



## Assessment of Marker Based Genotype Diversity of Saltol QTL in Relation to Seedling Stage Salinity Tolerance in Selected Rice Genotypes

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### ABSTRACT

Salinity stress is one of the major soil problems in relation to rice production in Sri Lanka. Development of salinity tolerant rice through rice breeding is a key solution for salinity problem. *Saltol* is the major Quantitative Trait Loci (QTL) responsible for salt tolerance at the seedling stage of the rice plants which is mapped on chromosome one of the rice genome. To select most salt tolerant rice genotypes for future breeding programmes and test the usefulness of the Microsatellite (SSR) markers associated with *Saltol* QTL, 18 rice varieties were screened using electrical conductivity 12 dSm<sup>-1</sup> nutrient solution for salt tolerance at the seedling stage using a visual score of 1-9 scale. Six polymorphic SSR markers linked to the *Saltol* QTL were used to determine the impact of these markers associated with salt tolerance in rice. Results of the phenotypic screening of 18 rice genotypes showed different responses for salt stress at the seedling stage. Based on the phenotypic screening rice genotypes were categorized into four groups, and *Pokkali* accessions were the most tolerant genotypes for salinity stress which can be useful as donor parents for future rice breeding programs. Microsatellite marker RM10843 showed highest allelic number while RM140 and RM10748 showed lowest allelic number. Polymorphism information content (PIC) value varied from 0.1780 for RM10748 to 0.7659 for marker alleles. Based on the six SSR markers, 18 rice genotypes were clustered into two groups and salinity tolerant *Pokkali* accessions were grouped in a separate cluster. Salinity tolerant and susceptible genotypes were clearly discriminated by RM10852 marker in 3% agarose gel and positively associated with the result of the phenotypic screening. Therefore, the marker will be useful in Marker Assisted Selection (MAS) in rice breeding programmes for salinity tolerance.

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## INTRODUCTION

Rice is one of the most important cereal crops which serves as the staple food for one-third of the global population. It is the world's third-largest cereal crop after wheat and maize (Mohammad-Nejad *et al.*, 2010). However, many abiotic stresses such as salinity, iron toxicity, acidity and drought greatly affect the rice production in these regions. At present, salinity is the second most widespread abiotic problem next to drought (Islam *et al.*, 2010). Around 21.5 million hectares of the land area is seriously affected by salinity in the south and south-east Asian region alone, where majority of the rice-growing areas of the world are situated (Li *et al.*, 2014). Salinity is a major constraint to expanding rice cultivation in Sri Lanka. Nearly 70% of the coastal rice lands are reported to be affected by salinity (De Costa *et al.*, 2012). Sirisena and Herath (2009) stated that salinity is gradually spreading in rice lands of Sri Lanka both in coastal and inland regions. Out of 12,000 ha of cultivated rice lands in Jaffna, about 3,000 ha are unproductive due to salinity (Rajadurai, 2003).

Rice plant is highly sensitive for salt stress, especially at early seedling and late reproduction stages (Zeng and Shannon, 2000), where salinity greatly affects rice yield. Vinod *et al.* (2013) reported a 12% yield reduction for every unit of  $\text{dSm}^{-1}$  of electrical conductivity (EC) above the threshold level of tolerance of  $3.0 \text{ dSm}^{-1}$ .

Breeding a salinity tolerant rice variety is one of the best approaches to overcome the effect of adverse saline soil condition. The rapid advancement of DNA based molecular marker technologies and their applications in plant breeding programmes were reported in the last two decades. Unravelling of rice genome has given an infinite opportunity to produce SSR markers throughout the rice genome (Thomson *et al.*, 2010). Therefore, a higher number of SSR markers are developed which are tightly linked to the important genes and QTLs regulating different important mechanisms in rice plant. *Saltol* is one of the major QTLs for salt tolerance at the seedling stage

(Bonilla *et al.*, 2002). Recently the *Saltol* QTL has been mapped by several studies (Bonilla *et al.*, 2002; Thomson *et al.*, 2010; Soda *et al.*, 2013). One of the studies reported that *Saltol* QTL is located between the SSR marker RM1287 and RM6711 (10.8Mb -16.4Mb) in the short arm of the rice chromosome one (Thomson *et al.*, 2010).

