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GENETIC DIVERSITY OF PIGMENTED RICE (*ORYZA SATIVA* L.) BASED ON DNA BARCODING AND ISSR ANALYSIS IN EAST AND NORTH KALIMANTAN, INDONESIA

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SUMMARY

Pigmented rice (*Oryza sativa* L.) genotypes are popular for their nutritional and functional benefits; however, exploring their genetic diversity has not received attention in Indonesia. The following study aimed to assess the genetic variation in 15 pigmented rice accessions from East and North Kalimantan using chloroplast DNA barcoding (*matK* and *rbcL*) and nuclear inter-simple sequence repeat (ISSR) markers. DNA barcoding revealed no nucleotide polymorphisms, suggesting a common ancestry for rice accessions. The chloroplast genes appeared unsuitable for distinguishing closely related cultivars with the same genetic backgrounds. In contrast, ISSR analysis disclosed substantial nuclear genomic diversity. Eleven primers generated polymorphic profiles that grouped the rice accessions into four distinct clusters based on the 70% genetic similarity threshold. Group I included 10 cultivars from overlapping agroecological regions, while Groups II–IV contained genetically distinct accessions, including a divergent red rice cultivar from Setulang, Indonesia. The results underscore the effectiveness of ISSR markers in detecting intraspecific variation and highlight the genetic richness of pigmented rice. The study highlighted the significant genetic diversity in these pigmented rice cultivars, offering insights for conservation strategies and the development of superior cultivars through breeding programs.

Keywords: Pigmented rice (*O. sativa* L.), genetic variation, DNA barcoding, genomic analysis, ISSR

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Key findings: Chloroplast DNA barcoding using *matK* and *rbcL* genes revealed no detectable genetic variation among the pigmented rice (*O. sativa* L.) accessions. However, ISSR markers detected substantial genomic polymorphism, and highlighting their potential for further improvement through breeding programs.

INTRODUCTION

Pigmented and traditional rice (*Oryza sativa* L.) germplasm are vital plant genetic resources, characterized by their red, purple, and black pericarp. They have gained considerable attention from consumers due to their nutritional benefits and potential health-promoting properties (Das *et al*., 2025). By comparing with non-pigmented rice, the pigmented cultivars contain significantly higher levels of antioxidants, phenolic compounds, anthocyanins, vitamins, and essential minerals (Yamuangmorn and Prom-u-Thai, 2021). Beyond their nutritional advantages, pigmented rice holds substantial cultural and economic importance, particularly in farming communities across Asia (Bhat *et al*., 2020).

In Indonesia, particularly in the regions of East and North Kalimantan, the indigenous communities are growing and using a rich diversity of pigmented rice cultivars over generations (Nurhasanah *et al*., 2018). The pigmented rice landraces have become deeply embedded in local diets, rituals, and agricultural traditions, and thus, represent a vital component of both cultural heritage and agrobiodiversity (Agnoletti and Santoro, 2022; Bai *et al*., 2024). Recent characterization of the genetic material has documented substantial phenotypic variation in local black/red rice genotypes and reaffirms their remarkable role in local foodways and agrobiodiversity (Hamidah *et al*., 2024).

Despite their significance, comprehensive studies on the genetic diversity and relationship among pigmented rice cultivars remain limited in Kalimantan, Indonesia. Global agricultural systems increasingly prefer a narrow range of high-yield commercial cultivars, and therefore, the genetic erosion of local landraces poses a serious threat to biodiversity and food system resilience (Khoury *et al*., 2022). Understanding the genetic basis of traditional pigmented rice is therefore vital for multiple reasons. Firstly, it supports the conservation of local germplasm, which is crucial for maintaining genetic diversity amid ongoing environmental challenges, such as climate change, emerging pests, and soil degradation (Salgotra and Chauhan, 2023). Secondly, genetic diversity provides the basis for successful crop improvement programs. Landraces often harbor alleles for stress tolerance, disease resistance, and nutritional enhancement, and such types of traits are increasingly valuable in modern rice breeding (Bohra *et al*., 2022). Lastly, investigating the genetic relationship among these rice cultivars can shed light on their evolutionary trajectories, domestication patterns, and ecological adaptations, offering insights that can considerably help in sustainable agriculture and resource management (Izawa, 2022).

