Molecular and phenotypic evaluation for heading date and panicle length on F\textsubscript{2} selected aromatic rice progenies derived from gene pyramiding effort

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Abstract.
Gene pyramiding, an attempt to combine many favourable genes from many parents into one single genotype, has been applied in recent years. Through hybridization of many rice genotypes including cv. Pandanwangi (fragrance rice), PTB33 (brown planthopper resistant), Ciapus (high yield and moderate amylose content) and KA (early maturity), some promising genotypes have been obtained. Thus, in order to select the best genotypes among the F\textsubscript{2} progenies derived from gene pyramiding effort, both phenotypic and molecular evaluations have to be done. These pyramided genotypes were evaluated with IFAP and ESP (fgr gene), RM19414 (Hd3), RM7601 (Hd2), and RM3600 (LP1) for molecular assessment, meanwhile morpho-agronomic traits were done to assess their performance. Aromatic trait was detected by 1.7% KOH. Meanwhile heading date and panicle length were measured phenotypically. Data obtained from molecular markers were scanned by visualizing DNA bands and calculating PIC (Polymorphism Information Content). Data from phenotypic markers were categorized based on IBPGR (International Board for Plant Genetic Resources) standards. Genotype #131 was supposed to have high yield potential, early maturity, aromatic, and intermediate amylose content. The selected will be recommended as promising genotype and continued as elite breeding materials and will be evaluated for their benefit.

Keywords: Molecular marker, morpho-agronomic traits, Rice, Simple Sequence Repeats

1. Introduction
The aroma is considered as one of the most significant grain quality traits in rice [1]. Strong aromatic expressions play an extraordinary role in the marketing of rice and its acceptance by consumers [2]. Aromatic rice is increasingly popular among consumers throughout the world [3], especially in the Middle East [4] and Western societies [5]. The superiority of aromatic rice should be accompanied by some valuable traits, such as, early maturity and high yield, in order to maximize farmers' income.

Currently, aromatic rice varieties have a relatively deep maturity such as cv. Pandanwangi which reach 155-168 DAP (days after planting, ICRR, 2011). Other aromatic varieties such as cv. Sintanur which have relatively early maturity, reaching 120 DAP, but still have an average yield of 6 ton/ha. Kusmana et al. [6] stated that to meet Indonesia's current rice food crop need, it is necessary to
increase rice productivity by more than 6 ton/ha in one season. According to Ya-dong et al. [7] and Sun et al. [8] the yield is mostly determined by the length of panicle. It is clear that aromatic trait, high yield which is represented by long panicle, and early maturity are important rice traits to be improved to fulfil farmers’ and consumers’ preference.

The aromatic character is controlled by a single recessive gene [1] [9] while characters related to early maturity and panicle length are controlled by many genes [10] [11]. Characters that are controlled by few genes will be easily passed on to offspring [12] and selected by applying both phenotypic and molecular approaches.

In the last decade, the success of molecular markers and phenotypic-based selection methods has been demonstrated by several researchers with various target characters such as leaf blight resistance, leaf rot, and blast in rice [13]. Meanwhile, for aromatic and non-aromatic molecular marker selection, IFAP (Internal Fragrant Antisense Primer) markers and ESP (External Sense Primer) will stick to the aromatic rice sequence, while the primary pair INSP (Internal Non-fragrant Primary Sense) and EAP (External Antisense Primary) will stick to non-aromatic rice sequences [14]. In addition, molecular markers that are closely related to fgr (fragrant) gene can be used to facilitate initial selection to detect the presence or absence of fragrance, and to identify locus (homozygous or heterozygous condition) [9]. Meanwhile, molecular markers of early maturity that have been known are RM19414, linked to the Hd3 gene (Heading date locus 3a) and RM7601 linked to Hd2 gene on chromosome 6 [15] and RM3600 linked to the LP1 (large panicle 1) gene [11]. Phenotypic evaluation includes observing morpho-agronomical characters are important to be done for confirming the expression of traits evaluated. Phenotypic testing for aromatic trait was carried out by sensory tests using 1.7% KOH [16], meanwhile both early maturity and panicle length were evaluated by phenotypic evaluation.

In order to combine the above-mentioned valuable traits into one genotype, four superior parents were hybridized as part of the gene pyramiding effort. They were cv. Pandanwangi (aromatic rice), PTB33 (resistant to brown plant hopper), Ciapus (high yield), and Kitaake (japonica, early maturity) [17]. Eleven F1 genotypes that have aromatic characters, early maturity, and high yield were selected and continued for consecutive generation. They were molecularly and phenotypically selected to obtain genotypes with aromatic trait, early maturity and high yield (long panicle).

