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Maliha A
PhD Scholar, Institute of biotechnology, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

M Balram
Professor and Head, Department of Genetics and Plant Breeding, College of Agriculture, Jagtial, Telangana, India

Ch V Durgarani
Principal Scientist (Oilseeds), Regional Agricultural Research Station, Palem, Telangana, India

Y Chandramohan
Senior Scientist, Genetics and Plant Breeding, Rice Research Centre, Agricultural Research Institute, Rajendranagar, Hyderabad, Telangana, India

T Ramesh
Associate Dean and University Head, Dept. of Crop Physiology, College of Agriculture, Warangal, Telangana, India

Corresponding Author:
Maliha A
PhD Scholar, Institute of biotechnology, PJTSAU, Rajendranagar, Hyderabad, Telangana, India

Assessment of genetic diversity of aerobic rice germplasm using SSR markers

Maliha A, M Balram, Ch V Durgarani, Y Chandramohan and T Ramesh

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Abstract
Rice is staple food for every second person on the globe but its cultivation demands a very high proportion of agricultural water available on earth. With the current trends of water usage and cultivation practices followed for rice, soon the water would become a scarce resource. Among the alternative methods available for cultivation of paddy with limited water, aerobic rice cultivation is the most economically and environmentally feasible option. Systematic breeding programs for aerobic rice varieties have been very few thus allowing a very narrow understanding about the genetic diversity present among rice lines that can be good candidates for aerobic cultivation. Since understanding the genetic diversity of a set of lines is pre-requisite for a successful breeding program, this study has been conducted using 110 germplasm lines collected from different parts of the country and a set of 58 SSR markers were used for polymorphism. Out of 58 markers used, 40 were found to be polymorphic thus making the overall polymorphism percentage 69.0%. The polymorphism percentage ranged from 55.6% (Chr. 12) to 100% (Chr. 8). The PIC (Polymorphism Information Content) values for the polymorphic markers ranged from 0.55 (RM525) to 0.98 (RM488) with an average of 0.84. Number of alleles recorded per marker ranged from minimum 2 to maximum 4 (average 2.63 alleles) and the total number of alleles scored is 105. A total of three markers (RM572, RM1367 and RM38) showed presence of alleles of four different sizes in the germplasm. Out of the remaining markers, 19 markers had 3 alleles each and 18 markers had 2 alleles each. As a result of diversity analysis the genotypes grouped into four distinct clusters (I, II, III, IV) with 22, 9, 65 and 14 genotypes respectively which further diverged into two sub-clusters each (Ia, Ib, IIa, IIb, IIIa, IIIb, IVa, IVb). Cluster I predominantly contained varieties and landraces for irrigated conditions that were tested under aerobic cultivation in this study. Cluster II had lines that have established under aerobic situations and are also being used as competent parents for aerobic rice breeding. The third cluster with maximum number of genotypes (65) was dominated by ARC (Assam Rice Collection) lines and the last cluster was a mixture of lines suitable for aerobic cultivation and landraces from Tamilnadu. The present study is thus an attempt to understand the genetic relatedness/ diversity of rice germplasm to enable selection of diverse lines from different clusters to be used as candidates in future research towards aerobic rice breeding.

Keywords: Aerobic rice, SSR markers, PIC, diversity analysis

Introduction
Rice being the staple food for every second person on the globe is also the single biggest consumer of freshwater diverted for agriculture. Production of 1 Kg rice requires around 4500 – 5000 litres of water (Bouman, 2009) [9]. Global agriculture now is witnessing a severe dearth of water availability and the water resources are depleting at a very rapid pace. At the same time sustainability of the irrigated rice systems is threatened by increasing costs of fresh water resources. It was estimated that 17 million ha of Asia's irrigated rice may experience "physical water scarcity" and 22 million ha may experience "economic water scarcity" by 2025 (Tuong and Bouman 2003) [28]. In the coming days cultivation of rice under traditional flooded conditions will not be possible and this demands implementation of certain water management techniques that will help reduce the usage of water for irrigation (Bouman, 2001) [8]. The most feasible among available alternatives is cultivation of aerobic rice. It involves growing of rice in aerobic soil, under non-flooded and non-puddled conditions by providing external inputs such as supplementary irrigation and fertilizers while aiming at high yields (Wang *et al*., 2002). As compared to the irrigated lowland method this method of growing rice utilizes almost 73% and 56% less water during land preparation and crop growth respectively (Castaneda *et al*., 2004). Due to reduced supply of water, yield obtained through this method of cultivation is significantly less compared to that obtained through lowland system. But this is not acceptable as the consumer demand for rice keeps increasing with increasing population.

