RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
57 (4) 1445-1457, 2025
http://doi.org/10.54910/sabrao2025.57.4.10
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

MOLECULAR CHARACTERIZATION OF ADVANCED RICE LINES TOLERANT TO LOW TEMPERATURE IN THE HIGHLANDS

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SUMMARY

The process of selecting rice (*Oryza sativa* L.) tolerant to low-temperature stress through phenotype selection activities is a time-consuming process. However, in rice (*Oryza sativa* L.) genotypes, the genetic diversity can be assessed rapidly using molecular marker-based characterization. This study aimed to evaluate the genetic diversity and cold tolerance of 15 F4 rice lines derived from crosses and their six parental genotypes using five SSR markers. The genetic diversity analysis employed the NTSYS pc 2.1 software. Results showed 21 rice genotypes had allele sizes of 150 to 230 bp, with an average number of alleles (5.4) and a polymorphism level of 0.79. The genetic similarity coefficient level of 0.37 contained three clusters. Four promising rice lines (F4UKIT102R-2-100, F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018) were classified as tolerant to low temperature and exhibited the best agronomic performance. The tolerant line F4UKIT102R-2-100 was notably very similar to the parental genotype Pare Bau. The three other lines, F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018 were similar to the parental genotypes Pare Lallodo, Pare Kombong, and Pare Ambo, respectively.

Keywords: Cold-tolerant rice (*O. sativa* L.) lines, molecular characterization, genetic similarity, new rice type

Communicating Editor: Prof. Naqib Ullah Khan

Manuscript received: February 06, 2025; Accepted: May 12, 2025.

Citation: Purnomo DW, Parari TY, Karamang S, Limbongan YL, Chadikun P, Mawikire NL, Syaranamual S, Wibawati Z, Nurlailah, Ayuningtias N, Rahim S (2025). Molecular characterization of advanced rice lines tolerant to low temperature in the highlands. *SABRAO J. Breed. Genet.* 57(4): 1445-1457. http://doi.org/10.54910/sabrao2025.57.4.10.

Key findings: The category as tolerant to low temperature, along with the best agronomic performance, was achieved by four promising rice (*O. sativa* L.) lines (F4UKIT102R-2-100, F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018))

INTRODUCTION

In Indonesia, rainfed rice (*Oryza sativa* L.) fields span four million hectares, comprising 3.2 million hectares of rainfed rice fields and 0.8 million hectares of dry land. These arable areas hold significant potential for supporting national food security, though their utilization remains suboptimal. However, these lands' potential varies based on factors, such as slope and elevation. Of the 3.2 million hectares of rainfed rice fields, 1.54 million hectares are located on slopes of 3%–15% in the mid-to-highland regions. Specifically, 1.13 million hectares are on slopes of 3%–10%, while the 0.50 million hectares are on slopes exceeding 15%, some of which are also in the highlands (Sulaiman *et al*., 2018). Rainfed rice fields in highlands and midlands have the potential to contribute to national food security. Although 0.50 million hectares of these high and midlands serve for rice cultivation, and their average production is still relatively low compared with the lowlands, ranging from 2.5 to 5.0 t ha^{-1} (Harahap *et al*., 1993; Zen, 2013).

In recent decades, rice breeding efforts have largely focused on lowland cultivars suited to optimal environmental conditions, with less attention to cultivars designed for specific challenges, such as drought, heavy metals, salinity, flooding, pest attacks, and low temperatures. Consequently, these breeding efforts have not comprehensively addressed the wide range of environmental conditions, the impacts of climate change, or the diversity of existing soil types (Limbongan *et al*., 2024). Therefore, the development of new high-yielding rice cultivars should incorporate prevailing environmental factors to enhance productivity and promote early maturity.

Highland areas hold considerable potential for rice cultivation, yet they are constrained by a range of production challenges. Upland rice production is contrained by, specific agro-climatic conditions, including low temperatures, relatively high rainfall, limited groundwater availability, extended photoperiods, and high air humidity, which can delay the harvest and induce flower sterility (Basuchaudhuri, 2014). Low-temperature stress greatly affects the vegetative phase of rice plants because it reduces the germination power, slows down the growth process, affects yellowing of leaves after transplanting, reduces the number of tillers, and stunts plants (Hsu and Hsu, 2019). If it occurs in the generative phase, it causes grain degeneration, incomplete panicle elongation, and increased grain sterility reducing production by 30%–40% (Zhao *et al.,* 2020). These challenges have driven breeding programs lowland rice toward developing varieties with tolerance to low-temperatures, efficient water and sunlight use, resistance to high humidity, pests and diseases, and the capacity to produce higher yields within a shorter growth period (Mara *et al*., 2015).

To address this challenge, a study was conducted by crossing five local Toraja rice cultivars, known for their low temperature tolerance and adaptation to highland areas above 1,000 meters above sea level (masl), with the superior cultivar Inpari-4. The resulting F2 population exhibited significant trait diversity (Parari *et al.*, 2022). This study has now progressed to the F4 generation, utilizing mass selection and single seed descent methods during the F2-F3 generations in North Toraja, Indonesia (1,400 masl). Several tolerant lines with desirable agronomic characteristics, such as early maturity and high productivity, have gained detection (Table 1) to achieve the main objective of the study. In developing highland rice cultivars tolerant to low temperatures, it is crucial to select for cold tolerance along with other specific traits.

Selecting genotypes based solely on agronomic traits may not guarantee their

Table 1. Characteristics of the genetic material used in the study.