Platten *et al.* (2006) reported *SKC1* (later named as gene *OshKT1;5*) is a causal gene underlying the *Saltol* QTL which is supported to control sodium/potassium ( $\text{Na}^+/\text{K}^+$ ) ratio. *SKC1* was found to encode a sodium transporter protein that helps control  $\text{Na}^+/\text{K}^+$  homeostasis through the unloading of sodium from the xylem, which has been functioning mainly in roots to reduce the amount of  $\text{Na}^+$  ions that are transported to the leaves (Ren *et al.*, 2005). There are many functional SSR markers which are tightly linked to the important loci of the *Saltol* QTL. Therefore, those markers will be very useful in genetic studies of the *Saltol* region and will provide significant assistance for future salinity tolerance rice breeding programmes. The objectives of the present study were as follows: The assessment of salinity tolerant ability of the popular local rice varieties and group those varieties based on their salinity tolerance level at the seedling stage, select the useful donor parents for future salinity tolerant rice breeding programmes, identification of positively associated SSR marker/markers for salinity tolerance with the results of phenotype screening for salinity tolerance, and identification of useful SSR markers for marker assisted selection to assist future salinity tolerant rice breeding programmes.

## MATERIALS AND METHODS

The laboratory work was conducted at the Rice Research and Development Institute (RRDI) at Bathalagoda from May 2016 to November 2017. Sixteen rice varieties and two *Pokkali* accessions were used for the study. Seeds of each variety were obtained from RRDI, Bathalagoda and Plant Genetic Resource Center, Gannoruwa, Sri Lanka.

### Morphological screening

The salinity tolerant ability at the seedling stage of the 16 rice varieties namely Bg369, Bg359, IR42, IR46, Bg250, Bg300, Bg360, Bg358, Bg370, Bg357, Bg310, Bg94/1, IR36, Bg380, At303, At362 and two *Pokkali* accessions were tested by using the method developed by Gregorio *et al.* (1997b) with some modifications. Varieties were selected based on different criteria such as reported salinity tolerant ability under field conditions, popularity of the variety and usability of each variety for future breeding programmes as donor and recipient parents. *Pokkali* IRRI accession, which is the most salinity tolerant IRRI accession and, *Pokkali* local accession 5557, which is one of the most salinity tolerant local accessions (Senanayake *et al.*, 2017a) were used as known salinity tolerant genotypes.

The morphological screening was done in the green house of the RRDI, Bathalagoda. Two replicates (two trays) were used for each variety in Complete Randomized Design (CRD) for the experiment. Each variety was separately planted in the each replicate with twenty-five seedlings. Rectangular Styrofoam sheets of 50 cm (Length, L) x 30 cm (Width, W) x 1.25 cm (Depth, D) were used for anchoring seedlings on the nutrition solution. Holes of 1 cm diameter were drilled, which were spaced 2.5 cm within the rows and 4 cm between the rows. A nylon net with sieve size less than 1 mm was fixed on one side of each styrofoam board. Plastic trays of 55 cm (L) x 35 cm (W) x 8 cm (D) were used to fill the nutrient solution and the prepared styrofoam board floated on these trays without letting them move.

### Seed germination and maintenance during the first twelve days

Sterilized seeds were placed in petri dishes on moistened filter paper and incubated for 48 hours to facilitate germination as in Gregorio *et al.* (1997b). Two pre-germinated seeds were inserted per each hole on the styrofoam seedling float and radicle was carefully inserted through nylon mesh.

Pre-germinated seeds were maintained under the water for three days to recover the damage of roots. After three days, the water was replaced by 2 dSm<sup>-1</sup> Albert solution at pH 6.5. The seedlings were maintained for nine days in these conditions. One well grown seedling was retained while removing the poorly grown seedling in each hole. Twenty-five healthy seedlings were remained in each replicate. Freshly prepared Albert solution at the 2 dSm<sup>-1</sup> was added to maintain the constant level of the nutrient solution to avoid plants getting dried in first 12 days.

