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Genetic diversity analyzed by microsatellite markers among rice (*Oryza sativa* L.) genotypes with different adaptations to saline soils

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Abstract

The success of salt tolerance breeding programs employing traditional screening and selection has been limited in the past decades. This study was designed to characterize the genetic diversity within a subset of rice germplasm with different adaptations to saline soils using microsatellite markers. Salt tolerance was then analyzed among molecularly characterized genotypes. Plants of 33 genotypes were grown in sand tanks under greenhouse condition and irrigated with Yoshida nutrient solution. Two salt treatments were imposed with electrical conductivities of 0.9 dS m^{-1} (control) and 6.5 dS m^{-1} (6:1 molar ratio of NaCl and $CaCl_2$). A total of 123 alleles were generated at 25 microsatellite loci among the 33 genotypes. Genotypes of japonica rice grouped into three clusters and those of indica rice grouped into two clusters based on microsatellite markers. Thirty percent of the alleles detected in 20 breeding lines were not identified in the cultivars analyzed. These alleles may provide favorable allelic combinations if the breeding lines are used for intercrosses. Physiological and morphological characters under salt stress were significantly ($P = 0.05$) different among microsatellite clusters. There was a highly significant correlation ($r = -0.25$; $P = 0.005$) between the matrices of Jaccard genetic similarity based on microsatellite markers and taxonomic distance based on ion data. These results indicate that the adaptation of rice to saline soils is different among genotypes with diverse genetic backgrounds. Implications for engineering salt tolerance are: (1) Improving salt tolerance can be achieved by selecting parental genotypes prior to intercrossing based on microsatellite markers. (2) Phenotypic variation of ion contents in segregating populations can be increased by selecting parental genotypes prior to intercrossing based on microsatellite markers. (3) Different salt tolerance components can be combined into a cultivar by intercrossing genotypes from different microsatellite clusters with diverse salt tolerance mechanisms.

Keywords: Salt tolerance; Microsatellite; Genetic similarity; Ion content; Ion selectivity; Rice

1. Introduction

Rice, one of the most widely cultivated crops, provides food for one-half of the world population. Rice breeders are increasingly challenged in the new century to meet the rapidly growing food demands of an increasing human population. Presently, growers in many regions have extended cultivation into marginal lands where salinity levels in soils are above thresholds affecting rice growth and yield. These instances, along with water conservation practices such as irrigating crops with marginal quality water [1,2], have increased the need for genetic improvement of salt tolerance in rice.

Unfortunately, rice is one of the most salt-sensitive cereal crops [3]. Previous studies of plant responses to salt stress have identified different physiological and morphological characters determining salt tolerance in plants [4]. For example Asch et al. [5] demonstrated that ion uptake in rice was influenced by the growth of root systems. Ion concentration in leaves, an important parameter for assessing salt damage, depends on ion uptake, translocation, and plant growth. Plant growth vigor, e.g. plant height or shoot biomass, was

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reported to have dilution effects on sodium accumulation in leaves of rice [4]. Leaf area can also affect sodium concentration in rice leaves by confounding effects of dilution and the transpirational driving force [5,6]. Additionally, leaf area has been shown to be highly correlated to grain yield in rice under salt stress [7]. Finally, panicle weight, tiller numbers per plant, and harvest index are important agronomic characters for the prediction of final yield in rice. These yield components are severely affected by salinity [8,9].

One major approach in plant breeding is to maximize the genetic diversity between parental genotypes for intercrosses. Genetic diversity between parental genotypes is usually estimated by measurements of physiological and morphological differences of quantitative and economically important traits. The disadvantages of this conventional approach are the cost of time and labor during the measurements, and the influences of environmental factors. Often, these disadvantages are exacerbated in salt-tolerance breeding. For example any change in environment such as temperature, light or humidity can dramatically change the transpirational driving forces and, subsequently, ion uptake [4,10]. Such changes may alter salt tolerance among genotypes. It is important to note that morphological characters are often limited in their numbers and may not adequately represent actual genetic relationships among genotypes.

Conversely, identified genetic variations based on DNA polymorphism are abundant and independent of environmental factors. Furthermore, a large sample size is usually required for the evaluation of genotypes when quantitative traits are measured. In contrast, a relatively small sample size can be informative for the evaluation when DNA polymorphisms are analyzed. Therefore, assays for DNA markers may be much less time-consuming and less labor intensive. DNA markers that differentiate genotypes are more reliable and convenient than physiological or morphological characters in the identification and characterization of genetic variation.

Microsatellite markers have been effectively used to identify genetic variation among rice cultivars [11–13]. Microsatellites are tandemly repeated sequence motifs that are ubiquitously distributed throughout the eukaryotic genome. They can be easily amplified by PCR reactions using DNA nucleotide primers, the unique sequences flanking the repeat motifs. Polymorphic DNA fragments can be produced due to differences in the number of the repeat units. A number of microsatellite markers have already been developed in rice and their primer sequences have been published [14–17].

The employment of genetic variation as identified by molecular markers to plant breeding programs may be useful in addressing agronomic problems such as abiotic stresses during crop production. Thanh et al. [18] have shown the genetic variation identified by microsatellite markers to be useful in evaluating upland rice accessions from Vietnam for drought-tolerance related morphology. Enoki et al. [19] also utilized the same approach to evaluate maize inbred lines adapted to cold regions of Japan. The objectives of this study were to characterize a subset of rice germplasm with different adaptations to saline soils for their genetic diversity using microsatellite markers, and analyze salt tolerance among molecularly characterized genotypes.

