SSR marker diversity analysis for WA cytoplasm based fertility restorer genes of hybrid rice (*Oryza sativa* L.)

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ABSTRACT

An understanding of genetic diversity among the parental lines is useful in hybrid rice breeding through informed selection of the parental lines to maximize heterosis. Present investigation was carried out to estimate genetic divergence among the 51 restorer and five CMS lines by using 16 simple sequence repeats (SSR) markers co-segregating with fertility restorer genes (*Rf_3*, *Rf_4* and *Rf_7*) of hybrid rice. Among them, 10 SSRs were found to be polymorphic and the number of amplified fragments ranged from one to five. The highest PIC value (more than 0.60) was observed for eight primers *viz.*, AB 443, RM 3, RM 29, RM 226, RM 228, RM 304, RM 1812 and RM 3873 with average PIC value of 0.444. Cluster analysis using NTSYS generated dendrogram divided all the 56 parental lines into thirteen different clusters 80 % coefficient of similarity. With greater polymorphism revealed by SSR markers, parental lines having the similar genetic background from pedigree information were grouped into different clusters. The combination of pedigree analysis and SSR markers could be a more reliable method to study the diversity and grouping of parental lines of hybrid rice.

Key words: SSR markers, Diversity, Restorer lines, Maintainer line

Hybrid rice breeding is considered as one of the promising, practical, sustainable and eco-friendly options to break the yield ceiling in rice (Sheeba *et al*., 2009). Production of rice hybrids using a CMS system is based on cytoplasmic male sterility and fertility restoration systems, hence it involves male sterile (A), maintainer (B) and restorer (R) lines. CMS is a maternally inherited trait characterized by inability of a plant to produce functional pollen grains which is associated with abnormal open reading frame (ORF) found in mitochondrial genome and male fertility can be restored by fertility restoration (*Rf)* genes associated with nuclear genes encoding pentatrico peptide repeats (PPR) proteins (Hanson and Bentolila, 2004). Several CMS / *Rf* systems *viz.*, Wild Abortive (WA), Dissi, Gambiaca, Boro Type II (BT) and Hongolian (HL) CMS systems have been identified. The WA - CMS system is a sporophytic system, in which pollen grains are aborted at uninucleate stage. Hence, it is more stable than other CMS systems. (Sattari *et al*., 2008).

CMS and restorer lines are difficult to identify by using conventional methods because the procedure for identification is labor intensive. Even though some morphological markers linked to CMS governing genes, their unstable pattern of inheritance limits its utility in hybrid rice breeding programme. Though morphological markers have been employed in assessing the underlying genetic diversity of a species, the accuracy of these markers is not always confirmative. The availability of low number of morphological and biochemical markers, their poor and unknown genetic control, environmental influence and phenotypic expression, stage specific identification and procedural difficulties are known impediments in using them alone as genetic markers for diversity analysis. Hence employment of molecular markers is the best strategy for discriminating and differentiating CMS line from restorer lines. Microsatellite or SSR markers are simple tandemly repeated, di to tetra nucleotide sequence motifs flanked by unique sequences. They

are valuable as genetic markers because they detect high levels of allelic diversity, co-dominant inheritance and are easily assayed by PCR (Jain *et al*., 2004). Previously, SSR markers have been used for studying genetic diversity and fine mapping of fertility restoration genes by Jing *et al.* (2001) and Ahmadikhah *et al*. (2006), Bazrkar *et al.* (2008), Sheeba *et al*. (2009), and Sattari *et al.* (2009). Hence, the present study was conducted with the objective of assessing the genetic divergence among the parental lines of hybrid rice in terms of fertility restorer genes using SSR markers which are co segregating with Rf_3, Rf_4 and Rf_7 genes.