DNA barcoding involves the use of specific gene regions, such as *matK* and *rbcL*, to accurately identify and classify the plant species at the molecular level. This technique provides a robust framework for assessing the genetic relationship, patterns of genetic similarity, and divergence among the closely related cultivars in field crops (Zhou *et al*., 2022). On the other hand, ISSR analysis is a molecular marker technique that amplifies random segments of the genome and offers insights into genetic variability (Amiteye, 2021). This method emerged to be particularly effective in distinguishing the Indonesian local rice genotypes that may exhibit subtle differences (Rini *et al*., 2023).

The primary objectives of this research were to explore the genetic diversity in the pigmented rice cultivars and the genetic relationship among these genotypes and to assess their potential for further breeding and conservation. By integrating DNA barcoding and ISSR analysis, the promising study aimed to provide a comprehensive overview of the genetic landscape of pigmented rice in

Kalimantan, Indonesia. These findings will support understanding the genetic makeup of the pigmented rice genotypes and the development of strategies for their conservation and sustainable utilization as valuable plant genetic resources (Sitaresmi *et al*., 2023).

MATERIALS AND METHODS

Plant material

The selection of pigmented and distinct rice (*O. sativa* L.) landraces for this study totaled 15, representing the diverse genetic and agroecological heritage of East and North Kalimantan, Indonesia. These rice genotypes came from field surveys and direct sampling in March 2024 from the farming communities who cultivate as part of local subsistence and cultural practices. The details about these rice accessions, including their origins and specific grain colors, are available in Table 1. The rice genotypes used in this study are the same as those previously characterized phenotypically by Hamidah *et al*. (2024). In this study, the genotypes incurred further analysis using molecular markers to complement and enhance the understanding of their genetic variation by providing more comprehensive insights at the genomic level.

DNA extraction

Genomic DNA extraction proceeded from approximately one cm segments of young leaf tissue collected from 15- to 20-day-old rice seedlings using the cetyltrimethylammonium bromide (CTAB)-free protocol (Chen *et al*., 2006), with minor modifications to optimize yield and purity. Grinding leaf samples in liquid nitrogen used a chilled mortar and pestle to ensure complete cellular disruption before DNA extraction.

The concentration and purity of the extracted DNA entailed assessment using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) by measuring absorbance at 260 and 280 nm. The A260/A280 ratio, as used, evaluated the protein contamination, considering the samples with ratios between 1.8 and 2.0 as acceptable. DNA integrity gained further verification by electrophoresis on a 1.0% (w/v) agarose gel to confirm the presence of high-molecular-weight genomic DNA without degradation. All the DNA samples acquired dilution to a working concentration of 20 ng/µL using nuclease-free water before storage at –20 °C until further use through polymerase chain reaction (PCR) amplification for DNA barcoding and ISSR analysis.

DNA barcoding

DNA barcoding succeeded in using chloroplast gene regions *matK* and *rbcL* (Table 2). Performing PCR amplification continued in a 65 µL reaction containing 65 ng of genomic DNA, 32.5 µL of OnePCR™ master mix (GeneDireX), 10 µM primers, 1.3 µL each of forward and reverse primers, and nuclease-free water. Thermocycling conditions included initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (gene-specific temperature, 30 s), and extension (72 °C, 1 min), with a final extension at 72 °C for 5 min.

Amplicons resolved ensued on a 1.5% agarose gel stained with RedSafe™ in 0.5× TAE buffer at 70 V for 60 min, with visualization using a BluPAD system (Bio-Helix). Clear, specific bands underwent selection for Sanger sequencing (Apical Scientific, Malaysia). Sequencing chromatograms obtained processing utilizing SnapGene for trimming, contig assembly, and alignment to reference sequences.

ISSR analysis

Eleven ISSR primers (Table 3) served to evaluate genetic variation among 15 pigmented rice accessions. PCR performed in 10 µL reactions contained 10 ng genomic DNA, 5 µL OnePCR™ master mix (GeneDireX, Taiwan), 0.2 µL of 10 µM primer, and nuclease-free water. Amplification proceeded using a Little Genius Thermal Cycler (Bioer Technology, China) under standard cycling conditions, including an initial denaturation at

Table 1. Pigmented rice cultivars from East and North Kalimantan, Indonesia used for genetic diversity studies.