Genotype Name	Type (F3 Line /Parental)	Source/Origin	Cold Tolerance Level (Phenotype)	Key Agronomic Traits
F3UKIT101-2-124	F3 Line	Pare Ambo ×Inpari-4	Medium	Early maturing, medium saplings, moderate quantity of grain per panicle, moderate yield
F3UKIT101-2-218	F3 Line	Pare Ambo ×Inpari-4	Medium	Early maturing, medium saplings, moderate quantity of grain per panicle, moderate yield
F3UKIT102-2-010	F3 Line	Pare Bau × Inpari-4	High	Early maturing, high saplings, high quantity of grain per panicle, high yield
F3UKIT102-2-056	F3 Line	Pare Bau × Inpari-4	High	Early maturing, high saplings, high quantity of grain per panicle, high yield
F3UKIT102-2-024	F3 Line	Pare Bau × Inpari-4	High	Early maturing, moderate saplings, high quantity of grain per panicle, moderate yield
F3UKIT103-2-100	F3 Line	Pare Kombong × Inpari-4	High	Super early maturing, high saplings, high quantity of grain per panicle, high yield
F3UKIT103-2-019	F3 Line	Pare Kombong × Inpari-4	Medium	Early maturing, moderate saplings, medium quantity of grain per panicle, medium yield
F3UKIT104-2-127	F3 Line	Pare Lallodo × Inpari-4	Medium	Early maturing, medium saplings, medium quantity of grain per panicle, medium yield
F3UKIT104-2-194	F3 Line	Pare Lallodo × Inpari-4	High	Super early maturing, medium saplings, medium quantity of grain per panicle, medium yield
F3UKIT104-2-089	F3 Line	Pare Lallodo × Inpari-4	Medium	Early maturing, medium saplings, high quantity of grain per panicle, medium yield
F3UKIT105-2-042	F3 Line	Pare Lea × Inpari-4	Medium	Medium maturing, high saplings, medium quantity of grain per panicle, high yield
F3UKIT105-2-039	F3 Line	Pare Lea × Inpari-4	High	Early maturing, high saplings, medium quantity of grain per panicle, high yield
F3UKIT102R-2-100	F3 Line	Inpari-4 ×Pare Bau (Reciprocal)	High	Super early maturing, high saplings, high quantity of grain per panicle, high yield
F3UKIT102R-2-078	F3 Line	Inpari-4 × Pare Bau (Reciprocal)	High	Super early maturing, high saplings,high quantity of grain per panicle, high yield
F3UKIT102R-2-018	F3 Line	Inpari-4 × Pare Bau (Reciprocal)	High	Early maturing, high saplings, high quantity of grain per panicle, high yield
Pare Bau	Parental	Origin	High	Long maturing, medium saplings, medium yield
Inpari-4	Parental	Superior varieties	Low	Early maturing, high saplings, medium quantity of grain per panicle, high yield
Pare Lallodo	Parental	Origin	High	Long maturing, small saplings, medium yield
Pare Kombong	Parental	Origin	High	Long maturing, small saplings, low yield
Pare Lea	Parental	Origin	Medium	Super long maturing, small saplings, medium yield
Pare Ambo	Parental	Origin	High	Long maturing, small saplings, low yield

(*Source: Limbongan et al., 2023)*

genetic suitability for the target environment. To address this issue, molecular analysis, such as simple sequence repeat (SSR) trials, can be employed for more effective genotype selection. This method can help identify target genotypes tolerant to low temperatures by assessing their genetic similarity to tolerant parent lines. Additionally, the SSR technique exhibits a high level of polymorphism, which can accelerate the selection process and reduce costs compared with repeated evaluation of agronomic traits in the field, a time-consuming process.

The SSR markers provide high reliability and reproducibility in allele identification, making them an effective tool for assessing genetic diversity in crop plants. Moreover, SSR markers enable efficient selection in large populations by offering a genetically based approach. These markers have been extensively used in rice breeding to characterize genetic diversity within the rice germplasm (Garris *et al*., 2005; Ladjao *et al.,* 2019), identify genetic bases related to resistance to pests, diseases, and environmental stresses (Usman *et al*., 2016), and detect the genes related to low-temperature tolerance (Wang *et al*., 2009; Bosetti *et al*., 2012; Artica, 2017). This aimed to evaluate the genetic diversity and tolerance to low-temperature of 15 F4 rice lines and their six parental genotypes using SSR markers.

MATERIALS AND METHODS

Breeding material and procedure

Six rice (*O. sativa* L.) parental cultivars (Pare Bau, Inpari-4, Pare Lallodo, Pare Kombong, Pare Lea, and Pare Ambo) sustained crossing to produce 15 hybrids (Table 1). Molecular analysis, as conducted in 2022, transpired at the laboratory of Molecular Biology, Indonesian Center for Agricultural Biotechnology Research and Development (ICABIOGRAD), in Bogor, Indonesia.

Laboratory material and tools

For molecular analysis, the materials used included samples of rice shoot leaves from all the genotypes (Table 1), along with liquid nitrogen, NaCl, EDTA, mercaptoethanol, Tris base, chloroform, ethanol, CTAB PCR buffer, ultrapure water, agarose, TBE buffer, TE 1x buffer, cold isopropanol, Taq DNA polymerase, and dNTPs. The five SSR primers used were RM5806, CT220, RM502, RM248, and RM5503 (Table 2). The use of the five primers correlated to local rice. For DNA analysis, the tools engaged were an analytical balance, spatulas, mortars, centrifuges, water baths, shakers, micropipettes, Eppendorf tubes (microtubes), spectrophotometers, microplates (PCR plates), PCR machines, horizontal electrophoresis equipment, and GelDoc systems.