MATERIALS AND METHODS

Plant materials

Five male sterile or CMS lines (COMS 23A, COMS 24A, COMS 25A, CRMS 31A and CRMS 32A) and fifty one tester lines (18 AICRIP parental lines, 13 recently stabilized breeding lines and 20 advanced cultures / lines under evaluation) formed the genetic material for the present study (Table 1). Pure seeds of CMS lines were obtained from Paddy Breeding Station, Centre for Plant Breeding and Genetics (CPBG), Coimbatore and tester seeds were obtained from Tamil Nadu Rice Research Institute, Aduthurai.

Isolation of genomic DNA

Total genomic DNA was isolated from 56 parental lines at tillering stage (45 DAS) from fresh leaves of each genotype following the CTAB (Cetyl Trimethyl Ammonium Bromide) procedure as described by Dellapota *et al.* (1983). Seventeen selected SSR markers co segregating with fertility restorer genes (Rf_3, Rf_4 and Rf_7) is given in Table 2. The isolated genomic DNA was quantified by NanoDrop^{TM} 1000 Spectrophotometer. Further, the isolated DNA was checked for intactness, homogeneity and purity by electrophoresis in 0.8 % agarose gel.

PCR amplification

PCR reactions were carried out in PTC (Programmable Thermal Cycler) MJ research Inc. USA. The reaction volume was 15 µl containing 2 µl of genomic DNA, 1X assay buffer, 200 µM of dNTPs, 2 µM $MgCl_2$, 0.2 µM each primer (Forward and Reverse) and 1 unit of *Taq* polymerase (Banglore Genei). The temperature cycles were programmed as 95^0C for 2 min, 94^0C for 45 sec, 55^0C for 1 min, 72^0C for 1.30 min for 35 cycles and additional temperature of 72^0C for 10 min for extension and 4^0C for cooling. The amplified PCR products were separated in 3% metamorpho agarose gel prepared in 1X TBE buffer stained with Ethidium bromide. The gel was run in 1X TBE buffer at a voltage of 90 V for a period of 45 min to 1 hr. The gel was visualized in UV trans-illuminator and photographs taken using Alpha Digidoc gel documentation instrument. Clearly resolved, unambiguous bands were scored visually for their presence or absence with each primer. The scores were obtained in the form of matrix with '1' and '0', which indicate the presence and absence of bands in each genotype.

Data analysis

Clear and distinct bands amplified by SSR primers were scored for the presence (1) or absence (0) of the corresponding bands among the genotype. The data were entered in to binary matrix and subsequently analyzed using NTSYSpc ver 2.2 (Rohlf *et al*., 1994). Coefficient of similarity was calculated by using Jaccard's coefficient by SIMQUAL sub-function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic mean) method by SAHN clustering sub-function of NTSYSpc. Relationships among rice genotypes were graphically represented in the form of dendrogram. The PIC value of each locus was calculated as,

$$\mathbf{PIC_J = 1 - \sum_{i-1\ to\ L} P_{ij}^2}$$

Where,

P_{ij} is the relative frequency of the i^{th} allele for the locus j and is summed across all the alleles (L) over all lines. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values may range from 0 (monomorphic) to 1 (very highly discriminative), with many alleles in equal frequencies.