The success of aerobic rice cultivation majorly depends on selection of appropriate cultivars (Wang *et al*., 2002) [30]. Aerobic rice should combine the features of upland rice (drought tolerance) and modern high-yielding rice varieties of the irrigated ecosystem (Lafitte *et al*., 2002, Atlin *et al*., 2006) [17, 4]. The aerobic rice breeding program when compared with lowland rice breeding is extremely small and was systematically started only in the recent past in a very few research centres worldwide which include IRRI (International Rice Research Institute, Philippines), NRRI (National Rice Research Institute, Cuttack) and UAS (University of Agricultural Sciences), Bengaluru. Though a good number of varieties were developed for aerobic cultivation, the yields obtained were 20% to 30% lower than that obtained with varieties grown under flooded conditions (Farooq *et al*., 2009, Prasad 2011) [12, 21]. Only a few successful aerobic rice cultivars with high-yield potential and broad biotic and abiotic stress tolerance are being grown as of now. But there is still a need to breed more varieties with less yield penalty in the aerobic ecosystem. Under such requirements, genetic diversity present among the genotypes acts as a very crucial tool in the process of selection of candidates for breeding program. Joshi and Dhawan (1966) [16] reported that genetic diversity was a very important factor for any hybridization program aiming at genetic improvement of yield especially in self-pollinated crops. Advances in the field of molecular biology and advent of molecular markers have made the understanding of genetic differences present between plants of same species very easy. Lower cost of inputs required to carry out genetic analysis and easy to handle protocols have made it even simpler to understand plants at DNA level. Among the available genetic markers, SSR (Simple Sequence Repeats) markers have been widely recognized for their co-dominant inheritance pattern, high informative power and transferability among the species, hence, is more preferred as a marker of choice for plant germplasm improvement program (Bligh *et al*., 1999 and Jeung *et al*., 2005) [6, 14]. The use of SSRs for rice diversity study is very crucial as it provides accurate and unbiased assessment and reveals in-depth information on the genetic divergence of a germplasm material (Ahmad *et al*., 2015) [1].

So far only a limited amount of research has been done towards understanding of diversity with the perspective of aerobic rice cultivation. These works were done either with an aim to compare efficiency of different genetic markers for estimation of genetic diversity (Mahajan *et al*., 2011) [18] or to validate the presence of desirable alleles for the markers reported earlier to be linked to aerobic adaptation traits (Rani *et al*., 2017) or for genetic differentiation and authentication of aerobic rice genotypes (Sweta kumari *et al*., 2018) [26]. Only one study involving 15 genotypes was carried out to compare diversity present between Basmati, aerobic and lowland (*indica* and *japonica*) lines (Sandhu *et al*., 2012) [23]. Hence, there is a need to understand the genetic diversity of more and more rice germplasm to improve breeding of aerobic rice varieties. This study was thus designed to understand genetic relatedness/ diversity of 110 rice germplasm lines collected from different parts of India with the help of genome wide SSR markers.

Materials and methods

Plant material

To understand the genetic diversity, experimental material (total 110 lines) for the present study was collected from Orissa (Cuttack) (60 lines), Tamilnadu (30 lines), Andhra Pradesh (8 lines), Telangana (6 lines) and Karnataka (2 lines). Apart from these lines, four entries used as national checks for water stress tolerance were also included in the plant material. Detailed list of plant material and respective source has been given in Table 1.

