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Research Article
Assessment of Genetic Diversity in Arsenic Contaminated Rice Using SSR Markers

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Abstract

Background and Objective: Arsenic phyto-toxicity tolerance of rice is largely controlled by rice genetics. The objective of present study was to evaluate the genetic variation and diversity of arsenic contaminated rice genotypes. **Materials and Methods:** A total of 295 SSR markers were used to study 15 arsenic contaminated rice genotypes. The molecular weights for each amplified allele were measured by using Alpha Ease. Summary statistics were determined by using Power Marker and the NTSYS-pc was used for cluster analysis. **Results:** Identified 191 polymorphic SSR markers detected 552 alleles among the tested genotypes of which 322 were common and the number of rare alleles was 230. Among the rare alleles, 141 were unique (genotype-specific) those could be used for varietal characterization. Polymorphism information content values ranged from 0.12-0.80, with an average of 0.37. Cluster analysis showed distinct two groups of tested genotypes. Cluster-I was constituted by the *japonica* subspecies US varieties 'Jefferson' and 'Priscilla' which were significantly different from all other genotypes. Cluster-II was comprised of *indica* subspecies, which were grouped into two different sub-clusters. **Conclusion:** It was concluded that high number of rare and unique alleles in the studied genotypes indicated their potentiality to use as a reservoir of rare genotypes.

Key words: Arsenic, jefferson, genetic diversity, priscilla, arsenic phyto-toxicity, rice genetics

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Arsenic contamination in groundwater has been reported from over 70 countries and its situation is the worst in Bangladesh and west Bengal in India[1,2]. In Bangladesh, arsenic concentration in groundwater has exceeded the safe level (0.05 mg L^{-1} is the Bangladesh standard) in 59 districts out of 64 and about 80 million people are exposed to arsenic poisoning[3]. This is mainly due to the fact that arsenic is accumulating in rice grown in arsenic contaminated soil or irrigating by contaminated water. Compared with other cereals (wheat, barley and maize) rice accumulates much higher levels of arsenic in the shoots and grain[4-7]. Arsenic contamination in rice is a major problem in south and southeast Asia[8,9]. The selection of rice cultivars that accumulate lower concentration of As in the grain and show high tolerance against arsenic phyto-toxicity, is an effective approach for reducing the As contamination in rice and also ensure safe crop production[10-12]. Large variations in grain arsenic concentration of Bangladeshi rice varieties sampled from rice fields have been observed[13,14]. A recent study indicated that variation in rice grain arsenic concentration in Bangladesh was largely controlled by rice genetics[15,16]. Moreover, knowledge regarding the amount of genetic variation in germplasm and genetic relationships between genotypes are required for the efficient breeding of commercial cultivars possessing low arsenic accumulation and good agronomic traits with high tolerance to arsenic photo-toxicity.

In the past, the characterization of germplasm diversity was carried out by morphological and biochemical markers which in many cases, did not have the resolution power for revealing polymorphisms in genetic analyses and/or for differentiating between closely related genotypes. Molecular marker technologies can assist conventional breeding efforts and are valuable tools for the analysis of genetic relatedness and the identification and selection of desirable genotypes for crosses as well as for germplasm conservation in gene banks[17]. Among different types of DNA markers, simple sequence repeats (SSRs) are co-dominant, hyper-variable, abundant and highly reproducible, exhibit a high degree of allelic variation and well distributed throughout the rice genome[18-20] and have been exclusively in rice for characterization of the genetic structure of the cultivated rice *O. sativa* at both the inter and intra-varietal level, genetic diversity and evolutionary analyses of landraces, weedy and wild rice germplasm[21], determination of the purity of breeding material or seed stocks[22], prediction of hybrid performance and heterosis and the analyses and tagging of valuable quantitative trait loci (QTL) and genes[23,24]. Studies showed that SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes. Cultivars collected from BRRI, Bangladesh was not examined previously in terms of genetic relatedness using high number of SSR molecular markers covering all the 12 chromosomes of rice genome. Hence, the present study had been undertaken to meet the following objectives: (1) To evaluate the genetic variation and diversity of 15 arsenic contaminated rice genotypes using SSR technique, (2) To suggest informative markers for marker assisted selection, (3) To determine the genetic relationship among these genotypes and (4) To characterize these rice genotypes.