Table 2. Description of the five molecular markers used.

Markers	Chromosomal Location	Sequence	Annealing Temperature (°C)	Reference
		Forward (5'-3') and Reverse (3'-5')		
CT 220	6	F: AAGTGGTTTCATGTTATGCTAATTTT R: GAAAGCAAGCGGCATTAGC	60	Andaya and Tai (2007)
RM 5806	10	F: CTAATTGCGGTTGAAGCCTC R: CCTCCCAATCTTTGCACATC	55	Gramene (2006)
RM 502	8	F: GCGATCGATGGCTACGAC R: ACAACCCAACAAGAAGGACG	55	Beccera *et al*. (2015)
RM 248	7	F: TCCTTGTGAAATCTGGTCCC R: GTAGCCTAGCATGGTGCATG	58	Anggraheni and Mulyaningsih (2017)
RM 5503	4	F: GGGAAGAAGATAGGAGATGG R: CTCTGGGTACACTTCACGAG	55	Gramene (2006)

DNA analysis

Performing DNA analysis used a modified version of the Murray and Thompson method (1980). The analyzed rice lines succeeded in planting at the target location (1,600 masl), with an area characterized by low-temperature stress. With budget limitations, this study focused on 15 high-yielding lines and six parental cultivars for molecular analysis. The characteristics and advantages of 15 productive F3 lines in the highlands are available in Table 1. The stages of SSR analysis are as follows.

DNA isolation

The extraction of total genomic DNA resulted from healthy, bright green, and unblemished young leaves of each rice genotype. The DNA isolation procedure using CTAB followed the mini-preparation extraction DNA CTAB of Saghai-Maroof *et al.* (1984) method, which yields small quantities of DNA suitable for subsequent use. The extracted DNA serves as the template for DNA amplification using a PCR machine. The isolation process began with cleaning 0.250 g of rice leaf samples using a tissue moistened with technical ethanol 70%. The leaf samples' grinding in a mortar received two liquid nitrogen (LN) treatments to prevent moisture. The extraction buffer (CTAB buffer, NaCl, EDTA, and Tris-HCl) attained preheating to 65 °C in a water bath before use. After grinding, the transfer of the powdered leaf tissue continued to a 2-mL microtube (Eppendorf), then added with 700 µL of extraction buffer and thoroughly mixed. The microtubes containing the samples sustained incubation at 65 °C for 20 min, with inversion every 10 min to aid in the lysis process.

After incubation, 700 µL of chloroform:isoamyl alcohol (Chisam) mixture (24:1 ratio) was added to all the samples. This step proceeded in a fume hood. Then, the samples' mixing by inversion occurred 60 times, followed by centrifuging at 10,000 rpm or 5 min. After centrifugation, 500 µL of the resulting supernatant was carefully transferred to a new 2 mL tube using a micropipette. The addition of cold isopropanol (500 µL) to the supernatant (1:1 ratio) ensued, with the mixture gently shaken. After DNA precipitation was visible, the samples proceeded centrifugation at 12,000 rpm for 10 min. The supernatant's careful discarding happened before inverting the microtube containing the DNA pellet to remove any residual liquid, as treated with 70% ethanol to remove any salt present. The DNA pellet then sustained re-suspension in 100 µL of TE buffer before measuring its concentration using a spectrophotometer.

DNA amplification

The DNA amplification process continued using a PCR machinethermocycler with a specific reaction composition and temperature cycling, known as the PCR profile. The PCR reaction mixture consisted of 2.0 µl DNA template (10-30 ng), 7.5 µl 2x MyTaq Red Mix, 1.5 µl SSR primer, and 4.0 µl H2O, for a total volume of 15.0 µl. The PCR cycling conditions were as follows: Pre-denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C (first six cycles) or 55 °C (remaining 24 cycles) for 15 s, and an extension at 72 °C for 10 s. A final extension step ensued at 72 °C for 7 min, with the reaction held at 15°C for 10 min.

Electrophoresis of PCR Products

Before DNA separation takes place on an 8% (w/v) polyacrylamide gel, the success of the PCR duplication requires verification. Preparing the 8% PAGE solution, mixing the following transpired: 400 ml of ddH2O, 100 ml of 10x TBE, and 200 ml of 40% acrylamide/bis-acrylamide (19:1) solution, bringing the total volume to 1000 ml. Next, pouring the 60 ml of the 8% PAGE solution succeeded into the IPC plate assembly. Just before pouring, the solution received 600 µl of 10% ammonium persulfate (APS) and 50–60 µl of TEMED to initiate polymerization.

For the sample preparation, adding 5 µl of loading dye occurred to each well containing 15 µl of the PCR product. Next, the careful loading of 3.5 µl of the prepared samples proceeded into the wells of the comb.

Table 3. Variance data from SSR analysis of 15 promising new type of rice lines with superior agronomic traits, five local parents, and national Inpari-4 cultivars, using five SSR markers.

