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MEDIUM-GRAIN RICE F_2 POPULATIONS SELECTION FOR LOW AMYLOSE CONTENT AND CHALKINESS THROUGH MARKER-ASSISTED SELECTION

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SUMMARY

Amylose content (AC) and percentage of chalkiness (PC) are the two most important quality parameters of rice (*Oryza sativa* L.). Breeding for high-quality medium-grain rice has been a decisive demand for the current market and consumption. This research aimed to study the crossing populations and the selection of early generations of promising combinations for evaluation in the next generations. The crossing populations' assessment ensued for genetic variability (PCV and GCV), heritability (h^2_{BS}), and genetic advance (GAM) for the related traits. The promising rice population's election for pure lines employed marker-assisted selection (MAS). The result showed three promising maternal cultivars (PY2, Sieu Ham Chau, and ML202) and paternal cultivars (M-202, Saturn, and Palmyra). Through the analysis of genetic parameters in the rice F_2 populations, the cross combination of ML202/M-202 was the choice because of its valuable genetic parameters expected with PCV and GCV ≥ 20%, h^2_{BS} ≥ 60%, and GAM ≥ 20% for both traits of AC and PC. The Chi-square test of the variables AC and PC in the cross-population ML202/M-202 indicated the segregated ratios of 3:1 in the phenotype and 1:2:1 in the genotype. Applying MAS to select the promising rice F_2 populations resulted in 92 individuals carrying genes identified with low AC and PC simultaneously. These individual rice genotypes became options for evaluation in the next generations.

Keywords: Rice (*Oryza sativa* L.), amylose content, chalkiness percentage, chi-square, genetic parameters, medium grain rice, molecular-assisted selection (MAS)

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Key findings: In rice (*Oryza sativa* L.) F_2 populations, the parameters with PCV and GCV ≥ 20%, h^2_{BS} ≥ 60%, and GAM ≥ 20% were foreseeable for the rice quality traits, i.e., AC and PC for the selected cross. The Chi-square test of the AC and PC in the cross-population showed the segregated ratios of 3:1 in the phenotype and 1: 2: 1 in the genotype. The genotyping strategy employed in the study also proved cost-efficient and effective in terms of identifying the F_2 individuals carrying genes with low AC and PC.

INTRODUCTION

In rice (*Oryza sativa* L.), the grain quality is one of the vital characteristics of breeding programs and an influential factor that affects the acceptability of rice in the market and by the end users (Kumar and Khush, 1986; Cramer *et al*., 1993). Rice grain quality includes physical and chemical properties related to grain shape, milling quality, cooking quality, and nutritional values (Juliano, 1985; Wang *et al*., 2007; Guo *et al*., 2011; Bao, 2014).

The rice grain trait chalkiness strongly links with the recovery rate (IRRI, 2006), and the amylose content mainly affects the softness and flexibility of the rice grains (Bao *et al*., 2006). According to genetic background, in rice, the major gene for amylose synthesis is the *Waxy* gene located on chromosome 6, which encodes the granule-bound starch synthase (Septiningsih *et al*., 2003; Fan *et al*., 2005; Tian *et al*., 2009). However, chalkiness is a complex trait controlled by multiple genes and, therefore, also gains effects from environmental factors (Zhu *et al*., 2018; Duoc *et al*., 2020). Past studies also proved that the *qPGWC-7* is the fine-mapped gene with a 44-kb region for chalky endosperm in rice (Zhou *et al*., 2009; Ha *et al*., 2020).

Selection for best-performing genotypes is an integral part of rice breeding. With the advent of DNA markers, the selection efficiency has significantly improved because the said task has environmental factors not affecting it. Use of these molecular markers could be at any stage of selection, and their utility is mainly practiced in selecting the desirable lines in early segregating generations because a genotype possessing all the desirable genes either in homozygous or heterozygous condition occurs most often in the early segregating generations (F_2) (Khan, 2015). MAS is a process in which a marker indirectly selects the genetic determinants of a trait of interest (Prabhu *et al*., 2009). It shortens the breeding period and lessens the laborious extensive assessment of the populations for the desired traits (Das *et al*., 2017).

Additionally, MAS has developed and improved numerous rice cultivars (Rao *et al*., 2014). Currently, simple sequence repeats (SSRs) markers' wide usage in molecular-assisted selection is due to their availability and comparatively cheaper than others, requiring a relatively simple technique with a higher polymorphism rate (Kumar *et al*., 2009; Khan, 2015; Gao *et al*., 2016). According to Wang *et al*. (1995), the *Wx* (*Waxy*) gene encoding the enzyme granule-bound starch synthase I (GBSSI) is the primary gene controlling the synthesis of amylose content in the endosperm. Cai *et al*. (2015) successfully developed the molecular marker WxIn 1 SNP, including four primers: GF, TR, GR, and TF. In particular, the primer pair GF-TR amplified the 387 bp in both varieties with low and high amylose content (> 20%). Primer pair GF-GR will identify rice varieties with high amylose content at 207 bp, and primer pair TF-TR will identify rice varieties with low amylose content (< 17%) with a 235 bp size. For chalkiness, Zhou *et al*. (2009) identified the *qPGWC-7* gene as the first fine-mapped gene for white-belly endosperm in rice. He proposed the RM21938 primer linked to the *qPGWC-7* gene (Zhou *et al*., 2009), and Lang *et al*. (2017) verified it. Lang *et al*. (2017) proved that RM21938 could recognize low (PC ≤ 10%) and non-low PC (PC > 10%) in rice, and the suitability with phenotypic assessment and genotyping with RM21938 was about 76%. In the presented study, the crossing of medium-grain rice proceeded between parental cultivars with opposite traits of AC and PC. The most

promising F_2 population for high-quality and better grain yield was a preference, selecting its sister lines possessing MAS.

