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SEGREGATION ANALYSIS OF MORPHOLOGICAL CHARACTER AND BIOACTIVE GENOTYPE OF RICE PLANT F3 (CEMPO SALAMET/IR64) POPULATION

Analisis Segregasi Karakter Morfologi dan Genotipe Bioaktif Tanaman Padi Populasi F3 Hasil Persilangan Cempo Salamet dan IR64

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ABSTRACT
Red rice contains high anthocyanin and bioactive antioxidant compounds that prevent free radical reactions. Cempo Salamet has potential as an antioxidant source, and the characteristics are red colored grains, 4–5 months old, 169 cm plant height, 7 productive tillers per plant, and resistance to blast disease. IR64 had been developed with the following characteristics: 3 months old, 85 cm plant height, 20–35 productive tillers per plant, resistance to brown leafhoppers pigment. This study aimed to obtain information on the segregation of the F3 population from crosses between the Cempo Salamet and IR64 varieties. Research methods included preparation and maintenance with genotype analysis. PCR analysis was conducted using SSR markers with primer RM346, RM316, RM228, and RM339. The segregation in F3 plants was 50% for >130 cm plant height, 51% for 10–19 tillers per plant, 67% for 2.2 g/100-grain weight, and 33% strong red for colour intensity. The findings demonstrated that SSR markers RM346, RM339, and RM228 could validate Cempo Salamet, IR64, and F3 DNA bands. However, RM316 could not validate all DNA bands in the research sample.

Keywords: *bioactive, crossing, molecular marker, morphology, red rice*

ABSTRAK
Beras merah mengandung antosianin yang tinggi dan senyawa bioaktif antioksidan yang mampu mencegah terjadinya reaksi radikal bebas. Cempo Salamet berpotensi sebagai sumber antioksidan yang memiliki karakteristik biji berwarna merah, umur 4–5 bulan, tinggi tanaman 169 cm, anakan produktif 7 batang, tahan terhadap penyakit blas. IR64 telah banyak dibudidayakan dengan karakteristik umur 3 bulan, tinggi tanaman 85 cm, anakan produktif 20–35 batang, tahan terhadap wereng coklat, akan tetapi tidak mengandung pigmen. Penelitian ini bertujuan mendapatkan informasi segregasi populasi F3 hasil persilangan antara varietas Cempo Salamet dan IR64. Metode penelitian meliputi persiapan dan pemeliharaan tanaman serta analisis genotipe bioaktif. Analisis PCR menggunaan marka SSR dengan primer RM 346, RM 316, RM 228, dan RM 339. Terjadi segregasi karakter morfologi pada populasi F3 yaitu diperoleh tanaman dengan tinggi >130 cm (50%), 10–19 anakan (51%), bobot 100 bulir dengan 2,2 g (67%), dan intensitas warna bulir dengan merah kuat (33%). Penelitian menunjukkan marker SSR yang dapat memvalidasi pita-pita DNA yaitu RM346, RM339, and RM228, sedangkan RM316 tidak dapat memvalidasi keseluruhan pita-pita DNA pada sampel penelitian.

Kata Kunci: bioaktif, marka molekuler, morfologi, padi beras merah, persilangan

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INTRODUCTION

Rice (*Oryza sativa L*.) is a food crop as an energy source generally consumed by people worldwide, including in Indonesia (Bommisetty et al. 2019). Local varieties of rice are genetic resources found in various regions. Based on the pigment content, rice is classified into pigmented and non-pigmented. Local varieties of pigmented rice will act as a functional food in the future. Functional food contains active components that are beneficial to health besides its nutrients. Pigmented rice is a functional food because it contains anthocyanins that function as antioxidants, namely bioactive nutrient components beneficial for health. Pigmented rice contains antioxidant compounds, including flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phenolic compounds (Chakuton et al. 2012). Then pigmented rice plants include brown rice, purple rice, and black rice. Brown rice contains bioactive antioxidant compounds that prevent free radical reactions (Priya et al. 2019).

The characteristics of brown rice, such as long life and high plant morphology, which make it easy to fall, have led to a lack of brown rice cultivation (Afza 2016). Furthermore, Ubaidillah and Siswoyo (2018) identified that the Cempo Salamet variety had a height of 169 cm, 4–5 months of age, 12 tillers, and is resistant to blast disease. Based on these characteristics, the Cempo Salamet variety can be used as the parent of a cross to produce superior varieties of brown rice. Crosses are conducted with the IR64 variety, which has relatively short-lived, highly adaptable to the environment, tolerant of pests and diseases, a high number of tillers but lack pigment to improve the shortcomings of Cempo Salamet (Mackill and Khush 2018).