### Initiating salt stress condition and evaluation of the seedlings

Pre-washed table salt was used to prepare a solution with electrical conductivity (EC) 10 dSm<sup>-1</sup>. Albert nutrient solution was added to the salt solution to increase the EC up to 12 dSm<sup>-1</sup> and pH was adjusted to 6.5 using 1M sodium hydroxide solution. This solution was used as the stress solution to evaluate the response of the rice seedlings. The initial nutrient solution (2 dSm<sup>-1</sup>) was replaced by using 12 dSm<sup>-1</sup> stress solutions to start the salt stress on rice seedlings, when seedlings were 12 days old. The rice seedlings were exposed for the salt stress condition for 14 days. The control experiment (unstressed seedlings) consisted of 10 plants of each variety which was maintained with Albert solution at 2 dSm<sup>-1</sup> EC and pH 6.5 simultaneously. The EC and pH of the stress solution were checked every 3 days and kept constant throughout the stress period by adding freshly prepared 12 dSm<sup>-1</sup> solution as same to the initial stress solution.

The seedlings were visually inspected for salt stress symptoms at 7, 10 and 14 days after imposing the stress solution. Salt stress symptoms in each plot were inspected separately and tolerance score was assigned based on modified standard evaluating score system (Table 1) developed by Gregorio *et al.* (1997b). Finally average tolerance score was given for each variety and categorized as in Table 1. To check the differences of response among varieties for salinity

tolerance at seedling stage, ANOVA was performed using General Linear Model (GLM) procedure followed by the Duncan

Multiple Range Test (DMRT). The evaluated results were used for the molecular marker-based genotype diversity analysis.

**Table 1: Modified standard evaluating score system for salinity screening at the seedling stage**

Tolerance Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant (HT)
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled	Tolerant (T)
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant (MT)
7	Complete cessation of growth; most leaves dry; some plants drying	Susceptible (S)
9	Almost all the plants dead or drying	Highly susceptible (HS)

Source: Gregorio et al. (1997b)

### SSR marker based analysis

Six polymorphic SSR markers: RM10852, RM10843, RM140, RM10748, RM3412, and RM8094 which are located in the *Saltol* QTL region were used to study the genetic diversity in the *Saltol* QTL in selected rice genotypes. These 6 markers were tightly linked to *Saltol* QTL and which are spanning from 11.2 Mbp to 14.2 Mbp in the *Saltol* region of the chromosome one of the rice genome (Thomson et al., 2010).

The total genomic DNA in 18 rice genotypes was extracted by using CTAB DNA extraction method. Polymerase Chain Reaction (PCR) was performed by using My cycler™ Thermal cycler (BIORAD, 2800 Woods, Madison, USA). The PCR amplification was done with Promega green PCR master mixture (PCR master mixture system, USA), with a total volume 15 µL, which contained 6 µL of the master mixture, 4 µL of nuclease-free water, 1 µL of the forward primer, 1 µL of reverse prime and 3 µL of Genomic DNA. PCR was conducted with an initial denaturation step at 94 °C for 5min followed by 35 thermal cycles of denaturation at 94 °C for 1min, annealing temperature 56 °C, 63 °C, 65 °C, 58 °C, 57 °C, 52 °C were respectively used for

RM10852, RM10843, RM140, RM10748, RM3412, and RM8094 primers for 1min, extension at 72 °C for 1min, terminated with a final extension of 5min at 72 °C. The PCR products were resolved with 3 % agarose gel electrophoresis for 2.5 hours and gels were stained by adding 10 µL of 10 mg/µL ethidium bromide stock solution.

SSR allelic composition for each genotype at every marker locus was determined by counting the frequency of major allele, number of genotype, number of alleles per locus, gene diversity, heterozygosity and polymorphic information content (PIC) value. Power marker version 3.25 (Liu and Muse, 2005) was used to perform those analyses. Dendrogram was constructed and cluster analysis was done with Un-weighted Pair Group Mean Algorithm (UPGMA) and similarity index developed by Nei et al. (1983) by MEGA 7.0 computer programme.

