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GENETIC VARIABILITY AMONG THE ADVANCED RICE (*ORYZA SATIVA* L.) POPULATIONS BASED ON MORPHOLOGICAL AND MOLECULAR MARKERS

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SUMMARY

Rice (*Oryza sativa* L.) production is vital to Indonesia and plays a key role as a staple food; however, its sustainable production faces threats from climate change. Genetic variability is crucial for developing environment-resilient rice cultivars. The following research aimed to evaluate six advanced F7 populations in comparison with three check cultivars using SSR primers. The experiment layout was in a randomized complete block design with three replications. The results revealed SSR markers showed higher genetic similarity than morphological traits, and leaf length and flag leaf length contributed most to variability. The SSR primer CMCT505 showed the highest polymorphism, with a PIC value of 0.6. The correlation between morphological traits and SSR markers provided a matrix correlation of 0.41483, Z = 22.3636, and P = 0.9883, suggesting a nonsignificant positive correlation. Principal component analysis (PCA) explained 87.56% of the total genetic variability, and leaf length and flag leaf length contributed considerably. Yield evaluation further revealed that F7 4-21-11-23-3-2 attained the maximum yield (13.51 t ha^{-1}), exceeding all three check cultivars. The correlation between SSR markers and morphological traits revealed a moderate relationship, indicating that one of the markers was better at explaining genetic variability.

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Keywords: Rice (*O. sativa* L.), genetic variability, morphological traits, yield components, SSR markers

Key findings: In studying rice (*O. sativa* L.) advanced populations in comparison with three check cultivars using SSR primers, PCA explained 87.56% of the total genetic variability, and the traits of leaf length and flag leaf length contributed significantly. The SSR primer CMCT505 appeared to be the most informative with a PIC value of 0.6. Among the advanced populations, F7 4-21-11-23-3-2 produced the highest grain yield (13.51 t ha^{-1}), confirming its breeding value.

INTRODUCTION

Rice (*O. sativa* L.) is central to Indonesia's socio-cultural, nutritional, and economic life, yet its production faces persistent threats from urbanization, industrialization, and climate change, all of which reduce the area available for cultivation (Ansari *et al.,* 2023). According to the Indonesian Central Bureau of Statistics (CBS, 2023), harvested rice area declined by 1.64% and production by 1.55% in 2024 compared with 2023. With the national rice demand exceeding 31 million tons annually against a production of about 30.9 million tons, the supply-demand gap underscores the need to broaden the genetic base of locally adapted cultivars.

Genetic variation influences individual genotypes, populations, subspecies, and species, reflecting genetic variability due to different alleles in the gene pool and genotypic differences among populations (Mukhopadhyay and Bhattacharjee, 2016). The genetic variability can be beneficial in developing superior rice cultivars through various breeding programs with sustainable enhanced production to address food security. Genetic variability helps in the selection success by reflecting a parent's climate adaptation and tolerance to diverse environmental conditions (Salgotra and Chauhan, 2023). In plant breeding, for assessing genetic variability, numerous conventional and molecular approaches are being practiced. Biotechnological techniques are effective for identifying crucial genetic differences, which also provide a more precise evaluation of the germplasm.

In this research, rice advanced populations selected through hybridization of stable genotypes, such as cultivars Cibogo and Situ Bagendit, showed considerable variability and similarity to their parental genotypes. However, previous studies exhibited that only some traits resemble either parent (Adiredjo *et al.*, 2019). In rice genotypes, the morphological traits can be optimal through molecular analysis. Integrating morphological selection with molecular approaches like simple sequence repeat (SSR) markers offers promising results. The SSR markers effectively explore the genetic variability due to their high polymorphism and codominance (Vieira *et al.*, 2016). The integration of the morphological traits with SSR markers provides essential genetic distance information and potential crop growth strategies in rice advanced populations.

MATERIALS AND METHODS

Experimental location and procedure

The research on rice (*O. sativa* L.) commenced from December 2023 to April 2024 in Jatimulyo Village, Lowokwaru District, Malang City, Indonesia. Molecular analyses proceeded at the Plant Biotechnology Laboratory, Department of Agronomy, Faculty of Agriculture, Brawijaya University, Malang, Indonesia.