Each individual from the hybridization program experiences a process of metallization in each generation. The heterozygous condition of a cross-population with maximum diversity in F2 turns into a homozygous population in F6–F7 (Devina et al. 2019). Various efforts have been made for breeding purposes, including conventional, molecular, and biotechnology. Furthermore, brown rice with superior characteristics should be crossed (Roy and Shil 2020). Abdullah (2017) explained that excellent brown rice has the characteristics of short-medium stems, early maturity, high production, disease resistance, and good rice quality.

Mahender et al. (2016) identified and characterized bioactive phenotype and genotype properties using molecular markers to develop rice varieties. Technology using markers determines genetic diversity, classification, and phylogeny related to germplasm management. Additionally, it is a tool for breeding and selection through gene markers (Chen et al. 2016). Identifying plant germplasm using molecular markers provides quick, effective, and accurate results. Therefore, the available markers, such as microsatellites or simple sequence repeats (SSRs), have become the most frequently used (Aljumaili et al. 2018). SSR markers also distinguish rice varieties. Therefore, they can be recommended to protect crop varieties (Miah et al. 2013).

Derivatives from crosses of local brown rice such as Cempo Salamet with IR64 are needed to obtain specific characteristics. Hence, they take advantage of their potential and eliminate unwanted features such as long life and high plant morphology (Rawte and Saxena 2018). Genetic differences between individuals are based on variations in genomic DNA sequences. Therefore, plant varieties should be identified by comparing DNA fingerprint profiles with SSR markers (Xu et al. 2016). SSR markers estimate genetic diversity between cultivars and plant parents of plants extracted from or between populations (Hue et al. 2018). This analyzes the F3 rice segregation from crosses of Cempo Salamet rice varieties with IR64 through morphological analysis and bioactive genotype using SSR molecular markers. This study aimed to obtain information on the segregation of the F3 population based on morphological characters and bioactive genotypes from crosses between the Cempo Salamet and IR64 varieties.

MATERIALS AND METHODS

Location and time

This research was conducted at Jubung Agrotechnopark Rice Fields (coordinate point: –8.1856983,113.633362) and Agrotechnology Laboratory (coordinate point: –8.1637602,113.7177487) Faculty of Agriculture, the University of Jember from August 2020 to March 2021.

Materials

Planting materials used were the seeds of the Cempo Salamet and the IR64 variety as parents with the F3 seeds from a cross between the Cempo Salamet variety (female) × IR64 (male).

DNA extraction and amplification

DNA of the rice plant samples were extracted using the CTAB method (Yang 2017). PCR amplification was then carried out with a total volume of 25 µL containing 12.5 µL of PCR master mix components, 1 µL of each forward and reverse primer, 9.5 µL of ddH2O, also 1 µL of target DNA. The PCR was programmed as follows: one cycle for pre-denaturation 95 ºC (5 minutes), 30 cycles for three stages of denaturation 95 ºC (30 seconds), annealing 55 ºC (30 seconds), extension 72 ºC (30 seconds), and one cycle for final-extension 72 ºC (5 minutes). The amplification products were stored at −20 ºC until ready for use. Electrophoresis was also conducted, and the results were observed with a UV Transilluminator.

Data analysis

The morphological character data and bioactive genotypes obtained were analyzed using frequency distribution analysis for quantitative data. Data collection was 10% of rice plants (Aedy and Mahmudin 2017). According to Sugiyono (2014), the steps for calculating frequency distribution analysis were as follow:

Counting the number of interval classes:
K = 1 + 3,3 log n

Calculating data range:
Data range =
(highest data – smallest data) + 1

Calculating class interval:
Class interval =
data range/number of classes

Plant height

Plant height was measured by cupping the plant, then measuring from the base of the stem to the tip of the highest pinnacle, using a ruler. The measurements were carried out in the ripening phase, and the criteria were classified into three, semidwarf (lowlands: less than 110 cm; highlands: less than 90 cm), intermediate (lowlands: 110–130 cm; highlands: 90–125 cm), and high (lowlands: more than 130 cm; highlands: more than 125 cm) (IRRI 2002).

Number of tillers

The number of tillers was counted per clump of rice plants in the pregnant phase. The criteria for the number of tillers were classified into four, including very high (more than 25 tillers/plants), tall (20/25 tillers/plants), medium (10–19 tillers/plants), and low (5–9 tillers/plants) (IRRI 2002).