2. Materials and methods

2.1. Plant materials

Seeds of 33 rice genotypes were received from the Field Crop Research Institute at Giza, Egypt; the International Rice Research Institute (IRRI), the Philippines; and California Rice Experiment Station, Biggs, CA. This collection of genotypes represents germplasm originating in Egypt, Philippines, India, and USA (Table 1). The genotypes obtained from Egypt and Philippines are breeding lines and cultivars related to the salt tolerance breeding programs at these two sites. Among these genotypes, ‘GZ178’, ‘Agami’, and ‘Daeyabyeo’ are locally improved cultivars for salt tolerance while ‘GZ177’, ‘Sakha101’, and ‘IR29’ are salt-sensitive cultivars (A.T. Badawi, Field Crop Research Institute, Giza, Egypt) (Table 1). Salt-tolerant landraces, ‘Pokkali’ and ‘Nona Bokra’, and five commonly cultivated salt-sensitive cultivars in California, ‘M-104’, ‘M-202’, ‘M-205’, ‘L-205’, and ‘S-102’, were included for comparison.

2.2. Plant culture and salinity treatments

The trials were conducted in a greenhouse at Riverside, CA (33°58′24″N latitude, 117°19′12″W longitude) between June and November, 2001. The plants were cultured using nutrient solution [20] in tanks (122 cm × 61 cm × 46 cm deep) filled with sand. The irrigation and nutrient solutions were maintained as previously described [7]. Seeds were planted in two rows per genotype with eight genotypes per tank. The rows were spaced 6–7 cm apart with 15 seeds per row. Water depth was controlled at 6–8 cm during the growing season. Air temperature ranged from 23 to 37 °C during the day and 17–23 °C during the night. Humidity ranged from 40 to 60%. Photosynthetically active radiation averaged 494 $\mu mol\,m^{-2}\,s^{-1}$ with a minimum of 30 and a maximum 1300 $\mu mol\,m^{-2}\,s^{-1}$ during the day. The experiment was designed as a randomized block in a split-plot with three replicates. Salt level was a main plot factor and genotype was a sub-plot factor. NaCl and $CaCl_2$ (6:1 molar concentration) were added to the nutrient solutions on the first day after planting (DAP). Salinity was maintained continuously until final harvest. Over the duration of stress, the salt level was maintained at an electrical conductivity (EC_w) of 6.5 $dS\,m^{-1}$. The control, i.e. nutrient solution without added salts, was maintained at 0.9 $dS\,m^{-1}$ during the trial.

Table 1
Genotypes with different origins, subspecies, and sensitivity to salinity

Entry number	Genotype	Country of origin	Classification of germplasm	Subspecies	Salinity tolerance[a]
1	M-104	USA	Cultivar	Jpn[b]	S
2	M-202	USA	Cultivar	Jpn	S
3	M-205	USA	Cultivar	Jpn	S
4	L-205	USA	Cultivar	Jpn	S
5	S-102	USA	Cultivar	Jpn	S
6	GZ177	Egypt	Cultivar	Jpn	S
7	GZ178	Egypt	Cultivar	Jpn/Ind	M
8	Sakha101	Egypt	Cultivar	Jpn	S
9	GZ5121-5-2-1	Egypt	Breeding line	Jpn/Ind	S
10	GZ5291-7-1-2	Egypt	Breeding line	Jpn	S
11	GZ5310-20-2-1	Egypt	Breeding line	Jpn	T
12	GZ5310-20-3-2	Egypt	Breeding line	Jpn	T
13	GZ5310-20-3-3	Egypt	Breeding line	Jpn	T
14	GZ5385-3-2-3-1	Egypt	Breeding line	Jpn	M
15	GZ5385-29-3-2	Egypt	Breeding line	Jpn	M
16	GZ5385-29-3-3	Egypt	Breeding line	Jpn	M
17	AC26	Egypt	Breeding line	Jpn	M
18	GZ1368-5-4	Egypt	Breeding line	Ind	M
19	Agami	Egypt	Cultivar	Jpn	T
20	Daeyabyeo	Korea	Cultivar	Jpn	T
21	IR4630-22-2-2-5-1-3	Philippines	Breeding line	Ind	T
22	IR50184-3B-18-2B-1	Philippines	Breeding line	Ind	M
23	IR51490-AC10	Philippines	Breeding line	Ind	M
24	IR61920-3B-15-2-2	Philippines	Breeding line	Ind	M
25	IR63352-AC202	Philippines	Breeding line	Jpn	S
26	IR63731-1-1-4-3-2	Philippines	Breeding line	Ind	T
27	IR70074-AC14	Philippines	Breeding line	Jpn	M
28	IR70080-AC1	Philippines	Breeding line	Jpn	M
29	IR29	Philippines	Cultivar	Ind	S
30	Nona Bokra	India	Landrace	Ind	T
31	Pokkali	India	Landrace	Ind	T
32	IR70077-AC2	Philippines	Breeding line	Jpn	M
33	IR71657-5R-B-12P	Philippines	Breeding line	Ind	S

[a] Genotypes were classified into three categories: S, salt sensitive; M, moderately tolerant; T, salt tolerant, based on their agronomic performance in saline soils from their origins and evaluation trials at George E. Brown, Jr., Salinity Laboratory.
[b] Jpn, japonica rice; Ind, indica rice; Jpn/Ind, genotypes derived from the crosses between japonica and indica rice.

