



Evaluation of the crossings between local and drought-tolerant rice varieties using simple sequence repeat (SSR) molecular marker

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Abstract

Two cultivars of local rice, namely Mentik Wangi and Mentik Susu, have been grown around west to south flank of Merapi volcano. They are highly valued for their good taste and cooking characteristics. An attempt to introduce drought-tolerant rice varieties has been conducted by crossing them with two sources, namely Kasalath and Bluebonnet. Molecular-assisted selection using a set of SSR markers was applied to check the F1 and F2 generation trueness and segregation. This research was conducted in the facilities of Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta. The research was a field experiment arranged in a completely randomized design, consisting of Mentik Wangi, Mentik Susu, Bluebonnet, and Kasalath, as well as their crossings' F1 and F2 plants as treatments. The SSR markers used are RM72, RM228, RM518, and RM20(A). Polymorphism test of the parents showed that polymorphism exists between local and donor parents, thus these markers were considered eligible for the F1 and F2 tests. The heterozygous individuals of F1 were 75 % for Bluebonnet × Mentik Wangi, 44.4 % for Bluebonnet × Mentik Susu, and 46.7 % for Mentik Wangi × Bluebonnet; all were consistent in every primer used. We could not confirm that the F2 populations showed segregation pattern that followed Mendelian segregation in some crosses due to too small size of the sample. Heterozygous individuals in F2 showed the differences pattern for each marker, indicating that the location of the SSR markers were far from each other in the genome.

INTRODUCTION

Southern and western flanks of Merapi volcano are the area in which local rice cultivars, including Mentik Wangi and Mentik Susu are grown (Yulianto et al., 2014). The cultivars have good taste and cooking characteristics, thereby being highly valued by consumers. They are included in late harvested cultivars, which need five months to harvest. They also have limited productivity and not adapted to limited water availability. Thus, their range of adaptation needs to be expanded, and the challenge of climate change also needs to be addressed. Crossing them with drought-tolerant cultivars can be a starting point

to obtain new cultivar with improved adaptability to limited water supply or even drought.

Bluebonnet is an Indica superior rice selected from the crossing of 'Rexoro' and 'Fortuna', drought-tolerant rice originating from the Philippines, which was released in Texas in 1944 (Tabien et al., 2008). Bluebonnet has been used as parent to develop new high-yielding varieties in Indonesia, Sigadis, which is the parent of high-yielding variety, Cisadane (Susanto et al., 2003). Kasalath is an Aus group of rice, which is known to be tolerant to drought and high temperature, and often used as material in plant breeding research (Thomson et al., 2007; Fukuta et al., 2012). In this study, both cultivars were used as donor parents for

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drought tolerance traits in crossing the forementioned local cultivars.

Crossing is an old method used to combine plant characteristics into one individual. This new individual is expected to have superior characteristics from both parents. After crossing, it is necessary to perform evaluation and selection to ensure that F1 resulting from the crossing carries the characteristics of both parents. F1 evaluation and selection can be performed effectively and efficiently using DNA markers. Further, in F2 generation, the genetic action needs to be studied whether a trait follows Mendelian inheritance pattern or merely quantitative in nature.

Specific DNA markers for drought-tolerant trait has been widely used, including SSR markers. SSR markers are codominant, and they may serve to assess allele diversity (Shehata et al., 2009). Al-Faifi et al. (2016) stated that the codominant nature of SSR also permits to detect high number of alleles per locus and contributes to reach higher levels of expected heterozygosity. The codominant marker system of SSR can recognize heterozygosity, and it would strongly enhance the effectiveness of determining genetic/molecular diversity (Thumilan et al., 2016). SSR or microsatellite markers are widely used because of their accuracy and high level of polymorphism, and spread evenly throughout the genome (Susanto et al., 2003). Selection using DNA markers is effective because it ensures that crossed individuals actually carry the target genes from both parents.