Weight of 100 rice grains

The calculation was conducted with 100 whole grains of rice taken at random and then weighed at post-harvest. The criteria for the weight of 100 grains were classified into three, hefty (>2.8 g), heavy (2.2–2.8 g), and light (<2.2 g) (Aryana et al. 2017).

Grain color intensity

Visual observations were conducted by observing the grains' color from the entire population's results classified based on color segregation (following the male and female parents) formed. The grain color characters were classified into four based on the colors of Cempo Salamet (female parent) and IR64 (male parent), including solid red, medium red, low red, and white.

PCR results

PCR results were observed using RM 346, RM 316, RM 228, and RM 339 (Table 1). Then, the electrophoresis results were observed under UV transilluminator.

Table 1. SSR markers related to bioactive genotype and nucleotide base sequence

Primer	Annealing Temperature (ºC)	Forward	Reverse	Source
RM339	55	GTAATCGATGCTGTGGGAAG	GAGTCATGTGATAGCCGATATG	Shao et al. (2011)
RM316	55	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	Shao et al. (2011)
RM228	55	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	Hue et al. (2011)
RM346	55	CGAGAGAGCCCATAACTACG	ACAAGACGACGAGGAGGGAC	Shao et al. (2016)

RESULTS AND DISCUSSION

Plant height

The Cempo Salamet variety (female parent) had an average height of 179.6 cm, whereas that of IR64 variety (male parent) was 85.74 cm (Figure 1). The height of the two varieties based on the description was 169 and 85 cm, respectively. Figure 2 shows the F3 rice plant height segregation from the cross of both varieties classified into several interval classes.

IRRI (2002) classified plant height into three groups, short (<110 cm), medium (110–130 cm), and tall (>130 cm). Based on this classification, the crosses between Cempo Salamet and IR64 resulted in F3 plants categorized as short (34%), medium (16%), and high (50%) (Figure 2). Short F3 plants consisted of those with the height of 80.1–93.1 cm (23%), 93.2–106.2 cm (7%), and 106.3–119.3 cm (4%). Medium F3 plants comprised of the plants having the height of 106.3–119.3 cm (13%) and 119.4–132.4 cm (3%). Lastly, tall F3 plants were made up of those plants with the height range of 132.5–145.5 cm (10%); 145.6–158.6 cm (23%); 158.7–171.7 (7%); and 171.8–185.8 cm (10%). The short plants (34%) followed the pattern of the IR64 parents, which had an average height of 89.2 cm. The plants with a medium height (16%) followed the combined pattern of the two Cempo parents (Salamet and IR64). The highest plant segregation (50%) followed the pattern of the Cempo Salamet parent with an average height of 181.2 cm, indicating the dominant parent. Limbongan et al. (2008) showed that the character of plant height in lowland rice is controlled by many genes (polygenes). In inheritance, there is a role for the environment that affects the appearance of the phenotype (Yue et al. 2015). Moreover, varying plant heights are influenced by each variety with different characteristics (Tiwari et al. 2020). Genetic factors, environmental factors, and cultivation systems influence plant growth and development (Luther et al. 2018).