Markers	Number of Allele	Major Allele Frequency	PIC	Allele Size (bp)
CT220	2	0.9524	0.7052	200-250
RM5806	4	0.5185	0.8807	150-230
RM502	6	0.8571	0.7430	250-375
RM248	4	0.8571	0.7778	250-360
RM5503	11	0.6154	0.8751	175-280

Electrophoresis was then successful with the samples, running at a voltage of 100 volts for 2 h. After the electrophoresis was complete, with the power turned off, removing the gel carefully ensued. The gel then underwent soaking in an ethidium bromide solution for approximately 10 min, followed by a 10-min rinse in water to remove the excess ethidium bromide (a known mutagen needing careful handling; lab safety guidelines require strict compliance, including wearing lab coats, gloves, and eye protection when working with ethidium bromide). The electrophoresis results gained visualization using a Chemidoc or Geldoc system. Finally, the DNA bands, captured and stored digitally, received scoring for the observed bands.

Data analysis

The obtained data underwent molecular analysis by scoring the DNA bands observed on the gel for the 21 rice genotypes. Scoring followed a binary system: a present band scored as 1, an absent band as 0, and uncertain bands recorded as 9 (missing data). The binary data from the scoring bore analysis were analyzed using the NTSys-pc 2.1 statistical software.

RESULTS

Polymorphism analysis

In 21 rice (*O. sativa* L.) genotypes, the polymorphism analysis using five SSR markers revealed the frequency of the major allele ranged from 0.6154 to 0.9524, with an average of 0.7601 (Table 3). The allele size range was smallest in marker RM5806 (150–230 bp), while the largest was in marker RM502 (250–375 bp). The highest polymorphic information content (PIC) values were evident for the markers RM5806 (0.8807) and RM5503 (0.8751), while the lowest PIC value was notable in the marker CT220 (0.7052).

PCR visualization, using the SSR marker RM5806, demonstrates that the primer effectively distinguished the rice parental genotypes and their F4 populations (Figure 1). The DNA banding patterns of lines 13 (F4UKIT102R-2-100) and 14 (F4UKIT102R-2-078) were similar to the parental genotype A (Pare Bau), while lines 1–11 were identical to parental cultivar E (Pare Lea). Line 15 (F4UKIT102R-2-018) appeared similar to the parent cultivar C (Pare Lallodo). Parental cultivars B (Inpari-4), D (Pare Kombong), and F (Pare Ambo) did not show any similarity to the analyzed rice F4 lines.

The PCR visualization for the SSR marker CT220 (Figure 2) showed that parental genotype A (Pare Bau) shares similarities with six rice lines, i.e., 3 (F4UKIT102-2-056), 4 (F4UKIT102-2-056), 5 (F4UKIT102-2-024), 9 (F4UKIT104-2-194), 13 (F4UKIT102R-2-100), and 14 (F4UKIT102R-2-078). Parental genotype E (Pare Lea) emerged similar to five F4 lines: 1 (F4UKIT101-2-124), 6 (F4UKIT103-2-100), 7 (F4UKIT103-2-019), 11 (F4UKIT105-2-042), and 12 (F4UKIT105-2-039). Parental genotype D (Pare Kombong) displayed a similarity with rice line 10 (F4UKIT104-2-089), while parental cultivar C (Pare Lallodo) exhibited likeness to line 15 (F4UKIT102R-2-018). The remaining two rice lines, 2 (F4UKIT101-2-218) and 8 (F4UKIT104-2-127), did not match any of the parental genotypes.

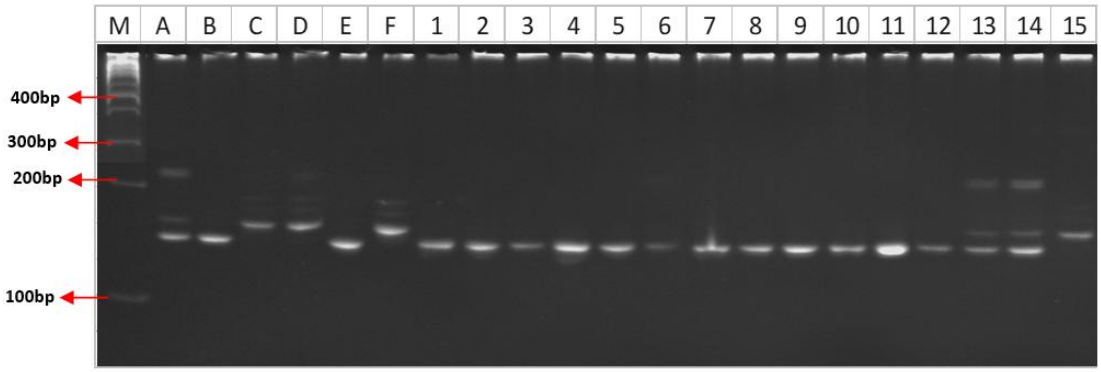

Figure 1. Visualization of SSR marker RM5806 analysis results for 21 rice genotypes. M: DNA Ladder (Promega ΦX165 DNA/HinfI), A = Pare Bau, B = Inpari-4, C = Pare Lallodo, D = Pare Kombong, E = Pare Lea, F = Pare Ambo, 1 = F4UKIT101-2-124, 2 = F4UKIT101-2-218, 3 = F4UKIT102-2-010, 4 = F4UKIT102- 2-056, 5 = F4UKIT102-2-024, 6 = F4UKIT103-2-100, 7 = F4UKIT103-2-019, 8 = F4UKIT104-2-127, 9 = F4UKIT104-2-194, 10 = F4UKIT104-2-089, 11 = F4UKIT105-2-042, 12 = F4UKIT105-2-039, 13 = F4UKIT102R-2-100, 14 = F4UKIT102R-2-078, and 15 = F4UKIT102R-2-018.

