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Research Article

Identifying Single Nucleotide Polymorphisms (SNPs) in *OsFER1* and *OsFER2* Genes Linked to Iron Accumulation in Pigmented Indonesian Rice (*Oryza sativa* L.)

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ABSTRACT

Iron (Fe) is an essential micronutrient for the well-being of plants, animals, and bacteria. In plants, iron plays a pivotal role in a myriad of metabolic processes, encompassing redox reaction, photosynthesis, respiration, chlorophyll synthesis, and nitrogen fixation. For humans, iron is indespensable for several metabolic functions, particularly in the synthesis of haemoglobin. Iron deficiency can lead to health issues on a global scale, therefore identifying key crops, such as rice for providing sufficient iron in diet intake is very important. In rice, the maintenance of iron homeostasis is orchestrated by various genes, with *OsFER1* and *OsFER2* acting as iron accumulator genes in leaves, stems, flowers, and grains. The primary objective of this study was to ascertain the single nucleotide polymorphisms (SNP) in the *OsFER1* and *OsFER2* and to assess the iron content in Indonesian local rice cultivars. To achieve this, we examined partial sequences of *OsFER1* and *OsFER2* to identify SNPs in the Indonesian rice cultivars used (Cempo Ireng, Pari Ireng, Hitam Kalsel, Merah Pari Eja, and Ciherang). Concurrently, the iron content in the seeds was quantified using Atomic Absorption Spectrophotometry (AAS). The analysis revealed that the *OsFER1* gene sequence, specifically exon 5, exhibited a SNP in the form of a transition. In contrast, the *OsFER2* gene sequences, specifically in intron 2 displayed SNPs in the form of insertions. Notably, the iron content in the seeds was highest in Cempo Ireng (black rice), while it was lowest in Merah Pari Eja (red rice) and Ciherang (non-pigmented rice). Importantly, the identified SNPs in these partial gene sequences did not exert any discernible influence on iron levels or the formation of ferritin protein.

INTRODUCTION

Iron (Fe) plays a vital role for plants, animals, and bacteria. In plants, iron is involved in various metabolic reactions, including electron transport chain redox processes, photosynthesis, respiration reactions, chlorophyll synthesis, and nitrogen fixation (Stein et al. 2009; Briat et al. 2010). Iron deficiency in plants can lead to chlorosis in the leaves, disrupting biosynthetic and photosynthetic processes due to low iron concentration. This chlorosis is especially concerning in young leaves, which act as strong absorbers, and require more iron compared to older leaves

(Kobayashi et al. 2019). On the other hand, excess iron can be toxic to plant cells as it can generate reactive hydroxyl radicals through the Fenton reaction (Liang 2022). Consequently, maintaining iron homeostasis in plants, particularly in rice, is essential. Achieving iron homeostasis in plants involves a dynamic process that employs proteins and small organic molecules to extract metals from the soil, transport them within plant tissues, sequester them intracellularly, act as buffers, and store excess iron (Briat et al. 2010).

The genes responsible for regulating the transport and accumulation of iron play a pivotal role in internal iron regulation within plants. In terms of iron accumulation, particularly in grain storage, *OsFER1* and *OsFER2* genes are of utmost importance (Briat et al. 2010). Plant cells typically store ferritin protein, often referred to as phytoferritin, in plastids and mitochondria. Around 80% of the iron content in leaves is stored in chloroplasts, while in seeds it is stored in leucoplasts and amyloplasts (Mauseth 2021). Remarkably, ferritin protein in plants has the capacity to store up to 4,500 iron atoms (Helmyati et al. 2014). Ferritin protein enables rice plants to withstand iron stress, enabling them to accumulate more iron by tolerating high Fe concentrations in the leaves. Higher ferritin gene expression was found in rice that was resistant to iron (Silveira et al. 2009).

Genotypic diversity among rice cultivars can be genetically identified by comparing and analyzing partial ferritin gene nucleotide sequences, examining the expression of ferritin protein-coding genes, and detecting polymorphisms (Utami et al. 2009; Herlinda et al. 2013). The analysis of Single Nucleotide Polymorphisms (SNPs) aims to generate molecular markers capable of distinguishing between rice cultivar genotypes. SNPs within the nucleotide sequences of the rice ferritin genes provide valuable information of the iron (Fe) content of rice and is useful in plant breeding programs (Collard & Mackill 2008). Research by Stein et al. (2009) revealed differences in the expression of *OsFER1* and *OsFER2* genes, while the study by Paul et al. (2012) demonstrated that overexpression of *OsFER2* led to increased level of Fe and Zn in transgenic plants. This study aims to establish a correlation between iron levels in rice grains form Indonesian rice cultivars and the presence of SNPs the sequences of each *OsFER1* and *OsFER2* gene.

