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Phylogenetic relationship of local rice from Central Java, Indonesia with Pokkali variety based on Single Nucleotide Polymorphism (SNP) markers

QORI NUR FAUZIAH, EDI PURWANTO♥, MUJI RAHAYU
Department of Agronomy, Faculty of Agriculture, Univeritas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia.
Tel: +62-271-646994, ♥email: edipurwanto@staff.uns.ac.id, qorinur00@student.uns.ac.id

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Abstract. *Fauziah QN, Purwanto E, Rahayu M. 2024. Phylogenetic relationship of local rice from Central Java, Indonesia with Pokkali variety based on Single Nucleotide Polymorphism (SNP) markers. Biodiversitas 25: 3965-3973.* Rice (*Oryza sativa* L.) is a major food crop in Asia particularly susceptible to the effects of climate change, with drought being a significant threat to productivity, potentially reducing yields by up to 50%. Indonesia is the largest producer of rice in Asia, with Central Java contributing up to 18% of the total, but the production of high-yielding varieties remains constrained. The objective of plant breeding is to ensure the sustainability of rice production by developing superior varieties. Therefore, this study aimed to develop local Central Java rice varieties tolerant to drought using the unique identification power of Single Nucleotide Polymorphism (SNP) markers. The potential impact of this research is the development of drought-tolerant rice varieties that could significantly improve rice production in Central Java. The experiment was conducted in Karanganyar and at the UGM campus, comprising several stages, including isolation, DNA amplification, and sequencing analysis. The results showed that based on sequencing analysis using BLAST, SNP was present in both the control and local rice varieties. The sequencing results were analysed for phylogenetic relationship using MEGA 11, showing thatPutih Mutiara (Klaten), Merah Sengreng (Boyolali), and Merah Wangi (Klaten) had the closest evolutionary relationship, and the most identical genetic structure to Pokkali varietycompared to the two controls (IR64 and Ciherang), and three local rice varieties namely Putih Mentikwangi (Karanganyar), Hitam Mutiara (Karanganyar), and Hitam Cempo (Boyolali).

Keywords: Central Java local rice, drought tolerance, OsDREB2A gene, rice breeding, Single Nucleotide Polymorphism

INTRODUCTION

Rice (*Oryza sativa* L.) is among the most widely cultivated cereal crops, particularly in Asia; it plays an important role as a staple food for approximately half of the global population. The growing human population requires a sustainable increase in rice productivity, which is highly dependent on climate patterns. Consequently, changes in climate patterns have the potential to threaten global food security. These factors drive the development of rice varieties that are compatible with climate variations and maintain yield while reducing the negative impacts of abiotic stresses (Saud et al. 2022; Hassan et al. 2023).

Drought represents a detrimental abiotic stress that reduces rice productivity by approximately 50% annually, with an estimated one-third of the global total agricultural land affected. It restricts the availability of water to plants, which in turn inhibits root growth and the uptake of nutrients. Consequently, plant growth and development are impaired due to unfavorable morphophysiological, biochemical, and molecular responses (Seleiman et al. 2021; Hrmova and Hussain 2021; Oguz et al. 2022; Hassan et al. 2023).

Indonesia is among the largest rice producers in Asia, with an annual production of 34.60 million tonnes. However, the country continues to experience several challenges, including those associated with climate change and other factors that impact rice productivity (Elvina et al. 2023). Central Java is one of the most developed provinces in rice commodity subsector. During the 2019-2021 period, Central Java accounted for up to 18% of the total national rice production (Badan Pusat Statistik Indonesia 2024). Conversely, the production of superior varieties continues to be constrained (Kamarudin et al. 2018; Auria et al. 2022).

The implementation of plant breeding initiatives can enhance the sustainability of rice production. The application of plant breeding techniques has led to the development of superior varieties that have contributed to a 56% increase in national productivity and played a significant role on a global scale (Purwanto et al. 2020). According to previous reports, superior varieties showed enhanced yield potential (Purwanto et al. 2020), the development of early-maturing varieties (Li et al. 2021), as well as increased resilience to biotic and abiotic stressors (Villalobos-López et al. 2022).

Pokkali variety (*O. sativa indica* cv Pokkali) is a superior rice variety, with high tolerance to drought conditions and has been the subject of numerous previous studies (Jadhao et al. 2014; Lathif et al. 2018; Herawati et al. 2021; Chrisnawati et al. 2022). Further studies show that drought tolerance in Pokkali variety is associated with the presence of the OsDREB2A gene. In the species *O. sativa*, many DREB genes, including OsDREB2A, have been showed to interact with the DRE element. Furthermore, in the model plant *Arabidopsis*, the DREB2A gene is induced in the presence of dehydration, high salinity, and heat stress.

Overexpression of DREB gene in *Arabidopsis* plants has been shown to result in increased drought tolerance (Cao et al. 2020).

Basic rice breeding can be initiated by identification through phylogenetic relationship analysis, which describes the evolutionary relationships between species based on similarities and differences in genetic traits. Phylogenetic trees consider changes in specific characters, such as nucleotides or amino acids, at each position in the genetic sequence (Zou et al. 2024). Therefore, to determine the phylogenetic relationship, molecular markers are needed that can identify nucleotide base sequences.

