COVERING LETTER

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Marker validation for salt tolerance in Indica rice

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This research has novelty in terms of using the germplasm under study. Research on molecular markers in rice plants has been widely used, but the application on Indica rice cultivars, especially rice from the plant breeder of Unversitas Jenderal Soedirman (Inpago Unsoed 1, Inpari 79 Agritan, and Unsoed Parimas).

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Ir. Suprayogi, M.Sc., Ph.D

Marker validation for salt tolerance in Indica rice

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Abstract. Development of salt tolerant rice variety is of importance with concern to the use of coastal area for rice production, and consequently development of rapid and accurate method of variety selection. Molecular markers associated with Quantitative Trait Loci (QTL) for salt tolerance in rice has been reported elsewhere. The objectives of this research is to identify molecular markers associated with salt tolerance in rice. The research was carried out from September 2020 to February 2021 at Plant Breeding and Biotechnology Laboratory, Faculty of Agriculture, Jenderal Soedirman University. Nine Simple Sequence Repeat (SSR) markers of Rice Microsatellite (RM) 212, RM 222, RM 223, RM 224, RM 225, RM 3412, RM 342, RM 8094, RM 8264 and one Sequence Tagged Site (STS) marker of Wn 11463 were evaluated for their association with salt tolerance. The rice varieties consisted of salt tolerant Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; salt sensitive IR 29, and commercial variety Cisadane, Pelopor, Dendang, Lambur, Siak Raya, Unsoed Parimas, Inpago Unsoed 1. Nona Bokra PCR amplicon is used as control for DNA band scoring. Binary data of the amplicon banding pattern were analyzed using Principal Component Analysis (PCA) to determine grouping of evaluated varieties in relation to their tolerance to assigned markers. The result demonstrated that the evaluated rice varieties fall into four group of marker resemblances. Among the evaluated SSR markers, RM 3412, RM 342, RM 8094 and Wn 11463 significantly contributed to the grouping. Based on the characteristics of salt tolerant and salt sensitive control varieties, RM 225, RM 3412, RM 342, RM 8094 and Wn 11463 markers could be concluded to be associated with salt tolerance in indica rice.

Key words: salt tolerance, molecular marker, SSR, STS, Principal Component Analysis,

Running title: marker validation for salt tolerance

INTRODUCTION

Rice is a major staple food crops of most Indonesian people (Suprayogi *et al*., 2019). Effort to maintain national self-sufficiency in rice production has been challenged by the decreasing area of arable paddy field due to land conversion to non-agriculture purposes (Suprayogi *et al*., 2019) which is estimated not less than 38.000 ha year^{-1} (Suprihanto *et al*., 2019; Nafisah *et al*., 2017). As such, to date, rice production area should be extended to marginal land that include coastal area which during dry season is affected by intrusion of sea water resulting in saline condition. Salt stress is one of the most severe problem for rice grown in coastal area. Response and adaptation of rice to salt stress is a complex process. Osmotic stress, ionic toxicity, and nutritional deficiencies causes root growth inhibition, leaf rolling, reduced plant height and tiller number, and spikelet sterility, which, ultimately, leads to a reduced yield (Qin *et al*., 2020). Among the solution for salinity problem of rice production in coastal area is the use of salt tolerant rice variety (Suprayogi *et al*., 2019; Nafisah *et al*., 2017).

Breeding for salt tolerant rice variety has been hindered by the limited availability of salt tolerant germplasm, lack of studies on the mechanism of genetic control of salt tolerance, and a lack of information of the linking between salt tolerance traits and desirable agronomic characters (Yen *et al*., 2011). Slow progress of breeding of salt tolerant rice is also due to the complexity of several tolerance mechanisms involved, inadequate screening techniques, low selection efficiency and poor understanding of salinity and environmental interactions (Lang *et al*., 2017).

Salinity tolerance in rice is a polygenic trait (Ismail and Horie, 2017). Recent development in molecular marker analysis has enabled rapid and accurate identification of individual genes or otherwise Quantitative Trait Loci (QTL) controlling a trait of interest. QTL mapping approach has been used to describe the genetic architecture of salt tolerant in rice using mapping populations derived from crosses between salt-sensitive and salt tolerant varieties (Cheng *et al*., 2015, Wang *et al*.. 2011, Liu *et al*., 2019). Some of the major salt tolerance QTLs included *qSKC-1* and *qSNC-7* which responsible for regulation of K^+/Na^+ homeostasis under salt stress and explain 48.5% and 40.1% of the total phenotypic variance, respectively; *qSKC-1* which encode HKT-type transporter family; *Saltol* which act to maintain shoot Na^+/K^+ homeostasis, *qSE3* which encode a K^+ transporter gene; *OsHAK21* promotes seed germination and seedling

establishment, *qST1* and *qST3* which conferr salt tolerance at the young seedling stage (Qin *et al*., 2020). *qST1.1 was reported to* play role in salt tolerance in *SR86*, explained 62.6% of the phenotypic variance (Wu *et al*., 2020)

Simple Sequence Repeat (SSR, microsatellite) marker has been used to identify salt tolerance or introgression genes to develop new cultivars (Mizan *et al*., 2015). Using SSR markers RM 3412, RM 510, and RM 336, Islam *et al*. (2015) identified five salt tolerant varieties out of the 25 evaluated varieties. Ali *et al*., (2014) identified four salt tolerant varieties out of 33 varieties using morphological markers and SSR markers RM 8046, RM 336, and RM 8094. (Emon *et al*., 2015) Reported STS (Sequence Tagged Sites) marker Wn 11463 in marker assisted selection (MAS) for rice with salt tolerant gene *SKC1*. Using 17 SSR markers, included a significantly associated salinity tolerance marker Wn11463 (https://archive.gramene.org/markers/microsat/all-ssr.html), saline tolerant variety Inpari Unsoed 79 Agritan could be separated distantly from the susceptible variety IR29 (Suprayogi, *et al*. 2021).

This study is aimed at identifying and validating molecular markers for salt tolerance in several commercial indica rice varieties. Such validated markers will later be used for rapid and accurate screening technique for salt tolerant rice genotypes.

MATERIALS AND METHODS

Materials

Eleven indica rice varieties were used for sources of DNA. These varieties included salt tolerant rice varieties Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; salt sensitive rice variety IR 29; and common commercial rice variety Cisadane, Pelopor, Dendang, Lambur, Siak Raya, Unsoed Parimas, Inpago Unsoed 1. Aluvial soils, organic manure, and rice husk were used as growth media. The chemicals used for markers analysis were Cetyl Trimethyl Ammonium Bromide (Amresco), NaCl (Merck), HCl, Tris base (Merck), Na EDTA, NaOH, polyvinyl Pyrrolidine (Amresco), β-Mercapto-ethanol (Merck), aquades (H_2O), liquid nitrogen, chloroform (Merck), ammonium asetat (Merck), isopropanol (Merck), ethanol 70%, ethanol 96% (Merck), RNAse (Geneaid), nuclease free water (Thermo Fisher Scientific), agarose, TBE (Tris-Boric-EDTA) 1x, DNA ladder 100 bp (Bioline), loading dye (Thermo Fisher Scientific), ethidium bromide (Invitrogen), 10 primer pairs (Table 1), and Dream Taq PCR Master Mix (Thermo Fisher Scientific).

Procedures

Preparation of Samples

The seeds was soaked in water for 24 hours to facilitate the germination. The media containing alluvial soils, organic manure, and rice husk (3:1:1) used for growth of the seeds. Two weeks old leaves were used as materials for DNA sources.

DNA Extraction and Quantification

DNA extraction was carried out using CTAB method with modification (Zannati *et al*., 2015). Samples of young leaves were ground using a mortar and pestle in the presence of liquid nitrogen. The sample was put into a 2 ml Eppendorf tube, added 1000µl of CTAB Buffer mix, and homogenized by vortexing for 10 seconds. The sample in the tube was incubated at 65^0C in a water bath, and was homogenized every 10 minutes. The chloroform (500 µl) was added to the tube, followed by 7 minutes centrifugation. The supernatant was transferred to a new 1.5 ml. The chloroform (500 µl) was added, then centrifuged again for 7 minutes. The supernatant was transferred to a new 1.5 ml Eppendorf tube. Ammonium acetate (0.8µl/10 µl) and cold isopropanol (5,4µl/10µl) were then added and homogenized. The tubes were stored in the refrigerator at 4^0C for 24 hours. After an overnight precipitation, the tube was allowed to stand at room temperature before centrifugation for 7 minutes. The supernatant was discarded, and the pellet was allowed to dry. The ethanol of 70% (700µl) was added, then centrifuged for 1 minute. The supernatant was discarded, and the pellet was allowed to dry. 90% ethanol (700µl) was added. The tube was centrifuged for 1 minute and allowed the pellet to dry. Tris-Cl-EDTA (TE) buffer (30 µl) and RNAse enzyme (1µl) were added to dilute the pellet. The tube containing DNA was then incubated at 37°C for 1 hour. For maintenance of DNA structure, DNA was stored in a deep freezer.

Concentration and purity of DNA were measured using nanophotometer. The DNA were diluted 100x using Tris-EDTA (TE) as solvent. A total of 3µl of diluted DNA was dropped on a nanophotometer. The purity and concentration numbers appeared automatically on the instrument.

Marker Amplification

Nine SSR markers RM 212, RM 222, RM 223, RM 224, RM 225, RM 3412, RM 342, RM 8094, RM 8264 and one STS marker Wn 11463 were evaluated for their possible association with salt tolerance (Tabel 1).

Table 1. List of primers used in this study

No	Classification	Primer name		Nucleotida sequences	Size of amplicon (bp)	Ta (°C)	Traits related	Literature review	Source of information
1.	SSR	RM	*forward*	CCACTTTCAGCTACTACCAG	136	53,5	Drought tolerance	Rana *et al.* (2009)	archive.gram

		212	*reverse*	CACCCATTTGTCTCTCATTATG				& Gomez *et al*. (2010)	ene.org
2.	SSR	RM 8264	*forward*	ACGCTCCTCGCTTTCTAC	206	55,5	Agronomic traits (total of secondary branch and *seed-set ratio)*	Kebriyaee *et al*. (2012) & Ma *et al.*, (2019)	archive.gram ene.org
			reverse	GCACCTCACACCAGTAATTC					
3.	SSR	RM 342	*forward*	CCATCCTCCTACTTCAATGAAG	141	58	Aromatic and drought tolerance	Shams *et al.* (2012) & Yue *et al.* (2005)	archive.gram ene.org
			reverse	ACTATGCAGTGGTGTCACCC					
4.	SSR	RM 225	*forward*	TGCCCATATGGTCTGGATG	140	53	Length of grain	Khatibani *et al*. (2019)	archive.gram ene.org
			reverse	GAAAGTGGATCAGGAAGGC					
5.	SSR	RM 222	*forward*	CTTAAATGGGCCACATGCG	213	56,3	Agronomic trait (numbers of tillers)	Chaudari (2018)	archive.gram ene.org
			reverse	CAAAGCTTCCGGCCAAAAG					
6.	SSR	RM 224	*forward*	ATCGATCGATCTTCACGAGG	157	56,3	Blast disease	Zarbafi *et al*. (2019)	archive.gram ene.org
			reverse	TGCTATAAAAGGCATTCGGG					
7.	SSR	RM 223	*forward*	GAGTGAGCTTGGGCTGAAAC	165	55	Not linked to specific trait	Jain *et al*. (2006)	archive.gram ene.org
			reverse	GAAGGCAAGTCTTGGCACTG					
8.	SSR	RM 8094	*forward*	AAGTTTGTACACATCGTATACA	209	55	Salinity tolerance	Niones (2004)	archive.gram ene.org
			reverse	CGCGACCAGTACTACTACTA					
9.	SSR	RM 3412	*forward*	AAAGCAGGTTTTCCTCCTCC	211	55	Salinity tolerance	Saha *et al.* (2016)	archive.gram ene.org
			reverse	CCCATGTGCAATGTGTCTTC					
10.	STS	Wn 1146 3	*forward*	TCCTCCTTCTCTCGCAAC	120	54,9	Salinity tolerance	Emon *et al*. (2015)	Emon *et al*. (2015)
			reverse	GATCCACTCGTCACAGG					

A total volume of 25 µl Polymerase Chain Reaction-mix solution was used for PCR reaction. The PCR mix comprises of 1 µl (50 ng) DNA samples. 1 µl forward primer, l µl reverse primer, 12.5 µl Dream Taq PCR Master-Mix, and 9.5 µl nuclease free water. The PCR following the program as seen in Table 2.

Table 2. PCR program

Stage	Temperature (^{0}C)	Time (minutes)	Cycle
Pre-denaturation	95^{0}C	5 minutes	1
Denaturation	95^{0}C	1 minute	35
Annealing	53,5-58^{0}C	1 minute	35
Extension	72^{0}C	1 minute	35
Final-extension	72^{0}C	5 minutes	1
Refrigation	4^{0}C	∞	

Amplicon Visualization

The DNA quality and amplicon visualization was checked by Agarose Gel electrophoresis. The DNA ladder was loaded into the first well. Meanwhile, 3µl of PCR product was mixed with 2µl of loading dye and loaded put into the well. Electrophoresis was carried out at 50 volt for 30 minutes. The electrophoretic gel was immersed in EtBr for 20 minutes, then visualized under UV-transilluminator.

