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Genetic diversity for fertility restoration and marker based screening of restorers for wild and dwarf abortive sterile lines

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ABSTRACT

Hybrid rice technology being one of the sustainable and eco-friendly options to break the yield ceiling in rice is currently adopted in different countries like China, India, Bangladesh, Myanmar, Philippines, Indonesia, Vietnam, etc. It is most popular in China covering more than 50% of its rice cultivation area. The three line system constituting cytoplasmic male sterile (CMS) line, a maintainer line and restorer line is more widely used for development of hybrid rice. CMS is often associated with the unusual open reading frames (ORFs) found in mitochondrial genomes leading to pollen sterility. The pollen fertility is restored by nuclear-encoded genes called fertility restorer (Rf) gene(s). Identification through molecular detection of the Rf genes/QTLs by using linked or direct markers is an easy method, after which phenotypic confirmation of Rf positive lines may be carried out. Many linked SSR markers are reported for different Rf genes/QTLs. In the present study, five linked markers namely RM228, RM202, RM35, RM18, RM17 for wild abortive (WA), boroII (BT) and dwarf wild abortive (DA) type restorers were used to identify presence of the fertility restorer QTLs qRf4, qRf11, qRf1 and qRf10-2. A moderate level of diversity parameters were observed from the set of the studied genotypes. The genotypes Krishnabhog, CR2690-3-2-1-1-1, Samba Mahsuri, Chandan, Saket4, Naveen, Lalat MAS, IR 84898-B-168-24-1-1-1, IET 22704 and Pusa 1592-6 showed presence of the fertility restoration QTLs qRF4, qRF11, qRF7 and qRF10-2. The genotypes like IR84887-B-156-17-1-1-1, Vandana, CO-41, Tapaswini MAS, Karjat-3, Tapaswini, Sonasali and BAS-366 observed to possess qRf4 and qRf10-2, whereas the genotypes Parijata, Annapurna, IR 84898-B-168-24-1-1-1, IR64 and CR3696-3-2-2-1-2 possessed qRf11 and qRf10-2. These lines, after further phenotypic confirmation for fertility restoration of WA, BT and DA type CMS lines can be used for future development of hybrid rice.

Key words: *Hybrid rice, fertility restoration, Rf genes, SSR markers*

Hybrid rice technology is considered to be one of the promising and sustainable and eco-friendly options to break the yield ceiling witnessed in rice. Hybrid rice technology is currently adopted in India, Bangladesh, Myanmar, Philippines, Indonesia and Vietnam. More than 50% of rice area in China covers hybrid rice cultivation. In India, around 2 million hectare area is used for hybrid rice cultivation (IIRR 2016). Two different approaches, *i.e.*, two-line system and three line system are being popular for hybrid rice development till date. The two-line system involves environmentally sensitive male sterility system. These kinds of hybrids are grown in limited areas due to their dependency on environmental conditions. The three-line system involves a cytoplasmic male sterile (CMS) line, a maintainer line and restorer line. This is the most popular method worldwide. Cytoplasmic male sterility (CMS) occurs widely in higher plants, and results in failure to produce functional pollens. CMS is a maternally inherited trait, and is often associated with the unusual ORFs found in mitochondrial genomes (Hanson and Bentolila 2004). Pollen fertility is restored by nuclear-encoded genes called fertility restorer (*Rf*) gene (Itabashi *et al*. 2011). In addition to the

commercial use of CMS/ *Rf* systems, they play important role in elucidating genetic interactions and cooperative functions of mitochondrial and nuclear genomes in plants (Fujii and Toriyama 2008).

