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# Genetics of Salt Tolerance in Cucumber (*Cucumis sativus* L.) Revealed By Quantitative Traits Loci Analysis

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## Abstract

To identify quantitative trait loci controlling salinity tolerance in cucumber, a total of 432 simple sequence repeat (SSR) markers were screened on two cucumber inbred parental lines, 11411S (salt tolerant) and 11439S (salt sensitive). Genotypic analysis was conducted using $F_2$ individuals while $F_{2:3}$ population was used for phenotypic evaluation and quantitative trait loci (QTL) analysis. Sixty polymorphic markers obtained from the parental screening were tested on the $F_2$ individuals along with parental lines. Six markers (SSR20710, SSR13312, SSR1667, SSR23627, SSR13021 and SSR 00398) with unambiguous banding patterns were subjected to simple regression analysis to determine the significant marker-trait association. Salinity tolerance was evaluated by visual scoring (TOL), the survival rate (SU) and relative leaf numbers (RLN14). The SU, TOL, and RLN14 were higher in the tolerant parent than sensitive one. The mean scores of $F_{2:3}$ families exhibited continuous variation and some had values outside the parental means. SSR20710 located on chromosome 3 explained 16.5%, 7.1%, 5.6% and 7.8% of variations observed in SU, TOL, RLN14 and percent green leaves (%GL), respectively. Markers SSR13312 and SSR16667 contributed 25% and 59% of RLN14 and TOL, respectively. This study provided valuable information for future genetic studies of salinity tolerance in cucumber and marker assisted selection for salinity tolerance improvement in cucumber.

## Introduction

Quantitative trait loci (QTL) analysis is one of the most powerful tools used to explain the inheritance of quantitative traits. The recent reports show that salt tolerance in cucumber and its component traits are controlled by both genetic and environmental factors [1]. The low heritability of the salt tolerance in cucumber in previous reports indicates that simple hybrid breeding for salinity tolerance improvement in cucumber is not feasible. Physiology of salt tolerance in cucumber is well documented [1, 2]. However, the genetics of salt tolerance in cucumber is not well understood. Salinity reduces germination, seedling growth, biomass production and yield of cucumber [3]. Although, there exists a genetic variation in salt tolerance of cucumber, the development of salt tolerant cucumber is difficult due to the narrow genetic base and lack of reliable morphological markers [4, 5]. In this regard, salinity tolerance of cucumber may be improved through marker-assisted selection (MAS) where QTLs associated with salt tolerance traits are stacked into one genotype.

Microsatellite markers are popular in plant genetic and genomic studies due to their multi-allelic nature, high reproducibility, chromosome specific location, genome-wide distribution and codominance inheritance [6]. The use of genomic microsatellite markers in biodiversity, cultivar identification, evolution studies, marker-trait association, and marker assisted selection is already documented [6, 7]. Unlike cereals such as rice, salt tolerant cucumber variety is not yet available commercially. Salt tolerance like other quantitative traits is controlled by a series of genes in the entire plant genome [8]. The traits underlying salt tolerance in cucumber can be screened in stressful conditions while the underlying QTLs could be achieved through mapping studies. Development of salt tolerant plants through conventional breeding is slow, partly due to lack of reliablility and quick selection methods [9]. To enhance selection efficiency, it is necessary to identify markers associated with genes or QTLs that control traits of interest [10]. QTL for salt tolerance is already reported in wheat [11]. Previous researchers documented QTLs for quantitative traits in cucumber such as waterlogging tolerance [12]. However, important QTLs for salt tolerance in cucumber are not available in the literature. Despite the discovery of DNA markers and full genome sequencing of major crops like cucumber, efforts to identify quantitative traits and practical application of MAS in breeding programs have yielded minimum success. Lack of appropriate mapping population, the absence of tight linkage between markers and QTLs of interest and QTL and environment interaction partly explains low success rate. To overcome the above problems, we used single marker analysis to study marker-salt tolerance association to decipher the underlying genetic basis of salt tolerance in cucumber.

