

# Diversity Analysis of Bangladeshi Coastal Rice Landraces (*Oryza sativa*) for Morpho-Physiological and Molecular Markers' Responses to Seedling Salinity Tolerance

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**ABSTRACT** Development of salt tolerance in rice through breeding program is mainly depends on the salinity responses of the potential rice germplasms. Coastal rice landraces of Bangladesh possess diverse morphological and physiological responses to salinity. Hence, our target is to identify candidate salt-tolerant coastal rice genotypes as a new source of salt tolerance (12 dS/m). Here, we annotated 20 Bangladeshi coastal *Aus* landrace rice regarding their phenotypic and genetic relatedness to salinity tolerance through multivariate analyses of five morpho-physiological traits namely, salt injury score (SIS), ion-leakage, chlorophyll concentration, root-shoot reduction percentage and profiling of DNA using simple sequence repeat (SSR). Based on the standard evaluation score (SES) the salt-induced coastal rice landraces were grouped into highly susceptible (HS), susceptible (S), moderately tolerant (MT), tolerant (T) and highly tolerant (HT). Besides, a canonical discrimination analysis of the mean trait values of five morpho-physiological parameters confirmed the above mentioned five categories of salinity tolerance. Based on all morpho-physiological parameters one genotype (*Kalihytta*) as highly tolerant (HT), two genotypes (*Manikmuri* and *Monsur IRRRI*) as tolerant (T) and five genotypes (*Nara*, *Iratom 27*, *Matichak*, *Abdul high IRRRI* and *Parija*) were identified as moderately tolerant (MT) against salinity. Finally, the molecular characterization using two SSR markers (RM493 and RM3412) revealed *Kalihytta*, *Nara*, *Iratom 27*, *Parija*, *Lal jamaibabu* and *Fullbadam*, as tolerant against salt stress. Our candidate salt tolerant *Aus* rice genotypes could be useful as novel sources of salt tolerance for thriving salt-tolerant high yielding varieties in the coastal ecosystem of Bangladesh.

**Keywords** Coastal rice, Morpho-physiological traits, Multivariate analyses, Seedling salinity tolerance, SSR markers

## INTRODUCTION

Salinity, among all the abiotic stresses is a highly prominent issue that severely affects plant growth and development (Nazar *et al.* 2011). Soil salinity is a global problem for producing crops, especially in coastal areas (De Leon *et al.* 2015). Rice (*Oryza sativa*) feeding almost 50% population of the world and considered one of the most important cereal crops. It is well-known as a model crop among the cereals (Eckardt 2000). Rice is generally a salt-sensitive crop (USDA, [http://www.Ars.Usda.Gov/services/docs](http://www.Ars.Usda.Gov/services/docs;);

Grover and Pental 2003). The reaction to the salt stress is variable among rice growth stages. For example, at seedling stage rice plants died at 10 dS/m (Munns *et al.* 2006), and at reproductive stage, up to 90% yield loss has been observed against a relatively low level of salt stress (3.5 dS/m) (Asch *et al.* 2000; Rahman and Ahsan 2001). Salinity tolerance is a composite trait and plant's reaction to salinity is largely depends on the environments (Gregorio and Senadhira 1993; Flowers 2004). Researchers already have shown much success in developing saline tolerant high yielding varieties (HYV) using several highly tolerant

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(HT) rice landraces (Gregorio *et al.* 2002).

Rice is the major staple food crop in Bangladesh, which covers about 81% of total cropped area (BBS 2012). The country ranked 4<sup>th</sup> position in 2014 both in area (11.77 million ha) and production (52.231 million MT) of rice, and 39<sup>th</sup> in the yield per hectare (4.42  $\text{tha}^{-1}$ ) among the rice growing countries (FAOSTAT, <http://faostat.fao.org/site/291/default.aspx>). In Bangladesh, *Aus*, *Aman* and *Boro*, are the three rice growing seasons which are respectively cultivated in *Kharif-1* (April–July), *Kharif-2* (July–November) and *Rabi* (November–April). There is approximately 2.85 million hectares (Mha) of coastal areas in Bangladesh which is seriously influenced by varying degrees of salinity (SRDI, 2010). Cropping intensity of this region is far below than the national standard mainly due to dry season salinity of soil, tidal flood, heavy clay basin, scarcity of quality irrigation water, late rainfall, low fertility status etc. In last four decades the saline area has increased to about 1.056 Mha from 0.833 Mha (SRDI 2010). Inclusion of short duration and salt tolerant HYV rice may be a scholastic approach towards increasing productivity and cropping intensity in coastal ecosystem (Hasan 2018).

However, development of salinity tolerance in crop plants through breeding programs largely depends on the good understanding of the tolerance mechanisms against salinity in the susceptible and tolerant genotypes (Cha-um *et al.* 2009). In fact, the abundance of suitable donor genotypes, and adequate knowledge on the hereditary and physiological mechanisms regarding salinity tolerance for appropriate screening methods are pivotal for developing a targeted trait (Negrão *et al.* 2011). For salinity tolerance study, the International Rice Research Institute (IRRI) proposed screening technique (Gregorio *et al.* 1997) but the lack of suitable growth environments in different laboratories limits its reproducibility (De Leon *et al.* 2015). Besides, several studies on screening techniques have already been reported (Yeo *et al.* 1990; Aslam *et al.* 1993; Asch *et al.* 2000; Kanawapeet *et al.* 2012; Mondal and Borromeo 2016). In most of the screening, salt-tolerant or susceptible rice genotypes have been identified either using visual salt injury scoring technique (Gregorio *et al.* 1997) or measuring the ion toxicity ( $\text{Na}^+/\text{K}^+$  content) levels in roots and shoots. Actually, salinity tolerance in plants is

polygenic and in a study of Yeo *et al.* (1990), proposed assembling of desired morpho-physiological attributes for enhancing salt tolerance in rice. In another study, Zeng *et al.* (2002) employed cluster group ranking of rice genotypes using different agronomic and physiological parameters to assess the salt tolerance. Recently, De Leon *et al.* (2015) proposed a much more efficient screening technique for salinity tolerance based on multiple morphological and physiological traits were used combining the cluster analysis and discriminant function analyses.