## RESULTS AND DISCUSSION

### Morphological Screening

Seedlings grew well without showing any abnormal conditions before commencing

the salt stress. One week after the salt stress, varieties differently responded for salt stress. *Pokkali* IRRI accession, *Pokkali* local accession 5557 and IR42 varieties did not show stress symptoms. However, during the last few days of the stress period, salinity susceptibility symptoms such as drying and rolling of mature leaves were gradually developed in both *Pokkali* accessions. However, majority of the plants of *Pokkali* variety grew well without showing salt stress symptoms. Based on the mean separation score, both *Pokkali* accessions were significantly different from other varieties at the end of the stress period (Table 2). Average tolerance score of 3 was given for those two varieties. Both IRRI and local *Pokkali* accessions were categorized under the salinity tolerant category (T) based on the modified standard evaluating score system for salinity screening at the seedling stage developed by Gregorio *et al.* (1997b). The same local *Pokkali* accession (accession no. 5557) and *Pokkali* IRRI accession were categorized under tolerant (T) category after screening in 12dSm<sup>-1</sup> stress solution for 14 days by Senanayake *et al.* (2017a and 2017b). However, Mohammadi-Nejadi *et al.* (2010) reported that the *Pokkali* IRRI accession showed highly tolerant (evaluating score 1) response reference to the same scoring system after following the same screening procedure after 14 days of stress period. However, based on this study, the *Pokkali* IRRI accession and *Pokkali* local accession (Bathalagoda accession no. 5557) were tolerant to salinity stress at the seedling stage and there was no significant difference in the tolerance level between the two varieties. Hence, those varieties can be used as donor parents for future salinity tolerance improvement programmes.

After one week of salinity stress, the varieties Bg369, Bg359, IR42 and IR46 began to gradually develop the salt stress symptoms. When it was 14 days of salinity stress, the symptoms could be well recognized. The symptoms based average tolerance score 5 was given for those varieties and it was significantly different

from the *Pokkali* accessions. Bg369 symptoms were significantly different from other varieties, which scored a value of 5 and categorized under moderately tolerant category (Table 2). However, Senanayaka *et al.* (2017b) reported that the variety Bg369 was categorized under the salinity tolerant category at seedling stage. Therefore, the popular local variety Bg369 will be useful for cultivation in moderately saline regions. However, the salinity tolerance response of these varieties both at the vegetative and reproductive stages should be evaluated to arrive at a more precise conclusion.

Most part of the leaves of the Bg357, Bg310, Bg94/1, IR36, Bg380, At303 and At362 varieties were dried and seedling growth was severely retarded with the salinity stress at the end of 12 days. The tolerance score of these varieties was significantly different from moderately tolerant varieties. Average evaluating score of 7 was given for these varieties based on the symptoms and categorized under the salinity susceptible (S) category. The varieties Bg250, Bg300, Bg360, Bg358 and Bg370 almost withered and dried during seedling stage itself and no vegetative growth could be observed. The tolerance score of these varieties was significantly different from susceptible varieties. Average evaluating score of 9 was given for those varieties and were categorized under highly susceptible (HS) group. None of the varieties in the control experiment show any salt stress symptom and hence their salinity tolerance score was 1. Salinity affects all growth stages of the rice plant in varying degrees from germination to maturation. The rice plant is especially very sensitive to salinity during the early seedling and late reproductive stages (Lafitte *et al.*, 2004). Therefore, the result of the morphological screening will be very useful for selecting the suitable varieties for the saline soils in Sri Lanka. Therefore, a prospective study to test the varietal response for salinity stress at the vegetative and reproductive stages would be helpful for further validation of the results. The final response of the 18 rice varieties for the salt stress after 14 days is shown in table 2.

**Table 2: Summary of the phenotypic screening of the 18 rice genotypes for salt stress**

Entry no	Variety	Pedigree	Evaluating Score	Reactions to salinity
1	<i>Pokkali</i> (IRRI) <sup>a</sup>	Not reported	3	T
2	<i>Pokkali</i> (5557) <sup>a</sup>	Traditional	3	T
3	Bg369 <sup>b</sup>	Bg94-2/ <i>Nonabokra</i>	5	MT
4	Bg359 <sup>c</sup>	Bg88-5089/Bg 379-2	5	MT
5	IR46 <sup>c</sup>	IR1416-131-5/IR1364-37-3-1 IR1366-120-3-1/IR1539-111	5	MT
6	IR42 <sup>c</sup>	IR1561-228-1-2/IR1737 CR94-13	5	MT
7	Bg380 <sup>d</sup>	Bg90-2*/Ob677	7	S
8	Bg357 <sup>e</sup>	BG797/BG300//85-1580/ <i>SenerangM-17</i>	7	S
9	At303 <sup>e</sup>	At66-2/Bg 276-5	7	S
10	Bg310 <sup>f</sup>	Bg 300/ <i>Pokkali</i>	7	S
11	Bg94/1 <sup>h</sup>	IR262/Ld66	7	S
12	IR36 <sup>h</sup>	Not reported	7	S
13	At362 <sup>g</sup>	At85-2/Bg380	7	S
14	Bg358 <sup>i</sup>	Bg12-1/Bg1492	9	HS
15	Bg360 <sup>i</sup>	84-3346/IR36// <i>Senarang</i>	9	HS
16	Bg370 <sup>j</sup>	IR555178/9-3R//IR65515-47-2-1/IR6551-47-2-1-91	9	HS
17	Bg300 <sup>k</sup>	Bg367-7//IR841/Bg276-5	9	HS
18	Bg250 <sup>k</sup>	Selection from farmer field	9	HS