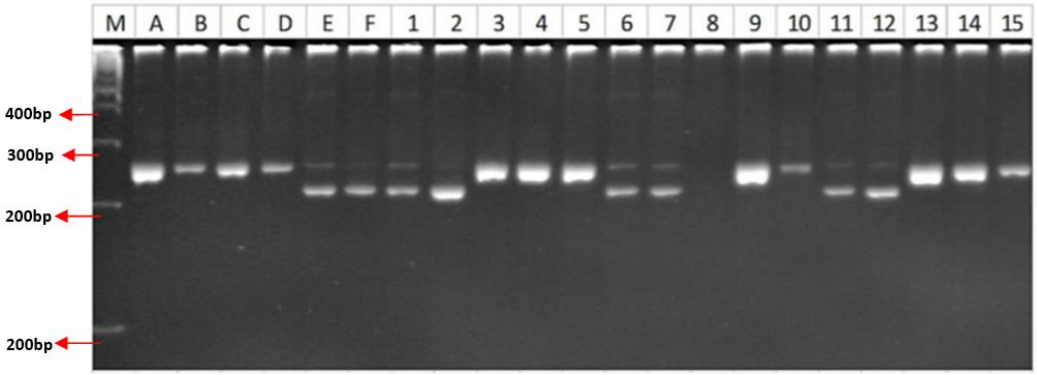

Figure 2. Visualization of SSR marker CT220 analysis results for 21 rice genotypes. M: DNA Ladder (Promega ΦX165 DNA/HinfI), A = Pare Bau, B = Inpari-4, C = Pare Lallodo, D = Pare Kombong, E = Pare Lea, F = Pare Ambo, 1 = F4UKIT101-2-124, 2 = F4UKIT101-2-218, 3 = F4UKIT102-2-010, 4 = F4UKIT102-2-056, 5 = F4UKIT102-2-024, 6 = F4UKIT103-2-100, 7 = F4UKIT103-2-019, 8 = F4UKIT104-2-127, 9 = F4UKIT104-2-194, 10 = F4UKIT104-2-089, 11 = F4UKIT105-2-042, 12 = F4UKIT105-2-039, 13 = F4UKIT102R-2-100, 14 = F4UKIT102R-2- 078, and 15 = F4UKIT102R-2-018.

Cluster analysis

The cluster analysis conducted engaged the NTSYS program and revealed all rice genotypes could undergo categorization into three main groups at a 37% coefficient. This classification is dependent on significant genetic differentiation values, indicating marked genetic differences among the groups. The grouping is distinctive of the genetic distance between the parent lines and the genotypes, suggesting that lines within the same group share close genetic proximity or tolerance. Conversely, if a line is far from the local parent group but closer to the Inpari-4 group, it indicates the line is sensitive. In Figure 3, it shows Group I included two genotypes, i.e., the parental cultivar Pare Bau and the lines F4UKIT102R-2-100 (G15). Group II comprised 13 genotypes, including one rice

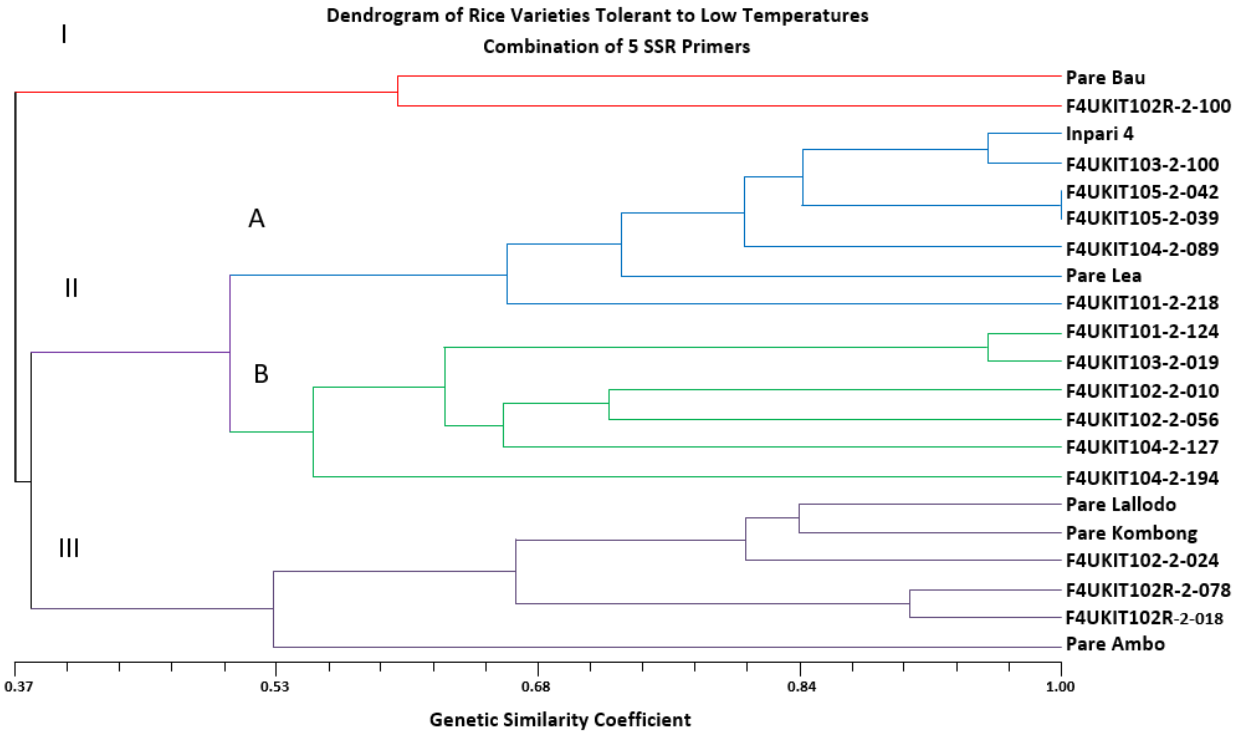

Figure 3. Cluster analysis results of 21 genotypes based on 5 SSR markers, conducted using the NTSYS pc 2.1 software.

