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Molecular diversity of rice germplasms from Thailand characterized by different pericarp colors using Inter Simple Sequence Repeat (ISSR) markers

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Abstract. *Saengprajak J, Phetsom J, Sangdee A, Atichart P, Theerakulpisut P. 2024. Molecular diversity of rice germplasms from Thailand characterized by different pericarp colors using Inter Simple Sequence Repeat (ISSR) markers. Biodiversitas 25: 2839-2848.* Studying genetic diversity in rice using molecular markers is necessary for varietal identification, better understanding of relationships and evolution, and conserving genetic resources. This study aimed to evaluate the genetic diversity of rice germplasms from Thailand characterized by different pericarp colors using ISSR markers. Thirty-two white and colored rice genotypes were assessed using 16 selected ISSR markers, which generated 179 alleles ranging from 210 to 1,374 bp, averaging 11.19 alleles per locus. The polymorphism percentage ranged from 40% to 100%, averaging 80%. The genetic similarity coefficient ranged from 0.458 to 0.855, while PIC values varied between 0.15 and 0.45, averaging 0.31. ISSR markers show a high Resolving Power (RP) of 12.15 and a Marker Index (MI) of 3.20, indicating their significant discriminatory power and effectiveness in detecting genetic diversity. The dendrogram grouped genotypes into five clusters: Cluster I included 5 red pericarp genotypes, Cluster II had 6 purple-black pericarp genotypes, Cluster III had 2 mixed white and purple-black pericarp genotypes, Cluster IV included 18 white pericarp genotypes, and Cluster V comprised 1 white pericarp genotype. PCA results corresponded to seed coat traits, forming eight grouping patterns similar to dendrogram clusters. These findings show that ISSR markers effectively assess genetic diversity and relationships in rice germplasms, providing valuable information for managing germplasm resources, developing variety-specific markers, and supporting rice breeding programs.

Keywords: Colored rice, DNA fingerprinting, genetic relationships, ISSR markers, *Oryza sativa* L.

INTRODUCTION

Rice (*Oryza sativa* L.) is widely grown worldwide and is a staple food for almost half of humans (Fukagawa and Ziska 2019). Rice accounts for over 21% of worldwide human calorific needs and constitutes 76% of Southeast Asia's calorie intake. Asia is the world's greatest rice producer, which constitutes 90.6% of global production, while within Southeast Asia, Thailand's production of 18.6 million Metric tons (Mt) is behind that of Vietnam, with the Philippines following closely behind (Mohidem et al. 2022). Rice cultivation has led to several different genotypes and phenotypes. Current technology advances and the need to improve the quality of life have led to a growing demand for food products that offer high nutritional value and appealing appearance.

Colored rice varieties are prevalent, with their unique color and aroma making them increasingly sought after. The color of rice, determined by flavonoid types and concentrations in the pericarp, impacts its antioxidant levels; red, purple, and black rice varieties offer more health benefits than white rice due to their higher antioxidant content (Sangma and Parameshwari 2023). These pigmented rice varieties contain beneficial compounds, including anthocyanins, flavonoids, and phenolic acids in their outer layers, particularly the bran and germ, which help protect cells from oxidative damage, support heart health, and offer potential effects against cancer and inflammation (Bhat et al. 2020).

Many rice breeding institutions focus on developing premium-quality rice varieties using artificial hybridization methods rather than transgenic approaches, crossing a diverse range of varieties for creation (Ashraf et al. 2024). Existing rice varieties exhibit significant genetic similarity due to shared genetic resources, leading to limited diversity and frequent seed disputes over genotype usage and breeder property protection (Park et al. 2019). To overcome such challenges, molecular markers are utilized to establish a database for each rice accession, enabling genetic data compilation for identified varieties. The International Union for the Protection of New Varieties of Plants (UPOV) provides guidelines for developing databases of varieties using molecular markers (Dutfield 2011). Assessing rice genetic diversity involves phenotype identification, biochemical analysis, and DNA diversity evaluation. However, methods for evaluating diversity through phenotypic and biochemical characteristics may not always be reliable due to environmental influences, labor intensity, and associated costs (Park et al. 2019).

Assessing genetic diversity in rice through DNA analysis is a widely utilized approach due to its repeatability, stability, and reliability. Various DNA-based molecular marker techniques are employed, including Restriction Fragment Length Polymorphism (RFLP) using

restriction enzymes, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), and Single Nucleotide Polymorphism (SNP) detection via Polymerase Chain Reaction (PCR) (Amiteye 2021). Despite the reproducibility issues and the dominance of markers in RAPD and AFLP techniques, ISSR remains effective by using a single 16-25 bp primer to target identical regions in microsatellites, focusing on regions with various repeating units such as tetra- or penta-nucleotides, trinucleotides, or dinucleotides (Xu et al. 2018). ISSR has been effectively used in various rice research studies, particularly for assessing genetic diversity and relationships (Al-Turki and Basahi 2015; Tanaporn et al. 2021; Al-Daej et al. 2023; Rini et al. 2023), as well as in species or variety identification (Haritha et al. 2016), phylogenetic analysis (Al-Turki and Basahi 2015), and hybridization analysis (Nivedha et al. 2024).

This study aimed to assess the genetic diversity and relationships within white-colored rice germplasm from Thailand and establish DNA profile databases utilizing ISSR markers. The results can serve as a foundation for implementing strategies to develop new variety-specific markers and assist in selecting parent plants for rice breeding programs.

MATERIALS AND METHODS

Plant materials

Thirty-two rice genotypes were obtained from farmer fields and various rice research institutes in Thailand, including 11 genotypes from farmers, 4 genotypes from Phitsanulok Rice Seed Center, and 17 genotypes from Pathum Thani Rice Research Center (Table 1; Figure 1). The color of the grain and pericarp (seed coat) are shown in Figure 2. Rice collection sites along with their respective GPS coordinates and altitude information are shown in Table 1. The seeds were sodden for 2 days and then placed in a culture tray filled with soil before germination and culture at the Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Fresh and young leaf samples were taken from seedlings that were 15 days old from five individual plants of each genotype and used for the extraction of DNA.