Table 1. Details of the parents used in the study

Tester lines			
Sl. No	**Name of the entry**	**Parentage**	**Source**
AICRIP parental lines			
1	IET 19863	GR 11 / Pusa Basmati 1 / 6-12-1-4-1-3	**AICRIP**
2	IET 19922	IET 12356 / IR 64	
3	IET 20601	OR 1530 – 8 / IR 68181 – B- 49	
4	IET 20605	SYE – 1 / Tadukan	
5	IET 20881	Abhaya / SKL – 8	
6	IET 20885	Improved White Ponni / Tetep	
7	IET 20888	ADT 38 / CR 1014	
8	IET 20890	GR – 11 / Pusa Basmati 1 / 6- 12- 1- 4 – 1 – 4 -3 -2	
9	IET 20895	Erramallelu / RNR – M7	
10	IET 20896	Erramallelu / Kavya	
11	IET 20897	NLR – 145 / Kavya	
12	IET 20898	NLR – 145 / Kavya	
13	IET 20899	Tella Hamsa / Erramallelu	
14	IET 20904	Kavya / RNR – M7	
15	IET 20932	Keshava / NLR – 33655	
16	IET 20937	OR – 1530 – 8 / IR 68181 – B – 49	
17	IET 20944	Jaya / Mahsuri	
18	IET 20945	Kavya / BPT 5204 // Vijetha	
Recently stabilized breeding lines			
19	AD 09522	Imp Rascadam / ASD 19 – 2-1-7-4-1	**TRRI, Aduthurai**
20	AD 09523	I.W.Ponni / Ratna – 1-1-2-3-1	
21	AD 09524	ASD 19 / WGL 32100 – 1-3-3-11-5	
22	AD 09525	ASD 19 / WGL 32100 – 13-2-2-1-1	
23	AD 09526	WGL 32100 / Swarna – 7-2-3-3-1	
24	AD 09527	BPT 5204 / Imp Rascadam – 4-7-1-2-1	
25	AD 09528	BPT 5204 / Imp Rascadam – 2-4-5-6-6	
26	AD 09529	I.W.Ponni / Kalajoha – 5 -1 – 5 - 4	
27	AD 09530	I.W.Ponni / Kalajoha – 21-3-3-3-1	
28	AD 09531	BPT 5204 / Azucena – 6-2-2-3-1	
29	AD 09532	ADT 43 / WGL 32100 – 7-3-3-2-2	
30	AD 09533	ADT 43 / WGL 32100 – 12-3-4-3-1	
31	AD 09534	ADT 43 / WGL 32100 – 3-3-7-10-1	
Advanced Cultures / lines under evaluation			
32	AD 04072	Improved White Ponni / P 1110 – 5 – 1 - 2	**TRRI, Aduthurai**
33	AD 06084	ADT 42 / JGL 384	
34	AD 07073	ADT 43 / JGL 384	
35	AD 07076	ADT 43 / JGL 384	
36	AD 07083	ADT 43 / I. W. Ponni	
37	AD 07158	BPT 5204 / AD 02235	
38	AD 07309	Ajaya / IET 11613 – 22 – 5 – 2	
39	AD 08005	ADT 43 / ACK 03002	
40	AD 08009	ADT (R) 45 / AD 01236	
41	AD 08010	ADT (R) 45 / AD 01236	
42	AD 08013	ADT (R) 45 / ADT (R) 47	
43	AD 09193	ADT 43 / JGL 384	
44	AD 09194	ADT (R) 47 / Karnataka Ponni	
45	AD 09203	ADT 43 / ADT 37	
46	AD 09206	ADT 43 / ADT 37	
47	AD 09216	ADT (R)45 / AD 01236	
48	AD 09222	ADT (R)45 / IET 18208	
49	AD 09223	ADT (R)45 / IET 18208	
50	AD 09231	PY 3 / ADT (R) 47	
51	AD 09241	AD 01236 / AD 01205	

Male sterile and Maintainer Lines		
52	COMS 23B	Coimbatore
53	COMS 24B	Coimbatore
54	COMS 25B	Coimbatore
55	CRMS 31B	Cuttack
56	CRMS 32B	Cuttack