Primer markers used to detect drought-tolerant traits include RM72, RM20(A), RM228 and RM518 (Lin et al., 2007 and Barik et al., 2018). Aboulila (2015) used RM228 and RM518 as markers for drought-tolerant traits, while RM72 and RM20 (A) were used in the identification of drought-tolerant traits in some rice cultivars (Ramadan et al., 2015 and Anupam et al., 2017) and in the detection of the location and distance of genes on chromosomes in drought-tolerant rice plants (Sahoo and Sharma, 2018).

DNA markers are used to facilitate the molecular selection process of F1 and to examine the segregation of F2. F1 showing polymorphism can be selected for further testing. The use of DNA markers can shorten the selection time so that the breeding process becomes faster. The objectives of this research were to determine the trueness of the heterozygosity

of F1 individuals using molecular markers and to examine the F2 segregation pattern of the crosses that were made.

MATERIALS AND METHODS

This research was conducted in research facilities of Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada (UGM). The research was conducted from February 2017 to March 2019. The research was arranged in a completely randomized design (CRD), consisting of two local rice cultivars (Mentik Wangi (MW) and Mentik Susu (MS)) and two drought-tolerant rice cultivars (Bluebonnet (BB) and Kasalath (KS)), the F1 materials (obtained from crossing BB × MW and its reciprocal, BB × MS and its reciprocal, KS × MW and its reciprocal, and MS × KS), and the F2 materials derived from the F1 generations as treatments. The F2 were marker-assisted selected, leaving only F2 generations of BB × MW and BB × MS populations and their respective reciprocals that were evaluated for genetic action.

SSR marker-assisted evaluation was performed using RM72, RM518, RM228 and RM20(A) primers to determine the trueness of F1 as heterozygous individuals, initiated with polymorphism test between parents, followed by the evaluation of F1 generation populations. The F1 selection was performed to determine the purity of the molecular crossing results of 15 selected samples. Every molecular evaluation performed always includes the parent's DNA used as a comparison. Then self-pollination was performed on samples that show polymorphic bands (heterozygotes) molecularly.

For the F2 generation populations, the marker series were used to examine the genetic pattern of the markers, and since the markers are associated with drought tolerant trait, it may be also used as indication for genetic action for the tolerance trait. F2 populations evaluation was performed on 20 randomly selected individual samples, continued with molecular genotyping.

Genotyping of molecular markers was conducted by running the PCR results on agarose (Metaphore) and polyacrylamide electrophoresis gel. Data analysis was done by identifying heterozygous F1 individuals and counting their percentage for each crossing population. The results of genotyping on F2 population were analyzed with chi-square test on hypothetical Mendelian segregation patterns at $\alpha = 1\%$.

RESULTS AND DISCUSSION

Polymorphism test between parents using four SSR primers

All the primer produced various amounts and locations of bands (polymorphic) in the parents used. RM72 primer generated two to three bands between 160 bp and 220 bp. RM518 generated three to four bands between 160 bp and 220 bp. RM228 generated five to six bands between 100 bp and 180 bp, and RM20(A) primer generated five to seven bands between 200 bp and 500 bp (Figure 1). The selection of male and female parents could be done based on the location of different DNA bands. All the primers could be used as markers on F1 crossing results evaluation.

In RM72 primer, each parent showed two bands (195bp and 210bp) in Bluebonnet cultivars, three bands (160bp and 170bp) in Kasalath cultivars, and three bands (165bp, 175bp and 180bp) in Mentik Wangi and Mentik Susu cultivars. In RM518 primer, each parent had four bands (170bp, 175bp, 180bp and 210bp) in Bluebonnet cultivars, three bands (165bp, 168bp and 174bp) in Kasalath cultivars, and three bands (170bp, 175bp and 180bp) in Mentik Wangi and Mentik Susu cultivars. SSR marker RM518 could not be used to differentiate Bluebonnet, Mentik Wangi and Mentik Susu cultivars because they had same size of bands. In RM228 primer, each parent showed six bands (120bp, 125bp, 130bp, 145bp, 160bp and 175bp) in Bluebonnet cultivars, five bands (100bp, 105bp, 115bp, 125bp and 140bp) in Kasalath cultivars, and five bands (105bp, 110bp, 118bp, 125bp and 140bp) in Mentik Wangi and