Data Analysis

Polymorphic Information Content (PIC) value was used as a standard for evaluating each genetic marker based on the amplified DNA bands. The PIC value is obtained by calculating the number of amplified alleles using application provided on https://gene-calc.pl/pic. The value of PIC is divided into three classes, PIC>0.5 = very informative, 0.25>PIC>0.5 = moderate informative, and PIC<0.25 = low informative (Carsono *et al*., 2014). PCR amplicon (DNA bands with different molecular weight) was converted into binary data by scoring. Notation 1 is given to DNA bands with similar molecular weight to Nona Bokra, 0 is given to DNA bands which is not similar to the molecular weight of Nona Bokra. The binary data were then used for PCA analyses using XLSTAT 2020 for grouping of the evaluated varieties (Vidal *et al*., 2020).

RESULTS AND DISCUSSION

Polymorphic Information Content (PIC) Value

PIC value describes level of informativeness of a marker. Marker with high PIC value is recommended to be used for molecular screening as this marker could better differentiate different genotypes. PIC analysis on the amplified alleles (Figure 1) shows that the PIC value of evaluated markers ranged from 0.1516 to 0.6781 (Table 3). Followings Carsono *et al*. (2014), the evaluated markers could be grouped into three classes, i.e.: RM 223, RM 3412, RM 342 as very informative markers, RM 212, RM 222, RM 223, RM 224, RM 225, RM 8264, Wn 11463 as moderate informative markers, and RM 8094 is low informative marker.

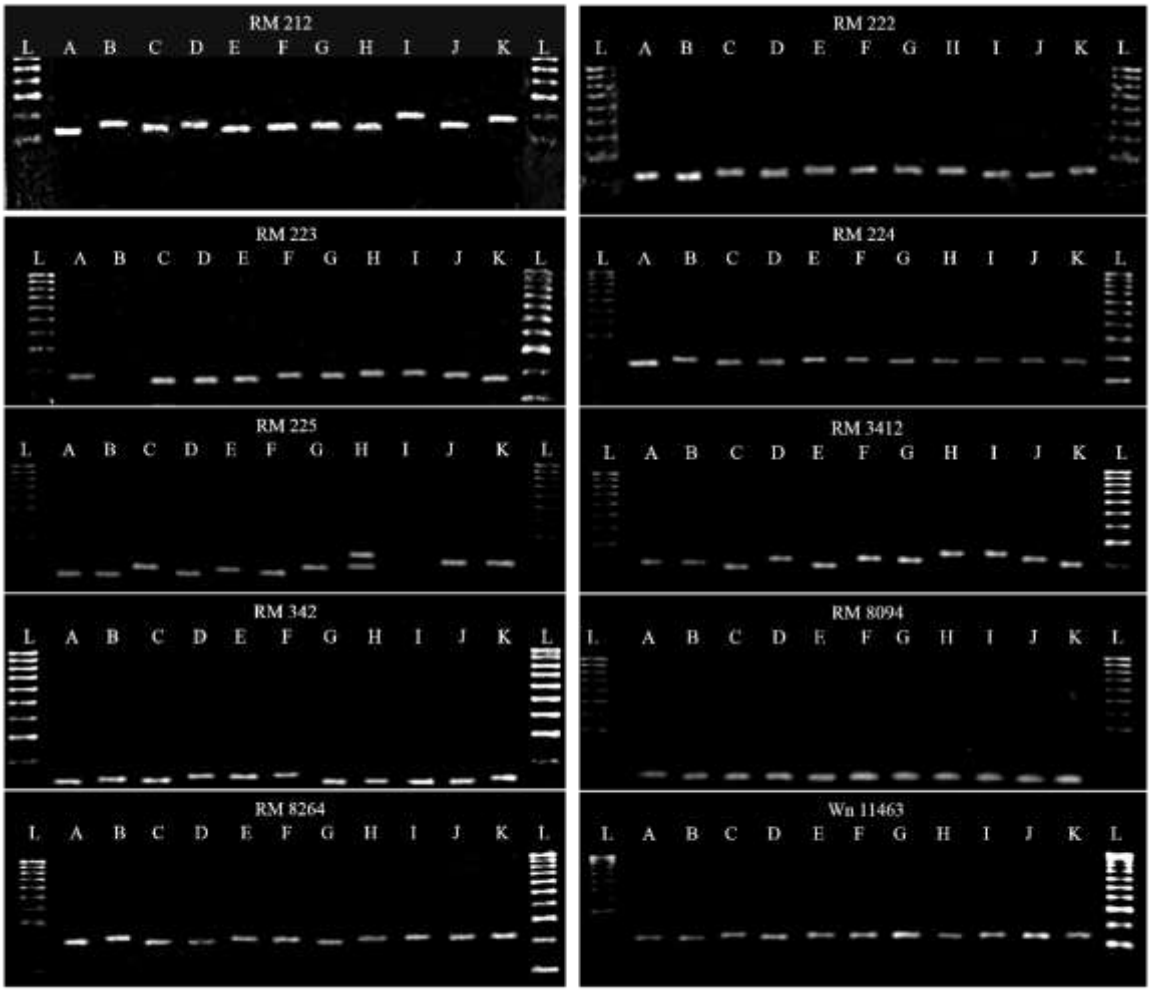

Figure 1. DNA band in 2% gel agarose (L= Ladder 100 bp; D = Atomita 2; H = Siak Raya; A = Nona Bokra; E = Pelopor; I = Unsoed Parimas; B = Inpari Unsoed 79 Agritan; F = Dendang; J = Inpago Unsoed 1; C = Cisadane; G = Lambur; K = IR 29).

Table 3. Chromosome location, number of alleles, and PIC of the evaluated markers

Marker	Chromosome location	Number of alleles	PIC
RM 212	1	3	0.4732
RM 222	10	2	0.3557
RM 223	8	3	0.5632
RM 224	11	2	0.318
RM 225	6	3	0.4762
RM 3412	1	4	0.6781
RM 342	8	3	0.5262
RM 8094	1	2	0.1516

RM 8264	8	2	0.3557
Wn 11463	1	2	0.318

PCA BiPlot Analysis of Degree of Resemblance

Analysis of degree of resemblance and identification of molecular markers for salt tolerance in rice was carried out using PCA Biplot analysis. Varieties are considered to have similar characteristics when they are at two adjacent points. The closer the distance between two points, the higher the level of similarity of two investigated varieties, based on a marker. Figure 2 shows the result of Biplot analysis of which the evaluated varieties are fallen into four quadrants. Cisadane, Unsoed Parimas, and Siak Raya are in one quadrant group and characterized by RM 224. No information of salt tolerance has been reported of these three varieties. Cisadane is a lowland rice variety that does not have resistance to any environmental stress conditions, except resistant to brown plant-hopper biotypes 1 and 2, and bacterial leaf blight (Suprihatno *et al*., 2009). Unsoed Parimas is a drought and aluminum tolerant variety, which is also resistant to *Pirycularia oryzae* race 0733 and somewhat resistant to *P. oryzae* race 133 (Balai Penelitian dan Pengembangan Pertanian, 2019). Siak Raya is a tidal swamp rice variety that tolerant Fe and Al (Dirgasan *et al*., 2019). Siak Raya is a moderate tolerant variety to salinity stress at seedling stage (Safitri *et al*., 2017).

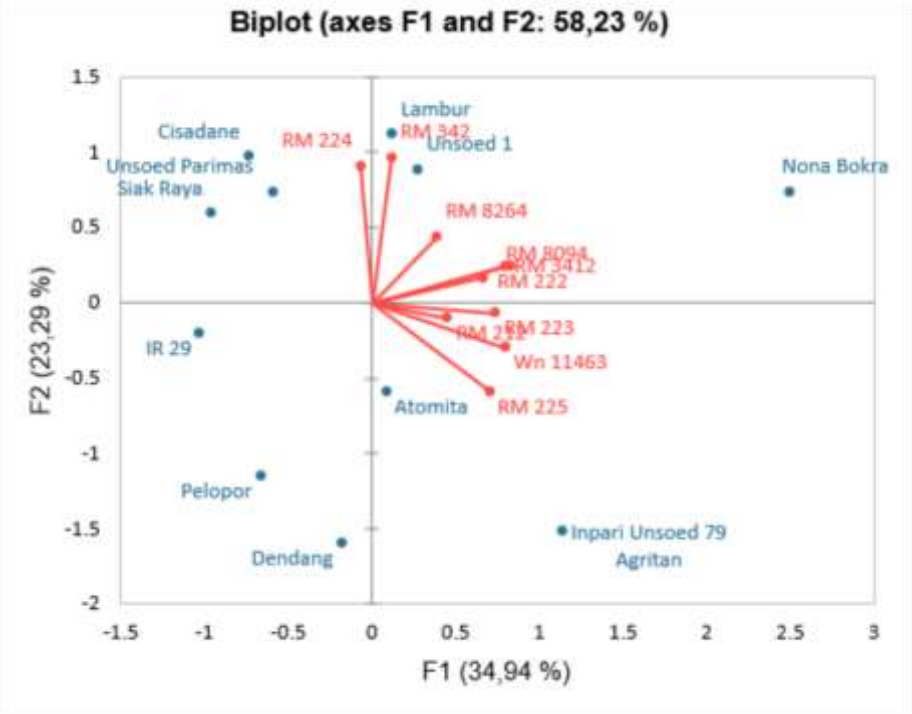

Figure 2. Varietal grouping based on PCA Biplot Analysis

Lambur, Inpago Unsoed 1, and Nona Bokra varieties were in one group and characterized by RM 222, RM 342, RM 8264, RM 8094, and RM 3412 markers. Based on that group, Lambur and Unsoed 1 varieties may be categorized as tolerant varieties as they were in the same group with Nona Bokra as positive control. Lambur is somewhat tolerant to salt, Al, and Fe. Inpago Unsoed 1 is a drought tolerant and moderately tolerant to iron (Fe) toxicity (Badan Penelitian dan Pengembangan Pertanian, 2019).

Atomita 2 is in the same group with Inpari Unsoed 79 Agritan, and characterized by RM 223, RM 212, Wn 1146, and RM 225 markers. Inpari Unsoed 79 Agritan is known to be tolerant to salt stress of 12 dSm^{-1} at seedling stage (Balai Besar Penelitian Tanaman Padi, 2020). Atomita 2 is a salt tolerant tidal swamp rice variety up to salinity of 4-6 mhos/cm (Balai Besar Penelitian Tanaman Padi, 2020). The similarity in the characteristics of these two varieties is also due to the fact that Atomita 2 is the parent of salt tolerant donor for Inpari Unsoed 79 Agritan. Wn 11463 marker was reported to be closely linked to the *SKC1* salt tolerance gene (Emon *et al*., 2015). Atomita and Inpari Unsoed 79 Agritan is also in the same quadrant group with STS marker Wn 11463.

IR 29, Pelopor, and Dendang were in one group but not characterized by any marker. Interestingly, PCA Biplot analysis placed IR 29, Pelopor, and Dendang at the opposite quadrant of Nona Bokra. IR 29 is a salt sensitive variety (Herlina *et al*., 2011). The use of IR 29 as a negative control and the characteristics of the three varieties which were the most different from Nona Bokra determined that Pelopor and Dendang are salt sensitive varieties. Pelopor was reported previously to be a salt sensitive variety (Zannati *et al*., 2015). Dendang, however, is a tidal swamp rice which is reported to

quite tolerant of Fe, Al, and salinity (Suprayogi *et al*., 2019). The disagreement between our result and that of (Zannati *et al*., 2015) on the grouping of Dendang still need to be investigated further using more salt tolerance markers published elsewhere.

Table 4. Contribution of marker in grouping of varieties (%)

Marker	1st Dimension (F1)	2nd Dimension (F2)
RM 212	5.384	0.398
RM 222	11.443	1.110
RM 223	14.337	0.186
RM 224	0.107	32.463
RM 225	12.924	13.511
RM 3412	16.813	2.346
RM 342	0.387	36.585
RM 8094	17.944	2.342
RM 8264	4.025	7.695
Wn 11463	16.635	3.362

The grouping of varieties based on the results of binary data analysis shows that markers used in this research could differentiate salt tolerant varieties from the sensitive ones. Each marker has a different contribution to grouping model (Figure 2). Table 2 shows that in the F1 (first) dimension, RM 8094 markers contributed the highest in the grouping of varieties, followed by RM 3412, and Wn 11463. Meanwhile, in F2 (second) dimension, RM 342 had the highest contribution followed by RM 224.

Analysis on contributing markers to the grouping of varieties (Table 4) and PCA Biplot (Figure 2) demonstrated that RM 8094, RM 3412, Wn 11463, and RM 342 significantly related to salt tolerant varieties of Nona Bokra, Atomita-2 and Inpari Unsoed 79 Agritan. This result is in accordance with previous report of (Emon *et al*., 2015). RM 3412 and RM 8094 were linked to Saltol QTL which is located on chromosome 1 (Niones, 2004). Main genes for salt tolerance traits (*Saltol*) is mapped on chromosomes 1 and 8 (Lang *et al*., 2017). The STS marker Wn 11463 is significantly linked to salt tolerant gene, *SKC 1* (Emon *et al*., 2015). The RM 342 marker is located on chromosome 8 (Jain *et al*., 2006). Salt tolerance gene on chromosome 8 controls tolerance at vegetative phase at EC (electrical conductivity) below 10 dSm^{-1} (Lang *et al*., 2017).