Single Nucleotide Polymorphism (SNP) is a game-changing molecular marker in rice research, especially when it comes to identifying changes in the sequence of a single nucleotide base in a population (Gunter 2024). These markers play an important role in identifying plant sensitivity to drought and can be found in normal individuals. SNP is often located in non-coding regions but affects protein changes (Degalez et al. 2021). Therefore, this study, which aims to develop local rice varieties in Central Java through SNP-based phylogenetic relationship analysis with Pokkali references, holds the promise of yielding local varieties that are genetically tolerant to drought, thereby revolutionizing rice breeding.

MATERIALS AND METHODS

Study area

The study was conducted from November 2023 to January 2024 at the Universitas Sebelas Maret (UNS) Agricultural Laboratory and the UGM Genetic Engineering Laboratory. The period of rice planting and sampling at the UNS Agricultural Laboratory, which occurred between November and December 2023, coincided with the rainy season. The average temperature was recorded at 32.67±0.68°C, with an average air humidity of 46.60% and light intensity of 14,407.67 lux. In January 2024, DNA extraction and amplification were conducted at the UGM Genetic Engineering Laboratory, also under rainy season weather conditions.

Procedures

Data collection

Rice seeds were sown and transplanted at 20 days after sowing (DAS), and data were collected from the flag leaf at 43 days after transplanting (DAT). The samples used were derived from two control and six local rice varieties, as detailed in Table 1.

DNA isolation

The DNA isolation techniques used in this study were modified and adapted to match the specifications outlined in the Genomic DNA Mini Kit (Plant) Protocol. Rice leaves aged 43 DAT from each variety were weighed, and a quantity of up to 100 mg was excised and macerated before being transferred to a microcentrifuge tube. Subsequently, 400 µL of GP1 buffer and 5 µL of RNase were added to each tube, which was then vortexed. The mixture was incubated at 60°C for 10 minutes, with the tubes gently inverted every 5 minutes. About 100 µL of GP2 buffer was added to the tube and vortexed once more. The filter column was positioned above the collection tube, and the mixture was transferred into the collection tube, followed by centrifugation at -40°C at 1,000× g for one minute. The pellet was discarded, and the supernatant in the collection tube was transferred into a microcentrifuge tube, followed by the addition of 1.50 volumes of GP3 buffer. Subsequently, the GD column was positioned above the collection tube, and 700 µL of the mixture was transferred into the GD column. This was followed by centrifugation at 16,000× g for 2 minutes, then the supernatant was discarded, and the GD column was returned to the collection tube. About 400 µL of W1 buffer was added to the GD column subjected to centrifugation at 16,000×g for 30 seconds, after which the supernatant was discarded. Furthermore, 600 µL of wash buffer was added to the GD column and subjected to another centrifugation at 16,000× g for 30 seconds. The supernatant was discarded, and the centrifugation was continued at 16,000× g for 5 minutes to ensure that the column was completely dry. The GD column was transferred into a new microcentrifuge tube, and 50 µL of the pre-incubated elution buffer was slowly added to the center of the matrix column. The mixture was left for 3-5 minutes to ensure complete absorption of the elution buffer by the matrix. Centrifugation was performed at 16,000× g for 30 seconds to collect the eluted DNA (Chrisnawati et al. 2022).

Table 1. A list of rice seeds collected is provided below

Sample	Species	Origin	Description
IR 64	*O. sativa*	Philippines	The control of paddy rice and drought-sensitive crops (Mackill and Khush 2018)
Ciherang	*O. sativa*	Indonesia	The control of upland rice (Hadjoeningtijas and Purnawanto 2013)
Hitam Cempo	*O. sativa*	Boyolali	Local rice
Hitam Mutiara	*O. sativa*	Karanganyar	Local rice
Merah Sengreng	*O. sativa*	Boyolali	Local rice
Merah Wangi	*O. sativa*	Klaten	Local rice
Putih Mutiara	*O. sativa*	Klaten	Local rice
Putih Mentikwangi Karanganyar	*O. sativa*	Karanganyar	Local rice

A quantitative and qualitative test of DNA isolation results

The results of the DNA isolation process were subjected to quantitative and qualitative analysis using a NanoDrop spectrophotometer. Measurements were taken at wavelengths of 260 nm and 280 nm. The initial step was the application of a 2 µL blank solution to the base of the spectrophotometer, after which the requisite measurements were taken to establish a calibration baseline. Subsequently, the top and bottom bases were cleaned with lint-free tissue to prevent contamination. The subsequent step was the application of 2 µL DNA sample solution to the lower base, after which spectral measurements were taken. The purity of the DNA was determined by comparing the absorbance values at wavelengths of 260 nm and 280 nm. A ratio of A260/A280 greater than 1.80 shows the presence of high-quality DNA (Aboul-Maaty and Oraby 2019).