MATERIALS AND METHODS

The experiment was conducted at Marker Assisted Selection (MAS) laboratory of Plant Breeding Division, Bangladesh Rice Research Institute (BRRI), Gazipur-1701, Bangladesh. The duration of this research work was 2 years, started from December, 2015 and ended at October, 2017.

Plant materials and markers: A total of 15 rice genotypes were used in the study (Table 1) collected from BRRI and abroad (IRRI Philippines, Cornell University, USA and China). All the genotypes were tested in hydroponic condition against sodium arsenate for arsenic phyto-toxicity tolerance. Some genotypes were identified as arsenic sensitive and some of them were arsenic tolerant. A total of 295 SSR markers were used in this study those were obtained from Gramene (http://www.gramene.org/). Markers were selected based on their location to cover all the chromosomes uniformly maintaining more or less similar distance in base pair (bp).

Isolation of genomic DNA: Genomic DNA was isolated from young leaves of 30 days old seedlings following modified Miniscale method[8]. The quality and quantity of the isolated concentrated DNA was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) using module Nucleic Acid, Software: 3.3.1.

SSR analysis: The PCR was performed[8] in a solution of 10 µL containing 30 ng of template DNA, 0.5 µM of each forward and reverse primer, 3 mM $MgCl_2$, 0.2 mM of dNTP mix, 1X PCR buffer and 1 unit of *Taq* DNA polymerase

Table 1: List of rice genotypes used in the study

Genotypes name	Subspecies	Country of origin/State from where collected
BRRI dhan28	*Indica*	BRRI, Bangladesh
BRRI dhan29	*Indica*	BRRI, Bangladesh
BRRI dhan33	*Indica*	BRRI, Bangladesh
BRRI dhan45	*Indica*	BRRI, Bangladesh
BRRI dhan47	*Indica*	BRRI, Bangladesh
BRRI dhan49	*Indica*	BRRI, Bangladesh
BRRI dhan50	*Indica*	BRRI, Bangladesh
BRRI dhan52	*Indica*	BRRI, Bangladesh
BRRI dhan54	*Indica*	BRRI, Bangladesh
BRRI dhan55	*Indica*	BRRI, Bangladesh
HUA 565	*Indica/japonica*	China
IR445955	*Indica*	IRRI, Philippines
Jefferson	*Japonica*	USA
Priscilla	*Japonica*	USA
Tie 90-1	*Indica*	China

(Bio basic, Canada). DNA Thermal Cycler, G-strom (Gene Technology Ltd., England) was used along with the following profile: Initial denaturation at 94°C for 5 min, 35 cycles of 45 sec denaturation at 94°C, 45 sec annealing at 55°C, 1 min and 30 sec extension at 72°C and additional temperature of 72°C for 7 min for final extension and 4°C indefinitely for cooling and storage. The amplified PCR products were separated in 8% polyacrylamide gel containing 0.5X TBE buffer at 100 V for 1.30-2.30 h along with a known DNA ladder (Bio basic, Canada). After completion of electrophoresis, gel was stained with ethidium bromide for 25-30 min and visualized under UV light and gel images were acquired under gel documentation system (BIO-RAD).