Table 1: List of Rice germplasm used in the present study

S. No.	Name	Source	S. No.	Name	Source	S. No.	Name	Source
1	ARC5922	Cuttack	38	ARC11553	Cuttack	75	Milagu Samba	Tamilnadu
2	ARC5973	Cuttack	39	ARC11566	Cuttack	76	Ottadam	Tamilnadu
3	ARC6096	Cuttack	40	ARC11611	Cuttack	77	Raja Mudi	Tamilnadu
4	ARC6101	Cuttack	41	ARC11691	Cuttack	78	Rattha Sali	Tamilnadu
5	ARC6143	Cuttack	42	ARC11702	Cuttack	79	Swarna Mashuri	Tamilnadu
6	ARC6153	Cuttack	43	ARC11750	Cuttack	80	Valan	Tamilnadu
7	ARC6180	Cuttack	44	ARC11861	Cuttack	81	Vandanj Samba	Tamilnadu
8	ARC6571	Cuttack	45	ARC11892	Cuttack	82	Bhavani	Tamilnadu
9	ARC6628	Cuttack	46	ARC12006	Cuttack	83	White ponny	Tamilnadu
10	ARC7071	Cuttack	47	Pyari	Cuttack	84	Ceeraga Samba	Tamilnadu
11	ARC7412	Cuttack	48	PR118	Cuttack	85	Elupa poo Samba	Tamilnadu
12	ARC10023	Cuttack	49	Brown gora	Cuttack	86	Karuvachi 140	Tamilnadu
13	ARC10222	Cuttack	50	Swarna sub-1	Cuttack	87	Mysore mally	Tamilnadu
14	ARC10223	Cuttack	51	Mahulata	Cuttack	88	Raja mannar	Tamilnadu
15	ARC10243	Cuttack	52	Vandana	Cuttack	89	Sinnar	Tamilnadu
16	ARC10248	Cuttack	53	Jaya	Cuttack	90	Thooya mally	Tamilnadu
17	ARC10258	Cuttack	54	Pathara	Cuttack	91	Swathi	A.P.
18	ARC10260	Cuttack	55	Pratikshya	Cuttack	92	Somasila	A.P.
19	ARC10405	Cuttack	56	Satari	Cuttack	93	NLR34449	A.P.
20	ARC10455	Cuttack	57	CR 143-2-2	Cuttack	94	Swarna	A.P.
21	ARC10612	Cuttack	58	Satyabhama	Cuttack	95	MTU1010	A.P.
22	ARC10625	Cuttack	59	Black gora	Cuttack	96	MTU1001	A.P.
23	ARC10655	Cuttack	60	RR 2-6	Cuttack	97	MTU1121	A.P.
24	ARC10689	Cuttack	61	Adukku Rice	Tamilnadu	98	MTU1156	A.P.
25	ARC10827	Cuttack	62	Arubadham kuruvai	Tamilnadu	99	JGL 11727	Telangana
26	ARC10838	Cuttack	63	Barani	Tamilnadu	100	JGL 24423	Telangana
27	ARC10843	Cuttack	64	Bommi	Tamilnadu	101	JGL 20171	Telangana
28	ARC10847	Cuttack	65	Gunducar	Tamilnadu	102	JGL 18047	Telangana

29	ARC10957	Cuttack	66	Kandha Sala	Tamilnadu	103	KNM 118	Telangana
30	ARC11064	Cuttack	67	Kala namak	Tamilnadu	104	RNR15048	Telangana
31	ARC11121	Cuttack	68	Kavuni	Tamilnadu	105	MAS26	Karnataka
32	ARC11211	Cuttack	69	Karuavury	Tamilnadu	106	MAS946-1	Karnataka
33	ARC11222	Cuttack	70	Kattu ponni	Tamilnadu	107	Sahbaghidhan	IRRI.
34	ARC11225	Cuttack	71	Karung Kuruvai	Tamilnadu	108	N22	UP
35	ARC11241	Cuttack	72	Kulla Khar	Tamilnadu	109	APO	IRRI
36	ARC11515	Cuttack	73	Kottha Malli samba	Tamilnadu	110	CRDhan 202	Cuttack
37	ARC-Y2a-536	Cuttack	74	Kuzhi Adichan	Tamilnadu			

A.P. – Andhra Pradesh, the last four varieties are national checks for water stress tolerance

Isolation of DNA and quality check

Isolation of DNA from leaf tissues of 110 samples individually was done following the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol given by Doyle and Doyle (1987) [11]. Once the obtained DNA pellet was dispersed in the TE (Tris-10mM, EDTA-1mM) buffer the quality of individual DNA samples was checked.