MATERIALS AND METHODS

Materials

The study utilized five local Indonesian rice cultivars: Ciherang, Merah Pari Eja, Hitam Kalsel, Cempo Ireng, and Pari Ireng (Table 1). The process sterilization and seed planting involved the use of various substances, including 5% sodium hypochlorite (NaClO), distilled water (aquadest), soil, goat manure, insecticide, fungicide, Nitrogen Phosphorus Potassium (NPK) fertilizer, Sulfur Phosphate (SP36) fertilizer, and Potassium Chloride (KCl) fertilizer. To prepare samples for iron content analysis through Atomic Absorption Spectrophotometry, we initially treated rice seeds with nitric acid (HNO_3), perchloric acid ($HClO_4$), and distilled water, following the metho described by Elango et al. (2021). Genomic DNA isolation was accomplished through a process that involved liquid nitrogen, Tris Boric EDTA (TBE) buffer, Na_2EDTA, 10% sodium dodecyl sulfate (SDS), sodium chloride (NaCl), isopropyl alcohol, ethanol (EtOH), and double-distilled water (ddH_2O). Subsequent to genomic DNA isolation, PCR amplification was carried out using GoTaq® Green Master Mix (Promega), along with forward and reverse primers (Table 2), Nuclease Free Water (NFW), and isolated genomic DNA sam-

ples.These primers were in silico designed via Primer3Plus, employing a DNA template sourced from the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/gene/9269178, with a target product size of 500 base pairs. The primer design was validated using Primer-BLAST, PCR Primer Stats, Oligo Calculator, and PrimerDimer software. Finally, the results of DNA genome isolation and amplification were evaluated using the electrophoresis method. The process involved materials such as a 1.2% agarose gel, 1x TBE buffer, a DNA ladder marker and ethidium bromide (EtBr).

Table 1. Rice sample cultivars used in this study.

No.	Cultivar	Origin	Pigment type
1.	Ciherang	Jawa Barat	White
2.	Merah Pari Eja	Gowa	Red
3.	Hitam Kalsel	Kalimantan Selatan	Black
4.	Cempo Ireng	Jawa Tengah	Black
5.	Pari Ireng	Sleman	Black

Table 2. Primers used for gene amplification in this study.

Gene	Primer	Sequence
OsFER1	Forward	TAGGAGAAAAGACACTGTGC
	Reverse	TAGCACACAGTAAGCAGAAG

Gene	*OsFER2*	
Product Size	582 bp	
Primer	**Forward**: 5'CCTTAGCTT-GTCATCCGTAG 3'	**Reverse**: 5'CAGACTAGCACACAG-TAAGG 3'
Start	1224	1805
Length	20 bp	20 bp
Tm	55.4 °C	55.2 °C
GC	50 %	50 %

Methods

Planting, cultivating and harvesting

The planting medium was prepared by homogenizing soil and goat manure in a 2:1 ratio. During the planting phase, rice pants were provided with nutrition through weekly fertilizer applications commencing at 30 days after planting (DAP). Harvesting was conducted at 78 DAP, where rice leaves were harvested by cutting and preserved at -20°C. For rice grains, they were harvested when they reached ripeness, characterized by their yellow-brown color and when approximately 50% of the flag leaves had turned yellow. The harvested grains were dried in an oven at a temperature of 30˚C to 40˚C for approximately three days and subsequently stored at 20˚C.

Iron concentration analysis

Samples of rice grains from each cultivar were weighed with a precision of ±3-5 grams. These samples were subsequently blended until achieving powder. Following this, the samples were homogenized and reweighed

with an accuracy of ±2 grams for each cultivar. They were then treated with a mixture of 15 mL of HNO_3 and $HClO_4$. The destruction was carried out on the heating plate until near to dryness, after which 10 mL of distilled water was added. The resulting solution was filtered into a 25 mL flask and topped up with distilled water to reach the mark. Finally, the prepared samples were subjected to iron (Fe) level anylysis using and Atom Absorption Spectrophotometer (AAS).