Data analysis and clustering: The molecular weights for each amplified allele were measured in base pairs using Alpha Ease FC 5.0 software (Alpha Innotech Corporation). One hundred and ninety one polymorphic SSR markers distributed across 12 chromosomes were used for diversity analysis. Data of non-amplified and monomorphic SSR markers were not used for diversity study. Summary statistics, including the number of alleles per locus, major allele frequency, gene diversity and PIC values were determined[25] by using Power Marker version 3.25. Polymorphic information content (PIC) values were calculated for each of the SSR loci using the following equation[26]:

$$PICi = 1 - \sum_{j=1}^{n} (Pij)^2$$

where, n is the number of marker alleles for marker i and Pij is the frequency of the jth allele for marker i. The allele frequency[25] data from Power Marker version 3.25 was used to export the data in binary format (allele presence = 1; allele absence = 0) for analysis with Numerical Taxonomy and Multivariate Analysis System[27] (NTSYS-pc) version 2.2. The binary data of microsatellite markers for 15 rice genotypes were calculated into similarity matrix using the Dice coefficient and clustered using an UPGMA with the module of SAHN in the NTSYS-pc package.

RESULTS

Selection of polymorphic markers: Status of SSR markers was presented in Fig. 1. A total of 295 SSR markers were used across the tested cultivars. Among them, 12 markers were not amplified at all and 92 SSR markers showed monomorphism. Therefore, these 104 markers do not have importance on diversity and genetic relationship study of the cultivars. The rest 191 SSR markers showed polymorphism among the tested cultivars which was used for diversity analysis.

Allelic variation and polymorphic information content (PIC) values: Allelic variation and polymorphic information content were presented in Table 2 . A total of 552 alleles were detected at 191 polymorphic SSR markers among the tested rice genotypes. The number of alleles per locus ranged from 2-7 with an average of 2.89 alleles across 191 loci. A total of 322 common alleles were detected with an average of 1.69 alleles per locus. A total of 230 rare alleles with an average of 1.20 alleles per locus were identified. The number of total unique alleles was 141 with a mean of 0.74 alleles per locus. The number of unique alleles per locus ranged from 0-4. The

Table 2: Summary of allelic variation and polymorphic information content (PIC) values among studied rice genotypes for 191 SSR markers

Variables	Total	Mean	Range
Alleles No.	552	2.89	2-7
Common alleles	322	1.69	1-4
Rare alleles	230	1.20	0-5
Unique alleles	141	0.74	0-4
Alleles size (bp)	-	-	74-316
Major allele frequency (%)	-	68.66	26.67-93.33
PIC value	-	0.37	0.12-0.80

PIC: Polymorphic information content

Table 3: Genotypes-specific alleles identified for 15 rice genotypes analyzed in this study