ID	Cultivars	Grain Color	Type	Village	Subdistrict
V1	Beras Hitam	Black	Low land	Laburan Baru	Paser Balengkong
V2	Beras Hitam	Black	Low land	L3 (Bangun Rejo)	Tenggarong Seberang
V3	Beras Hitam	Black	Up land	Mara I	Tanjung Palas Barat
V4	Beras Hitam	Black	Up land	Pa Payak	Krayan Barat
V5	Beras Hitam	Black	Up land	Lembudud	Krayan Barat
V6	Beras Hitam	Black	Up land	Buduk Kinangan	Krayan
V7	Beras Merah	Red	Up land	Santan Tengah	Marang Kayu
V8	Beras Merah	Red	Low land	L3 (Bangun Rejo)	Tenggarong Seberang
V9	Beras Merah (Big)	Red	Up land	Ombau Asa	Barong Tongkok
V10	Beras Merah (Small)	Red	Up land	Ombau Asa	Barong Tongkok
V11	Beras Merah	Red	Up land	Mara 1	Tanjung Palas Barat
V12	Beras Merah	Red	Up land	Setulang	Malinau Selatan Hilir
V13	Beras Merah	Red	Up land	Pa Payak	Krayan Barat
V14	Beras Merah	Red	Ladang	Lembudud	Krayan Barat
V15	Beras Merah	Red	Ladang	Buduk Kinangan	Krayan

Table 2. DNA barcoding primers used for genetic diversity studies of pigmented rice from East and North Kalimantan, Indonesia.

Primers	Primer sequences (5'-3')		Tm (°C)	Size (bp)*
matK-B	Forward	CGATCTATTCATTCAATATTTC	50	936
	Reverse	TCTAGCACACGAAAGTCGAAGT		
rbcL-A	Forward	ATGTCACCACAAACAGAGACTAAAGC	55	599
	Reverse	GTAAAATCAAGTCCACCRCG		

* Based on primary BLAST results (NCBI, https://www.ncbi.nlm.nih.gov/tools/primer-blast).

Table 3. ISSR primers used for genetic diversity studies of pigmented rice from East and North Kalimantan, Indonesia.

No.	Primer name	Primer sequences (5'-3')	Tm (°C)
1	UBC 807	(AG)8T	55
2	UBC 808	(AG)8C	52
3	UBC 811	(GA)8C	52
4	UBC 815	(CT)8G	52
5	UBC 823	(TC)8C	52
6	UBC 825	(AC)8T	49
7	UBC 862	(AGC)6	60
8	UBC 880	(GGAGA)3	55

94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 30 s), primer-specific annealing (30 s), extension (72 °C, 1 min), and a final extension at 72 °C for 5 min.

Amplicons' separation took place on 1.5% agarose gels stained with RedSafe™ in 0.5× TAE buffer at 70 V for 60 min, employing visualization using a BluPAD system (Bio-Helix). Clear and reproducible bands attained scores as present (1) or absent (0) before compilation into a binary matrix. Genetic similarity assessment engaged the Simple Matching coefficient in NTSYS-pc v2.02, with a UPGMA (unweighted pair group method with arithmetic mean) dendrogram constructed via the SAHN (sequential, agglomerative, hierarchical, non-overlapping) clustering.

The informativeness of each primer underwent evaluation using the polymorphic information content (PIC), effective multiplex

ratio (EMR), and marker index (MI). Higher PIC (>0.50) and MI values indicated greater primer efficiency in detecting polymorphism and distinguishing genetic relationships among the accessions (Sunaryo *et al*., 2020).

