *et al.*, 2019a; Pandey & Nichols, 2011). Based on this, *Drosophila* is very suitable to be used as a model organism in the process of discovering new drugs (Cagan, 2013; Nainu *et al.*, 2019a; Pandey & Nichols, 2011). In many studies, the use of fruit fly in the *in vitro* and *in vivo* experimental settings has been shown to produce some promising results in efforts to discover new drugs for cancer (Gladstone & Su, 2011; Willoughby *et al.*, 2013; Yadav *et al.*, 2016), cardiovascular diseases (Akasaka & Ocorr, 2009; Palandri *et al.*, 2018), epilepsy (Song & Tanouye, 2008), Parkinson's disease (Maitra & Ciesla, 2019), and antimicrobials (Ahsan *et al.*, 2019; Ekowati *et al.*, 2017; Nainu *et al.*, 2018; Nainu *et al.*, 2019a; Nainu *et al.*, 2020a). In fact, *D. melanogaster* has been proposed as a possible model organism to investigate potential host factor(s) responsible in the severity of coronavirus disease (COVID)-19 that are prospective as drug targets (Nainu *et al.*, 2020b).

One of the mutants currently available is *drpr*$^{\Delta 5}$ which has a disruption in the *drpr* gene in *Drosophila* (Tung *et al.*, 2013) making the fruit flies are unable to produce fully functional Draper protein that plays a role in the process of phagocytosis of apoptotic cells (Manaka *et al.*, 2004) and virus-infected cells (Nainu *et al.*, 2015). To determine the existence of mutations in experimental animals, the gold standard method that can be done is by sequencing method, which can provide genetic analysis at the nucleotide level so that it can detect all variations contained in a gene (Alberts *et al.*, 2017; Maslov *et al.*, 2015). But the application of this method requires specific instrument (DNA sequencer), expensive, and time-consuming (Buckingham, 2012). Therefore, we need a reliable and economical method, especially in the context of developing countries like Indonesia, to overcome these problems.

Real time polymerase chain reaction coupled with high resolution melting (real time PCR-HRM) method is one of the solutions in the detection of transgenic or mutant genotypes of certain species (Bancerz-Kisiel *et al.*, 2014; Ishikawa *et al.*, 2010; Lochlainn *et al.*, 2011) and also detection of pathogenic bacteria in certain sample (Nurhamidah *et al.*, 2018). This method is currently gaining its momentum in biomolecular sciences. With the real time PCR method, amplification of certain DNA segments can be carried out in a proper and rapid manner. In addition, real time PCR has a good sensitivity and reproducibility profiles (Alberts *et al.*, 2017; Buckingham, 2012). Subsequently after PCR, the detection of mutations in the amplified DNA or cDNA samples is analysed using the HRM method based on the different melting temperatures of the examined DNA/cDNA samples (Farrar & Wittwer, 2017). The real time PCR-HRM method offers a fast, cheap, and user-friendly genotyping analysis (Farrar & Wittwer, 2017). Based on the description above, this study was conducted with the aim of evaluating the real time PCR-HRM technique to determine the presence of the gene mutation of *drpr*$^{\Delta 5}$ line of *D. melanogaster*. Once succeed, this method can be widely used in the determination of mutant lines of *D. melanogaster* prior to sequencing to prevent unnecessary effort and cost which in turn presents a great economical advantage.

Materials and Methods

Fly stocks

The *w*^{1118} and *drpr*$^{\Delta 5}$ lines of *Drosophila* were used in this study (*Drosophila* lines were generously provided by The Laboratory of Host Defense and Responses Kanazawa University, Japan). Male flies at the age of 4-7 days were used in the entire experiments. These flies were bred and placed in a culture vial containing standard cornmeal-agar food. Flies were maintained in standard conditions (25°C, 12 hours light and 12 hours dark cycle).