In our present study, we tested salt tolerance and classified 20 coastal *Aus* landrace rice through several multivariate analyses and canonical discriminant functions analyses for validating the divergence among genotypes against salinity. And, finally to study the genotypic variation among these 20 rice genotypes, we employed microsatellite markers (SSR) to screen potential salt tolerant landraces suitable for developing HYV targeting coastal region of Bangladesh.

## MATERIALS AND METHODS

### Plant materials

We screened 20 coastal *Aus* landrace rice against salinity at their seedling stage (Supplementary Table 1). These local rice genotypes are cultivated in the coastal areas (south and south-west) of Bangladesh. In our study, we included a salt tolerant check Binadhan-10 collected from Bangladesh Institute of Nuclear Agriculture (BINA) and a sensitive check BRRI dhan48 from Bangladesh Rice Research Institute (BRRI).

To screen the salt tolerant coastal *Aus* landrace rice at their early growing stage, the experiment was conducted at Stress Agronomy Laboratory and Genetic Engineering and Molecular Breeding Laboratory of Patuakhali Science and Technology University, during the period from January 2019 to May 2019. The phenotypic study was done in Stress Agronomy Laboratory maintaining semi-control environment. The genotypic screening of the collected germplasm was done in Genetic Engineering and Molecular Breeding Laboratory using simple sequence

repeat (SSR) markers to find out salinity tolerant landraces.

### Phenotypic assessment on seedling salinity tolerance of coastal *Aus* landrace rice

For studying the seedling salinity tolerance of coastal *Aus* landrace rice the phenotypic screening was carried out following the protocol of International Rice Research Institute, in hydroponic culture medium (Yoshida *et al.* 1976) maintaining salinity level 12 dS/m (Gregorio *et al.* 1997). We germinated 10 seeds from each genotype in petri-dishes at 35°C for 48 hours. Then, we transferred them into styrofoam seedling floats and grew the seedlings in the hydroponic nutrient solution for two weeks. At seedling stage, we maintained saline condition at 12 dS/m adding NaCl and non-saline condition (control) without adding NaCl. We maintained pH between 5.0-5.1 using 0.01N of HCl or 0.1N NaOH. We performed our study following randomized block design with three replications. For data collection, from the 10 seedlings, only 5 plants having uniform growth per genotype were considered and their mean value of the targeted trait was considered as one replicate.

### Data collection

Data were recorded on salt injury score (SIS), ion-leak percent, reduction percent on chlorophyll concentration, shoot length and root length from three individual plants per experiment.

#### Ion leakage

Ion leakage from the leaf tissue as an indicator of early response of rice genotypes against salinity was measured following De Leon *et al.* (2015). The electrical conductance (using conductivity meter HI8733, HANNA, USA) for  $EC_{\text{initial}}$  and  $EC_{\text{final}}$  values of the solution was estimated before and after autoclaving, respectively. Finally, following Flint *et al.* (1967) the ion-leak percent of the genotypes were calculated as an indicator of salt injury index:  $\text{Ion-leakage} = 100 (R_1 - R_0) / (1 - R_0)$ ; here, Ion-leak is SIS (salt injury score),  $R_0$  is the quotient of initial and final values of EC of control plant,  $R_1$  is the quotient of initial and final values of EC of the saline treated plant.

### Chlorophyll content

After 4 days post-salinization (DPS) the leaf yellowing was noticed. The comparative chlorophyll intensity (SPAD value) was estimated from the central part of the second youngest leaf both in control and salt-induced rice genotypes using chlorophyll meter (SPAD-502 Plus, Konica Minolta, Japan) to find out differences among rice genotypes. The reduction percent (% R) of chlorophyll content was calculated by the formula:  $\text{Chl}_R = 100 (\text{Chl}_0 - \text{Chl}_t / \text{Chl}_0)$ ; here  $\text{Chl}_R$  means the percent reduction of chlorophyll concentration; ' $\text{Chl}_0$ ' denoted for the chlorophyll content in control condition; ' $\text{Chl}_t$ ' represent the chlorophyll content in saline treatment.

### Growth parameters

We monitored root length and shoot length both in control and salt-induced rice genotypes at 7 DPS. All assessments were done with regard to the control plant to find genotypic differences. The percent (%) reduction in root length and shoot length were measured employing similar formula as in the case of chlorophyll percent reduction.