Single marker analysis is the simplest QTL analysis method, but found to be effective in studying marker-trait association [13-15]. Single marker analysis is possible through multiple regression method to determine marker-trait association and the percent marker contribution estimated by $R^2$ value [16]. Various phenotypic traits used for the selection of salt tolerant crops include relative water content, germination rate, fresh and dry weight, survival rate, chlorophyll content and leaf number [3]. However, the above indices are not readily available in cucumber breeding programs and hence the increased efforts to link the traits with molecular markers [16]. The genetic dissection of salt tolerance is critical in breeding cucumber for saline environments for precise transfer of salt tolerance into popular commercial cucumber varieties. Identification of chromosomal regions associated with DNA markers would be useful in large-scale screening for cucumber salt tolerance. The objective of the current study was to identify DNA markers associated with salt tolerance and map salt tolerant QTL in cucumber at seedling stage.

## Materials and methods

### Development of mapping population for QTL analysis

The mapping population ($F_{2:3}$) was derived from a cross between salt tolerant, 11411S ($P_1$, female parent) and salt sensitive 11439S ($P_2$, male parent). The two parents were crossed in autumn 2010 to obtain $F_1$ plants. Selfing $F_1$ progeny generated 224 $F_2$ individuals during the spring season of 2011. Of the $F_2$

families planted, 102 individuals were selfed to produce 102 $F_{2:3}$ families during the autumn of 2011. We carried out pollinations in the plastic greenhouse at Jiangpu Farm, Nanjing Agricultural University, China. The two parents were involved in all the pollination exercise to ensure the production of seeds of the same age.

**Phenotypic evaluation of salinity tolerance**

The phenotypic evaluation was conducted in a glasshouse at Pailou Research Base, Nanjing Agricultural University during spring of 2012. The 98 $F_{2:3}$ derived lines, plus $P_1$ (tolerant) and $P_2$ (sensitive) parents were used to evaluate salt tolerance in cucumber. The seeds of each family were first pretreated in water at 55°C for 24 hours in darkness, then individually sown in pots filled with vermiculite and peat (2:1). Each family consisted of 6-10 individuals and planted in completely randomized pots. Salinity treatment commenced at the two leaf stage. The seedlings were irrigated with saline water containing full-strength Hoagland solution at an incremental rate of 20 mM NaCl until a concentration of 80 mM NaCl was attained. We did not include control experiment in the current experiment since the two parents did not show leaf chlorosis under salt-free pot medium in our previous preliminary study. The volume of the irrigation water per pot ranged from 100-150 ml. This volume was adequate to meet pot field capacity without causing waterlogging based on previous experience. Of the 98 $F_{2:3}$ families sown, 7 families that did not germinate or died before the start of salt treatment were omitted in the final phenotypic analysis. The means of the remaining $F_{2:3}$ families were used in subsequent analysis.

We estimated relative growth rate by calculating the ratio of the number of leaves at 0 and 14 days after the start of salt treatments. The percent survival rate was estimated by dividing the final number of living plants over the original number at the start of salt treatment then multiplied by 100. Plants without any green tissue were considered dead. We used scoring salt tolerance scale of 1-5: (1) no sign of injury; (2) > 90% green leaves; (3) 10-50% green leaves; (4) <10% green leaves and (5) dead, to assess salt tolerance of the cucumber at the end of the experiment as shown in Fig. 1. Tolerance score was based on survival, leaf chlorosis and vigor of the seedlings at three weeks after the start of the salt treatment. Tolerance scores of 1 to 3 represent dead and yellow leaves while scores 4-5 includes plant survival, dead and yellow leaves. Percent green leaves were estimated by the number of leaves without green color divided by the total number of leaves at the end of the experiment. The plants with a higher percentage of green leaves were deemed to have tissue NaCl tolerance.

**DNA extraction**

Leaf samples of parental lines and their $F_2$ progenies were collected and immediately frozen in liquid nitrogen and stored at -80°C for future use. Cetyl trimethyl ammonium bromide (CTAB) method was used to extract DNA [15]. Briefly, about 4 g of leaves of the respective $F_2$ individuals and the two parents were ground in liquid nitrogen and transferred into centrifuge tubes. Later, 900µl of preheated extraction buffer and 2 µl of β-mercaptoethanol were added then incubated in a water bath at 65°C for 30 minutes, then 1/3 volume of 5M Potassium acetate (300 µl) was added and the mixture was incubated further for 1 hour in water bath at 65°C before centrifuging at 12000 rpm for 10 minutes at 4°C. The supernatant was transferred into a 2 ml tube and 1/5 of 5% CTAB buffer was added and the mixture shaken, and then warmed in a water bath at 65°C for 20 min. The mixture was cooled to room temperature and then 1/5 volume of chloroform/isoamyl alcohol (24:1) was added. Extraction was conducted twice at 11000 rpm for 5 minutes at room temperature. The supernatant was transferred into clean tubes and 2/3 volume of isopropyl alcohol was added, shaken and cooled at room temperature for 10 min then centrifuged at 12000 rpm for 10 min at room temperature. The supernatant was discarded and the DNA pellet was washed twice with 70% ethanol. The DNA pellet was air dried after discarding the ethanol. 500 µl of TE was first added before adding 1/10 volume RNaseA (10 mg/ml). The DNA was transferred into a 1.5 ml tube and incubated overnight at 4°C overnight. 1/10 volume 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol were added and the mixture inverted

