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Molecular Diversity Analysis for Zinc Deficiency Tolerance under Aerobic Rice (*Oryza sativa* L.) Ecosystem

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Rice (*Oryza sativa* L.) is the "Global Grain" because of its use as prime staple food in about 100 countries of the world. Aerobic rice provides for effective use of water as the concept of flooding paddy fields is abandoned in this ecosystem. But due to the transition from flooding of paddy fields to aerobic system of rice cultivation many factors that determine nutrient (Zn) bioavailability changes in addition to water deficit. Achieving higher yields under aerobic conditions requires new varieties of "aerobic rice" combining both the water stress tolerance and nutrient (especially Zn) deficiency tolerance traits. Molecular marker technologies are the effective tools and they are used for the assessment of genetic variability because they are not influenced by the environment. Among the molecular markers, Simple Sequence Repeat (SSR) has proved to be the most powerful tool for variety identification in rice and has much potential in genetic and breeding studies. Among the 16 SSR markers specific to zinc deficiency tolerance used for assessing the genetic diversity, 13 primer pairs (81.25%) were polymorphic. The number of alleles per microsatellite locus ranged from 2 to 5, averaging 2.53 alleles per locus. Polymorphism information content (PIC) values ranged from 0.43 (RM242) to 0.69 (RM1089 and RM3331), with an average of 0.58. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram revealed two major groups with three clusters and the wide range of dissimilarity values (0.68-1.00) which showed a high degree of diversity among the germplasm lines. The results of the genetic diversity will be useful for the selection of the parents for developing zinc deficiency tolerant rice variety under aerobic condition through molecular breeding programme.

Key Words: DNA polymorphism, Genetic Diversity, Polymorphism information content (PIC), Rice, SSR markers.

Introduction

The wonder cereal, rice (*Oryza sativa* L.) is the heart of our culture and the staple food crop consumed by more than 50% of the world's population, which is cultivated over 163 million hectares with the production of 718 million tonnes (FAO, 2013). About 90% of the world's rice is produced in Asia, out of which 20% is produced in India. The area, production and productivity of rice in India is 427.53 lakh hectares, 105.24 million tonnes and 2462 kg/ha, respectively, in 2012-13 (DAC, 2014). India is the second largest producer and consumer of rice in the world, accounting for 22.3% of global production. In addition to food security, rice also plays an important role in socioeconomic and political stability in many countries in Asia, Africa and Latin America. Irrigated rice has very low water-use efficiency as it consumes 3000–5000 litres of water to produce one kg of rice. In rice, among the several production constraints, availability of irrigation water is a major factor as it consumes about 70% of the water available for agriculture (Vibhu Nayar and Ravichandran, 2012). Therefore, ways must be sought to reduce water requirements in rice and increase its productivity. The aerobic condition is maintained by using flush irrigation or sprinklers so that ponding occurs for only short periods of time just after irrigation or rain, if at all. The potential for water savings is large, but aerobic cultivation using conventional lowland rice varieties almost always leads to yield reduction (Atlin *et al*., 2004). Therefore achieving higher yields under aerobic conditions requires new varieties of "aerobic rice" combining both the water stress tolerance and nutrient (especially Zn) deficiency tolerance traits. Thus there is an immediate need to accelerate the genetic improvement of rice for aerobic cultivation, which until recently has received less attention compared to lowland rice. This could be hastened through the identification of molecular markers linked to traits associated with water use efficiency and zinc deficiency tolerance under aerobic conditions in rice and deployment *via* marker assisted breeding. It is difficult to classify the accessions solely

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based on their morphological characters (Gangashetty *et al*., 2013 and Singh *et al*., 2010). The development of reliable methods is necessary to allow for the assessment of genetic variability in germplasm collections or pedigree reconstruction. In various methodologies, DNA based technologies are the most reliable tools allowing for the assessment of genetic variability because they are not influenced by the environment. DNA markers *viz*., RFLP, RAPD, SSR, ISSR etc. can be used to assess the diversity studies (Venkatesan and Bhat, 2015; Kumbhar *et al*., 2013). Among them, SSR markers have great potential in genetic and breeding studies (Matin *et al*., 2012). The present investigation was made to identify the suitable SSR primers for genetic analysis of zinc deficiency tolerance under aerobic rice and to measure the genetic diversity and relatedness among 62 rice germplasm lines using SSR markers specific to zinc deficiency tolerance.

Materials and Methods

The experiments were conducted at Department of Rice, Tamil Nadu Agricultural University, Coimbatore during August/2013-January/2014.