This research also found that the drought tolerant rice variety Inpago Unsoed 1 fall in the same quadrant of salt tolerant variety Nona Bokra (Figure 2). This may suggest the drought tolerance and salt tolerance shares some common mechanisms in plant. This argument is based on (Huang *et al*., 2009) that *DST* gene of salt tolerance also relates to drought tolerance. This gene is located on chromosome 3 and functions to control the movement of stomata during drought and high salt conditions through the regulation of genes that involved in ROS (Reactive Oxygen Species) homeostasis (Huang *et al*., 2009).

This study demonstrated that markers RM 8094, RM 3412, Wn 11463, and RM 342 could be used to differentiate salt tolerant rice varieties from the sensitive ones. Based on previously reported salt tolerance markers, the present study also validated the strong relation between SSR marker RM 3412 and STS marker Wn 11463 to salt tolerance trait.

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[biodiv] Submission Acknowledgement

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Ahmad Dwi Setyawan <smujo.id@gmail.com> Tue, May 31, 2022 at 1:20 PM
To: Suprayogi Suprayogi <suprayogi@unsoed.ac.id>

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Thank you for submitting the manuscript, "Marker validation for salt tolerance in Indica rice" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

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2 messages

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To: Suprayogi Suprayogi <suprayogi@unsoed.ac.id>, Prita Sari Dewi <p_saridewi@yahoo.com>, Eka Oktaviani <oktaviani@unsoed.ac.id>, Alwa Widi Aisya <alwawidiaisyaa@gmail.com>

Suprayogi Suprayogi, Prita Sari Dewi, Eka Oktaviani, Alwa Widi Aisya:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Marker validation for salt tolerance in Indica rice".

Our decision is: Revisions Required

Reviewer A:

Only Only three markers used in this study are related to salt tolerance, i.e., RM 3412, Wn1134 and RM 8094. The use of other markers that are not related to salt tolerance to make the grouping of the tested varieties will make the conclusion that a variety with an unknown salt tolerance level is considered as salt tolerance since the variety is grouped together with a salt tolerance variety in a group (quadrant) is questionable. In practice, validation of salt tolerance markers just simply be made by observing the presence or absence of a specifically related salt tolerance marker band in a tested variety. Thus, in this study, it is better that the tested varieties are only validated for the presence or the absence of the three salt tolerance markers mentioned above. Involving other markers that are not related to salt tolerance will make the interpretation of the results more difficult. Please clarify and revise.

Recommendation: Revisions Required

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suprayogi 1 <suprayogi@unsoed.ac.id> Wed, Jul 20, 2022 at 9:34 AM
To: Smujo Editors <smujo.id@gmail.com>
Cc: Prita Sari Dewi <p_saridewi@yahoo.com>, Eka Oktaviani <oktaviani@unsoed.ac.id>, Alwa Widi Aisya <alwawidiaisyaa@gmail.com>

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Thank you for the fast forwarding manuscript after review. But you attached the wrong file. Please kindly send the reviewed version. Thank you very much. Best regards.

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COVERING LETTER

Dear **Editor-in-Chief,**

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Marker validation for salt tolerance in Indica rice

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This research has novelty in terms of using the germplasm under study. Research on molecular markers in rice plants has been widely used, but the application on Indica rice cultivars, especially rice from the plant breeder of Unversitas Jenderal Soedirman (Inpago Unsoed 1, Inpari 79 Agritan, and Unsoed Parimas).

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Place and date:

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Sincerely yours,

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Ir. Suprayogi, M.Sc., Ph.D

Marker validation for salt tolerance in Indica rice

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Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted: ..... 2016.

Abstract. Development of salt tolerant rice variety is of importance with concern to the use of coastal area for rice production, and consequently development of rapid and accurate method of variety selection. Molecular markers associated with Quantitative Trait Loci (QTL) for salt tolerance in rice has been reported elsewhere. The objective of this research was to identify molecular markers associated with salt tolerance in rice. The research was carried out from September 2020 to February 2021 at Plant Breeding and Biotechnology Laboratory, Faculty of Agriculture, Jenderal Soedirman University. Nine Simple Sequence Repeat (SSR) markers and one Sequence Tagged Site (STS) marker were evaluated for their association with salt tolerance. The rice varieties consisted of three salt tolerant varieties, salt sensitive variety, and six commercial varieties. Nona Bokra PCR amplicon is used as positive control for DNA band scoring. Binary data of the amplicon banding pattern were analyzed using Principal Component Analysis (PCA) to determine grouping of evaluated varieties in relation to their tolerance to assigned markers. The result demonstrated that the evaluated rice varieties fall into four group of marker resemblances. Among the evaluated SSR markers, RM 3412, RM 342, RM 8094 and Wn 11463 significantly contributed to the grouping. The present study confirmed previous report that Wn 11463 is a strong marker for salt tolerance in rice. Validation is need to be made on broader genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

Key words: salt tolerance, molecular marker, SSR, STS, Principal Component Analysis,

Running title: marker validation for salt tolerance 9

INTRODUCTION

Rice is a major staple food crop of most Indonesian people. Effort to maintain national self-sufficiency in rice production has been challenged by the decreasing area of arable paddy field due to land conversion to non-agriculture purposes (Suprayogi *et al*., 2019) which is estimated not less than 38.000 ha year^{-1} (Nafisah *et al*., 2017). As such, to date, rice production area should be extended to marginal land that include coastal area which during dry season is affected by intrusion of sea water resulting in saline condition. Salinity represents any unfavorable condition relates to excessive accumulation of salt (Sunusi *et al*., 2018). Salt stress promotes osmotic stress, ionic toxication, and nutrients deficiencies. Rice plants grown in saline condition experience growth retardant which is expressed in inhibition of root growth, plant height, number of tillers, promotes infertile spikelet, and ultimately, reduced yield (Qin *et al*., 2020). Among the solution for salinity problem of rice production in coastal area is the use of salt tolerant rice variety (Suprayogi *et al*., 2019; Nafisah *et al*., 2017).

Breeding for salt tolerant rice variety has been hindered by the limited availability of salt tolerant germplasm, limited information on genetic control of salt tolerance, and a lack of information of the relation between salt tolerance traits and specific agronomic characters (Sunusi *et al*., 2018). Slow progress of breeding of salt tolerant rice is also due to inadequate information on salt tolerance mechanisms, less information on screening techniques, inefficient selection techniques, and the complexity of effects of genetic x environment interactions (Lang *et al*., 2017).

Salt tolerance in rice is controlled by many genes (Ismail and Horie, 2017). Recent development in molecular marker analysis has enabled rapid and accurate identification of individual genes or otherwise Quantitative Trait Loci (QTL) controlling a trait of interest. QTL mapping approach has been used to describe the genetic architecture of salt tolerant in rice using a mapping population derived from the cross of salt sensitive and salt tolerant varieties (Cheng *et al*., 2015, Nakhla *et al*.. 2021, Liu *et al*., 2019). Some of the major salt tolerance QTLs included *qSKC-1* and *qSNC-7* which responsible for regulation of K^+/Na^+ homeostasis under salt stress which explain phenotypic variance of 48.5% and 40.1%, respectively; *qSKC-1* encode a major QTL for Shoot K^+ concentration and qSNC-7 for shoot Na+ concentration; *Saltol* which responsible for maintaining low Na+, high K+, to maintain shoot Na^+/K^+ homeostasis, *qSE3* which encode gene for K^+ transport; *OsHAK21* encodes gene responsible for seed germination and seedling establishment under salt stress, *qST1* and *qST3* which conferr salt tolerance at the young seedling stage (Qin *et al*., 2020). *qST1.1* was reported to be responsible to salt tolerance in SR86 and explained phenotypic variance of 62.6% (Wu *et al*., 2020)

Simple Sequence Repeat (SSR, microsatellite) marker has been used to identify salt tolerance or introgression genes to develop new cultivars (Mizan *et al*., 2015). Using SSR markers RM 3412, RM 510, and RM 336, Islam *et al*. (2015) identified five salt tolerant varieties out of the 25 evaluated varieties. Ali *et al*. (2014) identified four salt tolerant varieties out of 33 varieties using morphological markers and SSR markers RM 8046, RM 336, and RM 8094. Emon *et al*. (2015) Reported STS (Sequence Tagged Sites) marker Wn 11463 in marker assisted selection (MAS) for rice with salt tolerance gene *SKC1*. Using 17 SSR markers, included a significantly associated salinity tolerance marker Wn11463 (https://archive.gramene.org/markers/microsat/all-ssr.html), saline tolerant variety Inpari Unsoed 79 Agritan could be separated distantly from the susceptible variety IR 29 (Suprayogi *et al*. 2021).

This study is aimed at identifying and validating molecular markers for salt tolerance in several commercial indica rice varieties. Such validated markers will later be used for rapid and accurate screening technique for salt tolerant rice genotypes.

MATERIALS AND METHODS

Materials

Eleven indica rice varieties were used for sources of DNA. These varieties included salt tolerant rice varieties Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; salt sensitive rice variety IR 29; and common commercial rice variety Cisadane, Dendang, Inpago Unsoed 1, Lambur, Pelopor, Siak Raya, Unsoed Parimas. Aluvial soils, organic manure, and rice husk were used as growth media. The chemicals used for markers analysis were Cetyl Trimethyl Ammonium Bromide (Amresco), NaCl (Merck), HCl, Tris base (Merck), Na EDTA, NaOH, polyvinyl Pyrrolidine (Amresco), β-Mercapto-ethanol (Merck), aquades (H_2O), liquid nitrogen, chloroform (Merck), ammonium asetat (Merck), isopropanol (Merck), ethanol 70%, ethanol 96% (Merck), RNAse (Geneaid), nuclease free water (Thermo Fisher Scientific), agarose, TBE (Tris-Boric-EDTA) 1x, DNA ladder 100 bp (Bioline), loading dye (Thermo Fisher Scientific), ethidium bromide (Invitrogen), 10 primer pairs (Table 1), and Dream Taq PCR Master Mix (Thermo Fisher Scientific).

Methods

DNA Extraction and Quantification

DNA extraction was carried out using CTAB method with modification (Zannati *et al*., 2015). Leaves samples of two week-old seedling were ground using a mortar and pestle in the presence of liquid nitrogen. Powdery leaves sample was put into a 2 ml Eppendorf tube and added with 1000 µl of CTAB Buffer mix, and homogenized by vortexing for 5 seconds followed by incubation in a 65^0 C water bath and homogenized carefully every 10 minutes. Chloroform of 500 µl was then added to the tube and centrifuged for 7 minutes. The supernatant was transferred to a 1.5 ml Eppendorf tubes and added with 500 µl chloroform followed by centrifugation for 7 minutes. This step was repeated twice to allow better quality DNA. The supernatant was transferred to a new 1.5 ml Eppendorf tube. Ammonium acetate (0.8 µl/1 µl) and cold isopropanol (5,4µl/10µl) were then added followed by homogenization. The tubes were stored in the refrigerator at 4^0C for 24 hours. After an overnight precipitation, the tube was allowed to stand at room temperature. After 7 minutes centrifugation, the supernatant was discarded and let the DNA pellet to settle in the bottom of the tube. The DNA pellet was then washed with 70% Ethanol (700 µl) and precipitated by 1 minute centrifugation. The supernatant was discarded, and the pellet was allowed to dry. The pellet DNA was then washed with 90% ethanol (700 µl), and centrifuged for 1 minute and allowed the pellet to dry. Tris-EDTA (TE) buffer (30 µl) and RNAse (1 µl) were added to dilute the DNA pellet. The DNA solution was then incubated at 37°C for 1 hour. To keep the DNA from degradation, the DNA was stored in a -20^0 C freezer.

Concentration and purity of DNA were measured using nanophotometer. The DNA were diluted 100x using Tris-EDTA (TE) as solvent. A total of 3 µl of diluted DNA was used to determine the purity and concentration of the DNA.

Marker Amplification

Nine SSR markers and one STS marker Wn 11463 (Tabel 1) were evaluated for their possible association with salt tolerance.

Table 1. List of primers used in this study

No	Classification	Primer name		Nucleotide sequences	Size of amplicon (bp)	Ta (°C)	Traits related	Literature review	Source of information
1.	SSR	RM 212	*forward*	CCACTTTCAGCTACTACCAG	136	53,5	Drought tolerance	Roychowdhury *et al.* (2013)	archive.gramene.org
			reverse	CACCCATTTGTCTCTCATTATG					
2.	SSR	RM 8264	*forward*	ACGCTCCTCGCTTTCTAC	206	55,5	Agronomic traits (total of secondary branch and *seed-set ratio)*	Kebriyaee *et al.* (2012) & Ma *et al.*, (2019)	archive.gramene.org
			reverse	GCACCTCACACCAGTAATTC					
3.	SSR	RM	*forward*	CCATCCTCCTACTTCAATGAAG	141	58	Aromatic and	Shams *et al.*	archive.gram

		342	*reverse*	ACTATGCAGTGGTGTCACCC			drought tolerance	(2012) & Iqbal *et al.* (2015)	ene.org
4.	SSR	RM 225	*forward*	TGCCCATATGGTCTGGATG	140	53	Length of grain	Khatibani *et al*. (2019)	archive.gram ene.org
			reverse	GAAAGTGGATCAGGAAGGC					
5.	SSR	RM 222	*forward*	CTTAAATGGGCCACATGCG	213	56,3	Agronomic trait (numbers of tillers)	Chaudari (2018)	archive.gram ene.org
			reverse	CAAAGCTTCCGGCCAAAAG					
6.	SSR	RM 224	*forward*	ATCGATCGATCTTCACGAGG	157	56,3	Blast disease	Zarbafi *et al.* (2019)	archive.gram ene.org
			reverse	TGCTATAAAAGGCATTCGGG					
7.	SSR	RM 223	*forward*	GAGTGAGCTTGGGCTGAAAC	165	55	Not linked to specific trait	Supari *et al.* (2015)	archive.gram ene.org
			reverse	GAAGGCAAGTCTTGGCACTG					
8.	SSR	RM 8094	*forward*	AAGTTTGTACACATCGTATACA	209	55	Salinity tolerance	Ali *et al*. (2014)	archive.gram ene.org
			reverse	CGCGACCAGTACTACTACTA					
9.	SSR	RM 3412	*forward*	AAAGCAGGTTTTCCTCCTCC	211	55	Salinity tolerance	Saha *et al.* (2016)	archive.gram ene.org
			reverse	CCCATGTGCAATGTGTCTTC					
10.	STS	Wn 1146 3	*forward*	TCCTCCTTCTCTCGCAAC	120	54,9	Salinity tolerance	Emon *et al*. (2015)	Emon *et al*. (2015)
			reverse	GATCCACTCGTCACAGG					

A total volume of 25 µl Polymerase Chain Reaction-mix solution was used for PCR reaction. The PCR mix comprises of 1 µl (50 ng) DNA samples. 1 µl forward primer, l µl reverse primer, 12.5 µl Dream Taq PCR Master-Mix, and 9.5 µl nuclease free water. The PCR followed the program as seen in Table 2.