2.3. Measurements of morphological characters

Six seedlings of each genotype from each replicate were randomly sampled at 34 DAP (seventh to eighth leaf stage). Plants were measured for total leaf area per plant using a LI-3100 Area Meter (LI-COR Inc., Lincoln, Nebraska).[2] The same plants were dried in a forced-air oven (70 °C) until weights became constant. The samples were then measured for shoot dry weight, i.e. above ground biomass, and root dry weight. Data were averaged over the six sub-samples. When seeds on primary tillers matured, six plants of each genotype from each replicate were harvested by pulling up the roots. Plant height was measured from the base of stem to the tip of the flag leaf on the main culm. Plants were bagged individually after roots were removed. After oven-drying at 70 °C to constant weight, grain weight per panicle, grain weight per plant, and shoot dry weight (i.e. vegetative biomass above ground) were measured. Harvest index was calculated as grain weight per plant divided by the total above ground biomass (i.e. grain weight and shoot dry weight). Tiller numbers per plant were determined by the number of primary tillers with matured seeds. The data were averaged across the six sub-samples. Salt tolerance indexes were determined for the grain weight per plant of the salt-stressed plants. For calculations of salt tolerance, the measurements of grain weight per plant in each genotype under salt stress were divided by the mean of the same character in M-202 that was grown in each tank as a check cultivar. The cultivar, M-202, was consistently ranked as salt-sensitive for both agronomic and physiological characters in previous trials [7,21].

2.4. Ion analysis

The same seedlings that were sampled at 34 DAP (seventh to eighth leaf) were also used for ion analysis after the measurements of morphological characters. The seventh to eighth leaf growth stage was about 1 week before panicle initiation among early maturing genotypes originating from

[2] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Egypt and California and about 2–3 weeks before panicle initiation among late maturing genotypes originating from Philippines and India. The seventh to eighth leaf growth stage has been described as the maximum tillering stage [22]. Panicle initiation is a critical stage for early panicle development influencing grain yield under salt stress [23]. Zeng et al. [7] have shown ion selectivity at panicle initiation to be significantly correlated with grain yield in salt-stressed rice.

Ion contents in shoot (above ground biomass) were measured from dried tissues of leaves and shoots. The dried tissues were weighed and ground into fine powder by passing through a 60-mesh screen. Shoot concentrations of Na, K, Ca, Mg, P, and S were determined on nitric–perchloric acid digests by inductively coupled plasma optical emission spectrometry (ICP atomic emission spectrometer, Perkin-Elmer, Norwalk, CT, USA)[2]. Na–Ca selectivity ($S_{Na,Ca}$) was calculated using Gapon selectivity constant (K_g) [24]:

$$K_g = \frac{E_{Na}(A_{Ca})^{0.5}}{E_{Ca}A_{Na}}$$

where E represents the equivalent fraction of a given cation and A represents the activity of the ion in solution. In this way, K_g relates the equivalent fractions of the exchange ions to the activities of the ions in solution. The K–Na selectivity was calculated using the equation described by Pitman [25]:

$$S_{K,Na} = \left(\frac{\text{K content}}{\text{[K] medium}}\right) : \left(\frac{\text{Na content}}{\text{[Na] medium}}\right)$$

where $S_{K,Na}$ represents K–Na selectivity; K content and Na content represent the concentrations (mmol kg^{-1} dry weight) of K^+ and Na^+ in shoot.

2.5. DNA extraction and microsatellite markers

Leaf tissues (150–200 mg) were ground to a fine powder in liquid nitrogen and used for DNA extraction. Genomic DNA was extracted from the ground tissue following the procedure described by Dellaporta et al. [26]. The sequences of microsatellite primer pairs were downloaded from Genome Databases, RiceGenes Microsatellite Markers (http://ars-genome.cornell.edu/rice/microsats.html) or the reports by Akagi et al. [15] and Panaud et al. [16]. Primers were synthesized by Operon Technologies Inc., Alameda, CA, USA.

2.6. PCR amplification

PCR reactions were carried out on a PTC-100 Programmable Thermal Controller, MJ Research Inc., Watertown, MA, USA[2]. The reaction volume was 15 ul containing 50 ng genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 uM dinucleotides, 0.2 uM each primer, and 1 U of *Taq* DNA polymerase (Applied Biosystems, Foster, CA, USA)[2]. The temperature cycles were programmed as 94 °C for 5 min followed by 35 cycles of 94 for 1 min, 55 °C for 1 min, 72 °C for 2 min, and finally 5 min at 72 °C for final extension.