Genetic materials and experimental design

The rice experiment layout had a randomized complete block design (RCBD) with three replications. Each experimental unit comprised six polybags containing one plant per polybag. Likewise, an experimental unit comprising four sample plants resulted in sample plants totaling 108. In each experimental unit, three plants served for morphological observations,

with one plant used for molecular observations. The study included nine rice genotypes, including six advanced populations of the F7 generation obtained through crosses of cultivars Cibogo and Situ Bagendit and three check cultivars. The F7 populations received codes, namely, F7 4-21-11-23-3-2 (F7 3-2), F7 4-21-11-23-3-11 (F7 3-11), F7 4-21-11-23-3-12 (F7 3-12), F7 4-21-11-23-6-2 (F7 6-2), F7 4-21-11-23-6-11 (F7 6-11), and F7 4-21-11-23-6-17 (F7 6-17). The check cultivars Situbagendit (SB), Cibogo (CB), and Ciherang (CH) were specimens for comparison. The primers used in this study included CMAG59, CMCT505, and CMTA134a.

Morphological and yield observations

Observation of the morphological traits of rice plants took place under field conditions. The morphological analysis followed the guidelines set by the International Rice Research Institute (IRRI, 2013). For the principal component analysis (PCA), the qualitative traits entailed conversion into scores. For various traits, the score categories appear in Table 1. Yield-component traits comprised plant height (cm), tiller number, productive tillers, panicle length (cm), 50% and 100% flowering age (DAP), and harvesting age (DAP). Yield traits comprised the grain number per panicle, panicles per clump, fertile-grain percentage, grain weight per clump (g), 1000-grain weight (g), and grain yield (t ha^{-1}). Observations followed the IRRI (2013) Standard Evaluation System of Rice. Qualitative traits underwent conversion to scores for the PCA.

Genomic DNA isolation

DNA isolation

Fresh rice leaves used were from 21 days after planting (DAP). Following the Viogene Plant Mini Kit procedure, 0.1 g of leaf samples entailed grinding to a fine powder with liquid nitrogen. The powder succeeded in mixing with 400 µL of PX1 buffer and 4 µL of RNase A before being vortexed and incubated at 65 °C for 10 min. After adding 130 µL of PX2 buffer and vortexing, freezing the mixture took 5 min. The lysate's transfer to a shearing tube sustained centrifugation at 13000 rpm for 2 min. The flow-through gained mixing with 225 µL of PX3 buffer and 450 µL of 100% ethanol, with the 650 µL of the mixture transferred to a column and centrifuged at 10000 rpm for 1 min. The column received washing with 700 µL of WS buffer and centrifugation at 13000 rpm for 1 min, repeated for 3 min. Its transfer to a new tube continued, with 200 µL of TE buffer added before incubating for 5 min at room temperature. After a final centrifugation at 13000 rpm for 1 min, the obtained pure DNA could be used immediately or can be stored at -20 °C. Confirming the DNA quality used electrophoresis, where an obvious DNA band without smears indicated purity and the lack of contamination.

Table 1. Morphological traits and their scores used in PCA.

No.	Character	Type	Phase	Score
1	Leaf length (LL)	Very short (< 21 cm); Short (21–40 cm); Medium (41–60 cm); Long (61–80 cm); Very long (> 80 cm)	Heading	1, 2, 3, 4, 5
2	Leaf width (LW)	Narrow (< 1.3 cm); Wide (1.4–2 cm); Very wide (> 2 cm)	Heading	1, 2, 3
3	Flag leaf length (FLL)	Very short (< 11 cm); Short (11–20 cm); Medium (21–30 cm); Long (31–40 cm); Very long (> 40 cm)	Heading	1, 2, 3, 4, 5
4	Flag leaf width (FLW)	Narrow (< 1.2 cm); Wide (1.2–2 cm); Very wide (> 2 cm)	Heading	1, 2, 3
5	Leaf angle (LA)	Erect; Horizontal; Droopy	Booting	1, 5, 9
6	Flag leaf angle (FLA)	Erect; Intermediate; Horizontal; Descending	Booting	1, 3, 5, 7
7	Leaf blade color (LBC)	Light green; Green; Dark green; Purple tips; Purple margins; Purple blotch; Purple	Booting	1, 2, 3, 4, 5, 6, 7
8	Collar color (CC)	Light green; Green; Purple	Booting	1, 2, 3
9	Basal leaf sheath color (BLC)	Green; Purple lines; Light purple; Purple	Booting	1, 2, 3, 4