To check the quality of DNA in each sample, an agarose gel of 0.8% concentration was prepared by adding ethidium bromide and placed in the electrophoresis tank with buffer and samples were loaded in individual wells alongside uncut lambda DNA standard after mixing with the loading dye. The gel was then subjected to 80-100V of electric current for an hour and was then viewed under UV light to see the DNA (Fig. 1).

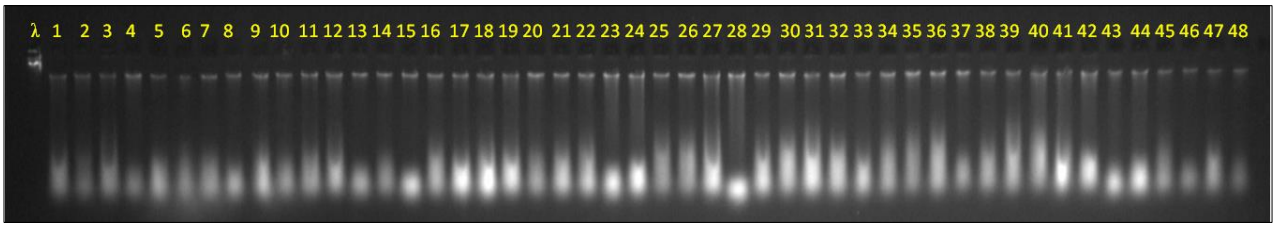

Fig 1: Representative gel picture showing the quality of DNA of germplasm lines

SSR markers and PCR analysis

To carry out the genotyping of germplasm a total of 58 SSR (Simple Sequence Repeats) markers (Table 2) were chosen covering the 10 chromosomes and PCRs (Polymerase Chain Reaction) were set up using a thermal cycler. The PCRs were set up using Emerald premix (TaKaRa) after adding the respective forward and reverse primers at the desired concentration and then adding this master mix to individual DNA samples (2 µl @ 50ng/ µl) to make up a volume of 10 µl. The PCR profile set up to carry out the reactions has been depicted in Fig. 2. The PCR products of the respective markers were then loaded in an ethidium bromide stained 3% agarose gel and subjected to electrophoresis. The gels were viewed and documented with the help of gel documentation system.

Table 2: List of SSR markers used for genotyping of the germplasm

S. No.	Marker	Chr.	S. No.	Marker	Chr.	S. No.	Marker	Chr.	S. No.	Marker	Chr.
1	RM 140	1	16	RM3212	2	31	RM1136	4	46	RM216	10
2	RM11943	1	17	RM71	2	32	RM317	4	47	RM304	10
3	RM12091	1	18	RM231	3	33	RM255	4	48	RM269	10
4	RM572	1	19	RM7332	3	34	RM16368	4	49	RM287	11
5	RM6703	1	20	RM545	3	35	RM3	6	50	RM28048	12
6	RM488	1	21	RM175	3	36	RM19367	6	51	RM511	12
7	RM212	1	22	RM186	3	37	RM510	6	52	RM28166	12
8	RM3825	1	23	RM416	3	38	RM407	8	53	RM28040	12
9	RM526	2	24	RM16030	3	39	RM38	8	54	RM7195	12
10	RM525	2	25	RM520	3	40	RM256	8	55	RM491	12
11	RM227	2	26	RM523	3	41	RM201	9	56	RM101	12
12	RM87	2	27	RM22	3	42	RM410	9	57	RM28130	12
13	RM5791	2	28	RM3387	3	43	RM496	10	58	RM28199	12
14	RM324	2	29	RM5686	3	44	RM228	10			
15	RM1367	2	30	RM349	4	45	RM147	10			