### Salt injury score (SIS)

Symptoms of salt injury in plants started to appear from 7 DPS. Here, standard evaluation scoring (SES) method of IRRRI was followed for visual scoring (Gregorio *et al.* 1997). The plants were given score 1 when normal growth continued with no leaf symptoms. The score of 3 was given if it showed nearly usual growth along with few leaves of white tips and rolled. A score of 5 was given if plants' growth severely retarded with rolled leaves with white tips. Plants viewing only green culm having desiccated leaves with complete cessation of growth were scored 7. Finally, the plants were scored 9 when all plants were dead. The average SIS score of a genotype was figured from the SIS values of 3 individual plants.

### Data analysis

To test the treatment effects on genotypes, morpho-physiological data were statistically analyzed and mean trait values were compared at 0.05%, 0.01% and 0.001% level of significance. Correlation among traits was also

computed based on the mean trait values of three replications. To classify the salinity tolerant genotypes, the mean trait values of genotypes for five traits were employed for multivariate cluster analysis using JMP8 software (JMP<sup>®</sup>8, SAS Institute Inc., Cary, NC, 1989-2019). The clustering of genotypes were done as moderately tolerant (MT), tolerant (T), highly tolerant (HT), highly sensitive (HS) and sensitive (S) based on the scoring of the group average SIS. Classification of genotypes was confirmed using discriminant analyses for each genotype with the same data used for clustering. To figure out the variation among salinity groups, multivariate analysis of variance (MANOVA) was run in JMP8. Thus, we classified 20 *Aus* landrace rice for seedling salinity tolerance combining both clustering and discriminant analyses.

### Genotyping of the rice genotypes using SSR marker

#### Genomic DNA isolation

We extracted genomic DNA from rice seedlings of 25-day-old using Promega A1120 DNA cleansing kit (Promega, USA) following the producer instructions. Extracted DNA was quantified using Nano-100 micro spectrophotometer (BOYN, China) and finally concentration was adjusted to 25 ng/μL through dilution.

#### PCR amplification of SSR marker and evaluation of genotypes

A set of 10 SSR primer pairs were primarily selected for the analysis of genotypic variation to salinity tolerance among the collected 20 rice genotypes (Supplementary Table 2). Based on banding patterns and potential for population discrimination two polymorphic SSR markers RM3412 and RM493 linked to *saltol* QTL (quantitative trait loci) of chromosome 1 (Ganie *et al.* 2014) were finally selected to screen coastal rice germplasm for salt tolerance.

PCR cocktail of 15 μL was prepared by mixing 1 μL forward primer, 1 μL reverse primer, 7.5 μL Go Taq<sup>®</sup> Green Master Mix (Promega, USA), 1.0 μL of extracted rice DNA (25 ng/μL), and 4.5 μL of nuclease free water. PCR (Applied Biosystems, USA) was done following the condition as follows: initialization for 5 minutes at 94°C and 35 cycles of amplification for 45 seconds at 94°C, for

45 seconds at 55°C, for 1 minutes at 72°C and final expansion at for 5 minutes at 72°C. To detect band, gel electrophoresis was done after PCR in 2% gel of agarose adding with ethidium bromide. Visualization of banding patterns was performed with UV transilluminator gel documentation unit. The banding patterns of 20 rice germplasm were genotyped comparing with tolerant and susceptible control variety. The genotypes having the same banding pattern to the salt tolerant check variety were considered as tolerant and similar to salt susceptible check variety were considered as salt susceptible. Thus, we genotyped 20 *Aus* rice varieties based on SSR genotyping to screen ideal tolerant genotypes.

## RESULTS

### Response against salt stress

Considering morpho-physiological responses of 20 *Aus* rice germplasms, significant genotypic differences were evident for many of the investigated attributes (Table 1).

#### Salt injury score (SIS)

A highly significant genotypic differences ( $P < 0.0001$ ) were observed with regard to the scores of salt injury under salinity. Among the check genotypes, Binadhan-10 had a mean SIS of 3. Among the 20 coastal germplasms, *Kalihytta* (SIS 1) was found as highly tolerant, two were recorded as salt tolerant namely, *Manikmuri*, and *Monsur IRRI* with SIS of 3, five were moderately tolerant *viz.* *Nara*, *Abdul high IRRI*, *Iratom 27*, *Parija*, *Matichak*, *Mohisur* with SIS of 5, three were highly susceptible *viz.* *Chawlmoni*, *China*, *Gota IRRI (Mota)*, *Gota IRRI (Chikon)* with SIS of 9, and the rest were susceptible to salt stress having a SIS 7.0 (Table 1).

#### Ion-leak percentage

A highly significant ( $P < 0.0001$ ) genotypic differences were noticed in case of ion-leakage. It ranged from 27.37 to 63.74%. Among the check cultivars, the susceptible check variety BRRI dhan48 showed high ion-leakage (50%) while the tolerant check, Binadhan-10 had 29.03%. Amongst the coastal genotypes, the highest ion-leak (%) found in