DNA amplification with SSR markers

The PCR reaction included a PCR tube, 25 µL of Nzytaq II Green Master Mix, 1 µL each of forward and reverse primers, and 1 µg of DNA template added to reach a total of 50 µL. The solution reached homogenization before its amplification in a PCR machine (Labnet Multigene Optimax Thermal Cycler) following Promega (2016), with pre-denaturation at 95 °C for 90 s, 35 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 60 s, and post-extension at 72 °C for 5 min. The amplified DNA either underwent direct use or storage at -20 °C. For electrophoresis, loading 5 µL of the DNA sample mixed with 1 µL of loading dye proceeded into the agarose gel well, running the machine at 50 volts for 45 min. The gel incurred soaking in 1x TAE buffer with ethidium bromide for 10–15 min, and using a gel documentation instrument (BIO-RAD Molecular Imager XR+), sought to visualize the DNA bands.

Band scoring and statistical analysis

The scoring of DNA bands consisted of binary data (1 = present, 0 = absent) and received analysis using NTSYS v2.02 (UPGMA [unweighted pair group method with arithmetic mean], SAHN [sequential, agglomerative, hierarchical, and nested] module, and SIMQUAL [similarity quality] subprogram) to construct similarity matrices. The measurement of band lengths used the Gel Analyzer. Genetic distance coefficients of 0–0.5 indicate lower similarity, while 0.5–1 indicate higher similarity (Wicaksono *et al.,* 2022). The calculation of PIC values followed Serrote *et al.* (2020), where PIC > 0.5 = highly informative, 0.25–0.5 = moderately informative, and < 0.25 = uninformative. Qualitative morphological data entailed conversion into multistate data upon analysis by UPGMA in NTSYS v2.02. The PCA performed in R-Studio had principal components retained for eigenvalues > 1, with contributing variables identified by factor loadings > 0.5 (Agustina and Waluyo, 2017). The correlation between morphological and SSR similarity matrices underwent testing using the Z-Mantel test (Mantel, 1967), categorizing *r* values as perfect (> 1), strongest (< 0.8), stronger ($0.6 \leq r < 0.8$), strong ($0.4 \leq r < 0.6$), weak ($0.2 \leq r < 0.4$), and weakest (< 0.2) (Sokal and Rohlf, 2009; Prayoga *et al.,* 2022).

RESULTS AND DISCUSSION

Genetic variability of morphological traits

Morphological scoring of nine rice genotypes (Table 2) revealed a significant variation in both quantitative and qualitative traits. F7 6-11 and F7 6-17 had shorter leaves than the check cultivars, whereas F7 3-2 and F7 3-12 showed leaf widths comparable to Cibogo and Ciherang. Flag leaf angle in F7 3-2, F7 3-11, F7 6-2, and F7 6-11 was similar to all check cultivars; F7 3-12 and F7 6-17 differed. Most genotypes exhibited medium leaves and narrow leaves, and erect flag leaves enhanced

Table 2. Morphological traits with scoring of rice genotypes observed in the field.