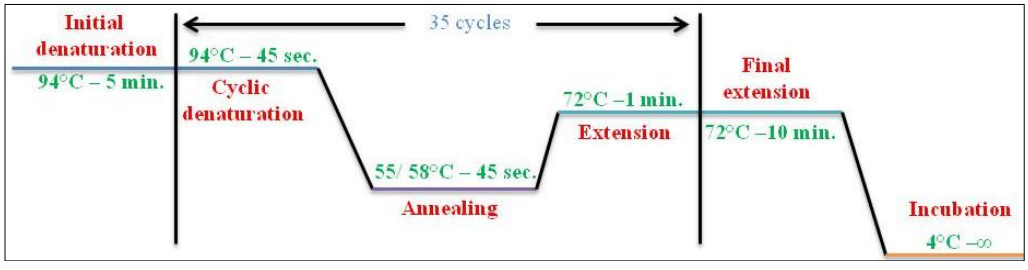

Fig 2: Thermal cycler profile for polymerase chain reactions

Scoring and diversity analysis

The documented gel images of respective markers (only polymorphic) were used to score the genotypes for each marker separately. The scoring was done as '1' and '0' for presence and absence of an allele respectively for each marker for as many alleles as obtained in the gel for that marker. After scoring gels pertaining to all the polymorphic markers, PIC (Polymorphism Information Content) values were calculated and the scoring data was used to assess the genetic diversity of the germplasm using DARwin 6.0 software (Perrier *et al*., 2003) [20]. The result was obtained in the form of a dendrogram.

Results and discussion

PCR analysis

The PCR analysis was done for all 110 samples with 58 different SSR markers. Out of these a total of 40 markers were found to be polymorphic. A representative gel picture showing the alleles obtained in the germplasm with marker RM16030 has been clearly depicted in Fig.3. The polymorphism percentage ranged from 55.6% (on chromosome 12) to 100% (on chromosome 8) and the overall polymorphism percentage obtained was 69.0% (Table 3).

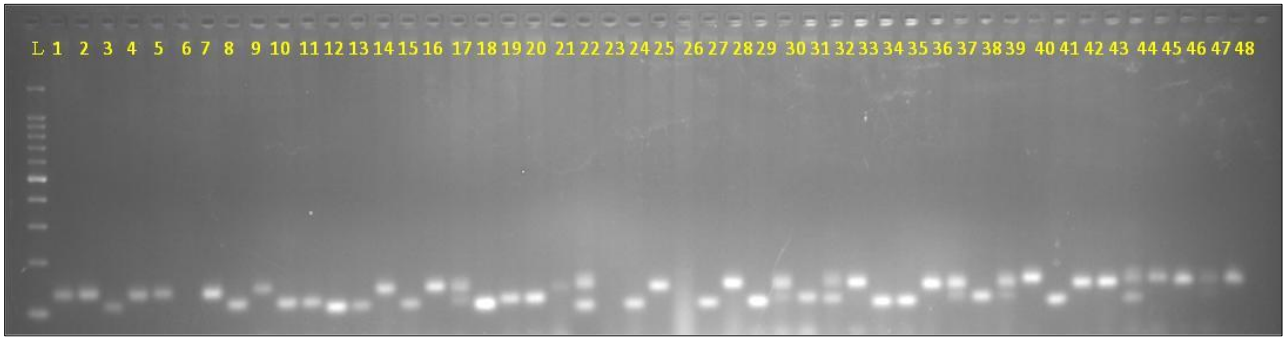

Fig 3: A representative gel picture showing amplification of germplasm DNA samples with marker RM16030

Table 3: Chromosome wise polymorphism percentage

Chr. No.	No. of Markers Tested	No. of Polymorphic Markers	Percent Polymorphism
1	8	7	87.5%
2	9	7	77.8%
3	12	9	75.0%
4	5	3	60.0%
5	0	0	0.0%
6	3	2	66.7%
7	0	0	0.0%
8	3	3	100.0%
9	2	0	0.0%
10	6	4	66.7%
11	1	0	0.0%
12	9	5	55.6%
Total	58	40	69.0%