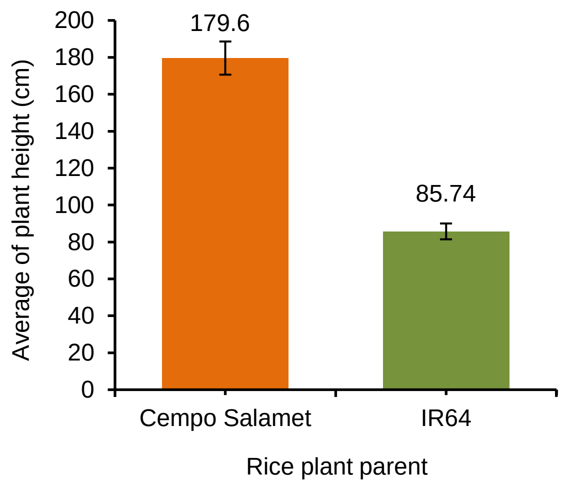

Figure 2. Average plant height (cm) of both parents as control plants

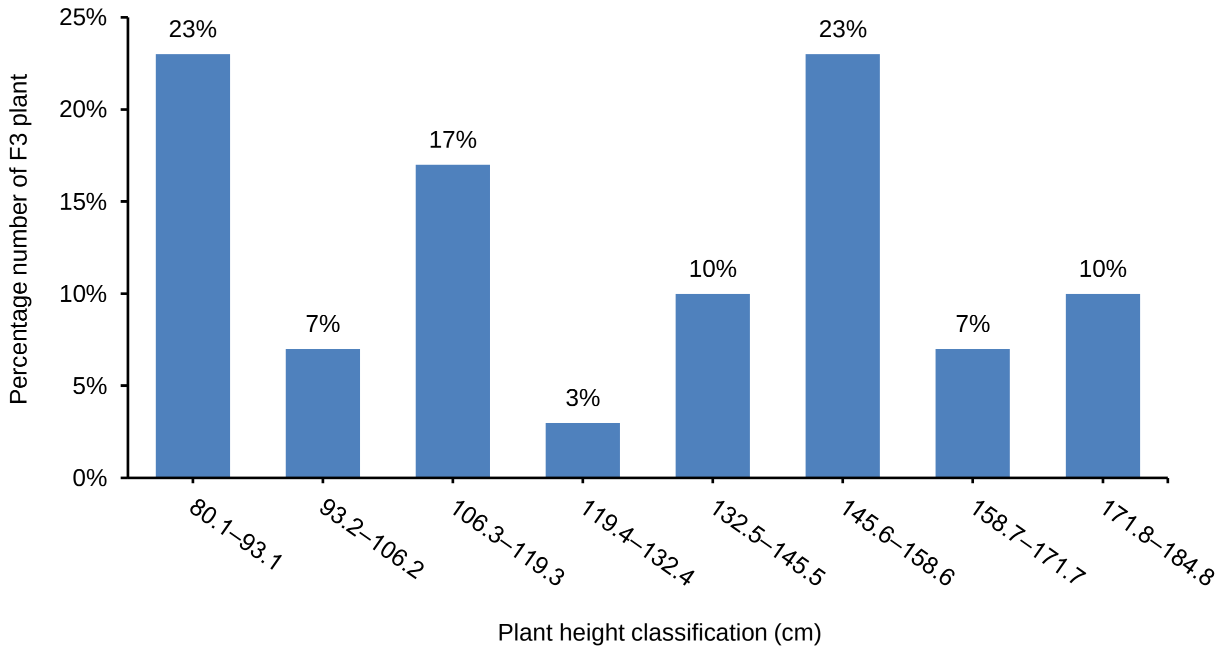

Figure 1. Frequency distribution of F3 plant height data from the crosses of Cempo Salamet and IR64

Number of tillers

The Cempo Salamet variety as female parents had an average of 17 tillers, while the IR64 variety as male parents had an average of 15 tillers (Figure 3). This indicates that the number of tillers of the two varieties based on the description was 12 tillers and 20–35 tillers. Figure 4 shows the segregation in the number of F3 rice tillers from a cross between Cempo Salamet and IR64.

IRRI (2002) classified the number of tillers into four types, very high (more than 25 tillers/plants), tall (20/25 tillers/plants), medium (10–19 tillers/plants), and low (5–9 tillers/plants). The data obtained for the number of tillers in the F3 derivatives indicated segregation within each plant. Therefore, it can be split into four categories: very high (21%), high (18%), medium (51%), and low (10%) (Figure 4). F3 plants with very high tillers had 23–27 tillers (8%), 28–32 tillers (3%); 33–37 tillers (3%); and 38–42 tillers (7%). Those of high tillers comprised of the F3 plants with 18–22 tillers (9%) and 23–27 tillers (9%). The medium classification was made up of F3 plants with 8–12 tillers (10%), 13–17 tillers (33%), and 18–22 tillers (8%). Lastly, those plants having low 8–12 tillers were only 10%. Plants with 39% of high tillers followed the IR64 parental pattern, with an average of 25 tillers. In comparison, plants with 51% of medium tillers followed the pattern of Cempo Salamet's parents, with an average of 16 tillers. Meanwhile, plants with a low number of tillers are 10% and did not follow the pattern of the two parents.

The highest population percentage of F3 plants was 51%, following the Cempo Salamet parent with an average number of 16 tillers, indicating the dominance of the parent. The inheritance of the number of tillers is controlled by one or two genes and many interacting genes (polygenes). A character controlled by many genes can be a quantitative trait because each gene contributes to the character's appearance being analyzed where the role is not significant (Devina et al. 2019).