In this study, the selection method begins with phenotypic selection related to aromatic characters controlled by single gene (fgr gene) will shorten and reduce population size for further molecular markers analysis related to other target characters i.e., early maturity and panicle length in early generations such as F2 progeny. Such methods have also been applied by Collard et al. [18], Lang and Buu [9], and Kiani [19] in selecting characteristics of some superior aromatic rice. However, in this study, we applied some molecular markers and morpho-agronomic traits which are significantly different with those mentioned papers. Therefore, the aim of this study was to obtain aromatic rice genotypes with early maturity (80-99 DAP), and long panicle (25-35 cm) in F2 populations.

2. Materials and Methods
2.1 Materials
The genetic materials used were consisted of one F2 generation derived from PP-51 x CKA-283. PP-51 is the F4 generation from cv. Pandanwangi x PTB-33, while the CKA-283 is the F4 generation from cv. Ciapus x Kitaake (Fig. 1) [17].
2.2. Methods
Phenotypic evaluation for aroma was conducted with 1.7% KOH for 314 F2 progenies derived from PP51 x CAKA283-2. Fifty genotypes were selected as aromatic rice by 1.7% KOH. The selected genotypes were then further confirmed by molecular markers using IFAP/ESP and INS/EAP markers. The selection was continued by molecular and phenotypic markers related to the target character, i.e., early maturity and panicle length.

2.2.1 Evaluation of phenotypic markers
Phenotypic markers were used to observe the heading date and panicle length and were then scored according to the International Rice Research Institute (IRRI) and International Board for Plant Genetic Resources standards [20]. Aromatic characters were observed based on sensory tests using 1.7% KOH [16].

2.2.2. Evaluation of Molecular Markers
DNA genomic was extracted by using the CTAB (Cetyl trimethylammonium bromide) method [21]. DNA amplification was carried out using a Polymerase Chain Reaction (PCR) machine with PCR components in the form of 9.5 μL Ready Mix DNA polymerases (0.25 U), dNTPs (0.2 Mm), MgCl2 (1.5 Mm)), 1 μL primer forward and reverse, as well as 1 μL template (20 ng DNA). Four molecular markers that closely related to the target trait were applied (Table 1). The product of PCR amplification was electrophoresed using 1.5% - 3% of agarose gel in a 0.5X TBE buffer solution at a certain time duration and amount of voltage (80-100 volt). Agarose gel then was immersed in EtBr solution for 10-15 min. UV transilluminator was applied to detect DNA band. As a standard, 100 bp DNA ladder (Promega) was used. The size of molecular markers was observed through DNA banding patterns with the help of Genetools software. Scoring was done by measuring the presence of allele, if presence was 1, and if not was 0.

3. Results and Discussion
3.1. Selection of aromatic genotypes based on phenotypic and molecular markers
KOH test for aromatic could select 50 genotypes, out of 314 genotypes. The fifty genotypes were then confirmed by molecular markers using IFAP/ESP and INS/EAP specific markers. Based on the DNA visualization, it was found that the banding pattern produced from the 50 genotypes had a PIC value of 0.69. PIC values above 0.5 contain high level of informativeness, so they can be used to identify genes associated with Aromatic characters in these pyramided genotypes. All pyramided genotypes had the same banding pattern as their parent (Pandanwangi, aromatic rice). It seems likely aromatic trait with homozygous gene appear in all pyramided genotypes. The aromatic character is a qualitative character.
so that it is easily inherited. It can be assumed that the aroma will also be present in the genotype of the next crosses [12].

Table 1. Molecular markers used in this research

| Traits                | Markers   | Linked to Chr. | Primer sequence | PCR product size | References |
|-----------------------|-----------|----------------|-----------------|------------------|------------|
| Panicle length        | RM3600    | 9              | tgcctcacatgtag  | 130bp            | [22]       |
| Heading date locus 2a | RM7601    | 6              | gcctgtcgcttca   | 133bp            | [15]       |
| Heading date locus 3a | RM19414   | 6              | gtcgaaactcaactac| 504bp            | [23]       |
| Aromatic              | RM223     | 8              | gaaggtacttggtgG| 139 – 163 bp     | Gramene    |
| Aromatic              | IFAP and  | 8              | 5’cataggagctctgaataatatac 3’ | 257bp         | [24]       |
| Aromatic              | ESP       | 8              | 5’tgttctgctctgctgtag 3’ | 257bp         |            |
| Aromatic              | INSP and  | 8              | 5’ctgatggtaagattaggctcta 3’ | 355bp         |            |
| Aromatic              | EAP       | 8              | 5’agttttacaaactcgttccgc 3’ | 355bp         |            |

Figure 2. Visualization of IFAP / ESP and INSP / EAP markers to distinguish aroma and non-aroma.