OsFER1 and *OsFER2* gene sequence analysis

The isolation of genomic DNA was carried out using an extraction buffer from 1.21 grams of Tris mixed with 5 mL of Na2EDTA (0.5M; pH 8) and 2.92 grams of NaCl. The pH of the mixture was adjusted to 8, followed by the addition of 100 mL of sterile distilled water. Next, 100 mg of leaf tissue was ground into a fine powder using liquid nitrogen. The leaf powder was subsequently transferred into a 1.5 mL microtube, and 500 µL of the extraction buffer was added to the tube. The sample was homogenized using a vortex. To this homogenized mixture, 66 µL of 10% SDS reagent was introduced, and the tube was then centrifuged at 13,000 rpm for 1 minute at 4 °C. Approximately 400 µL of the resulting supernatant was transferred to a new tube, and 400 µL of isopropyl alcohol was added. DNA precipitation was achieved by centrifugation at 13,000 rpm for 15 minutes at 4 °C, after which the supernatant was discarded, leaving the pellet DNA in the tube. To wash the pellets, 500 µL of 70% EtOH was added to the tube, which was then centrifuged again at 13,000 rpm for 1 minute at 4 °C. The pellet was dried by briefly inverting the tube, and 50 µL of ddH_2O was added. The rice DNA isolation was stored in a refrigerator at -20 °C. The DNA isolation results were quantified using a nanodrop spectrophotometer and visualized by electrophoresis.

Once the DNA genome was obtained, the results were amplified through PCR, with the *OsFER1* gene at an optimized annealing temperature of 58°C and the *OsFER2* gene at 59.2°C. The PCR product was then visualized by electrophoresis for further sequencing.

RESULTS AND DISCUSSION

Figure 1A depicts the growth status of five rice cultivars at 114 days after planting (DAP). Cempo Ireng and Merah Pari Eja cultivars exhibit the tallest plant heights among the cultivars, measuring over 230 cm, whereas Pari Ireng, Hitam Kalsel, and Ciherang reach a height of approximately 180 cm.

In Figure 1B, variations in seed morphology among the five rice cultivars are evident. Ciherang possesses the tallest seeds, while Merah Pari Eja shows the widest seed width. Cempo Ireng, Pari Ireng, and Hitam Kalsel display purplish-black seeds, Merah Pari Eja has reddish-brown seeds, and Ciherang features white seeds. This distinctive color result from varying concentrations of natural pigments in the seed coat called anthocyanins, which are water-soluble flavonoids (Devi & Badwaik 2022). Notably, white rice seeds (Ciherang) lack anthocyanins. Consequently, pigmented rice exhibits free radical scavenging activity, making it a potential source of antioxidants.

Figure 1C illustrates the average Fe content in different rice cultivars. Cempo Ireng black rice grains contain an average Fe content of 22.133 mg/kg ±10.87. Following closely is Pari Ireng with an average Fe content of 17.127 mg/kg ±2.95. Hitam Kalsel rank third, with an average Fe content of 16.815 mg/kg ±7.29. Merah Pari Eja red rice features an average Fe content of 13.913 mg/kg ± 1.35, and rice cultivars