RESULTS AND DISCUSSION

Genetic diversity of chloroplast genome

The PCR amplification of the chloroplast gene regions *matK* and *rbcL* yielded high-quality results across 15 pigmented rice (*Oryza sativa* L.) accessions (Figure 1). Each primer set successfully amplified the single, intense, and specific bands without non-specific amplification. The observed amplicon sizes (≈ 850–900 bp for *matK*, ≈ 550–600 bp for *rbcL*) were consistent with the canonical size ranges reported for plant DNA barcodes in past studies (Letsiou *et al*., 2024), supporting these primers' suitability for downstream Sanger sequencing and comparative analysis (Al-Shuhaib and Hashim, 2023).

Sanger sequencing further validated the integrity and specificity of the PCR products. The electropherograms displayed well-resolved and evenly spaced peaks with minimal background, indicating the high base-calling accuracy and sequencing. The absence of overlap peaks confirmed the fidelity of the amplification and sequencing. The high-quality chromatograms confirmed the generated sequence data were reliable for downstream applications, such as multiple sequence alignment and molecular characterization (Letsiou *et al*., 2024).

Despite the high quality of sequencing data, alignment of the *matK* and *rbcL* sequences enunciated an absence of significant nucleotide variations across 15 rice accessions (Figures 2A and B). No single nucleotide polymorphisms (SNPs), insertions, or deletions were evident in either gene region, suggesting the higher degree of sequence conservation within the sampled rice accessions. These results aligned with evidence that plastid coding barcodes (*rbcL*, *matK*) often lack resolving power at the species level, especially in crops with narrow plastid diversity (Xiong *et al*., 2022). The high sequence conservation of *matK* and *rbcL* was apparent for these plastid coding regions that reflect their limited intraspecific variability in rice, where the standard plant barcodes mostly lack resolving power among the closely related taxa (Zhang *et al*., 2021).

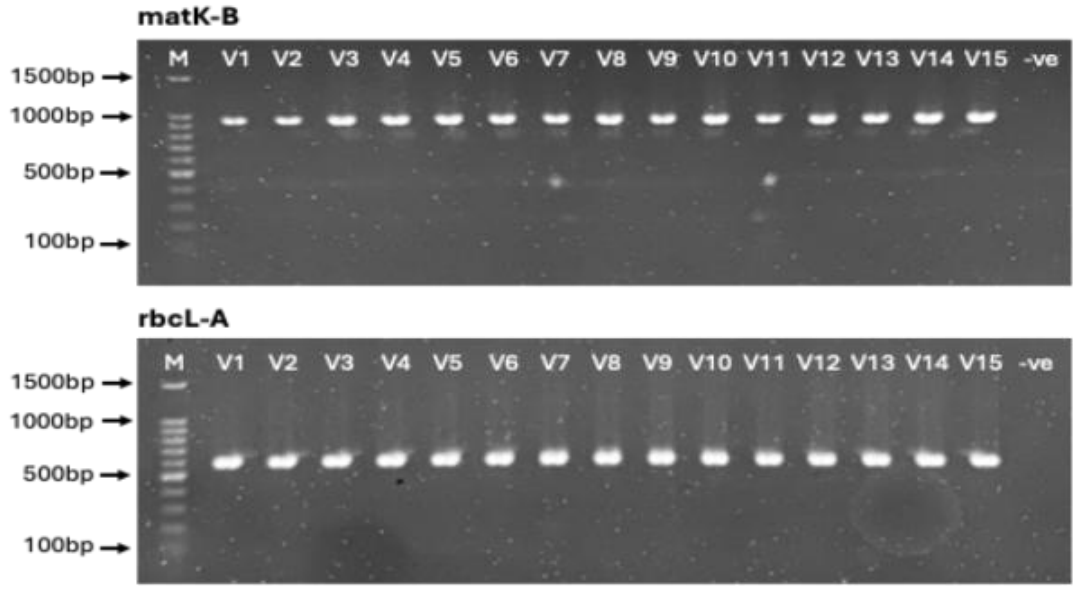

Figure 1. PCR results of 15 pigmented rice cultivars from East and North Kalimantan, Indonesia using *matK*-B and *rbcL*-A primers.