line, F4UKIT103-2-100, indicating similarity to the parental genotype Inpari-4. Within group II, at a 50% coefficient, the group further sustained dividing into two subgroups: Group A contains seven rice genotypes (Inpari-4, F4UKIT102R-2-100, F4UKIT105-2-042, F4UKIT105-2-039, F4UKIT104-2-089, Pare Lea, and F4UKIT101-2-218), and Group B comprises six lines (F4UKIT101-2-124, F4UKIT103-2-019, F4UKIT102-2-010, F4UKIT102-2-056, F4UKIT104-2-127, and F4UKIT104-2-194). Group III consists of six rice lines, viz., three parental cultivars (Pare Lallodo, Pare Kombong, and Pare Ambo) and three lines (F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018).

The kinship analysis of 21 rice genotypes (including six parental genotypes and 15 F4 populations) revealed a genetic distance range of 0.31 to 0.96 (Table 4). This information on kinship and genetic distance was remarkably crucial for selecting cross parents and assessing their potential for genetic diversity, especially when involving parental genotypes with distant genetic relationships. The 15 rice F4 populations derived through hybridization have a genetic distance greater than 0.70 from their parental cultivars.

DISCUSSION

Polymorphism analysis using five SSR markers revealed that the frequency of the major alleles ranged from 0.6154 to 0.9524, with an average of 0.7601 across the rice (*O. sativa* L.) populations. The analysis showed each SSR marker generated four distinct allele sizes, i.e., 100, 200, 300, and 400 bp. These PCR products reflect the variations in nitrogen-base sequences, referred to as polymorphism (Biswas *et al*., 2020). According to Ladjao *et al.* (2019), variations in allele size were due to repeated nitrogen-base sequences during analysis. The highest polymorphic information

Table 4. Genetic distance analysis of F6 lines and their parent comparisons.

Genotypes	A	B	C	D	E	F	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	0.00																				
B	0.55	0.00																			
C	0.58	0.55	0.00																		
D	0.55	0.44	0.89	0.00																	
E	0.44	0.82	0.37	0.48	0.00																
F	0.37	0.27	0.72	0.82	0.44	0.00															
1	0.55	0.65	0.62	0.51	0.62	0.48	0.00														
2	0.55	0.65	0.48	0.44	0.69	0.41	0.65	0.00													
3	0.65	0.62	**0.72**	0.69	0.51	0.51	0.75	0.69	0.00												
4	0.51	0.55	0.65	**0.75**	0.58	**0.72**	0.62	0.55	0.79	0.00											
5	0.62	0.58	**0.89**	**0.86**	0.48	0.69	0.65	0.58	0.82	0.75	0.00										
6	0.51	**0.96**	0.51	0.41	**0.86**	0.31	0.69	0.69	0.58	0.51	0.55	0.00									
7	0.58	0.69	0.65	0.55	0.58	0.44	0.96	0.62	0.79	0.65	0.69	0.72	0.00								
8	0.58	**0.75**	**0.72**	0.62	0.65	0.51	0.75	0.69	0.79	0.72	0.75	0.72	0.72	0.00							
9	0.65	0.48	0.51	0.62	0.51	0.51	0.69	0.55	0.72	0.72	0.62	0.44	0.72	0.51	0.00						
10	0.62	**0.86**	0.62	0.51	0.69	0.34	0.58	0.72	0.62	0.55	0.65	0.82	0.62	0.75	0.55	0.00					
11	0.48	**0.86**	0.55	0.44	**0.82**	0.41	0.72	0.79	0.62	0.55	0.58	0.89	0.69	0.82	0.41	0.86	0.00				
12	0.48	**0.86**	0.55	0.44	**0.82**	0.41	0.72	0.79	0.62	0.55	0.58	0.89	0.69	0.82	0.41	0.86	0.54	0.00			
13	**0.72**	0.62	0.58	0.62	0.58	0.51	0.48	0.41	0.58	0.65	0.62	0.58	0.51	0.58	0.51	0.48	0.48	0.48	0.00		
14	0.58	0.62	**0.79**	0.69	0.44	0.51	0.62	0.41	0.58	0.51	0.75	0.58	0.65	0.58	0.51	0.55	0.48	0.48	0.65	0.00	
15	0.51	0.62	**0.86**	**0.75**	0.44	0.58	0.62	0.41	0.58	0.51	0.75	0.58	0.65	0.58	0.51	0.55	0.48	0.48	0.58	0.93	0.00
Backcross Probability	1	5	3	3	3	1															
Total		16																			

A = Pare Bau, B = Inpari-4, C = Pare Lallodo, D = Pare Kombong, E = Pare Lea, F = Pare Ambo, 1 = F4UKIT101-2-124, 2 = F4UKIT101-2-218, 3 = F4UKIT102-2-010, 4 = F4UKIT102-2-056, 5 = F4UKIT102-2-024, 6 = F4UKIT103-2-100, 7 = F4UKIT103-2-019, 8 = F4UKIT104-2-127, 9 = F4UKIT104-2-194, 10 = F4UKIT104-2-089, 11 = F4UKIT105-2-042, 12 = F4UKIT105-2-039, 13 = F4UKIT102R-2-100, 14 = F4UKIT102R-2-078, and 15 = F4UKIT102R-2-01.

content (PIC) value was prominent for the marker RM5806 (0.8807), while the lowest PIC value resulted in the marker CT220 (0.7052).