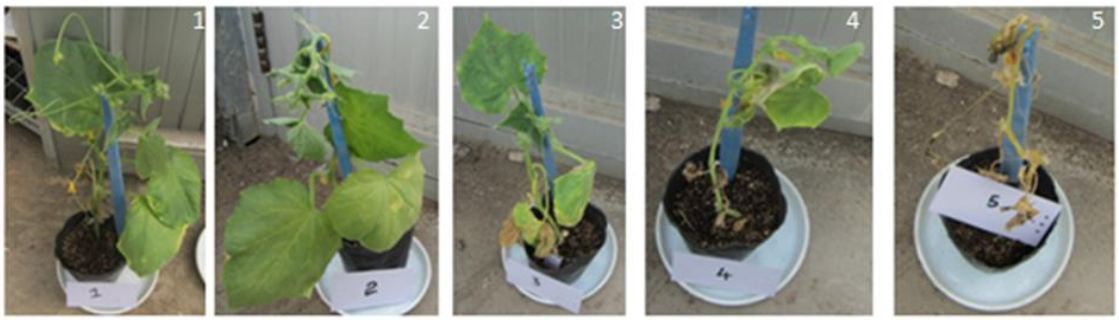


**Fig. 1** Salinity score of cucumber seedlings at 21 days after the application of 80 mM NaCl. The extent of leaf chlorosis was used to estimate salt tolerance. The salt tolerant seedlings with negligible signs of injury were scored 1 while the most salt sensitive seedlings that were completely dead were scored 5.

several times before incubating at -20°C for 10 min then centrifuged at 6000 rpm for 5 min at 4°C. The DNA pellet was washed twice with 70% ethanol and air dried after discarding ethanol. The DNA pellet was dissolved in TE buffer and the quality of the DNA was checked using Agar gel electrophoresis. The DNA was stored at -20°C for future use.

**SSR markers and QTL analyses**

We used SSR markers developed by Huang et al. [17]. To select polymorphic markers between the parental lines, we screened 432 SSR markers. Markers were generated by polymerase chain reaction (PCR) mixture consisting of 2 buffer solutions 10× (2.5 mM $Mg^{2+}$, 0.2 mM dNTPs), 0.2 um of each primer pair, 40 ng genomic DNA from either parent and 1 unit of Taq DNA polymerase (Takara) to make a final volume of 20 µl. PCR was performed in thermocycler (PTC-100; MJ Research Inc., Chatham, N.J., USA) with the following profile: 94°C for 3 min × 1 cycle; 94°C for 0.5 min, 58°C for 0.5 min, 72°C for 1 min × 35 cycles; the last cycle had an extra 10 min for the extension period at 72°C followed by 4°C. Three µl of each PCR product was separated by electrophoresis on gels containing polyacrylamide, TBE, TEMED and APS buffer at 120W. After complete electrophoresis, fixation was done in a mixture of 10% ethanol and 5% glacial acetic acid for 15 minutes then stained with 0.002 M $AgNO_3$ for 15 minutes. The gels were washed in distilled water for 30 seconds and rinsed with 200 ml distilled water containing 400 µl of 1% $Na_2S_2O_3$. Color development was achieved by agitating the gels in 0.375 M NaOH and 0.01M HCHO. The images of the bands were taken using a camera. Sixty polymorphic SSR markers with clear bands were selected for subsequent genotyping of the $F_2$ individuals. Each microsatellite marker was added to each PCR mixture containing respective genomic DNA of parental lines and $F_2$ individuals. PCR procedure, electrophoresis, staining and image acquisition was conducted as previously described. The banding patterns that were similar to parent 1 and 2 were A and B, respectively, while lanes that exhibited bands similar to both parental lines were scored H. Of the 60 polymorphic markers, only 6 with clear banding patterns were used for genotyping. We employed single marker analysis to determine the association between the markers and the four quantitative traits using simple regression methods. Marker effect was estimated using $R^2$ at probability level ($P < 0.05$).