Table 2. PCR program

Stage	Temperature (^{0}C)	Time (minutes)	Cycle
Pre-denaturation	95^{0}C	5 minutes	1
Denaturation	95^{0}C	1 minute	35
Annealing	53,5-58^{0}C	1 minute	35
Extension	72^{0}C	1 minute	35
Final-extension	72^{0}C	5 minutes	1
Refrigation	4^{0}C	∞	

Amplicon Visualization

The DNA quality and amplicon visualization was checked by Agarose Gel electrophoresis. The DNA ladder was loaded into the first well. Meanwhile, 3 µl of PCR product mixed with 2 µl of loading dye was loaded into the well. Electrophoresis was carried out at 50 volt for 30 minutes. For DNA visualization, the electrophoretic gel was immersed in EtBr for 20 minutes, then seen under UV-transilluminator.

Data Analysis

Polymorphic Information Content (PIC) value was used as a standard for evaluating each genetic marker based on the amplified DNA bands. The PIC value is obtained by calculating the number of amplified alleles using application provided on https://gene-calc.pl/pic. The value of PIC was grouped into three classes, namely: PIC > 0.5 = very informative, 0.25 > PIC > 0.5 = moderate informative, and PIC < 0.25 = low informative (Carsono *et al*., 2014). PCR amplicon (DNA bands with different molecular weight) was converted into binary data by scoring. Notation 1 is given to DNA bands with similar molecular weight to Nona Bokra, 0 is given to DNA bands which is not similar to the molecular weight of Nona Bokra. The binary data were then used for PCA analyses using XLSTAT 2020 for grouping of the evaluated varieties (Vidal *et al*., 2020).

RESULTS AND DISCUSSION

Polymorphic Information Content (PIC) Value

PIC value describes level of informativeness of a marker. Marker with high PIC value is recommended to be used for molecular screening as this marker could better differentiate different genotypes. PIC analysis on the amplified alleles (Figure 1) shows that the PIC value of evaluated markers ranged from 0.1516 to 0.6781 (Table 3). Followings Carsono *et*

al. (2014), the evaluated markers could be grouped into three classes, i.e.: RM 223, RM 3412, RM 342 as very informative markers, RM 212, RM 222, RM 223, RM 224, RM 225, RM 8264, Wn 11463 as moderate informative markers, and RM 8094 is low informative marker.

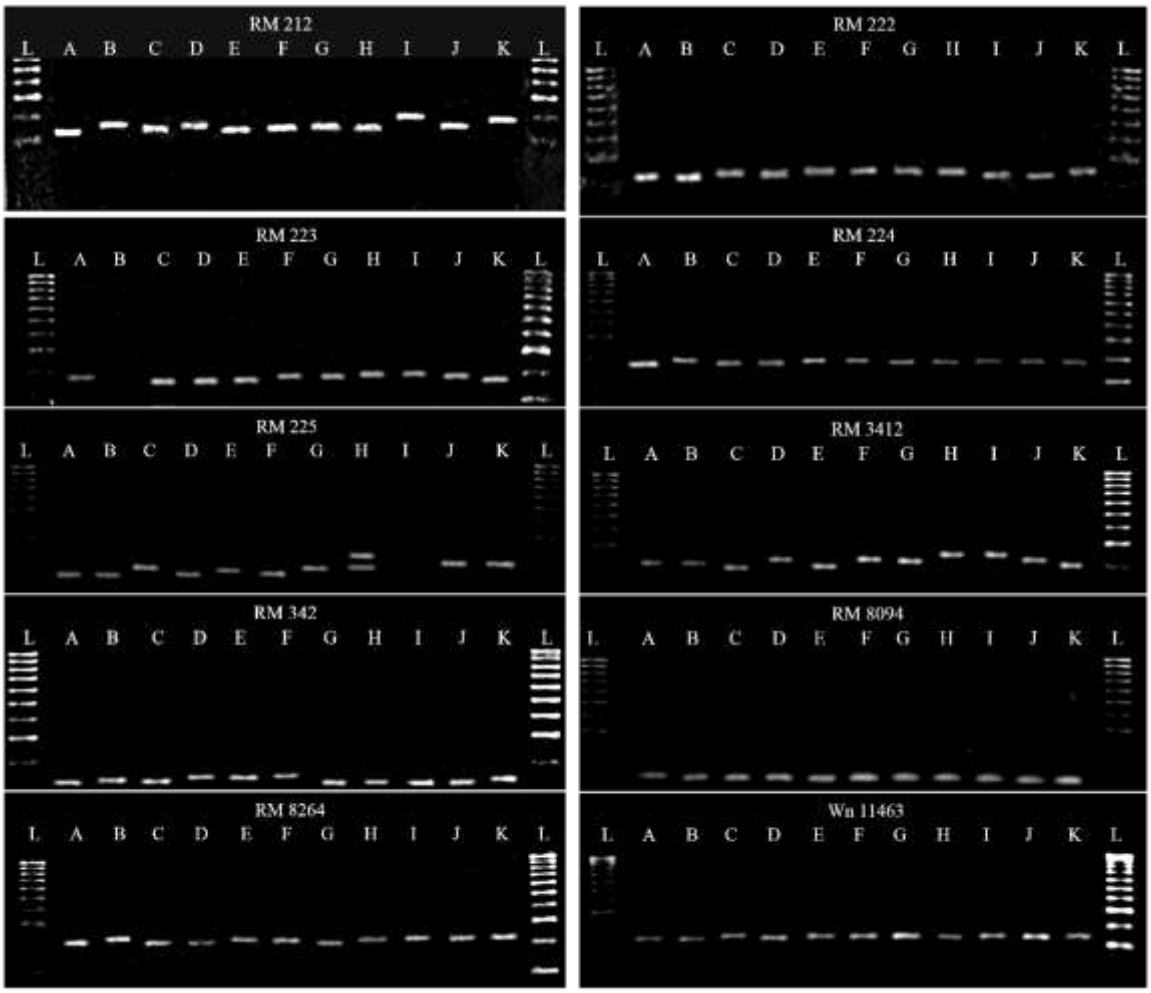

Figure 1. DNA band in 2% gel agarose (L= Ladder 100 bp; A = Nona Bokra; B = Inpari Unsoed 79 Agritan; C = Cisadane; D = Atomita 2; E = Pelopor; F = Dendang; G = Lambur; H = Siak Raya; I = Unsoed Parimas; J = Inpago Unsoed 1; K = IR 29).

Table 3. Chromosome location, number of alleles, and PIC of the evaluated markers

Marker	Chromosome location	Number of alleles	PIC
RM 212	1	3	0.4732
RM 222	10	2	0.3557
RM 223	8	3	0.5632
RM 224	11	2	0.318
RM 225	6	3	0.4762
RM 3412	1	4	0.6781
RM 342	8	3	0.5262
RM 8094	1	2	0.1516
RM 8264	8	2	0.3557
Wn 11463	1	2	0.318

PCA BiPlot Analysis of Degree of Resemblance

Analysis of degree of resemblance based on ten molecular markers was carried out using PCA Biplot analysis. Varieties are considered to have similar characteristics when they are at two adjacent points. The closer the distance between two points, the higher the level of similarity of two investigated varieties, based on a marker. Figure 2 shows the result of Biplot analysis of which the evaluated varieties are fallen into four quadrants. Cisadane, Unsoed Parimas, and Siak Raya are in one quadrant group and characterized by RM 224. No information of salt tolerance has been reported of these three varieties. Cisadane is a lowland rice variety that does not have resistance to any environmental stress conditions. Unsoed Parimas is a drought and aluminum tolerant variety, which is also resistant to *Pirycularia oryzae* race 0733 and somewhat resistant to *P. oryzae* race 133 (Agricultural Research and Development Agency, 2019). Siak Raya is a tidal swamp rice variety that tolerant Fe and Al (Dirgasan *et al*., 2019). Siak Raya is a moderate tolerant variety to salinity stress at seedling stage (Safitri *et al*., 2017).

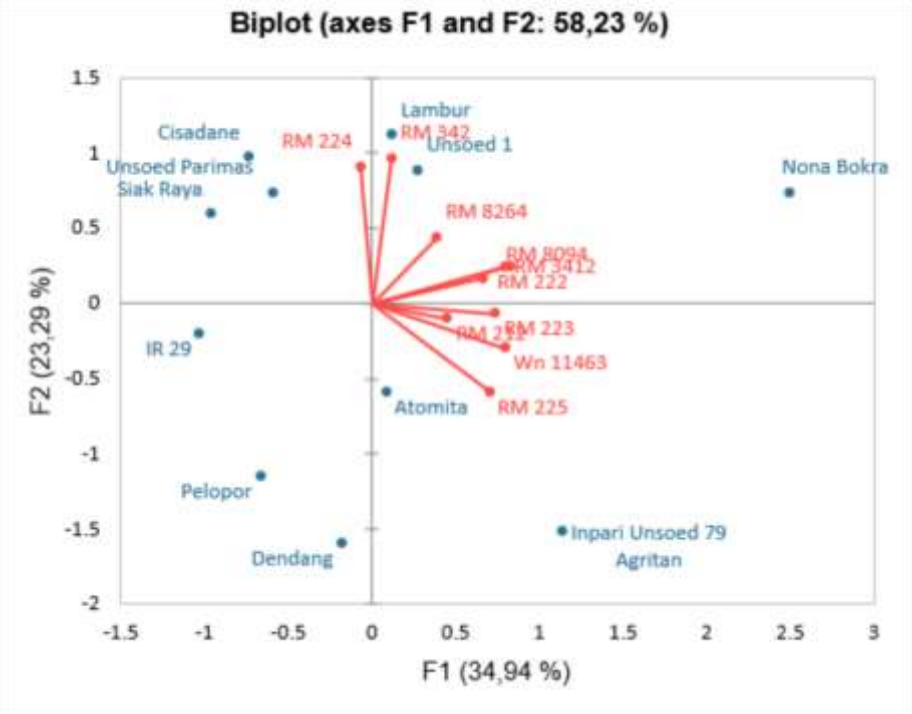

Figure 2. Varietal grouping based on PCA Biplot Analysis

Lambur, Inpago Unsoed 1, and Nona Bokra varieties were in one group and marked by RM 222, RM 342, RM 3412, RM 8264, and RM 8094. Among these SSR markers, RM 8094 was reported to be linked with salt tolerance (Ali *et al*., 2014). This result may suggest that Lambur and Inpago Unsoed may be also be salt tolerant varieties. Lambur is somewhat tolerant to salt, Al, and Fe. Inpago Unsoed 1 is a drought tolerant and moderately tolerant to iron (Fe) toxicity (Agricultural Research and Development Agency, 2019).

Atomita 2 is in the same group with Inpari Unsoed 79 Agritan, and marked by RM 223, RM 212, RM 225 and Wn 1146. Inpari Unsoed 79 Agritan is known to be tolerant to salt stress of 12 dSm^{-1} at seedling stage (Prasetia *et al*., 2022). Atomita 2 is a gamma-rays mutant rice variety which is known to be tolerant to salinity of 4-6 mhos/cm (Dewi *et al*., 2020). The similarity in the characteristics of these two varieties is also due to the fact that Atomita 2 is the parent of salt tolerant donor for Inpari Unsoed 79 Agritan. Wn 11463 marker was reported to be closely linked to the *SKC1* salt tolerance gene (Emon *et al*., 2015). Atomita and Inpari Unsoed 79 Agritan is also in the same quadrant group with STS marker Wn 11463.

IR 29, Pelopor, and Dendang were in the same quadrant with no marker. Interestingly, PCA Biplot analysis placed IR 29, Pelopor, and Dendang at the opposite quadrant of Nona Bokra. IR 29 is a salt sensitive variety (Ferreira *et al*., 2015), and consequently, Pelopor and Dendang, as being in the same quadrant, could be considered to be salt sensitive. Pelopor was reported previously to be a salt sensitive variety (Zannati *et al*., 2015). Dendang, however, is a tidal swamp rice which is reported to be quite tolerant to Fe, Al, and salinity (Suprayogi *et al*., 2019). The disagreement between our result and that of (Zannati *et al*., 2015) on the grouping of Dendang still need to be investigated further using more salt tolerance markers published elsewhere.