DNA amplification

The amplification of DNA was conducted using primer pairs specific to the OsDREB2A gene (forward 5'-CCT CAT TGG GTC AGG AAG AA-3' and reverse 5'-GGA TCT CAG CCA CCC ACT TA-3') (Jadhao et al. 2014; Lathif et al. 2018; Herawati et al. 2021). Polymerase chain reaction (PCR) reactions were conducted using MyTaq DNA Polymerase in a total volume of 50 µL. The reaction mixture consisted of 25 µL of MyTaq, 1 µL of forward primer, 1 µL of reverse primer, 21 µL of nuclease-free water (NFW), and 1 µL of DNA template. The PCR mixture was placed in the PCR apparatus, and the reaction was conducted with a total reaction volume of 50 µL for 30 cycles. The stages of the PCR process include an initial denaturation at 95°C for one minute, denaturation at 95°C for 15 seconds, annealing at 59°C for 15 seconds, extension at 72°C for 10 seconds, and a final extension at 72°C for 7 minutes (KAPA BIOSYSTEMS 2020). The results of the PCR amplification were subsequently 0visualized using a 1.20% agarose gel comprising 0.36 grams of agarose in 30 milliliters of 1x TBE buffer with the addition of 5 µl of DNA and 1 µL of loading dye. The visualization was conducted under a UV transilluminator. PCR samples that had distinct bands were subsequently subjected to sequencing through the Sanger method using the Applied Biosystem 3500 Genetic Analyzer 2500 at LPPT UGM.

Data analysis

The DNA amplification results were processed to establish a DNA consensus using BioEdit, and BLAST confirmation was performed on the NCBI website to assess the level of similarity or identity with Pokkali variety (KU.15973.1). The most identical consensus with the minimum error was matched with the DNA sequence of the OsDREB2A gene in Pokkali variety using BioEdit. The results showed whether there were differences in nucleotide bases or SNP identified in specific varieties. Next, a phylogenetic tree analysis was conducted using the UPGMA Maximum Composite Likelihood method, a specific and widely used approach, with MEGA 11 software to determine the genetic distance of each variety to Pokkali variety. In the final stage, the genetic distance was determined using the pairwise distance method in MEGA 11.

RESULTS AND DISCUSSION

The amplification of the OsDREB2A gene

DNA was successfully isolated from leaf samples of IR64, Ciherang, Hitam Cempo, Hitam Mutiara, Merah Sengreng, Merah Wangi, Putih Mutiara, and Putih Mentikwangi Karanganyar rice varieties. The quality of the isolated DNA was evaluated by spectrophotometry, which included the estimation of DNA concentration. The amount of light absorbed by the sample at a specific wavelength was measured with an absorption peak for nucleic acids at 260 nm. An 260/A280 ratio of 1.80 for dsDNA shows good DNA purity, while a ratio below 1.70 implies protein contamination (Gupta 2019). The study showed that the DNA purity of all samples was favorable, with a ratio of approximately 1.80 to 1.95, indicating the DNA was free from protein contamination (Table 2). Purity in these varieties was found to be consistent, suggesting that the isolation method was effective in maintaining genomic stability.

The DNA concentration was evaluated following Matlock (2015), stating that a good DNA concentration should be above 20 ng/uL. However, according toKAPA BIOSYSTEMS (2020), a concentration between 10 to 100 ng/uL is required for PCR analysis. The smallest concentration that can be detected is between 0.01 to 2.00 ng/uL (Sophian et al. 2021). The results showed that the concentration of DNA produced met the standard, with varying values observed. These include IR64 (76.09 ng/uL), Ciherang (38.66 ng/uL), Hitam Cempo (47.60 ng/uL), Hitam Mutiara (43.88 ng/uL), Merah Sengreng (27.70 ng/uL), and Putih Mutiara (12.60 ng/uL). The lowest concentration produced was 8.10 ng/uL in Merah Wangi, but it was still well detectable. The results of DNA isolation with good purity and concentration were subjected to further genetic analysis such as sequencing, SNP genotyping, or phylogenetic studies.

Table 2. Purity and concentration of DNA isolated from local Central Java rice

Sample	Average DNA purity (A260/280)	Average DNA concentration (ng/uL)
IR64	1.89±0.08	76.09±38.02
Ciherang	1.90±0.08	38.66±46.32
Hitam Cempo	1.95±0.03	47.60±35.00
Hitam Mutiara	1.85±0.08	43.88±23.53
Merah Sengreng	1.90±0.05	27.70±14.35
Merah Wangi	1.84±0.03	8.10±4.27
Putih Mutiara	1.86±0.04	12.60±4.91
Putih Mentikwangi Karanganyar	1.93±0.07	9.18±2.90

Good DNA purity and concentration are essential for successful PCR amplification. The PCR process includes three main steps, namely denaturation of the dsDNA template at 92-95°C, primer annealing at 50-70°C, and extension of the dsDNA molecule at approximately 72°C (Gupta 2019). Primers used in this study were sequences from the OsDREB2A gene in Pokkali variety (Lathif et al. 2018). The results of DNA amplification were evaluated using agarose gel electrophoresis, which is relatively inexpensive, easy to use, and provides a more accurate estimate of the amount of PCR product than other methods. Agarose gel electrophoresis separates nucleic acids based on the length of DNA fragments, with separable sizes ranging from 100 bp to 25 kb. The gel concentration used determines the agarose pore size, the higher the agarose concentration, the smaller the pore size (Lee et al. 2012). In this study, 1.20% agarose gel was used to produce 100 bp DNA fragments in each sample (Figure 2). The dye used in electrophoresis allows visualization under ultraviolet light, and the brightness of the resulting band shows the concentration of DNA in the sample. The higher the concentration of DNA, the brighter the band produced (Wittmeier and Hummel 2022). This study produced individual bands of high and consistent brightness, showing high DNA concentration within each band (Figure 1).