Genotypes	LL	LW	FLL	FLW	LA	FLA	LBC	CC	BLC
F7 4-21-11-23-3-2	3	2	2	1	1	1	2	1	1
F7 4-21-11-23-3-11	3	1	2	2	1	1	2	1	1
F7 4-21-11-23-3-12	3	2	3	2	1	3	2	1	1
F7 4-21-11-23-6-2	3	1	2	2	5	1	2	1	1
F7 4-21-11-23-6-11	2	1	3	2	1	1	3	1	1
F7 4-21-11-23-6-17	2	1	2	2	5	3	2	1	1
Situ Bagendit	3	1	3	2	1	1	1	1	1
Cibogo	3	2	3	2	5	1	2	1	1
Ciherang	3	2	2	2	1	1	2	1	1

LL = Leaf length, LW = Leaf width, FLL = Flag leaf length, FLW = Flag leaf width, LA = Leaf angle, FLA = Flag leaf angle, LBC = Leaf blade color, CC = Collar color, and BLC = Basal leaf sheath color.

Table 3. Average of yield traits.

Genotypes	Grain per panicle	Panicles per clump	Fertile grain (%)	Grain weight Clump^{-1} (g)	1000-grain weight (g)	Yield (t ha^{-1})
F7 4-21-11-23-3-2	108.38	18.28	77.83	27.23	31.48	13.51
F7 4-21-11-23-3-11	90.39	20.81	76.52	19.44	28.00	9.80
F7 4-21-11-23-3-12	89.65	20.44	79.90	21.70	29.75	10.85
F7 4-21-11-23-6-2	70.25	31.47	68.13	11.83	28.62	6.04
F7 4-21-11-23-6-11	85.85	24.44	68.80	17.14	28.00	8.57
F7 4-21-11-23-6-17	83.93	21.88	75.82	18.54	28.98	9.23
Situ Bagendit	77.32	21.59	79.55	15.81	25.33	8.15
Cibogo	84.50	21.63	87.66	19.69	27.25	9.84
Ciherang	90.08	18.91	77.59	20.35	28.50	10.17

light absorption for vegetative growth. Leaf dimensions are key determinants of canopy architecture and photosynthetic capture (Wahyuti *et al.,* 2013).

For flag leaf length, F7 3-12 and F7 6-11 matched Situ Bagendit and Cibogo, while F7 3-2, F7 3-11, F7 6-2, and F7 6-17 resembled Ciherang. All genotypes had similar flag leaf widths to the checks, except F7 3-2. Flag leaf characteristics influence light interception and grain yield, with smaller flag leaf area linked to higher canopy photosynthesis during ripening (Makino *et al.,* 2022). All genotypes showed green leaves with varying intensity; F7 6-11 had a dark green shade, indicative of higher photosynthetic capacity (Duraiswamy *et al.,* 2023). Uniform chlorophyll content and flag leaf color across populations suggest these traits had high heritability genes governing those (Sitaresmi *et al.,* 2013), reflecting the cumulative selection of stable traits in the F7 generation.

Yield component and yield performance

All genotypes differed significantly from Situ Bagendit in grain number per panicle (Table 3). The highest grain counts in F7 3-2 reflected combined inheritance from both parents and its superior panicle length and tiller productivity. Grain number, productive tillers, fertile grain percentage, and grain weight together form the principal yield components in rice (Abdullah *et al.,* 2008). F7 6-2 and F7 6-11 varied remarkably from all controls, with F7 6-11 producing the highest panicle count per clump (21.47).

The analysis of variance showed significant effects on flowering age (50% and 100%), harvesting age, plant height, productive tillers, panicle length, and yield traits. F7 3-2 reached the earliest harvesting age (≈112 DAP) and, together with F7 3-11 and F7 3-12, did not differ from Situ Bagendit in flowering, consistent with their Situ Bagendit × Cibogo descent. All genotypes produced upright flag leaves, a New-Type-Rice trait that promotes photosynthate translocation to panicles (Abdullah *et al.,* 2008). Plant heights ranged at 83–91 cm (short class, < 110 cm; IRRI, 2013), and tiller numbers ranged broadly, with F7 6-11 producing the most (≈26 tillers).