Scoring and diversity analysis

After scoring the genotypes for 40 polymorphic markers the information was used to calculate the PIC values of these markers. Detailed information of marker wise PIC values has been given in Table 4. A total of 105 alleles were scored with the number of alleles per marker ranging from minimum 2 to maximum 4 with an average of 2.63 alleles. A similar result of lower average allele number ranging between 2.4 to 3.35 was reported by Singh *et al*. (2000) [25] and Gowda *et al*. (2012) [13]. Another report of lower genetic diversity with an average of 2.75 alleles per locus was given by Shah *et al*., 2013. On the contrary a high average allele number per locus of 6.6 and 14.6 were also reported (Thomson *et al*., 2007 and Jin *et al*., 2010) [27, 15]. Total of three markers (RM572, RM1367 and RM38) showed presence of alleles of four different sizes in the germplasm. Out of the remaining markers, 19 markers had 3 alleles each and 18 markers had 2 alleles each. The PIC values for the markers ranged from 0.55 (RM525) to 0.98 (RM488) with an average of 0.84. In a study carried out by Anandan *et al*., 2016 [3] a nearly similar data for total alleles and average alleles per locus of 128 and 3.28 respectively and a lower average PIC value of 0.24 was reported with 39 SSR markers. In another study conducted by Aljumaili *et al*. (2018) [2] a total of 131 alleles with an average of 4.09 alleles per locus and average PIC value of 0.61 were reported using 32 highly polymorphic SSRs. On the other hand, a study carried out by Behera *et al*., 2012 [5] reported 4.69 alleles per locus with an average PIC value of 0.81 among the landraces having different therapeutic values with a set of 36 genome wide SSRs. In yet another study taken up by Sandhu *et al*., 2012 [23], a total of 260 alleles were detected with an average allele number of 5.1 alleles per locus and an average PIC value of 0.67 with the help of 51 markers (50 SSRs and 1 gene specific marker).

Table 4: Marker wise number of alleles and respective PIC values of polymorphic markers

Marker	Alleles	Pic	Marker	Alleles	Pic	Marker	Alleles	Pic
RM11943	2	0.72	RM231	3	0.89	RM407	2	0.87
RM12091	3	0.97	RM7332	2	0.75	RM38	4	0.94
RM572	4	0.88	RM545	3	0.86	RM256	3	0.85

RM6703	2	0.8	RM186	3	0.84	RM496	3	0.93
RM488	3	**0.98**	RM16030	3	0.85	RM228	3	0.89
RM212	2	0.7	RM523	3	0.57	RM216	3	0.91
RM3825	3	0.86	RM22	2	0.81	RM269	2	0.9
RM526	2	0.75	RM3387	3	0.85	RM28048	2	0.65
RM525	2	**0.55**	RM520	3	0.92	RM28166	3	0.93
RM5791	2	0.76	RM349	2	0.92	RM7195	2	0.87
RM324	3	0.91	RM317	2	0.92	RM28130	2	0.83
RM1367	4	0.84	RM255	3	0.89	RM28199	2	0.82
RM3212	2	0.76	RM3	3	0.96			
RM71	3	0.9	RM510	2	0.8			

The scoring data of all 40 polymorphic markers with 110 genotypes was then used to carry out diversity analysis using DARwin 6.0 software. The unweighted neighbour joining tree obtained as a result of analysis has been depicted in Fig.4. The genotypes grouped into four distinct clusters.

