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GENETIC DIVERSITY AMONG INDONESIAN RICE (*Oryza Sativa* L.) GENOTYPES FOR DROUGHT TOLERANCE

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SUMMARY

Rice is one of the most important staple foods for most people in the world. However, worldwide rice production is threatened by abiotic stress caused by global climate change. Drought is the most extensive abiotic stress for rice, because compared with the other cereal crops rice needs a vast amount of water for growth and development. The breeding of rice cultivars for drought stress is indispensable. The availability of broad genetic diversity, including drought tolerance, in rice is essential in crop genetic improvement programs. Thus, the evaluation of genetic diversity is a critical step in a breeding program. We report a genetic diversity analysis of Indonesian rice germplasm based on Simple Sequence Repeat (SSR) markers linked to drought tolerance traits in rice. Forty-five rice germplasm consisting of 41 genotypes and four check cultivars were used in this study. Nine markers consisting of eight SSR markers and one custom marker designed based on the deeper rooting gene were used to analyze rice germplasm. Results showed a low number of alleles and moderate genetic diversity. An unweighted pair group method with an arithmetic mean dendrogram generated by cluster analysis revealed four major clusters. A biplot generated via principal coordinate analysis divided rice germplasm into two major groups and confirmed the cluster analysis results. Rice genotypes in clusters I and II belonged to the first group. Similarly, the members of clusters III and IV belonged to the second group. The members of cluster I were predicted to be tolerant of drought stress because they belonged to the same cluster as the drought-tolerant check. This finding will be useful for the acceleration of rice breeding programs for drought stress tolerance.

Keywords: Genetic diversity, Indonesia, rice, SSR marker, drought

Key findings: Genetic diversity analysis based on simple sequence repeat markers linked to drought tolerance revealed relationships among Indonesian rice germplasm. Some of the germplasm possessed drought tolerance.

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INTRODUCTION

Crops are sessile organisms that are surrounded by environmental factors. Non-living variables that exist in the environment wherein the crop is grown are referred to as abiotic factors. These factors are erratic, and at some point they can induce stress and adversely influence crop growth and productivity (Zhu, 2016). Some examples of common abiotic stresses agriculture are drought, high in temperature; low temperature; nutrient deficits; and excess salt or toxic metals, such as aluminum, cadmium, and iron. Among these abiotic stresses, drought is considered as the most crucial abiotic stress given its worldwide coverage and impact on crop yield and productivity (Martínez-Fernández et al., 2016).

Rice (Oryza sativa L.) is one of the most important crops in the world, especially in Asia. Rice grains are utilized as a staple food by more than half of the human population world. In Southeast Asia, three-quarters of the daily calorie intake is provided by rice (Birla et al., 2017). Rice is grown on almost 153 million hectares of arable land on the earth's surface and occupies the third place as the crop with the largest cultivation area after wheat and maize (Liu et al., 2016). Rice is grown over a broad variability of locations and climatic conditions, i.e., from the driest areas where rainfall is less than a 100 mm per year to areas with more than 5000 mm of rainfall per year. Altitude, average temperature, and solar radiation also vary in areas that are used for rice

cultivation (Consultative Group on International Agriculture Research, 2013).

By 2050, the human population is estimated to multiply by two times from its current level. As а production. food consequence, including rice production, should be increased. Food production needs to increase by up to 60% to meet demand and ensure global food security (Silalertruksa et al., 2017). The agricultural sector, as the key player in food production, is facing various obstacles from many aspects, i.e., socioeconomics, agricultural field loss, environmental degradation, and global climate change (Pereira, 2016; Qi et al., 2018). Global climate change increases global temperatures and decreases average precipitation, thus resulting in the reduction of water availability for crops in manv agricultural production areas (Alvarez-Berríos et al., 2018).

Rice is grown mostly in irrigated paddy fields and is thus considered as one of the crops that requires a large amount of water for its cultivation. On average, 2500 liters of water is needed to produce 1 kg of rough rice in the field (Bouman et al., 2007). By contrast, other cereal crops, i.e., maize or wheat, only need half of this amount to produce the same quantity of grain. Rice is also more sensitive to soil water amount than other crops (Todaka et al., 2015). Undoubtedly, induced by drought stress will challenge rice production in upland and lowland areas.

