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Screening and Assessment of Genetic Diversity of Rice (*Oryza sativa* L.) Germplasm in Response to Soil Salinity Stress at Germination Stage

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Abstract: Salinity stress significantly affects rice yield, especially when it occurs during the germination stage. Direct seeding is an emerging method to conserve water in rice cultivation. However, to date, there have been limited efforts to screen rice germplasm for salt tolerance under this approach. In this study, 40 rice genotypes were evaluated for salt tolerance using a combination of germination and growth parameters. A total of 59 microsatellite markers were used to assess genetic diversity, revealing significant variation in both germination and growth traits. Based on germination parameters, IR36, Sri Malaysia 2, and MR185 performed well under saline conditions, while Hashemi Tarom and BAS2000 exhibited weak tolerance. MR219, MR211, and MR263 were identified as superior salt-tolerant genotypes against all growth parameters. BAS2000 and MCHKAB were identified as salt-sensitive, showing reduced growth in key traits, including root and shoot development. Marker-based genotyping identified a total of 287 alleles. The number of alleles per locus ranged from two to nine with an average of 4.86. The polymorphic information content (PIC) ranged from four to eight. The markers RM21, RM481 RM566, RM488, RM9, RM217, RM333, RM242, RM209, RM38, RM539, RM475, RM267, RM279, and RM430 were found highly polymorphic with PIC value > 0.7 and contain the highest number of alleles (≥6). Model- and distance-based population structures both inferred the presence of three clusters in the studied rice germplasm. Based on cluster analysis, Shiroodi, Hashemi Tarom, and BAS2000 were found as weak salt-tolerant varieties, whereas MR211 and MR219 are two Malaysian varieties found to be highly tolerant and have a high potential for direct seeding methods. An AMOVA test suggested that 95% genetic diversity was within the population, which implies that significant genetic variation was present in rice germplasm to be used to select parents for future breeding programs.

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1. Introduction

Rice is a staple crop that feeds half of the world's population [1]. The world's population is expected to rise to 9.7 billion by 2050 [2]. With this increasing population, it is imperative to increase global rice production to combat food security issues. This global demand needs to be achieved under increasing abiotic and biotic stresses caused by climate change. Among these abiotic stresses, salinity is a major factor that inhibits crop growth, development, and yield. More than 1000 million hectares of land are estimated to be saline or sodic, and between 25% and 30% of irrigated lands are salt-affected and commercially unproductive [3]. Currently, 4.03 billion people (over 50% of the world's population) living in 13 countries are severely affected by soil salinity. This number is expected to increase up to 5.02 billion by 2050 [4]. If the circumstances continue, half of the world's current agricultural landscapes will no longer be available for cultivation by the year 2050 [5]. To cope with this problem, there is a need to produce more food on limited agricultural land, land that is also seriously affected by salt.

In this context, Simple Sequence Repeat (SSR) markers play a crucial role in addressing the salt tolerance of rice. The SSR markers, known for their high polymorphism and co-dominant inheritance, have proven to be powerful tools in mapping quantitative trait loci (QTLs) associated with salt tolerance in rice [6]. Studies have shown that SSR markers linked to salt tolerance loci, such as *Saltol*, are useful for identifying rice genotypes that maintain high germination rates under saline conditions, facilitating marker-assisted selection (MAS) for breeding salt-resistant varieties [7,8]. Furthermore, SSR markers are critical for uncovering the genetic basis of salt tolerance by revealing genes involved in ion homeostasis and osmotic regulation during seed germination [9–11]. These markers have been influential in identifying key genes and genetic regions associated with salt stress tolerance, providing insights into the mechanisms that enable rice to adapt to saline environments [12,13]. As a result, SSR-based studies are vital for improving the salt tolerance of rice, offering valuable tools for the development of more resilient cultivars capable of thriving in saline soils [13,14].

The germination stage is one of the most critical periods in the life cycle of rice, particularly under salinity stress. Rice seeds are particularly vulnerable to salinity during germination and early seedling development, as high salt concentrations can inhibit seed imbibition, germination rates, and the establishment of healthy seedlings. The salt tolerance of rice varieties at germination is therefore a strong indicator of their potential performance in saline soils later in the growing season [15]. It was reported that rice growth and yield are affected by a minimum salinity stress of 30 mmol L^{-1} NaCl (electrical conductivity ~3 dS m^{-1}) [16]. Above 3 dS m^{-1}, most modern, high-yielding rice varieties display a 12% reduction in yield per dS m^{-1} while a 50% yield reduction has been documented at 6 dS m^{-1} [17]. By screening at this stage, a key vulnerability point was targeted where salinity stress can be mitigated early in the growth cycle, ensuring that only the most tolerant germplasm progress to later stages of development. This strategy can help to identify salt-tolerant varieties that are able to establish successfully even in saline conditions, which is essential for successful direct seeding practices in saline-affected paddy fields [18]. In conventional rice cultivation, seedlings are often grown in nurseries and then transplanted to the field. Hence, this negates the need for rice germplasm to be tolerant to salt stress during germination when using the transplantation method. In general, between 3000

and 5000 L of water are needed to produce 1 kg of rice, compared to other crops like 2000 liters for 1 kg of soy, or 900 L for 1 kg of wheat. Moreover, puddling and transplanting require an additional amount of water, which is becoming scarce and expensive, making rice production less profitable [19]. Consequently, the direct seeding method, where seeds are directly sown in the paddy field, is gaining popularity due to its labor-saving potential and suitability for regions with water scarcity [20]. However, direct seeding exposes seeds to a saline environment from the moment they are sown, which places additional stress on seed germination. Therefore, selecting rice germplasm that can tolerate salinity during the germination phase is critical for successful direct seeding in saline soils. By focusing on the germination stage, this study aimed to identify varieties that are best suited for early establishment in saline soils, which is a major challenge for rice cultivation in many coastal and inland saline areas [21].

The direct seeding approach is the main production strategy in countries like Malaysia, Thailand, Vietnam, the Philippines, and Sri Lanka [22]. Since the method was introduced in the late 1970s, it has successfully sustained rice production and has been favored over the transplantation approach. This exclusively emphasizes the importance of selection for germplasm with agronomically favorable traits like salt tolerance among local varieties. Hence, the performance of different varieties at the germination stage in a salinity stress environment must be screened effectively. Conversely, the transplantation approach requires less emphasis on rice growth abilities during its earliest vegetative stage. The aim of this study is to assess the salinity tolerance of rice germplasm at the seed germination stage and to analyze the diversity against salinity tolerance at seed germination for the direct seeding method. There are a number of tolerant varieties against salinity available for the transplantation approach, but very limited germplasm is available for salinity tolerance at the germination stage for the directed seeding method. So, another objective of the current study is to screen germplasm at the germination stage to be utilized for the direct seeding method in rice.

2. Materials and Methods

2.1. Plant Materials

In total, forty varieties were utilized in the current experiment including salt-tolerant, moderately tolerant, moderately susceptible, and susceptible. The *BAS2000* variety was used as a susceptible check while *MR219* was used as a salt-tolerant check variety. Twenty varieties were elite varieties from Malaysia and sourced from the Malaysian Agricultural Research and Development Institute (MARDI). Eight varieties originated from Pakistan, five varieties from Iran, five from Iraq, and one each from the Philippines and India (Table 1). The experiment was carried out at the laboratory of the Faculty of Biosciences and Medical Engineering (FBME), University Technology Malaysia, and analysis was performed at the Department of Plant Breeding and Genetics, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.

Table 1. List of rice germplasm used in the study.