Amplification of Target Gene

Twenty flies of both *w*^{1118} and *drpr*$^{\Delta5}$ *D. melanogaster* line were anesthetized with CO_2 and put into a Treff tube prior to DNA extraction. The extraction process was carried out per manufacturer's protocol using the Wizard® Genomic DNA Purification Kit (Promega). The obtained DNA subsequently analyzed quantitatively by qPCR-HRM method using Type-It HRM 2X Master Mix (Qiagen) and a set of *drpr* forward primer (5'-CGG AAT TCT CTG CCG CAG GGG TTA CAT AG-3'), and *drpr* reverse primers (5'-CCG CTG GAG CCG GCT CGA ATT TTC GCT T-3'). The process was conducted in a Rotor Gene Q thermal cycler (Qiagen, Germany) using a real time PCR protocol arranged for 40 cycles with the following settings: denaturation at 95°C for 10 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 10 seconds and followed by HRM protocol.

HRM Method Settings

In this study, gene mutation analysis in the *drpr* gene of *D. melanogaster* was conducted using HRM method towards DNA standard curve of the wild type and mutant flies. Previously, DNA of the wild type flies was isolated from *w*^{1118} *D. melanogaster* while its mutant sequence was isolated from the *drpr*$^{\Delta5}$ line of *D. melanogaster* which was known has have a deletion in the 5' untranslated region of the *drpr* gene (Freeman *et al.*, 2003).

HRM analysis was conducted soon after amplification by setting in the PCR cycle parameters into the Rotor-Gene Q thermal cycler software series (Qiagen, Germany) with HRM parameter profiles: temperatures from 65°C to 95°C with an increase of 0.1°C per minute. The real time PCR-HRM protocol then was started, and the machine automatically stopped after reaching temperature of 95°C as previously arranged. The data in the form of a curve displayed on the screen throughout the process.

The HRM method is used to detect differences in genotypes such as the length of the GC sequence in the nucleotide bases of each DNA samples (Farrar & Wittwer, 2017). The high content of G-C bonds will also require high temperatures to separate the bonds and will change the melting curve. HRM process was carried out with the combination of PCR method at first to amplify the sequence of targeted DNA. HRM analysis was then performed right after the completion of PCR process (Farrar & Wittwer, 2017). The examined DNA sequences were gradually denaturized by slowly increasing the temperature to acquire the DNA melting profile (Farrar & Wittwer, 2017). From the melting curve, the temperature ranges from 80°C to 90°C was selected as the melting-point location for defining the difference in the nucleotide bases sequence. HRM-location selection was attempted at the melting temperature which is most likely to have the highest GC bonds (Farrar & Wittwer, 2017). This approach has been used to identify *D. suzukii* (Dhami & Kumarasinghe, 2014) and to discriminate mutant variants from the wild type of *Yestinia enterocolitica* (Bancerz-Kisiel *et al.*, 2014).

Results and Discussion

Primer Specificity Testing

Both the *drpr* forward primer and the *drpr* reverse primer used in this study were constructed to identify the *drpr* target gene in *D. melanogaster*. To confirm the specificity of both primers, these primers subjected to specificity analysis using the Basic Local Alignment Search Tool (BLAST). This database search tool revealed the similarity between the sequence of our two *drpr* primers and the *drpr* sequence stored in BLAST database. This is an important step prior to qPCR experiments to demonstrate whether the *drpr* primer set will yield unintended PCR products that may affect our further analysis.

Based on the examined sequences, it can be seen in Figure 1 that both *drpr* primers used in this study were identical and aligned to the desired *drpr* target gene in *D. melanogaster* with the similarity of sequences were 96% and 100% for *drpr* forward primer and *drpr* reverse primer, respectively. To be more specific, the alimentation of primer NM 001274347.1 (*D. melanogaster drpr*, transcript variant E, mRNA) with our *drpr* forward primer showed that 25 out of 26 nucleotide bases were matched. Even all of 19 nucleotide bases of our *drpr* reverse primer

were totally matched with the primer NM_001274348.1 (*D. melanogaster draper* (*drpr*), transcript variant F, mRNA). These results suggested that our *drpr* primer set can specifically bind to the *drpr* gene of *D. melanogaster* thus shall produce a specific amplified *drpr* amplicon with expected size.