**Genetic linkage map construction and QTL analysis**

Based on genotype data of $F_2$ population, a molecular linkage map based on multipoint analysis was constructed using JoinMap 4.0 software. QTL analysis was conducted using WinQTL (version 2.0) software. Simple regression was used to determine the significant association between the markers and selected traits at $P<0.05$. $R^2$ was used to estimate marker contribution on the phenotypic variation.

**Statistical analysis**

Phenotypic data for mean trait values of parental lines and $F_{2:3}$ families were analyzed using the general linear model of MINITAB. Pearson's correlation analysis of phenotypic traits of the $F_{2:3}$ population was conducted to assess the relationships among traits. The mean value of each trait was used

for the identification of QTL associated with salt tolerance. Based on the bands analysis, the polymorphism information content (PIC) for each SSR was calculated with PIC-CALC software, according to the formula: PIC = 1 - $\sum pij^2$, where pi is the frequency of the $i^{th}$ allele of the j marker.

## Results

**Salinity tolerance of parental lines and $F_{2:3}$ families**

The analysis of variance showed highly significant (P < 0.0001) variation among the lines and parents with respect to salinity tolerance and relative leaf number of cucumber seedlings at day 14 after the start of salt stress treatment (Table 1). Fig. 2 shows the frequency distribution of the $F_{2:3}$. All the traits assessed showed a continuous distribution indicating their polygenic nature. As expected, $P_1$ had higher RLN14 than $P_2$. However, about 33 of the $F_{2:3}$ families had RLN14 outside the parental extremes. The tolerant parent had higher SU, RLN14, % GL and TOL than the sensitive parent. Only one $F_{2:3}$ family had better salt tolerance score than the tolerant parent, 11411S. Fourteen $F_{2:3}$ families showed a higher tolerance score than the salt sensitive parent 11439S, 38 (43%) of $F_{2:3}$ families had better percentage survival than the tolerant parent while 9 (10%) of them were worse than salt sensitive parent. None of the $F_{2:3}$ families had higher RLN14 than the tolerant parent, but eight of them had lower RLN14 than the sensitive parent. For %GL, nine families tended to be better than salt tolerant parent while 41 (46%) of them showed lower percentage green leaf than the salt sensitive parent.

**Phenotypic correlations**

Correlation analysis was done to determine the relationship of salt tolerant traits of $F_{2:3}$ derived families under salinity (Table 2). All the traits were significantly correlated except %GL and RLN14. TOL was negatively correlated to SU, %GL, and RLN14. SU was positively related to either %GL or RLN14. However, there was no correlation between RLN14 and %GL. TOL and SU were strongly correlated while the correlation between SU and %GL or TOL and %GL was moderate (or = 0.6). However, a weak correlation between RLN14 and SU (R = 0.3) was observed.

**Table 1** Analysis of variance for RLN14 and TOL of salt tolerant and sensitive cucumber and their $F_{2:3}$ families derived from a cross 11411S × 11439S under 80 mM NaCl for 14 days.

| Source | Degree of freedom | Sum of square | Mean square | P values |
|---|---|---|---|---|
| RLN14 | | | | |
| Family | 89 | 86.33 | 0.97 | 0.000 |
| Error | 716 | 170.91 | 0.2387 | - |
| Total | 809 | - | - | - |
| TOL | | | | |
| family | 89 | 145.07 | 1.63 | 0.000 |
| error | 719 | 337.93 | 0.47 | - |
| Total | 812 | - | - | - |

**Table 2** Correlation of survival (su), salt tolerance (TOL), relative leaf number (RLN14) and percentage green leaf (GL) of $F_{2:3}$ families from a cross 11411S (salt tolerant) and 11439S (salt sensitive).