Genotypes	No. of genotype-specific alleles	Locus (specific allele in base pair)
Jefferson	25	RM6672 (161 bp), RM8268 (233 bp), RM600 (230 bp), RM466 (227 bp), RM5638 (211 bp), RM7371 (149 bp), RM424 (234 bp), RM13129 (163 bp), RM1319 (158 bp), RM3033 (207 bp), RM1940 (172 bp), RM3180 (146 bp), RM7097 (180 bp), RM3708 (143 bp), RM3276 (161 bp), RM6748 (161 bp), RM6176 (156 bp), RM3207 (95 bp), RM4924 (119 bp), RM3215 (201 bp), RM6382 (147 bp), RM4771 (107 bp), RM7463 (193 bp), RM2191 (258 bp), RM27447 (261 bp)
BRRI dhan47	21	RM1282 (89 bp), RM8268 (218 bp), RM582 (218 bp), RM6141 (131 bp), RM2468 (109 bp), RM6959 (133 bp), RM6283 (87 bp), RM2416 (105 bp), RM5361 (123 bp), RM249 (145 bp), RM18271 (172 bp), RM1115 (171 bp), RM8200 (127 bp), RM3767 (148 bp), RM3215 (190 bp), RM331 (147 bp), RM3912 (181 bp), RM3283 (193 bp), RM27638 (228 bp), RM7003 (101 bp), RM17 (206 bp)
Priscilla	20	RM5638 (201 bp), RM6407 (129 bp), RM3688 (103 bp), RM3467 (125 bp), RM3033 (175 bp), RM3180 (139 bp), RM3601 (97 bp), RM6970 (109 bp), RM5503 (167 bp), RM4924 (114 bp), RM1111 (107 bp), RM310 (109 bp), RM331 (162 bp), RM2144 (114 bp), RM3225 (143 bp), RM7463 (189 bp), RM26482 (252 bp), RM4112 (205 bp), RM2191 (171 bp), RM27447 (271 bp)
Tie90-1	11	RM8268 (241 bp), RM582 (240 bp), RM3630 (136 bp), RM5916 (214 bp), RM5414 (114 bp), RM1359 (121 bp), RM3643 (111 bp), RM18271 (182 bp), RM586 (248 bp), RM2655 (158 bp), RM26501 (140 bp)
BRRI dhan33	10	RM8268 (230 bp), RM318 (140 bp), RM5412 (87 bp), RM3766 (134 bp), RM7097 (176 bp), RM2416 (117 bp), RM1111 (133 bp), RM26482 (246 bp), RM26501 (129 bp), RM7226 (179 bp)
HUA565	8	RM1211 (220 bp), RM1319 (150 bp), RM6914 (247 bp), RM6748 (188 bp), RM5361 (129 bp), RM1985 (170 bp), RM8258 (177 bp), RM28070 (205 bp)
BRRI dhan45	7	RM3766 (129 bp), RM5419 (180 bp), RM3125 (127 bp), RM3187 (132 bp), RM4584 (159 bp), RM4986 (104 bp), RM5436 (155 bp)
BRRI dhan49	7	RM6141 (124 bp), RM5361 (113 bp), RM5970 (108 bp), RM5770 (141 bp), RM8200 (116 bp), RM2229 (104 bp), RM1026 (157 bp)
BRRI dhan28	6	RM1843 (203 bp), RM5412 (80 bp), RM311 (185 bp), RM2935 (112 bp), RM28033 (112 bp), RM28050 (199 bp)
BRRI dhan52	6	RM2431 (153 bp), RM1985 (173 bp), RM5799 (186 bp), RM3912 (197 bp), RM3769 (74 bp), RM2863 (183 bp)
IR445955	6	RM1282 (140 bp), RM2468 (106 bp), RM2416 (107 bp), RM3643 (118 bp), RM310 (96 bp), RM1896 (79 bp)
BRRI dhan50	5	RM3521 (109 bp), RM2431 (168 bp), RM6202 (157 bp), RM310 (103 bp), RM3120 (123 bp)
BRRI dhan29	4	RM318 (147 bp), RM7279 (177 bp), RM1155 (161 bp), RM5799 (200 bp)
BRRI dhan54	4	RM1319 (135 bp), RM16296 (244 bp), RM1155 (147 bp), RM1985 (154 bp)
BRRI dhan55	1	RM3283 (180 bp)
Total	141	

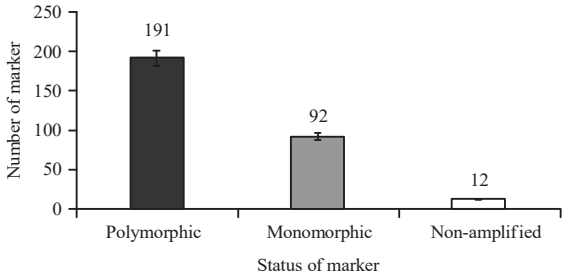

Fig. 1: Status of SSR markers used in diversity experiment among 15 genotypes

overall size of the amplified products ranged from 74 bp (RM3769) to 316 bp (RM26305). The frequency of the most common allele at each locus ranged from 26.67-93.33% with an average of 68.66%.The PIC values for the SSR loci ranged from 0.12-0.80 (RM6748), with a mean values of 0.37.

Genotype-specific alleles: Number of genotype-specific alleles and locus (specific allele in base pair) were presented in Table 3. Out of total 552 alleles, 141 alleles were specific for a given genotypes. The highest number of genotype-specific alleles (25) was observed in the genotype Jefferson followed by the genotypes BRRI dhan47; Priscilla; Tie90-1; BRRI dhan33; HUA565; BRRI dhan45 and BRRI dhan49; BRRI dhan28, BRRI dhan52 and IR445955; BRRI dhan50; BRRI dhan 29 and BRRI dhan54 while the lowest number of genotype-specific alleles (1) was recorded in BRRI dhan55.