Mentik Susu cultivars. While in RM20(A) primer, each parent had six bands (210bp, 235bp, 310bp, 345bp, 395bp and 550bp) in Bluebonnet cultivars, six bands (225bp, 245bp, 255bp, 295bp, 330bp and 445bp) in Kasalath cultivars, and five bands (230bp, 255bp, 280bp, 310bp and 400bp) in Mentik Wangi and Mentik Susu cultivars.

Parent's polymorphism testing was performed at the beginning of this research to maximize the use of molecular markers. Sraphet et al. (2015) stated that SSR has a high level of polymorphism, located spread among chromosomes and being able to detect the presence of loci associated with specific genes. Drought-tolerant traits can be detected molecularly using SSR markers. Primary markers used to detect drought-tolerant traits include RM72, RM20(A), RM228 and RM518 (Lin et al., 2007 and Barik et al., 2018).

F1 evaluation using SSR molecular markers

The results of the evaluation using SSR showed that the Bluebonnet × Kasalath crossing had the highest percentage of polymorphic F1 (100 %), consistent for the four primers used (Figure 2). Bluebonnet × Mentik Wangi crossing had as much as 75 % polymorphic F1 (Figure 3), Bluebonnet × Mentik Susu had as much as 44.44 % polymorphic F1 (Figure 4) and Mentik Wangi × Bluebonnet had as much as 46.67 % polymorphic F1 (Figure 5), consistent for the four primers used (Table 1). F1 samples from other crossings showed polymorphism, but some samples were not consistent in certain primers, so that further selection and observation were only performed for samples showing consistency in all primers.

The crossing of Kasalath × Bluebonnet produced three F1 samples. From the DNA band visualization,

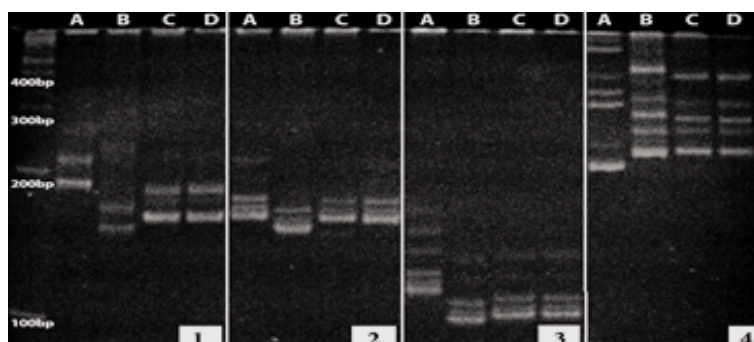


Figure 1. DNA band profiles from the amplification of four microsatellite markers using 8 % acrylamide gel of drought tolerant rice (A= Bluebonnet; B= Kasalath) and local rice (C= Mentik Wangi; D= Mentik Susu) using SSR molecular markers 1= RM72; 2 = RM518; 3= RM228; 4= RM20(A).