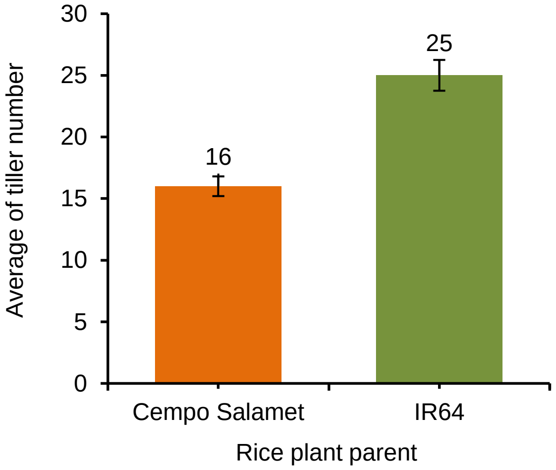

Figure 3. Average number of tillers of both parents as control plants

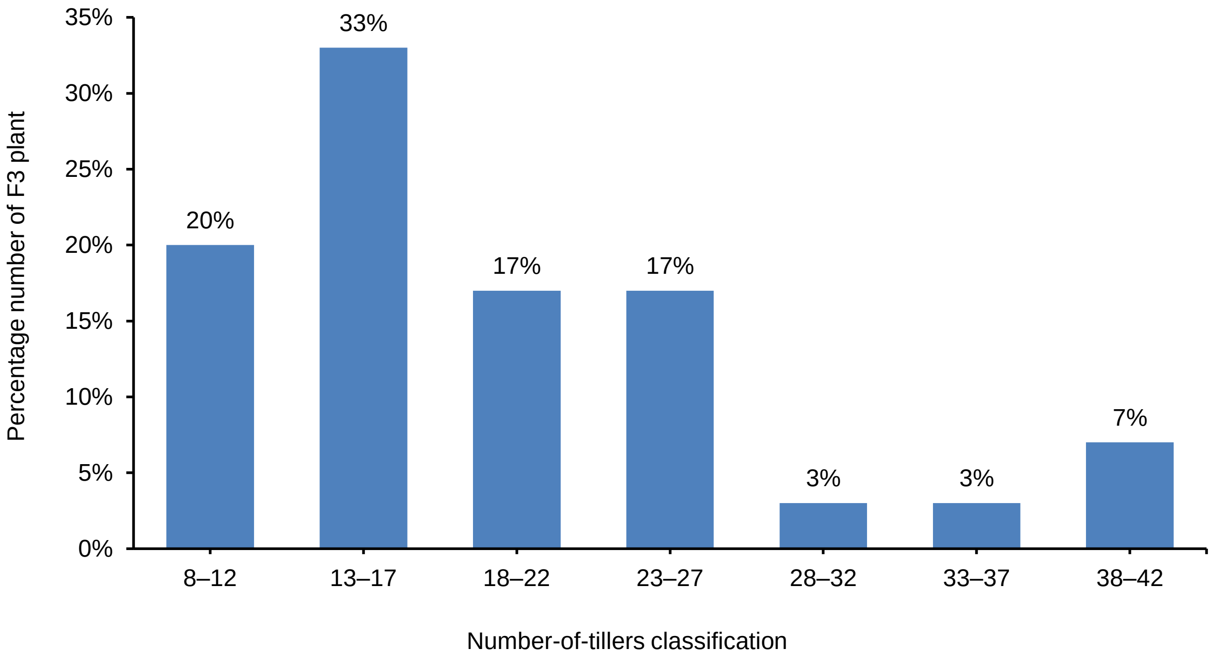

Figure 4. Frequency distribution of F3 plant tillers data from the crosses of Cempo Salamet and IR64

Weight of 100 rice grains

The Cempo Salamet variety had an average weight of 1.6 g/100 grains as female parents, while the IR64 variety as male parents had an average weight of 2.1 g/100 grains (Figure 5). The weights of 100 grains for the two varieties based on the descriptions were 1.92 and 2.41 g, respectively. Figure 6 shows the segregation of 100-grain weights in F3 rice plants from a cross between Cempo Salamet and IR64, classified into several interval classes.