Table 2. List of SSR markers used for restorer gene diversity

Sl. No	Primer	Sequences	Annealing Temperature (^{0}C)	Chromosomal Location	Linked genes	Genetic distance (cM)	Author
1	RM1	F - GCGAAAACACAATGCAAAAA R - GCGTTGGTTGGACCTGAC	54.0	1	*Rf*$_3$		
2	RM3873	F - GCTAGCTAGGACCGACATGC R - CCTCCTCCTTATCCTCCCTG	56.0	1	*Rf*$_3$	14	Alavi *et al.*, (2009)
3	RM3233	F - GTGGTGAGTAAACAGTGGTGG R - GAGAGCAGAGCAGAGGCAAC	57.0	1	*Rf*$_3$	14.5	Sattari *et al.*, (2008)
4	RM315	F - GAGGTACTTCCTCCGTTTCAC R - AGTCAGCTCACTGTGCAGTG	56.5	1	*Rf*$_3$	20.7	Bazrkar *et al.*, (2008)
5	RM490	F - ATCTGCACACTGCAAACACC R - AGCAAGCAGTGCTTTCAGAG	56.0	1	*Rf*$_3$	2.8	Sattari *et al.*, (2008)
6	RM443	F - GATGGTTTTCATCGGCTACG R - AGTCCCAGAATGTCGTTTCG	54.5	1	*Rf*$_3$	4.4	Bazrkar *et al.*, (2008)
7	RM171	F - AACGCGAGGACACGTACTTAC R - ACGAGATACGTACGCCTTTG	56.0	10	*Rf*$_4$	3.16	Ahmadikha *et al.*, (2006)
8	RM6737	F - CATTGGGGGTGGATAAAGAG R - TATCCTCTACTCCCTCGGCC	55.5	10	*Rf*$_4$	1.55	Ahmadikha *et al.*, (2006)
9	AB443-500	F - CACTCGTATAGTAACTTGAGACGAT R - CCAAACAAGTTCCCACAATAGA	53.4	10	*Rf*$_4$	1.03	Ahmadikha *et al.*, (2006)
10	RM304	F - TCAAACCGGCACATATAAGAC R - GATAGGGAGCTGAAGGAGATG	53.5	10	*Rf*$_4$	2.05	Ahmadikha *et al.*, (2006)
11	RM6100	F - TCCTCTACCAGTACCGCACC R- GCTGGATCACAGATCATTGC	56.5	10	*Rf*$_4$	2.3	Sattari *et al.*, (2008)
12	RM1108	F - GCTCGCGAATCAATCCAC R - CTGGATCCTGGACAGACGAG	55.5	10	*Rf*$_4$	1.6	Sattari *et al.*, (2008)
13	RM5841	F - CCTCTCTCTCTCTCCCCC R - TGTTATTGGCACGTGGTGTG	57.0	10	*Rf*$_4$	6.67	Sattari *et al.*, (2008)
14	RM6344	F - ACACGCCATGGATGATGAC R – TGGCATCATCACTTCCTCAC	55.5	7	*Rf*$_4$	13.3	Bazrkar *et al.*, (2008)
15	RM228	F – CTGGCCATTAGTCCTTGG R – GCTTGCGGCTCTGCTTAC	55.0	10	*Rf*$_4$	3.4	Jing *et al.*, (2001)
16	RM7003	F - GGCAGACATACAGCTTATAGGC R – TGCAAATGAACCCCTCTAGC	55.5	12	*Rf*$_7$	13.3	Bazrkar *et al.*, (2008)

RESULTS AND DISCUSSION

Analysis for *Rf*$_3$ and *Rf*$_4$ gene by SSR markers

In the amplification of genomic DNA of 56 hybrid rice parental (5 CMS and Restorer lines) genotypes using 16 SSR primers co segregating with *Rf*$_3$, *Rf*$_4$ and *Rf*$_7$ genes 10 SSRs were found to be polymorphic. In the present investigation, analysis for *Rf*$_3$ gene was performed with six SSR markers *viz.*, RM1, RM315, RM443, RM490, RM3233 and RM3873 and these SSRs amplified a total of 322 alleles with an average of 61.33 alleles per primer. Analysis for *Rf*$_4$ gene was performed with nine SSR markers *viz.*, RM171, RM228, RM304, RM1108, RM5841, RM6100, RM6344, RM6737, AB443-500 and *Rf*$_7$ gene was amplified using RM7003. SSRs co segregating with Rf$_4$ genes amplified 472 alleles with an average of 63.5 alleles per SSR. The number of amplified fragments ranged from one to five. Of the total amplified bands, the average polymorphic allele per primer is 2.7. Electrophoresis profile of RM 3873 is given in Fig 1**.** Among the 16 primers RM 304 (5), RM 228 (4), RM 171 and RM 3873 (3) produced the maximum number of alleles. The PIC values