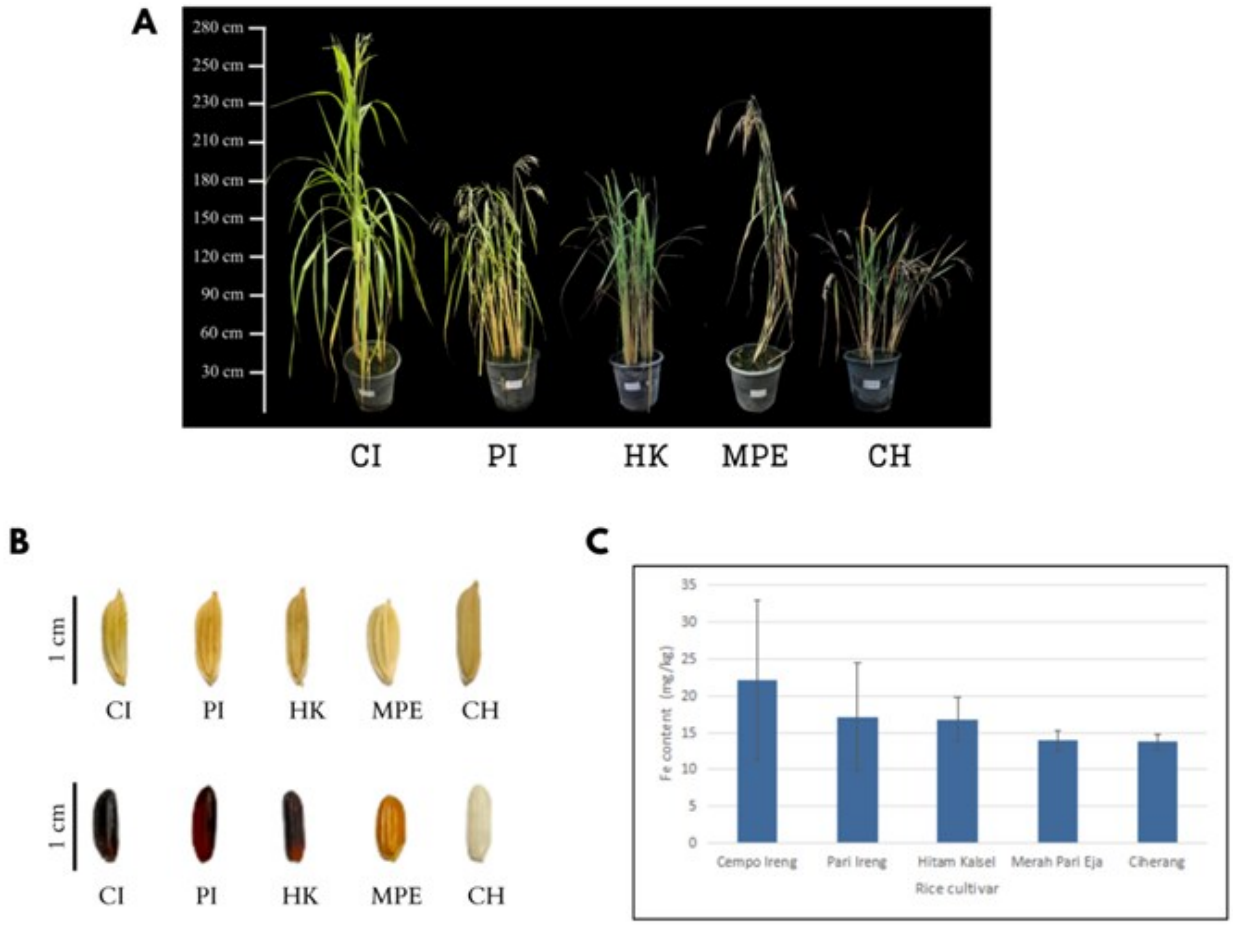

Figure 1. Rice plants morphology aged 114 DAP (A), undehulled (upper) and dehulled (lower) rice grains (B), iron content in rice grains from five cultivars used (C). (CI= Cempo Ireng, PI= Pari Ireng, HK=Hitam Kalsel, MPE= Merah Pari Eja, CH= Ciherang)

which has the lowest average Fe content is Ciherang white rice with an average of 13.756 mg/kg ± 1.06 (Figure 1C).

The isolated DNA sample from five cultivars exhibit a single band with a size of >10 kbp (Figure 2A). This result suggests a relatively high success rate for DNA isolation, affirming the suitability of the obtained genomic DNA as a template for amplifying the *OsFER1* and *OsFER2* genes.

Table 3. Quantification of genomic DNA from five rice cultivars used in this research.

Sample	Concentration (ng/µl)	A260/A230	A260/A280
Cempo Ireng	196.03	2.145	1.804
Pari Ireng	612.67	1.055	1.384
Hitam Kalsel	1190.72	1.095	1.206
Merah Pari Eja	293.25	2.050	1.848
Ciherang	518.86	0.917	1.166

The Table 3 reveals a wide range of DNA concentration, spanning from 196.03 ng/µl to 1190.72 ng/µl along with corresponding Optical Density (OD) values ranging from 0.917 to 2.145 (Table 3). The good ratio of OD A260/A280 and A260/A230 values ranging from 1.5 to 1.8 indicates that the level of DNA purity obtained is quite high (Ahmed et al. 2009). A low OD value at A260/A280 may suggest the presence of polyphenols contaminants, while a low OD at A260/A230 indicates the existence of salt residues (Aboul-Maaty & Oraby 2019).