**Table 1.** Mean trait values of coastal *Aus* rice genotypes against salinity.

Genotype	SIS	Ion-leakge (uS/cm)		Ion-leak (%)	Chlorophyll content (SPAD unit)		Chl-R (%)	Root length (cm)		RtL-R (%)	Shoot length (cm)		StL-R (%)
		Ro	Rt		Ctrl	Sal		Ctrl	Sal		Ctrl	Sal	
		<i>Nara</i>	5*	0.04	0.36	33.33**	17	12	29.41**	7	5	28.57	32
<i>Chawlmoni</i>	9	0.6	0.73	32.5**	12	4	66.67	8	6	25	30	12	60+++
<i>Gota IRRI (Mota)</i>	9	0.09	0.67	63.74	19	5	73.68	7	5	28.57	25	10	60+++
<i>Tepu</i>	7	0.05	0.38	34.74**	15	8	46.67	6	5	16.67**	26	13	50+++
<i>Sada jamaibabu</i>	7	0.06	0.37	32.98**	14	9	35.71*	7	6	14.29**	29	18	37.93+
<i>China</i>	9	0.09	0.58	53.85	18	4	77.78	6	5	16.67**	27	14	48.15++
<i>Lal jamaibabu</i>	7	0.09	0.39	32.97**	12	5	58.33	7	7	0***	21	19	9.52
<i>Fullbadam</i>	7	0.05	0.57	54.74	18	7	61.11	8	7	12.5**	27	17	37.04+
<i>Manikmuri</i>	3***	0.07	0.33	27.96***	18		5.56***	8	7	12.5**	30	24	20
<i>Benamuri</i>	7	0.04	0.55	53.13	14	6	57.14	9	8	11.11**	30	22	26.67
<i>Sochwala</i>	7	0.04	0.53	51.04	18	517	72.22	9	7	22.22	26	20	23.07
<i>Iratom 27</i>	5*	0.61	0.75	35.90**	20	15	25**	7	6	14.29*	21	19	9.53
<i>Kalihytta</i>	1***	0.05	0.31	27.37***	18	16	11.11***	8	6	25	32	25	21.88
<i>Parija</i>	5*	0.07	0.32	26.89***	14	13	7.14***	7	4	42.86	23	21	8.69
<i>Abdul high IRRI</i>	5*	0.03	0.33	30.93**	15	13	13.34**	6	5	16.67*	23	22	4.35
<i>Monsur IRRI</i>	3**	0.05	0.32	28.42***	19	17	10.53***	9	8	11.11**	19	18	5.26
<i>Kutiogroni</i>	7	0.06	0.49	45.74*	14	8	42.86	8	7	12.5**	32	16	50+++
<i>Gota IRRI (Chikon)</i>	9	0.07	0.61	58.06	16	9	43.75	6	4	33.33	33	12	63.63+++
<i>Matichak</i>	5*	0.08	0.37	31.52**	15	12	20**	7	6	14.29*	25	21	16
<i>Mohishur</i>	3**	0.04	0.32	29.17***	16	13	18.75***	6	5	16.67*	27	24	11.11
BRR1 dhan48	7	0.11	0.56	50.56	22	11	50	5	4	20	16	10	37.5+
Binadhan-10	3**	0.07	0.34	29.03***	25.9	18.7	27.79**	8	7	12.5**	22	19	13.64
Genotypic effect (Pr > F)	< .0001			< .0001			< .0001			< .0001			< .0001

SIS: salt injury score, Ion-leak: index of injury by ion leakage, Ro: ion leakage in control treatment, Rt: ion leakage in saline treatment, Ctrl: control, Sal: saline treatment, Chl-R: % chlorophyll reduction, Shl-R: % shoot length reduction, Rt-R: % root length reduction, \*: Significantly different to BRR1 dhan48 at 0.05 probability level, \*\*: Significantly different to BRR1 dhan48 at 0.01 probability level, \*\*\*: Significantly different to BRR1 dhan48 at 0.001 probability level, +: Significantly different to Binadhan-10 at 0.05 probability level, ++: Significantly different to Binadhan-10 at 0.01 probability level, +++: Significantly different to Binadhan-10 at 0.001 probability level.

*Gota IRRI (mota)* 63.73% and the lowest ion-leak was recorded in *Parija* (26.89%), followed by *Kalihytta* (27.37%), *Manikmuri* (27.96%), and *Monsur IRRI* (28.42%); hence, these genotypes were predicted to be salt-tolerant as they had lower percentages of ion-leak at 12 dS/m EC of the salinity level. Moderately salt-tolerant lines were *Abdul high IRRI*, *Matichak*, *Nara* with ion leakage values of 30.93, 31.52, and 33.33%, respectively (Table 1).

#### Chlorophyll reduction percentage

Highly significant ( $P < 0.0001$ ) %R of chlorophyll concentration (Chl-R) also indicate about the higher

genotypic differences among coastal genotypes under salt stress. Among the check genotypes, susceptible check BRR1 dhan48 had the highest chlorophyll reduction (50%) and tolerant check Binadhan-10 exhibited the lowest chlorophyll reduction (27.79%). Among the coastal genotypes, *Manikmuri* (5.56%), *Parija* (7.14%), *Monsur IRRI* (10.53%), *Kalihytta* (11.11%), *Abdul high IRRI* (13.33%), *Mohishur* (18.75%), *Matichak* (20%), *Iratom 27* (25%) showed relatively greater tolerance against salinity with much lower %R of chlorophyll content than the susceptible check BRR1 dhan48 (Table 1).

**Table 2.** Pearson correlation matrix of five seedling traits against salinity (12 dS/m) in 20 *Aus* rice genotypes.

Traits	SIS	Ion-leak	Chl-R	RtL-R	ShL-R
SIS	1				
Ion-leak	0.6968***	1			
Chl-R	0.7721***	0.7048***	1		
RtL-R	-0.1606	0.0273	-0.0927	1	
ShL-R	0.6586***	0.6521***	0.6385***	0.2289	1

SIS: salt injury score, Chl-R: % chlorophyll reduction, ShL-R: % shoot length reduction, Rt-R: % root length reduction, \*: Significant at the 0.05 probability level, \*\*: Significant at the 0.01 probability level, \*\*\*: Significant at the 0.001 probability level.

