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Research Article
SSR Molecular Marker Application in Selection of Hybrid Rice Lines with Salinity Tolerant Gene of Back Crossing Generation

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Abstract

Background and Objective: The breeding and selection of high-quality rice cultivars that have tolerant traits with salinity are involved in adapting to climate changes. Marker assisted selection (MAS) played a vital role in accurate and reliable screening methods. By applying SSR markers, the present study aimed to characterize the salt tolerance, aroma and amylose content of the BC_3F_6 generation, which is the hybrid between Jasmine 85 and Pokkali cultivars. **Materials and Methods:** The BC_3F_6 generation of the backcrossing hybrid from Jasmine 85 and Pokkali cultivars was evaluated with salt tolerance, aroma and amylose content. The SSR markers were employed to determine the relation to morphological characteristics. GelAnalyzer software was employed to determine the band sizes of each primer. **Results:** Based on the RM206 and RM201 markers, seven rice lines (P6, P7, P8, P9, P10, P11 and P12) carried salt-tolerant genes by specific band patterns. In terms of aromatic traits, all twelve rice lines contain 2 alleles of the recessive gene. Furthermore, there were 8 rice lines (P1, P2, P3, P4, P5, P6, P7 and P8) containing high levels of amylose, indicated by a Waxy marker. Thus, our results illustrated that three rice lines namely P6, P7 and P8 can tolerate and grow in salt environments. Moreover, yield trait genotypes such as aroma and amylose were also detected in such lines. **Conclusion:** The selected lines were potential breeding materials and should be evaluated in rice field conditions.

Key words: Aroma rice, amylose content, salt tolerance, SSR marker, rice

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the main crops served by more than 3 billion people, mainly in Asia[1]. Many aromatic rice cultivars have become popular with high economic value[2]. Rice is grown in more than 100 countries around the world across continents[3]. In particular, Vietnam is one of the five countries that had the highest rice production along with the following countries: India, Pakistan, Thailand and China[4]. However, soil salinity and ecosystem degradation have negatively affected agricultural productivity and food security. According to Qin *et al.*[5], improving the salt-tolerant characteristic of rice will not only increase the potential use of saline-alkaline land for rice cultivation but also be one of the effective solutions to solve the food problem. This problem requires the breeding of new rice varieties capable of growing in coastal lands and adverse environments. It is crucial to understand the stress tolerance mechanism of rice plants[6] through simultaneous analysis of several factors: Physiological responses, genetic variation, genomic changes and molecular mechanisms[7]; identify quantitative trait loci and markers associated with significant QTLs. There have been many extensive studies on mapping QTL, but very few QTL can be effectively applied in practical breeding. However, these investigations still bring excellent value in identifying QTLs for salt tolerance or related genes in potential gene sources to select and create new species[8].

Saltol is a major QTL for salt-tolerant characteristics at the seedling stage[9]. The QTL Saltol located in chromosome 1 was discovered from a hybrid of IR29 (sensitivity) and Pokkali (tolerance). Based on the results of the correlation between survival days of seedlings (SDSs) and complex salt-tolerance physiological characteristics in rice under the influence of salinity in a hybrid between Indica (Nona Bokra-tolerance) and Japonica (Koshihikari-sensitivity), Lin *et al.*[10] suggested that leaf damage is due to high Na^+ accumulation in shoot, in which Na^+ transports from root to bud when external Na^+ concentration is high. There are two influential QTL factors, qSNC-7 for Na^+ concentration and qSK-1 for K^+ concentration in shoot accounted for 48.5 and 40.1% of the total phenotypic variance, respectively. The QTLs detected between shoot and root are not located in the same location on the genetic map, the genes controlling Na^+ and K^+ transport between shoot and root might different[10]. Another QTL that has been reported is qSNC11, which was identified to reduce Na^+ concentration in the shoot, plays an important role in salt tolerance of rice and can be used to improve the salt-tolerant ability of commercial rice lines using molecular marker-assisted selection (MAS), investigated by Wang *et al.*[11].