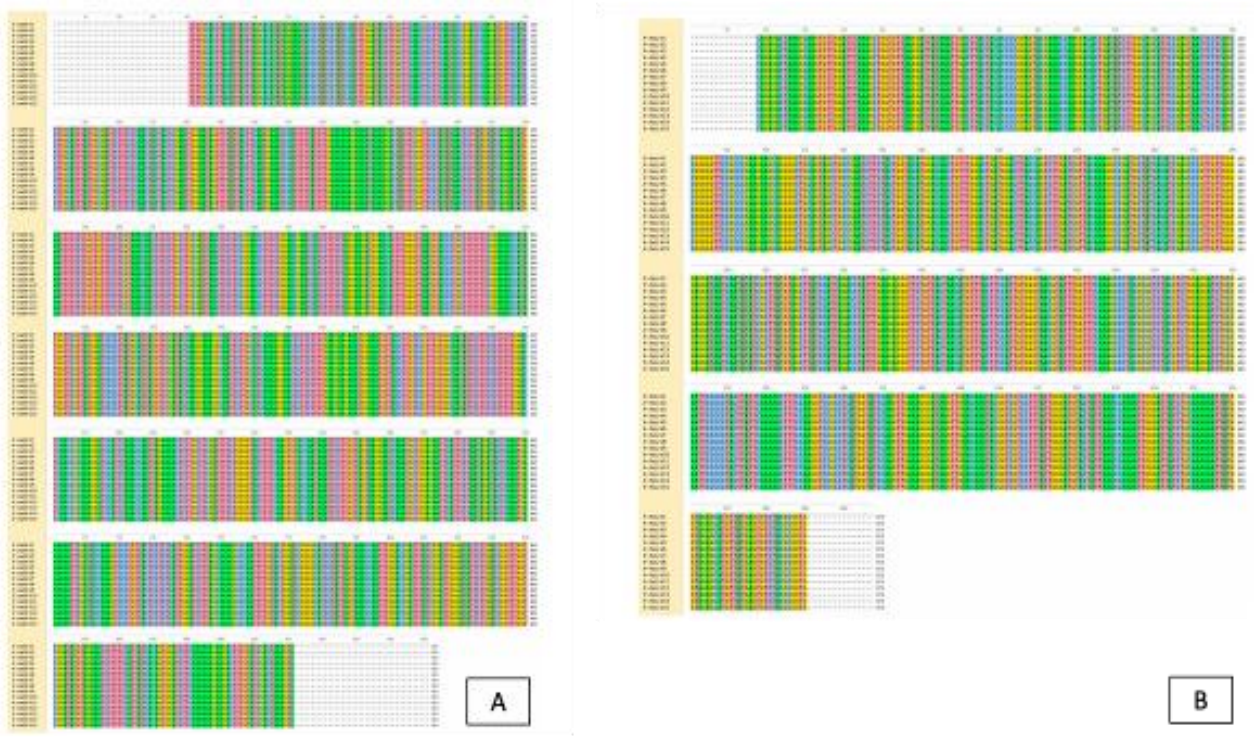

Figure 2. Alignment results of the (A) *matK* and (B) *rbcL* sequence segment for 15 pigmented rice cultivars from East and North Kalimantan, Indonesia. The blue, orange, green, and pink colors represent nucleotides Cytosine, Guanine, Adenine, and Timin, respectively.

The *matK* and *rbcL* genes with an absence of variations suggested these genes may not be sufficiently polymorphic to identify diversity among pigmented rice accessions. Plant chloroplast genomes evolve more slowly than nuclear genomes, and plastid coding sequences are typically under considerable selection, yielding limited variations within species (Zhou *et al*., 2022). In the chloroplast genome, the low level of variation is often insufficient to differentiate genotypes of the same species, such as local rice cultivars that tend to have scarce genetic diversity due to domestication and adaptation to the same environments. Rice-specific population work further revealed cultivated species *O. sativa* carries reduced plastome diversity relative to wild relatives due to domestication, founder effects, and maternal lineage sorting, which together depress the discriminatory signals of plastid barcodes among rice genotypes (Gao *et al*., 2019).

The absence of genetic variation suggested these pigmented rice accessions, despite their different endosperm colors, may have diverged from a common ancestor recently. Consequently, it may also refer to insufficient evolutionary time having passed for the development of significant genetic variations to accumulate in the chloroplast gene regions analyzed. Moreover, among these local rice accessions, the phenotypic divergence in endosperm pigmentation reached nuclear encoding. Hence, chloroplast markers need not track those visible color differences, and the plastid barcodes fail to differentiate the accessions of Southeast Asian weedy rice (Cui *et al*., 2016).