Procedures

Genomic DNA isolation

The molecular analysis was performed in the Laboratory of Molecular Genetics, Department of Biology, the Faculty of Science, Mahasarakham University, Thailand. Approximately 0.2 g of fresh young leaf samples for each cultivar was finely ground into a powder using liquid nitrogen. Genomic DNA was extracted from the homogenized mixture using PureDireX Genomic DNA Isolation Kit (Thermo Fisher Scientific Co., Ltd.) following the manufacturer's instructions meticulously.

Quantification of the extracted genomic DNA was performed by running the dissolved DNA in 1.0% agarose gel in 1X TBE (Tris-Borate-EDTA) buffer alongside uncut λ DNA of known concentration. The genomic DNA was diluted using nuclease-free H_2O (Invitrogen™, Thermo Fisher Scientific Co., Ltd.) to a concentration of 50 ng/µl to be prepared for ISSR analysis.

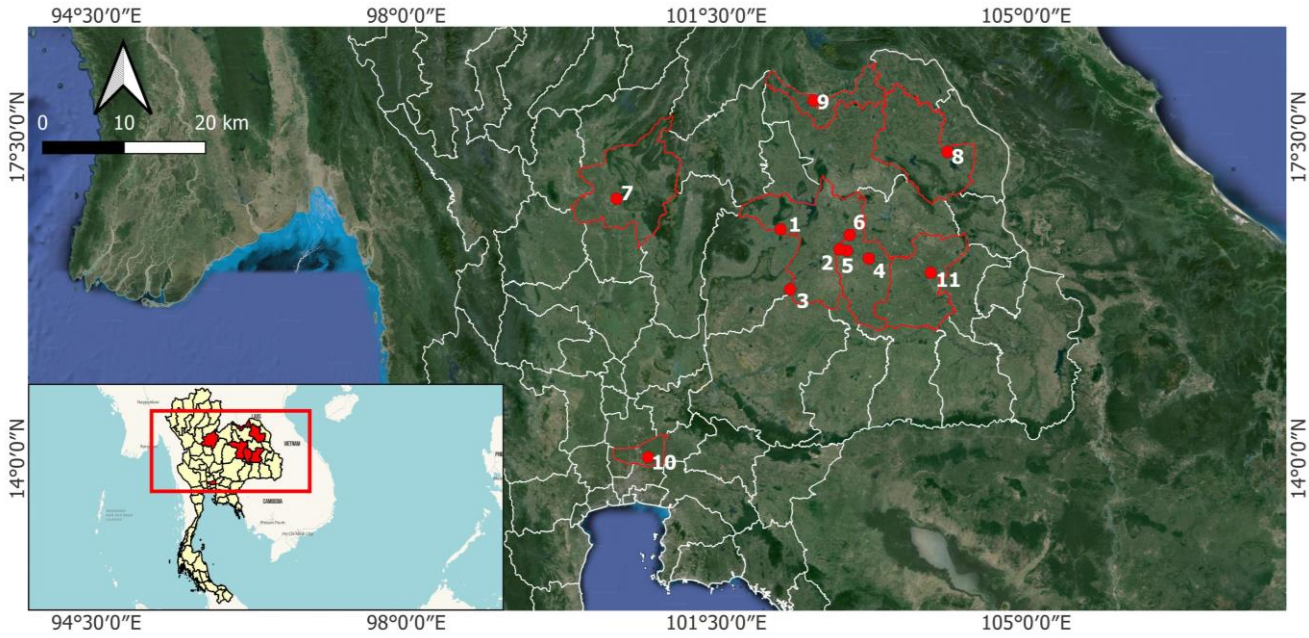

Figure 1. Sample collection sites used in the study. 1. R1, R2 and R4; 2. R3; 3. R5; 4. R6; 5. R7; 6. R8; 7. R10 and R12-14, 8. R17; 9. R18; 10. R9, R11, R15-16, R19-27 and R29-32, and 11. R28 genotype. ID codes refer to Table 1

Table 1. ID codes, names, and collection locations of the thirty-two rice genotypes used in this study

ID code	Name	Location	Coordi”Nates (Latitude-”N, Lo”Ngitude-E)	Altitude (m asl.)	Group*
R1	Thabthim Chumpae	Ban Non u-dom, Chumphae, Khon Kaen Province	16°30'18.612”N, 102°13'37.59”E	210	II
R2	Riceberry	Ban Non u-dom, Chumphae, Khon Kaen Province	16°30'18.612”N, 102°13'37.59”E	210	III
R3	Hom Nin	Ban Non Rasi, Khwao Rai, Kosum Phisai, Maha Sarakham Province	16°17'21.5”N, 102°53'33.0”E	156	IV
R4	Kam Sirithon	Ban Non u-dom, Chumphae, Khon Kaen Province	16°30'18.612”N, 102°13'37.59”E	210	III
R5	Kam Luempua	Tha Nang Naeo, Wang Noi Khon Kaen Province	15°51'11.376”N, 102°20'02.72”E	177	III
R6	Khokho 6	Ban Tha Song Khon, Mueang Maha Sarakham, Maha Sarakham Province	16°11'17.376”N, 103°13'26.616”E	153	I
R7	Nheuw Daeng	Ban Paeng, Kosum Phisai, Maha Sarakham Province	16°16'22.583”N, 102°58'35.83”E	156	II
R8	Mali 105	Ban Ku Thong, Chiang Yuen, Maha Sarakham Provinve	16°26'33.395”N, 103°00'29.19”E	165	I
R9	Daeng Noi	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R10	Khao Kam Nai	Phitsanulok Rice Seed Center, Wang Thong, Phitsanulok Province	16°50'11.9”N, 100°22'28.5”E	47	I
R11	Hom Chan	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R12	Malinil Surin	Phitsanulok Rice Seed Center, Wang Thong, Phitsanulok Province	16°50'11.9”N, 100°22'28.5”E	47	IV
R13	Khao Kam Nok	Phitsanulok Rice Seed Center, Wang Thong, Phitsanulok Province	16°50'11.9”N, 100°22'28.5”E	47	I
R14	Champalai	Phitsanulok Rice Seed Center, Wang Thong, Phitsanulok Province	16°50'11.9”N, 100°22'28.5”E	47	I
R15	Taban	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R16	Lon Krok	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R17	Somali	Ban Khok sa-at, Aumjarn, Kusuman, Sakon Nakhon Province	17°20'33.0”N, 104°06'42.2”E	176	I
R18	Dho Luang	Ban Kong Nang, Tha Bo, Nong Khai Province	17°53'51.6”N, 102°35'57.6”E	172	I
R19	Dho Dum	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	II
R20	Hom Phama	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R21	Mali Hom	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R22	Pueng Ngein	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R23	Luang Yai	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	II
R24	Kaew	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R25	Luang Thong	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	II
R26	Luang Boonma	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	II
R27	Nheuw Dhodaeng	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	II
R28	Khaw Nheuw Dum	Selaphum, Roi Et Province	16°02'00.8”N, 103°55'25.3”E	4	IV
R29	Sao Hai	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R30	Khaw Hom	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R31	Khaw Mali	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I
R32	Khaw Klang	Pathum Thani Rice Research Center, Rangsit, Thanyaburi, Pathum Thani Province	14°01'00.8”N, 100°43'46.5”E	4	I