#	Varieties	Origin	Salinity Tolerance Status
1	POKKALI	India	Tolerant
2	LOCAL TAROM	Iran	Tolerant
3	SHIROODI	Iran	-

Table 1. *Cont.*

#	Varieties	Origin	Salinity Tolerance Status
4	FADJIR	Iran	-
5	NEDA	Iran	Moderately Tolerant
6	HASHEMI TAROM	Iran	Susceptible
7	YASMIN	Iraq	-
8	ANBIR BARAKA	Iraq	-
9	MCHKAB	Iraq	-
10	FIRAT1	Iraq	-
11	ANBIR33	Iraq	-
12	KADARIA	Malaysia	-
13	MR159	Malaysia	-
14	MR220	Malaysia	-
15	SRI MALAYSIA 2	Malaysia	-
16	MANIK	Malaysia	-
17	SRI MALAYSIA 1	Malaysia	-
18	MR185	Malaysia	-
19	RIA	Malaysia	-
20	MR232	Malaysia	Tolerant
21	MAHSURI	Malaysia	Tolerant
22	MR211	Malaysia	Tolerant
23	MASRIA	Malaysia	-
24	MR253	Malaysia	Tolerant
25	PANDERAS	Malaysia	-
26	MR127	Malaysia	-
27	MR219	Malaysia	Tolerant
28	PULUT SIDING	Malaysia	-
29	JAYA MALAYSIA	Malaysia	Susceptible
30	MR263	Malaysia	Tolerant
31	BAHAGIA	Malaysia	-
32	BAS515	Pakistan	Tolerant
33	BAS385	Pakistan	Moderately Tolerant
34	BAS386	Pakistan	-
35	SRI8	Pakistan	Moderately Tolerant
36	KP2	Pakistan	-
37	4365	Pakistan	Moderately Susceptible
38	BAS2000	Pakistan	Susceptible
39	PAKBAS	Pakistan	Moderately Susceptible
40	IR36	Philippines	Sensitive

2.2. Germination and Growth Parameters

To calculate the germination and its related parameters, seeds were grown in 5 mL of 175 mM salt solution in a 6 cm petri dish. Ten seeds for each replication were used to calculate the average data and there were three replications. The complete randomized design was opted to conduct the experiment. Salinity concentration was determined by previous findings [23], which found the threshold to affect the germination and growth of rice. A total of eight germination and related parameters were recorded, namely, final germination percentage (FGP), mean germination time (MGT), germination index (GI), germination energy (GE), peak value (PV) (or peak period of germination in other literature), germination speed (GS), germination rate (GR), and germination capacity (GC). The formula for measuring each parameter is given in the Supplementary File (Supplementary Table S1; Supplementary Figure S1). All the germination parameters were recorded after 7 days of sowing.

For growth parameters, seeds were grown in greenhouse conditions between 27 °C to 30 °C and subjected to 175 mM NaCl treatment in order to assess the effect of salt stress on rice growth parameters. Eight growth parameters were calculated, namely, Total length (TL), shoot length (SL), root length (RL), shoot and root fresh weight (SFW and RFW), shoot

and root dry weight (SDW and RDW), and vigor index (VI). The formula for measuring each parameter is given in the Supplementary File (Supplementary Table S1; Supplementary Figure S1). All the growth parameters were recorded after 14 days of sowing.

2.3. Statistical Analysis

The germination parameters and growth parameters were calculated using Microsoft Excel. The data were then statistically analyzed using R 3.4.1 by running an analysis of variance (ANOVA) test (R Core Team 2017 R, Vienna, Austria).

2.4. Genomic DNA Isolation

Rice germplasm was germinated in soil (a mixture of soil and perlite). Before sowing, the rice seeds were surface-sterilized to eliminate potential microbial contaminants. The sterilization process involved soaking the seeds in 70% ethanol for 2 min, followed by treatment with a 1% sodium hypochlorite solution for 15 min. After sterilization, the seeds were thoroughly rinsed with sterile distilled water (at least three times) to remove any remaining sterilizing agents. The sterilized seeds were then sown in pots. The pots were placed in a growth chamber (28 °C and a 16 h light/8 h dark photoperiod). Soil moisture was maintained by regular watering to ensure optimal germination conditions. Germination was monitored, and seeds were allowed to sprout for 5–7 days until the seedlings reached the 2–3 leaf stage. A total of 20 mg of leaf tissue was then collected from each seedling. The collected tissue was immediately frozen in liquid nitrogen to preserve DNA integrity.

The genomic DNA (gDNA) was extracted using optimized protocols according to a previous study [24]. The quality was determined by running DNA on agarose gel and quality was measured by nanodrop (ND1000, Thermo Scientific, Waltham, MA, USA).

2.5. Genotyping by SSR

Polymerase chain reaction (PCR) was performed using 59 microsatellite markers distributed across all twelve chromosomes. The markers were selected based on their relatedness to salt tolerance in rice (Supplementary Table S3). The PCR was run on ABI thermal cycler (ProFlex PCR) with the following conditions: initial denaturing temperature at 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, specific temperature 55–60 °C for each primer for 30 s, 72 °C for 60 s with a final extension at 72 °C for 10 min. The efficiency of primer pairs in terms of their ability to yield unique and genotype-specific allele(s) was assessed by computing the Polymorphism Percent as PP = [un/tn] × 100, where un and tn represent the number of unique alleles and the total number of alleles detected amongst the varieties, respectively [25] Amplified PCR products were run on 2–3% agarose gel and bands were visualized under UV light. The amplified products were then recorded in the binary data format: 1 for presence and 0 for absence. Each amplified band against each marker is termed an allele. These binary data were further used for assessment of the genetic diversity in the form of allelic variation, which was assessed by calculating the polymorphic information content [26] of the primer pairs as PICi = 1 − ∑j = 1kP2ij, where, k is the total number of alleles detected for a marker, Pij is the frequency of the jth allele for ith marker, and summation extends over k alleles.

2.6. Population Structure

The binary genotypic data and the phenotypic parameters were analyzed for identification of the population structure. These data were then further interrogated for Jaccard's genetic distance matrix using DARwin 6.0.014. Population structure was assessed using (i) model-based and (ii) distance-based approaches [27]. The former approach was addressed using STRUCTURE 2.3.4 software [28]. STRUCTURE Harvester was then used to

extract the results for consecutive interpretation [29]. The run length and Markov Chain Monte Carlo (MCM) length were set to 150,000 and the burning length to 150,000 replications, as described by [27].

Conversely, the distance approach utilized a pairwise distance algorithm to calculate the dissimilarity matrix using DARwin 6 software. The genetic dissimilarity was calculated from the binary genotypic data using the Dice coefficient with 1000 bootstraps. The bootstrap value followed the same recommendation suggested by a previous study [27]. An unweighted neighbor-joining tree was constructed under the same bootstrap conditions to determine the association and hence validate the number of subpopulations in the germplasm. Analysis of Molecular Variance (AMOVA) between the clusters identified by the distance-based approach was analyzed using GenAlEx 6.503 [30]. The populations were set based on the k value obtained, with the number of regions set as 1 and 999 permutations, following the default setting for binary haploid input data.

3. Results

3.1. Germination Parameters

The variation in the phenotypes is a key factor for crop improvement. The phenotypic traits were divided into two groups i.e., germination parameters and growth parameters.