representing allelic diversity and frequency among genotypes varied from one locus to another. The PIC values of these markers ranged from 0.038 (RM443) and 0.640 (RM3873) respectively (Table 3). The PIC values for these markers which were reported earlier as flanking markers for Rf_4 gene were 0.336 (RM1) and 0.691 (RM304) respectively. Results of this study, *i.e.*, number of alleles and PIC value are comparable to those reported by Cho *et al.* (2000), Yu *et al.* (2003), Thomson *et al.* (2007), Lapitan *et al.* (2007) and Seetharam *et al*. (2009). The above results provided an overview of the genetic diversity in all the genotypes. From this study it has been concluded that, greater diversity has been observed for Rf_4 genes rather Rf_3 and Rf_7.

Cluster analysis of molecular data

The cluster analysis using NTSYS generated dendrogram (**Fig 2**) throws light on genetic similarity among the genotypes. It divided 56 parental lines into seventeen distinct clusters at 85% coefficient of similarity (**Table 4**).

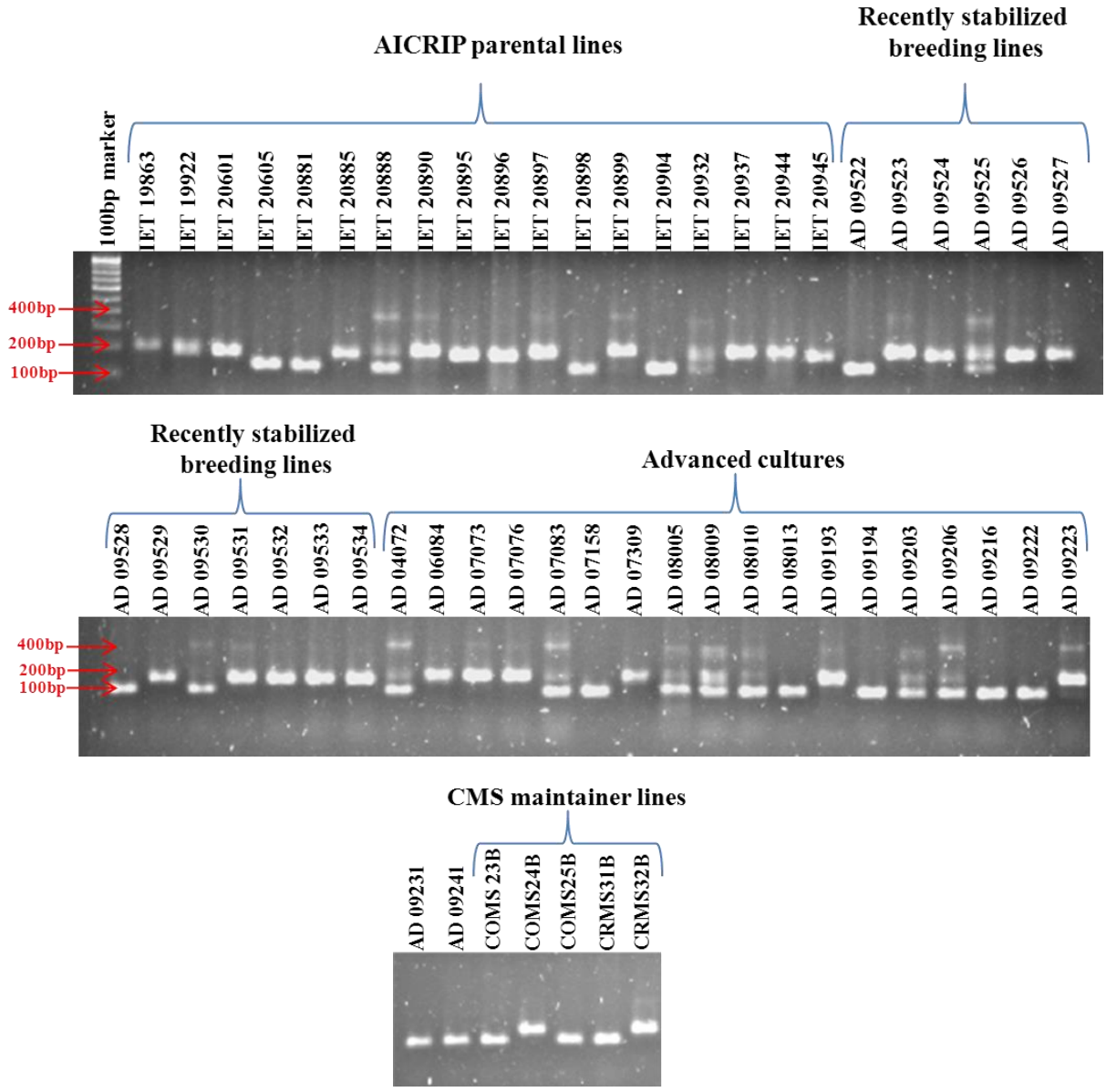

Fig 1. SSR polymorphism observed among the parental lines using RM 3873

Table 3. Number of alleles, percentage of polymorphism and PIC value of 10 polymorphic SSR markers

Sl. No	Primer	Number of alleles	Polymorphic bands	Percentage of polymorphism	PIC value
1	AB 443	2	2	100	0.497
2	RM1	2	2	100	0.336
3	RM171	3	2	66.67	0.492
4	RM228	4	3	75	0.666
5	RM304	5	4	80	0.691
6	RM443	2	2	100	0.038
7	RM490	2	1	50	0.338