Notes: * Group I: white-yellow rice samples; II: red pericarp rice samples; III: purple rice samples; and IV: purple-black pericarp rice samples

DNA amplification and electrophoresis

Sixteen ISSR primers from the University of British Columbia (UBC) and three PCP primers were selected from previous research based on their Polymorphism Information Content (PIC) values after primer screening (Table 2). Polymerase Chain Reaction (PCR) was performed in thermal cyclers (Biometra TAdvanced, Bio-Active Co., Ltd.). The PCR mixtures were prepared in a total volume of 20 µL, containing 2 µL of 50 ng/µL genomic DNA, 0.8 µL of 50 mM $MgCl_2$, 2.0 µL of 10X PCR buffer, 0.4 µL of 10 mM dNTPs, 0.05 µL of 100 µM each ISSR primer, and 1.0 U of *Taq* DNA Polymerase (nano*Taq* hot-start DNA polymerase, Bio-Helix Co., Ltd., Taiwan). The volume was adjusted to 20 µL by adding nuclease-free H_2O. The amplification program was 4 min preheating with initial denaturation at 94°C; followed by 40 cycles of 1 min at 94°C denaturation step, 1 min primer annealing at 51-55°C, and 2 min extension at 72°C; a final extension of 10 min at 72°C; then kept at 4°C until electrophoresis. The amplified DNA fragments were then fractionated by electrophoresis on 1.0% (w/v) agarose gel with 1X TBE buffer. Electrophoresis was carried out with a 100 V for 60 to 90 min. The size of each DNA band produced was estimated using a 100 bp DNA ladder RTU (Bio-Helix Co. Ltd., Taiwan). The gel electrophoresis results were visualized using Visafe Green Gel Stain (Vivantis®, Malaysia), and photographed under an ultraviolet transilluminator. All PCR assays were performed twice to validate the results.

Data analysis

The profiling pattern of the accessions developed by each ISSR primer was scored separately. The size of the amplified fragments was determined by comparing the migration distance of amplified fragments relative to the molecular weight of known size markers (100 bp DNA ladder) and calculated using PhotocaptMW software. Bands produced by each ISSR primer were counted for every rice genotype. Band sizes were tracked, and monomorphic and polymorphic bands were identified among all genotypes for each primer. A base pair position was scored as "1" for presence and "0" for absence of a band at that position.

Table 2. ISSR primers used in the study

Primer	Sequence (5' - 3')	Annealing temperature (°C)	Reference
PCP-1	GAC GAC GAC GAC GAC	55	Meena et al. (2021)
PCP-2	AGG AGG AGG AGG AGG AGG	55	Meena et al. (2021)
PCP-3	GTG CGT GCG TGC GTG C	55	Meena et al. (2021)
UBC 810	GAG AGA GAG AGA GAG AT	53	Al-Turki and Basahi (2015)
UBC 811	GAG AGA GAG AGA GAG AC	53	Kshirsagar et al. (2014)
UBC 813	CTC TCT CTC TCT CTC TT	53	Rini et al. (2023)
UBC 815	CTC TCT CTC TCT CTC TG	53	Rini et al. (2023)
UBC 817	CAC ACA CAC ACA CAC AA	52	Rini et al. (2023)
UBC 819	GTG TGT GTG TGT GTG TA	52	Rini et al. (2023)
UBC 820	GTG TGT GTG TGT GTG TC	54	Rini et al. (2023)
UBC 827	ACA CAC ACA CAC ACA CG	54	Kshirsagar et al. (2014)
UBC 834	AGA GAG AGA GAG AGA GT	52	Kshirsagar et al. (2014)
UBC 835	AGA GAG AGA GAG AGA GYC	52	Kshirsagar et al. (2014)
UBC 848	CAC ACA CAC ACA CAC ARG	52	Kshirsagar et al. (2014)
UBC 888	BDB CAC ACA CAC ACA CA	51	Kshirsagar et al. (2014)
UBC 890	CTC TCT CTC TCT CTV HV	51	Kshirsagar et al. (2014)