To overcome this problem, the genetic improvement of rice is needed

to increase tolerance to drought stress. The genetic improvement of crop mainly consists of three steps: (1) the creation of broad genetic variability, (2) selection for traits of interest, and (3) the fixation of desirable genotypes for the dissemination of planting material (Lee et al., 2015). Advancements in molecular genetics and biotechnology have allowed plant geneticists to develop various tools to support plant breeding programs. In recent decades, powerful tools, namely molecular markers, have been developed. By molecular markers, using various genes and quantitative trait loci (QTL) responsible for important traits related to drought tolerance in rice have been identified and used in various ways to support plant breeding programs.

Simple sequence repeat (SSR) markers are molecular markers based motifs of 2-5 repeating on nucleotides. This type of marker has been applied in various activities related to drought tolerance rice, i.e., improvement in gene discovery (Prince et al., 2015; Zhou et al. 2016; Sabar et al., 2019), prebreeding (Singh et al., 2015; Anupam 2017), et al., and selection (Shamsudin et al., 2016). In addition to genetic diversity analysis, the most important activity in pre-breeding involves the identification of germplasm that has desirable traits or genes as a potential donor for genetic improvement. Thus, prebreeding serves to fill the gap between genetic utilization resource and crop improvement (Sharma et al., 2013). When combined with the application of prebreeding molecular markers, activity can provide a solid foundation for rice improvement toward drought tolerance. Anupam *et al.* (2017) provided an excellent example for the

identification of rice germplasm from Northeast India that carries the beneficial allele for grain yield under drought stress by using SSR markers linked to responsible QTL.

Screening for drought tolerance considerably rice is laborious in because specialized procedures to induce stress and identify stress tolerance levels are needed. The of application molecular markers linked to the trait of interest will increase the affectivity and efficiency activities of pre-breeding in the improvement drought of stress tolerance in rice. In this study, we analyzed the genetic diversity of rice germplasm by using nine molecular markers linked to drought stress tolerance; these markers comprised eight SSR markers and one marker designed on the basis of the deeper rooting gene in rice (OsDRO1) (Uga et 2013). A total of 45 rice al., germplasm consisting of local varieties, cultivars, and check cultivars were genotyped. The aims of this study were to evaluate aenetic diversity of Indonesian rice germplasm and identify potential germplasm with drought tolerance.

MATERIALS AND METHODS

Genetic material

Forty-five rice germplasm consisting of 41 genotypes and four check cultivars were used as genetic materials. The check cultivars were as follows: Bluebonnet, Ciherang, Kasalath, and FR13A. The detailed information about the rice germplasm used in this study is presented in Table 1. The seeds of each germplasm were obtained from genebank of the Department of Agronomy, Faculty of Agriculture,