A wide range of diversity was observed across various germination parameters. Final germination percentage is a critical parameter to check the germination rate of seeds. Analysis of FGP data revealed a significant genetic diversity within the rice germplasm studied. Phenotypic variations were visually represented through histograms. FGP ranged from 10 to 100% and peaked in frequency between 90 and 100% (Figure 1a). Notably, Hashemi Tarom and BAS2000 exhibited the lowest FGP values, recorded at 23.33 $\pm$ 11.55% and 30.00 $\pm$ 17.32%, respectively. Furthermore, twelve varieties demonstrated a perfect FGP score of 100%, namely, IR36, Sri Malaysia 2, Manik, MR185, BAS386, Sri8, Ria, MR232, Pulut Siding, Firat1, Jaya Malaysia, and MR263 (Supplementary Figure S2a). Mean germination time (MGT) spanned from 1 to 8 days, with a peak frequency observed between 3 and 4 days (Figure 1b), the Fadjir and Mahsuri varieties exhibited the shortest MGT of 2.12 days. Conversely, Anbir Baraka, BAS2000, MCHKAB, and Hashemi Tarom displayed the longest MGT durations, surpassing 6 days. Notably, sixteen varieties demonstrated above-average MGT scores (Supplementary Figure S2b). The Germination Index (GI) exhibited a wide range of genetic diversity, ranging from 0.1 to 1, with the highest frequency observed between 0.9 and 1 (Figure 1c). Hashemi Tarom and BAS2000 had the lowest average GI scores, recorded at 0.23 $\pm$ 0.12 and 0.30 $\pm$ 0.17, respectively. Conversely, twelve varieties achieved the highest average GI score, reaching the maximum value of 1. These varieties were IR36, Sri Malaysia 2, Manik, MR185, BAS386, Sri8, Ria, MR232, Pulut Siding, Firat1, Jaya Malaysia, and MR263. Moreover, GI values for twenty varieties surpassed the median value (Supplementary Figure S2c). The Germination Energy (GE) ranged from 0 to 100%, with the highest frequency observed between 90 and 100% (Figure 1d). The Hashemi Tarom and BAS2000 varieties showed no GE. Conversely, Manik, Sri8, and Jaya Malaysia exhibited an average GE score of 100% (Supplementary Figure S2d). The Peak Value (PV) ranged from 0 to 3.5, with an average frequency between 2 and 2.5 (Figure 1e). The Kadaria and Fdjir varieties both recorded the highest PV of 3.33 $\pm$ 0.12, while Hashemi Tarom had the lowest PV value of 0.17 $\pm$ 0.1 (Supplementary Figure S2e). The Germination Speed (GS) ranged from 0 to 7 seeds/day, with the highest frequency observed between 2 and 3 seeds/day (Figure 1f). Sri Malaysia 1 exhibited the highest GS, with a count of $\geq$5.67 seeds per day. Conversely, Hashemi Tarom (0.38 $\pm$ 0.16), BAS2000 (0.48 $\pm$ 0.25), and Shiroodi (0.72 $\pm$ 0.52) recorded among the lowest GS scores (Supplementary Figure S2f). The Germination Rate (GR) ranged from 0 to 7% per day, with the highest frequency

observed between 6 and 7% per day (Figure 1g). Shiroodi, MR127, and MCHKAB recorded the lowest GR at 3.56% per day. Meanwhile, seven varieties achieved the highest GR at 6.67% per day, namely, BAS385, Sri8, Pulut Siding, Pokkali, Jaya Malaysia, Hashemi Tarom, and Bahagia (Supplementary Figure S2g). The Germination Capacity (GC) ranged from 10 to 100%, with the highest frequency observed between 90 and 100% (Figure 1h). Hashemi Tarom and BAS2000 documented among the lowest GC scores of 20 ± 10 and 23.33 ± 15.28%, respectively. IR 36 showed the highest GC scores of 90 ± 10. All the data were validated by the normality test (p-value < AD-value) (Table 1). Based on these results, the null hypothesis, if the data follows a normal distribution, is rejected. So it was concluded that there is a wide range of genetic diversity present in the studied rice germplasm. All these parameters were statistically tested, and the results showed that germination parameters (FGP, MGT, GI, GE, GS, GR, and GC) were found to be statistically significant at $\alpha = 0.1\%$ (Table 2). However, there are some variations observed and outliers for FGP, GI, GS, GR, and GC were identified and illustrated by a boxplot (Supplementary Figure S3). Based on the above results, it was concluded that compared with the control variety BAS2000, Hashemi Tarom expressed weak-tolerant phenotypes (high MGT; low GI, GS, and FGP; nil GE). While the other varieties i.e., IR36, Sri Malaysia 2, Manik, MR185, BAS386, Sri8, Ria, MR232, Pulut Siding, and Jaya Malaysia, on average performed well in all germination parameters and hence can be considered salt-tolerant varieties in the germination stage.

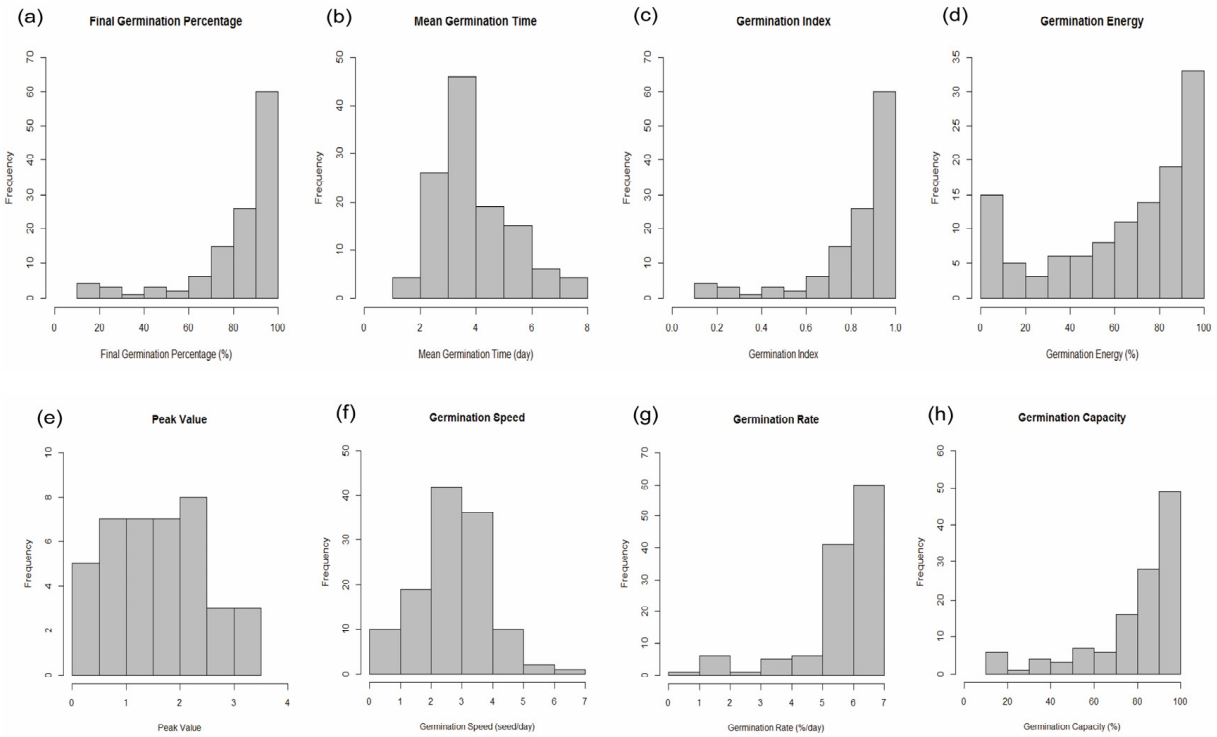

Figure 1. Histograms generated using R, representing the distribution of 40 varieties for eight germination parameters. These parameters are: (**a**) Final Germination percentage (FGP), which ranged from 10–100% and peaked at 90–100%; (**b**) mean germination time (MGT), which ranged from 2–8 days and peaked at 3–4 days; (**c**) germination index (GI), which ranged from 0.1–1 and peaked at 0.9–1; (**d**) germination energy (GE), which ranged from 0–100% and peaked at 90–100%; (**e**) peak value (PV), which ranged from 0–3.5 and peaked at 2–2.5; (**f**) germination speed (GS), which ranged for 0–7 and peaked at 2–3 seed/day; (**g**) germination rate (GR), which ranged from 0–7 and peaked at 6–7%; and (**h**) germination capacity (GC), which ranged from 0–100% and peaked at 90–100%. n = 120 for all parameters excluding PV where n = 40.