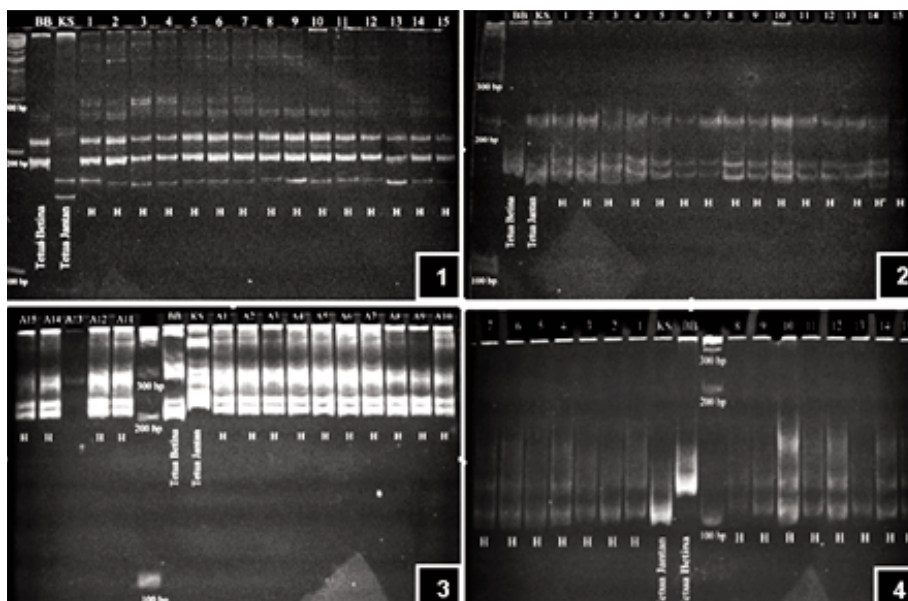


Figure 2. DNA band profiles from the amplification of four microsatellite markers using 8 % acrylamide gel on F1 resulted from crossing Bluebonnet vs Kasalath. Scale 100 bp; 1. RM72; 2. RM20(A); 3. RM228; 4. RM518. A= Band patterns follow the female parents, B= Band patterns follow the male parents, H= Band patterns follow both parents.

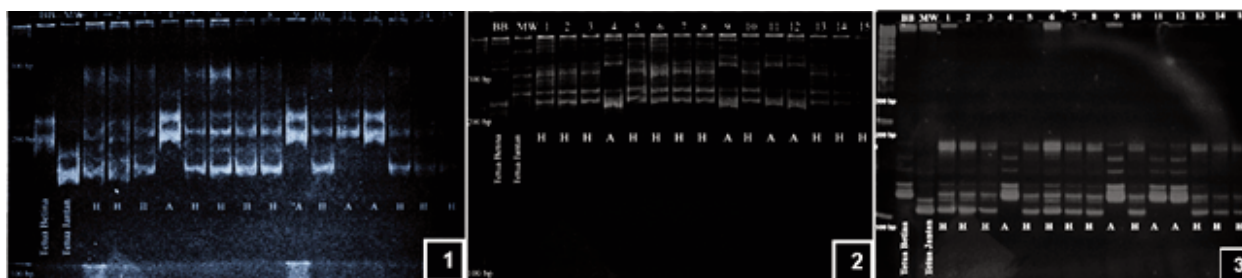


Figure 3. DNA band profiles from the amplification of four microsatellite markers using 8 % acrylamide gel on F1 resulted from crossing Bluebonnet × Mentik Wangi. Scale 100 bp; 1. RM72; 2. RM20(A); 3. RM228. A= Band patterns following the female parents, B= Band patterns following the male parents, H= Band patterns following both parents.