In rice, various types of CMS/Rf systems have been reported (Kinoshita 1997; http:// www.gramene.org/index. html). They include WA-type, DA-type, BT-type, HL-type, etc. WA-CMS originating from a wild abortive line (Lin and Yuan 1980) needs two major genes, *Rf*3 and *Rf*4 mapped to chromosomes 1 and 10, respectively to restore pollen fertility (Yao *et al*. 1997; Zhang *et al*. 1997; Ahmadikhah and Karlov 2006). HL-CMS (Honglian type) originated from red-awned wild rice (Rao 1988). Two fertility restorer genes, *Rf*5 and *Rf*6(t), mapped on chromosome 10 (Liu *et al*. 2004) are used for fertility restoration of HL-CMS. The BT-CMS was developed from Chinsurah Boro II, an *indica* variety (Shinjyo 1975). The *Rf* genes responsible for restoring the BT-CMS are *Rf*1 and *Rf*11 located on chromosome 10 and 11, respectively (Itabashi *et al*. 2011; Guang-Xian *et al*. 2005). CW-CMS originated from *Oryza rufipogon* Griff. and the gene *Rf*17 (previously designated *Rf*cw) located on chromosome 4 (Fujii and Toriyama 2005) is used for restoration of its pollen fertility. This is clear from above that many major genes along with many QTLs control the trait of fertility restoration in different type of CMS system. Even multiple genes and QTLs are involved for a single cytoplasm in different restorer lines and each restorer line may have a different combination of alleles of these genes and QTLs which ultimately decide the degree of fertility restoration ability of that particular line.

Identification of restorers that can efficiently restore the fertility of F_1 is the pre-requisite for developing high yielding heterotic hybrids. The process of phenotypic screening for fertility restoration trait is laborious and time consuming as it involves test crossing with a set of CMS lines and evaluation of genotype for pollen and spikelet fertility. The use of molecular markers linked to *Rf* genes can enhance the selection efficiency, save time and avoid the complications associated with phenotype-based screening. The present study aims to identify efficient restorer lines possessing different *Rf* genes using linked SSR markers and study the genetic diversity existing between them.

MATERIALS AND METHODS

Plant materials

The seeds of forty three selected genotypes along with two cytoplasmic male sterile lines CRMS32A and CRMS31A and their restorer line were collected from gene bank of National Rice Research Institute, Cuttack and were germinated in tray under controlled condition of RGA-cum-Phytotron facility (Table 1). The selected genotypes were better yielders with early to mid maturing type expected to give high standard heterosis with CMS lines.

Genomic DNA Extraction

Genomic DNA was extracted from the leaves of 30 days old seedlings. The leaf samples were crushed in liquid nitrogen with the help of tissue lyser II (Qiagen). The total genomic DNA was extracted using CTAB buffer (2% CTAB, 100mM Tris-HCl, 20mM EDTA, 1.3M NaCl with pH adjusted to 8.0) along with phenol-chloroform-isoamyl alcohol followed by RNase treatment and isopropanol precipitation. The DNA was washed with 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA). The DNA was quantified by agarose gel electrophoresis using commercial Lambda DNA as standard.

Amplification of markers by polymerase Chain Reaction

The amplification of target markers were performed in a reaction volume of 20µl containing 30ng of genomic DNA, 1 unit of Taq Polymerase, 200µM each of dATP, dCTP, dTTP, dGTP, 4pMole of each forward and reverse primers (Table 2), 1.5mM Tris HCL (pH 8.5), 50mM KCL, 2mM $MgCl_2$, and 0.1% TritonX-100 in a Gradient Thermal Cycler (Peqlab, Sigma). The temperature profile applied to the reaction mixture is as follows: an initial denaturation at 94^{0}C for 4mins followed by 35 cycles of 1 min denaturation at 94^{0}C, 1 min annealing at 55-59^{0}C, and 1 min extension at 72^{0}C, then final extension at 72^{0}C for 10 mins. The product was hold at 4^{0}C till removal of the sample from thermal cycler and then stored at -20^{0}C till further use.

Gel electrophoresis and documentation of amplified products

Agarose gel (3%) containing 0.8µg/ml Ethidium Bromide was used for electrophoresis of the PCR

Table 1. Genotypes used for molecular diversity for fertility restoration and restorers identification