Table 2. Analysis of variance (ANOVA) results for germination parameters of forty rice varieties.

	FGP	MGT	GI	GE	PV	GS	GR	GC
Mean	86.92	3.95	0.87	68.08	1.61	2.73	5.80	83.17
Median	95.00	3.60	0.95	80.00	1.67	2.85	6.34	90.00
				Anderson-Darling Normality Test				
AD-value	13.8920	2.1206	13.8920	6.4223	0.3258	0.7669	13.8550	10.6010
p-value	$<2.20 \times 10^{-16}$	2.03×10^{-5}	$<2.20 \times 10^{-16}$	7.48×10^{-16}	0.5110	0.0450	$<2.20 \times 10^{-16}$	$<2.20 \times 10^{-16}$
				ANOVA				
				Varieties (DF = 39)				
SS	42492	198.44	4.249	122126	30.3100	152.40	108.2000	52397
MS	1089.6	5.088	0.10896	3131.4	0.7773	3.908	2.7740	1344
F-value	11.9900	24.3	11.99	32.39	n/a	42.03	1.9570	13.44
p-value	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	n/a	$<2 \times 10^{-16}$ ***	0.0058 **	$<2 \times 10^{-16}$ ***
				Error (DF = 80)				
SS	7267	16.75	0.727	7733	n/a	7.44	113.4000	8000
MS	90.8	0.209	0.00908	96.7	n/a	0.093	1.4170	100

DF = Degree of Freedom, SS = Sums of Square, MS = Means of Square. n/a= not applicable, ** and *** means significant and highly significant, respectively, at $\alpha = 0.001$.

3.2. Growth Parameters

A wide range of diversity was observed in growth parameters. The trait-wise phenotypic variation was depicted by a histogram (Figure 2). Total Length (TL) ranged from 1 to 10 cm and peaked in frequency between 5 and 6 cm (Figure 2a). The MR219 and MR211 varieties were among those with the highest average TL at 8.40 ± 1.39 and 8.39 ± 0.62 cm, respectively. BAS2000 demonstrated the shortest average TL of 1.26 ± 0.34 cm (Supplementary Figure S4a). Shoot Length (SL) ranged from 1 to 6.5 cm, with the highest frequency observed between 4.5 and 5 cm (Figure 2b). The MR232 variety had the highest average SL of 6.13 ± 0.12 cm. Conversely, the BAS2000 and MCHKAB varieties demonstrated the shortest average SL at 1.26 ± 0.34 and 1.27 ± 0.30 cm, respectively (Supplementary Figure S4b). Root Length (RL) ranged from 0 to 3 cm, with the highest frequency observed between 0.5 and 1 cm (Figure 2c). The MR211 variety had the highest average RL of 3.32 ± 0.58 cm. However, BAS2000 showed nil RL and 4365 and Pulut Siding demonstrated the shortest average RL at 0.08 ± 0.02 and 0.18 ± 0.12 cm, respectively (Supplementary Figure S4c). The Vigor Index (VI) ranged from 0 to 1000, with the highest frequency observed between 500 and 600 (Figure 2d). The MR219 variety had the highest average VI of 812.03 ± 145.07. Conversely, BAS2000 demonstrated the lowest average VI of 36.00 ± 16.70 (Supplementary Figure S4d). Shoot Fresh Weight (SFW) ranged from 0 to 35 mg, with the highest frequency observed between 15 and 20 mg (Figure 2e). Pokkali and MR219 showed the highest average SFW of 28.55 ± 3.62 and 27.33 ± 1.79 mg, respectively. Conversely, MCHKAB and BAS2000 had the lowest average SFW of 3.52 ± 0.27 mg and 5.11 ± 0.67 mg respectively (Supplementary Figure S4e). Root Fresh Weight (RFW) ranged from 0 to 25 mg, with the highest frequency observed between 0 and 5 mg (Figure 2f). MR219 recorded the highest average RFW score of 22.36 ± 1.40 mg while, five varieties (BAS385, Pulut Siding, 4365, and BAS2000) recorded a nil RFW value (Supplementary Figure S4f). Shoot Dry Weight (SDW) ranged from 0.5 to 6 mg, with the highest frequency observed between 4 and 5 mg (Figure 2g). The MR220, MR219, and IR126 varieties showed the highest average SDW at 5.40 ± 0.23, 5.34 ± 0.29, and 5.18 ± 0.23 mg, respectively. Conversely, BAS2000 and MCHKAB measured the lowest average SDW at 0.74 ± 0.17 and 0.79 ± 0.16 mg, respectively (Supplementary Figure S4g). Root Dry Weight (RDW) ranged from 0 to 2.5 mg, with the highest frequency observed between 1 and 1.5 mg (Figure 2h). The MR219 variety had the highest average RDW of 3.06 ± 0.80 mg. Similarly, as described previously for RFW, the same five varieties (BAS385, Pulut Siding, 4365, and BAS2000) recorded a nil RDW value (Supplementary Figure S4h). Although some outliers were also observed which can be seen in (Supplementary Figure S5).