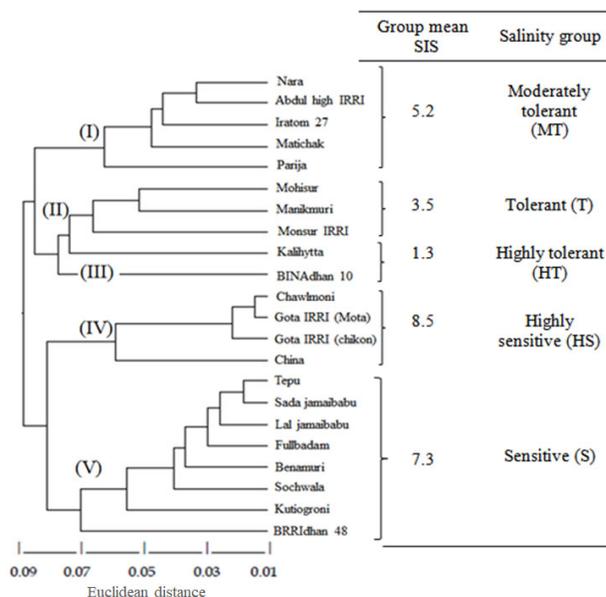
### Shoot length and root length reduction percentage

Percent (%) root length reduction (RtL-R) and shoot length reduction (ShL-R) revealed the differences among genotypes ( $P < 0.0001$ ). Several coastal genotypes showed higher root length reduction such as *Parija* (42.86%), *Gota IRRI (Chikon)* of 33.33%, *Gota IRRI (Mota)* (28.57%), *Nara* (28.57%), indicating their susceptibility against salinity. On the other hand, some genotypes, such as *Monsur IRRI* (11.11%), *Benamuri* (11.11%) showed the lowest root length reduction indicating higher tolerance of these genotypes against salinity (Table 1).

Among the check varieties, BRRIdhan48 showed highest shoot length reduction (37.5%), while Binadhan-10 showed lowest shoot length reduction (13.64%). Among the coastal genotypes, *Gota IRRI (Chikon)* showed highest shoot length reduction (63.63%) followed by *Gota IRRI (Mota)* (60%), *Chawlmoni* (60%), suggesting the susceptibility of this genotypes to salt stress and the lowest shoot length reduction (4.35%) was found in *Abdul high IRRI*, followed by *Monsur IRRI* (5.26%), *Parija* (8.69%) indicating their higher tolerance to salt stress (Table 1).

### Correlation analysis among the traits for salt tolerance

From the individual relationship of physiological traits, a positive and high correlation in case of SIS was observed with %R of chlorophyll, ion-leak, and shoot length but negatively correlated with %R of root length (Table 2). Ion-leak was positively correlated with percent reduction of chlorophyll, root length, shoot length, and SIS. Percent of chlorophyll reduction was negatively correlated with %R of root length and positively correlated with SIS, ion-leak, as well as %R of shoot length. Root length %R



**Fig. 1.** Clustering of 20 *Aus* rice genotypes by UPGMA based on Euclidean distance of five morphological and physiological trait responses against salinity.

was positively linked with %R of shoot length, ion-leak but negatively correlated with SIS, chlorophyll %R.

### Clustering analysis of salt tolerance

Clustering study through dendrogram revealed 2 highly tolerant (HT) genotypes including one local genotype (Binadhan-10 and *Kalihytta*), 4 tolerant (T) genotypes including 3 local genotypes viz. *Mohishur*, *Manikmuri* and *Monsur IRRI*; 4 moderately tolerant (MT) viz. *Nara*, *Abdul high IRRI*, *Iratom 27* and *Matichak*, *Parija*; 8 susceptible (S) genotypes including eight local genotypes viz. *Tepu*, *Fullbadam*, *Sada jamaibabu*, *Lal jamaibabu*, *Benamuri*, *Sochwala*, *Kutiogroni* and BRRIdhan48 and 4 local highly

susceptible (HS) genotypes viz. *Chawlmoni*, *Gota IRR1 (Mota)*, *Gota IRR1 (Chikon)* and *China* (Fig. 1).

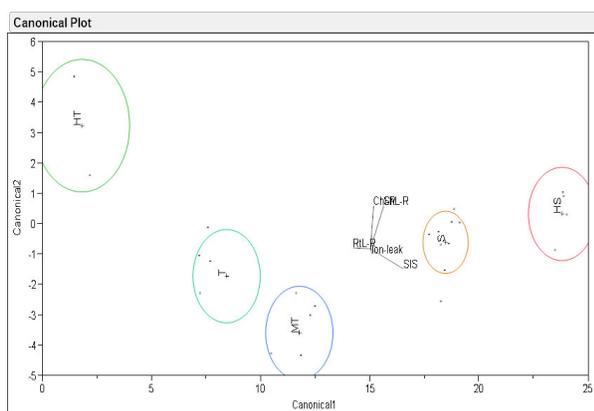
### Canonical discriminant analysis for salt tolerance