MATERIALS AND METHODS

Plant material and site

The material in this study included six rice (*O. sativa* L.) parental cultivars comprising PY2, Sieu Ham Chau, ML202, M-202, Saturn, and Palmyra. The said rice cultivars also served as check genotypes in the presented experiments. Nine F_2 rice populations included PY2/M-202, PY2/Saturn, PY2/Palmyra, Sieu Ham Chau/M-202, Sieu Ham Chau/Saturn, Sieu Ham Chau/Palmyra, ML202/M-202, ML202/Saturn, and ML202/Palmyra that were developed and studied. The experiments started from March 2020 to August 2021 at the Experimental Station, Can Tho University, Can Tho City, Vietnam.

Analysis of amylose content (AC)

A collection of 50 samples from each F_2 population underwent analysis of AC. Determining the AC of the milled rice samples followed the methods of Juliano (1971) and Graham (2002). Milled rice flour (100 mg) bore soaking in 1 mL of 95% ethanol and 9.0 mL of 1 N NaOH in a 50-mL glass test tube. Then, leaving it undisturbed overnight took 16 hours. Afterward, adding distilled water (90 mL) brought the solution to 100 mL, with 0.5 mL aliquot transferred into a 20-mL test tube containing 5 mL distilled water. Then, adding 0.1 mL of 1 M CH_3COOH, the solution was mixed thoroughly using a vortex mixer, then added with 0.2 mL of iodine solution (0.15% I_2 in 1.5% KI). The solution's dilution to 10 mL used 4.2 mL distilled water. In developing the calibration curves for the amylose content determination in a rice sample, a 40 mg Avebe potato amylose (standard amylose) placed in a 50 mL test tube bore the same process described above. Then, transferring 0.1, 0.2, 0.3, 0.4, and 0.5 ml of the standard amylose sample solution into 20 mL test tubes proceeded similarly to the trial sample. Construction of the calibration curve continued by converting from the spectral reading to the percentage of amylose content according to the following formula: ($y = ax + b$, where y is the absorbance OD, and x is the amount of amylose in the measured sample [mg/L]). Rice cultivars' classification was high (> 25%), intermediate (20.1% - 25%), low (12.1% - 20%), lowest (5.1% - 12%), and waxy (0% - 5%) amylose classes (Juliano and Villareal, 1993).

Analysis of the percentage of chalkiness (PC)

Each F_2 population had 50 samples collected for analysis of the PC. Grain chalkiness visual assessment followed the Standards Evaluation System for Rice (SES) of the International Rice Research Institute (IRRI). Based on the chalky spot in white rice grain, four chalk categories became measures for classification, i.e., scale 0 (non-chalkiness), scale 1 (chalkiness area less than 10%), scale 5 (chalkiness area from 11% to 20%), and scale 9 (chalkiness area more than 20%) (IRRI, 2014). For each seed sample, 100 g of rice grains reached milling and classifying for each grain of rice. The percentage of chalkiness determination used the formula:

PC (%) = (Weight of chalkiness of grains in scale 9/Weight of milled rice) × 100.

According to the modified Standard Evaluation System for Rice (SES) (IRRI, 2014), the percentage of chalkiness (PC) belonging to grain weight in rice cultivars comprised classification into four groups, i.e., non-chalkiness (PC = 0%), low chalkiness (PC = 0.1%–10%), intermediate chalkiness (PC = 10.1%–20%), and high chalkiness (PC > 20%).

DNA extraction

Genomic DNA extraction used the modified cetyltrimethylammonium bromide (CTAB) method based on the protocol of Murray and Thompson (1980).

Table 1. Information of molecular markers in the study.

Marker	Trait	Chr.	Sequence (5'-3')	Reference
Wx-In1	AC	6	GF: tacaaatagccacccaca TR: gatcagcctaaccaaaca GR: gggaaacaaagaattataaacatatatgtacac TF: catcaggaagaacatctgcaagt	Cai *et al*., 2015
RM21938	PC	7	F: ccaaattgcttcctcggatatagg R: cggatttagggagttcgtgttcg	Zhou *et al*., 2009

Notes: Chr.: Chromosome; AC: Amylose content; PC: Percentage of chalkiness.

Polymerase-Chain-Reaction (PCR) Protocol

PCR amplification of markers transpired in a Mastercycler (Eppendorf, Germany) in a total volume of 20 µL, with the following genotyping PCR reagents: 2 µL of DNA at 20 ng/µL, 2 µL of 10× buffer containing 25 mM $MgCl_2$, 1 µL of 2.5 mM dNTPs, 1 unit of Taq Polymerase (Bioline, England), and 1 µL each of forward and reverse primers/markers (10 µM). All performed amplifications comprised 35 cycles of 1 min at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. Amplified PCR products' analysis engaged electrophoresis on 2.5% agarose with the GelRed fluorescent nucleic acid stain (Merck, Germany). Molecular markers related to AC and PC used in this study appear in Table 1.