| | SU | TOL | % GL |
|---|---|---|---|
| TOL | -0.8 (P<0.001) | - | - |
| %GL | 0.6 (P<0.0001) | -0.6 (P<0.0001) | - |
| RLN14 | 0.4 (P<0.001) | -0.4 (P<0.0001) | -0.04 (P = 0.9) |

**QTLs associated with salt tolerance traits**

The single marker analysis using simple regression method is shown in Table 3. Six markers with the unambiguous banding system were used to screen for polymorphism in 89 $F_2$ individuals and to establish the association between the markers and the phenotypes. Simple regression analysis detected three markers with significant association with at least one trait measured. Marker SSR20710 had a significant association with the four traits, TOL, SU, RLN14, and %GL. It contributed 16.5, 7.1, 5.6 and 7.8 of variations observed in SU, TOL, RLN14, and %GL, respectively. Markers SSR00398, SSR23627, and SSR13021 had no significant association with all the traits. Marker SSR13312 was strongly associated with RLN14 with a 25 % contribution to the phenotypic variation observed. SSR 16667 accounted for the 58.7% of the TOL but was not related to the remaining phenotypes. This study reveals that TOL and RLN14 are controlled by two loci on chromosome 3. Of the six informative markers, three markers had highly distorted segregation ratio, hence could not be used for linkage map construction. We, therefore, constructed a linkage map consisting of three markers. Only one marker, SSR16667 with LOD score of 2.5 was detected a significant QTL explaining 13.10% of salt tolerance trait (Fig. 4). Additive and dominance effects were 0.21 and 0.28,

**Table 3** SSR markers associated with NaCl salinity tolerance traits in $F_{2:3}$ population of the cucumber cross 11411S and 11439S based on single marker analysis using simple regression at $P<0.05$. A, b and c denote the coefficient of determination, probability and non-significant, respectively.

| Marker | SU | | TOL | | RLN14 | | % GL | |
|---|---|---|---|---|---|---|---|---|
| | $R^2$ (%)[a] | P[b] | $R^2$ (%) | P | $R^2$ (%) | P | $R^2$ (%) | P |
| SSR20710 | 16.5 | 7.827E-05 | 7.1 | 0.0084 | 5.6 | 0.01 | 7.8 | 0.006 |
| SSR13312 | - | ns[c] | - | ns | 25.05 | 7.49E-07 | 0.2 | ns |
| SSR23627 | - | ns | - | ns | - | ns | 0.4 | ns |
| SSR16667 | - | ns | 58.7 | 4.13E-17 | - | ns | 0.6 | ns |
| SSR13021 | 0.5 | ns | 0.6 | ns | 0.5 | ns | 0.7 | ns |
| SSR00398 | 0.1 | ns | 0.5 | ns | 0.7 | ns | | |

Survival (su); salt tolerance (TOL); relative leaf number (RLN14); percentage green leaf (GL)

**Table 4** Allele variation and PIC values for SSR markers identified in 89 $F_2$ individuals obtained from a cucumber cross 11411S ×11439S.

| Marker locus | Chromosome | No. of alleles | PIC values |
|---|---|---|---|
| SSR00398 | 5 | 4 | 0.67 |
| SSR13021 | 4 | 3 | 0.58 |
| SSR13312 | 3 | 4 | 0.69 |
| SSR20710 | 3 | 3 | 0.57 |
| SSR16667 | 3 | 5 | 0.75 |
| SSR23627 | 3 | 2 | 0.37 |

PIC = polymorphism information content

Respectively. The number of alleles and PIC values are shown in Table 4. The total numbers of alleles detected by the six polymorphic markers were 21 with a mean of 3.1 alleles per marker. The PIC values varied from 0.37 to 0.75.

## Discussion

Salinity remains a serious problem in crops, especially in irrigated agriculture. A lot of work on salinity tolerance in crops has continued for a very long time. However, genetic characterization of this complex trait has only been successful in few crops [19, 20]. Salt related QTLs are already reported in crops such as rice [16]. However, it is difficult to compare these results due to varying screening, materials and genotyping methods employed. Currently, salinity tolerance of most crops is estimated using several indices such as morphological, physiological and biochemical [3, 17, 3, 18]. A critical review by Munns and Tester [1] showed that no single selection criterion is effective for salt tolerance. The inheritance of phenotypic salinity tolerance is modified by environment and gene-environment interaction. To minimize environmental effects, we conducted the experiment in pot mix arranged within a greenhouse where temperature and humidity were regulated.