Table 4. Contribution of marker in grouping of varieties (%)

Marker	1st Dimension	2nd Dimension

	(F1)	(F2)
RM 212	5.384	0.398
RM 222	11.443	1.110
RM 223	14.337	0.186
RM 224	0.107	32.463
RM 225	12.924	13.511
RM 3412	16.813	2.346
RM 342	0.387	36.585
RM 8094	17.944	2.342
RM 8264	4.025	7.695
Wn 11463	16.635	3.362

Grouping of the evaluated varieties as shown in the PCA Biplot demonstrated that markers used in this study could differentiate salt tolerant varieties from the sensitive ones. Each marker relates to different variety in different quadrant (Figure 2). Table 2 showed that in the F1 (first) dimension, RM 8094 markers contributed the highest in the grouping of varieties, followed by RM 3412, and Wn 11463. Meanwhile, in F2 (second) dimension, RM 342 had the highest contribution followed by RM 224.

Analysis on contributing markers to the grouping of varieties (Table 4) and PCA Biplot (Figure 2) demonstrated that RM 8094, RM 3412, Wn 11463, and RM 342 significantly related to salt tolerant varieties of Nona Bokra, Atomita-2 and Inpari Unsoed 79 Agritan. This result is in accordance with previous report of (Emon *et al*., 2015). RM 3412 and RM 8094 were linked to Saltol QTL which is located on chromosome 1 (Geetha *et al*., 2017). Main genes for salt tolerance traits (*Saltol*) is mapped on chromosomes 1 and 8 (Lang *et al*., 2017). The STS marker Wn 11463 is significantly linked to salt tolerant gene, *SKC 1* (Emon *et al*., 2015). The RM 342 marker determine salinity tolerance at reproductive stage and is located on chromosome 8 (Iqbal *et al*., 2015). Salt tolerance gene on chromosome 8 controls tolerance at vegetative phase at EC (electrical conductivity) below 10 dSm^{-1} (Lang *et al*., 2017).

This study also found that the drought tolerant rice variety Inpago Unsoed 1 fall in the same quadrant of salt tolerant variety Nona Bokra (Figure 2). This may suggest the drought tolerance and salt tolerance shares some common mechanisms in plant. This argument is based on (Shuyu *et al*., 2013) that *DST* gene of salt tolerance also relates to drought tolerance. This gene is located on chromosome 3 and functions to control the movement of stomata during drought and high salt conditions through the regulation of genes that involved in ROS (Reactive Oxygen Species) homeostasis (Ar-Rafi *et al*., 2017).

Apart from the result of marker-based genotypes grouping, validation was carried out for the salt tolerance markers Wn 11463 (Emon *et al*., 2015), RM 8094 and RM 3412 (Lang *et al*., 2017). In practice, validation could be made by simply observing the presence/absence of specifically related salt tolerance marker band in the evaluated genotypes. Such a method, however, could only be applied for validated dominant-type molecular marker, or otherwise a specific gene sequence. For co-dominant-type markers, such as STS marker Wn 11463, and SSR markers RM 8094 and RM 3412, validation should be based on molecular size, and its consistency across salt tolerant genotypes.

In the present study, polymorphic bands of Wn 11463 with consistent molecular size could be seen across the reference tolerant genotypes Nona Bokra, Inpari Unsoed 79 Agritan, Atomita-2, Dendang, Lambur, and Siak Raya (Figure 1.). This result confirms that Wn 11463 is a strong marker for salt tolerance in rice (Emon *et al*., 2015). RM 8094 was polymorphic but the tolerant genotypes did not necessarily have the same molecular size. RM 3412 produced 3 (three) polymorphic band (Table 3.) with inconsistent molecular size across salt tolerant genotypes. In contrast with Lang et al. (2017), the present study suggests that validation is need to be made on broader genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant marker.

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Marker validation for salt tolerance in Indica rice

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Abstract. *Suprayogi, Dewi PS, Oktaviani, E, Aisya AW. 2022. Marker validation for salt tolerance in Indica rice. Biodiversitas 23: xxxx.* The development of salt-tolerant rice variety is of importance with concern to the use of the coastal area for rice production and, consequently, the development of a rapid and accurate method of variety selection. Molecular markers associated with Quantitative Trait Loci (QTL) for salt tolerance in rice have been reported elsewhere. This research aimed to identify molecular markers associated with salt tolerance in rice. The research was carried out from September 2020 to February 2021 at Plant Breeding and Biotechnology Laboratory, Faculty of Agriculture, Jenderal Soedirman University. Nine Simple Sequence Repeat (SSR) markers and one Sequence Tagged Site (STS) marker were evaluated for their association with salt tolerance. The rice varieties consisted of three salt-tolerant varieties, one salt-sensitive variety, and six commercial varieties. Nona Bokra PCR amplicon was used as a positive control for DNA band scoring. Binary data of the amplicon banding pattern were analyzed using Principal Component Analysis (PCA) to determine the grouping of the evaluated varieties about their tolerance to assigned markers. The result demonstrated that the evaluated rice varieties fell into four groups of marker resemblances. Among the evaluated SSR markers, RM 3412, RM 342, RM 8094, and Wn 11463 significantly contributed to the grouping. The present study confirmed that Wn 11463 is a strong marker for salt tolerance in rice. However, validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

Keywords: salt tolerance, molecular marker, SSR, STS, Principal Component Analysis

INTRODUCTION

Rice is a major staple food crop of most Indonesian people. However, the effort to maintain national self-sufficiency in rice production has been challenged by the decreasing area of arable paddy fields due to land conversion to non-agriculture purposes (Suprayogi et al. 2019), which is estimated not less than 38.000 ha year^{-1} (Nafisah et al. 2017). To date, the rice production area should be extended to marginal land that includes coastal areas which, during the dry season, are affected by seawater intrusion resulting in saline conditions. Salinity represents any unfavorable condition of excessive salt accumulation (Sunusi et al. 2018). Salt stress promotes osmotic stress, ionic toxication, and nutrient deficiencies. Rice plants grown in saline condition experience growth retardant expressed in inhibition of root growth, plant height, number of tillers, promotes infertile spikelets, and ultimately, reduced yield (Qin et al. 2020). Among the solution to the salinity problem of rice production in the coastal areas is using salt-tolerant rice varieties (Suprayogi et al. 2019; Nafisah et al. 2017).

Breeding for salt-tolerant rice variety has been hindered by the limited availability of salt-tolerant germplasm, limited information on the genetic control of salt tolerance, and a lack of information on the relation between salt tolerance traits and specific agronomic characters (Sunusi et al. 2018). Slow progress in the breeding of salt-tolerant rice is also due to inadequate information on salt tolerance mechanisms, less information on screening techniques, inefficient selection techniques, and the complexity of effects of genetic x environment interactions (Lang et al. 2017).

Salt tolerance in rice is controlled by many genes (Ismail and Horie 2017). Recent development in molecular marker analysis has enabled rapid and accurate identification of individual genes or otherwise Quantitative Trait Loci (QTL) controlling a trait of interest. QTL mapping approach has been used to describe the genetic architecture of salt tolerance in rice using a mapping population derived from the cross of salt sensitive and salt tolerant varieties (Cheng et al. 2015, Nakhla et al. 2021, Liu et al. 2019). Some of the major salt tolerance QTLs included *qSKC-1* and *qSNC-7*. These QTLs are responsible for the regulation of K^+/Na^+ homeostasis under salt stress, which explains phenotypic variance of 48.5% and 40.1%, respectively; *qSKC-1* encodes a major QTL for Shoot K^+ concentration and qSNC-7 for shoot Na+ concentration; *Saltol* which is responsible for maintaining low Na+, high K+, shoot Na^+/K^+ homeostasis; *qSE3* encodes the gene for K^+ transport; *OsHAK21* encodes gene responsible for seed germination and seedling establishment under salt stress, *qST1* and *qST3* conferred salt tolerance at the young seedling stage (Qin et al. 2020). *qST1.1* was reported to be responsible for salt tolerance in SR86 and explained a phenotypic variance of 62.6% (Wu et al. 2020)

Simple Sequence Repeat (SSR, microsatellite) marker has been used to identify salt tolerance or introgress genes to develop new cultivars (Mizan et al. 2015). Using SSR markers RM 3412, RM 510, and RM 336, Islam et al.

(2015) identified five salt tolerant varieties out of the 25 evaluated varieties. Ali et al. (2014) identified four salt tolerant varieties out of 33 varieties using morphological markers and SSR markers RM 8046, RM 336, and RM 8094. Emon et al. (2015) Reported STS (Sequence Tagged Sites) marker Wn 11463 in marker-assisted selection (MAS) for rice with salt tolerance gene *SKC1*. Using 17 SSR markers, including a significantly associated salinity tolerance marker Wn11463 (https://archive.gramene.org/markers/microsat/all-ssr.html), saline tolerant variety Inpari Unsoed 79 Agritan could be separated distantly from the susceptible variety IR 29 (Suprayogi et al. 2021).

This study aimed at identifying and validating molecular markers for salt tolerance in several commercial indica rice varieties. The validated markers will later be used for rapid and accurate screening techniques for salt-tolerant rice genotypes.

MATERIALS AND METHODS

Materials

Eleven indica rice varieties were used as DNA sources. These varieties included salt-tolerant rice varieties Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; a salt-sensitive rice variety IR 29; and common commercial rice varieties Cisadane, Dendang, Inpago Unsoed 1, Lambur, Pelopor, Siak Raya, Unsoed Parimas. Alluvial soils, organic manure, and rice husk were used as growth media. The chemicals used for markers analysis were Cetyl Trimethyl Ammonium Bromide (Amresco), NaCl (Merck), HCl, Tris base (Merck), Na EDTA, NaOH, polyvinyl Pyrrolidine (Amresco), β-Mercapto-ethanol (Merck), aquades (H_2O), liquid nitrogen, chloroform (Merck), ammonium acetate (Merck), isopropanol (Merck), ethanol 70%, ethanol 96% (Merck), RNAse (Geneaid), nuclease-free water (Thermo Fisher Scientific), agarose, TBE (Tris-Boric-EDTA) 1x, DNA ladder 100 bp (Bioline), loading dye (Thermo Fisher Scientific), ethidium bromide (Invitrogen), 10 primer pairs (Table 1), and Dream Taq PCR Master Mix (Thermo Fisher Scientific).

Methods

DNA Extraction and Quantification

DNA extraction was carried out using CTAB method with modification (Zannati et al. 2015). Leaves samples of two-week-old seedlings were ground using a mortar and pestle in the presence of liquid nitrogen. Powdery leaves sample was put into a 2 ml Eppendorf tube, added with 1000 µl of CTAB Buffer mix, and homogenized by vortexing for 5 seconds, followed by incubation in a 65^0 C water bath and homogenized carefully every 10 minutes. Chloroform of 500 µl was added to the tube and centrifuged for 7 minutes. Next, the supernatant was transferred to a 1.5 ml Eppendorf tube and added with 500 µl chloroform, followed by centrifugation for 7 minutes. This step was repeated twice to allow better-quality DNA. Next, the supernatant was transferred to a new 1.5 ml Eppendorf tube. Ammonium acetate (0.8 µl/1 µl) and cold isopropanol (5,4µl/10µl) were then added, followed by homogenization. The tubes were stored in the refrigerator at 4^0C for 24 hours. After overnight precipitation, the tube was allowed to stand at room temperature. After 7 minutes of centrifugation, the supernatant was discarded, and the DNA pellet was allowed to settle in the bottom of the tube. The DNA pellet was then washed with 70% Ethanol (700 µl) and precipitated by centrifugation for one minute. The supernatant was discarded, and the pellet was allowed to dry. The pellet DNA was then washed with 90% ethanol (700 µl), centrifuged for 1 minute, and let the pellet dry. Tris-EDTA (TE) buffer (30 µl) and RNAse (1 µl) were added to dilute the DNA pellet. The DNA solution was then incubated at 37°C for 1 hour. The DNA was stored in a -20^0 C freezer to keep the DNA from degradation.

The concentration and purity of DNA were measured using a nanophotometer. The DNA was diluted 100x using Tris-EDTA (TE) as a solvent. A total of 3 µl of diluted DNA was used to determine the purity and concentration of the DNA.

Marker Amplification

Nine SSR markers and one STS marker Wn 11463 (Table 1) were evaluated for their possible association with salt tolerance.

A total volume of 25 µl PCR-mix solution was used for PCR reaction. The PCR mix comprised 1 µl (50 ng) DNA samples. 1 µl forward primer, l µl reverse primer, 12.5 µl Dream Taq PCR Master-Mix, and 9.5 µl nuclease-free water. The PCR followed the program as seen in Table 2.

Amplicon Visualization

The DNA quality and amplicon visualization was checked by Agarose Gel electrophoresis. First, the DNA ladder was loaded into the first well. Meanwhile, 3 µl of PCR product mixed with 2 µl of loading dye was loaded into each well. Electrophoresis was carried out at 50 volt for 30 minutes. For DNA visualization, the electrophoretic gel was immersed in EtBr for 20 minutes, then seen under UV-transilluminator.