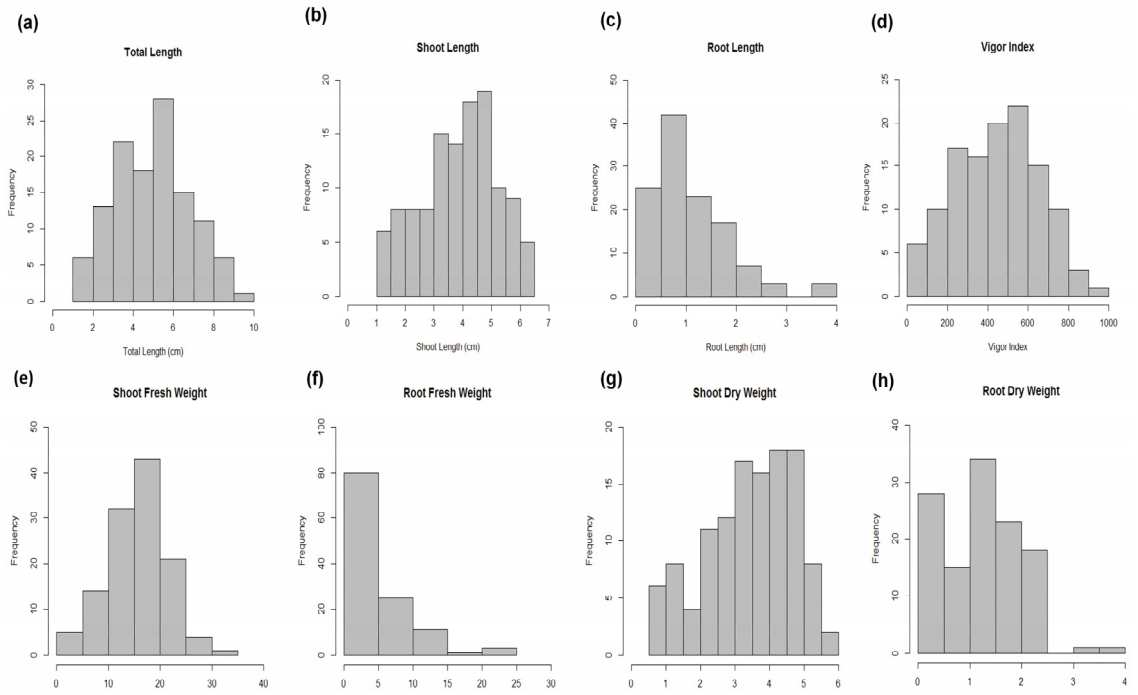

Figure 2. Histograms generated using R, representing the distribution of 4 varieties for eight growth parameters. These parameters are: (**a**) Total length (TL), which ranged from 1–10 cm and peaked at 5–6 cm; (**b**) shoot length (SL), which ranged from 1–6.5 cm and peaked at 4–5 cm; (**c**) root length (RL), which ranged from 0–3 cm and peaked at 0.5–1 cm; (**d**) vigor index (VI), which ranged from 0–1000 and peaked at 500–600; (**e**) shoot fresh weight (SFW), which ranged from 0–35 mg and peaked at 15–20 mg; (**f**) root fresh weight (RFW), which ranged from 0–25 mg and peaked at 0–5 mg; (**g**) shoot dry weight (SDW), which ranged from 0.5–6 mg and peaked at 4–5 mg; and (**h**) root dry weight (RDW), which ranged from 0–2.5 mg and peaked at 1–1.5 mg.

All growth parameters (TL, SL, RL, VI, SFW, RFW, SDW, and RDW) exhibited statistically significant variation at α = 0.1% (Table 3). It was concluded from the above results that the MCHKAB variety was severely affected by salt stress and hence, was categorized as a salt-susceptible variety. Conversely, MR varieties, namely, MR219, MR263, and MR211, performed better in all growth parameters and hence, were categorized as salt-tolerant varieties.

Table 3. Analysis of variance (ANOVA) results for growth parameters of forty rice varieties.

	TL	SL	RL	VI	SFW	RFW	SDW	RDW
Mean	4.96	3.88	1.07	444.29	15.80	4.63	3.45	1.19
Median	5.02	4.04	0.92	453.60	16.36	3.35	3.68	1.24
Anderson-Darling Normality Test								
AD-value	0.2618	0.6469	2.2196	0.2902	0.5547	4.7936	1.2093	1.8591
p-value	0.6997	0.0893	1.16×10^{-5}	0.6061	0.1494	6.15×10^{-12}	0.0036	8.93×10^{-5}
ANOVA								
Varieties (DF = 39)								
SS	375.4481	180.2389	58.25	4,633,768	3385.1960	2470.3946	175.1349	62.2502
MS	9.6270	4.6220	1.49	118,815	86.8000	53.3400	4.4910	1.5962
F-value	15.81	13.71	15.25	15.36	36.06	43.58	20.65	8.37
p-value	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	$<2 \times 10^{-16}$ ***	8.93×10^{-16} ***
Error (DF = 80)								
SS	48.7166	26.9623	7.8339	618,981	192.5450	116.2765	17.3964	15.2516
MS	0.6090	0.3370	0.0979	7737	2.4100	1.4500	0.2170	0.1906
RSE	0.7804	0.5805	0.3129	87.9617	1.5514	1.2056	0.4663	0.4366

DF = Degree of Freedom, SS = Sums of Square, MS = Means of Square, RSE = Residual Standard Error, *** means significant and highly significant, respectively, at α = 0.01.

Additionally, clustering analysis based on normalized phenotypic traits suggested significant variation between three subgroups. However, in this clustering, the weak and highly tolerant varieties grouping was significantly smaller (Figure 3, Table 4). Shiroodi, Hashemi Tarom, and BAS2000 were clustered together, and presented as weak tolerant varieties. MR211 and MR219 are two Malaysian varieties that were clustered as highly tolerant traits. The remaining 87.5% of varieties clustered into moderately tolerant varieties.

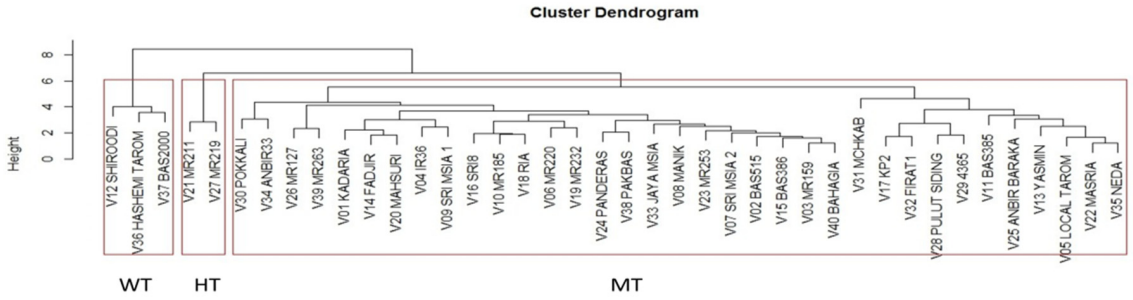

Figure 3. Cluster dendrogram based on normalized phenotypic traits. Based on phenotypic traits, the germplasm was divided into 3 groups viz. weakly tolerant (WT), which contained three varieties, highly tolerant (HT), which contained two varieties, and moderately tolerant (MT), which contained 35 varieties.

Table 4. Normalized aggregate for different phenotypic parameters regarding their salt tolerance.

Germination Parameters								
Group	**FGP**	**GR**	**MGT**	**GI**	**GE**	**GS**	**GC**	**PV**
WT	−2.93	−0.40	1.73	−2.93	−2.07	−1.93	−2.72	−1.48
MT	0.23	0.01	−0.10	0.23	0.13	0.13	0.20	0.10
HT	0.42	0.44	−0.75	0.42	0.83	0.54	0.56	0.50
Growth Parameters								
Group	**TL**	**SL**	**RL**	**SFW**	**RFW**	**SDW**	**RDW**	**VI**
WT	−1.21	−1.09	−1.16	−1.37	−0.79	−1.28	−1.07	−1.78
MT	−0.01	0.02	−0.06	0.03	−0.10	0.05	−0.03	0.05
HT	1.93	1.21	2.76	1.54	3.00	1.01	2.14	1.77

Note: WT: weakly tolerant; MT: moderately tolerant; HT: highly tolerant.

3.3. Genotyping Analysis

These genotyping markers collectively find 287 alleles. The number of alleles ranged from two to nine, among which, RM403, RM559, RM343, RM596, and RM479 each had the lowest number of alleles (two), while RM481 and RM21 both shared the highest number of alleles (nine). The number of alleles averaged at 4.86 per locus (Table 3). Fifty markers were assigned as highly polymorphic (PIC > 0.50), seven as moderately polymorphic (PIC < 0.50) and two markers were not categorized as no PIC value was calculated for them. RM21 had the highest PIC value i.e., 0.88, while RM462 had the lowest PIC value i.e., 4.0 (Table 5). The average PIC value is 0.69.