Hereditary analysis of F_2 populations

The variance components' computation of the phenotypic coefficient of variation (PCV) and the genotypic coefficient of variation (GCV) was according to Burton and Devane (1953).

$$\sigma_g^2 = (MSG - MSE)/r ;$$

$$\sigma_e^2 = MSE/r ;$$

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2 ;$$

$$PCV = (\sqrt{\sigma_p^2}/\bar{X}) \times 100 ;$$

$$GCV = (\sqrt{\sigma_g^2}/\bar{X}) \times 100$$

where σ_g^2 is the genotypic variance, σ_p^2 is the phenotypic variance, $\overline{X}$ is the general mean, MSG is the mean square of accessions, MSE is the mean square of error, and r is the number of replications.

PCV and GCV classification followed Sivasubramanian and Madhavamenon (1973), i.e., low (<10%), moderate (10%–20%), and high (>20%).

Calculating the broad-sense heritability (h_{BS}^2) followed the formula according to Allard's (1960):

$$h_{BS}^2 = (\sigma_g^2/\sigma_h^2) \times 100$$

The h_{BS}^2 classification was according to the scoring of Johnson *et al*. (1955), i.e., low (0%–30%), moderate (31%–60%), and high (>60%).

Genetic advance (GA) estimation followed the formula given by Allard (1960):

$$GA = (k \times h_{BS}^2 \times \sqrt{\sigma_p^2})$$

where k is selection differential at 5% selection intensity which accounts to a constant value 2.06.

Genetic advance over mean (GAM) computation used the following formula, then expressed in percentage.

$$GAM = (GA/\bar{X}) \times 100\%$$

The genetic advance as a percent over the mean had categories as suggested by Johnson *et al*. (1955), i.e., low (<10%), moderate (10%–20%), and high (>20%).

A population's consideration to be effectively capable of inheriting desired traits occurs when its genetic parameters satisfy the

following values, i.e., PCV and GCV are greater than 20%, h^2_{BS} is greater than 60%, and GAM is greater than 20% (Johnson *et al*., 1955; Allard, 1960; Sivasubramanian and Madhavamenon, 1973).

Chi-square test (χ^2)

In the presented study, the chi-square statistic computation followed the below formula (Pearson, 1900):

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where:

χ_c^2 is the chi-square test statistic; c is the degrees of freedom; O is the observed value; E is the expected value; and I is the "i^{th}" position in the table. The wider the difference between the observations and the expectations (O - E in the equation), the bigger the chi-square.

RESULTS AND DISCUSSION

Parental genotypes selection

In the rice (*Oryza sativa* L.) breeding programs for cross combinations, choosing the parental material came from the existing cultivars and elite lines. Parents should be better donors of one or more of the targeted traits, while new cultivars should have those traits' superior combinations (Yan and Frégeau-Reid, 2008). For the crossing block, early selection of the parental genotypes can offer an unparalleled advantage for enhancing genetic gain (Heffner *et al*., 2009). The parental medium-grain rice cultivars with grain yield and quality traits are available in Table 2.

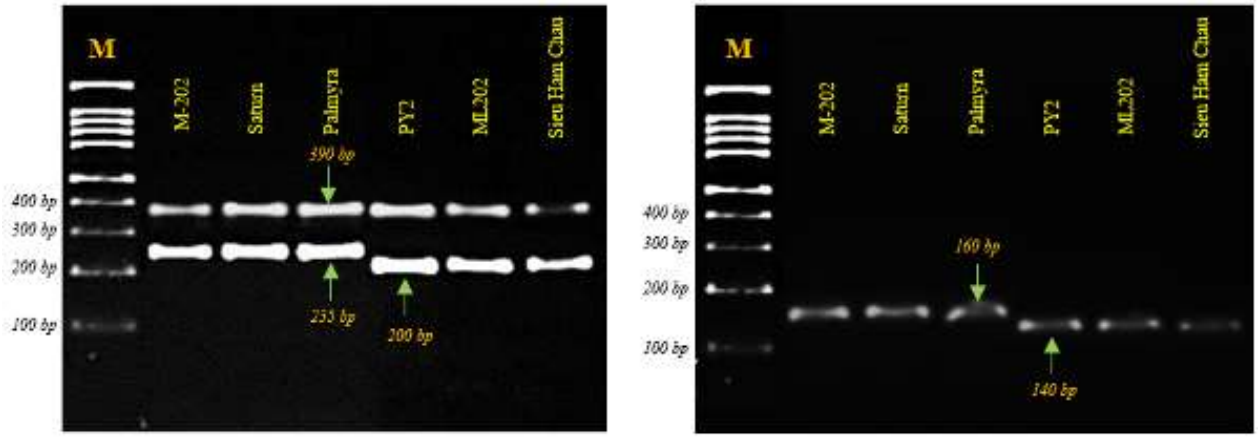
The three rice cultivars, viz., PY2, Sieu Ham Chau, and ML202, were a group of genotypes with high amylose content (AC > 25%), high percentage of chalkiness (PC > 20%), and the highest grain yield (higher than 5 t/ha). These rice cultivars' AC, PC, and grain yield traits were significantly ($p \leq 0.05$) higher than other cultivars. In addition, these three cultivars have broader cultivation in the Mekong Delta, in particular, and Vietnam, in general; hence, recognizing these genotypes as better adapted to local farming conditions, with a proposal as maternal parents in breeding high-quality medium-grain rice. The three other rice cultivars, viz., M-202, Palmyra, and Saturn were options as paternal rice cultivars. These cultivars gave a record low AC (10.83% to 15.38%) and had no chalkiness in grains.