The genotypic analysis of $F_2$ population derived from a cross between salt tolerant (11411S) and salt sensitive (11439S) cucumber inbred lines is that salt tolerance is polygenic. The continuous phenotypic variation with concomitant transgressive segregation in the $F_{2:3}$ progenies, especially for salt tolerance score (TOL), survival (SU) and percentage of green leaves (%GL), confirmed the quantitative nature of the traits. The transgressive segregation observed in this study showed that either parent contributes alleles for increasing or decreasing the corresponding traits. Transgressive segregation may also be due to epistasis as observed in our previous experiment [19]. Although this observation is common for both biotic and abiotic stresses where the progenies performance falls outside the parental ranges, fewer reports for genotypes developed from the selection of superior segregants are currently available. In our study, we found that TOL, RLN14, and SU were negatively correlated, thus segregants with lower TOL indices, higher SU/RLN14 could be selected at advanced generations and crossed to concentrate the favorable genes. Our findings parallel the report by Lexer et al. [20] who reported that progenies of wild sunflower showed 5-14% of salinity tolerance better than the wild sunflower parents. Recently, Wang et al. [21] reported higher root-knot resistance in progenies of cotton derived from susceptible parents.

We identified significant marker-trait association in this study. Three markers had a significant association with at least one trait. Markers that show significant association with more than one trait

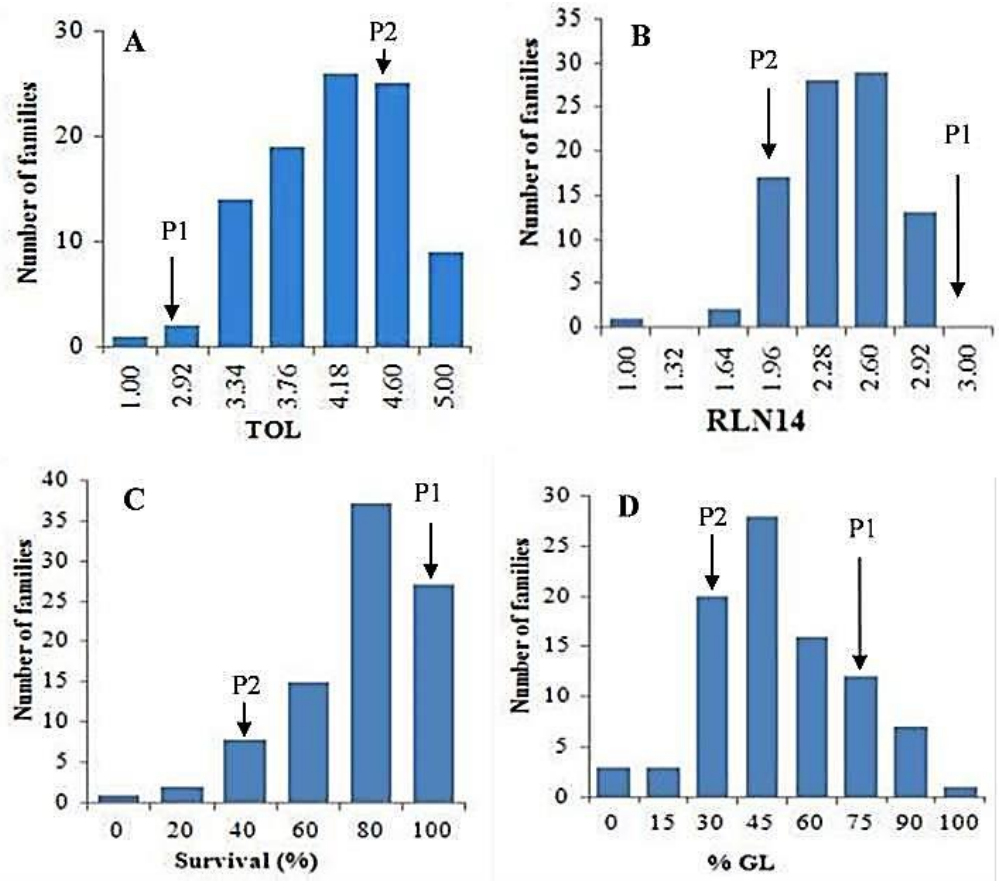


**Fig. 2** Frequency distribution of salt tolerance (TOL, 2A), relative leaf number at 14 days, percentage survival (2C) and percentage green leaf (%GL, 2D) of F2:3 population (n = 91) derived from a cross 11411S × 11439S. The arrows indicate the mean values of the respective traits for each parent (P1and $P_2$ denotes salt tolerant parent (11411S) and salt sensitive parent (11439S), respectively.