F7 3-11, F7 3-12, and F7 6-2 had notably more productive tillers than Cibogo and Ciherang, with F7 3-12 having the highest (≈23 per hill). All six F7 genotypes had considerably longer panicles than Cibogo and Ciherang; the longest panicle (24.50 cm) and highest grain number per panicle (108.38) both resulted in F7 3-2. Grain number, productive tillers, fertile grain percentage, and grain weight together constitute the principal yield components in rice. F7 3-2 reached the ultimate 1000-grain weight (31.48 g) and the maximum yield (13.51 t ha^{-1}), reflecting superior nutrient uptake and photosynthesis. Among the F7 lines, F7 3-2 and F7 3-12 exceeded all three check varieties in yield, while F7 3-11, F7 6-11, and F7 6-17 surpassed only Situ Bagendit, supporting the advancement of F7 3-2 toward new high-yielding variety development.

Table 4. Similarity matrix of morphological traits in rice genotypes using NTSYS 2.02.

Genotypes	F7 3-2	F7 3-11	F7 3-12	F7 6-2	F7 6-11	F7 6-17	SB	CB	CH
F7 3-2	1.0000								
F7 3-11	0.7778	1.0000							
F7 3-12	0.8519	0.7778	1.0000						
F7 6-2	0.8148	0.8148	0.8148	1.0000					
F7 6-11	0.7037	0.7778	0.7778	0.7407	1.0000				
F7 6-17	0.6667	0.7407	0.7407	0.8519	0.7407	1.0000			
SB	0.7407	0.7407	0.6667	0.7037	0.7037	0.6296	1.0000		
CB	0.6667	0.6296	0.6667	0.7037	0.6667	0.7037	0.5556	1.0000	
CH	0.7407	0.7407	0.7407	0.7037	0.6667	0.7037	0.7037	0.6667	1.0000

SB = Situ Bagendit, CB = Cibogo, CH = Ciherang.

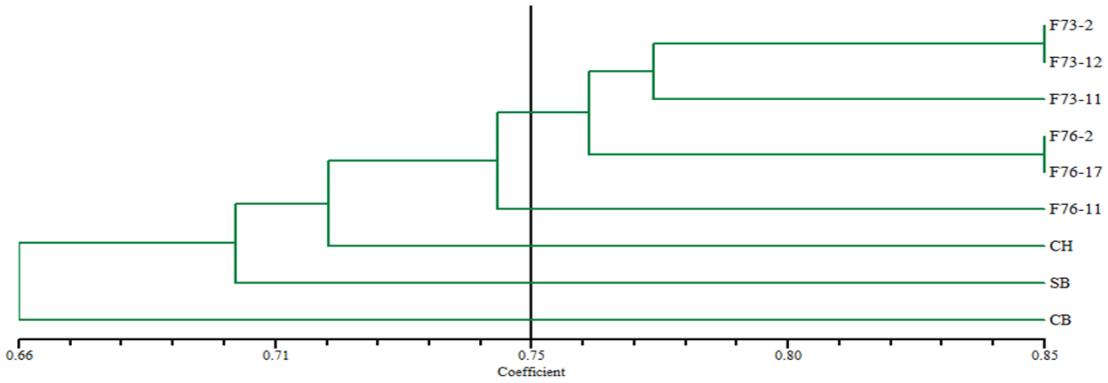

Figure 1. Dendrogram obtained from the analysis of rice genotypes based on morphological traits.

Clustering analysis

The morphological trait observations on various rice genotypes based on the scores succeeded in their analysis through NTSYS 2.02 to obtain a similarity matrix (Patel *et al.,* 2018). The similarity matrix exhibited similarities of rice genotypes with one another (Table 4). The morphological data of the nine rice genotypes underwent assessment through NTSYS 2.02, with the dendrogram constructed (Figure 1).

The dendrogram revealed two main clusters: Cluster I comprising eight genotypes and Cluster II containing the check cultivar Cibogo. Cluster I incurred further division into subgroups IA and IB, with subgroup IA including F7 populations and the check genotype Ciherang, while subgroup IB contained only Situ Bagendit. Although Cibogo was genetically similar to Ciherang, it clustered separately from the diverse F7 populations, indicating a complex genetic inheritance. Closer proximity on the dendrogram signifies greater genetic similarity and shared ancestry (Kimwemwe *et al.,* 2023). Genotypic correlation, influenced by pleiotropy and linkage disequilibrium, explained the inheritance and linkage of various traits (Sary *et al.,* 2022). High similarity among certain F7 populations indicates a shared genetic background from the same parental cross, producing morphological uniformity in the advanced generation.