Notes: B, D, H, R, V, Y: IUB nucleotide code

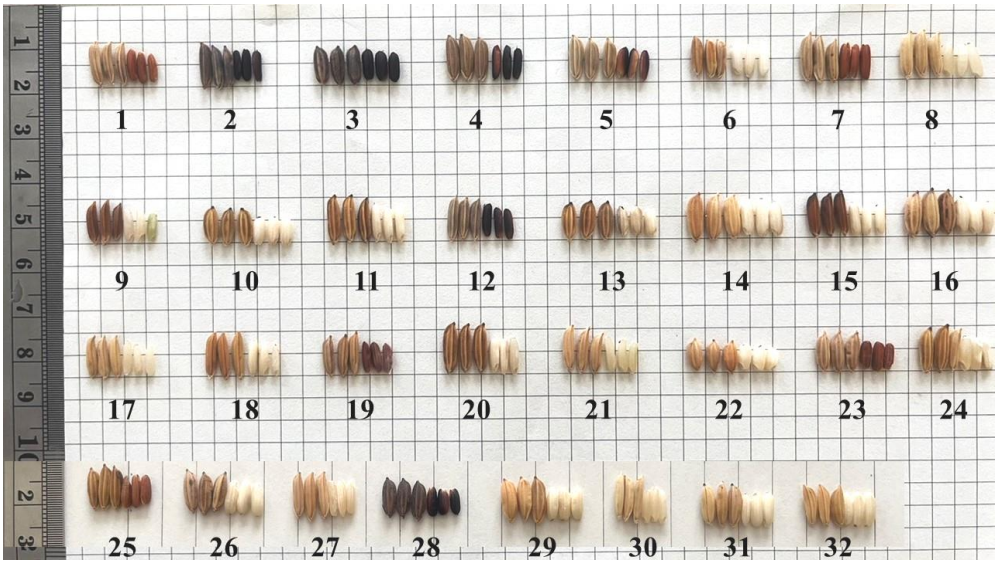

Figure 2. Grain color and pericarp color (seed coat color) of 32 rice genotypes were evaluated in this study. The sample number refers to Table 1

Among the parameters assessed was the Number of Total Bands (NTB), Number of Polymorphic Bands (NPB), Percentage of Polymorphism (PP), Number of Monomorphic Bands (NMB), Polymorphic Information Content (PIC), Resolving Power (RP), and Marker Index (MI). NTB was determined by counting the total number of clear DNA bands produced through PCR amplification. NPB was calculated by observing the different positions of DNA bands across all lanes, and PP was calculated as the ratio between NPB and NTB. The total number of amplified bands across all samples was used to determine NMB. The PIC value was estimated for each ISSR locus using the formula created by Roldan-Ruiz et al. (2000) as PIC=2fi(1-fi), where fi is the frequency of the marker bands which were present and 1-fi is the frequency of the absent marker bands. RP was estimated by the summation of each band informativeness, which was calculated using the formula RP=Σ Ib, where Ib is given by 1-(2×|0.5-p|), with p representing the proportion of total genotypes containing the band. MI was obtained using the formula MI=PIC×npi, where npi represents the number of polymorphic bands, as described by Powell et al. (1996).

A binary matrix was calculated based on the DNA banding data. This matrix was then used to construct a dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method through the SAHN (Sequential, Hierarchical, Agglomerative, and Nested Clustering) module of the NTSYSpc 2.1 software (Rohlf 2001). Additionally, the covalent structures of the 32 rice genotypes were determined using three-dimensional principal component analysis (3D-PCA) with the same software.

RESULTS AND DISCUSSION

Genetic polymorphisms

All isolated genomic DNAs showed sufficient quality to serve as PCR templates. The PCR amplification produced the appropriate profiling patterns for DNA samples of rice genotypes. The band patterns produced by ISSR primers PCP-2 and UBC 815 are depicted in Figures 3.A and 3.B. The 16 ISSR primers generated a total of 179 amplified fragments, ranging from 210 to 1,374 bp. Among these primers, the number of bands varied from 4 to 21, averaging 11.19 bands per primer. Primer UBC 834 produced the highest band count with 21 bands, while UBC 819 yielded the lowest with 4 bands. Notably, NPB presented 144 bands (80.45%), whereas NMB had 35 bands (19.55%), averaging 9.00 and 2.19 bands, respectively. The percentage of polymorphism (PP) varied from 40% (UBC 820) to 100% (PCP-1, UBC 819, and UBC 835), with an average polymorphism rate of 80% (Table 3).

Polymorphism information content

A high PIC value suggests that these primers are effective in distinguishing between different genotypes. The PVP 3 and UBC 810 primers generated the highest PIC value of 0.45 among the ISSR primers utilized, while the UBC 820 primer had the lowest value of 0.15. The average PIC value was moderate at 0.31. The average values for Resolving Power (RP) and Marker Index (MI) were 12.15 and 3.20, respectively (Table 3). The informative indices, RP and MI indicated the potential of markers to distinguish between diverse genetic resources (El-Esawi et al. 2022). The high significance of these results suggests that the primers were highly effective and resolved, which could be important for future investigations into rice species.

Table 3. Band variation, PIC, RP, MI values, and amplified product size for the 16 ISSR markers utilized in this study

Primer	NTB	NPB	NMB	PP	PIC	RP	MI	Product size (bp)
PCP-1	10	10	0	100.00.00	00.34	0,4270833	03.39	250-1255
PCP-2	9	8	1	88.89	00.30	0,3395833	02.38	240-1220
PCP-3	17	16	1	94.12.00	00.45	16.56	07.13	245-1342
UBC 810	18	16	2	88.89	00.45	18.13	07.13	217-1374
UBC 811	10	9	1	90.00.00	00.32	10.25	0,14375	275-657
UBC 813	14	11	3	78.57.00	00.36	16.13	0,1909722	365-1267
UBC 815	6	5	1	83.33.00	00.23	07.13	01.15	338-789
UBC 817	8	4	4	50.00.00	00.20	10.06	0,0569444	267-1355
UBC 819	4	4	0	100.00.00	00.20	0,16875	0,0569444	468-1280
UBC 820	5	2	3	40.00.00	00.15	07.38	00.31	320-976
UBC 827	9	5	4	55.56.00	00.23	12.50	01.15	268-1190
UBC 834	21	15	6	71.43.00	00.43	26.00.00	06.45	320-890
UBC 835	5	5	0	100.00.00	00.23	0,21875	01.15	380-1090
UBC 848	15	14	1	93.33.00	00.41	14.25	0,2631944	244-1310
UBC 888	12	10	2	83.33.00	00.34	11.44	03.39	210-1280
UBC 890	16	10	6	62.50.00	00.34	0,80625	03.39	211-987
Total	179	144	35			194.44.00		
Mean	11.19	09.00	02.19	80.00.00	00.31	12.15	03.20	