No.	Accession name	ID	Origin	No.	Accession name	ID	Origin
1.	Ciherang	CHR	Indonesia	24.	99	BP-L-116	Bantul (SPY)
2.	Bluebonnet	BB	United States	25.	Batang Rimbang	BP-L-118	Magelang(CJ)
3.	FR13A	FR13A	India	26.	Janur	BP-L-120	Bantul (SPY)
4.	Kasalath	KAS	Bangladesh	27.	Minang	BP-L-126	Padang (WS)
5.	Rokan	BP-R-1	Indonesia	28.	Merah	BP-L-133	Bantul (SPY)
6.	Widas Sawah 1	BP-R-9	Indonesia	29.	Lokal Merah	BP-L-134	Indonesia
7.	Widas Sawah 2	BP-R-10	Indonesia	30.	Cempo Lombok	BP-L-135	Bantul (SPY)
8.	Dewi Sri	BP-R-117	Indonesia	31.	Rojolele Gepyok	BP-L-137	Bantul (SPY)
9.	Rojolele	BP-R-124	Klaten(CJ)	32.	Andel Abang	BP-L-144	Magelang(CJ)
10.	Slegreng 3	BP-R-127	Indonesia	33.	Saodah	BP-L-150	Magelang(CJ)
11.	Pandan Wangi	BP-R-130	Cianjur (WJ)	34.	Sedani Cendek	BP-L-152	Sleman (SPY)
12.	Cempo Merah	BP-R-131	SPY	35.	Melik Ireng	BP-L-158	Sleman (SPY)
13.	Mentik Wangi	BP-R-132	Magelang (CJ)	36.	Raket Putih	BP-L-166	Mataram (WNT)
14.	Anak Daro	BP-R-161	Solok (WS)	37.	Padi Beton	BP-L-167	Mataram (WNT)
15.	Batang Gadis	BP-R-218	Indonesia	38.	Reket Dodoh	BP-L-170	Mataram (WNT)
16.	Mayangan	BP-R-260	Indonesia	39.	Padi Lobak	BP-L-173	Mataram (WNT)
17.	Danau Gaung	BP-R-304	Indonesia	40.	HitamTemen	BP-L-180	Wonosobo(CJ)
18.	Gogo Rencah	BP-L-7	Indonesia	41.	IrengBulu	BP-L-181	Wonosobo(CJ)
19.	H3	BP-L-12	Indonesia	42.	Rening	BP-L-187	Indonesia
20.	Merah Panjang	BP-L-94	Magelang(CJ)	43.	Lembayung Gogo	BP-L-220	Indonesia
21.	Gadung Mlathi	BP-L-56	Indonesia	44.	Putho	BP-L-235	Indonesia
22.	Ketan Salome 1	BP-L-114	Pandak (SPY)	45.	CempoBulu	BP-L-237	Indonesia
23.	Ketan Salome 2	BP-L-115	Bantul (SPY)				

Table 1. Detailed information on the rice germplasm used in this study.

Note: Letters inside the bracket in the Origin column indicates the Indonesian province of origin of the accession. Accessions abbreviations are as follows CJ: Central Java Province, WJ: West Java, SPY: Special Province of Yogyakarta, WS: West Sumatera, WNT: West Nusa Tenggara.

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Extraction of genomic DNA

Genomic DNA from each genotype was isolated from fully expanded leaves taken from plants aged 21 days after planting (DAP). The CTAB method (Murray and Thompson 1980) was used for DNA extraction and was followed by the addition of RNAseA (Fermentas, Thermo Fisher Scientific). The purity and concentration of the extracted DNA were measured by using NanoVuePlusTM (Biochrom), and DNA quality was checked on 1.5 % agarose (w/v). DNA was diluted to 40 ng/µL concentration of the working solution and stored at -20 °C.

Marker genotyping assay

The markers used in this study were genotyped on a T100 thermal cvcler (BioRad) with a 12.5 µL final volume consisting of 6.25 µL of GoTag® Green master mix (Promega), 1 µL of each primer (10 µM), 40 ng of DNA genomic DNA template, and nucleasefree water up to the final volume. The PCR profile was follows: as predenaturation at 94 °C for 4 min. followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54.2 °C-60.0 °C for 1 min, elongation at 72 °C for 2 min, and final elongation at 72 °C for 10 min. The information (nucleotide sequence, linked trait, and reference) of the nine markers used in this study are shown in Table 2. PCR amplification products were MetaPhor[™] electrophoresed on agarose (Lonza) stained by using FloroSafe DNA Stain (1st Base) in Tris-Borate EDTA buffer and then visualized bv usina а UV transilluminator. Α standard DNA ladder was included to determine the

size of amplified products. The absence (0) or presence (1) of each amplicon was scored.

Data analysis

DNA band data were stored in a matrix and used for the analysis of genetic diversity among 45 rice germplasm. POPGENE software version 1.32 (Yeh et al., 1999) was used to calculate the observed number of alleles per locus (Na), effective alleles (Ne), and Shannon Information Index (I). Nei's gene diversity (He) and polymorphic information content (PIC) were calculated on the basis of overall marker diversity among germplasm usina GeneCalc by (https://gene-calc.pl/).

coefficient The of genetic similarity among rice germplasm used in this study was estimated by using Jaccard's coefficient. Clusterina analysis was performed through the unweighted paired group method (UPGMA) based on the previously generated genetic similarity coefficients. NTSYS software version 2.02 (Exeter) was used to perform aenetic similarity and clusterina analyses. Lastly, principal coordinate analysis (PCoA) was performed by using GenAlEx software version 6.5 (Peakall and Smouse, 2012).