Sl. No.	Name of the genotype	Remarks	Sl. No.	Name of the genotype	Remarks
1	CRMS31A	CMS line developed by ICAR-NRRI, Cuttack	24	Samba Mashuri	High yielding and MS grained variety of Andhra Pradesh
2	CRMS32A	Sterile line developed by ICAR-NRRI, Cuttack	25	Chandan	Cold tolerant variety of ICAR-NRRI, Cuttack
3	CRL32R	Irrigated rice culture developed by RRLRRS, Gerua	26	Saket4	Irrigated variety released by ICAR-NRRI,Cuttack
4	CR Dhan 601	Boro variety adapted to Odisha, West Bengal and Assam states	27	Parijata	Irrigated variety released by ICAR-NRRI,Cuttack
5	CR DHAN 300	Fine grain variety released for Odisha, Bihar, Maharashtra and Gujarat	28	Naveen	Irrigated variety released by ICAR-NRRI,Cuttack
6	IR84887-B-156-17-1-1-1	Early maturing and high yielding culture of IRRI	29	Tapaswini	Irrigated variety released by ICAR-NRRI,Cuttack
7	VLDhan-82	Irrigated early duration rice variety developed by VPKAS, Almora	30	Lalitgiri	Upland variety released by OUAT, Bhubaneswar
8	IR84899-B-183-6-1-1-4	Early maturing and high yielding culture of IRRI	31	Sonasali	
9	IR84894-B-140-6-1-1-1	Early maturing and high yielding culture of IRRI	32	Annapurna	Irrigated variety released by ICAR-NRRI,Cuttack
10	IR36	Irrigated rice variety	33	BAS-366	
11	RP 5311 (IET22729)	Irrigated rice culture	34	Lalat MAS	Irrigated variety released by ICAR-NRRI,Cuttack
12	Vandana	Upland variety of ICAR-NRRI, Cuttack	35	IR 84898-B-168-24-1-1-1	Early maturing and high yielding culture of IRRI
13	CO-41	High yielding variety of TNAU, Coimbatore	36	MGD-1104 (IET 22704)	
14	Pyari	Aerobic variety of ICAR-NRRI, Cuttack	37	Panindra	High yielding lowland variety of Assam
15	Tapaswini MAS	Irrigated variety of ICAR-NRRI, Cuttack	38	Swarna-Sub1	High yielding lowland variety of Odisha
16	Karjat-3	Rice variety for Maharashtra state	39	IR64	High yielding variety of Odisha
17	Satabdi	Early maturing and fine grained variety of ICAR-NRRI,Cuttack	40	CR2996-1-14-29-3-1 (IET 22731)	Irrigated culture of ICAR-NRRI, Cuttack
18	IR84899-B-179-16-1-1	Early maturing and high yielding culture of IRRI	41	Pusa 1592-6	Basmati rice culture of IARI, New Delhi
19	PTB 10	Pureline selection from Thekkencheera, Pattambi, Kerala	42	MTU1071	Mid duration rice culture from Maru Teru, Andhra Pradesh
20	Ranbir basmati	Long grained basmati rice cultivar	43	CR3696-3-2-2-1-2	Irrigated high yielding culture of ICAR-NRRI, Cuttack
21	Krishnabhog	Small grained aromatic rice cultivar	44	Sampad	High yielding rice variety for Andhra Pradesh
22	MAS-946	Aerobic variety of UAS, Bangalore	45	Padmanava	Lowland rice variety
23	CR2690-3-2-1-1-1	Irrigated culture of ICAR-NRRI, Cuttack	46	IET 21287	Irrigated variety CR Dhan 305 of ICAR-NRRI, Cuttack

products. 10µl of sample was loaded onto the gel and electrophoresed in 1X TBE (pH 8.0). 50bp DNA ladder was loaded at least to one lane to know the size of the amplicons. Electrophoresis was carried out at 3V/cm for 3.5 hrs and photographed using a Gel Documentation System (SynGene).

Scoring and analysis of data

The data were scored in a binary matrix on the basis of the presence (1) or absence (0) of amplified products for each genotype-primer combination. Data were analyzed and similarity matrix was constructed from binary data with Jaccard's coefficients and dendrogram was generated with unweighted pair group method arithmatic average (UPGMA) algorithm, with bootstrap value of 100 by using FreeTree software (Hampl *et al.* 2001; Pavalicek *et al*. 1999) and the dendrograms were visualized by Treeview 32 software (Page 1996).