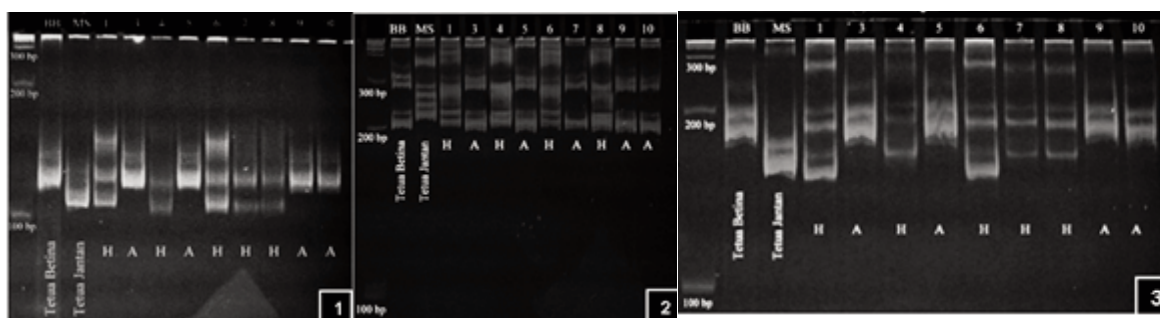


Figure 4. DNA band profiles from the amplification of four microsatellite markers using 8 % acrylamide gel on F1 resulted from crossing Bluebonnet × Mentik Susu. Scale 100 bp; 1. RM228.; 2. RM20(A); 3. RM72. A= Band patterns following the female parents, B= Band patterns following the male parents, H= Band patterns following both parents.

it was known that there was no consistent sample in the four primers used, so none of the observed sample could proceed to F2 generation. The crossing of Mentik Susu × Bluebonnet produced three

consistent F1 samples showing polymorphism on 17 samples from the three markers used. F1 from the crossing of Kasalath × Mentik Wangi (two out of six samples) and Mentik Susu × Kasalath (one

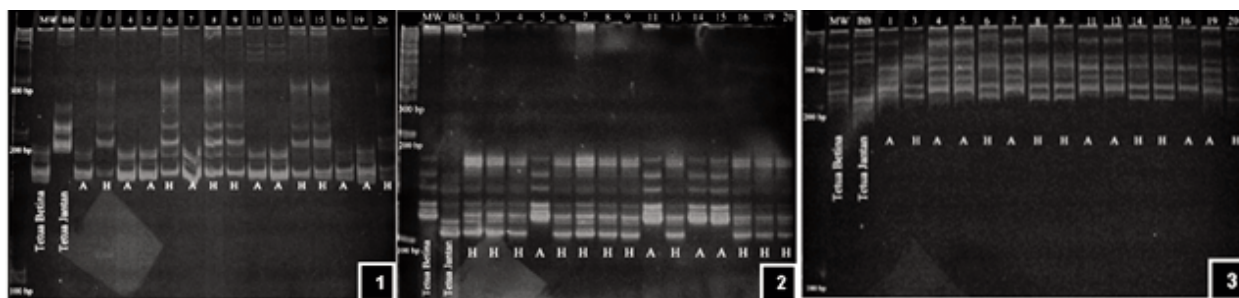


Figure 5. DNA band profiles from the amplification of four microsatellite markers using 8 % acrylamide gel on F1 resulted from crossing Mentik Wangi × Bluebonnet. Scale 100 bp; 1. RM72; 2. RM228.; 3. RM20(A). A= Band patterns following the female parents, B= Band patterns following the male parents, H= Band patterns following both parents.

Table 1. Percentage of polymorphic F1 resulted by crossing 4 selected parents using 4 primary SSR markers of drought-tolerant traits

Crossing combinations (♀ female × ♂ male)	Sample	Percentage of polymorphic F1 (%)			
		RM 72	RM 228	RM 20A	RM 518
Bluebonnet × Mentik Wangi	15	75.00	75.00	75.00	00.00
Bluebonnet × Mentik Susu	9	44.44	44.44	44.44	00.00
Kasalath × Mentik Wangi	6	00.00	33.33	83.33	83.33
Mentik Wangi × Bluebonnet	15	46.67	46.67	46.67	00.00
Mentik Wangi × Kasalath	8	12.50	00.00	00.00	37.50
Mentik Susu × Bluebonnet	15	60.00	66.67	46.67	00.00
Mentik Susu × Kasalath	0	00.00	00.00	00.00	00.00

out of eight samples) showed consistency on the primers used. Observation of F2 generation from these two crosses could not be performed because the samples died before they produced F2 seeds.