2.7. Non-radioactive detection

The amplified PCR products were separated in 6% denaturing acrylamide gels containing 7 M urea using a DNA sequencing system (FisherBiotech)[2]. Prior to electrophoresis, a short glass plate was treated with bind silene (Promega Corp., Madison, WI, USA)[2] and a long glass plate treated with SigmaCote (Sigma)[2]. PCR products were denatured at 94 °C for 2 min before loading. The gel was run in 1× TBE buffer (0.089 M Tris–borate, 0.002 M EDTA, pH 8.3) at constant power of 60 W for 1–1.5 h. After electrophoresis, the plates were separated and the short plate was processed for staining using a silver sequencing system (Promega). The gel was fixed for 20 min in 10% acetic acid, rinsed with water three times for 2 min each, and stained in staining solution (0.2% (w/v) silver nitrate, 0.05% formaldehyde) for 30 min with agitation. The gel was rinsed 5–10 s and developed in developing solution (6% sodium carbonate, 0.05% formaldehyde, 0.0002% sodium thiosulfate) at 10–12 °C until bands were visible. The gel was dried overnight at room temperature and photographed by exposure to APC films (Promega) using a white fluorescent light box. The sizes of the amplified DNA fragments were determined using Quantity One software (Bio-Rad, Hercules, CA, USA)[2].

2.8. Data analysis

Polymorphic information content (PIC) values were calculated for each of the microsatellite loci using the formula developed by Nei [27]

$$\text{PIC} = 1 - \sum x_k^2$$

where x_k represents the frequency of the kth allele. The data of both microsatellite markers and morphological characters were analyzed using the NTSYS-pc statistical package, version 2.1 (Exeter Software, Setauket, NY). The bands on APC films were scored for each of the microsatellite primer pairs in each genotype based on presence or absence of bands, generating a matrix of 1 and 0. Informative bands were used to calculate genetic distance based on Jaccard's similarity coefficients using SIMQUAL procedure. The DNA data of microsatellite markers for 33 rice genotypes were clustered using an unweighted pair group method (UPGMA) with the module of SAHN in the NTSYS-pc package.

2.9. Matrix comparison

A similarity distance matrix was calculated based on the means of the quantitative variables for the ion contents and ion selectivity. The data were standardized by subtracting the means from the original values and dividing by the standard deviation using STAND procedure. The similarity distance was calculated from the standardized data using SIMINT procedure of NTSYS-pc based on Average Taxonomic Distance (i.e. DIST coefficient in the procedure). A

matrix of similarity was created from all pairs of rice genotypes. Jaccard's genetic distance matrix based on microsatellite markers and the matrix based on ion data were compared using MXCOMP procedure of NTSYS-pc. The significance of the correlation between the matrices was tested using the normalized Mantel *Z*-statistics.

3. Results

3.1. Polymorphism of microsatellite markers

All of the primer pairs used in this study generated polymorphic bands among the genotypes. A total of 25 loci were assigned to the 23 miscrosatellite primer pairs. As previously reported, RM4 and RM20 each detected two loci [16]. A total of 123 alleles were detected among the 33 rice genotypes with an average of 4.9 alleles per locus (Table 2). The number of alleles per locus ranged from 2 (in OSR12) to 9 (in OSR1). The PIC values for the microsatellite loci ranged from 0.06 to 0.85 with an average of 0.57 (Table 2). The low PIC values were observed for the primers of RM6 (0.06), RM16 (0.21), RM17 (0.27), RM174 (0.22), and OSR6 (0.39). The PIC values of the remaining microsatellite loci were all above 0.50.

The total alleles identified in the 33 genotypes were classified into two categories: (1) shared alleles (i.e. identified in commercial cultivars) and (2) unique alleles (i.e. not identified in commercial cultivars) (Table 3). The 20 breeding lines evaluated by SSR markers contain 74 (87%) of 85 alleles, the total number of the alleles identified in the commercial cultivars. The number of unique alleles identified in breeding lines and landraces amounted to 30 and 38% of the total alleles detected in the two types of germplasm, respectively.

Table 2
Allele variation and PIC values for microsatellite loci (SSR) identified in 33 rice genotypes

SSR locus	Chromosome positions	Number alleles	Size ranges (bp)	PIC values
RM1	1	7	90–115	0.800
RM4A	12	3	138–154	0.512
RM4B	11	3	103–110	0.527
RM6	2	3	150–170	0.056
RM7	3	6	120–170	0.644
RM8	2	3	243–252	0.430
RM11	7	5	140–160	0.575
RM14	1	5	170–191	0.575
RM16	3	3	165–180	0.207
RM17	12	4	148–182	0.273
RM19	12	5	203–238	0.681
RM20A	12	4	250–268	0.703
RM20B	11	4	196–227	0.634
RM22	3	5	179–194	0.736
RM122	5	5	215–233	0.604
RM163	5	7	129–175	0.634
RM164	5	7	240–294	0.784
RM174	?	3	201–216	0.219
OSR1	11	9	170–284	0.846
OSR4	?	4	220–260	0.538
OSR6	11	4	110–164	0.390
OSR7	8	5	170–190	0.724
OSR9	2	5	97–130	0.769
OSR12	9	2	297–315	0.463
OSR14	?	6	173–203	0.656
OSR20	12	5	150–204	0.769

3.2. Cluster analysis of DNA polymorphism and morphological characters

The genetic relationships among rice genotypes are presented in a dendrogram based on informative microsatellite alleles (Fig. 1). All genotypes clearly grouped into two major branches in the dendrogram with less than 10% similarity based on Jaccard similarity index. One branch unambiguously represents the subspecies, japonica rice. Another branch represents either the subspecies, indica, or the hybrids between japonica rice and indica rice. The only exception was a cultivar of japonica rice, 'Daeyabyeo', which grouped with the genotypes of indica rice. Below the main japonica branch in the dendrogram, most genotypes grouped into three clusters, A1, A2, and A3, at 57, 47, and 48% similarity, respectively. Below the main Indica branch in the dendrogram, most genotypes grouped into two clusters, B1 and B2, at about 30% similarity.