Data Analysis

Polymorphic Information Content (PIC) value was used to evaluate each genetic marker based on the amplified DNA bands. The PIC value is obtained by calculating the number of amplified alleles using the application on https://gene-calc.pl/pic. The value of PIC was grouped into three classes, namely: PIC > 0.5 = highly informative, 0.25 > PIC > 0.5 = moderately informative, and PIC < 0.25 = poorly informative (Carsono et al. 2014). PCR amplicon (DNA bands with different molecular weights) was converted into binary data by scoring. Notation 1 was given to DNA bands with similar molecular weight to Nona Bokra, 0 was given to DNA bands that are not similar to the molecular weight of Nona Bokra. The binary data were then used for PCA analyses using XLSTAT 2020 to group the evaluated varieties (Vidal et al. 2020).

Table 1. List of primers used in this study

No	Classification	Primer name		Nucleotide sequences	Size of amplicon (bp)	Ta (°C)	Traits related	Literature review	Source of information
1.	SSR	RM 212	*forward*	CCACTTTCAGCTACTACCAG	136	53,5	Drought tolerance	Roychowdhury et al. (2013)	archive.gramene.org
			reverse	CACCCATTTGTCTCTCATTATG					
2.	SSR	RM 8264	*forward*	ACGCTCCTCGCTTTCTAC	206	55,5	Agronomic traits (total of secondary branch and *seed-set ratio)*	Kebriyaee et al. (2012) & Ma et al., (2019)	archive.gramene.org
			reverse	GCACCTCACACCAGTAATTC					
3.	SSR	RM 342	*forward*	CCATCCTCCTACTTCAATGAAG	141	58	Aromatic and drought tolerance	Shams et al. (2012) & Iqbal et al. (2015)	archive.gramene.org
			reverse	ACTATGCAGTGGTGTCACCC					
4.	SSR	RM 225	*forward*	TGCCCATATGGTCTGGATG	140	53	Length of grain	Khatibani et al. (2019)	archive.gramene.org
			reverse	GAAAGTGGATCAGGAAGGC					
5.	SSR	RM 222	*forward*	CTTAAATGGGCCACATGCG	213	56,3	Agronomic trait (numbers of tillers)	Chaudari (2018)	archive.gramene.org
			reverse	CAAAGCTTCCGGCCAAAAG					
6.	SSR	RM 224	*forward*	ATCGATCGATCTTCACGAGG	157	56,3	Blast disease	Zarbafi et al. (2019)	archive.gramene.org
			reverse	TGCTATAAAAGGCATTCGGG					
7.	SSR	RM 223	*forward*	GAGTGAGCTTGGGCTGAAAC	165	55	Not linked to specific trait	Supari et al. (2015)	archive.gramene.org
			reverse	GAAGGCAAGTCTTGGCACTG					
8.	SSR	RM 8094	*forward*	AAGTTTGTACACATCGTATACA	209	55	Salinity tolerance	Ali et al. (2014)	archive.gramene.org
			reverse	CGCGACCAGTACTACTACTA					
9.	SSR	RM 3412	*forward*	AAAGCAGGTTTTCCTCCTCC	211	55	Salinity tolerance	Saha et al. (2016)	archive.gramene.org
			reverse	CCCATGTGCAATGTGTCTTC					
10.	STS	Wn 11463	*forward*	TCCTCCTTCTCTCGCAAC	120	54,9	Salinity tolerance	Emon et al. (2015)	Emon et al. (2015)
			reverse	GATCCACTCGTCACAGG					

Table 2. PCR program

Stage	Temperature (^{0}C)	Time (minutes)	Cycle
Pre-denaturation	95^{0}C	5 minutes	1
Denaturation	95^{0}C	1 minute	35
Annealing	53,5-58^{0}C	1 minute	35
Extension	72^{0}C	1 minute	35
Final-extension	72^{0}C	5 minutes	1
Refrigeration	4^{0}C	∞	

RESULTS AND DISCUSSION

Polymorphic Information Content (PIC) Value

PIC value describes the level of informativeness of a marker. A marker with a high PIC value is recommended for molecular screening as this marker could better differentiate different genotypes. PIC analysis on the amplified alleles (Figure 1) shows that the PIC value of evaluated markers ranged from 0.1516 to 0.6781 (Table 3). Followings Carsono et al. (2014), the evaluated markers could be grouped into three classes, i.e.: RM 223, RM 3412, RM 342 as highly informative markers, RM 212, RM 222, RM 223, RM 224, RM 225, RM 8264, Wn 11463 as moderately informative markers, and RM 8094 is poorly informative marker.

PCA BiPlot Analysis of Degree of Resemblance

Analysis of the degree of resemblance based on ten molecular markers was carried out using PCA Biplot analysis. Varieties are considered to have similar characteristics when they are at two adjacent points. The closer the distance between two points, the higher the similarity between two investigated varieties based on a marker. Figure 2 shows the result of Biplot analysis in which the evaluated varieties fell into four quadrants. Cisadane, Unsoed Parimas, and Siak Raya are in one quadrant group characterized by RM 224. No information on salt tolerance has been reported for these three varieties. Cisadane is a lowland rice variety that does not have resistance to any environmental stress conditions. Unsoed Parimas is a drought and aluminum-tolerant variety, which is also resistant to *Piryculariа oryzae* race 0733 and moderately resistant to *P. oryzae* race 133 (Agricultural Research and Development Agency 2019). Siak Raya is a tidal swamp rice variety that is tolerant Fe and Al (Dirgasan et al. 2019). Siak Raya is a moderately tolerant variety to salinity stress at the seedling stage (Safitri et al. 2017).

Lambur, Inpago Unsoed 1, and Nona Bokra varieties were in one group and marked by RM 222, RM 342, RM 3412, RM 8264, and RM 8094. Among these SSR markers, RM 8094 was reported to be linked with salt tolerance (Ali et al. 2014). This result may suggest that Lambur and Inpago Unsoed may also be tolerant to salinity stress. Lambur is somewhat tolerant to salt, Al, and Fe. Inpago Unsoed 1 is drought tolerant and moderately tolerant to iron (Fe) toxicity (Agricultural Research and Development Agency 2019).

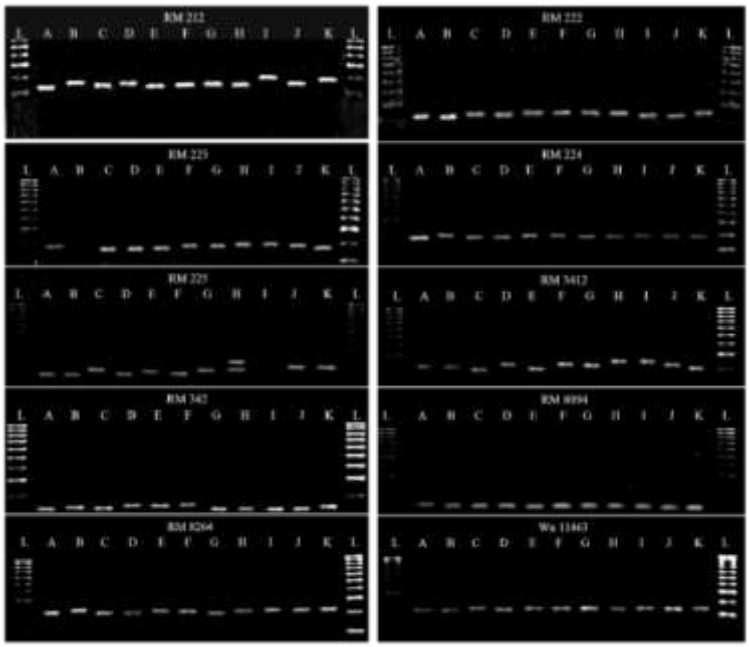

Figure 1. DNA band in 2% gel agarose (L= Ladder 100 bp; A = Nona Bokra; B = Inpari Unsoed 79 Agritan; C = Cisadane; D = Atomita 2; E = Pelopor; F = Dendang; G = Lambur; H = Siak Raya; I = Unsoed Parimas; J = Inpago Unsoed 1; K = IR 29)

Table 3. Chromosome location, number of alleles, and PIC of the evaluated markers

Marker	Chromosome location	Number of alleles	PIC
RM 212	1	3	0.4732
RM 222	10	2	0.3557
RM 223	8	3	0.5632
RM 224	11	2	0.318
RM 225	6	3	0.4762
RM 3412	1	4	0.6781
RM 342	8	3	0.5262
RM 8094	1	2	0.1516
RM 8264	8	2	0.3557
Wn 11463	1	2	0.318

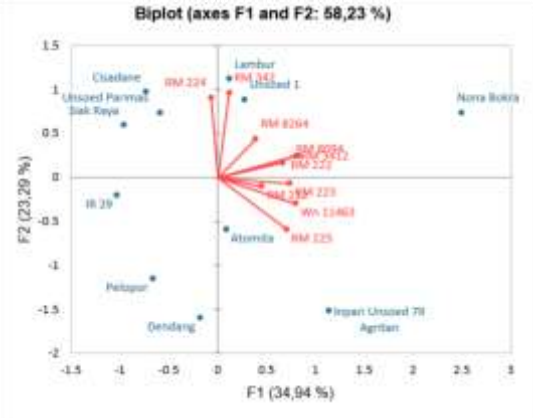

Figure 2. Varietal grouping based on PCA Biplot Analysis

Table 4. Contribution of the marker in the grouping of varieties (%)

Marker	1st Dimension (F1)	2nd Dimension (F2)
RM 212	5.384	0.398
RM 222	11.443	1.110
RM 223	14.337	0.186
RM 224	0.107	32.463
RM 225	12.924	13.511
RM 3412	16.813	2.346
RM 342	0.387	36.585
RM 8094	17.944	2.342
RM 8264	4.025	7.695
Wn 11463	16.635	3.362

Atomita 2 is in the same group as Inpari Unsoed 79 Agritan, marked by RM 223, RM 212, RM 225, and Wn 1146. Inpari Unsoed 79 Agritan is known to be tolerant to salt stress of 12 dSm^{-1} at the seedling stage (Prasetia et al. 2022). Atomita 2 is a gamma-rays mutant rice variety known to be tolerant to the salinity of 4-6 mhos/cm (Dewi et al. 2020). The similarity in the characteristics of these two varieties is also due to the fact that Atomita 2 is the parent of the salt-tolerant donor for Inpari Unsoed 79 Agritan. Wn 11463 marker was closely linked to the *SKC1* salt tolerance gene (Emon et al., 2015). Atomita and Inpari Unsoed 79 Agritan is also in the same quadrant group with STS marker Wn 11463.

IR 29, Pelopor, and Dendang were in the same quadrant with no marker. Interestingly, PCA Biplot analysis placed IR 29, Pelopor, and Dendang in the opposite quadrant of Nona Bokra. IR 29 is a salt-sensitive variety (Ferreira et al. 2015), and consequently, Pelopor and Dendang, as being in the same quadrant, could be considered salt sensitive. Pelopor was previously reported as a salt-sensitive variety (Zannati et al. 2015). Dendang, however, is tidal swamp rice that is reported to be quite tolerant to Fe, Al, and salinity (Suprayogi et al. 2019). The disagreement between

our result and that of (Zannati et al. 2015) on the grouping of Dendang still needs to be investigated further using more salt tolerance markers published elsewhere.

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Grouping of the evaluated varieties, as shown in the PCA Biplot demonstrates that markers used in this study could differentiate salt tolerant varieties from the sensitive ones. This is because each marker relates to a different variety in a different quadrant (Figure 2). Table 2 shows that in the F1 (first) dimension, RM 8094 markers contributed the highest in the grouping of varieties, followed by RM 3412, and Wn 11463. Meanwhile, in F2 (second) Dimension, RM 342 had the highest contribution, followed by RM 224.

Analysis of contributing markers to the grouping of varieties (Table 4) and PCA Biplot (Figure 2) demonstrated that RM 8094, RM 3412, Wn 11463, and RM 342 significantly related to salt-tolerant varieties of Nona Bokra, Atomita-2, and Inpari Unsoed 79 Agritan. This result follows the previous report (Emon et al. 2015). RM 3412 and RM 8094 were linked to Saltol QTL, located on chromosome 1 (Geetha et al. 2017). The main genes for salt tolerance traits (*Saltol*) are mapped on chromosomes 1 and 8 (Lang et al. 2017). The STS marker Wn 11463 is significantly linked to the salt-tolerant gene, *SKC 1* (Emon et al. 2015). The RM 342 marker determines salinity tolerance at the reproductive stage and is located on chromosome 8 (Iqbal et al. 2015). The salt tolerance gene on chromosome 8 controls tolerance at the vegetative phase at EC (electrical conductivity) below 10 dSm^{-1} (Lang et al. 2017).

This study also found that the drought-tolerant rice variety Inpago Unsoed 1 fell in the same quadrant as the salt-tolerant variety Nona Bokra (Figure 2). This may suggest that drought tolerance and salt tolerance share some common mechanisms in the plant. This argument is based on (Shuyu et al. 2013) that *DST* gene of salt tolerance also relates to drought tolerance. This gene is located on chromosome 3 and controls stomata's movement during drought and high salt conditions through the regulation of genes involved in ROS (Reactive Oxygen Species) homeostasis (Ar-Rafi et al. 2017).

Apart from marker-based genotypes grouping, validation was carried out for the salt tolerance markers Wn 11463 (Emon et al. 2015), RM 8094, and RM 3412 (Lang et al. 2017). In practice, the marker validation could be made by observing the presence/absence of specifically related salt tolerance marker bands in the evaluated genotypes. However, such a method could only be applied for the validated dominant-type molecular marker or a specific gene sequence. Therefore, for co-dominant-type markers, such as STS marker Wn 11463, and SSR markers RM 8094 and RM 3412, validation should be based on molecular size and its consistency across salt tolerant genotypes.