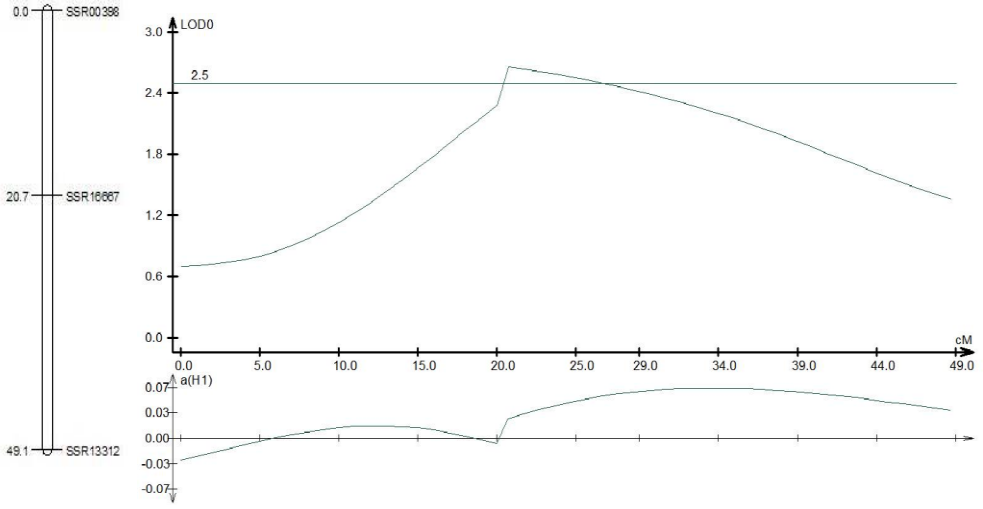


**Fig. 3** Quantitative trait loci mapping of salinity tolerance in cucumber under salinity stress.

indicates the pleiotropic effect implying that molecular mechanisms controlling the traits are similar. In this study, SSR20710 was significantly associated with improvement of the four traits simultaneously under SU, TOL, RLN14 and %GL indicating pleiotropy. It is, therefore, plausible that it would facilitate salinity stress [22]. Our study showed both the loci SSR16667 and SSR20710 both control TOL while SSR20710 and SSR13312 were highly linked to RLN14. Although we identified a potential QTL for TOL, the linkage map obtained was sparse with average marker interval of 24.6 cM, larger than the desirable interval required for QTL detection in interval mapping [23]. A total of 35% of the polymorphic markers used in this study exhibited segregation distortion hence omitted in the linkage map. A similar observation was observed in a study involving maize [24]. In the future, more markers including those with distorted segregation should be included in order to cover the seven cucumber chromosomes. The high segregation distortion revealed in our genotypic data compromised the QTL information observed in this study. Allele segregation distortion in the genotypic analysis is due to several factors ranging from pollen tube competition and selective fertilization [25, 26]. The exact reason for segregation observed in this study requires further detailed investigation. The relatively high PIC values observed in the two markers, SSR16667 and SSR20710 coupled with their corresponding trait association indicates their potential use in marker assisted selection. The lack of significant association between the markers, SSR00398, SSR23627 and SSR13021 with the selected traits indicate that they are associated with other traits.

## Conclusions

The study confirmed that phenotypic markers alone do not fully explain the underlying genetic factors affecting salinity tolerance in cucumber. The thrust of this study was to identify QTL underlying TOL, SU, RLN14 and %GL of cucumber under salinity stress. Whereas single marker analyses showed a significant association between the traits and corresponding loci, the multiple interval mapping detected only one QTL for TOL with relatively low LOD (2.5). This study provided valuable information for future genetic studies of salinity tolerance in cucumber. We identified three microsatellite markers with significant association with specific quantitative traits. These markers could be used in marker-assisted-selection for salinity tolerance improvement in the cucumber breeding program.

## Acknowledgements

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## Conflict of interest

The authors declare that there is no conflict of interest.

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