Among the genotypes of japonica rice, most genotypes derived from Egypt grouped into Cluster A1 while those derived from Philippines and California grouped into Cluster A2 and A3, respectively. Among the genotypes of indica rice, Clusters B1 and B2 consist primarily of the genotypes derived from Egypt and Philippines, respectively. The genotypes of 'GZ5291-7-1-2', 'Agami', 'L205', and 'Pokkali' were not included into these clusters.

Means of the ion contents and morphological characters under salt stress were calculated among the clusters grouped by microsatellite markers (Tables 4 and 5). There were wide ranges of the means for most characters analyzed among the microsatellite clusters. Significant ($P = 0.05$) differences of the all characters except S and shoot/root were observed among the clusters. Na content and ion selectivity, especially Na–Ca selectivity, in the genotypes of Cluster A3 are significantly different from the other genotypes (Table 4). Low salt tolerance in terms of morphological characters was also observed for the genotypes of Cluster A3 (Table 5). The best ion selectivity, i.e. the highest K–Na selectivity and the lowest Na–Ca selectivity, and the lowest Na content were observed in indica rice (Table 4). However, grain yield was significantly lower for the genotypes of Cluster B1 than those of Cluster A1 (Table 5). Although ion selectivity was similar between Cluster B1 and B2, Na content was significantly lower in Cluster B2 than Cluster B1. This difference in ion content may be caused by strong growth vigor, i.e. height, leaf area and tiller number, which may have diluted ion contents in shoot.

Table 3
Alleles detected by microsatellite markers in cultivars, breeding lines, and landraces

Type of germplasm	Total alleles	Shared allele[a]	Unique allele[b]	Percentage of unique allele
Cultivars	85	85	–	
Breeding lines	105	74	31	30
Landraces	40	25	15	38

[a] The alleles detected in commercial cultivars.
[b] The alleles not detected in commercial cultivars.

3.3. Relationships between genetic similarity and ion contents

Relationships between Jaccard similarity index based on mircrosatellite markers and Average Taxonomic Distance, based on ion data were analyzed by comparisons between the two different matrices (Fig. 2). The correlation between genetic similarity and taxonomic distance was highly significant ($r = -0.25$; $P = 0.005$).