8	RM1108	2	2	100	0.495
9	RM3873	3	3	100	0.640
10	RM6100	2	2	100	0.113
	Average	2.7	2.3	87.18	0.431

Table 4. Clustering pattern of 56 rice genotypes based on SSR markers co segregating with Rf_3, Rf_4 and Rf_7 genes (at 85 % similarity)

Cluster	Genotypes	No. of genotypes
I	IET19863,IET20605,AD09528,IET20881	4
II	IET19922,IET20601,IET20888	3
III	IET20890	1
IV	IET20896,IET20937,AD09534,IET20897,IET20945,AD09523,AD09525	7
V	IET20898,AD09222	2
VI	IET20904,AD09241,COMS23A,CRMS31A,COMS25A	5
VII	IET20885	1
VIII	IET20895,AD09223,IET20899,CRMS32A,COMS24A	5
IX	IET20932,AD08005,AD08009,AD09203,AD09206,AD08010,AD09193, AD09104,AD09216,IET20944,AD09532,AD09533,AD08013	13
X	AD09526,AD09531	2
XI	AD04072,AD07083	2
XII	AD06084,AD07076,AD07309	3
XIII	AD09522,AD09524	2
XIV	AD07158,AD09231	2
XV	AD09529	1
XVI	AD09530,AD07073	2
XVII	AD09527	1

Among the clusters, XI was the largest which contains 13genotypes (IET20932, AD08005, AD08009, AD09203, AD09206, AD08010, AD09193, AD09104, AD09216, IET20944, AD09532, AD09533, AD08013) and belonged to advanced cultures originated from TRRI, Aduthurai. Among the thirteen genotypes of cluster XI, six and four had ADT 43 and ADT (R) 45 as the female parents respectively. Recently stabilized breeding lines were grouped in nine different clusters, among them cluster IV contains a maximum of 3 genotypes (AD09534, AD09523, AD09525). Cluster X, XII contains two genotypes each and remaining six clusters had one recently stabilized breeding line each.