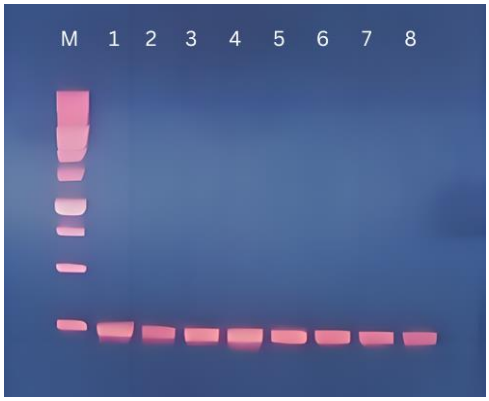

Figure 1. Visualization of PCR results from isolation of sample leaves showing the presence of the OsDREB2A Gene. M: Marker using 1 kb DNA ladder from SMOBIO; 1: IR64 (control sensitive variety); 2: Ciherang (upland rice control); 3: Hitam Cempo; 4: Hitam Mutiara; 5: Merah Sengreng; 6: Merah Wangi; 7: Putih Mutiara; 8: Putih Mentikwangi Karanganyar

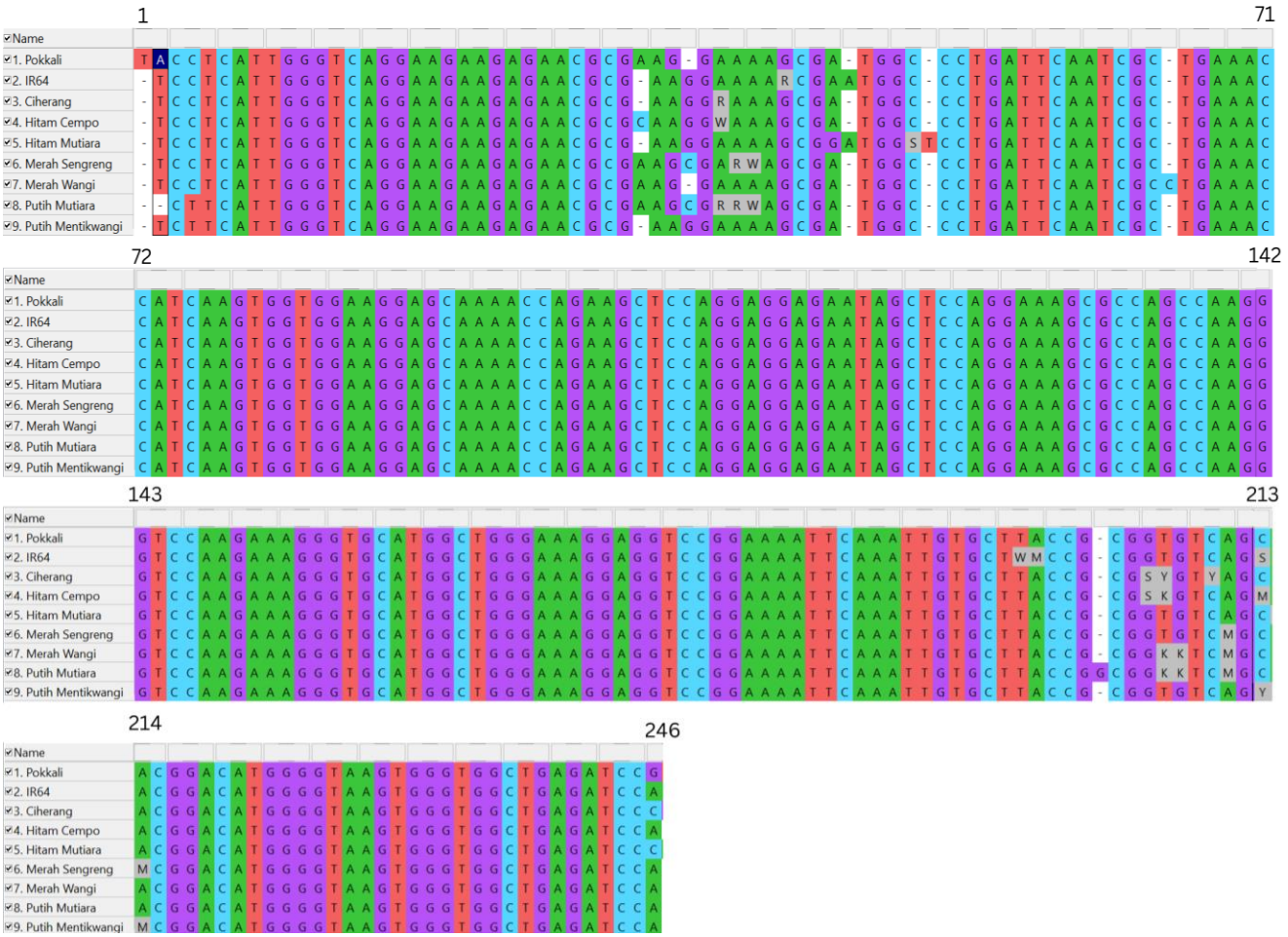

Figure 2. Multiple alignment analysis of OsDREB2A in each sample with control and Pokkali varieties