Note: the mean followed by the same letter are not significantly different at p=0.05

T-Tolerance, MT-Moderately Tolerance, S-Susceptible, HS-highly Susceptible

### Molecular marker analysis

The six markers were spanning for a 3.0 Mbp length in the *Saltol* region of the chromosome one (Thomson *et al.*, 2010). The SSR markers were based on the allele diversity which ranged from one hexa-allelic marker RM10843, one penta-allelic marker RM3412, one quad-allelic marker RM10852 and three bi-allelic markers RM8094, RM140 and RM10748. The RM10843 marker will be useful to perform a molecular diversity analysis between different *Pokkali* accessions in Sri Lanka, because *Pokkali* alleles were slightly distinguishable from other alleles of the different genotypes. Nineteen separate alleles were identified for

six SSR markers with 3.5 average alleles per marker. Highest number of genotype number was six and it was given by RM 10843 markers. Mohommad-nejad *et al.* (2010) and Chowdhury *et al.* (2016) reported that RM8094 marker showed highest PIC value and highest number of marker alleles. The present study did not agree with those studies where RM8094 showed only two alleles for eighteen genotypes with low PIC value. Chowdhury *et al.* (2016) also reported that RM 10843 and RM3412 showed only three marker alleles among 54 rice genotypes in 6% poly acrylamide gel electrophoresis. In the present study same markers have given a

high number of alleles even in 3% agarose gel electrophoresis.

The polymorphic information content (PIC value) in the present study varied from 0.1780 to 0.7659 with an average 0.4650. Based on the PIC value three markers, i.e. RM3412, RM10843 and RM 10852, were considered as highly informative because the PIC value was greater than 0.5. The highest PIC value was reported by RM10843 SSR marker as 0.7659. RM8094 SSR maker showed a PIC value of 0.3711 and it was considered as an informative marker because PIC ranges were 0.25-0.5. The markers RM140 and RM 10748 were considered as less informative because they showed a PIC value less than 0.25 (Table 3).

The marker alleles of RM10852 (Figure 1) was positively associated with the salinity tolerance genotypes. The marker alleles of the *Pokkali* IRRI accession and local accession were unique and discriminated salt tolerant and susceptible genotypes. The

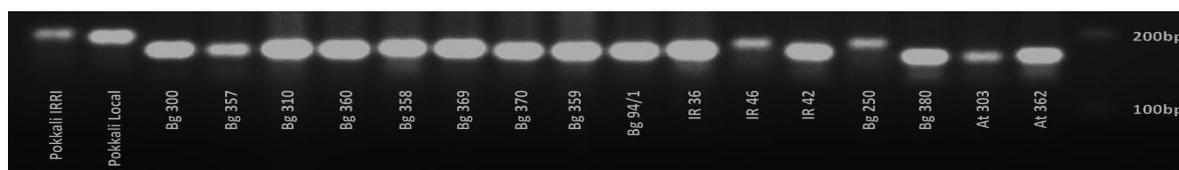
RM10852 markers fairly discriminated the salinity tolerance genotypes even in 3% Agarose gel. It is easy and less laborious compared with the polyacrylamide gel electrophoresis. Hence, this marker would be an effective tool for marker-assisted back-crossing. Huyen *et al.* (2013) reported RM10852 marker could discriminate the salinity tolerance and susceptible genotypes in 6% polyacrylamide gel electrophoresis. Therefore, further validation with polyacrylamide gel electrophoresis will be helpful to re-confirm the results of the present study. Furthermore, *Pokkali* marker allele RM10852 will be useful to select the back-crossed population, if the same *Pokkali* accessions is used as donor parents in marker assisted back-crossed programmes. The *Pokkali* allele of the RM10843 marker showed some separation from other alleles except for a few exceptions. However, polyacrylamide gel electrophoresis is needed to confirm whether it is clearly discriminate two salinity tolerance genotypes form others (Figure 2).