Notes: NTB: Number of Total Bands; NPB: Number of Polymorphic Bands; PP: Percentage of Polymorphism; NMB: Number of Monomorphic Bands; PIC: Polymorphic Information Content; RP: Resolving Power; MI: Marker Index

Genetic similarity and clustering

Genetic similarity coefficients showed variations among rice genotypes from 0.458 to 0.855 (Table 4). The highest value was 0.855 between two genotypes pair including; Riceberry (R2) and Hom nin (R3), and Mali hom (R21) and Pueng ngein (R22), with the lowest value being 0.458 between Kam luempua (R5) and Khokho 6 (R6). A dendrogram generated by UPGMA was constructed of 32 white and colored rice into five clusters with a similarity coefficient of 0.68 (Figure 4). Cluster I comprised 5 genotypes: Thubthim chumphae (R1), Nheuw daeng (R7), Luang boonma (R26), Nheuw dhodaeng (R27), and Luang thong (R25). Cluster II included 6 genotypes: Riceberry (R2), Hom nin (R3), Kam Sirithon (R4), Malinil Surin (R12), Dho dum (R19), and Khaw nheuw dum (R28). Cluster III contained 2 genotypes: Kam luempua (R5) and Luang yai (R23). Cluster IV consisted of 18 genotypes: Khokho 6 (R6), Champalai (R14), Taban (R15), Lon krok (R16), Khaw mali (R31), Khaw klang (R32), Khaw kam nok (R13), Somali (R17), Dho luang (R18), Kaew (R24), Mali 105 (R8), Daeng noi (R9), Khaw kam nai (R10), Hom phama (R20), Mali hom (R21), Pueng ngein (R22), Hom chan (R11), and Khaw hom (R30). Cluster V comprised only 1 genotype, Sao hai (R29).

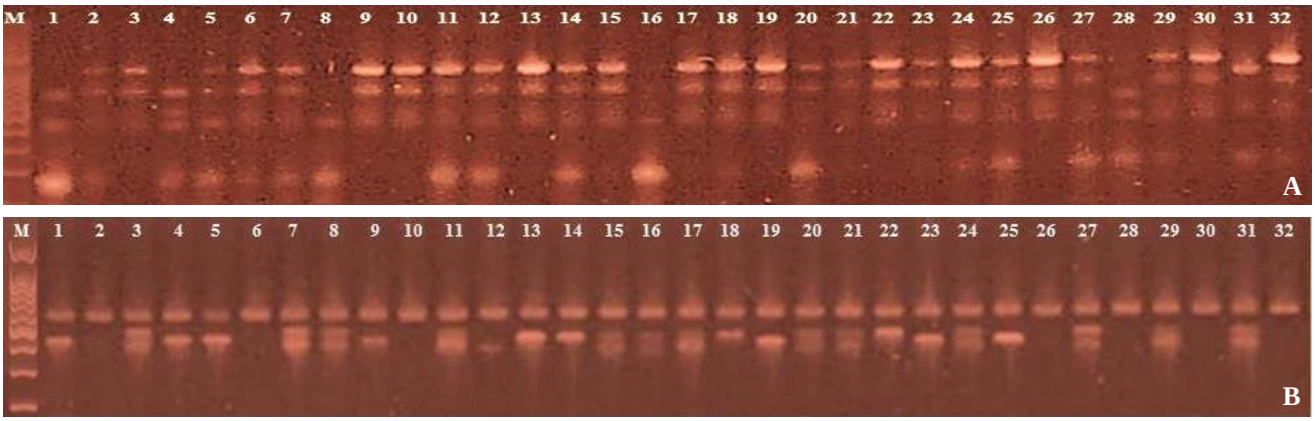

Figure 3. Results of DNA amplification with ISSR primers: A. PCP 2 and; B. UBC 815. Lanes: M: 100 bp DNA ladder; R1: Thabthim chumpae; R2: Riceberry; R3: Hom nin; R4: Kam Sirithon; R5: Kam luempua; R6: Khokho 6; R7: Nheuw daeng; R8: Mali 105; R9: Daeng noi; R10: Khao kam nai; R11: Hom chan; R12: Malinil Surin; R13: Khao kam nok; R14: Champalai; R15: Taban; R16: Lon krok; R17: Somali; R18: Dho leang; R19: Dho dum; R20: Hom phama; R21: Mali hom; R22: Pueng ngein; R23: Luang yai; R24: Kaew; R25: Luang thong; R26: Luang boonma; R27: Nheuw dhodaeng; R28: Khaw nheuw dum; R29: Sao hai; R30: Khaw hom; R31: Khaw mali; and R32: Khaw klang