Sequence analysis

Samples identified as containing the OsDREB2A gene were then analyzed by sequencing. The sequences obtained were processed into consensus DNA and confirmed using BLAST (Basic Local Alignment Search Tool) to determine the degree of similarity between the nucleotide bases in the sample and *O. sativa* Indica Group (KU159743.1).The statistical calculation results obtained include e-value, alignment length, query coverage, and percent similarity. The e-value has a threshold for statistical significance ranging from 1×10^{-2} to 1×10^{-20}, where the lower the e-value, the higher the significance. Query coverage above 50-80% and percent similarity above 50% also produced statistical significance to the BLAST confirmation results (Nestor et al. 2023). This study showed that all samples had e-values between 1×10^{-114} to 8×10^{-116}, query coverage of 97-99%, and percent similarity of 96.68-98.74%. The e-values obtained suggest the confirmation results are quite significant, with query coverage that exceeds the threshold and a very high percent similarity, showing the presence of a gene shared with Pokkali or homologous sites (Table 3).

Following BLAST analysis, homologous sites in the sample sequences were further processed using multiple sequence alignment in MEGA11 by aligning the nucleotide base sequences of all samples and Pokkali variety to identify SNP. The results showed the presence of SNP in all the sample sequences tested, as shown in Figure 2. These SNP suggest the presence of genetic variation among the samples, although BLAST results showed high genetic similarity which is characterized by shared genes: Pokkali or the presence of homologous sites. The findings of this research are crucial for rice breeding and genetic research.

In all varieties except Putih Mutiara, the SNP detected a substitution mutation at base 2, and except Merah Wangi at base 35. The SNP also detected a substitution mutiation in Hitam Cempo at base 32, in Hitam Mutiara at base 44 and 50, in Merah Wangi at base 65, as well as in Putih Mutiara at base 203. The substitution mutation also occurred in IR64, Ciherang, Hitam Cempo, Hitam Mutiara, and Putih Mentikwangi at base 34, as well as in IR64 and Hitam Mutiara at base 46. The presence of gaps indicates deletions therefore, the SNP detected was a deletion mutation, which occurred the deletion of base A in Putih Mutiara at base 2, as well as in IR64, Ciherang, Hitam Mutiara, and Putih Mentikwangi at base 32. In all varieties, the deletion mutation also occurred at base 50 (except Hitam Mutiara), base 65 (except Merah Wangi), and base 203 (except Putih Mutiara). In Pokkali and Merah Wangi at base 35, as well as in Pokkali, Ciherang, Hitam Cempo, Merah Sengreng, Merah Wangi, Putih Mutiara, and Putih Mentikwangi at base 45, were also a deletion mutation.

Phylogenetic relationship

Homologous sites in matched sequences form the basis for the analysis of phylogenetic relationships through the construction of trees. In general, phylogenetic trees describe the evolutionary relationship between species, represented as strands of DNA containing the nucleotide bases A, T, C, and G (A: Adenine, T: Thymine, C: Cytosine, and G: Guanine). It uses a root role that describes the direction of relationship with the branching sequence. The earliest branch shows the Last Common Ancestor (LCA) with the earliest evolutionary divergence, while the last branch indicates the LCA with the most recent divergence. Branches that form LCAs are separated from the same clade (Edwards 2019; Munjal et al. 2019). This separation is based on the degree of genetic similarity and difference between the sequences. It also shows the possibility of significant functional or structural similarity between two sequences (Munjal et al. 2019). Therefore, the presence of the SNP in the sequence may be responsible for the formation of distinct clades based on evolutionary time.

The construction of phylogenetic trees in this study was based on a pairwise distance matrix that grouped related plant species sets. Clustering was performed based on pairwise distance values. Specifically, for sequences with high similarity , grouped into the same branch. Meanwhile, for sequences with high difference within a set of plants on the sam branch resulted in differences in branch lengths on the tree (Munjal et al. 2019). The phylogenetic tree was visualized using MEGA 11 software with the Kimura 2-parameter evolutionary model approach, maximum likelihood algorithm, and 1000 bootstrap replicates. Bootstrap values were categorized as strong (>85%), moderate (70-85%), low (50-69%), and very low (<50%) (Kress et al. 2002).

Table 3. BLAST confirmation result of OsDREB2A in *O. sativa* indica group cultivar Pokkali dehydration responsive element binding protein 2A (DREB2A), complete cds (KU159743.1)

Sample	Max score	Total score	Query cover	e-value	Per. ident	Acc. len	Accession
IR64	425	425	97%	1×10^{-114}	97.93%	849	KU159743.1
Ciherang	431	431	97%	2×10^{-116}	98.34%	849	KU159743.1
Hitam Cempo	425	425	99%	1×10^{-114}	97.93%	849	KU159743.1
Hitam Mutiara	427	427	97%	3×10^{-115}	98.35%	849	KU159743.1
Merah Sengreng	425	425	99%	1×10^{-114}	97.93%	849	KU159743.1
Merah Wangi	429	429	99%	8×10^{-116}	98.35%	849	KU159743.1
Putih Mutiara	411	411	98%	3×10^{-110}	96.68%	849	KU159743.1
Putih MentikWangi Karanganyar	431	431	98%	2×10^{-116}	98.74%	849	KU159743.1