RESULTS

Marker polymorphism

The results of marker polymorphism analysis are tabulated in Table 3. All of the nine markers used in this study generated DNA bands with various sizes ranging from 90 to 700 base pairs (bp). The average number of alleles was 3.111, the highest was 5, and the lowest was 2. The number of alleles that might be present in the population was shown by *Ne* value. The *Ne* value obtained in this study ranged from 1.471 to 3.575 with an average of 2.360. The average value of *I* observed in this study was 0.904,

the lowest was 0.5000, and the highest was 1.319. *He* ranged from 0.320 to 0.720 with an average of 0.539. PIC values were calculated and ranged from 0.269 to 0.667 with an average value of 0.469.

Table 2. List of names, nucleotide sequences, and annealing temperatures of the primers used in this study.

No.	Markers	Sequence	Linked traits	Reference
1	20A	F: ATCTTGTCCCTGCAGGTCAT	Drought	Barik <i>et al.,</i>
		R: GAAACAGAGGCACATTTCATTG	tolerance	(2019)
2	RM72	F: CCGGCGATAAAACAATGAG	Leaf rolling	Anupam <i>et al.,</i>
		R: GCATCGGTCCTAACTAAGGG		(2017)
3	RM228	F: CTGGCCATTAGTCCTTGG	Leaf rolling	Anupam <i>et al.,</i>
		R: GCTTGCGGCTCTGCTTAC		(2017)
4	RM518	F: CTCTTCACTCACTCACCATGG	Grain yield under	Anupam <i>et al.,</i>
		R: ATCCATCTGGAGCAAGCAAC	drought stress	(2017)
5	RM7424	F: AGAAGCCCATCTAGCAGCAG	Deeper rooting	Uga <i>et al.,</i>
		R: TCAAGCTAGCCACACAGCTG		(2013)
6	RM6909	F: AAGTACTCTCCCGTTTCAAA	Grain yield under	Deshmukh <i>et</i>
		R: CCTCCCATAAAAATCTTGTC	drought stress	<i>al.,</i> (2018)
7	RM29433	F: TAGCTGCTTAGCTTTGACTTGG	Deeper rooting	Uga <i>et al.,</i>
		R: ATGTAATCCTACGAGGAGATCG		(2013)
8	RM27933	F: TCCTCTGTCATATGGCTGTAAACG	Yield under water	Boopathi <i>et al.,</i>
		R: GGACAAGGAGGAACTATTGATTGG	stress	(2013)
9	DRO	F: GTC CAC GAG AGA GCA TGG	Deeper rooting	Uga <i>et al.,</i>
		R: ACG AAC GCG AAT TAT TTC TG		(2013)

Table 3. Names, product size ranges, *Na*, *Ne*, *I*, *He*, and PIC of the nine markers used in this study.

No.	Marker name	Marker Product size range (base pair)		Ne	Ι	He	PIC
1	RM20A	200-330	2	1.800	0.637	0.444	0.346
2	RM72	100-200	3	2.467	0.988	0.595	0.521
3	RM228	100-180	4	2.770	1.114	0.639	0.570
4	RM518	100-180	3	2.152	0.885	0.535	0.458
5	RM7424	90-150	3	2.299	0.918	0.565	0.477
6	RM6909	100-400	4	3.575	1.319	0.720	0.667
7	RM29433	200-600	2	1.471	0.500	0.320	0.269
8	RM27933	250-700	5	3.179	1.248	0.685	0.623
9	DRO	400-500	2	1.528	0.530	0.346	0.286
Means			3.111	2.360	0.904	0.539	0.469
St. Dev			1.054	0.722	0.299	0.142	0.144

Note: *Na*: number of allele, *Ne*: effective number of allele, *I*: Shannon information index, *He*: Nei gene diversity, and PIC: Polymorphic information content.



Figure 1. Distribution of the genetic similarity of the rice germplasm used in this study.