In the present study, polymorphic bands of Wn 11463 with consistent molecular size could be seen across the reference tolerant genotypes Nona Bokra, Inpari Unsoed 79 Agritan, Atomita-2, Dendang, Lambur, and Siak Raya (Figure 1). This result confirms that Wn 11463 is a strong rice salt tolerance marker (Emon et al. 2015). RM 8094 was polymorphic, but the tolerant genotypes did not necessarily have the same molecular size. RM 3412 produced 3 (three) polymorphic bands (Table 3) with inconsistent molecular size across salt tolerant genotypes. In contrast with Lang et al. (2017), the present study suggests that validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

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Commented [Ma1]: ", and Alwa Widi Aisya for her assistance in the molecular works" is deleted as she is a co-author

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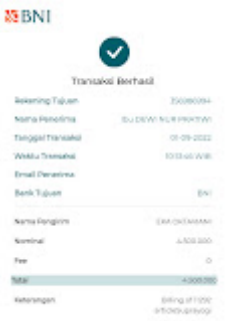

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Marker validation for salt tolerance in Indica rice

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Abstract. *Suprayogi, Dewi PS, Oktaviani, E, Aisya AW. 2022. Marker validation for salt tolerance in Indica rice. Biodiversitas 23: 4517-4523.* The development of salt-tolerant rice variety is of importance with concern to the use of the coastal area for rice production and, consequently, the development of a rapid and accurate method of variety selection. Molecular markers associated with Quantitative Trait Loci (QTL) for salt tolerance in rice have been reported elsewhere. This research aimed to identify molecular markers associated with salt tolerance in rice. The research was carried out from September 2020 to February 2021 at Plant Breeding and Biotechnology Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Indonesia. Nine Simple Sequence Repeat (SSR) markers and one Sequence Tagged Site (STS) marker were evaluated for their association with salt tolerance. The rice varieties consisted of three salt-tolerant varieties, one salt-sensitive variety, and six commercial varieties. Nona Bokra PCR amplicon was used as a positive control for DNA band scoring. Binary data of the amplicon banding pattern were analyzed using Principal Component Analysis (PCA) to determine the grouping of the evaluated varieties about their tolerance to assigned markers. The result demonstrated that the evaluated rice varieties fell into four groups of marker resemblances. Among the evaluated SSR markers, RM 3412, RM 342, RM 8094, and Wn 11463 significantly contributed to the grouping. The present study confirmed that Wn 11463 is a strong marker for salt tolerance in rice. However, validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

Keywords: Molecular marker, Principal Component Analysis, salt tolerance, SSR, STS

INTRODUCTION

Rice is a major staple food crop of most Indonesian people. However, the effort to maintain national self-sufficiency in rice production has been challenged by the decreasing area of arable paddy fields due to land conversion to non-agriculture purposes (Suprayogi et al. 2019), which is estimated not less than 38.000 ha year^{-1} (Nafisah et al. 2017). To date, the rice production area should be extended to marginal land that includes coastal areas, which during the dry season, are affected by seawater intrusion resulting in saline conditions. Salinity represents any unfavorable condition of excessive salt accumulation (Sunusi et al. 2018). Salt stress promotes osmotic stress, ionic toxication, and nutrient deficiencies. Rice plants grown in saline condition experience growth retardant expressed in inhibition of root growth, plant height, number of tillers, promotes infertile spikelets, and ultimately, reduced yield (Qin et al. 2020). Among the solution to the salinity problem of rice production in the coastal areas is using salt-tolerant rice varieties (Nafisah et al. 2017; Suprayogi et al. 2019).

Breeding for salt-tolerant rice variety has been hindered by the limited availability of salt-tolerant germplasm, limited information on the genetic control of salt tolerance, and a lack of information on the relation between salt tolerance traits and specific agronomic characters (Sunusi et al. 2018). Slow progress in the breeding of salt-tolerant rice is also due to inadequate information on salt tolerance mechanisms, less information on screening techniques, inefficient selection techniques, and the complexity of effects of genetic x environment interactions (Lang et al. 2017).

Salt tolerance in rice is controlled by many genes (Ismail and Horie 2017). Recent development in molecular marker analysis has enabled rapid and accurate identification of individual genes or otherwise Quantitative Trait Loci (QTL) controlling a trait of interest. QTL mapping approach has been used to describe the genetic architecture of salt tolerance in rice using a mapping population derived from the cross of salt sensitive and salt tolerant varieties (Cheng et al. 2015; Liu et al. 2019; Nakhla et al. 2021). Some of the major salt tolerance QTLs included *qSKC-1* and *qSNC-7*. These QTLs are responsible for the regulation of K^+/Na^+ homeostasis under salt stress, which explains phenotypic variance of 48.5% and 40.1%, respectively; *qSKC-1* encodes a major QTL for Shoot K^+ concentration and qSNC-7 for shoot Na+ concentration; *Saltol* which is responsible for maintaining low Na+, high K+, shoot Na^+/K^+ homeostasis; *qSE3* encodes the gene for K^+ transport; *OsHAK21* encodes gene responsible for seed germination and seedling establishment under salt stress, *qST1* and *qST3* conferred salt tolerance at the young seedling stage (Qin et al. 2020). *qST1.1* was reported to be responsible for salt tolerance in SR86 and explained a phenotypic variance of 62.6% (Wu et al. 2020)

Simple Sequence Repeat (SSR, microsatellite) marker has been used to identify salt tolerance or introgress genes to develop new cultivars (Mizan et al. 2015). Using SSR markers RM 3412, RM 510, and RM 336, Islam et al.

(2015) identified five salt tolerant varieties out of the 25 evaluated varieties. Ali et al. (2014) identified four salt tolerant varieties out of 33 varieties using morphological markers and SSR markers RM 8046, RM 336, and RM 8094. Emon et al. (2015) Reported STS (Sequence Tagged Sites) marker Wn 11463 in marker-assisted selection (MAS) for rice with salt tolerance gene *SKC1*. Using 17 SSR markers, including a significantly associated salinity tolerance marker Wn11463 (https://archive.gramene.org/markers/microsat/all-ssr.html), saline tolerant variety Inpari Unsoed 79 Agritan could be separated distantly from the susceptible variety IR 29 (Suprayogi et al. 2021).

This study aimed at identifying and validating molecular markers for salt tolerance in several commercial indica rice varieties. The validated markers will later be used for rapid and accurate screening techniques for salt-tolerant rice genotypes.

MATERIALS AND METHODS

Materials

Eleven indica rice varieties were used as DNA sources. These varieties included salt-tolerant rice varieties Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; a salt-sensitive rice variety IR 29; and common commercial rice varieties Cisadane, Dendang, Inpago Unsoed 1, Lambur, Pelopor, Siak Raya, Unsoed Parimas. Alluvial soils, organic manure, and rice husk were used as growth media. The chemicals used for markers analysis were Cetyl Trimethyl Ammonium Bromide (Amresco), NaCl (Merck), HCl, Tris base (Merck), Na EDTA, NaOH, polyvinyl Pyrrolidine (Amresco), β-Mercapto-ethanol (Merck), aquades (H_2O), liquid nitrogen, chloroform (Merck), ammonium acetate (Merck), isopropanol (Merck), ethanol 70%, ethanol 96% (Merck), RNAse (Geneaid), nuclease-free water (Thermo Fisher Scientific), agarose, TBE (Tris-Boric-EDTA) 1x, DNA ladder 100 bp (Bioline), loading dye (Thermo Fisher Scientific), ethidium bromide (Invitrogen), 10 primer pairs (Table 1), and Dream Taq PCR Master Mix (Thermo Fisher Scientific).

Methods

DNA extraction and quantification

DNA extraction was carried out using CTAB method with modification (Zannati et al. 2015). Leaves samples of two-week-old seedlings were ground using a mortar and pestle in the presence of liquid nitrogen. Powdery leaves sample was put into a 2 mL Eppendorf tube, added with 1000 µL of CTAB Buffer mix, and homogenized by vortexing for 5 seconds, followed by incubation in a 65ºC water bath and homogenized carefully every 10 minutes. Chloroform of 500 µL was added to the tube and centrifuged for 7 minutes. Next, the supernatant was transferred to a 1.5 mL Eppendorf tube and added with 500 µL chloroform, followed by centrifugation for 7 minutes. This step was repeated twice to allow better-quality DNA. Next, the supernatant was transferred to a new 1.5 mL Eppendorf tube. Ammonium acetate (0.8 µlL1 µL) and cold isopropanol (5,4µL/10µL) were then added, followed by homogenization. The tubes were stored in the refrigerator at 4ºC for 24 hours. After overnight precipitation, the tube was allowed to stand at room temperature. After 7 minutes of centrifugation, the supernatant was discarded, and the DNA pellet was allowed to settle in the bottom of the tube. The DNA pellet was then washed with 70% Ethanol (700 µL) and precipitated by centrifugation for one minute. The supernatant was discarded, and the pellet was allowed to dry. The pellet DNA was then washed with 90% ethanol (700 µL), centrifuged for 1 minute, and let the pellet dry. Tris-EDTA (TE) buffer (30 µL) and RNAse (1 µL) were added to dilute the DNA pellet. The DNA solution was then incubated at 37°C for 1 hour. The DNA was stored in a -20ºC freezer to keep the DNA from degradation.

The concentration and purity of DNA were measured using a nanophotometer. The DNA was diluted 100x using Tris-EDTA (TE) as a solvent. A total of 3 µL of diluted DNA was used to determine the purity and concentration of the DNA.

Marker amplification

Nine SSR markers and one STS marker Wn 11463 (Table 1) were evaluated for their possible association with salt tolerance.

A total volume of 25 µL PCR-mix solution was used for PCR reaction. The PCR mix comprised 1 µL (50 ng) DNA samples. 1 µL forward primer, l µL reverse primer, 12.5 µL Dream Taq PCR Master-Mix, and 9.5 µL nuclease-free water. The PCR followed the program as seen in Table 2.

Amplicon visualization

The DNA quality and amplicon visualization was checked by Agarose Gel electrophoresis. First, the DNA ladder was loaded into the first well. Meanwhile, 3 µL of PCR product mixed with 2 µL of loading dye was loaded into each well. Electrophoresis was carried out at 50 volts for 30 minutes. For DNA visualization, the electrophoretic gel was immersed in EtBr for 20 minutes, then seen under UV-transilluminator.

Data analysis

Polymorphic Information Content (PIC) value was used to evaluate each genetic marker based on the amplified DNA bands. The PIC value is obtained by calculating the number of amplified alleles using the application on https://gene-calc.pl/pic. The value of PIC was grouped into three classes, namely: PIC>0.5: highly informative, 0.25>PIC>0.5: moderately informative, and PIC<0.25: poorly informative (Carsono et al. 2014). PCR amplicon (DNA bands with different molecular weights) was converted into binary data by scoring. Notation 1 was given to DNA bands with similar molecular weight to Nona Bokra, 0 was given to DNA bands that are not similar to the molecular weight of Nona Bokra. The binary data were then used for PCA analyses using XLSTAT 2020 to group the evaluated varieties (Vidal et al. 2020).

Table 1. List of primers used in this study

Classi-fication	Primer name		Nucleotide sequences	Size of amplicon (bp)	Ta (°C)	Traits related	Literature review	Source of information
SSR	RM 212	Forward	CCACTTTCAGCTACTACCAG	136	53.5	Drought tolerance	Roychowdhury et al. (2013)	archive.gramene.org
		Reverse	CACCCATTTGTCTCTCATTATG					
SSR	RM 8264	Forward	ACGCTCCTCGCTTTCTAC	206	55.5	Agronomic traits (total of secondary branch and *seed-set ratio)*	Kebriyaee et al. (2012); Ma et al. (2019)	archive.gramene.org
		Reverse	GCACCTCACACCAGTAATTC					
SSR	RM 342	Forward	CCATCCTCCTACTTCAATGAAG	141	58	Aromatic and drought tolerance	Shams et al. (2012); Iqbal et al. (2015)	archive.gramene.org
		Reverse	ACTATGCAGTGGTGTCACCC					
SSR	RM 225	Forward	TGCCCATATGGTCTGGATG	140	53	Length of grain	Khatibani et al. (2019)	archive.gramene.org
		Reverse	GAAAGTGGATCAGGAAGGC					
SSR	RM 222	Forward	CTTAAATGGGCCACATGCG	213	56.3	Agronomic trait (numbers of tillers)	Chaudari (2018)	archive.gramene.org
		Reverse	CAAAGCTTCCGGCCAAAAG					
SSR	RM 224	Forward	ATCGATCGATCTTCACGAGG	157	56.3	Blast disease	Zarbafi et al. (2019)	archive.gramene.org
		Reverse	TGCTATAAAAGGCATTCGGG					
SSR	RM 223	Forward	GAGTGAGCTTGGGCTGAAAC	165	55	Not linked to specific trait	Supari et al. (2015)	archive.gramene.org
		Reverse	GAAGGCAAGTCTTGGCACTG					
SSR	RM 8094	Forward	AAGTTTGTACACATCGTATACA	209	55	Salinity tolerance	Ali et al. (2014)	archive.gramene.org
		Reverse	CGCGACCAGTACTACTACTA					
SSR	RM 3412	Forward	AAAGCAGGTTTTCCTCCTCC	211	55	Salinity tolerance	Saha et al. (2016)	archive.gramene.org
		Reverse	CCCATGTGCAATGTGTCTTC					
STS	Wn 11463	Forward	TCCTCCTTCTCTCGCAAC	120	54.9	Salinity tolerance	Emon et al. (2015)	Emon et al. (2015)
		Reverse	GATCCACTCGTCACAGG					

Table 2. PCR program

Stage	Temp. (°C)	Time (minutes)	Cycle
Pre-denaturation	95	5	1
Denaturation	95	1	35
Annealing	53.5-58	1	35
Extension	72	1	35
Final-extension	72	5	1
Refrigeration	4	∞	

RESULTS AND DISCUSSION

Polymorphic Information Content (PIC) value

PIC value describes the level of informativeness of a marker. A marker with a high PIC value is recommended for molecular screening as this marker could better differentiate different genotypes. PIC analysis on the amplified alleles (Figure 1) shows that the PIC value of evaluated markers ranged from 0.1516 to 0.6781 (Table 3). Followings Carsono et al. (2014), the evaluated markers could be grouped into three classes, i.e.: RM 223, RM 3412, RM 342 as highly informative markers, RM 212, RM 222, RM 223, RM 224, RM 225, RM 8264, Wn 11463 as moderately informative markers, and RM 8094 is poorly informative marker.