The phylogenetic tree shown in Figure 3 shows the evolutionary relationship between local rice and control with Pokkali variety as a reference or called outgroup. Based on the results, the outgroup (Pokkali variety) shows the most distant evolutionary relationship with the ingroup, which includes local rice and control. However, based on the branching length that shows a closer evolutionary relationship with the outgroup, the phylogenetic tree divides into two clades. Clade 1 shows a closer evolutionary relationship with the outgroup (Pokkali variety), where the local rice comprises Putih Mutiara, Merah Sengreng, and Merah Wangi, supported bylow genetic data (bootstrap values of 53%). Meanwhile, clade 2 formed a branch that shows the most distant evolutionary relationship with the outgroup (Pokkali variety), where the control includes IR64 and Ciherang, and local rice comprises Putih Mentikwangi, Hitam Mutiara, and Hitam Cempo, supported by moderate genetic data (75% bootstrap values). These results suggest that although Hitam Mutiara has a greater branch length between IR64 and Hitam Cempo, it remains within the same branch group and shows a close relationship among the tree. This implies that based on phylogenetic tree, the most distant evolutionary relationship to Pokkali variety is IR64 and Hitam Cempo.

Genetic or pairwise distance is an important tool to ascertain the phylogenetic relationship between local rice and Pokkali varieties. A pairwise distance value of at least 0 indicates an identical genetic structure between two varieties. Based on Table 4, Pokkali variety with Merah Sengreng, Merah Wangi, and Putih Mutiara had a very low pairwise distance value (0.0084), showing that Pokkali and these three local rice had identical genetic structures. This result was consistent with phylogenetic tree results, where Merah Sengreng, Merah Wangi, and Putih Mutiara had the closest evolutionary relationship with Pokkali among the varieties studied. The control and the remaining local had higher pairwise distance values (0.0169-0.0295) than Pokkali showing that these varieties have lower genetic similarity to Pokkali. This may be attributed to the origin of the same population or similar genetic selection. The result was consistent with a phylogenetic tree where IR64, Ciherang, Putih Mentikwangi, Hitam Cempo, and Hitam Mutiara formed clade 2.

Discussion

Extensive efforts have been made in rice breeding to develop improved varieties that have a positive impact on yield while being able to cope with changing climatic factors (Wang et al. 2021). This study used local rice from Central Java to increase productivity and develop drought-tolerant varieties. The breeding method used was to analyze the phylogenetic relationship based on SNP between local Central Java rice and Pokkali variety containing the OsDREB2A gene (accession number KU159743.1). Local rice was sourced from three districts, namely Boyolali, Klaten, and Karanganyar. The three areas are the centers of rice production in Central Java, with the topography including lowlands, hills, and mountains, which support the availability of water sources for agriculture (Dhamira and Irham 2020; Auria et al. 2022).

Phylogenetic relationship analysis requires nucleotide base sequences with homologous sites that go through the steps of DNA extraction, amplification, sequencing, BLAST confirmation, and alignment. Efficient extraction leads to good DNA quantity and quality, pure and free from contaminants, as well as appropriate concentration. Good DNA extraction determines the success of amplification. The quality of DNA pure and free from protein or phenol contamination has a purity value of 1.80, and a slightly higher or lower value shows slight contamination or variation in the isolation process. The DNA quality in this study produced values of 1.84 to 1.95, showing adequate purity and freedom from contaminants (Table 2).

The DNA concentration variations, with the highest concentration found in IR64 at 76.09 ng/uL and the lowest in Merah Wangi at 8.10 ng/uL, are influenced by several key factors. The efficiency of the isolation method, the extraction kit used, and intrinsic tissue variations such as cell number and size, nuclear ratio during mitosis and interphase, and the amount of extra-nuclear DNA all contribute to these variations. Certain differences in the structure or biochemical composition of the tissues, such as polysaccharides, polyphenols, and other secondary metabolites, also play a role in differences in DNA concentration. The growth stage of the plant, particularly when leaves are taken for DNA isolation, also contributes to the variation in concentration. Young leaves tend to have higher DNA concentrations compared to older, coarser-grained roots or stems due to more intense cell division activity (Kefelegn et al. 2021). In this study, both young and old leaves were collected, which may explain the observed variation in DNA concentration. This highlights the importance of considering the stage of plant growth when conducting DNA isolation for accurate results.

Table 4. Pairwise distance matrix based on the number of substitutions per site in Pokkali sequences

	Pokkali	**IR64**	**Ciherang**	**Hitam Cempo**	**Hitam Mutiara**	**Merah Sengreng**	**Merah Wangi**	**Putih Mutiara**	**Putih Mentikwangi**
Pokkali									
IR64	0.0295								
Ciherang	0.0253	0.0127							
Hitam Cempo	0.0169	0.0000	0.0042						
Hitam Mutiara	0.0294	0.0083	0.0127	0.0085					
Merah Sengreng	0.0084	0.0086	0.0129	0.0128	0.0171				
Merah Wangi	0.0084	0.0043	0.0085	0.0085	0.0128	0.0000			
Putih Mutiara	0.0085	0.0131	0.0173	0.0172	0.0217	0.0043	0.0043		
Putih Mentikwangi	0.0170	0.0042	0.0085	0.0042	0.0127	0.0128	0.0085	0.0086	