Principal component analysis (PCA)

The principal component analysis of the nine rice genotypes with morphological traits in the PCA biplot revealed the genotypes' distribution across four plots (Figure 2). The eigenvalue, loading factor, proportion of variance, and cumulative proportion are available in Table 5. Plot I included F7 3-2, F7 6-2, and Cibogo, contributing significantly to leaf length (LL).

Table 5. Loading factor, eigenvalue, proportion of variance, and cumulative variance of rice genotypes based on morphological traits.

No.	Characters	PC1	PC2
1	LL	0.62	0.41
2	LW	0.01	0.06
3	FLL	0.41	0.90
4	FLW	0.05	-0.04
5	LA	-0.15	0.20
6	FLA	0.12	-0.13
7	LBC	-0.01	0.05
8	CC	0	0
9	BLC	0	0
	Eigenvalue	6.46	3.80
	Proportion of Variance	65.10%	22.46%
	Cumulative Proportion	65.10%	87.56%

LL = Leaf length, LW = Leaf width, FLL = Flag leaf length, FLW = Flag leaf width, LA = Leaf angle, FLA = Flag leaf angle, LBC = Leaf blade color, CC = Collar color, and BLC = Basal leaf sheath color.

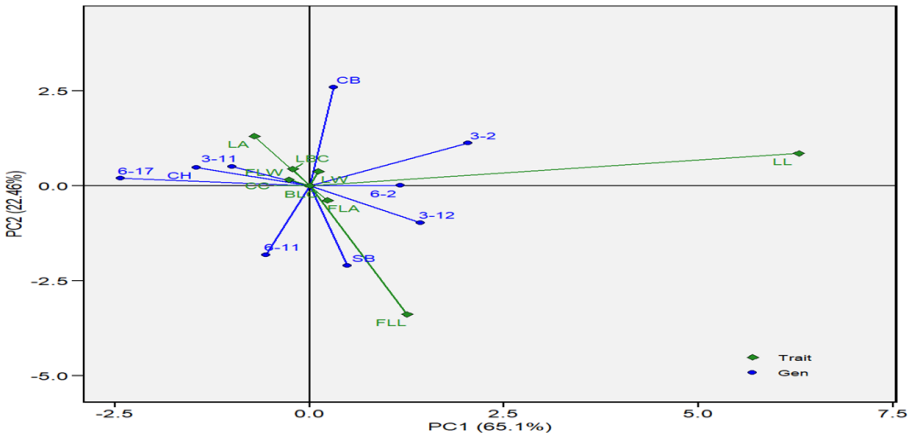

Figure 2. Rice genotype distribution based on morphological traits with principal component analysis (PCA).

Plot II contained F7 3-11, F7 6-17, and Ciherang, while Plot III had F7 6-11. Finally, plot IV included F7 3-12, F7 6-2, and Situ Bagendit, contributing considerably to flag leaf length (FLL).

PC1 had an eigenvalue of 6.46 and explained 65.1% of the total variation, while PC2, with an eigenvalue of 3.80, accounted for 22.46%. The traits of leaf length, leaf width, flag leaf length, flag leaf width, and leaf angle positively influenced genetic variability in PC1, while PC2 exhibited a positive effect from leaf length, leaf width, leaf angle, and leaf blade color. PC1 represents significant morphological trait variability among the rice genotypes (Priyanga *et al.,* 2020). The PCA biplot indicated clusters of closely related genotypes, with stability represented by central traits and high variability shown by the distance from the axis (Dewi *et al.,* 2014).

SSR markers' banding pattern analysis

The DNA band length displayed a marker ladder, ranging from 100 to 1500 bp, which indicated the total number of alleles for each primer. The allele frequency and PIC value for each primer appear in Table 6. The SSR markers revealed polymorphic band patterns across the nine rice genotypes; the primers CMAG59, CMCT505, and CMTA134a showed 17, 9, and 11 alleles, respectively.