The maternal and paternal rice cultivars had opposite traits of AC and PC and could reach crossing with each other. Ali *et al*. (2021) argued that selecting parental lines with maximum genetic distance might help breed better rice combinations with desirable traits. Additionally, a rice cross combination with more genetically diverse parents will have higher hybridization efficiency and more variation in successive segregating populations (Kumar *et al*., 2007).

Table 2. Grain yield and quality of the parental medium-grain rice.

Name of cultivar	Grain yield (t/ha)	AC (%)	PC (%)
PY2	6,05^{a}	25,30^{c}	46,7^{a}
Sieu Ham Chau	5,88^{a}	30,12^{a}	23,3^{c}
ML202	5,40^{a}	28,40^{b}	33,3^{b}
M-202	3,95^{b}	10,83^{f}	0,0^{d}
Palmyra	3,60^{b}	15,38^{d}	0,0^{d}
Saturn	3,83^{b}	13,63^{e}	0,0^{d}
P	**	**	**
CV (%)	14.85	1.11	14.25
LSD 5%	1.29	0.42	4.46

Notes: AC: Amylose Content; PC: Percentage of Chalkiness; P: probability; **: significant difference of data at 1% level; CV: Coefficient of Variation; LSD: Least significant difference; Different superscripts in the same column indicate significantly different at 5% level.



(A) (B)

Figure 1. Testing allele polymorphisms of parent cultivars by molecular markers related to AC (Wx-In1) (A) and PC (RM21938) (B). *Notes: PCR products in the 2.5% agarose gel; M: ladder (100-1000 bp).*

Testing the parental rice cultivars for allelic polymorphism continued by the Wx-In1 marker related to AC (Cai *et al*., 2015) and marker RM21938 linked to PC (Zhou *et al*., 2009). The result of PCR products appears in Figure 1. The PCR products by molecular markers showed allele polymorphisms in the maternal and paternal cultivars. For the AC trait, the Wx-In1 marker amplified PCR results of 200 bp in the high AC maternal cultivars (PY2, Sieu Ham Chau, and ML202) and 235 bp in the low AC paternal cultivars (M-202, Saturn, and Palmyra). However, in the PCR products, the 390-bp bands appeared in all the rice cultivars. For the PC trait, the marker RM21938 provided the PCR results in the maternal and paternal genotypes at 140 bp and 160 bp positions, respectively.

Genetic parameters in F_2 populations

In the F_2 populations, the AC values varied greatly, and this diversity might relate to the genetic makeup of the parental genotypes and their genome combination in the progeny (Figure 2). Differences in AC were evident between individuals of the same population and among the populations (Figure 3). In the F_2 generation, individuals with low AC (<20%) occurred primarily in the populations including the cross combination, i.e., ML202/M-202, PY2/M-202, PY2/Saturn, and Sieu Ham Chau/M-202. In these four populations, the AC had an average value of 17.6%, 17.8%, 18.3%, and 19.1%, respectively. In the presented study, for all the evaluated traits, the estimates of PCV were better than their corresponding GCV. These results were analogous to those of Souroush *et al*. (2004), Singh *et al*. (2013), and Singh *et al*. (2018). These studies showed that PCV was higher than GCV in agronomic traits and grain quality attributes.

In diversity of variance, the population ML202/M-202 had the highest PCV and GCV, and their values were more than 20%. For heritability, the said cross combinations providing high heritability included ML202/M-202 (h^2_{BS} = 0.89) and Sieu Ham Chau /Palmyra (h^2_{BS} = 0.67). However, the other combinations had heritability coefficients ranging from 0.25 to 0.57. For the selective efficiency (GAM), three cross combinations, i.e., ML202/M-202, Sieu Ham Chau/Saturn, and Sieu Ham Chau/M-202, emerged with higher values than 20% (Table 3). In predicting a population with efficient heritability of traits, their genetic parameters satisfy that PCV and GCV are greater than

Figure 2. Analysis of AC by the biochemical method in the F_2 populations.

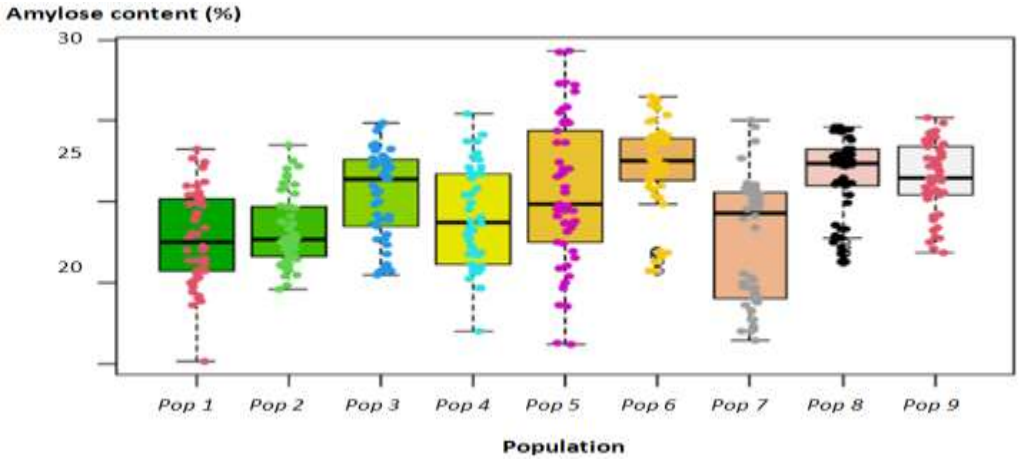


Figure 3. Variation of AC in the F_2 populations. *Notes: Pop 1: PY2/M-202; Pop 2: PY2/Saturn; Pop 3: PY2/Palmyra; Pop 4: Sieu Ham Chau/M-202; Pop 5: Sieu Ham Chau/Saturn; Pop 6: Sieu Ham Chau/Palmyra; Pop 7: ML202/M-202; Pop 8: ML202/Saturn; Pop 9: ML202/Palmyra.*

Table 3. Hereditary parameters related to AC and PC in the F_2 populations.