Plant Material

A total of 62 rice germplasm lines (Table 1) comprising of upland and lowland rice cultures were selected and sown directly under aerobic conditions.

Extraction of Genomic DNA

DNA was extracted from the leaf samples of the two parents and the progenies following CTAB method developed by Saghai-Maroof *et al*. (1984) with suitable modifications suggested by Hoisington *et al*. (1994).

DNA Quality and Quantity Check

The nucleic acids were quantified by the methods of Spectrophotometric determination and gel electrophoresis (0.8% agarose gel). DNA concentration for PCR amplification was estimated by comparing the band intensity of a sample with the band intensities of known dilutions that gave good amplifications. The dilutions were carried out by dissolving the genomic DNA in appropriate volume of TE buffer.

PCR Amplification

SSR Markers

The parental survey was performed for 62 rice germplasm lines using 16 SSR markers specific to zinc deficiency tolerance (Bashir *et al*., 2012; Vivek, 2012) for amplifying genomic DNA fragment by polymerase chain reaction. The parental polymorphism survey identified 13 polymorphic markers between germplasm lines. The list of polymorphic markers used for surveying the 62 rice germplasm lines is furnished in Table 2.

SSR Analysis

PCR amplifications were performed in Bio-Rad (MyCycler thermal cycler) and AB PCR machine. About 25 ng of genomic DNA was used as the template. The reaction was carried out in a total reaction volume of 20 µl containing the following components.

Components	Volume (µl)
DNA	2.00
Forward primer	1.00
Reverse primer	1.00
Sterile water	13.6
DNTP's	0.6
Taq buffer	1.5
Taq enzyme	0.3
Total	20.00

The thermal cycler was programmed as follows.

Step 1	Initial denaturing step	94°C for 5 minutes
Step 2	Denaturing	94°C for 1 min.
Step 3	Annealing	55°C for 1 min.
Step 4	Extension	72°C for 2 min.
Step 5	Repeat steps 2 through 4 for a total of 35 cycles (34 times)	
Step 6	Final extension	72°C for 5 min.
Step 7	4°C hold until sample retrieval	
Step 8	End	

PCR products were kept at 4ºC until further use.

Agarose gel electrophoresis was performed at constant power 120 volts for about 3 h to separate amplification products.

Analysis of Genetic Diversity

Genetic diversity among the 62 rice genotypes were evaluated using 13 SSR primers. Each fragment size was treated as a unique characteristic and scored as present (1) or absent (0). Genetic similarity index (UPGMA cluster analysis of the Jaccard's similarity coefficient) was used to construct a dendrogram which illustrated the genetic relationship among the 62 genotypes of