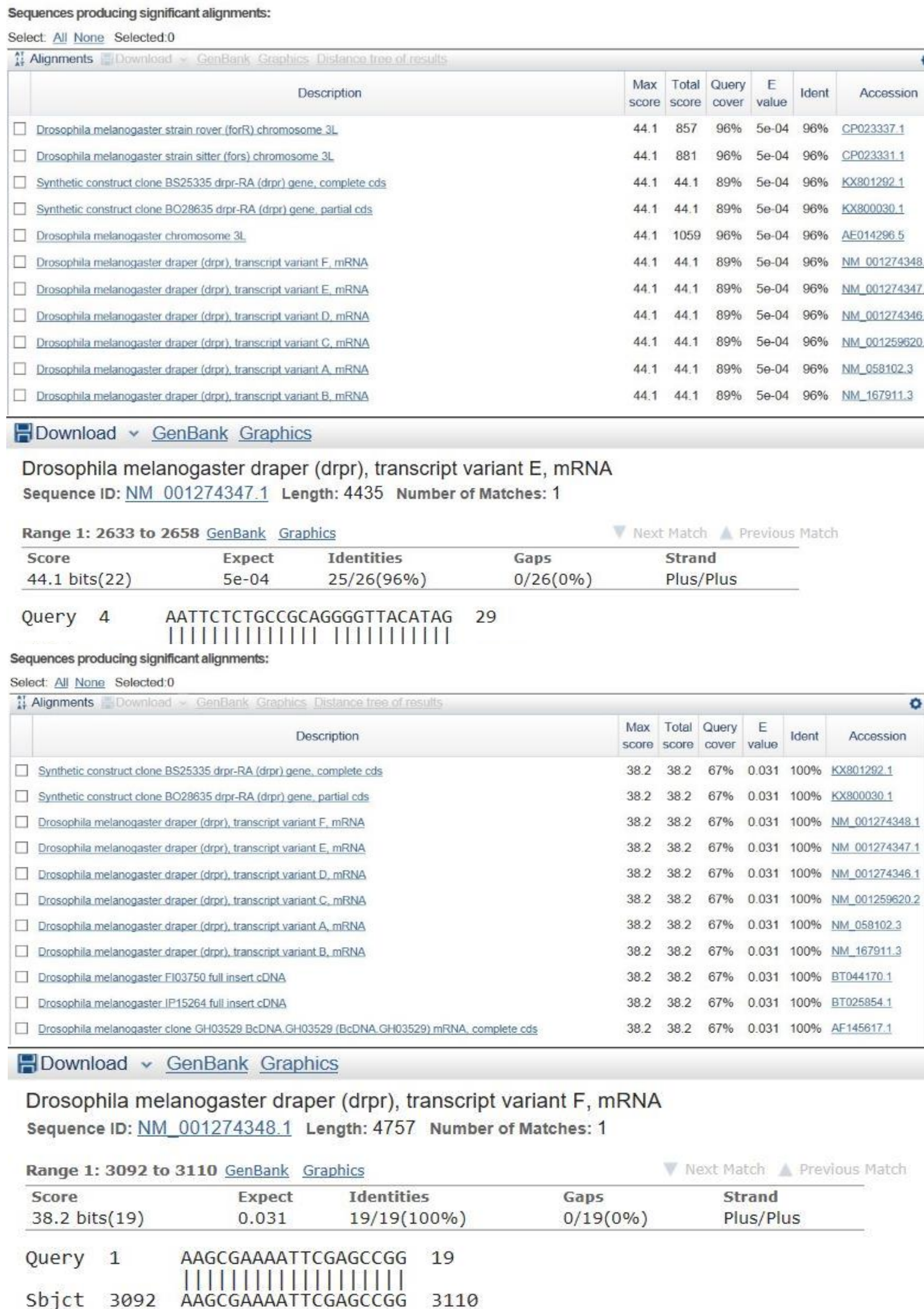

Figure 1. The results of *drpr* forward primer and *drpr* reverse primer specificity testing through Primer-BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide BLAST was performed for testing both primers. The sequence of *drpr* forward primer was inserted to the query sequence and then followed by a similar analysis on the *drpr* reverse primer. The displayed results demonstrated the high specificity of *drpr* primers used in this study.

Amplification using Real Time PCR

occurred at an early cycle (data not shown) which represents the sufficient amount of this gene in *D. melanogaster* amplification of *drpr* DNA was occurred in a proper manner as samples yielded positive result of amplification and there was no positive signal given by the no template control (NTC) sample simply due to the absence of DNA template to be amplified through PCR cycles.