Trait	Population	Min	Max	Mean	SD	PCV (%)	GCV (%)	h^2_{BS} (%)	GAM (%)
AC	Pop1	10,17	23,29	17,82	4,00	22,4	11,3	0,26	11,8
(%)	Pop2	14,65	23,49	18,29	2,63	14,4	10,4	0,52	15,4
	Pop3	15,47	24,86	20,53	3,56	17,4	11,0	0,40	14,3
	Pop4	12,03	25,41	19,11	3,71	19,5	14,7	0,57	23,0
	Pop5	11,19	29,34	20,55	5,51	26,9	19,0	0,50	27,7
	Pop6	15,72	26,47	22,21	3,06	13,8	11,3	0,67	19,1
	Pop7	11,43	25,00	17,65	3,93	22,4	21,2	0,89	41,2
	Pop8	16,32	24,60	21,61	3,33	15,4	7,7	0,25	7,9
	Pop9	16,89	25,23	21,53	2,70	12,6	8,7	0,48	12,5
PC	Pop1	0,0	31,2	10,7	7,95	75,1	55,5	0,55	84,5
(%)	Pop2	0,0	25,1	12,2	7,90	65,0	52,3	0,65	86,6
	Pop3	0,0	18,4	8,3	6,39	77,6	51,0	0,43	68,9
	Pop4	2,6	19,4	7,8	4,67	59,9	42,2	0,50	61,2
	Pop5	0,0	20,0	8,1	5,73	71,0	48,9	0,47	69,4
	Pop6	1,2	22,4	12,3	7,21	58,8	50,1	0,73	88,0
	Pop7	0,0	20,0	8,7	5,94	68,2	54,2	0,63	88,7
	Pop8	1,2	23,5	10,9	7,12	65,6	55,4	0,71	96,2
	Pop9	1,7	22,2	10,1	6,28	62,6	49,9	0,64	81,9

Notes: Min: Minimum; Max: Maximum; Mean: Average; SD: Standard Deviation; PCV: Phenotypic coefficient of variation; GCV: Genotypic coefficient of variation; h^2_{BS}: Heritability in broad sense; GAM: Genetic advance over mean; Pop1: PY2/M-202; Pop2: PY2/Saturn; Pop3: PY2/Palmyra; Pop4: Sieu Ham Chau/M-202; Pop5: Sieu Ham Chau/Saturn; Pop6: Sieu Ham Chau/Palmyra; Pop7: ML202/M-202; Pop8: ML202/Saturn; Pop9: ML202/Palmyra.

20%, h^2_{BS} is more than 60%, and GAM is higher than 20% (Johnson *et al*., 1955; Allard, 1960; Sivasubramanian and Madhavamenon, 1973). It was also better that the paternal parents transferred the desirable genes, with the ability of the maternal parents to receive the genetic materials and to transfer the same to the offspring. Thus, the cross combination ML202/M-202 was the selection because of its optimum genetic parameters of AC.

Granule chalkiness was also different among the rice populations (Figure 4). The cross combinations, i.e., Sieu Ham Chau/M-202, Sieu Ham Chau/Saturn, PY2/Palmyra, and ML202/M-202, were visible with low chalkiness (7.8%, 8.1%, 8.3%, and 8.7%, respectively) (Table 3, Figure 5). For the PC trait in all the F_2 populations, the values of PCV and GCV were significantly higher than 20%. The cross combinations with higher heritability consisted of Sieu Ham Chau/Palmyra, ML202/Saturn, PY2/Saturn, ML202/Palmyra, and ML202/M-202. The higher heritability inferred that PC got fixed early in the segregating generations. Jennings *et al*. (1979) also considered the crucial evaluation and strict selection with the F_3 grains from F_2 plants in single crosses because the chalky endosperm attains establishment in the early generations. By evaluating the genetic parameters, the hybrid combinations with desirable genetic values (PCV and GCV ≥ 20%, h^2_{BS} ≥ 60%, and GAM ≥ 20%) consisted of PY2/Saturn, Sieu Ham Chau/Palmyra, ML202/M-202, ML202/Saturn, and ML202/Palmyra.

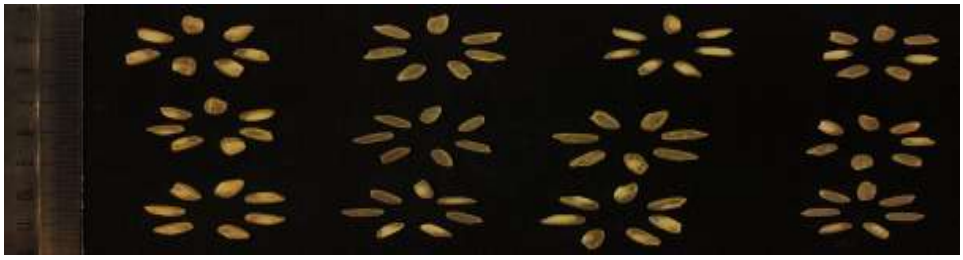


Figure 4. Diversity of kernel chalkiness of the F_2 populations.