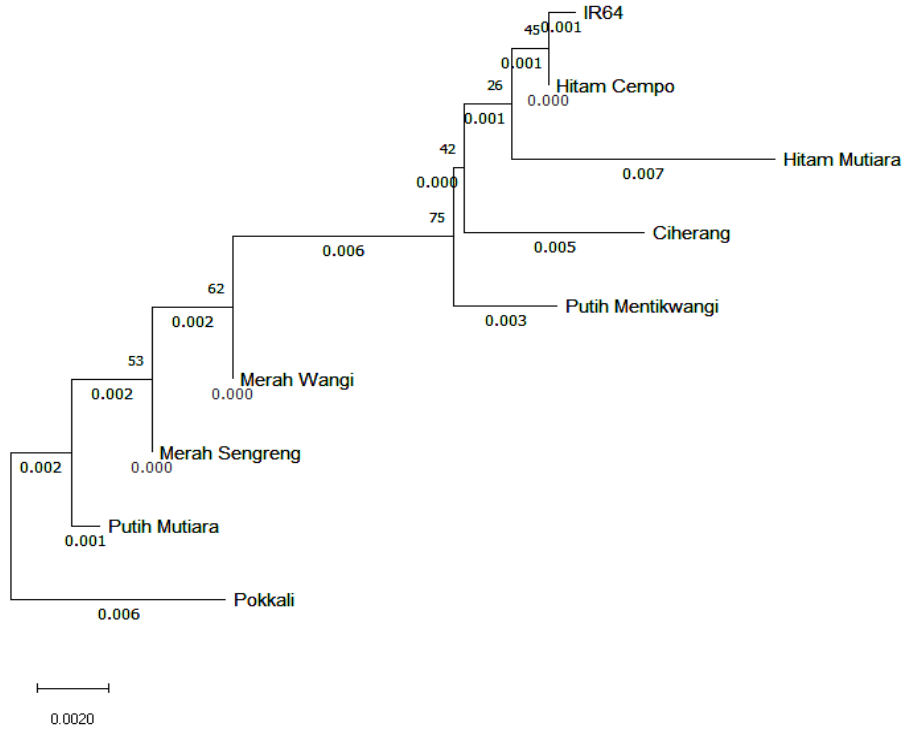

Figure 3. Phylogenetic tree by UPGMA and evolutionary distance calculated by maximum parsimony

DNA amplification was successfully carried out and resulted in an OsDREB2A gene size of 100 bp based on agarose gel electrophoresis analysis (Figure 1). Sequencing results using the Sanger method showed a size of 246 bp (Figure 2). This sequencing method is the latest technique with high convenience, reliability, and harmlessness (Eren et al. 2023). The genome length of OsDREB2A in Pokkali variety was 846 bp, and the successfully matched sample sequences represent 246 bp of the total genome. The number of sequences greatly affects the interpretation and significance of the identified SNP. The greater the number of sequences used, the more representative the SNP detected, providing a comprehensive understanding of the genetic variation in a population (Alqahtani and Almutairy 2023). The number of sequences covered in this study was greater compared to Chrisnawati et al. (2022) at 204 bp, showing that the SNP detected are representative enough to show the presence of genetic variation among the samples tested.

The phenomenon of drought is controlled by a multitude of genes with a diverse range of functions, which in turn leads to a variety of physiological and biochemical responses. The molecular mechanisms associated with drought stress are controlled by two principal factors namely signal transduction and functional factors. Signal transduction factors include protein kinases, transcription factors, and ABA receptors, while functional factors are implicated in metabolism, osmotic regulation, protein conversion, protein modification, and ROS transport. DREB is one of the transcription factors that show differential expression in response to drought. This transcription factor acts as a molecular switch that binds directly to OsDREB2A, encoding ERF/AP2 and binding to other transcription factors (Cao et al. 2020). This generally leads to the establishment of a pathway through ABA-independent signaling, enabling plants to sense and respond to drought. The signal produced then causes an excessive expression and accumulation of ROS and Ca^{2+} in plant tissues and cells. Additionally, osmolytes such as sugars, polyols, and amino acids, including proline, are produced. The accumulation of ROS leads to the production of enzymatic antioxidants, including peroxidase (POD) and superoxide dismutase (SOD), as well as non-enzymatic antioxidants, namely ascorbic acid, carotenoids, and phenolic compounds (Oguz et al. 2022). These compounds The phenomenon of drought is controlled by many genes with diverse functions, which in turn give rise to a variety of physiological and biochemical responses. The molecular mechanisms associated with drought stress are controlled by two main factors, namely signal transduction and functional factors. Signal transduction factors include protein kinases, transcription factors, and ABA receptors, while functional factors are involved in metabolism, osmotic regulation, protein conversion, protein modification, and ROS transport. DREB, a transcription factor, plays a pivotal role as a molecular switch that shows differential expression in response to drought. It binds directly to OsDREB2A, encoding ERF/AP2, and binds to other transcription factors (Cao et al. 2020). This generally leads to the establishment of pathways through ABA-independent signaling, which allows plants to sense and respond to drought. The resulting signal then causes excessive expression and accumulation of ROS and Ca^{2+} in plant tissues and cells. In addition, osmolytes such as sugars, polyols, and amino acids, including proline, are produced. The accumulation of ROS leads to the production of enzymatic antioxidants, including peroxidase (POD) and superoxide dismutase (SOD), as well as non-enzymatic

antioxidants, namely ascorbic acid, carotenoids and phenolic compounds (Oguz et al. 2022). This compound experienced a significant increase during drought stress in plants.