Self-pollination was performed to see the segregation pattern in F2 generation of the crossing results, which showed consistent polymorphism. The crossings observed up to F2 generation were Bluebonnet × Mentik Wangi, Bluebonnet × Mentik Susu, Mentik Wangi × Bluebonnet and Mentik Susu × Bluebonnet. The crossing of Bluebonnet × Kasalath showed molecular consistent results, but no F2 evaluation was performed because they did not carry local rice parent traits.

F2 segregation pattern

The results of molecular evaluation of F2 showed various types of segregation. The crossing of Bluebonnet × Mentik Wangi and the combined (pooled) population in RM72 markers showing a chi square value smaller or equal to 2.7 indicate an agreement with the Mendelian segregation law at a ratio of 1:2:1, while the other markers did not follow

Mendelian segregation law because the chi square value was greater than 2.7. Chi square value of Mentik Wangi × Bluebonnet, Bluebonnet × Mentik Susu and Mentik Susu × Bluebonnet crossings also showed segregation patterns that did not follow Mendelian segregation law in all markers used (Table 2 and Table 3).

The results of gel electrophoresis visualization using RM20(A) marker showed the presence of more than one DNA band fragment. In gel electrophoresis visualization, each parent’s genotype showed two DNA fragments, and each crossed F2 genotype had two to four DNA band fragments. Because of the presence of two DNA band fragments in the parent’s genotype, it was assumed that the RM20(A) marker is related to genes at 2 different loci. Testing was performed with two assumptions. The first assumption was that locus scores on the first and second row band, while locus 2 scores on the third and fourth bands.

The Chi-Square analysis results for the first and second assumption in F2 from Bluebonnet × Mentik Wangi crossing and combined (pooled) population at locus 1 and 2 showed that the population segregation

Table 2. Chi-square test of F2 population segregation using SSR marker

SSR marker	Cross combined (♀ female × ♂ male)	F2 Genotype			X ²	p-value
		A	H	B		
M72	Bluebonnet × Mentik Wangi	5	13	2	2.70	0.259200
	Mentik Wangi × Bluebonnet	3	6	11	9.60	0.008230**
	combined (Pooled)	8	19	13	1.35	0.509200
RM228	Bluebonnet × Mentik Wangi	11	7	2	9.90	0.007083**
	Mentik Wangi × Bluebonnet	4	4	12	13.60	0.001114**
	combined (Pooled)	15	11	14	8.15	0.016990**

Remarks: A= Band patterns following the Bluebonnet, B= Band patterns following the Mentik Wangi, H= Band patterns following both parents. (**) indicates a significant p-value at α= 1 %.

Table 3. Chi-square test of F2 population segregation using SSR marker

SSR marker	Cross combination (♀ female × ♂ male)	F2 Genotype			X ²	p-value
		A	H	B		
M72	Bluebonnet × Mentik Susu	10	6	4	6.8000	0.03337**
	Mentik Susu × Bluebonnet	1	2	4	3.8571	0.14540
	combined (Pooled)	11	8	8	5.1481	0.07622
RM228	Bluebonnet × Mentik Susu	2	9	9	5.1000	0.07808
	Mentik Susu × Bluebonnet	0	2	5	8.4286	0.01478**
	combined (Pooled)	2	11	14	11.5930	0.00304**

Remarks: A= Band patterns following the Bluebonnet, C= Band patterns following the Mentik Susu, H= Band patterns following both parents. (**) indicates a significant p-value at α= 1 %.

was in accordance with Mendelian segregation law at a ratio of 1:2:1. Meanwhile, the crossing of Mentik Wangi × Bluebonnet did not show results that are in accordance with the hypotheses (Table 4).