Table 1. Parentage of 62 germplasm lines used in the study

S. No.	Germplasm lines	Parentage	Origin
1	CB-08-702	IR 75417-R-R-R-R-267-3	TNAU, Tamil Nadu, India
2	CB-06-803	PMK3 × Norungan	TNAU, Tamil Nadu, India
3	CB-08-701	IR80013-B-141-4-1	TNAU, Tamil Nadu, India
4	CB-07-701-252	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
5	CB-07-701-274	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
6	CB-07-701-256	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
7	CB-07-701-143	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
8	CB-07-701-279	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
9	IR84895-B-127-CRA-5-1-1	IR77080-B-34-3 × IRRI123	IRRI, Philippines
10	IR83381-B-B-18-3	IR72022-46-2-3-3-2 × IR77080-B-34-1-1	IRRI, Philippines
11	IR83387-B-B-110-1	IR72022-46-2-3-3-2 × Sambha mahsuri	IRRI, Philippines
12	CB-09-512	OR1797-4/Varapukudanchan	TNAU, Tamil Nadu, India
13	CB-09-516	RR4065-381-245/UPR2893-97-5-27	TNAU, Tamil Nadu, India
14	CB-07-701-12	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
15	CO51	ADT43 × RR272-1745	TNAU, Tamil Nadu, India
16	CB-07-701-23	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
17	CB-07-701-115	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
18	CB-07-701-126	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
19	CB-07-701-128	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
20	CB-07-701-129	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
21	CB-07-701-146	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
22	CB-07-701-148	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
23	CB-07-701-150	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
24	CB-07-701-151	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
25	CB-07-701-174	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
26	CB-07-701-199	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
27	CB-07-701-218	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
28	CB-07-701-230	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
29	CB-07-701-255	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
30	CB-07-701-262	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
31	CB-07-701-264	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
32	CB-07-701-265	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
33	CB-07-701-268	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
34	CB-07-701-278	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
35	CB-07-701-280	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
36	CB-07-701-283	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
37	CB-07-701-284	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
38	CB-07-701-181	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
39	CB-07-701-288	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
40	CB-00-11-4	IR50 × Norungan	TNAU, Tamil Nadu, India
41	CB-00-11-7	IR50 × Norungan	TNAU, Tamil Nadu, India
42	CB-00-11-19	IR50 × Norungan	TNAU, Tamil Nadu, India
43	CB-00-11-21	IR50 × Norungan	TNAU, Tamil Nadu, India
44	CB-00-11-22	IR50 × Norungan	TNAU, Tamil Nadu, India
45	CB-00-11-23	IR50 × Norungan	TNAU, Tamil Nadu, India
46	CB-00-12-91	Norungan × IR50	TNAU, Tamil Nadu, India
47	CB-00-12-192	Norungan × IR50	TNAU, Tamil Nadu, India
48	ARB-6	Budda × IR64	UAS, GKVK, Bangalore
49	CB-06-803-2	PMK3 × Norungan	TNAU, Tamil Nadu, India
50	Anna-4	Pantdhan10 × IET9911	TNAU, Tamil Nadu, India
51	CB-00-15-10	IR64 × Norungan	TNAU, Tamil Nadu, India
52	CB-00-15-23	IR64 × Norungan	TNAU, Tamil Nadu, India
53	CB-00-15-44	IR64 × Norungan	TNAU, Tamil Nadu, India
54	CB-00-755-2	IR64/Norungan/IR64	TNAU, Tamil Nadu, India
55	CB-00-15-24	IR64 × Norungan	TNAU, Tamil Nadu, India
56	CB-08-709-2	RR4065-381-245 × UPR2893-99-5-2-1	TNAU, Tamil Nadu, India
57	Apo (IR55423-01)	UPLRI5 × IR12979-24-1	IRRI, Philippines
58	PSBRC-80	IR64 x Moroberekan	Philippine seed board rice, Philippine rice research institute, Philippines.
59	PSBRC-82	IR47761-27-1-3-6 × PSB RC 28	Philippine seed board rice, Philippine rice research institute, Philippines.
60	CB-07-701-22	Norungan/TKM9/Norungan	TNAU, Tamil Nadu, India
61	IR64	IR5857-33-2-1 × IR2061-465-1-5-5	IRRI, Philippines
62	CB-09-123	BPT5204 × CO50	TNAU, Tamil Nadu, India

Table 2. List of polymorphic markers and Polymorphic Information Content (PIC) value

S. No.	Primers	Chromosome No.	Forward sequence	Reverse sequence	No. of alleles	PIC value
1	RM263F	1	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	2	0.49
2	RM234F	1	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	2	0.49
3	RM21F	2	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	2	0.64
4	RM212F	2	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG	2	0.50
5	RM499F	3	TACCAAACACCAACACTGCG	ACCTGCAGTATCCAAGTGTACG	2	0.50
6	RM1089F	5	CAGAAGGATTATCTCGATACC	AATAGGGCTTGAAATAAATTG	2	0.69
7	RM296F	5	CACATGGCACCAACCTCC	GCCAAGTCATTCACTACTCTGG	3	0.49
8	RM164F	5	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	2	0.52
9	RM3331F	7	CCTCCTCCATGAGCTAATGC	AGGAGGAGCGGATTTCTCTC	5	0.69
10	RM6832F	9	GTTGTAAATGCCTGAGTGC	AAAGAGCTAAACCGCTAGG	2	0.61
11	RM26334F	11	GACTCCCTACTAGTGGTTCTGATTCG	CCTTTGACGATTGTGATGCTACG	2	0.62
12	RM242F	11	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	3	0.43
13	RM87F	12	CCTCTCCGATACACCGTATG	GCGAAGGTACGAAAGGAAAG	3	0.57

rice used in the study. A dendrogram was constructed using similarity index adopting Sequential Hierarchial and Nested (SAHN) using the NTSYS-pc 2.2 (Rohlf, 2005).

Results and Discussion

Molecular markers are a useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of tissue or environmental effects and allow cultivar identification early in plant development. The molecular markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and protein based markers (Mignouna *et al*., 1998; Tanksley *et al*., 1989). Simple Sequence Repeats (SSR) is used as a primer to amplify regions between the microsatellites. This marker reveals a much larger number of fragments per primer than RAPD analysis (Bajpai *et al*., 2008).