Genetic relationship and cluster analysis

The genetic relationship between rice germplasm was measured on the basis of Jaccard's coefficient. The values of genetic similarity coefficient ranged from 0 to 1. A value of 0 indicates no similarity between genotypes. By contrast, a value of 1 indicates similar genotypes. The distribution of the genetic similarity coefficients of the rice germplasm used in this study is shown in Figure 1. The genetic similarity coefficients of the rice germplasm in this study ranged from 0.4043-0.9167 with an average of 0.4044. Cluster analysis performed to elucidate the was genetic relationships among rice Figure 2 germplasm. shows the dendrogram generated by using the UPGMA method. The average value of genetic similarity coefficient (0.4044) was used as the cutoff point for the dendrogram. As a result, four different clusters were established.

The first, second, third, and fourth groups comprised 6, 6, 30, and respectively. genotypes, Four 3 genotypes, i.e., BP-L-166, BP-L-167, BP-L-170, and BP-L-173, originating from Mataram belonged to cluster I together with Bluebonnet and BP-L-235. Cluster II was divided into two subclusters, i.e., IIA and IIB with three genotypes each. Cluster IIA consisted of BP-L-180, BP-R-124, and BP-L-134, whereas cluster IIB comprised of BP-L-181, BP-R-260, and BP-L-187. Two genotypes originating from Wonosobo belonged to the second group. Cluster III, the most notable cluster, possessed the highest number of genotypes (30). Two of the check cultivars, i.e., Ciherang and Kasalath, belonged to cluster III but in different subclusters. Lastly, cluster IV, with the smallest number of genotypes, comprised three genotypes, i.e., BP-L-126, FR13A, and BP-R-1.

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Figure 2. UPGMA dendrogram of the 45 rice germplasm used in this study. Note: Dotted line indicates the cutoff to the dendrogram based on the average of genetic similarity coefficient (0.40)



Coord. 1

Figure 3. PCoA biplot based on SSR marker scores.

ΡϹοΑ

PCoA was performed to resolve the relationship between rice germplasm on the basis of molecular marker data. PCoA explained 55.01% of the total variation. The first axis explained 32.42%, followed by 11.92 %, and 10.67%. The biplot analysis results for the first two PC revealed that rice germplasm was clustered into two major groups (Figure 3). The first group consisted of 12 genotypes, whereas the second group consisted of 33 genotypes. Interestingly, the members of the first group were the same genotypes in clusters I and II in the previously generated dendrogram. Moreover, the second group consisted of genotypes from clusters III and IV.

DISCUSSION

Molecular markers have advantages morphological characters for over analyzing the genetic diversity of crop species. The phenotype of an organism is the result of three aspects, i.e., genetic, environment, and genetic environment X molecular interactions. By using effects markers, the of the environment and growth phase of an organism can be neglected. Linked molecular markers significantly increase the efficiency and effectiveness of identifying potential germplasm that possesses favorable traits that are difficult to observe by the naked eye or under normal conditions, i.e., nutritional content and biotic or abiotic stress tolerance. The choice of molecular markers plays a critical role in genetic diversity analysis. Markers that can generate a high level of polymorphism are desired because they can capture the variability of an organism at the DNA level. SSR markers exhibit a high level of polymorphisms, including in rice (Nachimutu et al., 2015).

The observed number of alleles in this study was lower than that in studies using other molecular marker systems, i.e., RAPD or ISSR (Qian et al., 2001). This result might be caused by the nonrandom selection of SSR markers used in this study. They were selected on the basis of their association with drought tolerancerelated traits in rice. Given this approach, their locations on the rice genome are highly specific and linked to the genes that responsible for drought stress adaptation. However, high-yielding cereal most crops cultivars were developed for favorable environments (Landi et al., 2017). Several types of alleles, i.e., droughttolerant alleles, may have been discarded during selection in cultivar development because they may be linked to the other alleles that control undesirable traits (Vikram et al., 2015). As a result, low variability was observed at the locus amplified by the markers used in this study.