Chi-Square Analysis of F2 population genotype resulted from the crossing of Bluebonnet × Mentik Susu and combined (pooled) population in the first assumption locus number one showed a probability value of > 0.05 (Table 5.). This value shows that the genotype follows Mendelian segregation law. Chi-Square analysis in the first assumption locus number two and second assumption locus number one and two genotypes from the Bluebonnet × Mentik Susu crossing, reciprocal and combined (pooled) population showed a segregation ratio that was not in accordance with the Mendelian segregation law ratio of 1: 2: 1.

Contingency test was then performed to the scoring results of locus 1 and locus 2 for each F2 population. Contingency test aims to determine the independence of locus 1 and locus 2 in each individual. The results of the contingency test in F2 population showing the probability value of > 0.05

stated that the locus 1 and locus 2 in the individual were not mutually independent (linked) (Table 6.). The linked genes, or alleles, mean that they are on the same chromosome, in a close distance. According to Sutton (1903), genes and chromosomes are physically related to more than one gene on each chromosome. Segregation that occurs at both loci at close range is called co-segregation. Contingency test results that showed a probability value of < 0.05 means that locus 1 and locus 2 are mutually independent.

The results of the contingency test showed that in the first assumption, the only population stating that locus 1 and locus 2 are mutually independent was the F2 population resulted from the crossing of Mentik Wangi × Bluebonnet. Results of Bluebonnet × Mentik Wangi, Bluebonnet × Mentik Susu and Mentik Susu × Bluebonnet crossings showed linkage of both loci. The results of the contingency test in the second assumption stated that locus 1 and locus 2 of all F2 are linked or interlinked.

The RM20(A) marker on chromosome 12 in rice genome is polymorphic. Research conducted by Lin

Table 4. Chi-square test of F2 population segregation using SSR marker RM20(A)

Assumption	Locus	Cross combination (♀ female × ♂ male)	F2 Genotype			X ²	p-value
			A	H	B		
I	Locus 1	Bluebonnet × Mentik Wangi	7	11	2	2.70	0.25920
		Mentik Wangi × Bluebonnet	1	7	12	13.90	0.00096**
		Combined (Pooled)	8	18	14	2.20	0.33290
	Locus 2	Bluebonnet × Mentik Wangi	5	12	3	4.40	0.54880
		Mentik Wangi × Bluebonnet	1	7	12	17.60	0.00096**
		Combined (Pooled)	6	19	15	4.15	0.12560
II	Locus 1	Bluebonnet × Mentik Wangi	5	11	4	0.30	0.86070
		Mentik Wangi × Bluebonnet	2	6	12	13.20	0.00136**
		Combined (Pooled)	7	17	16	4.95	0.08416
	Locus 2	Bluebonnet × Mentik Wangi	9	8	3	4.40	0.11080
		Mentik Wangi × Bluebonnet	1	6	13	17.60	0.00015**
		Combined (Pooled)	10	14	16	5.40	0.06721

Remarks: A= Band patterns following the Bluebonnet, B= Band patterns following the Mentik Wangi, H= Band patterns following both parents. (**) indicates a significant p-value at α= 1 %.

Table 5. Chi-square test of F2 population segregation using SSR marker RM20(A)

Assumption	Locus	Cross combination (♀ female × ♂ male)	F2 Genotype			X ²	p-value
			A	H	B		
I	Locus 1	Bluebonnet × Mentik Susu	7	9	4	1.10	0.57690
		Mentik Susu × Bluebonnet	0	3	4	4.71	0.09469
		Combined (Pooled)	7	12	8	0.40	0.81570
	Locus 2	Bluebonnet × Mentik Susu	9	5	6	5.90	0.05234
		Mentik Susu × Bluebonnet	1	0	6	14.10	0.00085**
		Combined (Pooled)	10	5	12	11.00	0.00409**
II	Locus 1	Bluebonnet × Mentik Susu	10	6	4	6.80	0.03337**
		Mentik Susu × Bluebonnet	1	2	4	3.80	0.14540
		Combined (Pooled)	11	8	8	5.10	0.07622
	Locus 2	Bluebonnet × Mentik Susu	7	6	7	3.20	0.20190
		Mentik Susu × Bluebonnet	0	1	6	13.80	0.00098**
		Combined (Pooled)	7	7	13	8.90	0.01153**

Remarks: A= Band patterns following the Bluebonnet, C= Band patterns following the Mentik Susu, H= Band patterns following both parents.(**) indicates a significant p-value at α = 1 %.