The level of polymorphism among rice germplasm lines was evaluated by calculating allelic number and PIC values for each of the 13 SSR loci specific to zinc deficiency tolerance. A total of 36 alleles were detected at the loci of 13 microsatellite markers across 62 rice germplasm lines. The results revealed that all the primers showed distinct polymorphisms among the germplasm lines studied indicating the robust nature of microsatellites in revealing polymorphism for zinc deficiency tolerance under aerobic condition. Among the polymorphic markers, eight generated two alleles each, four produced three alleles each and only one produced 5 alleles (Table 3). The number of alleles per locus ranged from 2 (RM234, RM3331, RM164, RM212, RM263, RM499, RM296 and RM279) to 5 alleles (RM 1089 and RM3331) with an average of 2.53 alleles across the 13 primers. Similar number of microsatellite markers previously used as subset for genetic diversity analysis of *O. sativa* (Garris *et al*., 2005; Thomson *et al*., 2007; Kanawapee *et al*., 2011; Kumbhar *et al*., 2013; Venkatesan and Bhat, 2015). The Value is comparable to 2-5 allele per SSR locus with an average number of alleles of 2.53 per locus for various classes of microsatellite (Siwach *et al*., 2004; Matin *et al*., 2012). Maximum number of polymorphic alleles (5) was obtained with the marker RM 1089, while the minimum numbers of polymorphic alleles (2) was obtained by using RM234, RM3331, RM164, RM212, RM263, RM499, RM296 and RM279 (Fig. 1).

Polymorphism information content (PIC) value is a reflection of allele diversity and frequency among the germplasm lines. PIC values ranged from 0.43 to 0.69 with an average of 0.58 (Table 3). The highest PIC value 0.69 was obtained for RM1089 and RM3331 followed by respectively RM21 (0.64), RM26334 (0.62) and RM6832 (0.61). PIC value revealed that RM1089 and RM3331 were considered as best markers for 62 rice germplasm lines for zinc deficiency tolerance under aerobic condition. Similar results were reported by Alvarez *et al*., 2007; Matin *et al*., 2012; Kumbhar *et al*., 2013.

Cluster Analysis

The cluster analysis based on unweighted paired group method of arithmetic means (UPGMA) with 13 SSR

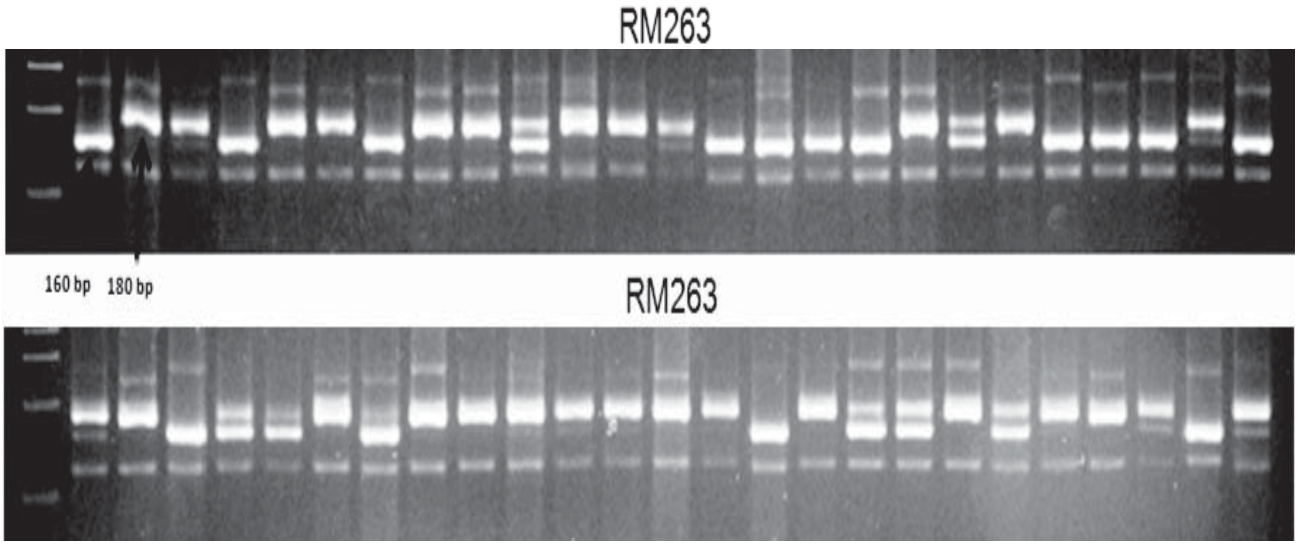

Fig. 1. Representative SSR profile of rice germplasm lines obtained with RM263

Table 3. Distribution of rice germplasm lines into different clusters based on SSR analysis