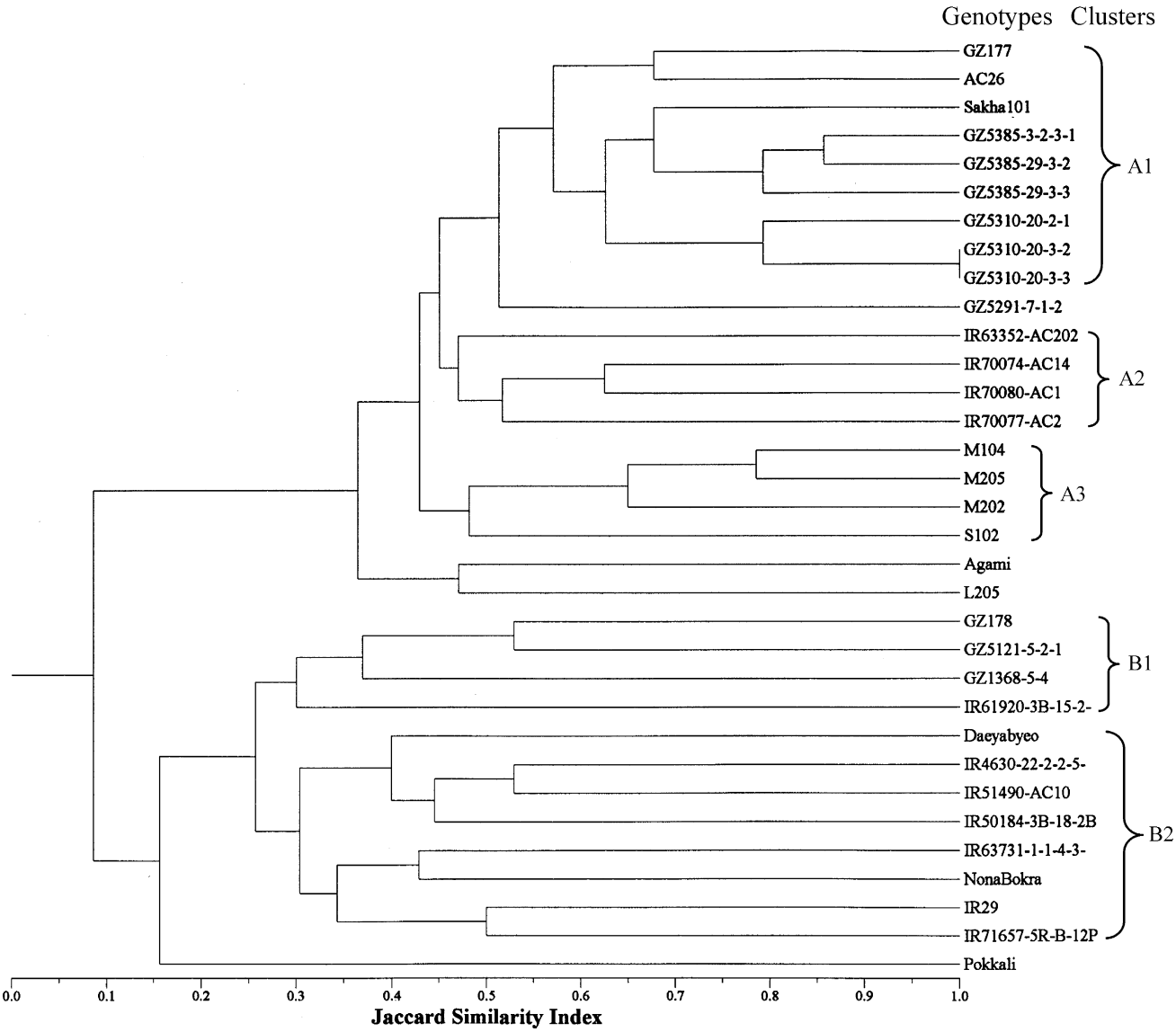

Fig. 1. Clusters of 33 rice genotypes based on Jaccard's similarity index calculated from data matrix of 25 microsatellite loci. The dendrogram was created by UPGMA.

Table 4
Cluster means of ion contents in rice shoots and ion selectivity under salt stress among the clusters of microsatellite markers

Cluster	Ion contents (mmol kg^{-1} dry wt.) and ion selectivity								
	Genotypes/cluster	Na	Ca	K	Mg	P	S	$S_{K,Na}$[a]	K_g[b]
A1	9	497 b[c]	131 bc	675 abc	99 d	114 a	104 a	87 b	2.81 b
A2	4	530 ab	144 b	745 a	116 bc	106 ab	108 a	93 b	2.69 bc
A3	4	578 a	120 cd	646 bc	104 cd	108 ab	102 a	70 b	3.57 a
B1	4	443 c	153 a	713 ab	131 a	113 ab	116 a	111 a	2.10 c
B2	8	371 d	129 bcd	743 a	118 b	104 b	106 a	130 a	2.30 c

[a] $S_{K,Na}$, K–Na selectivity; the preference for K over Na.
[b] K_g, Gapon selectivity constant representing Na–Ca selectivity; the lower values indicate the lower Na content in shoot.
[c] Means within columns followed by the same letter were not different at $P = 0.05$ based on Duncan's multiple range test.

Table 5
Cluster means of the morphological characters under salt stress among the clusters of microsatellite markers

Cluster	Genotypes/ cluster	SWPL (g per plant)	SWP (g per panicle)	TLR (number per plant)	HVI	HT (cm)	LA (cm^2 per plant)	SH/RT	STI
A1	9	3.94 a[a]	1.17 a	3.33 b	0.47 a	66.9 bc	61.2 b	3.56 a	1.38 a
A2	4	3.69 ab	0.82 b	4.35 a	0.38 b	77.7 a	80.1 ab	3.88 a	1.29 a
A3	4	2.81 b	0.88 b	2.98 b	0.41 ab	65.2 bc	71.6 b	3.17 a	0.98 b
B1	4	2.83 b	0.83 b	3.36 b	0.35 b	60.3 c	79.0 ab	3.29 a	0.98 b
B2	8	3.27 ab	0.77 b	4.35 a	0.24 c	73.1 ab	103.8 a	3.36 a	1.14 ab

The morphological characters of the plants under salt stress: SWPL, seed weight per plant; SWP, seed weight per panicle; TLR, tillers per plant; HVI, harvest index; HI, plant height; LA, leaf area; SH/RT, shoot and root ratio; STI, salt tolerance index derived from seed weight per plant under salt stress divided by the mean of the same character in M-202.
[a] Means within columns followed by the same letter were not different at $P = 0.05$ based on Duncan's multiple range test.