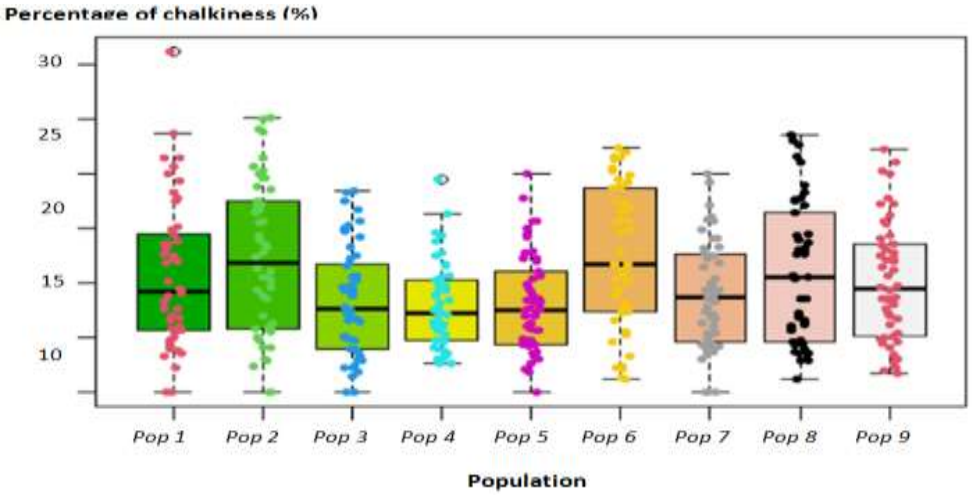


Figure 5. Variation of PC in the F_2 populations. *Notes: Pop 1: PY2/M-202; Pop 2: PY2/Saturn; Pop 3: PY2/Palmyra; Pop 4: Sieu Ham Chau/M-202; Pop 5: Sieu Ham Chau/Saturn; Pop 6: Sieu Ham Chau/Palmyra; Pop 7: ML202/M-202; Pop 8: ML202/Saturn; Pop 9: ML202/Palmyra.*

Table 4. Phenotyping Chi-square (χ^2) testing on AC and PC segregation in the F_2 generation.

Trait	Total of individuals	Low content /percentage	Intermediate or high content /percentage	χ^2(3:1)	P (α = 0.05)
AC	150	105	45	2.000**	0.25 - 0.10
PC	150	103	47	3.209**	0.10 - 0.05

Notes: AC: Amylose content; PC: Percentage of chalkiness; χ^2: Chi-square test statistic; **: significant difference of data at 1% level.

Therefore, through the analysis of genetic parameters in the F_2 generation, the cross combination ML202/M-202 was the selection to develop rice cultivars with low amylose content and grain chalkiness. Besides, the PC had a highly diversified variance in phenotype, genotype, and selection efficiency, while the genetic parameters of the AC were lower. It explains that grain chalkiness is a complex trait controlled by multiple genes and influenced by many external factors; thus, the data variance has diversity, and the selection efficiency of the trait of PC is high. Nakata and Jackson (1973) and Zhu *et al*. (2018) also claimed that these genes are also relative to the effect of the endosperm and maternal cytoplasm. The heritability of AC and PC from populations in the study is medium to high. The average heritability index of AC and PC was at 0.50 and 0.59, respectively. The results suggested that, in rice populations, the granule chalkiness and amylose content traits could appear stable earlier in the segregating generations and, therefore, require choosing in a pure-line selection in early generations. Jennings *et al*. (1979) and IRRI (1996) affirmed that the fixing of grain chalkiness early in the segregating generations, it is vital to evaluate and begin strict selection with the F_3 grain from F_2 plants in single crosses or with the grain of F_1 plants from individual backcrosses or three-way crosses. Karladee *et al*. (2012) also suggested that when attributing the segregants to gene dosage effects in the endosperm, selection for amylose levels was effectively happening in an early generation.

Selection of F_2 population by MAS

In the F_2 generation, 150 F_2 rice lines attained growth in the field. These lines' analysis for AC, PC, and their genotype transpired. The individuals carrying the heterozygous or paternal-like homozygous genes acquired selection for the next generation. For amylose content, the cultivars had distinct segregation into two categories, i.e., low (<20%) and intermediate to high (> 20%) amylose content. Overall, 105 individuals plotted in Category 1, while 45 in Category 2. This distribution showed a bimodal pattern (Table 4) in a 3:1 ratio (χ^2 = 2.000, P > 0.05). This segregation in the F_2 generation of AC also had reports from Kumar and Kush (1986). When crossing between the maternal variety BPI 121-407 with high AC and the paternal variety IR37307-8 with low AC, Kumar and Khush (1986) proved that four groups were identifiable from the F_2 seed analyses: those with 1%–7%, 8%–12%, 13%–18%, and 19%–25% AC, corresponding to 97, 100, 100, and 92 seeds, respectively. Thus, the segregation ratio of low and non-low (intermediate to high) AC in F_2 generation was equivalent to a 3:1 ratio, as in this study. Through testing genes associated with AC by the Wx-In1 marker, besides a mutual band of 390 bp, maternal-like and paternal-like homozygotes were at the positions of 200 bp and 235 bp, respectively (Figure 6). The result of the Chi-square test in genotyping showed three categories, i.e., maternal-like homozygotes (M), heterozygotes (H), and paternal-like homozygotes (P), separated belonging to the ratio of M:H:P = 1:2:1 (Table 5). A combination of the Chi-square test in AC phenotyping and genotyping inferred that the paternal gene identified low AC (AC < 20%) completely dominates over the maternal gene identified intermediate to high AC (AC > 20%).