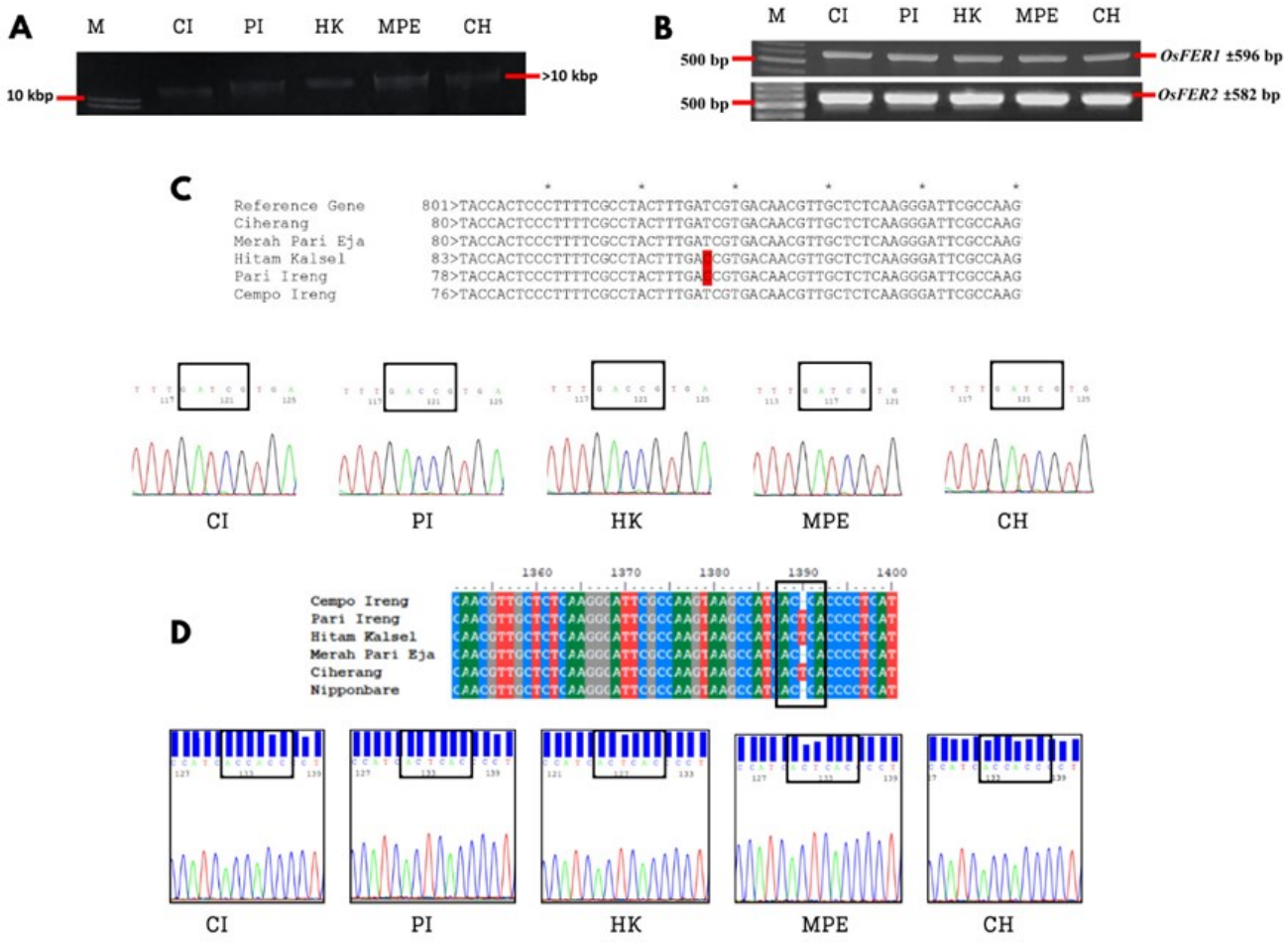

Figure 2. Visualization of genomic DNA isolation (A), Amplicon of *OsFER1* and *OsFER2* gene (B), Sanger sequencing and alignmnent result in partial *OsFER1* (C) and *OsFER2* (D) gene.
(M= Marker, CI= Cempo Ireng. PI= Pari Ireng, HK= Hitam Kalsel, MPE= Merah Pari Eja, CH= Ciherang)

Sanger sequenching was employed to decode the nucleotide sequences of partial *OsFER2* genes from the five rice cultivars. The sequencing results, represented in chromatograms, unveil the positions containing SNPs and are presented in Figure 2C and 2D. Alignment analysis using the BioEdit and MEGA11 software applications allowed a comparison of the nucleotide sequences of the five rice cultivars with the reference Nipponbare cultivar. High nucleotide sequence similarity indicates a close common ancestor (Kemena & Notredame 2009). The results of this analysis confirm the presence of 3 SNPs in the partial *OsFER2* gene sequence alignment confirms that there were 3 SNPs detected in the partial *OsFER2* gene sequence at the 1390 bp position.