PCA Biplot analysis of degree of resemblance

Analysis of the degree of resemblance based on ten molecular markers was carried out using PCA Biplot analysis. Varieties are considered to have similar characteristics when they are at two adjacent points. The closer the distance between two points, the higher the similarity between two investigated varieties based on a marker. Figure 2 shows the result of Biplot analysis in which the evaluated varieties fell into four quadrants. Cisadane, Unsoed Parimas, and Siak Raya are in one quadrant group characterized by RM 224. No information on salt tolerance has been reported for these three varieties. Cisadane is a lowland rice variety that does not have resistance to any environmental stress conditions. Unsoed Parimas is a drought and aluminum-tolerant variety, which is also resistant to *Pirycularia oryzae* race 0733 and moderately resistant to *P. oryzae* race 133 (Agricultural Research and Development Agency 2019). Siak Raya is a tidal swamp rice variety that is tolerant Fe and Al (Dirgasan et al. 2019). Siak Raya is a moderately tolerant variety to salinity stress at the seedling stage (Safitri et al. 2017).

Lambur, Inpago Unsoed 1, and Nona Bokra varieties were in one group and marked by RM 222, RM 342, RM 3412, RM 8264, and RM 8094. Among these SSR markers, RM 8094 was reported to be linked with salt tolerance (Ali et al. 2014). This result may suggest that Lambur and Inpago Unsoed may also be tolerant to salinity stress. Lambur is somewhat tolerant to salt, Al, and Fe. Inpago Unsoed 1 is drought tolerant and moderately tolerant to iron (Fe) toxicity (Agricultural Research and Development Agency 2019).

Atomita 2 is in the same group as Inpari Unsoed 79 Agritan, marked by RM 223, RM 212, RM 225, and Wn 1146. Inpari Unsoed 79 Agritan is known to be tolerant to salt stress of 12 dSm^{-1} at the seedling stage (Prasetia et al.

2022). Atomita 2 is a gamma-ray mutant rice variety known to be tolerant to the salinity of 4-6 mhos/cm (Dewi et al. 2020). The similarity in the characteristics of these two varieties is also due to the fact that Atomita 2 is the parent of the salt-tolerant donor for Inpari Unsoed 79 Agritan. Wn 11463 marker was closely linked to the *SKC1* salt tolerance gene (Emon et al. 2015). Atomita and Inpari Unsoed 79 Agritan is also in the same quadrant group with STS marker Wn 11463.

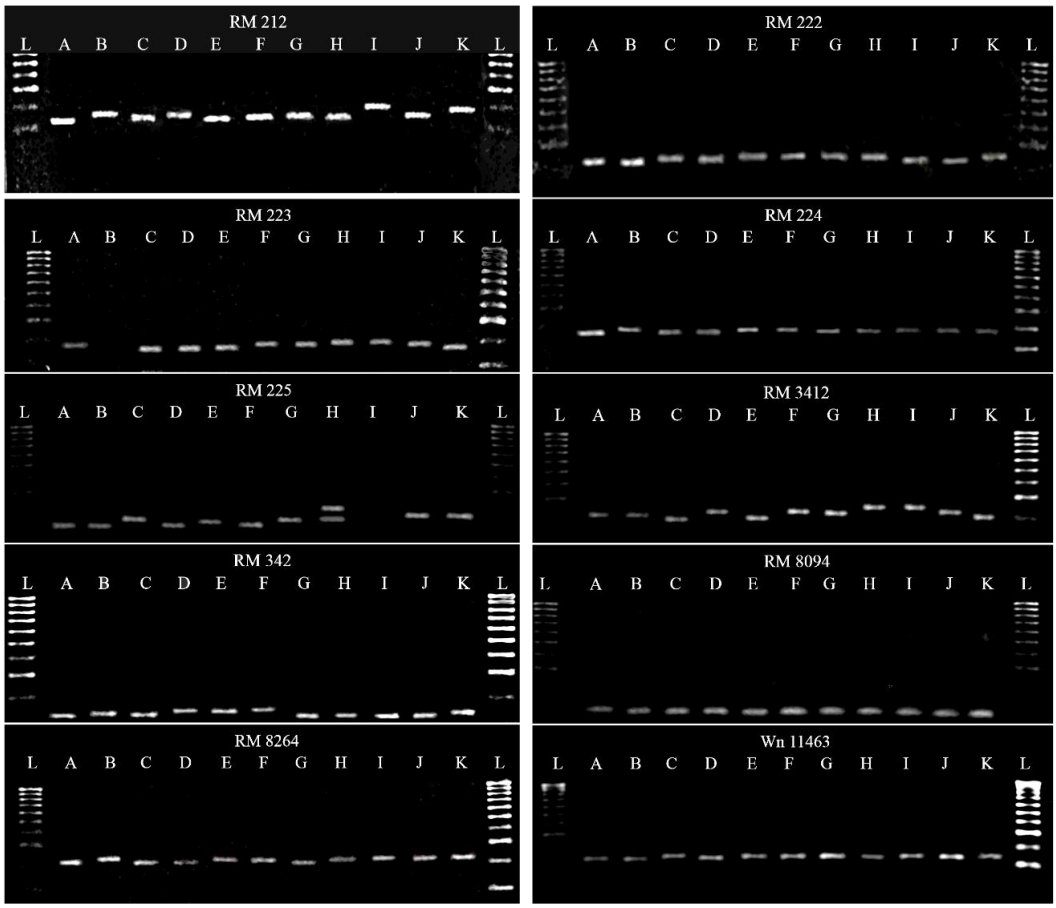

Figure 1. DNA band in 2% gel agarose. A: Nona Bokra, B: Inpari Unsoed 79 Agritan, C: Cisadane, D: Atomita 2, E: Pelopor, F: Dendang, G: Lambur, H: Siak Raya, I: Unsoed Parimas, J: Inpago Unsoed 1, K: IR 29, L: Ladder 100 bp

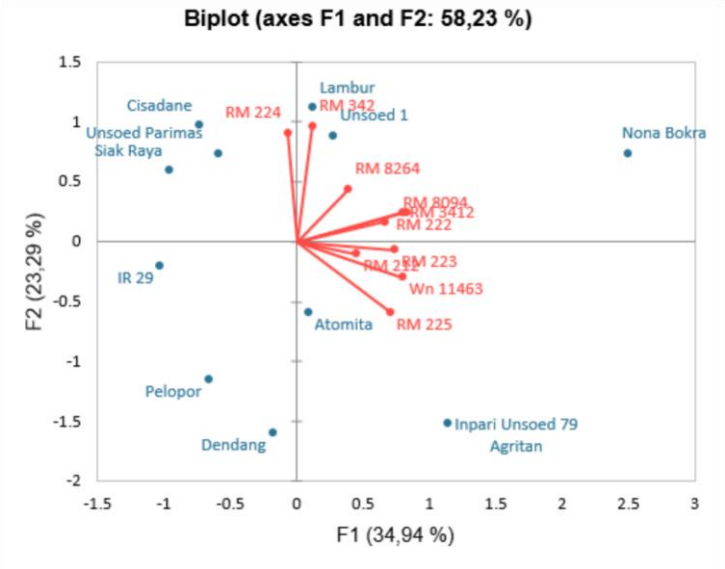

Figure 2. Varietal grouping based on PCA Biplot analysis

Table 3. Chromosome location, number of alleles, and PIC of the evaluated markers

Marker	Chromosome location	Number of alleles	PIC
RM 212	1	3	0.4732
RM 222	10	2	0.3557
RM 223	8	3	0.5632
RM 224	11	2	0.318
RM 225	6	3	0.4762
RM 3412	1	4	0.6781
RM 342	8	3	0.5262
RM 8094	1	2	0.1516
RM 8264	8	2	0.3557
Wn 11463	1	2	0.318

Table 4. Contribution of the marker in the grouping of varieties (%)

Marker	1st Dimension (F1)	2nd Dimension (F2)
RM 212	5.384	0.398
RM 222	11.443	1.110
RM 223	14.337	0.186
RM 224	0.107	32.463
RM 225	12.924	13.511
RM 3412	16.813	2.346
RM 342	0.387	36.585
RM 8094	17.944	2.342
RM 8264	4.025	7.695
Wn 11463	16.635	3.362

IR 29, Pelopor, and Dendang were in the same quadrant with no marker. Interestingly, PCA Biplot analysis placed IR 29, Pelopor, and Dendang in the opposite quadrant of Nona Bokra. IR 29 is a salt-sensitive variety (Ferreira et al. 2015), and consequently, Pelopor and Dendang, as being in the same quadrant, could be considered salt sensitive. Pelopor was previously reported as a salt-sensitive variety (Zannati et al. 2015). Dendang, however, is tidal swamp rice that is reported to be quite tolerant to Fe, Al, and salinity (Suprayogi et al. 2019). The disagreement between our result and that of (Zannati et al. 2015) on the grouping of Dendang still needs to be investigated further using more salt tolerance markers published elsewhere.

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Grouping of the evaluated varieties, as shown in the PCA Biplot demonstrates that markers used in this study could differentiate salt tolerant varieties from the sensitive ones. This is because each marker relates to a different variety in a different quadrant (Figure 2). Table 2 shows that in the F1 (first) dimension, RM 8094 markers contributed the highest in the grouping of varieties, followed by RM 3412, and Wn 11463. Meanwhile, in F2 (second) Dimension, RM 342 had the highest contribution, followed by RM 224.

Analysis of contributing markers to the grouping of varieties (Table 4) and PCA Biplot (Figure 2) demonstrated that RM 8094, RM 3412, Wn 11463, and RM 342 significantly related to salt-tolerant varieties of Nona Bokra, Atomita-2, and Inpari Unsoed 79 Agritan. This result follows the previous report (Emon et al. 2015). RM 3412 and RM 8094 were linked to Saltol QTL, located on chromosome 1 (Geetha et al. 2017). The main genes for salt tolerance traits (*Saltol*) are mapped on chromosomes 1 and 8 (Lang et al. 2017). The STS marker Wn 11463 is significantly linked to the salt-tolerant gene, *SKC 1* (Emon et al. 2015). The RM 342 marker determines salinity tolerance at the reproductive stage and is located on chromosome 8 (Iqbal et al. 2015). The salt tolerance gene on chromosome 8 controls tolerance at the vegetative phase at EC (electrical conductivity) below 10 dSm^{-1} (Lang et al. 2017).

This study also found that the drought-tolerant rice variety Inpago Unsoed 1 fell in the same quadrant as the salt-tolerant variety Nona Bokra (Figure 2). This may suggest that drought tolerance and salt tolerance share some common mechanisms in the plant. This argument is based on (Li et al. 2013) that *DST* gene of salt tolerance also relates to drought tolerance. This gene is located on chromosome 3 and controls stomata's movement during drought and high salt conditions through the regulation of genes involved in ROS (Reactive Oxygen Species) homeostasis (Ar-Rafi et al. 2017).

Apart from marker-based genotypes grouping, validation was carried out for the salt tolerance markers Wn 11463 (Emon et al. 2015), RM 8094, and RM 3412 (Lang et al. 2017). In practice, the marker validation could be made by observing the presence/absence of specifically related salt tolerance marker bands in the evaluated genotypes. However, such a method could only be applied for the validated dominant-type molecular marker or a specific gene sequence. Therefore, for co-dominant-type markers, such as STS marker Wn 11463, and SSR markers RM 8094 and RM 3412, validation should be based on molecular size and its consistency across salt tolerant genotypes.

In the present study, polymorphic bands of Wn 11463 with consistent molecular size could be seen across the reference tolerant genotypes Nona Bokra, Inpari Unsoed 79 Agritan, Atomita-2, Dendang, Lambur, and Siak Raya (Figure 1). This result confirms that Wn 11463 is a strong rice salt tolerance marker (Emon et al. 2015). RM 8094 was polymorphic, but the tolerant genotypes did not necessarily have the same molecular size. RM 3412 produced 3 (three) polymorphic bands (Table 3) with inconsistent molecular size across salt tolerant genotypes. In contrast with Lang et al. (2017), the present study

suggests that validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

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