We performed canonical discriminant analysis to evaluate the level of diversity among salinity groups (Fig. 2). We found two canonical discriminant functions. Canonical discriminant function 1 (Can 1) was positively correlated to SIS, Ion-leak, Chl-R, RtL-R, ShL-R. In contrast, a negative correlation with SIS, Ion-leak and RtL-R was evident in case of canonical discriminant function 2 (Can 2). Therefore, this result indicates Can 1, Can 2 differentiates genotypes based on their ion-leak (%), percent reduction of chlorophyll concentrations, root length, and shoot length. In Can 1, the largest detachment of group means regarding HT and S (4.18 and 3.59) was observed, while mean separation between HS and T was 3.99 vs. 2.22 (Fig. 2). Examination of Can 2 indicated the detachment of group HT from the T (4.18 vs. 2.22) and differences of MT from the S group (3.31 vs. 3.59). Four groups (T, MT, S and HS) except HT had negative mean values to Can 2. In contrast, positive mean scores were found for each of the group to Can 1. In canonical plot, MT group was located in the middle of the T and S groups (Fig. 2). The HT group with positive value (+4.18) in both the case for Can1 and Can 2 indicating their

low values in SIS, Chl-R, RtL-R, and ShL-R. The T group with positive mean value to Can 1 (+2.20) and negative mean value to Can 2 (-2.20), suggesting their proximity to the HT group, but it has higher shoot length and chlorophyll reduction as opposed to HT categories. For the T and MT, a positive and negative mean values for T group were observed, respectively in the Can 1 and Can 2. Finally, a positive mean value to Can 1 (+3.59) and negative mean value to Can 2 (-3.59) were found in case of the sensitive (S) group.

### Multivariate analysis of variance for salt tolerance

From a multivariate analysis of variance (MANOVA), significant differences were evident for salinity tolerance groups (HS, HT, MT, S and T) against five variable parameters (ion-leak, SIS, Chl-R, RtL-R and ShL-R). Moreover, comparison study for each of the trait between groups through LS mean values indicated significant variations between HT vs. S & HS groups in all the cases (Table 3). Similarly, a significant difference was evident for the group T from HT in case of RtL-R, ShL-R and from MT in ion-leak, Chl-R, RtL-R. Besides, the group S and MT were significantly different due to having significantly different values for ShL-R, SIS, Chl-R and the group S differed with HS only for the significant difference in ShL-R. Thus, the whole pairwise comparison is highly significant indicating complete partitioning among groups based on the five important quantitative parameters.



**Fig. 2.** Canonical discriminant analysis showing population structure of 20 *Aus* rice genotypes in consideration of the morphological and physiological traits responses to salinity, where genotypes grouped as HT: highly tolerant, T: tolerant, MT: moderately tolerant, S: susceptible, HS: highly susceptible.

**Table 3.** Least square (LS) means of salinity groups in five parameters.

Group	SIS	Ion-leak (%)	Chl-R (%)	RtL-R (%)	ShL-R (%)
HS	9	52.04	65.47	25.89	57.95
HT	1	27.37	11.11	25.00	21.88
MT	5	33.37	22.05	26.19	13.81
S	7	43.08	54.50	13.73	33.07
T	3	28.47	14.63	12.04	12.97

SIS: salt injury score, Chl-R: % chlorophyll reduction, ShL-R: % shoot length reduction, Rt-R: % root length reduction, HS: highly sensitive, HT: highly tolerant, MT: moderately tolerant, S: sensitive.

### Screening of *Aus* landrace rice for salinity tolerance using SSR markers

From the morpho-physiological and genotypic performance, we considered Binadhan-10 as tolerant and BRRI dhan48 as susceptible. Coastal genotypes were identified as tolerant having banding pattern similar to Binadhan-10 and as salt susceptible alike to banding pattern of BRRI dhan48 (Supplementary Figs. 1, 2 and Table 4). In respect to primer RM493, we found 3 coastal genotypes viz. *Kalihytta*, *Iratom 27*, *Sochwala* as tolerant, and 11 genotypes viz. *Matichak*, *Kutiogroni*, *Monsur IRRI*, *Gota IRRI (Chikon)*, *Benamuri*, *Manikmuri*, *China*, *Sada jamaibabu*, *Tepu*, *Gota IRRI (Mota)*, *Chawlmoni* as susceptible with compared to the salt-tolerant check Binadhan-10 and susceptible check BRRI dhan48. Additionally, 5 genotypes viz. *Abdul high IRRI*, *Fullbadam*, *Lal jamaibabu*, *Nara*, *Parija* had similar banding patterns to both tolerant check Binadhan-10 and susceptible check BRRI dhan48 (Supplementary Fig. 1). Besides, with the reaction with RM412, 6 coastal genotypes viz. *Kalihytta*, *Fullbadam*, *Lal jamaibabu* were detected as tolerant, 7 genotypes viz. *Gota IRRI (Chikon)*, *Kutiogroni*, *Monsur IRRI*, *Sochwala*, *Benamuri*, *Abdul high IRRI*, *Sada jamaibabu* showed susceptibility comparing with tolerant and susceptible check Binadhan-10 and BRRI dhan48, respectively. And, 5 genotypes viz. *Matichak*, *Parija*, *Iratom 27*, *Manikmuri*, *china*, *Nara*, *Chawlmoni* appeared with banding patterns of both the tolerant and susceptible check (Supplementary Fig. 2).