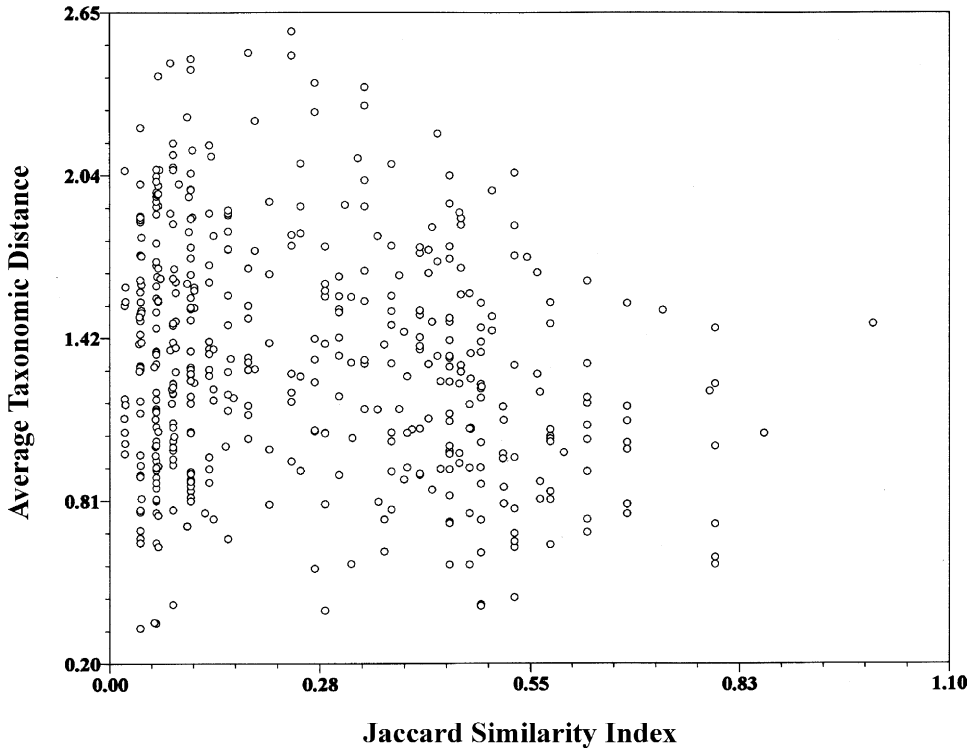

Fig. 2. Correlation between genetic similarity identified by microsatellite markers and taxonomic distance measured by ion contents and ion selectivity. Comparisons of genotype pairs for Jaccard Similarity Index and Average Taxonimic Distance were represented by the symbol (○).

4. Discussion

The genotypes used in this study were collected from different regions of the world. However, the genetic diversity in this subset of rice germplasm was relatively low compared to other reports. Although the average of the PIC values in this study (0.57) was higher than that determined by Akagi et al. [12] in 59 japonica cultivars in Japan (0.37), it was lower than that determined by Panaud et al. [16] in 24 rice cultivars and wild rice species (0.69) and Garland et al. [13] in 43 Australian cultivars (0.74). The cause of low PIC in this collection of germplasm is unknown.

Breeders often have to deal with the tasks of genetic improvement in crops for tolerance to abiotic stress when the related mechanisms are not well characterized. One of the major approaches in molecular breeding is selection with the aid of molecular markers linked to the quantitative trait loci (QTLs) underlying the physiological or agronomical performance under stress when the candidate genes are not available. The QTLs controlling salt tolerance have been identified using molecular markers in tomato [28,29] and rice [30,31]. Although this approach remains promising, its application to complicated traits such as salt tolerance in terms of physiological characters may be limited due to wide confidence intervals [32], close genetic relationships between parental populations, large sample size required for screening in segregating populations [33], and possible significant interactions between environment and genotype for QTL analysis.

This study, based on our knowledge, is the first to analyze salt tolerance using molecularly classified germplasm. Molecular characterization of germplasm prior to crosses of parental lines can increase genetic diversity among parental genotypes, maximize genetic variation present in breeding populations, and minimize the efforts in the screening, for either direct selection in traditional breeding or indirect selection through QTLs. Another important aspect of the rationale utilizing molecularly characterized germplasm for salt tolerance breeding is the fact that salt tolerance in plants is a multigenic phenotype because of the complicated nature of salinity stress in soil. Salt stress is not due to single ions. Instead, it may be the result of different cations (Na^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^- and $SO_4{}^{2-}$) as discussed by Gorham and Jones [34]. These ions may all contribute to salinity stress. Salinity stress may also be compounded with other stresses such as nutritional deficiencies and toxicities [35]. The selection for salt tolerance in field trials is actually a process of screening for desired recombinant genotypes in segregating populations responding to these complicated stresses. At the genome level, among the cDNA libraries established from salt-stressed rice, 26% of the unique transcripts were functional based on the change in transcript abundance in salt-stressed tissues relative to unstressed tissues [36]. Although the function of these transcripts is unknown, it would be reasonable to hypothesize that the genes controlling salt tolerance may be wide spread in the rice genome. Thus, increased genetic diversity will undoubtedly increase the probability of identifying desirable recombinant genotypes during screening for salt tolerance.

In this study, 30% of the alleles detected in 20 breeding lines were not identified in the gene pool composed of the 11 commercial cultivars. Although only two landraces were screened, 38% of the alleles detected in these land races were not identified in the cultivars. This indicates that the genetic diversity in these breeding lines and landraces may be useful in the genetic improvement of rice. One of the fundamentals in breeding is the search for new genes and gene combinations through intercrosses. The unique alleles, i.e. the alleles not detected in gene pools of commercial cultivars as defined in this study, may provide favorable allelic combinations at the same locus through allelic interactions or at different loci through epistatic interactions [37,38]. Plants in segregating populations may be screened for favorable allelic recombinants in response to salt stresses. Although more unique alleles may exist in landraces than in commercial cultivars, the elimination of unfavorable agronomic characters such as tall stature and long growing season from landraces is usually difficult in breeding programs. Development of salt tolerance germplasm, i.e. breeding lines, through crosses with salt tolerant landraces is an intermediate step before the final transfer of salt tolerance genes into commercial cultivars. There have been tremendous efforts in the breeding programs at IRRI to develop salt tolerance germplasm from landraces. Among the breeding lines obtained from IRRI for this study, IR4630-22-2-2-5-1-3, IR71657-5R-B-12P, and IR50184-3B18-2B-1 were derived from Pokkali and IR63731-1-1-4-3-2 was derived from Nona Bokra. Those lines derived from Pokkali were not identical to this parent based on microsatellite clusters. It appears that the genes from Pokkali were lost during multiple crosses and subsequent selections for favorable agronomic traits. In contrast, the transfer of salt tolerance genes from Nona Bokra to IR63731-1-1-4-3-2 was more successful. This breeding line was ranked at the top for its salt tolerance in previous evaluation trials [21]. Moreover, IR63731-1-1-4-3-2 and Nona Bokra grouped into the same cluster. This indicates that the genetic backgrounds between the two genotypes are identical.