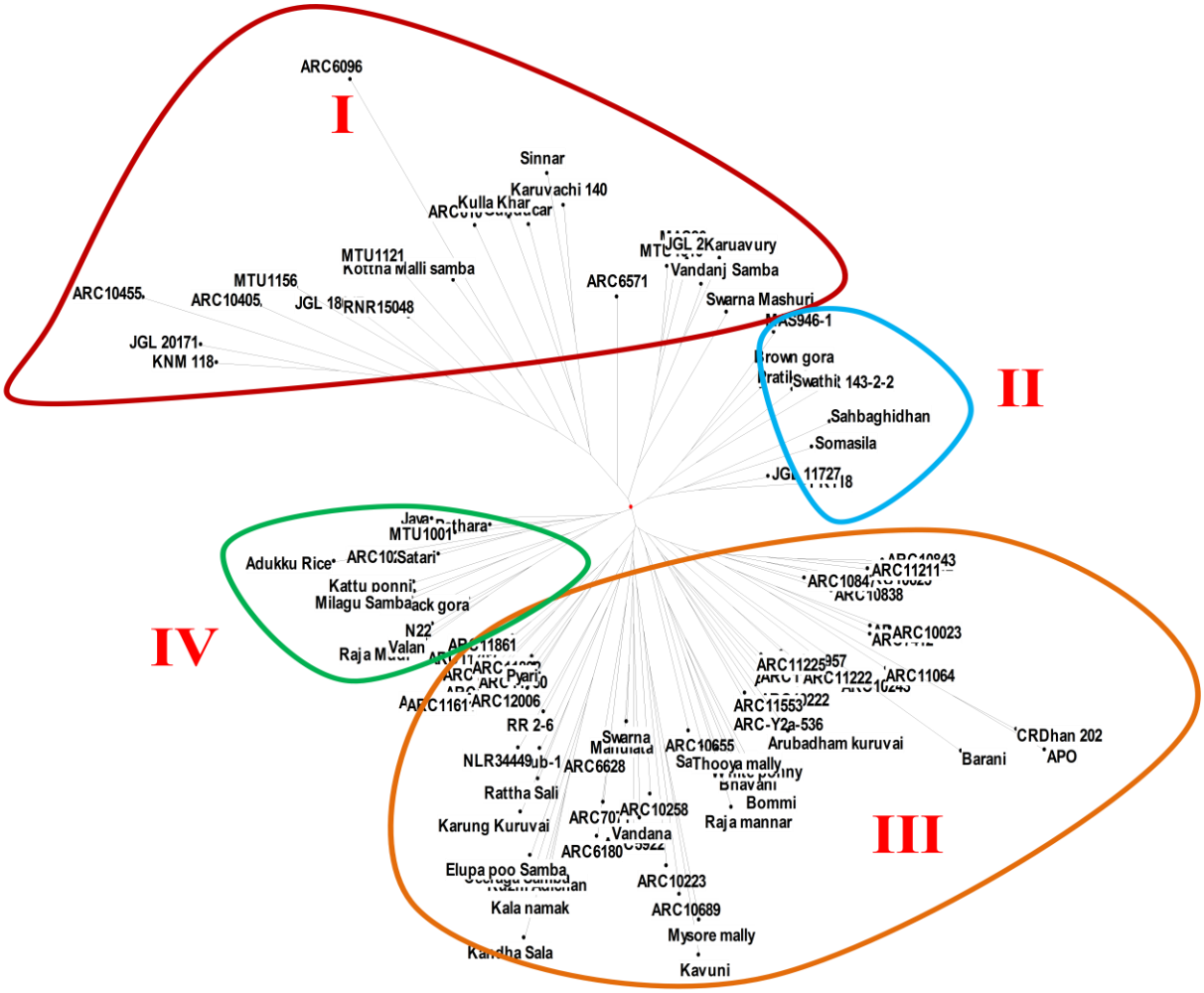

Fig 4: Unweighted neighbour joining tree depicting the genotypic diversity among germplasm

Which further diverged into two sub-clusters each. The clusters I, II, III and IV contain 22, 9, 65 and 14 genotypes respectively. Detailed information regarding the cluster composition has been given in Table 5. Cluster I predominantly contains varieties and landraces for irrigated conditions that were tested under aerobic cultivation in this study. The genetic relatedness of these lines can be reasoned by repeated involvement of similar parents in the process of their development (mostly in case of established varieties). Cluster II has lines that have established under aerobic situations and are also being used as competent parents for aerobic rice breeding. The reason that these lines formed a same cluster may also be their genetic relatedness in terms of their origin and parentage. The third cluster with maximum number of genotypes (65) is dominated by ARC (Assam Rice Collection) lines, the reason being their common geographical origin, thus the genetic relatedness. The last cluster is a mixture of lines suitable for aerobic cultivation and a landraces from Tamilnadu. This cluster does not follow geographical discrimination, implying that there may be an ancestral relatedness existing in these lines in terms of their genome composition.

Many researchers have relied on SSR markers to understand the diversity present in a wide variety of rice accessions all over the globe. In a very similar study involving 15 aerobic and lowland Basmati, *indica* and *japonica* rice varieties carried out by Sandhu *et al.* (2012) [23], a set of 51 genetic markers (50 SSRs and one *BADH2* gene specific marker) was

used to assess the genetic diversity. Cluster analysis resulted information of two distinct groups, first one including only Basmati genotypes and the second one further divided into sub-groups having *indica*, aerobic and *japonica* genotypes. The study concluded that there was more genetic divergence between Basmati and aerobic rice varieties as compared to lowland *indica* and aerobic rice varieties.