Currently, improving the ability of rice plants to adverse conditions with little impact on yield and quality remains a challenge for breeders[5]. Many floating rice cultivars have high living capacity in saline environments such as Pokkali and Nona Bokra. However, the Pokkali's agronomic characteristics are not effective, Pokkali currently plays a role as a salinity tolerance gene in breeding programs[12]. Productivity and quality are two important criteria to ensure food security in domestic and international markets. According to Ganie *et al.*[7], applying molecular markers for assisted breeding (MAB) is one of the leading preferences in assessing genetic diversity, supporting the identification of QTL and markers associated with salt-tolerant ability. In particular, simple sequence repeats (SSR) is a molecular marker closely linked to QTL saltol, which will make the breeding selection more effective. Based on the practical demand, this research was conducted to identify some species with optimal salt-tolerant genotypes and high-quality yield to serve as the foundation for selecting promising salt-tolerant cultivars.

MATERIALS AND METHODS

Materials: The BC3F6 generation of the hybrid between Jasmine 85 and Pokkali.

Duration and location: The research was conducted from March to July 2023 at the Laboratory of the Institute of Food and Biotechnology, Can Tho University, Vietnam.

Methods

Evaluation of agronomic characteristics

Quality criteria analysis: Rice seed length and rice seed width based on IRRI standards[13]. Analyze amylose content according to Graham's method[14], gel form stability analysis method and aroma sensory evaluation method using 1.7% KOH combine with *fgr* genotype test.

Salinity screening: Rice at the seedling stage is purified in an artificial saline environment and NaCl environment with a concentration of 4% (EC = 8 dS/m) according to IRRI standards.

Evaluation of salt-tolerant genotypes of rice lines

Analysis of salt-tolerant genotype: The DNA materials of 12 rice lines were extracted using the modified CTAB method[15]. The DNA extraction solution was CTAB 2X (2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl). In addition, β-mercaptoethanol, chloroform: Isoamyl alcohol (24:1),

Table 1: List of SSR primer pairs associated with salt-tolerant QTL located on 12th chromosomes

Primers	Nucleotide sequences	Motif	Annealing temperature (°C)	Product size (bp)
RM206	F-5'CCCATGCGTTTAACTATTCT3'	$(CT)_{21}$	55	147
	R-5'CGTTCCATCGATCCGTATGG3'			
RM225	F-5'TGCCCATATGGTCTGGATG3'	$(CT)_{18}$	55	140
	R-5'GAAAGTGGATCAGGAAGGC3'			
RM223	F-5'GAGTGAGCTTGGGCTGAAAC3'	$(CT)_{25}$	55	165
	R-5'GAAGGCAAGTCTTGGCACTG3'			
RM201	F-5'CTCGTTTATTACCTACAGTACC3'	$(CT)_{17}$	55	158
	R-5'CTACCTCCTTTCTAGACCGATA3'			
RM535	F-5'ACTACATACACGGCCCTTGC3'	$(AG)_{11}$	55	138
	R-5'CTACGTGGACACCGTCACAC3'			

Table 2: BADH2 primer pairs recognize waxy and fragrant genes

Primer	5'→3' sequence	Product	Band length (bp)
Waxy G-T identification primer[17]			
WxGF	TACAAATAGCCACCCACA	GF-TR (common band)	387
WxGR	GGGAAACAAAGAATTATAAACATATATGTACAC		
WxTF	CATCAGGAAGAACATCTGCAAGT	GF-GR (G type)	207
WxTR	GATCAGCCTAACCAAACA	TF-TR (T type)	235
Aroma identification primer[17]			
EAP	AGTGCTTTACAAAGTCCCGC	ESP-EAP (common band)	580
ESP	TTGTTTGGAGCTTGCTGATG		
INSP	TGGTAAAAAGATTATGGCTTCA	ESP-IFAP (aroma)	257
IFAP	CATAGGAGCAGCTGAAATATATACC	EAP-INSP (non- aroma)	355

isopropanol and ethanol (70%) support DNA extraction efficiency. After extraction, DNA was dissolved in 40 µL TE (pH 8.0) and stored at -20°C.