**Table 3: Summary of the molecular marker analysis**

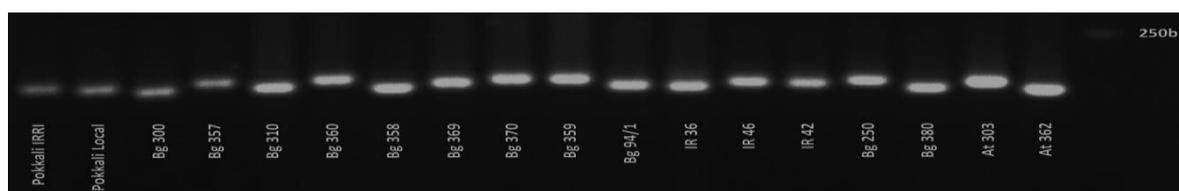
Marker	Major Allele Frequency	No of genotypes	Allele No	Gene Diversity	Polymorphic Information Content (PIC)
<b>RM3412</b>	0.2222	5.0	5.0	0.7963	0.7637
<b>RM10748</b>	0.8889	2.0	2.0	0.1975	0.1780
<b>RM10843</b>	0.2778	6.0	6.0	0.7963	0.7659
<b>RM140</b>	0.8889	2.0	2.0	0.1975	0.1780
<b>RM10852</b>	0.6111	4.0	4.0	0.5741	0.5332
<b>RM8094</b>	0.5625	2.0	2.0	0.4922	0.3711
<b>Mean</b>	0.5752	3.5	3.5	0.5090	0.4650

RM 8094 is one of the most tightly linked markers in *Saltol* QTL with highest LOD peak value and the maximum alleles were found for the RM8094 marker (Thomson *et al.*, 2010; Islam *et al.*, 2011). The previous studies conducted by Mohammad-Nejad *et al.* (2010), Thompson *et al.* (2010) and Babu *et al.* (2014) used RM8094 and RM3412 to test the discriminating ability of salinity

tolerance and susceptible genotype. In those studies, maximum alleles were found for the RM8094 marker. Rice genotypes having *Pokkali* allele for RM8094 marker were strongly associated with tolerant or highly tolerant phenotypes during the morphological screening. However, in this study, this positive association was not identified for the RM8094 marker.



**Figure 1: Amplified RM10852 marker alleles of 18 rice genotypes on 3% Agarose Gel**



**Figure 2: Amplified RM10843 marker alleles of 18 rice genotypes on 3% Agarose Gel**

### Cluster analysis

According to the similarity matrix introduced by Nei *et al.* (1983), lowest genetic distance (0.333) was reported between *Pokkali* IRRI and *Pokkali* local accession which reflected the genetic closeness between the *Pokkali* local and *Pokkali* IRRI varieties. The UPGMA analysis defined all 18 rice genotypes into two clusters (Figure 3). Cluster one consisted of the salinity tolerance genotypes which consisted of only the *Pokkali* IRRI accession and *Pokkali* local accession. These two varieties were clearly divergent from the moderately tolerant, susceptible and highly susceptible varieties during the phenotypic screening too. Therefore, phenotypic screening results were further confirmed by salinity tolerance accessions by the cluster analysis. The results also showed that the salinity tolerance of the *Pokkali* accessions share a common allele for the markers.

Therefore, screening of all local *Pokkali* accessions and genotyping with more tightly linked SSR markers to *Saltol* region will be useful to further explain the relationship between *Pokkali* allele of the marker and salinity tolerance response during the seedling stage.

The second cluster was divided into two sub-clusters (Figure 3). Moderately salinity tolerant and susceptible varieties were distributed in these two clusters and it was difficult to explain the common relationship of the varieties in cluster 02 and sub-cluster of the cluster 02. The moderately tolerant varieties Bg359 and Bg369 were clustered in sub cluster 02. Therefore, the salinity tolerance relationship among these varieties at the seedling stage is difficult to explain by these markers, because the salinity tolerance mechanism of seedling stage regulation is done by many other QTLs in addition to *Saltol* QTLs. Gregorio (1997a)



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