Table 5. Genotyping analysis across twelve chromosomes using 59 microsatellite markers.

#	Marker	Chr	Repeat Motif	Number of Alleles	PIC
1.	RM462	1	(GA)12	3	0.40
2.	RM283	1	(GA)18	3	0.61
3.	RM493	1	(CTT)9	6	0.72

Table 5. *Cont.*

#	Marker	Chr	Repeat Motif	Number of Alleles	PIC
4.	RM9	1	(GA)15GT(GA)2	8	0.83
5.	RM488	1	(GA)17	8	0.84
6.	RM403	1	(GA)8	2	0.50
7.	RM279	2	(GA)16	6	0.73
8.	RM561	2	(GA)11	4	0.70
9.	RM526	2	(TAAT)5	4	0.63
10.	RM327	2	(CAT)11(CTT)5	3	0.64
11.	RM475	2	(TATC)8	6	0.77
12.	RM423	2	(TTC)9	4	0.70
13.	RM231	3	(CT)16	5	0.70
14.	RM489	3	(ATA)8	4	0.60
15.	RM517	3	(CT)15	5	0.76
16.	RM7	3	(GA)19	5	0.75
17.	RM563	3	(CCT)6	3	0.66
18.	RM261	4	C9(CT)8	5	0.70
19.	RM317	4	(GC)4(GT)18	4	0.60
20.	RM471	4	(GA)12	5	0.75
21.	RM559	4	(AACA)6	2	0.50
22.	RM267	5	(GA)21	6	0.77
23.	RM574	5	(GA)11	5	0.63
24.	RM430	5	(GA)25	6	0.72
25.	RM161	5	(AG)20	6	0.64
26.	RM343	6	(CAT)5(CAC)5CAT(CAC)4	2	0.50
27.	RM539	6	(TAT)21	6	0.87
28.	RM527	6	(GA)17	5	0.72
29.	RM540	6	(AG)16	5	0.72
30.	RM454	6	(GCT)8	4	0.67
31.	RM217	6	(CT)20	8	0.83
32.	RM481	7	(CAA)12	9	0.86
33.	RM500	7	(AAG)9	5	0.70
34.	RM560	7	(CT)12	4	0.66
35.	RM505	7	(CT)12	3	0.54
36.	RM18	7	(GA)4AA(GA)(AG)16	4	0.68
37.	RM502	8	(TG)10	4	0.66
38.	RM404	8	(GA)33	5	0.72
39.	RM433	8	(AG)13	5	0.50
40.	RM337	8	(CTT)4-19-(CTT)8	3	0.66
41.	RM38	8	(GA)16	7	0.73
42.	RM316	9	(GT)8-(TG)9(TTTG)4(TG)4	5	0.79
43.	RM105	9	(CCT)6	5	0.76
44.	RM566	9	(AG)15	8	0.85
45.	RM242	9	(CT)26	7	0.81
46.	RM201	9	(CT)17	4	0.65
47.	RM333	10	(TAT)19(CTT)19	8	0.83
48.	RM269	10	(GA)17	5	0.64
49.	RM474	10	(AT)13	5	0.78
50.	RM596	10	(GAC)10	2	0.47
51.	RM20B	11	(ATT)n	3	N/A
52.	RM332	11	(CTT)5-12-(CTT)14	5	0.65
53.	RM479	11	(TC)9	2	0.50
54.	RM209	11	(CT)18	7	0.77
55.	RM21	11	(GA)18	9	0.88
56.	RM19	12	(ATC)10	6	N/A
57.	RM117	12	(AG)7	3	0.70
58.	RM463	12	(TTAT)5	3	0.60
59.	RM512	12	(TTTA)5	3	0.57

Chromosome (Chr): represents the position in which the respective marker is mapped on the chromosomes.

3.4. Population Structure Analysis

The model-based analysis using STRUCTURE HARVESTER successfully interrogated the dataset using the evano approach, benefiting from the presence of 10 replicate sets ($\geq$3), which allowed for the analysis of k. The result indicated that the number of k groups best fitting the dataset was three, as the maximum Δk was observed at 33.64. The k versus Δk graph illustrated the presence of three groups in the rice germplasm (Figure 4a). Furthermore, the presence of these groups was confirmed by a triangle cluster diagram

(Figure 4b). Additionally, the QQ plot showed clear indications of three groups represented by different colors (Figure 4). These groups represented moderately tolerant (Group A), highly tolerant (Group B), and weakly tolerant (Group C) subpopulations.