Cluster	Subcluster	No.of genotypes	Genotypes
I	A	2	CB-08-702 and CB-07-701-12
	B	4	CB-07-701-143,CB-07-701-150, PSBRC-80 and PSBRC-82
II	A	6	CB-06-803, IR83381-B-B-18-3, IR83387-B-B-110-1, CB-07-701-264, CB-00-15-44 and CB-09-123
	B	4	CB-00-11-23, CB-00-755-2, CB-00-15-24 and CB-08-709-2
	C	7	CB-09-512, CB-09-516, CB-07-701-146, CB-00-12-91, Anna-4, CB-00-15-10 and Apo
	D	1	IR84895-B-127-CRA-5-1-1
	E	5	CB-00-12-192, ARB-6, CB-06-803-2, CB-00-11-22 and IR64
	F	4	CB-00-11-4, CB-00-11-7, CB-00-11-19 and CB-00-11-21
III	A	5	CB-08-701, CB-07-701-274, CB-07-701-256, CB-07-701-279 and CB-07-701-218
	B	1	CB-00-15-23
	C	7	CB-07-701-23, CB-07-701-199, CB-07-701-262, CB-07-701-278, CB-07-701-283, CB-07-701-284 and CB-07-701-288
	D	5	CB-07-701-22, CB-07-701-174, CB-07-701-255, CB-07-701-265 and CB-07-701-280
	E	2	CB-07-701-230 and CB-07-701-252
	F	3	CB-07-701-151, CB-07-701-268 and CO51
	G	6	CB-07-701-115, CB-07-701-126, CB-07-701-128, CB-07-701-129, CB-07-701-148 and CB-07-701-181

primers specific to zinc deficiency tolerance allowed the discrimination of cultivars. The UPGMA based clustering grouped 62 rice germplasm lines into three major clusters (Table 3, Fig. 2). Cluster III had maximum number of genotypes (29) followed by cluster II (27), whereas cluster I had six genotypes. Cluster III was the largest and most diverse cluster consisting of 29 genotypes. This cluster was subgrouped into seven sub-clusters (A, B, C, D, E, F and G) at varying degree of similarity. Subgroup B contain highest number of genotypes (7) *viz*., CB-07-701-23, CB-07-701-199, CB-07-701-262, CB-07-701-278, CB-07-701-283, CB-07-701-284 and CB-07-701-288 followed by subgroup G (6) *viz*., CB-07-701-115, CB-07-701-126, CB-07-701-128, CB-07-701-129, CB-07-701-148 and CB-07-701-181, subgroup A (5) *viz*., CB-08-701, CB-07-701-274, CB-07-701-256, CB-07-701-279 and CB-07-701-218, subgroup D (5) *viz*., CB-07-701-22, CB-07-701-174, CB-07-701-255, CB-07-701-265 and CB-07-701-280, subgroup F (3) *viz*., CB-07-701-151, CB-07-701-268 and CO51, subgroup E (2) had CB-07-701-230 and CB-07-701-252 and subgroup B (1) had only one genotype *ie*., CB-00-15-23. Cluster II contained 29 germplasm lines and subclustered into six groups (A, B, C, D, E and F). In this subgroup C contains highest number of genotypes (7) *viz*., CB-09-512, CB-09-516, CB-07-701-146, CB-00-12-91, Anna-4,

CB-00-15-10 and Apo followed by subgroup A (6) *viz*., CB-06-803, IR83381-B-B-18-3, IR83387-B-B-110-1, CB-07-701-264, CB-00-15-44, CB-09-123, subgroup E (5) *viz*., CB-00-12-192, ARB-6, CB-06-803-2, CB-00-11-22 and IR64, subgroup B (4) *viz*., CB-00-11-23, CB-00-755-2, CB-00-15-24 and CB-08-709-2, subgroup F (4) *viz*., CB-00-11-23, CB-00-755-2, CB-00-15-24 and CB-08-709-2, D (1) had only one genotype *ie*., IR84895-B-127-CRA-5-1-1. Cluster I was the smallest group had six genotypes which contains two subclusters (A and B). Subgroup B had 4 genotypes *viz*., CB-07-701-143,CB-07-701-150, PSBRC-80 and PSBRC-82 followed by subgroup A (2) namely CB-08-702 and CB-07-701-12. Similar results were reported by Matin *et al*., 2012 and Kumbhar *et al*., 2013, Venkatesan *et al*., 2011 and Venkatesan and Bhat, 2015. Hence microsatellite marker based molecular fingerprinting could serve as a potential basis in the identification of genetically distance genotypes as well as in sorting of duplication for morphologically close accession. The present study is an attempt at characterizing diversity at the molecular level in this set of germplasm lines for zinc deficiency tolerance under aerobic conditions. Moreover, the cultivars with wide genetic distance can be crossed to widen the genetic base and exploit heterosis. The informative primers would prove useful in marker assisted selection, linkage mapping and gene tagging for zinc deficiency tolerance under aerobic conditions.