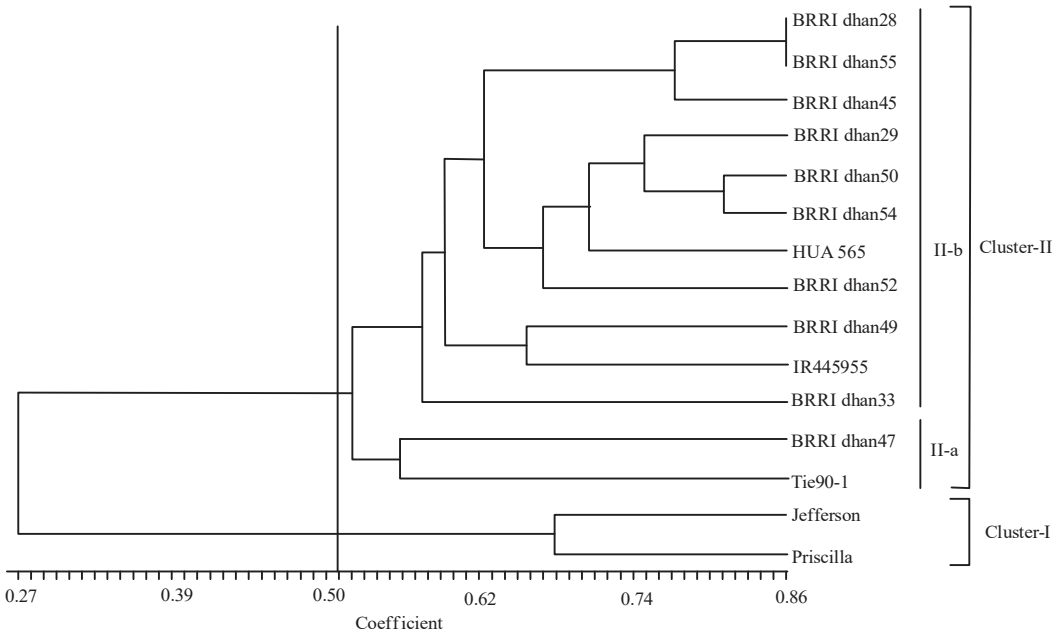

Fig. 2: Dendrogram showing the genetic relationships among 15 rice cultivars using UPGMA cluster analysis of Dice genetic similarity coefficients generated from 191 SSR markers

Genetic distance based analysis: The results in Fig. 2 showed the genetic relationships among the tested genotypes. Two major clusters were observed at the similarity coefficient of 0.51. Cluster-I was constituted by *japonica* subspecies 'Jefferson' and 'Priscilla' while Cluster-II was comprised of *indica* subspecies. Sub-cluster-IIa consisted of BRRI dhan47 and Tie90-1 while sub-cluster-IIb included the remaining eleven genotypes.

DISCUSSION

A total of 552 alleles were found among the tested genotypes by using 191 polymorphic SSR markers, of which 322 were common and 230 were rare alleles. Among the rare alleles, 141 were genotype-specific. The PIC values ranged from 0.12-0.80 (average 0.37). Cluster analysis showed distinct two groups of tested genotypes.

In this study, 295 SSR markers were evaluated among 15 rice cultivars; 10 of these genotypes represent *indica* varieties commercially cultivated in Bangladesh. The other five genotypes represented two *indica*, two *japonica* and one *indica* and *japonica* cross. Among the tested markers, 12 markers were not amplified which might be due to the selection of these markers based on published *japonica* rice base sequences that might differ from *indica* type rice cultivars studied here[28]. These markers might be useful for other *japonica* type rice genotypes rather than *indica* rice[20]. In addition, 92 marker showed monomorphic allele among the cultivars which means that these markers were not useful for the study of genetic diversity and genetic relationship of the studied cultivars[29].