HRM Analysis

The result of HRM analysis showed curve differences between wild type w^{1118} *D. melanogaster* and its $drpr^{\Delta 5}$ mutant (Figure. 2). As can be seen in Figure 2, each increase in temperature of 0.1°C/min was constantly causing a decrease in the melting temperatures of both curves. That reduction was occurred due to the turning of the double-stranded DNA strand into single-stranded DNA due to the heating process. Therefore, the dyes bound to the double stranded were also came apart which then reducing the level of fluorescence (Farrar & Wittwer, 2017). The difference curve reduction between w^{1118} and $drpr^{\Delta 5}$ *D. melanogaster* was a proof that the nitrogen base arrangements amid their gene sequences are also different. For sure, this indicates that a true mutation has occurred in $drpr^{\Delta 5}$ line of *D. melanogaster*.

To support or assumption on the HRM result, we carried out additional step to assign the examined samples based on the confidence percentage level. As shown Table 1, the confidence intervals of more than 90% were reached by all HRM results, signifying that HRM method could really distinguish between wildtype w^{1118} flies and its $drpr^{\Delta 5}$ mutants. Overall, our results provide evidence that PCR-HRM analysis can be used to identify the presence of gene mutation in the model animals without the need of sequencing procedure. This would be a helpful feature in the detection of mutant animals when one need to screen mutant genotypes from their wildtype counterparts in large number of stocks (genotyping). To improve the detection of mutant genotypes, gene sequencing can be done after initial screening using PCR-HRM. This is important to locate the location of mutation and to identify what type of mutation is occurred (deletion, insertion, or others). In addition, examination on whether detection of single nucleotide polymorphisms (SNPs) in the *drpr* gene can be assessed and visualized using our current real time PCR-HRM protocol would be useful to perform in the near future.

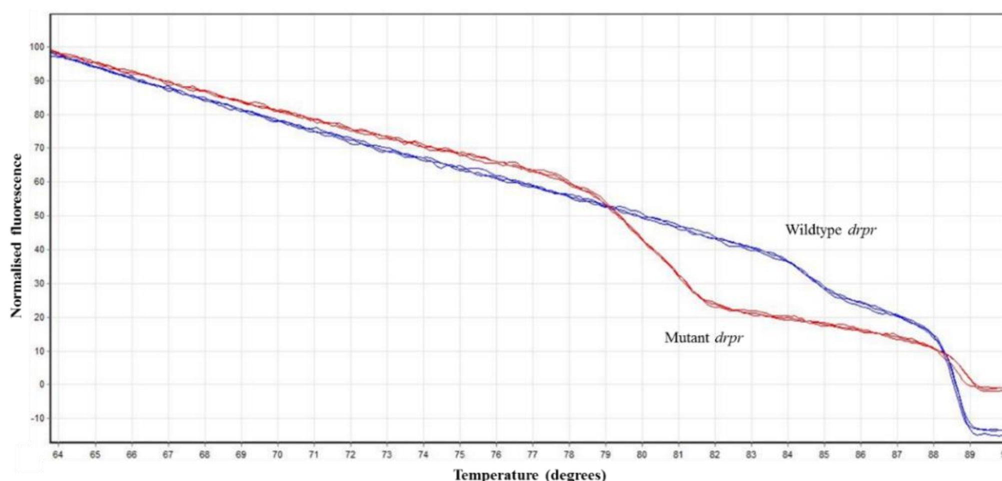

Figure 2. Species differentiation using HRM method by analysing the melting profile. Mutation in the *drpr* gene resulted in the decreased melting temperature of *drpr* sequence upon HRM analysis. The fluorescence curves of w^{1118} (blue line) and $drpr^{\Delta 5}$ (red line) were normalised using Rotor-Gene Q series software.

Table 1. The confidence level of HRM analysis based on the genotype differences.

No	Samples	Genotypes	Confidence %
1	w^{1118}	wildtype	90.39
2	w^{1118}	wildtype	91.27
3	w^{1118}	wildtype	91.89
4	*drpr*	mutant	90.13
5	*drpr*	mutant	96.05
6	*drpr*	mutant	92.67

Conclusion

This study has provided the first evidence that our real time PCR-HRM protocol can be used in the genotyping effort to discriminate the *drpr* mutant from their wildtype control genotype in a fast, specific (with confidence level more than 90%), and robust manner.

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