DNA barcoding using *matK* and *rbcL* genes emerged to be more effective at higher taxonomic levels, such as categorizing the different species. However, in this study, the results revealed these markers were not very effective for diversity analysis among closely

related rice genotypes. This study reinforces a well-established pattern: core plastid barcodes excel at interspecific diagnosis; however, they frequently underperform for cultivar, population, and level resolution in field crops, including rice (Zhou *et al*., 2022). Finer resolution, more informative markers, and wider genomic targets are vital in crop plants (Letsiou *et al*., 2024). Therefore, for enhancing the accuracy of diversity analysis, the ISSR markers could be more applicable. The ISSR markers technique can easily distinguish cultivars with significant genetic differences, making it more suitable for analyzing diversity among the local accessions with very similar genetic backgrounds.

Genetic diversity in rice nuclear genomes using ISSR

The ISSR profiling exhibited substantial nuclear genomic polymorphism across the 15 East and North Kalimantan pigmented rice accessions (Figure 3). The UPGMA dendrogram at the 70% similarity threshold resolved four well-supported clusters (Groups I–IV) (Figure 4), a pattern that highlights the high resolution within species commonly reported for ISSR in Asian rice landraces (Rini *et al*., 2023; Saengprajak *et al*., 2024). Group I was the largest, comprising 10 rice accessions (five black and five red) originating from the locations Laburan Baru, Bangun Rejo, Pa Payak, Lembudud, and Buduk Kinangan. It was consistent with ISSR's capacity to capture fine-scale variation among closely related rice cultivars (Rini *et al*., 2023; Saengprajak *et al*., 2024). The genetic proximity among these accessions within this group was evident. Particularly, the close similarity observed between the black rice cultivars from Pa Payak and Lembudud (accessions 4 and 5) and the red rice genotypes from Lembudud and Buduk Kinangan (genotypes 14 and 15) suggested a shared genetic origin, a dynamic frequently detected in rice metapopulations (Cui *et al*., 2021; Liu *et al.*, 2022).