In rice plants (*Oryza sativa, L.*), the ferritin gene consists of *OsFER1* on chromosome 11 and *OsFER2* on chromosome 12 (Herman et al. 2014). This study utilized the gene sequence of LOC4351264 *Fer2, chloroplastic Oryza sativa Japonica Group* from the Nipponbare cultivar (Gene ID: 4351264), located on chromosome 12, as the reference sequence. Chromosome 12 is linear DNA segment with a length of 27,531,856 bp (Nugraha et al. 2014).

The *OsFER2* gene is located at position 320,417 to 323,918 encompassing a length of 3,502 bp. Based on NCBI Refseq data NC_029267.1 with *accession* NC_029267 *REGION*: complement (320417..323918), the *OsFER2* gene (LOC4351264) is 3,502 bp long and consists of 8 exons and 7 introns (1..587, 588..1293, 1294..1377, 1378..1457, 1458..1518,

1519..1620, 1621. .1708, 1707..2460, 2461..2522, 2523..2655, 2656..2721, 2722..2834, 2835..2898, 2899..3004, 3005..3502).

The alignment analysis results reveal the presence of 3 SNPs in the sequence of *OsFER2* gene, specifically at the 1390 bp position. Pari Ireng, Hitam Kalsel, and Ciherang sequence exhibit a single thymine (T) insertion at 1390 bp. According to NCBI Refseq data NC_029267.1 from *Oryza sativa var. Nipponbare*, this 1390 bp region corresponds to intron 2 of the *OsFER2* gene, spanning from 1378 to 1457 bp. Importantly, intron regions are not part of the coding sequence (CDS) and are subsequently excised through mRNA splicing during post-translational modification. As a result, nucleotide changes within introns do not impact ferritin protein domain, amino acid composition, or variations in the Fe content of rice seeds.

Table 4. SNPs Positions in *OsFER1* and *OsFER2*.

Gene	Cultivar	SNP	Position (bp)	Nucleotide Base
OsFER1	Pari Ireng	Transition	104	T → C
	Hitam Kalsel	Transition	109	T → C
OsFER2	Pari Ireng	Insertion	1390	T
	Hitam Kalsel	Insertion	1390	T
	Ciherang	Insertion	1390	T

In contrast, the analysis also identifies 2 SNP in the sequence of the *OsFER1* gene, spesifically at the 104 bp and 109 bp positions. Pari Ireng and Hitam Kalsel both exhibit a single transition of thymine (T) to cytosine (C) at these locations. It's worth nothing that all of the identified SNPs in *OsFER2* are located in intron 2, while the SNPs in *OsFER1* are located in exon 5.

Previous studies (Nugraha et al. 2014; Roslim et al. 2016) have reported the presence of several SNPs in the *OsFER2* gene sequence from various local rice cultivars, including Siam Sintanur, Bakung, Mahsuri, Amat Candu, Sadani, and IR64, when compared with Nipponbare reference sequence. According to the findings of this study, there were at least 5 SNPs in intron 1 and 12 SNPs in intron 2. Furthermore, 2, 19, and 3 SNPs were detected in exon 2, exon 3, and exon 4, respectively. The increased number of SNPs reported in previous studies may be attributed to the greater diversity among the rice cultivars under investigation. This studies likely involved more contrasting traits, such as groups of rice cultivars with varying resistance or susceptibility to Fe stress, which could account for the heightened differences in nucleotide sequence observed.

Figure 3 presents a phylogenetic tree illustrating the evolutionary relationship among the plant organisms under investigation. This phylogenetic tree was constructed using the MegaX software with the Neighbor-joining statistical method. This statistical method used a distance-based algorithm to assess sequence similarity. Based on the dendogram, it become evident that the five studied cultivars (Pari Ireng, Hitam Kalsel, Ciherang, Cempo Ireng, and Merah Pari Eja) share a very close relationship by comparing the horizontal branch with the scale bar below. The distance between the nodes of each cultivar and the common ancestor node is shorter than the scale bar (0.20). More specifically, the distances are as follows: Merah Pari Eja 0.00; Cempo Ireng 0.05; Ciherang 0.08; Hitam Kalsel 0.09; and Pari Ireng 0.10. The phylogenetic tree construction employed a standard of 1000 bootstrap replications. A reliable interpretation for the percentage of confidence in the Bootstrap

Support (BS) value is typically set at 95% (Patrick et al. 2018). Nodes with a bootstrap value below 80% have been collapsed (Brandis 2021). Reviewing the dendogram's structure, it is evident that the BS value for each node exceeds 80%. This indicates that the constructed phylogenetic tree offers a high level of confidence in accurately depicting the relationships among the organisms under study.