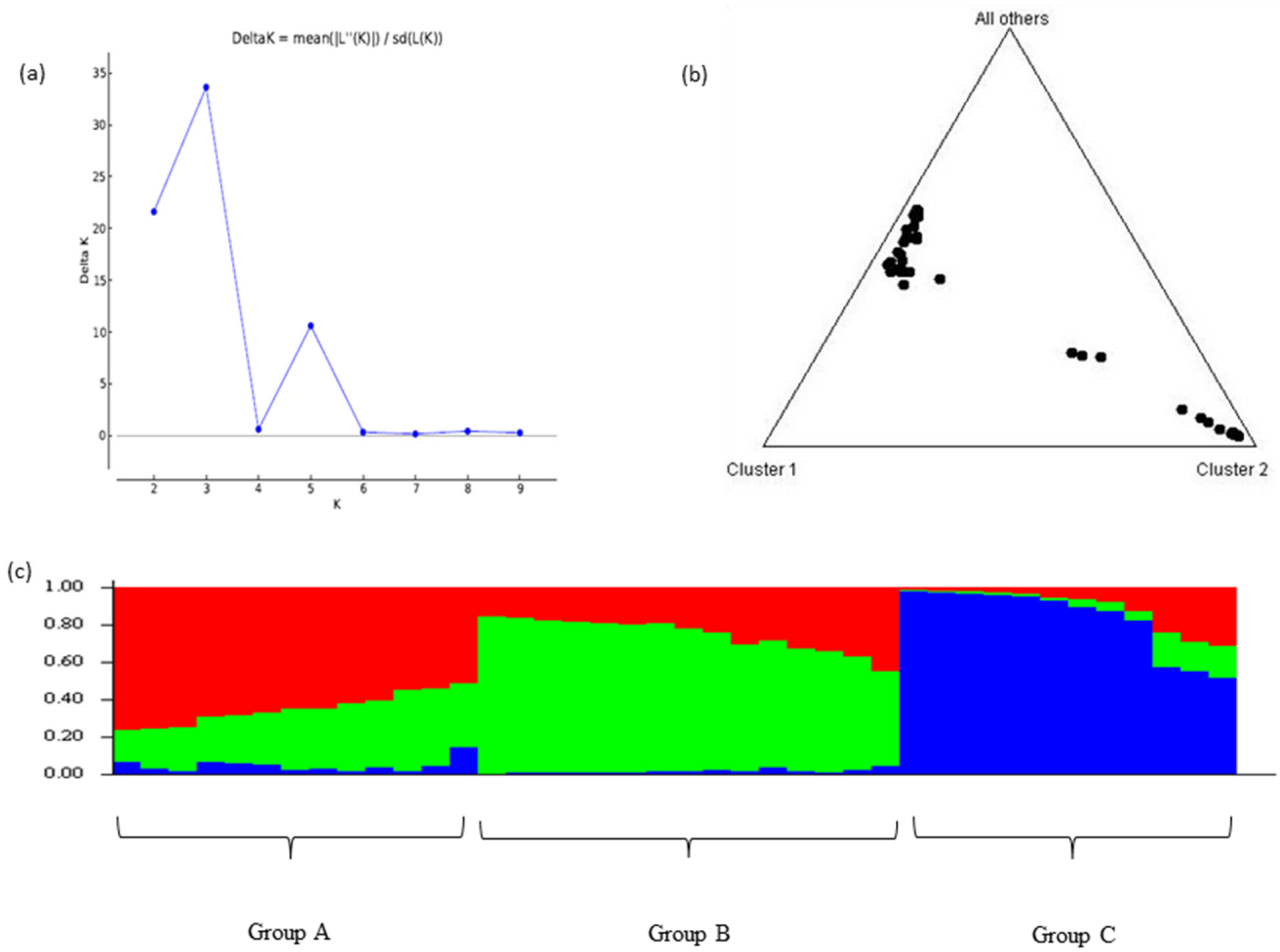

Figure 4. Depiction of genetic diversity analysis using a model-based approach. (**a**) Triangle plot generated by STRUCTURE showing the presence of three subpopulations, i.e., k = 3. (**b**) The ΔK against k (1 to 9) plot showing a clear peak at 3 which showed the presence of three subgroups within the studied rice germplasm. (**c**) QQ matrix plot showing clear convergence into 3 groups. Group A represents the moderately tolerant varieties, Group B represents the highly tolerant varieties, and Group C represents the weakly tolerant varieties.

Comparatively, the dendrogram generated by DARwin using the distance-based approach established a similar conclusion regarding the population structure among the varieties (Figure 5). Clear sub-clades were present, with increasing dissimilarity between them. Therefore, similar to the findings of a previous study [27], both population structure approaches and phenotypic clustering demonstrated similarities between the genotype clustering. The dendrograms labeled as Group I represented weakly tolerant, Group II represented highly tolerant, and Group III represented moderately tolerant varieties.

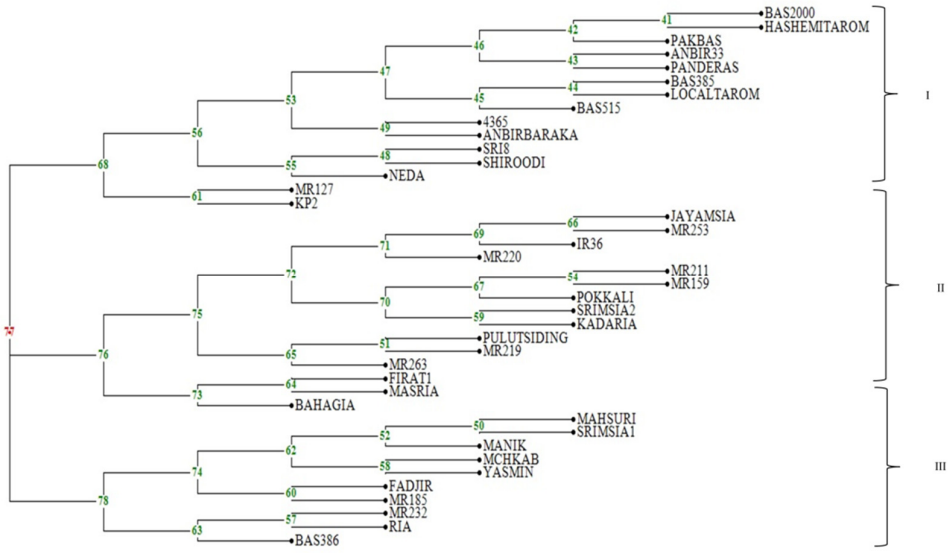

Figure 5. Depiction of clustering using the distance-based approach. The dendrogram shows the presence of three groups and the distribution of varieties in each group in the studied rice germplasm. Group I represents the weakly tolerant varieties, corresponding to Group C in Figure 4; Group II represents the highly tolerant varieties and corresponds to Group B in Figure 4; and Group III represents moderately tolerant varieties and corresponds to Group A in Figure 4.

3.5. Genetic Diversity

An AMOVA test suggested that there was 95% genetic diversity within the population, which implies significant genetic variation between each grouped subpopulation with salinity-tolerant characteristics (Figure 6). The variation between all forty varieties was significantly smaller (5%) given a relatively high PIC average and wide geographical origin of the cultivars tested but this can be accounted for by the large variation between those exhibiting similar salinity tolerances.

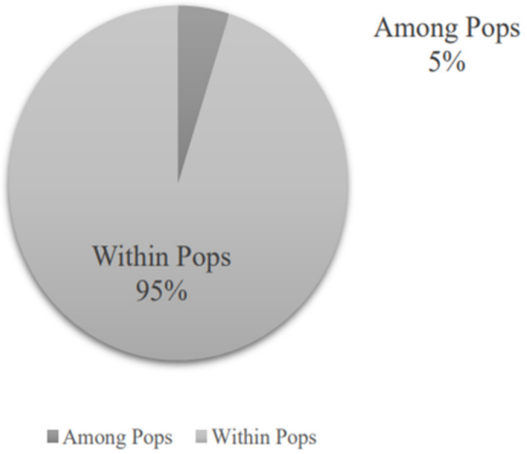

Figure 6. Analysis of molecular variance (AMOVA) results generated by GenAlEx. The results indicate a high variance (95%) within each population and a small variance (5%) among populations.

4. Discussion

Genetic variation is crucial for species to withstand and adapt to adverse environmental conditions [13,31,32]. The greater the genetic diversity, the greater the phenotypic variation in the particular trait [13,33]. Furthermore, Genetic diversity is also the foundation for heterosis and a crucial element in the breeding of high-yielding hybrid rice varieties [34]. So, there is always a dire need to explore the genetic diversity of crop germplasm. There are limited studies available for screening rice germplasm for salt tolerance at the germination

stage. In this study, we conducted a comprehensive assessment of the phenotypic and genetic diversity of rice germplasm under salinity stress conditions, for germination and growth parameters. Based on germination parameter analysis, it was found that compared with susceptible control (BAS2000), Hashemi Tarom showed weak-tolerant phenotypes, as they showed high MGT, low GI, GS, and FGP, and nil GE. While the other varieties, i.e., IR36, Sri Malaysia 2, Manik, MR185, BAS386, Sri8, Ria, MR232, Pulut Siding, and Jaya Malaysia, on average performed well in all germination parameters. Hence, the latter were considered the salt-tolerant genotypes at the germination stage. However, MR varieties, including the MR219 control and MR263 and MR211, performed better in all growth parameters suggesting that these varieties showed salt tolerance characteristics. Overall, results showed that MR219 and MR211 exhibit the greatest potential to be salt-tolerant varieties suitable for a direct seeding approach. Conversely, BAS2000, Hashemi Tarom, and Shiroodi were found to be salt-susceptible, indicating their unsuitability for direct seeding in saline conditions. BAS2000 and Hashemi Tarom are known to be susceptible to salinity. The study revealed that Shiroodi also exhibits high susceptibility to salt tolerance at the germination stage. Additionally, based on growth parameters compared with a susceptible control, MCHKAB was found moderately susceptible to salt tolerance. However, MR219 exhibited high TL, SL, RL, VI, SFW, RFW, SDW, and RDW, thus suggesting high salt-tolerance characteristics. In comparison with a tolerant control, MR219, the other MR varieties, MR263 and MR211, consistently performed better in germination and growth-related parameters. Thus, these varieties can be used as promising parental lines for future breeding programs aimed at developing salt-tolerant varieties suitable for direct seeding approaches.