The PIC values reflect the level of polymorphism, and the average PIC value across the five SSR markers ranged from 0.7 to 0.8, with an overall average of 0.79. These results indicate the markers used were highly informative, as a PIC value above 0.5 is considerably very informative. According to Lei *et al*. (2023), markers with a PIC value above 0.5 had a classification as highly informative, those with values between 0.25 and 0.50 as moderately informative, and those below 0.25 as low. Rohaini *et al*. (2016) also noted a higher PIC value signifies a better marker for detecting a wider range of alleles, making it particularly useful for analyzing the genetic relationship among the genotypes.

The outcomes revealed marker RM5806 produced the highest PIC value (Table 3), resulting in a highly informative allele pattern that helps visualize the kinship between the rice F4 lines and their parental cultivars. Moreover, the marker CT220 provided allele visualizations that offered useful insights into the similarity between the rice lines and their parental genotypes. The markers RM502, RM248, and RM5503 also produced informative PIC values; however, their allele pattern did not clearly distinguish the genetic similarity among the rice F4 lines. The markers with higher PIC values proved informative; although, the banding pattern may not always be consistent across the genotypes (Wang *et al*., 2015; Sha *et al*., 2021). Based on these results, it is evident that using SSR markers with a high level of polymorphism can make the selection of cold-tolerant rice more efficient and faster. This approach allows for earlier detection of the genetic relatedness of breeding outcomes with cold-tolerant genetics compared with traditional repeated selection methods taking longer periods of results.

The PCR visualization using the SSR marker RM5806 disclosed that the DNA banding patterns of rice lines 13 (F4UKIT102R-2-100) and 14 (F4UKIT102R-2-078) were similar to parental genotype A (Pare Bau) (Figure 1). Lines 1–11, including F4UKIT101-2-124, F4UKIT101-2-218, F4UKIT102-2-010, F4UKIT102-2-056, F4UKIT102-2-024, F4UKIT103-2-100, F4UKIT103-2-019, F4UKIT104-2-127, F4UKIT104-2-194, F4UKIT104-2-089, and F4UKIT105-2-042, exhibited the banding patterns found identical to parental cultivar E (Pare Lea). However, rice line 15 (F4UKIT102R-2-018) showed a pattern similar to parental cultivar C (Pare Lallodo). Parental genotypes B (Inpari-4), D (Pare Kombong), and F (Pare Ambo) did not show similarity with any tested lines. The results indicate that using SSR markers with a high level of polymorphism significantly enhances the efficiency and speed of selecting cold-tolerant rice varieties. This method enables earlier detection of genetic relationships in breeding outcomes that exhibit cold-tolerant traits, in contrast to traditional selection methods, which typically require longer periods to achieve results.

The PCR image visualization using the SSR CT220 marker revealed that parental genotype Pare Bau appeared similar to six F4 rice lines, namely, 3 (F4UKIT102-2-010), 4 (F4UKIT102-2-056), 5 (F4UKIT102-2-024), 9 (F4UKIT104-2-194), 13 (F4UKIT102R-2-100), and 14 (F4UKIT102R-2-078) (Figure 2). Parental genotype E (Pare Lea) was alike with five rice lines: 1 (F4UKIT101-2-124), 6 (F4UKIT103-2-100), 7 (F4UKIT104-2-127), 11 (F4UKIT105-2-042), and 12 (F4UKIT105-2-039). Parent D (Pare Kombong) matches the rice line 10 (F4UKIT104-2-089), and parent C (Pare Lallodo) was similar to line 15 (F4UKIT102R-2-018). The two rice lines, 2 (F4UKIT105-2-218) and 8 (F4UKIT104-2-127), did not show any similarity to the parental cultivars. Visualization using both markers RM5806 and CT220 indicates several lines were identical to their parents. On average, 13 promising lines exhibited genetic similarities with two parental cultivars, Pare Bau and Pare Lea.

The cluster analysis signified four rice lines have a tolerant-to-low temperature category (Figure 3). The rice line F4UKIT102R-2-100 had a close relation to the parental genotype Pare Bau in cluster 1, while three other rice lines (F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018) were notably similar to the parental genotypes Pare Lallodo, Pare Kombong, and Pare Ambo in cluster 3. These findings attained support from the genotype's-genotype's performance at the test site in Sesean District, located at 1,400 masl, with an average daily temperature of 14 °C–16 °C. Limbongan *et al*. (2023) also reported that several local Toraja rice accessions can adapt to altitudes above 1,000 meters with average temperatures below 20 °C, classifying them in the cold temperature stress resistance category. Among the lines showing the best productivity, based on results from molecular analyses, were F4UKIT102R-2-100, F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018 (Table 5). These lines demonstrated a high percentage of filled grains

Table 5. Percentage of filled grains per panicle and potential yield (t/ha) of F4 lines planted in the highlands of Sesean Village (1,600 masl).