The genotype AD 09529 (I.W.Ponni × Kalajoha) and AD09527 (BPT 5204 / Imp Rascadam – 4-7-1-2-1) alone fell in a single cluster XV and XVII respectively. The genotypes from AICRIP were grouped in 9 clusters. Among the nine clusters, IV contains a maximum of four genotypes (IET20896, IET20937, IET20897 and IET20945) and cluster I and II had two genotypes each. Cluster III and VII exclusively contains AICRIP parental line. Interestingly, cytoplasmic male sterile (CMS) lines were grouped in cluster VI (COMS23A, COMS25A and COMS31A) and VIII (COMS24A and COMS32A) and the same clusters contains some of the restorer lines also. It reveals that with the use of

molecular markers linked or co-segregating with fertility restorer genes are not useful to discriminate the male sterile lines from restorer lines. In contrast to the morphological or biometrical markers, the molecular or geneic markers reveal polymorphism at the DNA level, suggesting a very powerful tool for characterization of genotype and estimation of genetic diversity. Among the molecular markers, microsatellite or SSR (simple sequence repeats) markers showed a high potential for identification and estimation of genetic diversity (Hashimoto *et al.*, 2004). In this study, newly stabilized breeding lines *viz.*, AD 09527 and AD 09528 with the same genetic background (AD 09527: BPT 5204 × Imp Rascadam – 4-7-1-2-1, AD 09528: BPT 5204 × Imp Rascadam – 2-4-5-6-6) were grouped into separate clusters *viz.*, I (AD 09528) and XVII (AD 09527). Likewise, AD 09529 and AD 09530 had the same genetic background (AD 09529: I.W.Ponni × Kalajoha – 5 -1 – 5 – 4, AD 09530: I.W.Ponni × Kalajoha – 21-3-3-3-1) but were also grouped into separate clusters (XV: AD09529 and XVI: AD09530). These results indicated that SSR analysis could be a better method to study the diversity among the parental lines of hybrid rice. Presently, molecular marker techniques have been widely used in studying genetic variation and diversity among populations, species and varieties (Hashimoto *et al.*, 2004, Yu *et al.*, 2005). In the present study, uses of SSR markers in hybrid rice breeding indicated that tester lines had wider genetic bases than the CMS maintainer lines.

In addition, there were clear and wider genetic differences between the CMS and restorer lines. The original two parental groups *viz.*, CMS lines and testers have become more complex, through the emergence of new patterns of heterotic combinations. Therefore, the partitioning of parental lines to new parental groups and subgroups (AICRIP cultures, stabilized breeding lines and advanced cultures) is more important than the recognition of two parental groups (*i.e.*, maintainer and tester / restorer group). Also SSR markers are an indispensable complementation to pedigree analysis in the identification of parental groups. In general, the pedigree analysis is considered to have no effect on selection and mutation. Therefore, pedigree analysis cannot reveal the relationship between progeny and their parents exactly.