Differential responses to salt stress were observed among microsatellite clusters. Poor ion selectivity, i.e. the low K–Na selectivity and the high Na–Ca selectivity, in the genotypes of Cluster A3 was consistent with their high Na content and low K content compared with the other japonica rice genotypes. High Na content and low K content in genotypes of Cluster A3 were consistent with their low means of yield related parameters. In contrast, Na content in Clusters B1 and B2 was significantly ($P = 0.05$) lower than those in Cluster A3, whereas the means of grain yield were not significantly different from that in Cluster A3. Salt tolerance in the genotypes of Clusters B1 and B2 could not be explained simply by the ion contents. Unequal Na distribution between old and young leaves or tissue tolerance are important mechanisms

controlling salt tolerance [4,39]. It is possible that tissue tolerance is low in Clusters B1 and B2 because these genotypes are less stressed internally than those of japonica rice genotypes. Although ion selectivity was similar between Cluster B1 and B2, Na content was significantly ($P = 0.05$) lower in Cluster B2 than Cluster B1. Other mechanism such as direct apoplastic leakage [40] or growth vigor such as height and leaf area may contribute to the variation of Na content in these genotypes. Therefore, salt tolerance among the genotypes of different microsatellite clusters must be controlled by diverse mechanisms.

The rice germplasm used in this study was characterized at 25 microsatellite loci. Certainly, these loci could not include all salt tolerance genes. It is not surprising to see some sensitive genotypes mixing with tolerant ones in the clusters. Even though, among indica rice genotypes, all tolerant ones grouped into Cluster B2 while genotypes in Cluster B1 were either sensitive or moderately tolerant to salt stress. Among japonica rice genotypes, all tolerant ones grouped into Cluster A1 while genotypes in Cluster A3 were all sensitive (Fig. 1). Statistically, physiological and morphological characters were significantly ($P = 0.05$) different among microsatellite clusters. It is very interesting to see a highly significant ($P = 0.005$) correlation between genetic similarity based on microsatellite markers and taxonomical distance based on ion data (Fig. 2). That is, the genotypes with closer genetic relationships will be more similar for ion contents and ion selectivity. It is obvious that the adaptation of rice to saline soils is different among the genotypes with diverse genetic backgrounds.

We are aware that the number of genotypes used for microsatellite clustering in this study was relatively small. In addition, physiological responses to salinity were measured solely based on ion contents. Other aspects of salinity effects such as osmotic response are also known to be important [41]. These considerations may limit the scope of our conclusions. However, the major finding in this study, the new approach to enhance salt tolerance in rice by association of genetic similarity from microsatellite clusters with salt tolerance phenotypes, remains intact. Our new approach could be applied to breeding programs with larger number of genotypes. To be cautious, microsatellite clusters were tested by changing the number of genotypes for cluster analysis. The roots of the clusters were generally unchanged with different number of genotypes during cluster formation. For example most japonica genotypes derived from Egypt and California grouped into A1 and A3, respectively, regardless of the joining of other genotypes.

In summary, the results in this study provide some implications for engineering salt tolerance using microsatellite clusters.

1. A close genetic relationship between parental populations is a common problem in salt tolerance breeding programs and limits the success of selection in segregating populations, especially when the underlying physiological characters are targeted [33]. Improving salt tolerance can be achieved by selecting parental genotypes before intercrossing based on microsatellite markers. For example salt tolerance of California rice cultivars could be improved by intercrossing with those genotypes in Cluster B2 or A1 because of the significant difference for physiological characters among these clusters. A tremendous number of breeding lines have been developed through multiple crosses with salt-tolerant land races of ‘Pokkali’ and ‘Nona Bokra’ or anther culture at the International Rice Research Institute [35]. Many of these lines are either genetically similar or difficult to trace the parentage among these lines because of the complex crossing schemes used during their development or somaclonal variation generated during tissue culture. The identification of the genetic distances among the breeding lines or cultivars will be important to maximize their use in the breeding for salt tolerance.
2. Evaluating and selecting salt tolerance among genotypes are not easy tasks because measurements of physiological and morphological phenotypes are highly affected by environmental factors. Highly significant correlation between genetic similarity and taxonomic distance of ion contents suggests that physiological phenotypes among different genotypes can be predicted based on their genetic similarity characterized by microsatellite markers. Since ion contents among genotypes tend to be similar within microsatellite clusters, the variation of physiological phenotypes in segregating populations can be increased by choosing parental genotypes between microsatellite clusters prior to intercrossing. Genetic variation of ion contents within segregating populations may be further maximized.
3. The diverse salt tolerance mechanisms among microsatellite clusters indicate that these mechanisms are genetically controlled. Identifying different salt tolerance components and pyramiding them into a salt-tolerant cultivar have been suggested [39]. The success in pyramiding different salt tolerance components into a cultivar can be increased using microsatellite markers because of the difficulty in identifying these tolerance components using conventional methods. With the aid of microsatellite makers, different salt tolerance components may be combined by intercrossing the genotypes from different clusters. For example genotypes in Cluster A1 may have better tissue tolerance than those in Cluster B1 while the genotypes in Cluster B1 possess better ion selectivity than Cluster A1. Crosses between these two clusters could have segregants with complementary genes controlling both tissue tolerance and ion selectivity.

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