Molecular markers, particularly SSRs and SNPs, have emerged as powerful tools for elucidating genetic diversity in various crops, including rice [33,35–37]. Compared with other markers, SSR markers have remarkable potential for discriminating against rice genotypes [38–41]. In this study, we employed 59 polymorphic SSR markers to assess the genetic diversity of rice germplasm under salinity stress conditions. Our findings revealed a substantial degree of allelic variation, with a total of 287 alleles detected across the genotyped markers. Notably, the average number of alleles per locus (4.86) indicated a favorable level of genetic diversity within the studied rice germplasm. The Polymorphic Information Content (PIC) is a key factor in assessing the allelic diversity and in our study, it ranged from 0.5 to 0.85. Markers with PIC values exceeding 0.7 are considered highly informative and valuable for expanding the genetic base of crop germplasm [33]. In our dataset, 50% of the markers exhibited PIC values exceeding 0.7. This might suggest their potential utility in future breeding efforts aimed at identifying salt-tolerant rice varieties for direct seeding methods. Furthermore, SSR markers with PIC values equal to or greater than 0.5 are regarded as effective tools for evaluating genetic divergence and polymorphism rates [33,42]. Our study demonstrated that over 90% of the SSR markers possessed PIC values of 0.5 or higher, with an average PIC value of 0.69. This indicates ample genetic diversity within the rice germplasm, providing promising prospects for breeding programs focused on selecting parental lines with enhanced salt tolerance for direct seeding methods. These findings corroborate previous studies that have highlighted the importance of genetic diversity in enhancing crop resilience to environmental stresses [35,37,43,44]. Our results highlighted the potential of utilizing SSR markers with high PIC values to expedite the development of salt-tolerant rice varieties tailored for direct seeding practices, thereby contributing to sustainable agricultural production in saline-affected regions.

In the study, the marker RM493 was identified to be closely linked with the *Saltol* QTL located on chromosome 1 [45]. The *Saltol* QTL plays a crucial role in maintaining Na^+/K^+ homeostasis within the cell, particularly during the seedling stage. Previous research revealed that *Saltol* QTL possesses five alleles with a PIC value of 0.69 [45]. Our study

corroborates these findings, with marker RM493 exhibiting six alleles and a slightly higher PIC value of 0.72. These results highlight the importance of markers with higher PIC values and a greater number of alleles, as they exhibit enhanced diversity and discriminatory power in distinguishing between salt-tolerant and salt-susceptible genotypes. Furthermore, recent studies have shed light on the significance of molecular markers in elucidating genetic diversity and identifying loci associated with salt tolerance in rice. Several studies demonstrated the utility of SNP and SSR markers in pinpointing genomic regions linked to salt-tolerance traits [36,37,46]. Additionally, the authors of one study conducted a comprehensive analysis of SSR markers in rice, highlighting their role in assessing genetic diversity and population structure. Remarkably, in our investigation, marker RM21 displayed the highest number of alleles (nine) and the highest PIC value (0.88), followed closely by RM481 (nine alleles, PIC value of 0.86). Similarly, markers RM566, RM488, RM9, RM217, RM333, RM242, RM209, RM38, RM539, RM475, RM267, RM279, and RM430 exhibited high levels of polymorphism, with PIC values exceeding 0.7 and containing six or more alleles (Table 3). These markers hold significant importance for future breeding programs aimed at selecting salt-tolerant genotypes for direct seeding methods. Overall, the identification of highly polymorphic markers with substantial allelic diversity underscores their potential utility in enhancing the efficiency and precision of breeding efforts. Therefore, these markers pave the way for developing salt-tolerant rice varieties tailored for direct seeding approaches.

A normalized aggregate between the 40 varieties based on germination and growth parameters categorizes the germplasm into three subpopulations (Figure 3). The results showed that MR211 and MR219 were highly salt tolerant and can be used for the direct seeding method. The Darwin-based dendrogram (based on genotypic analysis) showed similar results but interestingly, showed more varieties having highly tolerant genetic characteristics. In total, 15 varieties showed high tolerance genotypic characteristics (Figure 5). The clustering pattern in the present study indicated the existence of variability among rice genotypes. There were sufficient variations between the varieties for the development of newly improved crosses sharing similar salinity tolerance criteria. Furthermore, population structure analysis confirmed the clustering of the sampled genotypes in a similar group, suggesting that enough diversity is present for the selection of parents for direct seeding methods. Future studies are required to test these genotypes in soil or field conditions for direct seeding methods. Although significant variation in studied rice germplasm revealed that these varieties have the potential to germinate under saline conditions. Crosses of unrelated parents can be practiced to enlarge the genetic diversity to develop breeding populations; however, AMOVA revealed highly significant genetic differences ($p \leq 0.001$) among the populations, among individuals (95%), and within individuals (5%) (Figure 6). Variations with a similar pattern have been reported in previous studies on rice germplasm [47,48]. AMOVA results suggest that a small collection within a given source will capture the genetic diversity present in the test genotypes. The presence of variability within and between the populations suggests the possibility of making wide crosses for population development and enhancing genetic divergence in rice.

5. Conclusions

Salinity stress has a significant impact on crop yields. This study screened germplasm for salinity tolerance during germination, which might be suitable for direct seeding methods. Based on phenotypic and genotypic clustering, MR211 and MR219 may hold promise for direct seeding methods, although field trials are needed to confirm their suitability for direct seeding under natural conditions. A significant variation was revealed based on genotypic analysis. Fifty markers, particularly RM21, RM481, RM566, and RM9, exhibited strong polymorphism (PIC > 0.7), providing useful tools for the genetic

characterization of rice germplasm under salinity stress. Population structure analysis identified three subpopulations, indicating sufficient genotypic diversity for developing new salt-tolerant varieties. The AMOVA test showed 95% genetic diversity within the studied population, indicating a high level of intra-population variation. This substantial genetic variation highlights the potential for selecting diverse parental lines for future breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15020376/s1, Table S1: Formula used to measure the germination and growth parameters. Table S2: List of primers used in the study. Figure S1: Measurement of germination and growth parameters under saline conditions. Figure S2: Graph of the average value for seven germination parameters: Final Germination percentage (FGP) it ranges from 10–100%. Mean germination time (MGT) ranged from 2–8 days. Germination index (GI) ranged from 0.1–1. Germination energy (GE) ranged from 0–100% peak value (PV) ranged from 0–3.5. Germination speed (GS) ranged from 0–7 days. Germination rate (GR) ranged from 0–7 %. Germination capacity (GC) ranged from 0–100%. The error bar represents the standard deviation between the triplicates. Figure S3: Boxplot generated using R for different germination parameters (a) Final germination percentage (FGP). There were 8 outliners (b) germination index (GI). There were 11 outliers (c) germination speed (GS). There were 3 outliers (d) germination rate (GR). There were 8 outliers (e) germination capacity (GC). There were 11 outliers. Figure S4: Graph of the average value for all eight growth parameters. These parameters are: (a) TL ranged from 1–10 cm (b) SL ranged form 1–6.5 cm (c) RL) ranged from 0–3 cm (d) VI ranged from 0–1000 (e) SFW ranged from 0–35 mg (f) RFW ranged from 0–25 mg (g) SDW ranged from 0.5–6 mg and (h) RDW ranged form 0–2.5 mg. The error bar represents the standard deviation between the triplicates. Figure S5: Boxplot generated using R for different growth parameters (a) Root length (RL). There were 2 outliers, (b) shoot fresh weight (SFW). There was 1 outlier, (c) root fresh weight (RFW). There were 4 outliers and (d) root dry weight (RDW). There was 1 outlier.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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