Table 2. List of primers used in the study

Name	Sequence (5' -> 3')	Tm (^{0}C)	Linked QTL	Marker type	Repeat motif	Expected amplicon size (bp)	Reference
RM228	CTGGCCATTAGTCCTTGG (F) GCTTGCGGCTCTGCTTAC (R)	57.3 59.58	qRf4	flanking	(CA)6(GA)36	154	Ahmadikhah and Karlov, 2006; gramene.org
RM202	CAGATTGGAGATGAAGTCCTCC (F) CCAGCAAGCATGTCAATGTA (R)	60.07 55.75	qRf11	flanking	(CT)30	189	Guang-Xian *et al.* 2005; gramene.org
RM35	TGGTTAATCGATCGGTCGCC (F) CGACGGCAGATATACACGG (R)	59.85 59.72	qRf1	flanking	(GA)19	207	gramene.org
RM18	TTCCCTCTCATGAGCTCCAT (F) GAGTGCCTGGCGCTGTAC (R)	57.8 61.86	qRf7	flanking	(GA)4AA(GA)(AG)16	157	gramene.org
RM258	TGCTGTATGTAGCTCGCACC (F) TGGCCTTTAAAGCTGTCGC (R)	59.85 57.5	qRf10-2	flanking	(GA)21(GGA)3	148	gramene.org
RM17	TGCCCTGTTATTTTCTTCTCTC (F) GGTGATCCTTTCCCATTTCA (R)	56.35 55.75	qRf10-2	flanking	(GA)21	184	gramene.org

RESULTS AND DISCUSSION

SSR markers have been used reliably for studying genetic diversity and fine mapping of fertility restoration genes in earlier studies by Ahmadikhah and Karlov (2006), Jing *et al*. (2001), Bazrkar *et al*. (2008), Sheeba *et al.* (2009), and Sattari *et al*. (2008). In the present investigation, five linked markers namely RM228, RM202, RM35, RM18, RM17 for detection of qRf4, qRf7 and qRf11 containing restorers for WA cytoplasm;qRf1 for BoroII and qRF10-2 for DA type sterile cytoplasm . RM35 showed monomorphic amplification among all the genotypes tested, whereas rest of the markers could show polymorphism. An 110bp amplicon was observed with RM228 in CRMS31A, CRMS32A, CRL32R and another 27 lines under study (Figure 1). The genotype IR84894-B-140-6-1-1-1, Satabdi and Annapurna showed a band of 145bp, whereas 155bp was observed in the genotypes Satabdi, Parijata, Lalitgiri, IR 84898-B-168-24-1-1-1, Panindra,

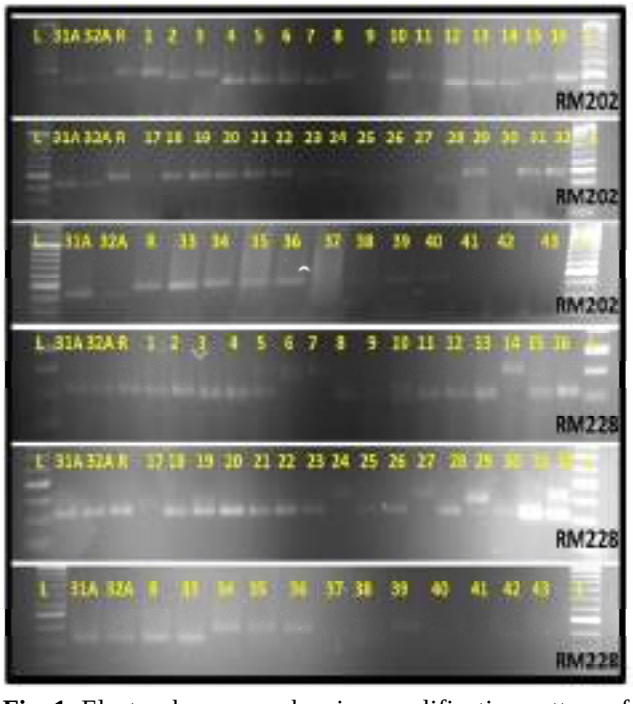

Fig. 1. Electrophoregram showing amplification pattern of the genotypes with RM202 and RM228 markers. The numbers denote the genotypes presented in Table 1. L: 50bp ladder