In a nutshell as represented in Table 4, 7 genotypes viz. *Nara*, *Lal jamaibabu*, *Fullbadam*, *Iratom 27*, *Kalihytta*, *Parija* including tolerant check Binadhan-10 were found as tolerant and 7 genotypes viz. *Sada jamaibabu*, *Monsur IRRI*, *Kutiogroni*, *Benamuri*, *Gota IRRI (Chikon)* including susceptible check BRRI dhan48 were found as sensitive against both the markers. However, 8 genotypes viz. *Abdul high IRRI*, *Matichak*, *Manikmuri*, *Chawlmoni*, *Gota IRRI (Mota)*, *China*, *Tepu* and *Sochwala* were evident to have both tolerant and susceptible banding patterns (as Binadhan-10 and BRRI dhan48) in reaction with RM3412 and RM49.

**Table 4.** Genotypic profiles of 19 coastal *Aus* rice genotypes using SSR markers.

Sl No.	Genotype	Predicted salt tolerance levels using SSR	
		RM 493	RM3412
1	<i>Nara</i>	T	T
2	<i>Chawlmoni</i>	S	T
3	<i>Gota IRRI (Mota)</i>	S	T
4	<i>Tepu</i>	S	T
5	<i>Sada jamaibabu</i>	S	S
6	<i>China</i>	S	T
7	<i>Lal jamaibabu</i>	T	T
8	<i>Fullbadam</i>	T	T
9	<i>Abdul high IRRI</i>	T	S
10	<i>Manikmuri</i>	S	T
11	<i>Benamuri</i>	S	S
12	<i>Sochwala</i>	T	S
13	<i>Iratom 27</i>	T	T
14	<i>Kalihytta</i>	T	T
15	<i>Parija</i>	T	T
16	<i>Monsur IRRI</i>	S	S
17	<i>Kutiogroni</i>	S	S
18	<i>Gota IRRI (Chikon)</i>	S	S
19	<i>Matichak</i>	S	T
20	BRRI dhan48	S	S
21	Binadhan-10	T	T

T: tolerant, S: susceptible.

## DISCUSSION

Here, we have done genotypic diversity analysis of 20 *Aus* rice genotypes against salinity based on morpho-physiological responses. Screening of potential germplasm is making efforts for enhancing their utility in the development of HYV with desired trait (Ismail *et al.* 2007). Coastal landraces having salt tolerance ability may be considered as important resources of salt tolerance in the breeding of salt tolerant HYV. To characterize salt-tolerant rice genotypes, several attempts have been made based on morpho-physiological parameters against salinity (Caldo *et al.* 1996; Zeng *et al.* 2005; Sanni *et al.* 2012). For identifying potential coastal genotypes, we used visual salt injury scoring technique in our study as it represents the overall plant's reaction to salinity (Gregorio *et al.* 1997). We used five quantitative traits for genotypic classification and to

determine their salinity tolerance levels. As reported by many researchers, here we also observed different injuries induced by salt stress for example, yellowing and drying of leaves, reduction of shoot and root length, stunted growth and drying of seedlings (Bonilla *et al.* 2002; Islam 2004; Niones 2004; Bhuiyan 2005). Different agronomic traits like chlorophyll content, root length, and shoot length of tolerant genotypes had lesser reduction percentages than sensitive genotypes (Table 1). No salt injury symptoms were observed in case of highly tolerant genotypes. Based on visual salt injury we used 1-9 scale for grading the genotypes (Gregorio *et al.* 1997). Primarily, in our phenotypic assessment following the modified SES system the ion-leak (%) values were used to categorize 20 coastal genotypes where 3 genotypes namely, *Kalihytta*, *Manikmuri*, and *Monsur IRRI* were found tolerant under salt stress. And, at the same salinity level, 6 genotypes *viz.* *Nara*, *Abdul high IRRI*, *Iratom 27*, *Parija*, *Matichak*, *Mohisur* were evident as moderately tolerant (Table 1). The above results suggest low ion-leak and low SIS in these genotypes increased tolerance under salt stress.

We observed that genotypes were significantly different in case of shoot parameters than that of roots (Table 1), indicating about greater role of shoots in salt tolerance. This might be the fact that shoots confronted higher levels of salinity provoked DNA methylation compared with roots in many of the tested rice varieties under salt stress (Karan *et al.* 2012). We also observed highest shoot growth reductions from salt sensitive groups, which corroborate the findings of Apse *et al.* 1999; Lin *et al.*, 2004 and Munns and Tester 2008. In our present study, the highest shoot length reduction (63.63%) was found in *Gota IRRI (Chikon)*, followed by *Gota IRRI (Mota)* (60%), *Chawlmoni* (60%), that revealed the susceptibility of these genotypes to salt stress and the lowest shoot length reduction (4.35%) was found in *Abdul high IRRI*, followed by *Monsur IRRI* (5.26%), *Parija* (8.69%), indicating about higher tolerance ability of these genotypes against salt stress. We used Pearson correlation matrix to identify associations among five traits that represents salinity tolerance. And, three traits e.g., ShL-R, ChL-R, Ion-leak were found significant having positive correlation with SIS (Table 2). This phenomenon suggests about decreased photosynthetic ability of

salt-sensitive plants under salinity which is symptomatically represented by chlorosis and reduced shoot growth.