Table 6. Four crossed and reciprocal contingency tests

Assumption	Cross combination (♀ female × ♂ male)	X ²	p-value
I	Bluebonnet × Mentik Wangi	7.937220	0.159729
	Mentik Wangi × Bluebonnet	22.066330	0.000509**
	Bluebonnet × Mentik Susu	10.055550	0.073676
II	Mentik Susu × Bluebonnet	1.555550	0.906571
	Bluebonnet × Mentik Wangi	4.549242	0.473322
	Mentik Wangi × Bluebonnet	10.961540	0.052148

et al. (2007) mentioned that RM20(A) marker was associated with leaf-rolling traits as an indicator of drought tolerance. This marker is very effective for molecular-based selection in the formation of drought-tolerant rice. RM20(A) markers have the ability to amplify two DNA fragments of different sizes (Freeg et al., 2016). Drought-tolerant QTL on chromosome 12 that has been identified is qDTY12.1. According to Mishra et al. (2013), qDTY12.2 is one of QTL showing a large influence in increasing rice yields on drought stress. On chromosome 12, QTL qDTY12.1 is located at 10.2, cm and RM20(A) marker is at 9.8 cm (Bernier et al., 2007). The use of RM20(A) marker can be associated with QTL qDTY12.1, which is a tolerant gene.

The difference between the F2 segregation ratio and the Mendelian segregation law ratio was due to the small sample size, so that it is not able to represent the entire population. Different ratio at each marker was caused by the location of the traits controller on different chromosomes. The ideal population size for F2 population testing is 50–1000 individuals or all individuals in F2 population. According to Lestari et al. (2018), population size that is too small in the analysis also causes fragmentation of the linkage groups and inaccurate arrangement of loci on the genetic map.

Molecular observations on F2 showed different segregation patterns between primers. This was due to the location of genes of different chromosomes, so that when segregation and pairing during meiosis occurred, the random separation caused molecular differences in F2 heterozygous individuals. This opinion is in accordance with Solis et al. (2018), stating that RM72 marker sized 60.9 cM in chromosome 8 was located very close to QTL qDTY8.1, and RM228 marker sized 130.3 cM in chromosome 10 was located on QTL qDTY10.1. According to the study by Swamy et al. (2013), QTL qDTY10.1 was identified as having additives and high phenotype variants in drought tolerance characteristic. The RM20(A) marker on chromosome 12 in the rice genome was polymorphic. A study conducted by Lin et al. (2007) stated that RM20(A) marker was associated with leaf rolling trait as an indicator of drought tolerance, while RM518 marker was used to mark QTL Ir8.1 (in chromosome 8 that is close to RM72) and QTL Ir4.1 (in chromosome 4 that is close to RM518) and were mapped by using QTLs for the leaf rolling trait (Lin et al., 2007).

CONCLUSIONS

Polymorphism test of parental using SSR markers (RM72, RM228, RM518 and RM20(A)) showed polymorphism between parental of local and drought-tolerant rice, then these markers can be used for the markers in F1 and F2. The heterozygous individuals of F1 based on SSR markers showed that the percentage in Bluebonnet × Mentik Wangi (75 %), Bluebonnet × Mentik Susu (44.44 %) and Mentik Wangi × Bluebonnet (46.67 %) was consistent in each primer used. We could not confirm that the F2 populations of some crossings following Mendelian segregation pattern because sample size was not adequate. The heterozygous individuals in F2 showed the different pattern for each marker, indicating that SSR markers used positioned far to each other in the genome.

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