The weight of 100 grains is divided into three groups, namely very heavy (>2.8 g), heavy (2.2–2.8 g), and light (<2.2 g) (Lim and Heong 1985). The results obtained for the weight of 100 grains in the F3 derivative showed segregation. Therefore, this was classified into three: very heavy (0%), heavy (11%), and light (89%). There was no F3 plants that produced grains classified as very heavy. The heavy 100 grains weighed in the range of 2.03–2.36 g (8%) and 2.37–2.70 g (3%), whereas the light ones in the range of 1.35–1.68 g (27%), 1.69–2.02 g (40%), and 2.03–2.36 g (22%). The results showed that 11% follow the pattern of the IR64 parent, with an average weight of 2.1 g. Meanwhile, 89% followed the pattern of Cempo Salamet's with an average weight of 1.6 g. The highest population percentage is 89%, indicating the weight character of the 100 F3 plants followed the Cempo Salamet parent. The shape and size affect the weight of the grain. Therefore, the resulting weight varies (Hasan et al. 2020). The 100-grain weight character is included in the quantitative features controlled by many genes that have a negligible effect on a character's expression (Zhang et al. 2015). The agro-morphological character of F3 population still highly segregated. Other research Aryana et al. (2017) reported on the cross of black rice also showed the same pattern where the dominant trait was indicated by the size of the individual class in the population.

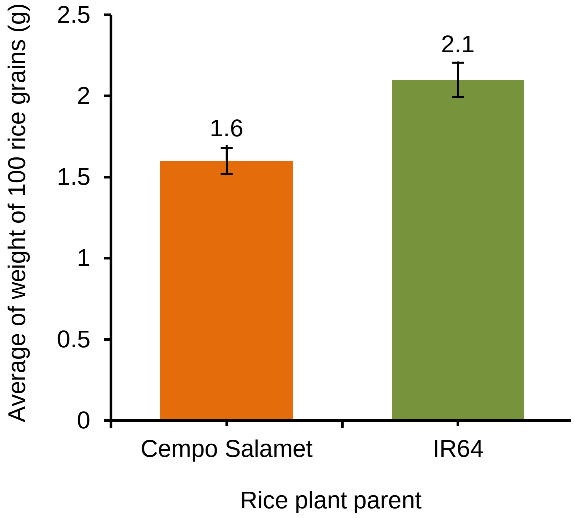

Figure 5. Average weight of 100 rice grains of both parents as control plants

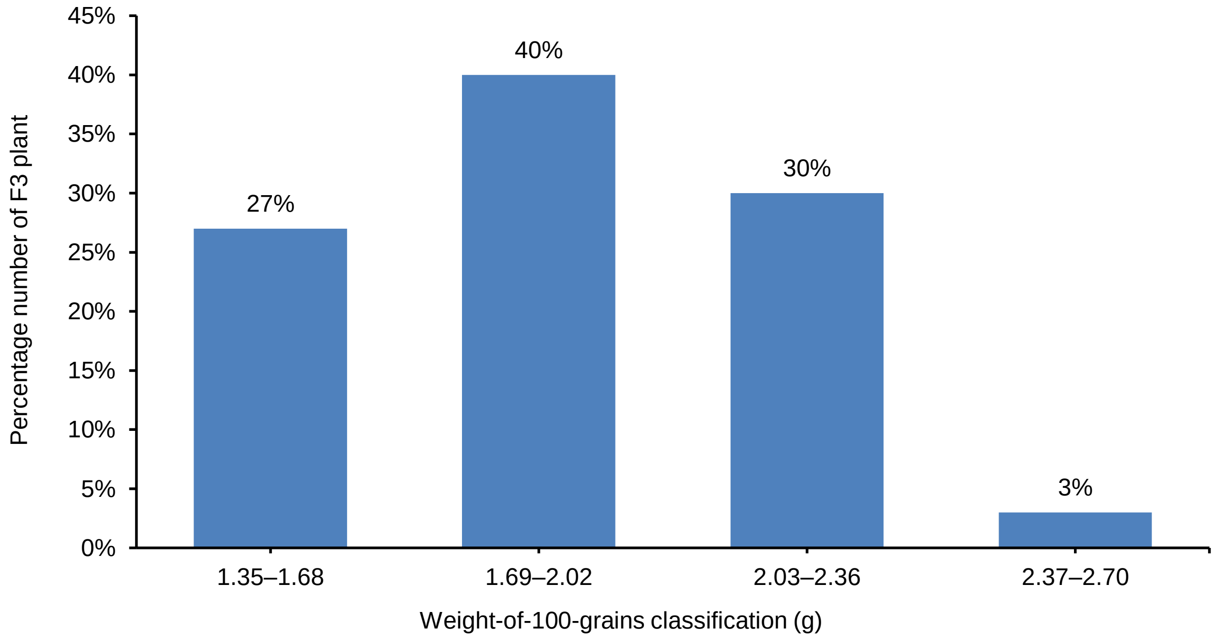

Figure 6. Frequency distribution of the data of weight average of 100 rice grains (g) from the crosses of Cempo Salamet and IR64