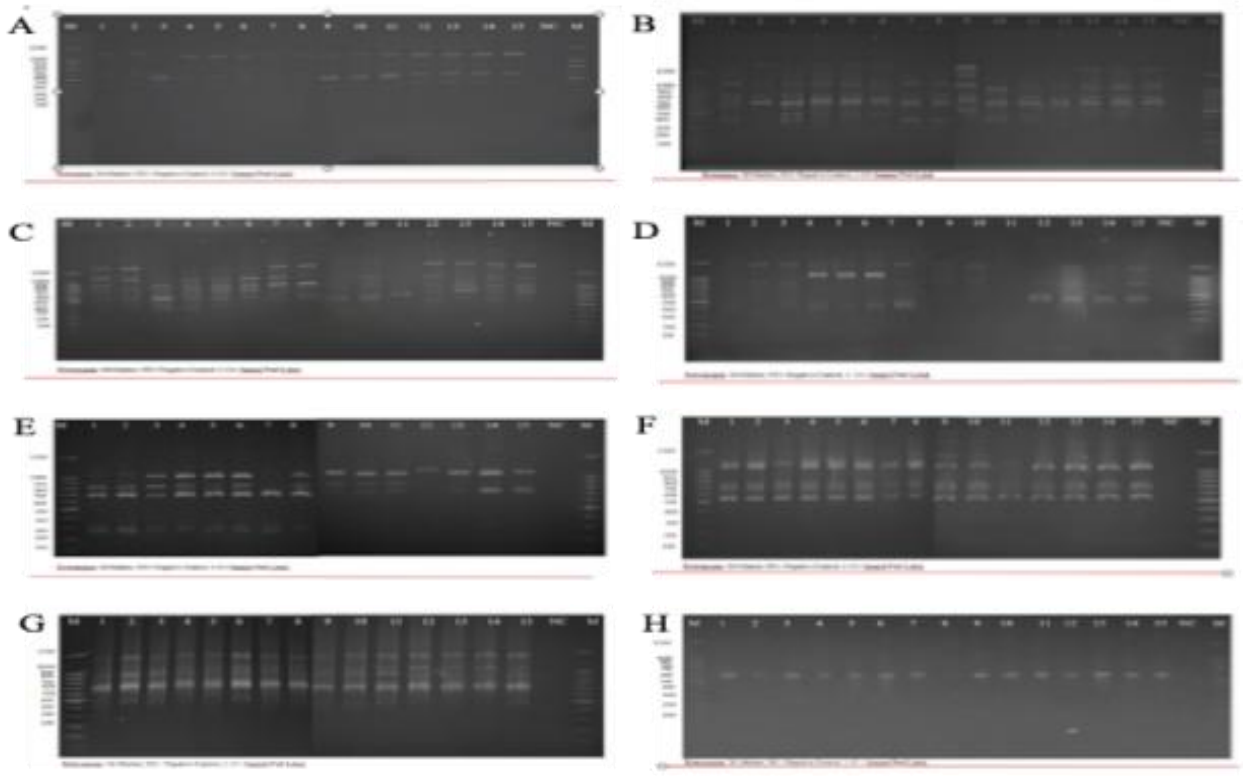

Figure 3. ISSR analysis of 15 pigmented rice cultivars from East and North Kalimantan, Indonesia.

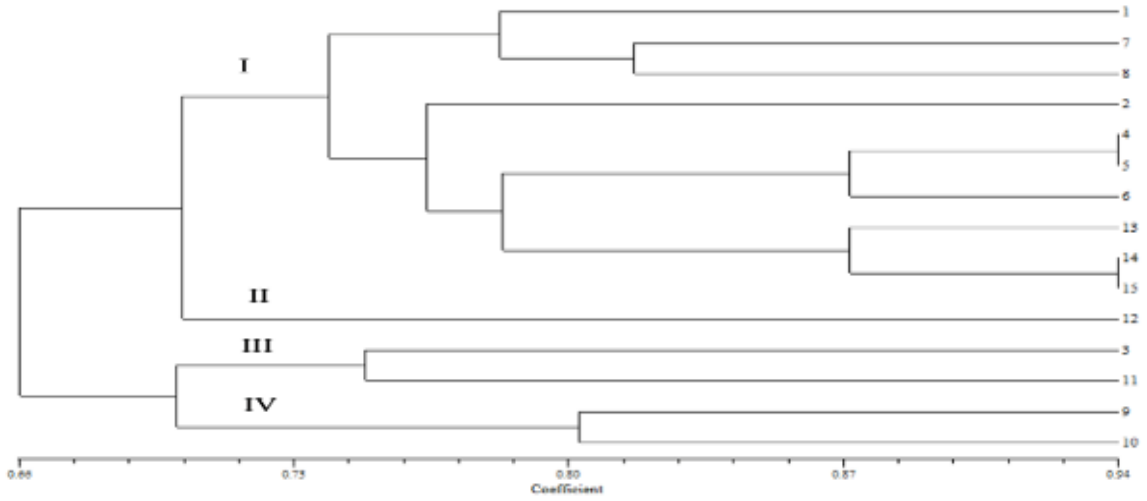

Figure 4. Dendrogram of 15 pigmented rice cultivars based on ISSR marker analysis from East and North Kalimantan, Indonesia.

In contrast, group II had a single red-pericarp rice accession representative from Setulang, forming a long branch separated from all other genotypes, a configuration consistent with geographic and cultural isolation reducing effective gene flow (Luong *et al*., 2021). Group III comprised two rice accessions from Mara Satu (West Tanjung Palas District), having one black (accession 3) and one red (accession 11) rice genotype. Group IV included two red rice cultivars from Ombao Asa (Barong Tongkok District), also differentiated by grain size: large red rice (accession 9) and small red rice (accession 10). They created coherent, locality-linked units, aligning with prior observations that farmer selection in distinct agroecologies can reinforce local genomic structure even under short spatial scales (Luong *et al*., 2021). These clusters collectively highlighted appreciable nuclear diversity within a small geographic region and underscore the value of pigmented rice landraces in Kalimantan, Indonesia, as pivotal and adaptive plant genetic resources (Lestari *et al*., 2022).