The variations of allele number per locus were within the range from 2-7 with an average of 2.89 which was quite comparable to the values reported by Joshi and Behera[30] and Pachauri *et al.*[31] but quite low compared with other reports by Rahman *et al.*[8], Prabakaran *et al.*[32] and Herrera *et al.*[33]. Comparatively low allele per locus might be due to the use of lower number of genotypes in the present study which was also reported by Prabakaran *et al.*[32]. Close genetic makeup would be another cause of low allele number[33].

Several SSR markers were identified that could readily distinguish BRRI released cultivars from the rest of genotypes. A total of 141 unique (accession-specific) alleles were observed that might be useful for identification of a particular type of genotype. More specifically, 57 SSR markers produced 71 genotype-specific alleles that distinguished 10 BRRI rice cultivars (Table 3); these markers could be used for molecular characterization of BRRI collected huge native germplasms. The occurrence of a relatively high number of rare alleles and unique alleles in Jefferson, Priscilla and BRRI dhan47 indicated the potentiality as a reservoir of novel alleles for varietal improvement[34]. In this study, lower PIC values (0.37)

indicated that the genotypes were not much diverged and were closely related[30,31]. The PIC value observed in the present study was comparable to many of the previous reports by Wankhade *et al.*[35] and Islam *et al.*[36]. High PIC values could be attributed to the use of more informative markers[8].

The dendrogram obtained using the UPGMA method revealed cultivars that were genetically similar and thus clustered together and explained the relationship among the test rice varieties. Rice varieties were separated into two major groups (*indica* and *japonica* subspecies) at similarity coefficients of 0.51. About 191 SSR markers also allowed the distinction among *indica* accessions; all 13 *indica* cultivars were clearly distinguished even though a high relatedness or similarity was measured between cultivar pairs. Similar observations were made by other scientist which was in agreement with the present study by Rahman *et al.*[8] and Prabakaran *et al.*[32]. Though the genetic divergence was not very high[33], but cultivars of the present study showed considerable genetic diversity. However, among the studied genotypes, the most arsenic (As) tolerant cultivar, BRRI dhan47 and the most As sensitive cultivar BRRI dhan45 occupied distinct different sub-cluster at the molecular level[37]. The identified highly informative SSRs markers might be useful in genetic studies of rice germplasm, especially, in arsenic tolerant marker assisted breeding and QTL identification in future. There is also a great scope to get further improved materials by crossing with distant genotypes identified during the present study.

CONCLUSION

The current study suggested a considerable level of micro satellite allelic variation among rice genotypes. A total of 230 rare alleles were identified, among which 141 were unique alleles which has potentiality to use as a reservoir of rare genotypes. Cluster-I was constituted by the *japonica* subspecies US varieties 'Jefferson' and 'Priscilla' cultivars which were significantly different from all other genotypes. Cluster-II was comprised of *indica* subspecies varieties, which were grouped into two different sub-clusters. Molecular identification of 15 rice cultivars were done with 99 primers pairs which produced 141 genotype-specific-alleles. Those genotype-specific-alleles were important to identify a particular type of genotype, background selection in backcross breeding and arsenic tolerant QTL identification.

SIGNIFICANCE STATEMENT

This study discovers a considerable level of microsatellite allelic variation among rice genotypes. Highly informative polymorphic markers and genotype specific alleles identified during the present study will be beneficial for DNA fingerprinting, marker assisted selection and genetic dissection of traits. This study will help the researchers to use the most arsenic (As) tolerant rice cultivar, BRRI dhan 47 and the most arsenic sensitive rice cultivar, BRRI dhan45 occupied distinct different sub-cluster at the molecular level. Therefore, the studied polymorphic SSRs could be effectively used for QTL identification to arsenic phyto-toxicity tolerance in rice by crossing between these two cultivars.

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