Salt-tolerant genotype was evaluated based on the association of 12 SSR primer pairs with QTL located on 12 chromosomes according to Lin *et al.*[10] and Thomson *et al.*[16] using the PCR technique. The PCR reactions were performed in 15 µL of PCR mixture, using 2x PCR solution (MyTaq Bioline, UK) including 5X MyTaq Buffer, MyTaq DNA polymerase, molecular grade water, primers and extracted DNA. The solution was mixed well before running on the GeneAmp PCR System 9700 PCR system. The PCR reaction was performed in 35 cycles, including 5 min at 95°C , 30 sec at 95°C, 30 sec at 55-70°C (depending on the temperature of each primer), 30 sec at 72°C, extension for 5 min at 72 and the product stored at 10°C for 20 min and the order of 12 primer pairs with 12 chromosomes is listed in Table 1 and primers for screening waxy and fragrant genes were shown in Table 2. After electrophoresis, the gel was stained with ethidium bromide (10 mg/mL). The PCR results were recorded based on the electrophoresis spectrum for band sizes that match the cultivar served as positive control (tolerance), or the negative control cultivars (sensitivity).

Data analysis methods: Excel 2013 software was used to analyze quality criteria. The DNA band size for genotype assessment was calculated using GelAnalyzer software. The UPGMA method was used through NTSYS pc 2.1 software to create a graph showing the genetic relationship.

RESULTS AND DISCUSSION

Results of assessing salt-tolerant genotypes: In the study of salt-tolerant genotypes evaluation, the rice lines used as the tolerant control was Doc Phung, numbered 2 and the sensitive control was IR29 cultivar.

The RM206 primer pair was located on chromosome 11 linked to QTL qSNC11 and showed the ability to excrete Na^+ in the shoot. Effective elimination will reduce the concentration of Na^+ in the shoot, giving better salt-tolerance[11]. The amplification reaction was successful in all samples and amplicon sizes ranged from 150 to 200 bp. Gel patterns indicated that there were differences between rice lines (Fig. 1). It was observed polymorphic bands among individuals of P2, P3 lines and P6, P7, P8, P9, P10, P11 and P12 lines when nine samples showed 147 bp bands, similar to the Pokkali and Doc Phung cultivars, which were all positive standards for salt-tolerant rice. However, other samples in the P1, P4 and P5 lines had distinct electrophoretic bands from Pokkali and Doc Phung with band-matching IR29. Thus, the purity of the P1, P4 and P5 lines was not stable to use for the breed development in salinity areas. The RM206 marker had been confirmed to have a close correlation with the salt-tolerant ability of rice plants. Rana *et al.*[18] identified the SSR