3.2. Aromatic rice genotypes have early maturity based on phenotypic and molecular markers
Based on the IBPGR classification [20], the early maturity was found in genotype #044 (88 DAP) (Fig. 3).

Figure 3. Heading date (DAP) of parents and pyramided genotypes derived from gene pyramiding effort.
Using both molecular markers i.e., RM7601 and RM19414 which are associated with the $Hd2$ and $Hd3$ genes, both showed polymorphism with PIC values of 0.38 and 0.25, respectively. PIC values ranged from 0.25 to 0.5, which categorized as good polymorphism. Meanwhile by using RM7601, 27 genotypes, out of 50, were having the band size as the same as parent of early-maturity donor cv. Kitaake (Fig. 4), while using RM19414, there were 22 genotypes having the same banding pattern as Kitaake's (Fig. 5).

![Figure 4. Agarose gel visualization of RM7601 which is associated with early maturity of eight pyramided genotypes derived from gene pyramiding effort](image)

The difference in the number of genotypes selected based on phenotypic and molecular markers for heading date, it could be due to the trait is a quantitative which is influenced by many genes and is strongly influenced by photoperiodism [25]. $Hd2$ and $Hd3$ genes are two major genes that control the heading date. The presence of these genes in a plant can regulate the heading date [25].

![Figure 5. Agarose gel visualization of RM1941 which is associated with early maturity of eight pyramided genotypes derived from gene pyramiding program](image)

According to Yamamoto et al. [26] there were five QTL genes related to early maturity in rice namely $Hd1$, $Hd2$, $Hd3$, $Hd5$, and $Hd6$ showing the presence of sensitivity to photoperiodism. The $Hd3$ gene is located in the short arm of chromosome 6, and is involved in the photoperiodic response and in stimulating flowering under short day conditions [27]. $Hd3$ gene is possibly found in Pandanwangi but it has deep maturity phenotypically, probably because the ability of $Hd3$ to stimulate flowering must be mediated by the $Hd1$ gene.
3.3. Aromatic Genotypes Have Long Panicles based on Phenotypic and Molecular Markers

Based on the IBPGR [20] about a classification for panicle length, there are three class categories, the first class is the eight percent long class, the second class is 90%, and the third class is two percent short panicle length in the pyramid genotype. Long panicles were found in genotypes #313, #219, #178, and #280, reaching 30 cm (Fig. 6).

![Panicle length (cm) graph]

**Figure 6.** Panicle length of parents and pyramided genotypes

Based on the selection of molecular markers related to panicle length using RM3600 marker, polymorphism between the DNA bands of the parents and genotypes tested was 0.69. PIC with more than > 0.5, the marker has a high level informativeness. It can be concluded that all genotypes derived from gene pyramiding are thought to have LP1 gene linked to long panicle (Fig. 7).

![Agarose gen visualization of RM3600](image)

**Figure 7.** Agarose gen visualization of RM3600, associated with panicle length on eight pyramided genotypes

Fifty genotypes were selected based on phenotypic and molecular markers. Although early maturity and panicle length are quantitative traits controlled by many genes, they have been detected on F2. According to Fehr [28] this is due to the selection of the previous population, so the chances of detecting these characters are high. Based on observations, it was found that genotypes identified as
having aromatic characters, early maturity (heading date was 98 HST), and long panicles (25 cm) were genotype #131.

From these data, some phenotypically selected genotypes were identified as having target characters but not molecularly, except qualitative characters, aromatic. This is because phenotypic selection on quantitative characters is fully influenced by the environment [29] [30] [18], so that the appearance of the phenotype can be different from the selection of molecular markers that suspect genotypes based on their genetic constitution [31].

4. Conclusion
Genotype #131 was identified as having aromatic character, early maturity and long panicle based on phenotypic and molecular markers. Genotype #131 will be further investigated for evaluation.

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