Table 6. Allele frequency and polymorphism information content (PIC) of each primer analyzed in rice genotypes.

No.	Primer	Allele Frequency	PIC	Category
1	CMAG59	0.91	0.1	Not informative
2	CMCT505	0.42	0.6	Very informative
3	CMTA134a	0.83	0.2	Not informative

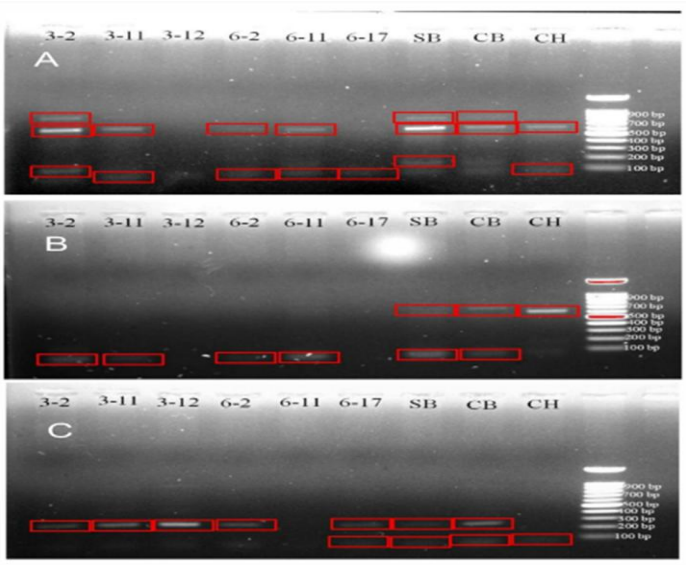

Figure 3. Visualized electrophoresis result of rice genotypes using a) CMAG59 primer, b) CMCT505 primer, and c) CMTA134a primer.

Electrophoresis results are available in Figure 3. According to Dalimunthe *et al.* (2020), markers generate distinct banding patterns that reflect genetic variability; however, missing bands may result from primer-template mismatches that inhibit amplification. Korzh and Dubina (2022) reported that molecular markers bind only when DNA has close links with the marker and remain unaffected by environmental factors. The high level of polymorphism was largely due to the influence of the primers' ability to bind their complementary DNA sequences in the given template.

Morphological and SSR markers' correlation

The morphological similarity matrix came from scoring traits directly observable in rice genotypes, while the SSR marker similarity matrix was reliant on DNA band patterns converted into binary data. The correlation analysis between these matrices yielded an r-value of 0.42483, indicating a moderate positive relationship between the genetic similarity assessed by morphological and SSR markers. Morphological traits provide a direct assessment of observable characteristics, which can also gain influences from environmental factors, affecting their reliability in genetic studies. In contrast, SSR markers target specific DNA sequences unaffected by environmental variation, making them more consistent in assessing genetic variability.

Differences between morphological and SSR marker dendrograms highlighted distinct aspects of the genetic variability assessment. The morphological dendrogram captures sensitive traits influenced by additive genetic effects and environmental conditions, whereas the SSR markers focus on specific genomic

regions. The considerable Mantel statistic (Z = 22.3636, P = 0.9883) underscores the significant relationship between morphological traits and SSR marker variations, reinforcing their complementary role in understanding genetic diversity and informing breeding programs (Prayoga *et al.,* 2022).

CONCLUSIONS

Genetic variability analysis of nine rice genotypes—six F7 advanced populations and three check cultivars—revealed an obvious differentiation through both morphological and molecular assessments. The six F7 populations expressed considerable variation in agronomic and yield-related traits, with F7 4-21-11-23-3-2 producing the highest yield (13.51 t ha^{-1}). PC1 and PC2 jointly explained 87.56% of the total morphological variance, and leaf length and flag leaf length contributed most to the variability. The SSR primer CMCT505 was the most informative (PIC = 0.6). The moderate positive correlation between morphological and SSR similarity matrices (r = 0.42) confirms that integrating both methods provides a robust strategy for characterizing rice genetic diversity and selecting promising lines for cultivar development.

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