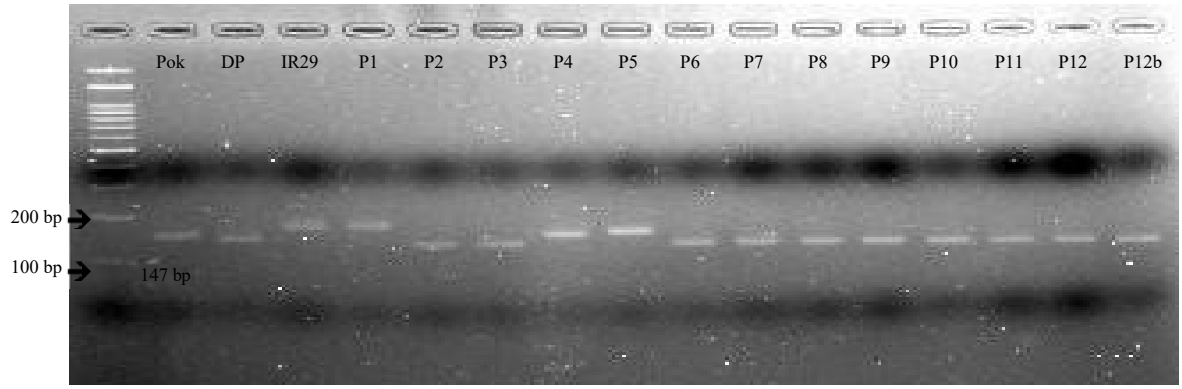

Fig. 1: RM206 primer confirmed the salt-tolerant genotype of rice lines

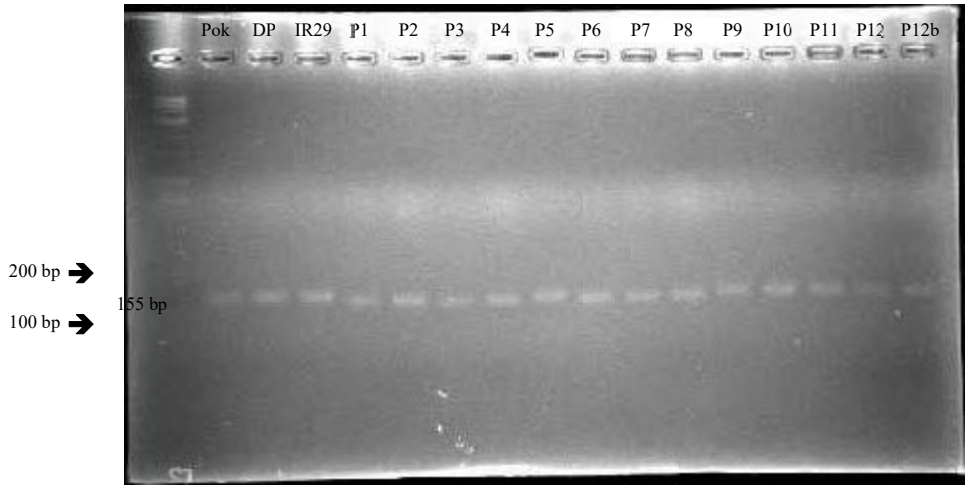

Fig. 2: RM201 primer confirms the salt-tolerant genotype of rice lines

marker for salt-tolerant characteristics in the F3 of the CSR10 rice population and concluded that RM206 was significantly associated with the Na/Ka phenotype. Furthermore, Hasan *et al.*[17] also stated that this marker is also a useful marker for bacterial leaf blight disease resistance based on 1.9 cM near the Xa23 locus.

The amplification reaction was successful in all samples and amplicon sizes ranged from 150 to 200 bp. The gel pattern indicated that there were differences among rice lines (Fig. 2). It was observed polymorphic bands among individuals of P6, P7, P8, P9, P10, P11 and P12 lines while seven samples showed band 155 bp, similar to Pokkali and Doc Phung cultivars, these were positive standards for salt-tolerant lines. However, other samples in P1, P4 and P5 lines had different electrophoretic bands from Pokkali and Doc Phung, these cultivars had bands matching with IR29. Thus, the purity of P1, P2, P3, P4 and P5 lines was not stable to use for breed development in salinity areas. The RM201 marker has been confirmed to have a close correlation with the salt-tolerant capacity of rice plants. Rana *et al.*[18] identified the SSR marker for salt-tolerance in hybrid populations of G46A and IR25571R, concluding that RM201 was significantly associated with Na/Ka phenotype. Furthermore, this marker is also useful for drought resistance[19].

Results of aromatic genotype assessment: The scent of rice is controlled by the *frg* gene on chromosome 8, the presence of the BADH2 enzyme will reduce the content of 2AP, one of the main compounds in aromatic rice. Meanwhile, removing the nucleotide pairs of this gene leads to the inactivation of the BADH2 enzyme and the 2AP compound accumulates enough to create a scent for rice[19]. Four specific primers amplified 2 bands 577, 355 and 257 bp for homozygous dominant, indicating the results of aromatic gene experiments on the BC3F6 generation (Fig. 3) for all rice lines. Thus, hybrid rice cultivars carried both 2 alleles of the recessive gene. In 2021, Carsono *et al.*[20] used two pairs of primers, ESP and IFAP,