In this study, the results of multiple sequence alignment showed that the upland rice control variety (Ciherang) and the drought-sensitive control (IR64) had the OsDREB2A gene, but the presence of SNP was observed (Figure 2). This was consistent with the biochemical response of both varieties to drought, where the Ciherang had increased levels of malondialdehyde (MDA) and SOD compared to Situ Bagendit (drought-tolerant variety) (Refli et al. 2015). The results suggested that Ciherang had a less effective antioxidant defense capacity than Situ Bagendit, reflected in the high level of ROS-induced cell damage. Based on the results of multiple sequence alignment, local rice with genetic similarity to the control variety Ciherang was IR64 variety, Hitam Cempo, and Hitam Mutiara. It is hypothesized that both Ciherang, IR64, Hitam Cempo, and Hitam Mutiaramay be more susceptible to oxidative stress under drought stress conditions, affecting resistance to water-stressed environments (Salsinha et al. 2022).

As reported by Chrisnawati et al. (2022), IR64 showed the presence of SNPs, specifically a mutation from guanine (G) to adenine (A), which has been identified as a factor contributing to poor adaptation to water-deficient environments. Miftahudin et al. (2020) showed that IR64 had impeded primary root growth in comparison to Situbagendit. Additionally, it showed the most pronounced reduction in leaf relative water content and a significant elevation in MDA concentration. An increase in proline levels and a significant decrease in leaf chlorophyll content were also observed in IR64. In the context of this study, IR64 showed genetic similarities with several local rice varieties, including Hitam Cempo, and Hitam Mutiara. The Hitam Cempo mutant strain 51, as reported by Patmi et al. (2020), was observed to show a significant elevation in proline levels, impeded root growth, a reduction in the number of leaves, and a decline in plant biomass. These results suggest that this mutant is susceptible to drought conditions. However, the morphophysiological responses of other local rice varieties have not been extensively documented in the scientific literature.

Phylogenetic analysis grouped rice varieties into two main clades (Figure 3). Clade 1 consists of Pokkali, and the three local rice comprises Putih Mutiara, Merah Sengreng, and Merah Wangi, while Clade 2 comprises the two control Ciherang and IR64, and the three local rice includes Putih Mentikwangi, Hitam Mutiara, and Hitam Cempo. In general, clade 1 has a closer evolutionary relationship with Pokkali varieties, among others. This evolutionary relationship suggests that the three local rice comprises Putih Mutiara, Merah Sengreng, and Merah Wangi may have inherited some adaptation mechanisms from a common ancestor, which could be the basis for the development of drought-tolerant varieties using the OsDREB2A gene. The outgroup separates Pokkali varieties from the two control comprises IR64 and Ciherang, and the three local rice comprises Putih Mentikwangi, Hitam Mutiara, and Hitam Cempo by branching to form clade 2, indicating that despite similar evolutionary pathways, there are evolutionary differences. The separation between the outgroup Pokkali and IR64 clades was also reported in a study by Chrisnawati et al. (2022), showing a very distant evolutionary relationship between the two.

Pairwise distance plays an important role in confirming evolutionary relationships, specifically the results of multiple sequence alignments, which are non-deterministic polynomial-time complete (NP-complete) and cannot be estimated computationally (Domazet-Lošo and Haubold 2009). In other words, the role of multiple sequence alignment is to provide information on genetic similarity, while pairwise distance clarifies genetic relationships precisely. Using these two methods helps to provide a more comprehensive understanding of the evolutionary relationship and divergence between varieties, which is important for the development of drought-tolerant varieties. Based on the pairwise distance results, Pokkali, Putih Mutiara, Merah Sengreng, and Merah Wangi have lower values compared to others (Table 4), showing an identical genetic structure. This also supports clade 1, where the varieties share a common ancestor or a similar evolutionary pathway. Meanwhile, clade 2 has a higher pairwise distance value relative to Pokkali, showing that IR64, Ciherang, Putih Mentikwangi, Hitam Mutiara, and Hitam Cempo have different genetic structures. This was supported by a phylogenetic tree, which showed the same clade. These studies confirm the role of SNP in determining plant response to drought in varieties studied, and evolutionary closeness, facilitating the application as molecular markers for drought resistance and plant breeding.

In conclusion, based on the phylogenetic tree, local rice with the closest evolutionary relationship to Pokkali varieties were Putih Mutiara, Merah Sengreng, and Merah Wangi (clade 1). The most distant evolutionary relationships were found in IR64, Ciherang, Putih Mentikwangi, Hitam Cempo, and Hitam Mutiara which showed similarities in genetic structure. Therefore, Putih Mutiara, Merah Sengreng, and Merah Wangi can be used as a plant breeding object to improve drought-tolerant traits by exploiting the presence of the OsDREB2A gene.

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