For the chalkiness of rice grains, the cultivars also sustained segregation into two categories, i.e., low (≤10%) and intermediate to high (>10%) percentage of chalkiness in the

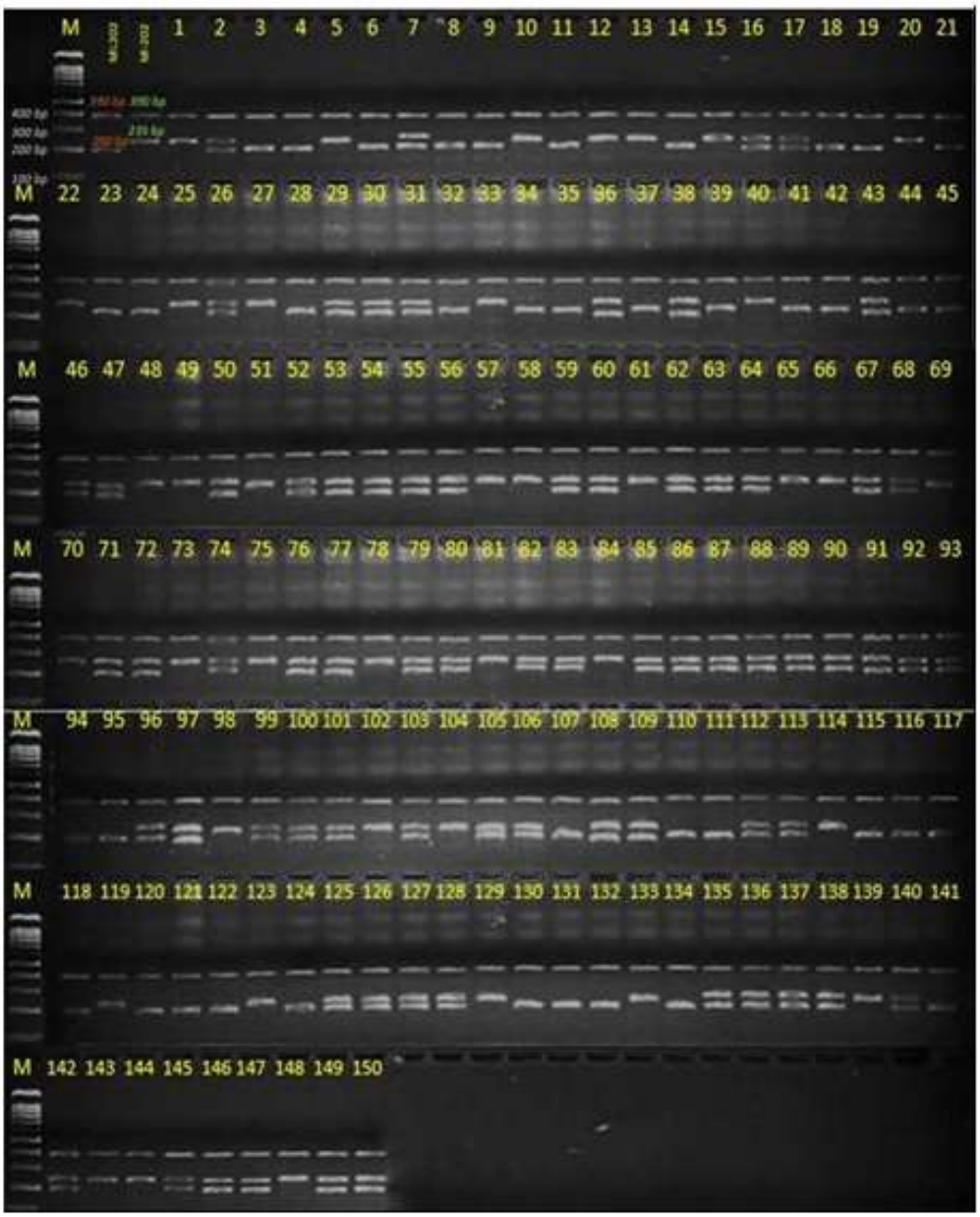


Figure 6. Evaluation of gene related to AC with the Wx-In1 marker in the F_2 population. *Notes: M: Ladder (100-1000 bp); 1-150: F_2 individuals in the ML202/M-202 population.*

Table 5. Genotyping Chi-square (χ^2) testing on AC and PC segregation in the F_2 generation.

Trait	Total of individuals	Maternal-like Homozygote	Heterozygote	Paternal-like Homozygote	χ^2(1:2:1)	P (α = 0.05)
AC	150	40	71	39	0.440**	0.90 - 0.75
PC	150	51	70	29	7.120*	0.05 - 0.01

Notes: AC: Amylose content; PC: Percentage of chalkiness; χ^2: Chi-square test statistic; **: significant difference of data at 1% level.