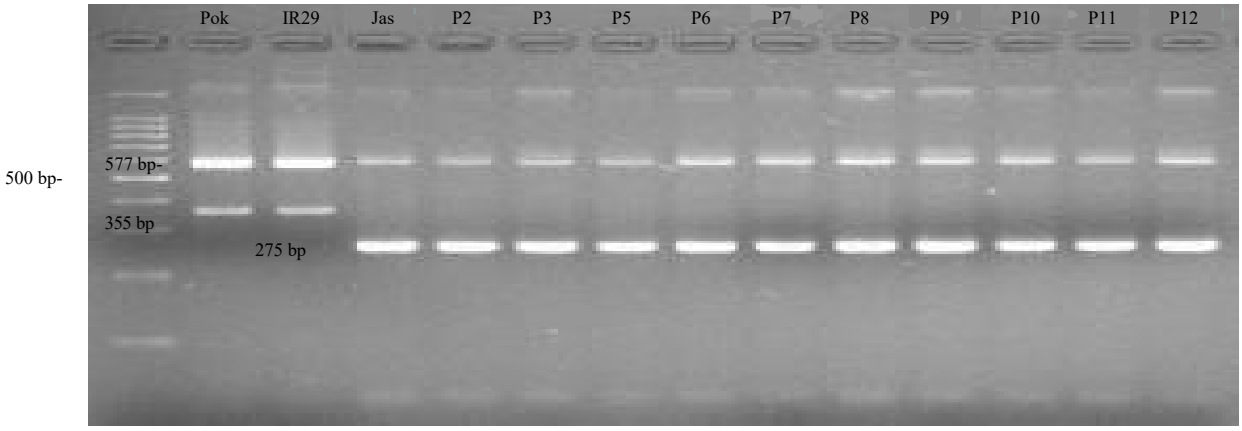

Fig. 3: *frg* primer confirmed aromatic genotype on chromosome 8th of improved rice lines

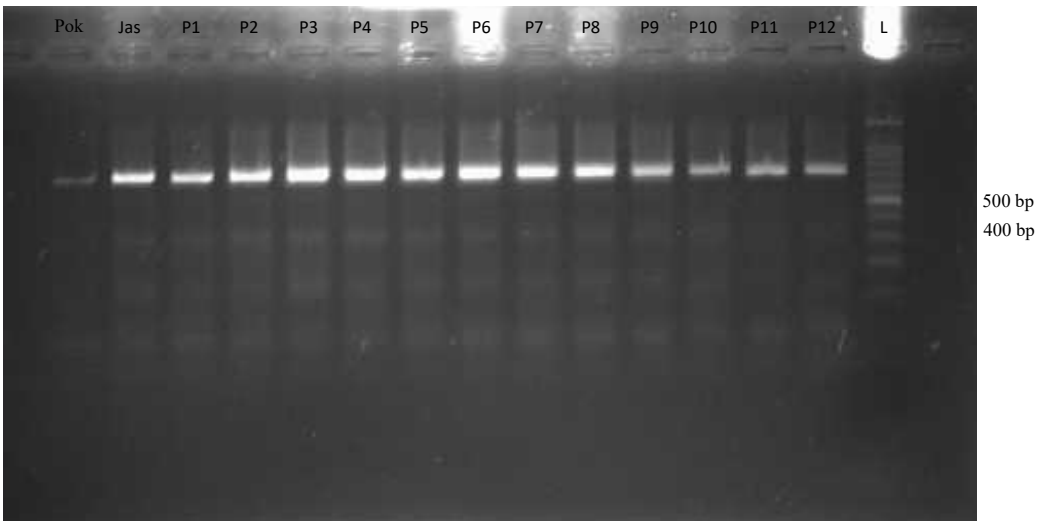

Fig. 4: WAXY primer confirmed the starch expression genotype on chromosome 6th of rice lines on 8% acrylamide gel

to quickly and effectively identify the aromatic lines of the F2 generation when overlapping genes between Pandanwangi (aromatic rice), PTB33 (brown planthopper-resistance), Ciapus (high yield and low amylose) and KA (short-day growing). This study also illustrated similar results with Bradbury *et al.*[21]. The molecular markers demonstrated that in the backcrossing method to the BC3F6 generation, the possibility of the offspring gene source being the same as the mother plant, specifically in this experiment was Jasmine 85.

Results of amylose genotype assessment: Wang *et al.*[22] determined the sequence of the waxy gene in the *Oryza sativa* (*Japonica* Heng-feng) species, showing that wx is 5499 bp long, including 14 exons and 13 introns. Chang-Jie *et al.*[23] research shows that there are 3 alleles at locus wx: Wxa, Wxb and wx are recessive alleles.

Zhao *et al.*[24] suggested that the Wxa allele regulates high amylose content, is incompletely dominant over the Wxb allele, which regulates low amylose content. The Wxa is present in most rice lines of the *Indica* subspecies and Wxb in *Japonica* variety. The results of waxy primer analysis of 12 hybrid lines showed that the lines had different band lengths compared to the Pokkali parental cultivar and similar band lengths to the maternal variety Jasmine 85 (Fig. 4).