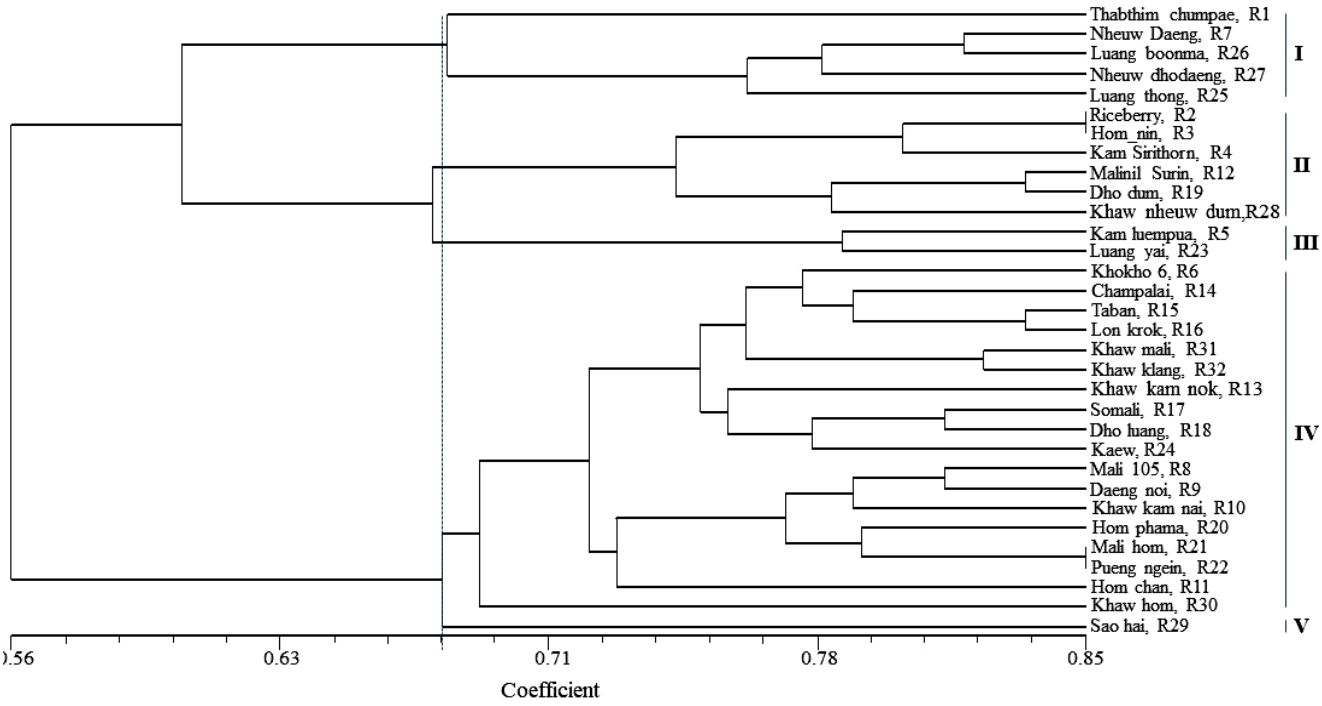

Figure 4. A dendrogram among 32 rice genotypes using 16 ISSR markers constructed by UPGMA cluster analysis

Table 4. Genetic similarity among 32 rice genotypes based on the 16 ISSR markers

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18	R19	R20	R21	R22	R23	R24	R25	R26	R27	R28	R29	R30	R31	R32
R1	1.000																															
R2	0.615	1.000																														
R3	0.626	**0.855**	1.000																													
R4	0.553	0.799	0.810	1.000																												
R5	0.620	0.603	0.637	0.693	1.000																											
R6	0.603	0.620	0.609	0.598	**0.458**	1.000																										
R7	0.670	0.553	0.564	0.609	0.682	0.564	1.000																									
R8	0.570	0.575	0.587	0.503	0.570	0.687	0.542	1.000																								
R9	0.587	0.536	0.525	0.520	0.587	0.715	0.547	0.816	1.000																							
R10	0.525	0.553	0.575	0.564	0.592	0.732	0.564	0.799	0.782	1.000																						
R11	0.575	0.615	0.592	0.665	0.609	0.704	0.615	0.715	0.709	0.737	1.000																					
R12	0.503	0.743	0.799	0.799	0.726	0.564	0.642	0.553	0.525	0.564	0.637	1.000																				
R13	0.631	0.615	0.637	0.620	0.542	0.726	0.547	0.749	0.765	0.704	0.709	0.570	1.000																			
R14	0.620	0.637	0.648	0.603	0.542	0.771	0.514	0.726	0.788	0.771	0.687	0.547	0.765	1.000																		
R15	0.587	0.626	0.637	0.603	0.486	0.760	0.559	0.693	0.732	0.704	0.631	0.525	0.732	0.788	1.000																	
R16	0.592	0.587	0.587	0.575	0.469	0.799	0.553	0.754	0.782	0.743	0.659	0.520	0.737	0.793	0.838	1.000																
R17	0.609	0.570	0.581	0.570	0.475	0.749	0.570	0.749	0.754	0.704	0.687	0.525	0.754	0.732	0.743	0.804	1.000															
R18	0.615	0.631	0.587	0.536	0.514	0.743	0.531	0.732	0.737	0.676	0.670	0.531	0.760	0.715	0.715	0.754	0.816	1.000														
R19	0.542	0.737	0.760	0.726	0.687	0.570	0.603	0.559	0.497	0.547	0.575	0.838	0.531	0.508	0.553	0.514	0.486	0.525	1.000													
R20	0.547	0.553	0.564	0.581	0.559	0.721	0.598	0.799	0.782	0.777	0.749	0.587	0.749	0.715	0.693	0.743	0.726	0.732	0.615	1.000												
R21	0.581	0.553	0.575	0.559	0.581	0.698	0.553	0.788	0.749	0.754	0.726	0.598	0.715	0.726	0.715	0.721	0.749	0.732	0.592	0.821	1.000											
R22	0.547	0.564	0.587	0.570	0.570	0.676	0.587	0.777	0.782	0.743	0.715	0.587	0.693	0.726	0.726	0.743	0.715	0.709	0.559	0.765	**0.855**	1.000										
R23	0.531	0.603	0.637	0.654	0.788	0.492	0.704	0.581	0.508	0.570	0.587	0.715	0.508	0.486	0.497	0.469	0.464	0.514	0.721	0.503	0.559	0.592	1.000									
R24	0.620	0.659	0.693	0.615	0.508	0.749	0.525	0.737	0.743	0.715	0.665	0.559	0.754	0.777	0.765	0.771	0.777	0.782	0.564	0.749	0.704	0.693	0.497	1.000								
R25	0.676	0.581	0.603	0.570	0.631	0.570	0.749	0.615	0.598	0.648	0.631	0.592	0.553	0.564	0.587	0.547	0.587	0.547	0.575	0.547	0.570	0.570	0.676	0.575	1.000							
R26	0.682	0.531	0.542	0.564	0.670	0.542	0.821	0.542	0.570	0.587	0.581	0.575	0.514	0.581	0.570	0.553	0.547	0.508	0.581	0.564	0.553	0.575	0.670	0.492	0.771	1.000						
R27	0.687	0.570	0.592	0.603	0.709	0.514	0.760	0.547	0.553	0.592	0.542	0.615	0.497	0.531	0.587	0.559	0.542	0.492	0.642	0.570	0.570	0.581	0.687	0.475	0.765	0.804	1.000					
R28	0.542	0.704	0.693	0.715	0.687	0.525	0.648	0.514	0.508	0.525	0.575	0.782	0.531	0.486	0.520	0.480	0.464	0.514	0.788	0.547	0.536	0.570	0.732	0.464	0.587	0.637	0.687	1.000				
R29	0.603	0.587	0.620	0.592	0.536	0.709	0.575	0.642	0.704	0.631	0.670	0.553	0.726	0.659	0.693	0.709	0.737	0.698	0.536	0.676	0.631	0.654	0.547	0.670	0.570	0.553	0.559	0.547	1.000			
R30	0.620	0.581	0.570	0.553	0.520	0.715	0.581	0.670	0.687	0.682	0.642	0.525	0.732	0.721	0.698	0.715	0.665	0.670	0.553	0.682	0.659	0.626	0.542	0.709	0.598	0.581	0.520	0.520	0.659	1.000		
R31	0.615	0.598	0.598	0.581	0.492	0.732	0.553	0.698	0.682	0.698	0.693	0.542	0.726	0.782	0.749	0.765	0.704	0.709	0.469	0.676	0.687	0.732	0.514	0.737	0.581	0.542	0.503	0.503	0.631	0.704	1.000	
R32	0.598	0.626	0.637	0.598	0.475	0.737	0.536	0.760	0.709	0.737	0.709	0.559	0.788	0.788	0.765	0.771	0.788	0.771	0.520	0.726	0.726	0.737	0.508	0.765	0.564	0.525	0.508	0.520	0.693	0.709	0.827	1.000