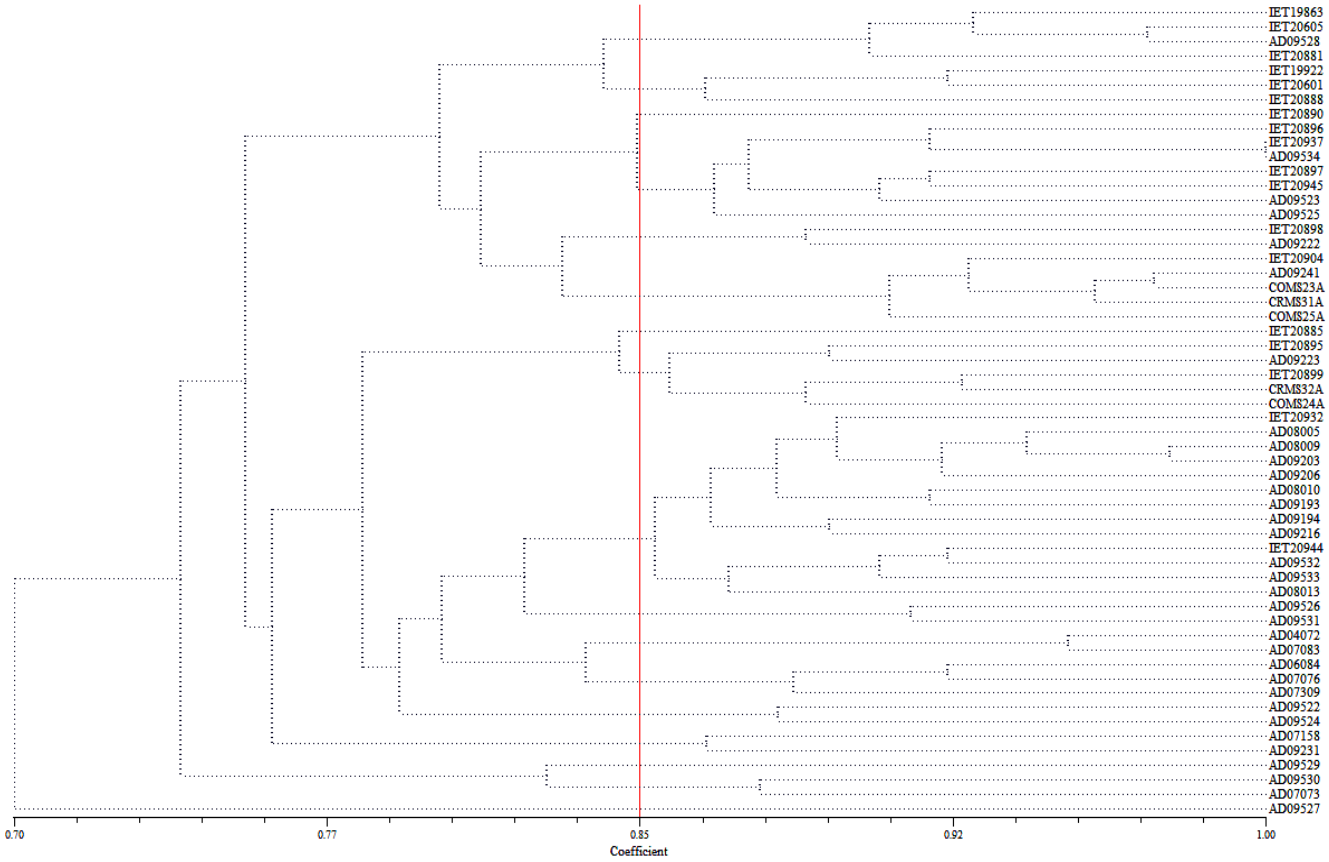

Fig 2. Dendrogram generate for fifty six genotypes using fifty five SSR markers

Also SSR markers are an indispensable complementation to pedigree analysis in the identification of parental groups. In general, the pedigree analysis is considered to have no effect on selection and mutation. Therefore, pedigree analysis cannot reveal the relationship between progeny and their parents exactly. On the contrary, SSR markers can detect genetic variation at DNA level. A combination of pedigree analysis and SSR markers will be helpful in more reliable grouping of hybrid rice parental lines.

It was observed form this investigation that the SSR markers involved could reveal the latent, diversity present in rice genotypes. Regarding fertility restorer genes, the study revealed that the highest allelic frequencies were observed in genotypes which are non-restorer. Similarly for allele length and allelic frequencies, the restorer failed to amplify maximum number of occasion when screened with different SSR markers. It was inferred that allelic length may not have any significant correlations with fertility restoration, since the sterility and restoration are controlled by a combination of cytoplasmic and nuclear genes, the expression of restorer genes may be influenced by large number of genetic and epigenetic factors. Exact complimentarity between the primers and genotypes may, to a certain extent help in identifying and characterizing fertility restorer genes.

CONCLUSION

During this experiment, the maintainer lines and tester lines of rice were clearly discriminated on the basis of SSR analysis. Furthermore, with higher polymorphism revealed by SSR markers, some of the parental lines having the similar genetic background from pedigree information were also grouped into different clusters. The identification of heterotic groups and patterns among breeding populations and lines provides fundamental information in order to help the plant breeders to gain more information on heterosis. Systematic studies on classifying the breeding lines into heterotic groups are limited. Therefore, the grouping result based on SSR analysis might be helpful to identify heterotic groups for hybrid rice breeding.

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