Grain color intensity

Based on Figure 7, the grain color intensity class is 33% strong red pigment, which is indicated by seeds number 7 and 15, 17% medium red pigment from seeds number 9 and 16, 27% low red pigment indicated by numbers 3 and 20, and 23% unpigmented indicated by numbers 4 and 21. The color of

Figure 7. Intensity of color in the rice grains of both parent (Cempo Salamet and IR64) and F3 plants (3, 4, 7, 9, 15, 16, 20, and 21)

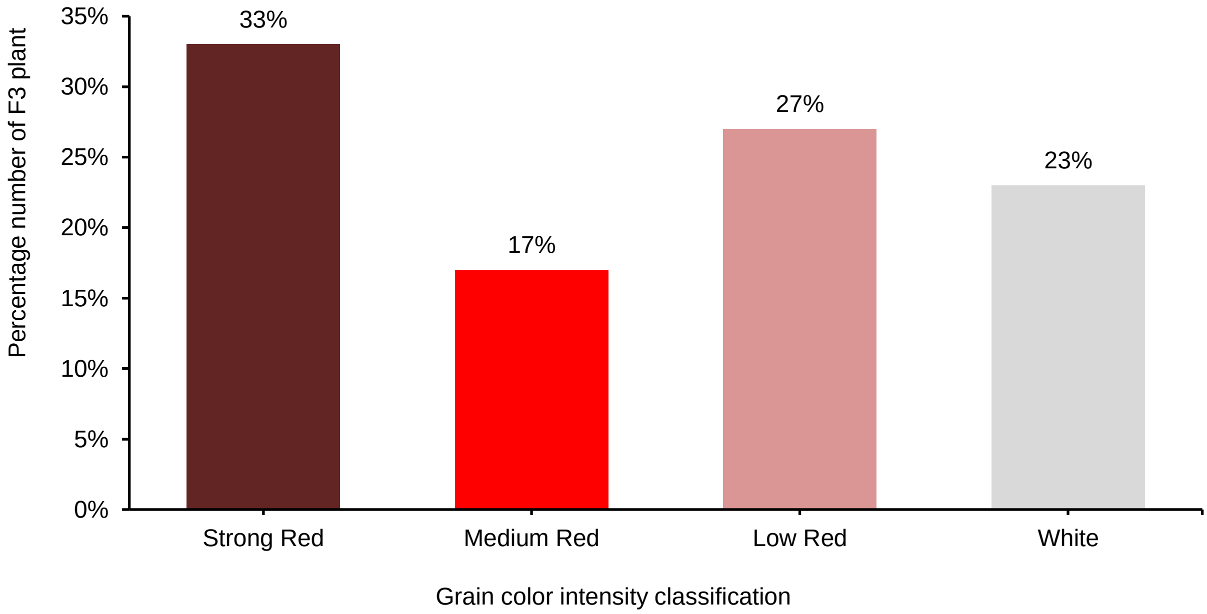

Figure 8. Frequency distribution of rice grain color intensity data of F3 plant from the crosses of Cempo Salamet and IR64

rice is a trait inherited by the parents, where those with white pigment in the grain were IR64 and red pigments were Cempo Salamet. Figure 8 shows the colors of the two parents (control plants), Cempo Salamet is red, and IR64 is white. According to the anthocyanin content, F3 produced color variations ranging from red solid at 33%, medium red 17%, and low red 27% to white at 23%. Pigmented rice contains a class of flavonoid compounds such as proanthocyanidins and anthocyanins (Mbanjo et al. 2020). Qualitative characteristics included fundamental factors that affect grain color (Sholikhah et al. 2019). From these results, 33% red color indicates the dominant gene with the most significant percentage.

PCR results

PCR analysis used four primers, namely RM 346, RM 339, RM 228, and RM 316. These primers were utilized on samples from the Cempo Salamet and IR64 parents and F3, the offspring of the parents' cross. The analysis results showed that the DNA band character in the F3 sample followed the parent Cempo Salamet (red pigmented) and IR64 (unpigmented).

SSR markers were used to analyze the genetic diversity of colored rice, such as local brown, colored, and brown rice (Kristamtini et al. 2018). Shao et al. (2011) explained that two loci are identified from genetic analysis, namely Rc (pericarp and brown seed coat) and Rd (red pericarp and seed coat). The presence of both produces a red color in the seeds. RM339 and RM316 identify phenolic content, flavonoids, and antioxidant capacity. RM228 identifies flavonoid content and antioxidant capacity, while RM346 identifies the phenolic content.