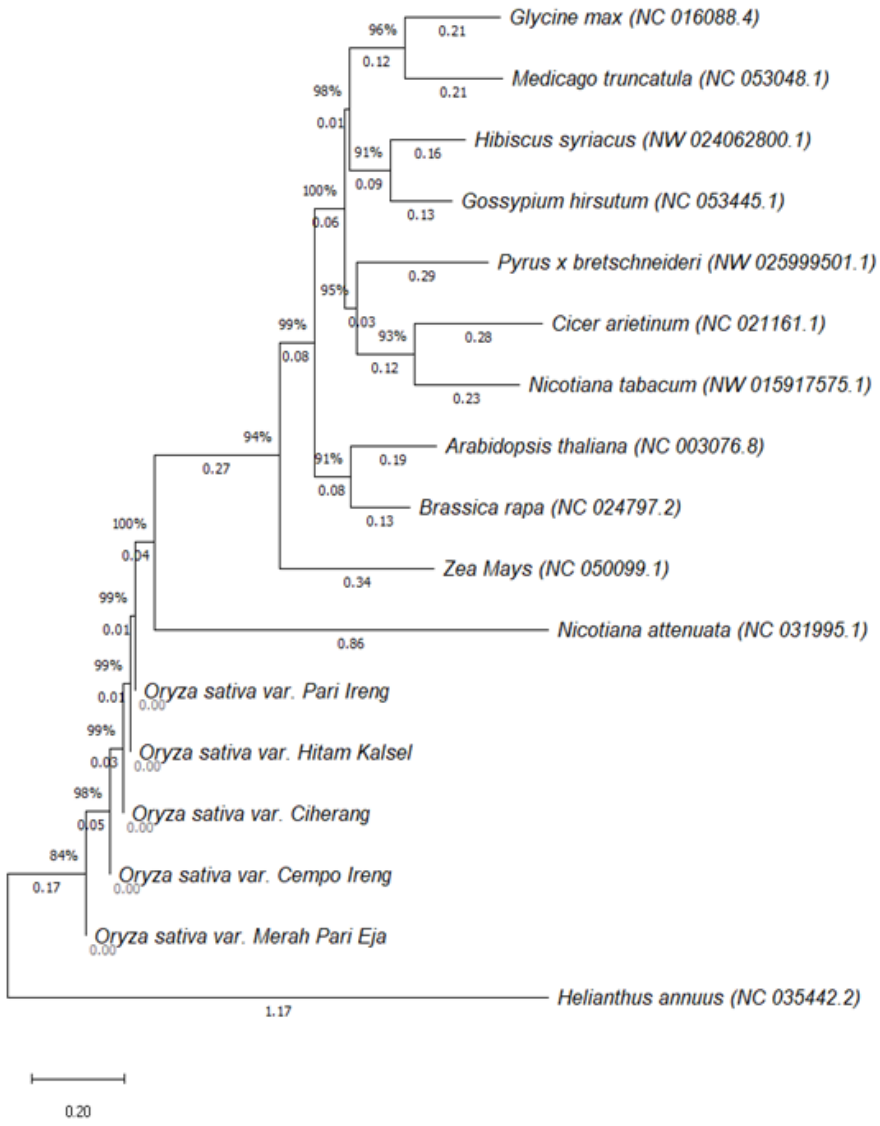

Figure 3. *OsFER1* phylogenetic tree of rice cultivars and out-group.

The dendrogram (Figure 4) constructed based on the *OsFER2* gene from various plants reveals that the five rice cultivars share the closest relationship with *O. sativa* var. *Nipponbare*. The distances between the nodes for each cultivar and the common ancestor node are remarkably close, measuring 0.00 for Ciherang, Hitam Kalsel, Pari Ireng, and Cempo Ireng, and 0,01 for Merah Pari Eja. In particular, the Ciherang cultivar is closely related to Nipponbare, forming the rice group. Pari Ireng, Hitam Kalsel, and Cempo Ireng cultivars exhibit a close relationship, constituting the black rice group. Meanwhile, Merah Pari Eja cultivar emerges as an outgroup in relation to the other rice cultivars. Furthermore, the *OsFER2* sequences of the five studied cultivars show a close relationship with the *OsFER1* sequence of *O. sativa var. Nipponbare*.