Principal component analysis (PCA)

PCA is essential in crop breeding for assessing genetic diversity and evaluating genetic divergence among genotypes. The cluster analysis in our results was verified by carefully analyzing the covariance displacement and structure provided by 3D-PCA (Figure 5), by grouping the 32 accessions into 8 groups. Group I included 5 genotypes: R1, R7, R25, R26, and R27, all characterized by red pericarp rice. Group II comprised 4 colored rice genotypes: R2, R3, R12, and R19, with one red seed coat, one purple, and two purple-black pericarps. Group III consisted of two genotypes: R5 and R23, both with red seed coats. Group V had one genotype with a purple seed coat (R4). Group VI contained a single genotype with a purple-black pericarp (R28). Group VII featured one white rice genotype (R11), while Group VIII also had a single white rice genotype (R16). Group IV included 17 rice genotypes: R6, R8-R10, R13-R15, R17-R18, R20-R22, R24, and R29-R32, all with white rice pericarps, showing a high membership level. The PCA results indicated a consistent clustering of seed coat traits corresponding to the grouping patterns of the accessions, except for the white rice genotype that was intermixed in group 2. PCA serves as a tool to classify genotypes of colored rice genetic resources by utilizing molecular morphological changes and plant morphological data.

Discussion

Evaluating genetic diversity is important for conserving and breeding rice germplasm to develop ideal and desired crop varieties for present and future needs. The sequences of ISSR primers were longer and bound to conserved areas between SSR regions, meaning that this method had superior characteristics such as simplicity and high reproducibility (Amiteye 2021). The ISSR method effectively distinguishes genotypes with high genetic similarity due to the high mutation rate commonly seen in ISSR loci (Verma et al. 2017). This marker has been previously reported in several studies. Al-Turki and Basahi (2015) assessed genetic diversity in Hassawi rice in Saudi Arabia using ISSR primers, enhancing rice germplasm authentication. Moonsap et al. (2019) studied Indochina rice varieties with ISSR markers, indicating high polymorphism and effective clustering. Rini et al. (2023) reported rice genotypes from Simeulue and Sumba Islands, Indonesia, indicating that ISSR markers showed greater resolving power and marker index compared to Sequence-Related Amplified Polymorphism (SRAP) markers. Additionally, Al-Daej et al. (2023) identified significant marker-trait associations in indica, japonica, and indica-japonica rice varieties. In maize, Amoon and Abdul-Hamed (2020) evaluated genetic diversity among five inbred lines and hybrids using ISSR markers, showing high polymorphism and genetic similarity. Furthermore, Iqbal et al. (2023) demonstrated the effectiveness of ISSR markers in assessing genetic variation in Pakistani Bt and non-Bt cotton varieties.

The high polymorphism rate of ISSR markers indicates their effectiveness in assessing genetic diversity in rice genotypes (Rini et al. 2023). Our results indicated an average polymorphism rate of 80% using ISSR markers, similar to findings by Al-Turki and Basahi (2015), who reported a 90.02% polymorphism rate in hassawi rice. Wan et al. (2008) showed a high polymorphism rate of 80.36% in wild rice, while Iqbal et al. (2023) reported a moderate polymorphism rate of 60% in cotton genotypes. Many studies, including those on wild rice *Oryza rufipogon* Griff. (Haritha et al. 2016), corn (Uslan and Jannah 2020), cotton (Ashraf et al. 2016), and soybean (Arslan et al. 2020) have demonstrated the effectiveness of the ISSR technique in analyzing genetic diversity and germplasm.