The percentage distribution of genotype analysis with markers RM346, RM339, RM228, and RM316 validated pigment color but could not validate color degradation in pigmented rice. The results showed that the marker genotype in pigmented and non-pigmented rice was 77% and 23%. SSR markers that could validate the DNA bands of Cempo Salamet and IR64 parents with F3 were RM346, RM339, and RM228. Therefore, the segregation of the F3 samples followed the character of the Cempo Salamet or IR64 parent, but RM316 could not validate all the DNA bands in the sample. Further research is necessary since there is still a significant level of segregation in the F3 plant population. However, by continuing this research, offspring with stable and superior traits similar to the two parents might be obtained. The expected excellent characteristics are short plant morphology, many tillers, a pithy grain containing pigment,

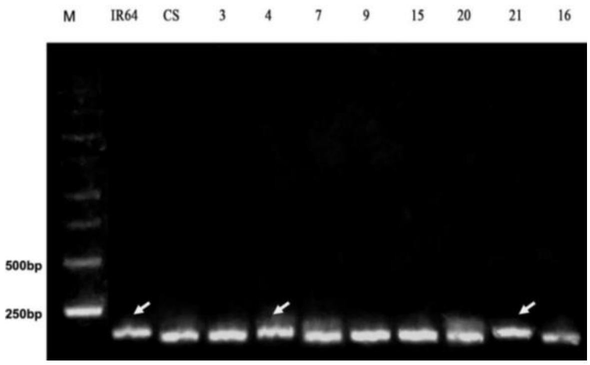

Figure 9. PCR amplification product of the parental cross and F3 samples using RM346 M SSR primer. (M: 1 kb marker; white arrows: bands in non-pigmented parental and tiller populations)

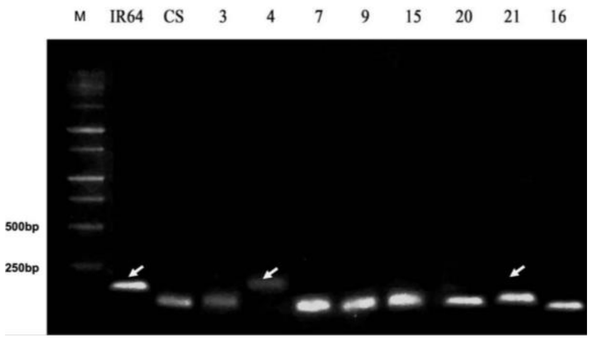

Figure 10. PCR amplification product of **the** parental cross and F3 samples using RM339 SSR primer. (M: 1 kb marker; white arrows: bands in non-pigmented parental and tiller populations)

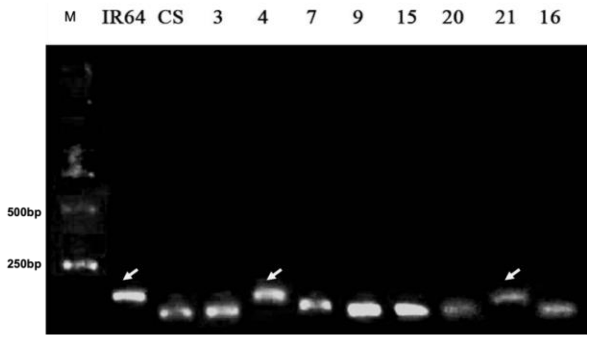

Figure 11. The PCR amplification product of the parental cross and F3 samples used the RM228 SSR primer. (M: 1 kb marker; white arrows: bands in non-pigmented parental and tiller populations)

and resistance to pests and diseases. It can also add research parameters on bioactive genotype characters such as phenolic content, flavonoids, and anthocyanins.

CONCLUSION

There was segregation of morphological characters in the F3 population resulting from a cross between Cempo Salamet and IR64. F3 plant height was short (34%), medium (16%), and tall (50%). The number of tillers in F3 plants categorized as very high, high, medium, and low number of tillers were 21%,18%, 51%, and 10%, respectively. The average weight of 100 grains in F3 plants was found to be very heavy, heavy, and light with the percentage of 0%, 33%, and 67%, respectively. The intensity of the color was red solid (33%), medium red (17%), low red (27%), and white (23%). The SSR markers that could validate the Cempo Salamet and IR64 parents' DNA bands and F3 were RM346, RM339, and RM228. The segregation of the F3 samples was observed to inherit the character of the Cempo Salamet or IR64 parent. However, RM316 could not validate all the DNA bands in the research sample.

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