The dendogram also proves that organisms from the same genus have the closest relatives. It can be seen from *O. branchyantha* which is the

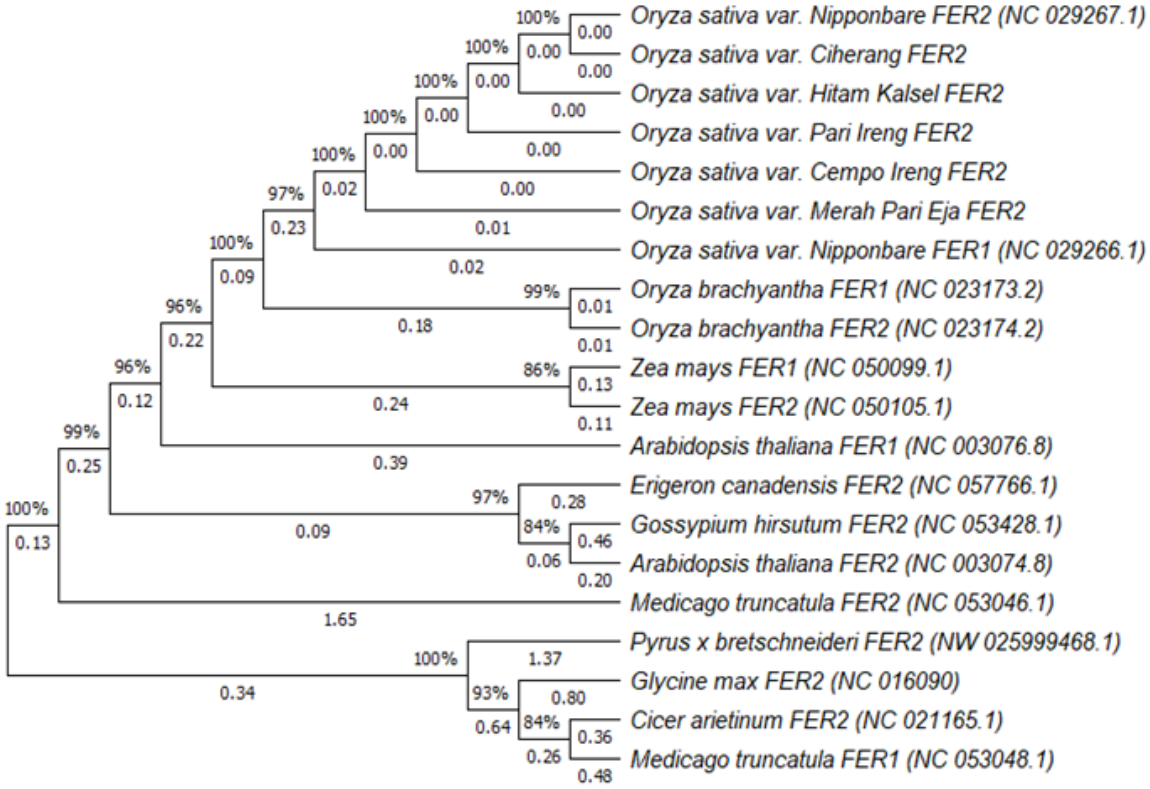

Figure 4. *OsFER2* phylogenetic tree of rice cultivars and out-group.

closest relative of *O. sativa*. Moreover, organisms within the same order also exhibit close relationships, as exemplified by the grouping of *O. sativa*, *O branchyantha*, and *Zea mays* which form a Poales group. Additionally, *FERI* and *FER2* gene sequences from the same organism demonstrate close relationships and form groups within the species, as observed in *O. branchyanta*, *O. sativa*, and *Z. mays*. The BS value for each node is >80% (Brandis 2021), confirming the phylogenetic tree's robust confidence in accurately depicting the relationship between organisms considered.

CONCLUSIONS

The target sequences in the *OsFER1* and *OsFER2* genes are known to have SNPs. However, the presence of SNPs in each of these gene sequences did not have a significant impact on the Fe concentration in the rice grains of each cultivar.

AUTHOR CONTRIBUTION

A.P. and R.F.B. contributed equally to this paper. Y.A.P supervised all the research process and writing the manuscript, A.P. designed the manuscript and analyzed the *OsFER1* gene data, R.F.B. analyzed the *OsFER2* gene data, reviewed, and edited the manuscript, I.S.E. assisted the isolation process, A.S. assisted the research process.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this research.

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