The results of PCR products from the RM230 marker were almost similar to Jasmine 85, specifically in P1, P2, P3, P4, P5, P6, P7, P8 lines; while P10, P11 and P12 lines had a similar band to the parental Pokkali (Fig. 5). This result was also consistent with the phenotypic contribution of the QTL marked by RM203, which expressed a closer linkage to rice shape and size traits than to amylose content traits[25]. The RM203 marker was determined to be located on QTL *Btemp*,

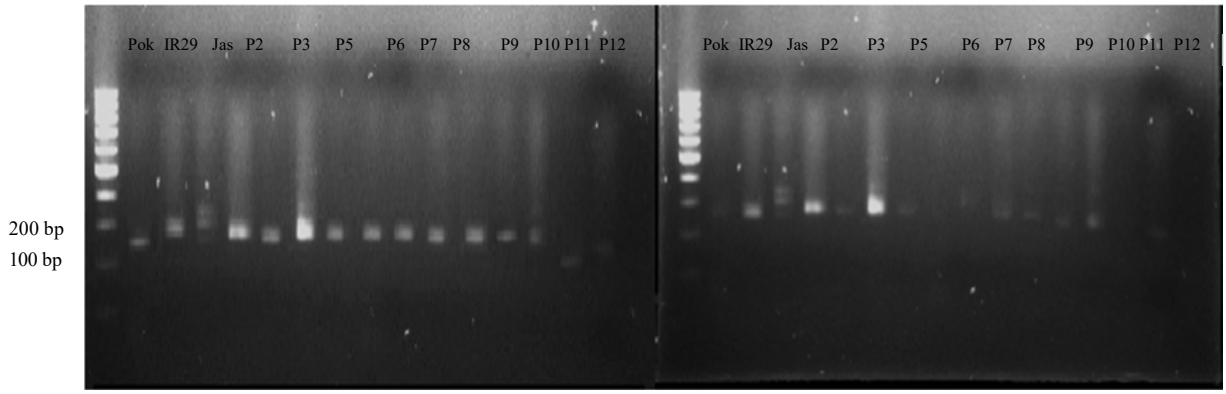

Fig. 5: Electrophoretic spectrum of PCR products of rice lines using RM230 and RM203 molecular markers

contributing 9.3% of the phenotypic variation in lake temperature trait in a recombinant inbred population (RIL) with 188 individuals from the hybrid combination between Zhenshan 97/Delong 208[26].

Starch is the main component stored in the endosperm of cereal grains in the form of glucid. Starch granules are mainly composed of two forms of polysaccharide: Amylose (0-30%) and amylopectin (>70)[20]. The amylose content determines the softness and elasticity of the rice grain: The higher the amylose content, the drier and harder the rice grain is. Amylose molecule has a straight, unbranched chain structure, made up of 300-1000 glucose residues by the β-(1-4) glucan bond with a molecular weight of 100-200 kDa. The amylose chain has a spring helical shape with 6 glucose bases on one ring, each spiral absorbs one iodine molecule to form a bluish-black solution. When heated, the iodine molecule separates and loses its color[27]. This characteristic is used to determine the amylose content in starch.

CONCLUSION

The evaluation result of salt-tolerant genotypes and traits of 12 notable lines with salt-tolerant genes, 7 lines were identified with electrophoretic bands matching with the control Doc Phung cultivar and the parental Pokkali cultivar. Regarding the aromatic trait via molecular analysis, it was shown that all 12 lines carried the aromatic genotype. Amylose content in rice grains is controlled by Waxy genes. The PCR product results from the RM230 marker were almost similar to Jasmine 85 cultivar, specifically in P1, P2, P3, P4, P5, P6, P7 and P8 lines; while the P10, P11 and P12 lines gave a similar appearance to the parental Pokkali cultivar. This result was also consistent with the QTL contribution of the RM203 marker.

SIGNIFICANCE STATEMENT

The study aimed to determine the effectiveness of SSR markers in selecting rice lines with desirable traits including salinity tolerance, amylose content and aroma. The used primers consisting of RM201, RM206, RM223, RM225 and RM535 could be distinguished the rice lines based on salinity tolerance trait. Based on selecting criteria, P6, P7 and P8 rice lines obtained expected characteristics. The findings suggest that the SSR markers were reliable in selecting rice lines with expected traits and could be applied in other characteristics.

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