In another study carried out by Upadhyaya *et al*. (2011) [29] a set of 29 rice varieties from India were tested for their diversity with the help of 20 genome wide SSRs. These varieties divided into two distinct clusters based on their parental origin. In yet another study carried out by Bonny *et al*., 2015 [7] a set of 99 genotypes from Africa were tested using 10 highly polymorphic SSR markers that were multiplexed to increase their throughput. The dendrogram detected 5 distinct genetic groups and analysis of molecular variance indicated that 97% of the diversity observed was explained by differences in the genotypes themselves, and only 3% was due to the sources from which the genotypes were obtained.

On the other hand, in a study done by Ahmad *et al*., 2015 [1], 42 coloured rice genotypes were selected for determination of their genetic divergence using 25 simple sequence repeat (SSR) primers, out of which 20 showed distinct and reproducible polymorphism. A dendrogram constructed using the SSR primers clustered the 42 coloured rice genotypes into 7 groups and the clustering was mainly based on the country and region of origin. In another study taken up by

Table 5: Detailed cluster information of the Dendrogram

S. No.	Cluster	Sub-Cluster	No. of Entries	Names Of Entries
1.	Cluster I	Cluster Ia	16	ARC6096, ARC6101, ARC6571, ARC10405, ARC10455, JGL 20171, JGL 18047, KNM 118, Gunducar, Kulla Khar, Kottha Malli samba, MTU1121, MTU1156, RNR15048, Karuvachi 140, Sinnar
		Cluster Ib	6	MAS26, MTU1010, JGL 24423, Karuavury, Swarna Mashuri, Vandanj Samba
2.	Cluster II	Cluster IIa	5	Brown gora, Pratikshya, CR 143-2-2, MAS946-1, Swathi
		Cluster IIb	4	PR118, Somasila, JGL 11727, Sahbaghidhan
3.	Cluster III	Cluster IIIa	33	ARC6143, ARC7412, ARC10023, ARC10222, ARC10243, ARC10260, ARC10612, ARC10625, ARC10655, ARC10827, ARC10838, ARC10843, ARC10847, ARC10957, ARC11064, ARC11121, ARC11211, ARC11222, ARC11225, ARC11241, ARC-Y2a-536, ARC11553, Satyabhama, APO, CRDhan 202, Arubadham kuruvai, Barani, Bommi, Ottadam, Bhavani, White ponny, Raja mannar, Thooya mally
		Cluster IIIb	32	ARC5922, ARC6180, ARC6628, ARC7071, ARC10223, ARC10258, ARC10689, ARC11515, ARC11566, ARC11611, ARC11691, ARC11702, ARC11750, ARC11861, ARC11892, ARC12006, Pyari, Swarna sub-1, Mahulata, Vandana, NLR34449, RR 2-6, Swarna, Kandha Sala, Kala namak, Kavuni, Karung Kuruvai, Kuzhi Adichan, Rattha Sali, Ceeraga Samba, Elupa poo Samba, Mysore mally
4.	Cluster IV	Cluster IVa	8	ARC5973, ARC6153, Black gora, N22, Kattu ponni, Milagu Samba, Raja Mudi, Valan
		Cluster IVb	6	ARC10248, Jaya, Pathara, Satari, MTU1001, Adukku Rice

Masuduzzaman *et al*. (2016), 160 rice varieties from the tidal and flood prone areas of south and south East Asian countries were analyzed using 30 polymorphic SSRs. Cluster analysis divided the genotypes into four main clusters and six sub-clusters. The basis of grouping of these genotypes was their geographical origin and their ecotype. In yet another recent work done by Aljumaili *et al*., 2018 [2], a set of 53 rice accessions were genotyped with 32 polymorphic SSR markers. Cluster analysis grouped the 53 accessions into 10 distinctive clusters. The result of PCA showed clear geographical correspondence to the accessions with grouping pattern.

As evident from the literature, a systematic, intensive and product oriented breeding for aerobic rice cultivars is just beginning and needs more and more exploration of germplasm to understand the diversity that can be captured and moulded into a product that would be useful for predominantly marginal farming community of the country who will be facing both physical and economic scarcity of water in near future. This study thus helps the rice researchers to get a reasonable insight into the genetic diversity present among different rice lines collected from various parts of the country. This piece of research would help a breeder choose genetically diverse candidates while designing a breeding program with an aim to develop varieties suitable for aerobic cultivation without compensating the yield.

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