Genetic diversity and marker utility in the study are determined using PIC values, which assess power of marker based on allele numbers and frequencies, indicating the informativeness of each marker. The study showed PIC values ranging from 0.15 to 0.45, indicating that PCP 3 and UBC 810 were suitable for genetic diversity analysis. According to Botstein et al. (1980), a PIC value of ≥0.5 is classified as very high information, 0.5>PIC≥0.25 as medium information, and PIC<0.25 as providing little information (Tran et al. 2022). The PIC values of the ISSR markers utilized in this study were moderate but higher than those reported by Al-Daej et al. (2023); the average was 0.222, while Iqbal et al. (2023) reported a value of 0.25 for ISSR. To gain more genetic information, either increase the number of primers or consider alternative DNA markers such as SSR, SNP, InDel (Insertion Deletion), or SCoT (Start Codon Targeted) (Amiteye 2021). A higher PIC value indicates better discrimination among alleles, signifying increased genetic diversity at that locus. Our results also support RP and MI as informative indices, indicating significant discriminatory power and effectiveness in detecting genetic diversity. Similar to our findings, studies by Reddy et al. (2009) and Al-Daej et al. (2023) have demonstrated high RP and MI values, suggesting the reliability and utility of ISSR markers.

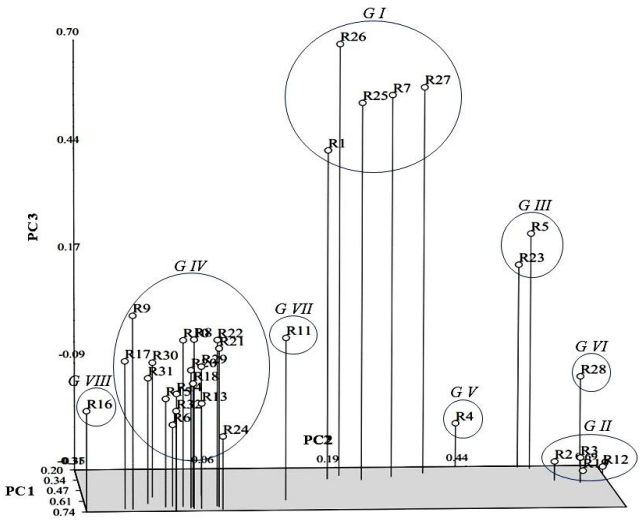

Figure 5. Three-dimensional PCA of the 32 rice genotypes based on 16 SSR markers

The results revealed significant genetic variation among the genotypes, as indicated by the diverse range of similarity coefficient values. Al-Turki and Basahi (2015) reported a wide range of similarity coefficients from 0.208 to 1.000, indicating high diversity among germplasm resources. In contrast, Shirmohammadli et al. (2018) showed a genetic similarity index varying from 0.208 to 0.661, indicating a lower variability range than this study. Additionally, Kumbhar et al. (2015) observed similarity coefficients ranging from 0.25 to 0.85, Moonsap et al. (2019) reported a genetic similarity range of 0.428 to 0.980, and Talamphai et al. (2021) showed coefficients ranging from 0.60 to 1.00. These values are similar to our results, which showed genetic similarity coefficients ranging from 0.458 to 0.855. This indicates a degree of genetic similarity among the accessions and suggests relatively similar levels of genetic diversity.

The genetic relationships among the 32 rice accessions revealed five clusters. Cluster I included genotypes with red pericarp rice grains, while Cluster II comprised 3 genotypes with purple-black seed coats. Cluster III contained 3 genotypes with a mix of white and purple-black pericarp rice. Cluster IV had the largest number of genotypes, with 17 white rice seed coats. Cluster V comprised one white rice pericarp. Most groups distinguished between colored and white rice, although some groups were intermixed. The clustering based on the seed coat colors corresponded closely with the ISSR profiles of the rice genetic resources. Most of them can distinguish between different groups of colored rice and white rice, except in some groups that are mixed. The five clusters represented based on the colors of the seed coat corresponded closely with the ISSR profiles of the rice genetic resources. Additionally, previous studies on rice conducted by Park et al. (2019), Sarif et al. (2020), and Al-Daej et al. (2023) have also grouped accessions based on similar morpho-agronomic characteristics associated with ISSR and SSR markers.

PCA is an analytical method that categorizes data based on individual elements and identifies optimal representations of variations within each dataset. In rice and other crop breeding programs, PCA is utilized to precisely assess genetic diversity and investigate genetic divergence among different genotypes. In the PCA method, the axis with the highest variance is chosen as the first main component, and the axis with the second greatest variance is displayed as a diagram with the data shown as axes. This study revealed significant variability, aiding in identifying related rice genotypes and distinguishing between genotypes during classification. The PCA results demonstrated similar tendencies to the dendrogram, with comparable overall and group compositions. Moreover, various studies employed PCA for rice genotype classification. Park et al. (2019) compared PCA with the dendrogram, resulting in similar tendencies in general and group compositions. Al-Daej et al. (2023) observed distinct genotype groupings based on their origin varieties.

In conclusion, this study revealed significant genetic variability among 32 white and colored rice genotypes using 16 ISSR markers. The results demonstrated a high polymorphism rate, with PIC values confirming the markers' efficacy in assessing genetic diversity. The effectiveness of the markers was further validated by informative indices such as RP and MI. UPGMA clustering analysis grouped the genotypes into five clusters corresponding to pericarp color traits, providing valuable insights into the genetic relationships among the rice genotypes. Additionally, PCA analysis aided in genotype classification, establishing its utility in genetic diversity assessment. These findings enhance our understanding of genetic diversity and relationships within white and colored rice germplasm using ISSR markers. The results could potentially lead to the development of new genotype-specific markers for rice and assist in the selection of parent plants for rice breeding programs.

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