Code	Genotypes	Key Agronomic Trait	
		Percentage of Grainy Grains in Each Panicle	Potential Yield (tons per hectare)
1	F4UKIT101-2-124	69.3	4.36
2	F4UKIT101-2-218	61.0	3.91
3	F4UKIT102-2-010	93.5	6.73
4	F4UKIT102-2-056	89.4	6.13
5	F4UKIT102-2-024	97.1	7.16
6	F4UKIT103-2-100	54.9	4.47
7	F4UKIT103-2-019	91.0	6.10
8	F4UKIT104-2-127	91.8	6.13
9	F4UKIT104-2-194	94.2	6.46
10	F4UKIT104-2-089	90.00	6.05
11	F4UKIT105-2-042	84.5	5.00
12	F4UKIT105-2-039	83.5	5.51
13	F4UKIT102R-2-100	98.7	7.74
14	F4UKIT102R-2-078	96.7	7.35
15	F4UKIT102R-2-018	94.8	7.60
A	Pare Bau	98.8	6.42
B	Inpari-4	48.5	3.44
C	Pare Lallodo	98.8	5.51
D	Pare Kombong	97.5	5.77
E	Pare Lea	84.7	4.47
F	Pare Ambo	96.5	5.43
	LSD Test (0.05)	4.2	0.59
	CV (%)	11.4	7.48

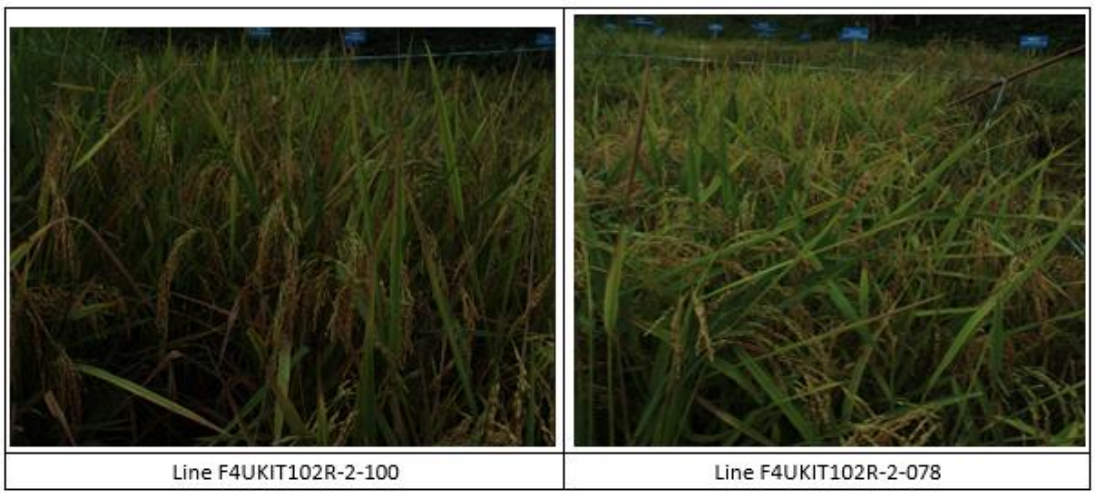

Figure 4. Agronomic performance of low temperature-tolerant rice lines.

and productivity, as well as exhibited an excellent phenotypic appearance even under cold stress conditions (Figure 4). Consistent with the previous study (F3), these four lines canachieve a higher production potential per hectare than the other lines and Inpari-4, as their flower germination ability remained normal in cold conditions.

Rice genotypes with close genetic distances came from similar genetic backgrounds, and the recombinations through crossing proved ineffective due to their strong genetic similarity. In contrast, a greater genetic distance between the rice genotypes leads to a higher heterotic effect when crossed. Streck *et al*. (2020) also emphasized the importance of selecting hybrid parents with large genetic distances for hybrid variety formation. Similarly, Toriyama (2021) and Wang *et al*. (2024) reported crossing parents with significant genetic distance were notably more likely to produce offspring with high diversity, offering the potential for superior traits through heterosis. In this context, the lines' crossing with their parental genotypes through backcrossing can enhance the genetic quality of the resulting offspring. As the selection process advances, occasions can exist when the phenotypes generated by breeding lines do not align with the breeder's objectives. In these instances, backcrossing with the parent plant becomes essential for enhancing genetic diversity. This technique is crucial for breeders aiming to identify lines resilient to environmental stress and exhibit increased productivity. Rohaeni and Permadi (2012) recommended lines with a genetic distance coefficient greater than 0.7 were recognizably most suitable for crossing with their parental genotypes.

CONCLUSIONS

Based on the study results, it was concluded that the SSR markers used are effective in detecting genetic diversity among different genotypes of several lines and local rice parental varieties under cold stress. The markers revealed allele sizes ranging from 150 to 375 bp, with a polymorphism level exceeding 0.50. The most effective markers for visualizing DNA banding patterns are RM5806 and CT220. Among the F4 rice lines (*O. sativa* L.), lines F4UKIT102R-2-100, F4UKIT102-2-024, F4UKIT102R-2-078, and F4UKIT102R-2-018 are the most tolerant to low temperatures. These lines have close linkages to local highland rice parental genotypes and revealed the best agronomic performance.

ACKNOWLEDGMENTS

The authors would like to thank the Center for Standard Testing of Biotechnology Instruments and Agricultural Genetic Resources, Republic of Indonesia, for assistance with the molecular analysis.

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