Notably, the pericarp pigmentation did not predict cluster membership: black and red rice accessions co-occurred in Groups I and III. It highlights multi-omics and population-genomic evidence that pigmented rice spans multiple varietal groups and that pigmentation loci explain only a small fraction of genome-wide structure (Sedeek *et al*., 2023). However, this decoupling was biologically plausible because the grain color has few key regulators managing it, whereas ISSR markers sample many anonymous loci across the nuclear genome in pigmented rice (Chen *et al*., 2023). Accordingly, color differences among the rice accessions need not imply broad genomic divergence, and conversely, accessions with similar colors may be genomically distinct (Yang *et al*., 2022).

From a breeding perspective, the pronounced separation of Group II and the locality-structured Groups III and IV identifies these rice accessions as strategic donors to broaden the genetic base of pigmented rice. Likewise, it helps to introgress the alleles linked to local adaptation (Cui *et al*., 2021). Group IV, with two Ombao Asa red lines that differed in grain size, provides a practical axis for managing grain-quality attributes and nutritional pigmentation. This outcome is consistent with past findings on marker-trait association linking pericarp-color loci to micronutrient and flavonoid profiles in rice genotypes (Chen *et al*., 2023). Coupled with emerging clinical evidence that pigmented rice can benefit selected cardio-metabolic endpoints, albeit with inter-trial heterogeneity, these lineages constitute compelling candidate

genotypes for nutraceutical-oriented improvement pipelines that balance quality, nutrition, and adaptation (Mendoza-Sarmiento *et al*., 2023).

The clustering patterns highlighted the rich genetic diversity among the local pigmented rice cultivars found in East and North Kalimantan, with the same findings also reported in previous studies on local rice germplasm (Saengprajak *et al*., 2024). Additionally, studies on local pigmented rice from Indonesia have revealed considerable genetic diversity, as demonstrated not only through morphological characterization (Hamidah *et al*., 2024; Husnah *et al*., 2024) but also through molecular analyses (Andarini *et al*., 2022; Husnah *et al*., 2025). The variations observed reflect differences in geographic origins as well as influences from farmer selection practices, environmental adaptation, and limited seed exchange across the different regions. The results emphasized their potential for conservation and breeding initiatives, particularly in developing cultivars adapted to specific agroecological conditions and further tailored for market preferences (Dempewolf *et al*., 2023). Furthermore, the genetic uniqueness observed in Groups II, III, and IV suggests the opportunities for targeted breeding programs to improve specific traits and broaden the genetic base of cultivated local rice germplasm.

Methodologically, in the presented study, the observed considerable resolution was consistent with past reports that ISSR loci deliver high percentages of polymorphic bands and robust marker indices in rice, often complementing other dominant systems within species diversity (Rini *et al*., 2023). However, the study recommends further exploration of the observed molecular basis of genetic diversity. Advances in genome sequencing and molecular marker technologies could facilitate the identification of specific genes associated with traits, such as stress tolerance, grain yield, and nutritional quality in pigmented rice (Sedeek *et al*., 2023). Such efforts would enhance the utility of these plant genetic resources in breeding programs and contribute to sustainable agricultural development.

CONCLUSIONS

Chloroplast DNA barcoding using *matK* and *rbcL* genes revealed no sequence variation among the 15 pigmented rice (*O. sativa* L.) accessions from Kalimantan, Indonesia, reflecting high plastid genome conservation. It also suggests a recent common ancestry among the accessions. In contrast, ISSR analysis uncovered substantial nuclear genomic diversity, clustering the accessions into four distinct groups and highlighting the effectiveness of ISSR markers for fine-scale genetic differentiation. The identified genetic structure did not align with pericarp color; however, it considerably corresponded to geographical origins and local adaptation, indicating the complexity of varietal diversity beyond visible traits. These findings highlighted the considerable genetic diversity in Indonesia's local pigmented rice germplasm, providing valuable insights for conservation strategies and supporting improved cultivar development through targeted breeding programs.

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