Genetic diversity refers to the existence of different alleles in a gene in different individuals within the same species (Verma et al., 2019). In this study, two approaches, i.e., I and He, used to measure aenetic were diversity of rice germplasm. The I and He values obtained in this study could be classified as moderate but were lower than the overall gene diversity reported by Tu et al. (2007) and Thomson et al. (2007) for germplasm

in this study were collected from several areas and thus did not represent the overall genetic diversity of Indonesian rice. PIC indicates the informativeness and power discriminatory capability of molecular marker. The PIC value is useful for measuring allelic diversity. The average PIC value of the markers used in this study was considered as highly informative (> 0.50)accordance with Botstein et al. (1980).The values of Na, He, and PIC obtained in this study were similar to the results reported by other authors.

from Yunnan Province and Indonesia,

respectively. The rice germplasm used

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Nachimutu et al. (2015) analyzed the genetic diversity of rice germplasm from various origins and reported the average Na, He, and PIC values of 3, 0.52, and 0.468, respectively. Salgotra et al. (2015) reported the genetic diversity of Basmati rice from North germplasm Western Himalayas and obtained Na and PIC values of 3 and 0.405, respectively. Anupam et al. (2017) analyzed the genetic diversity of rice germplasm from Tripura State, Northeast India via an approach similar to that used in this study, i.e., by using droughtlinked markers. Surprisingly, the average Na, He, and PIC obtained in the aforementioned study were closely similar to the results of this study, i.e., 2.9, 0.551, and 0.469, respectively. These findings suggested a similar pattern of rice genetic diversity assessed by SSR markers.

This study included four check cultivars, i.e., Bluebonnet, Ciherana, Kasalath, and FR13A. Each of the check cultivars is known as a source of favorable traits in various breeding programs. Bluebonnet is the principal from the US rice cultivar that possesses drought tolerance (Yoshida and Hasegawa, 1982; Chang and Loresto, 1985). Uga et al. (2013) reported that Bluebonnet has a higher deeper rooting ratio than other rice cultivars, i.e., IR64. Ciherang is a derivative of IR64 and has become the most widely grown rice cultivar in Indonesia due to its grain quality. In addition, Ciherang has been improved in terms of various traits, i.e., heading date (Prasetiyono et al., 2013), flood tolerance (Nugraha et al., 2017), and grain aroma (Cing et al., 2017). Kasalath is famous for its Phosporus uptake 1 gene that can increase rice yield under phosphorus deficiency (Chin et al., 2011). The last check cultivar, FR13A, is the source of the Submergence1 gene that confers flooding resistance. This gene has been introgressed into many rice for India, Philippines, cultivars Indonesia, and Bangladesh (Bailey-Serres et al., 2010).

The availability of broad genetic diversity is useful in a breeding program because it allows breeders to select the genotypes that possess the traits of interest. Group establishment in cluster analysis and PCoA is based on the genetic similarity coefficient between rice germplasm. Genotypes that belong to the same cluster exhibit high genetic similarity. The genetic information from genotypes in this study was obtained from SSR markers that were linked to traits related to drought tolerance. Thus, the genotypes that belonged to the same cluster might possess similar drought stress tolerance.

Bluebonnet, the droughttolerant check, belonged to the first group along with the other five

genotypes. This result indicated that the other genotypes in the cluster I miaht be drought tolerant. Interestingly, four genotypes from Mataram clustered in the same group. Mataram is located in Lombok Island. In this area, rice is cultivated by using the *gogo rancah* cultivation system in soil with limited water. This cultivation system has been practiced for almost 40 years in Lombok Island (Fagi and Kartaatmadia, 2002). The rice genotypes in this area may possess drought tolerance because they have been selected either naturally or artificially to adapt the gogo rancah system.

Breeding programs for drought stress tolerance must be started with pre-breeding activities focusing on identifying germplasm that possess drought-tolerant trait. Frequently, numbers of genotypes with unidentified stress tolerance levels are involved. Screening by using conventional methods is costly and highly laborious because it needs specialized procedures. Moreover, plant response to drought stress is affected by arowth phase and environmental conditions, thus increasing complexity (Widyawan et al., 2018). The use of molecular markers that are linked to drought stress tolerance can overcome these problems. By using marker analysis, the results can be obtained as soon as the DNA of the plant can be extracted. The time and labor spent on the prebreeding programs can be reduced. The results of this study may provide insight into the stress tolerance levels of Indonesian rice germplasm and thus accelerate rice breeding programs for drought stress tolerance.

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