The present study revealed a considerable genetic diversity present in the 62 rice genotypes mainly belongs to Tamil Nadu state of India at molecular level and a limited number of SSR markers efficiently grouped these genotypes into 6 clusters on basis of zinc deficiency tolerance. SSR markers such as RM1089 and RM3331 could be useful to breeders for discriminating different rice genotypes for zinc deficiency tolerance under aerobic conditions.

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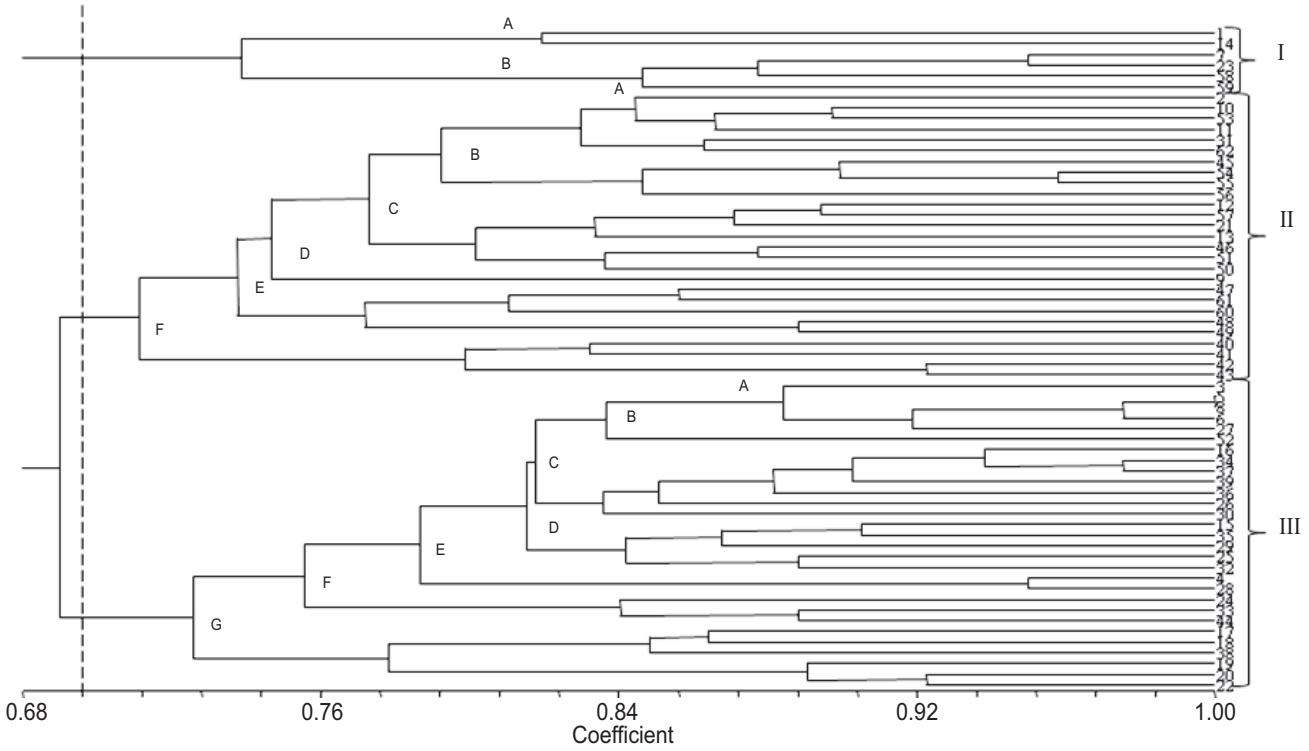

Fig. 2. UPGMA based dendrogram of rice germplasm lines using SSR markers specific to zinc deficiency tolerance

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