We employed cluster analysis to identify the HT, T, MT, S and HS salinity group (Fig. 1). Discriminant analyses along with multivariate analysis of the target traits were also conducted in investigating the phenotypic and genetic diversity of the salt induced coastal genotypes. From the discriminant analysis and multivariate analysis of traits at seedling stage, highly significant genotypic differences and correlations among ion-leakage, SIS, chlorophyll content, shoot length and root length reduction were observed and thus, delineated the differences in salt tolerance among 20 coastal genotypes which coincides the results of Yeo *et al.* (1990). Tolerant genotypes apparently had lower salt injury value, chlorophyll reduction, ion-leakage, root length and shoot length reduction thus providing a clue on salt tolerance potential of a genotype. Actually, genetic diversity and salt responsiveness of the adapted donor rice germplasm are prerequisite for successful rice breeding program in developing salt-tolerant HYV (De Leon *et al.* 2015). In our present study, we found that tolerant group was superior to moderately tolerant with regard to their significant variation in chlorophyll reduction. The highly tolerant group was statistically higher up to the sensitive and highly sensitive groups with respect to all traits, whereas the tolerant and moderately tolerant groups were statistically superior to sensitive and highly sensitive groups with regard to visual salt injury score and chlorophyll concentration. Except in the case of shoot length reductions, all trait responses between sensitive and highly sensitive groups were not significantly different and presumably the sensitive and highly sensitive group could be considered as one group (Table 3). The tabulated results indicated that highly tolerant group showed lowest ion-leakage indicating lowest leaf injury, while the highly sensitive group had highest leaf injury mainly induced by highest ion-leakage. De Leon *et al.* (2015) also reported different salinity tolerance groups based on six important quantitative traits which also corroborate our findings in this study.

In the final validation study, we used SSR marker for determining any genetic variation and unraveling cultivars' identity (Ni *et al.* 2002; Samal *et al.* 2019). SSR screening

technique is considered very useful for their robustness and higher potentials to differentiate individual germplasm accessions (Xu *et al.* 1996; Jeung *et al.* 2005) and additionally, they have higher coverage for rice genome (McCouch *et al.* 2002).

For final molecular validation two polymorphic SSR markers *viz.* RM3412 and RM493 linked to *saltol* QTL in the chromosome 1 of rice (Ganie *et al.* 2014) were used here. These two SSRs showed higher polymorphisms through different banding patterns and successfully discriminated tolerant genotypes from susceptible with relation to the tolerant check Binadhan-10 and sensitive check BRRI dhan48 (Supplementary Figs. 1 and 2). Krishnamurthy *et al.* (2014) also used these two SSR markers in screening 94 rice genotypes and reported different combinations of alleles (haplotypes) for efficient genotyping of those rice genotypes. RM3412 and RM493 were also employed by Mohammadi-Nejad *et al.* (2008) and Ganie *et al.* (2014), where they stated higher number of allelic variations for successfully discriminating the rice genotypes.

Further, Babu *et al.* (2014) and Thomson *et al.* (2010) reported about RM493 and RM3412 for their discerning efficiency against salt stress. And, recently Islam *et al.* (2020) performed an extensive screening of 46 Bangladeshi rice landraces using RM3412 and RM493 for profiling salt tolerance of these genotypes. In our present study, in consideration of banding profiles of both RM3412 and RM493 we found 6 genotypes *viz.* *Nara*, *Lal jamaibabu*, *Fullbadam*, *Iratom 27*, *Kalihytta*, *Parija* and Binadhan-10 were found as tolerant, and 5 genotypes *viz.* *Sada jamaibabu*, *Benamuri*, *Monsur IRRI*, *Kutiogroni*, *Gota IRRI (Chikon)* and BRRI dhan48 were found as susceptible.

To compare the data on multivariate analyses of several morpho-physiological traits regarding seedling salinity tolerance of coastal rice with their subsequent validation using RM3412 and RM493 reveals 4 genotype *viz.* *Nara*, *Iratom 27*, *Parija* and *Kalihytta* consistently showed varying levels of tolerance (moderately to highly tolerant) against salt stress (Fig. 1 and Table 4). While, *Monsur IRRI*, a coastal rice genotype was evident as tolerant in the morpho-physiological salinity screening and subsequently it's DNA profiling using both the SSRs designated it as susceptible to salt stress. On the contrary, *Lal jamaibabu*

and *Fullbadam* were identified as sensitive to salinity at seedling stage screening but their SSR based screening using RM3412 and RM493 grouped them into the tolerant category. These inconsistent data between the two studies might be related with the fact that RM493 and RM3412 may not strongly linked with QTL or the genes which are associated with salt tolerance.

## CONCLUSIONS

Here we successfully identified the diversity among 20 Bangladeshi coastal *Aus* rice genotypes for salinity tolerance through several multivariate analyses. In a morpho-physiological study we monitored responses of multiple traits of these coastal genotypes against salinity and based on their cumulative tolerance values effectively screened potential genotypes for salt tolerance. We also performed molecular validation of their salt tolerance by two SSRs. In a nutshell, our coastal genotypes *viz.* *Nara*, *Lal jamaibabu*, *Fullbadam*, *Iratom 27*, *Kalihytta*, *Parija* could be the new sources of seedling salinity tolerance and also be used in marker-assisted backcrossing in developing salt-tolerant high-yielding rice varieties for the coastal ecosystem of Bangladesh.

## AUTHOR CONTRIBUTIONS

GS, SCS and AKC conceived the concept. HS carried out research work under supervision of GS, SCS and AKC. HS and US analyzed data and drafted the manuscript. GS, SCS and AKC critically edited the manuscript. All authors read and approved the final version of the manuscript.

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**CONFLICT OF INTEREST**

The authors declare that there is no potential conflict of interest relevant to this article.

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