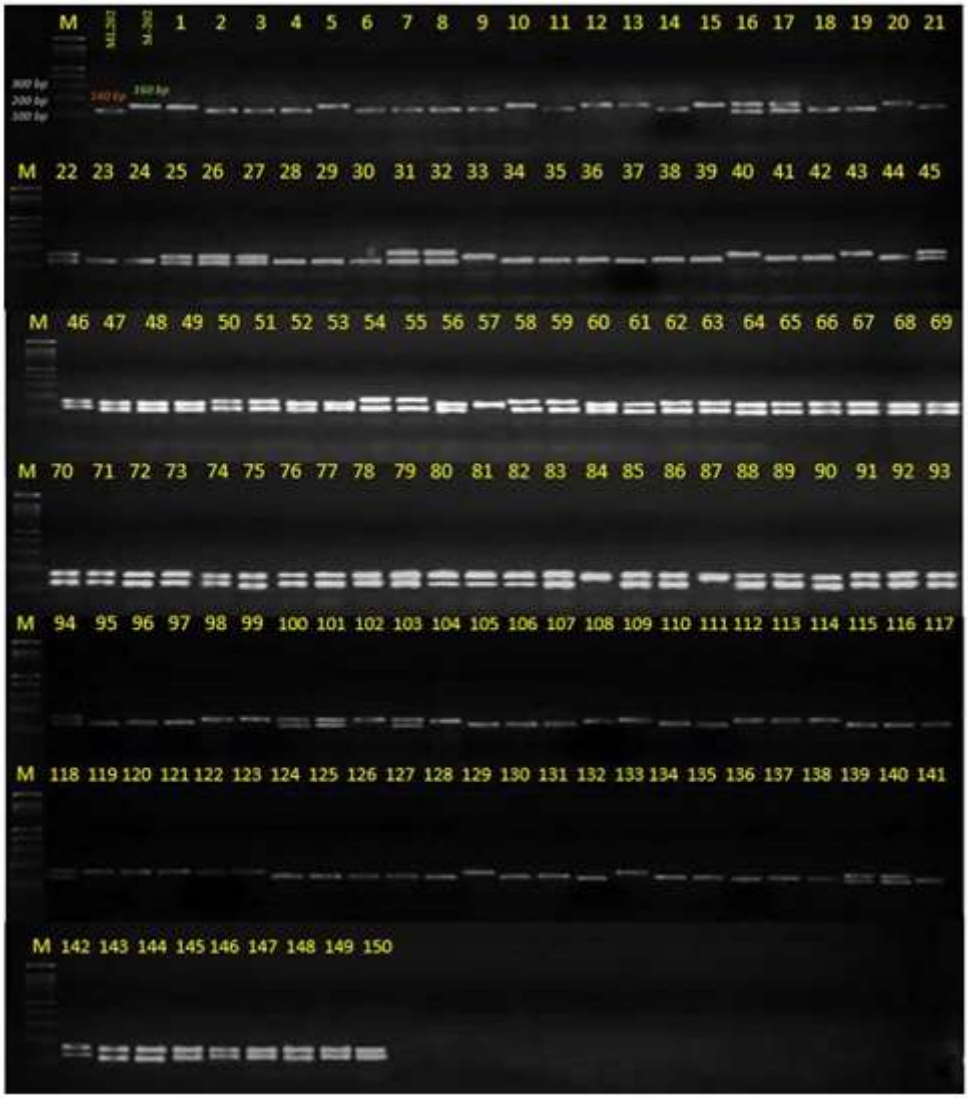


Figure 7. Evaluation of gene related to PC with the RM21938 marker in the F_2 population. *Notes: M: Ladder (100-1000 bp); 1-150: F_2 individuals in the ML202/M-202 population.*

grains. The low and intermediate to high PC categories had 103 and 47 individuals, respectively (Table 4), and in F_2 generation, their segregating ratio was 3:1 (χ^2 = 3.209, P > 0.05). Genotype testing associated with PC by the marker RM21938 recorded individuals expressing maternal-like and paternal-like homozygous genes at the position of 140 bp and 160 bp, respectively (Figure 7). However, when classifying the genotype of PC into three categories, M, H, and P, the distribution of alleles attained segregation following a ratio of M: H: P = 1:2:1 (Table 5).

The Chi-square test showed a low probability. It infers that the paternal gene identified low PC (PC ≤ 10%) non-completely dominates over the maternal gene identified intermediate to high AC (PC > 10%). Besides, it can be due to the genes controlled by the endosperm and maternal cytoplasm (Zhu *et al*., 2018), plant nutrition (Sun *et al*., 2014), high-temperature stress during seed ripening (Ishimaru *et al*., 2009), and the source and the capacity of rice plant (Zhou *et al*., 2009). In F_2 generation, the cross population ML202/M-202 included 110 individuals carrying alleles

identifying with low AC (235 and 390 bp) and with the Wx-In1 marker (Figure 6), and 99 individuals carrying alleles identifying low PC (160 bp) with the marker RM21938 (Figure 7). By combining phenotyping and genotyping of AC and PC, 92 individuals had considerations of carrying the genes identified with low AC and low PC simultaneously. These individuals entailed continuous growing and selection for the next generations.

CONCLUSIONS

The maternal rice cultivars with high yield included PY2, Sieu Ham Chau, and M202. The paternal cultivars with low AC and PC were M-202, Palmyra, and Saturn, and these genotypes became choices for crossing material for high-quality medium-grain rice breeding program. The F_2 hybrid combination ML202/M-202 with the expected genetic parameters was the option to continue for pure-line selection in the next generations. The marker Wx-In1 related to AC and marker RM21938 related to PC could be applicable for selecting segregating rice lines through MAS. Results of Chi-square testing of phenotype and molecular-marker testing of genotypes showed that the distribution of both AC and PC alleles had segregation following a ratio of 3:1. In the F_2 generation, 92 individuals carrying genes identified with low AC and PC were the choices. These individuals became preferences for study in the next generations.

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