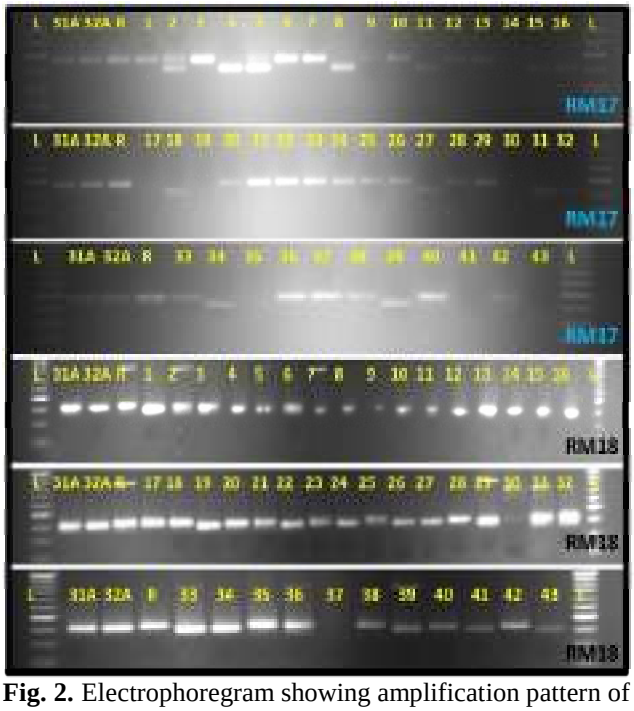

Fig. 2. Electrophoregram showing amplification pattern of the genotypes with RM17 and RM18 markers. The numbers denote the genotypes presented in Table 1. L: 50bp ladder

Swarna Sub-1, IR64 and MTU1071. RM202 could clearly distinguish between A and R-line. A-lines showed 165bp allele and R-line showed 200bp allele (Figure 1). The lines that showed 200bp allele are CR Dhan 601, IR84887-B-156-17-1-1-1, IET22729, IR84899-B-179-16-1-1, PTB 10, Ranbir basmati, Krishnabhog, MAS-946, CR2690-3-2-1-1-1, Samba Mahsuri, Chandan, Saket4, Parijata, Naveen, Annapurna, Lalat MAS, IR 84898-B-168-24-1-1-1, IET 22704, Panindra, Swarna-Sub1, Pusa 1592-6, MTU1071 and CR3696-3-2-2-1-2. The genotypes VLDhan-82, IR84899-B-183-6-1-1-4, IR84894-B-140-6-1-1-1, IR36, IET22729, Tapaswini MAS, Karjat-3, Satabdi, Tapaswini and Sonasali showed the R-type 165bp allele. Another allele of 185bp was observed in the genotypes CR Dhan 300, CO-41, Pyari, Saket4 and Naveen.

Two markers RM17 and RM18 were used for q*Rf*10-2 (Figure 2). RM17 showed 190bp allele in 28 genotypes, whereas a 155bp amplicon was obtained in 13 genotypes. These include CR Dhan 300, VLDhan-82, IR84899-B-183-6-1-1-4, IET22729, Pyari, IR84899-B-179-16-1-1, PTB 10, Krishnabhog, Lalitgiri, Lalat MAS, IR 84898-B-168-24-1-1-1, Panindra and MTU1071. RM18 showed two alleles of 150bp and 165bp for A and R-line respectively. Eighteen number of genotypes namely CRL32R, Ranbir basmati, Krishnabhog, CR2690-3-2-1-1-1, Samba Mahsuri, Saket4, Naveen, Sonasali, BAS-366, Lalat MAS, IR 84898-B-168-24-1-1-1, Swarna-Sub1, IR64, Pusa 1592-6, CR3696-3-2-2-1-2, Sampad, Padmanava and IET 21287 showed 165bp band, whereas the genotypes CR Dhan 601, CR Dhan 300, IR84887-B-156-17-1-1-1, VLDhan-82, IR84899-B-183-6-1-1-4, IR84894-B-140-6-1-1-1, IR36, IET22729, Vandana, CO-41, Pyari, Tapaswini MAS, Karjat-3, Satabdi, IR84899-B-179-16-1-1, PTB 10, MAS-946, Chandan, Parijata, Tapaswini, Lalitgiri, Annapurna, IET 22704, Panindra, MTU1071, Sampad and IET 21287.

Sheeba *et al.* (2009) and Suresh *et al*. (2012) validated the earlier reported markers in two mapping populations. Hu *et al*. (2016) studied fertility restoration by use of linked markers in BIL population to restore the fertility of dwarf wild abortive (DA), Indonesia paddy (ID) and Dongxiang wild rice (DWR) -type CMS.

The details of the markers used for genotyping the set of 46 genotypes and their genetic diversity parameters obtained are presented in Table 3. A total of 13 alleles were amplified with the five co-dominant and linked markers with different QTLs for fertility restoration. The number of alleles per marker varied from 1 to 3 with an average of 2.6 per locus. The mean PIC value was found to be 0.319 with minimum value of 0.00 (RM35) and maximum of 0.461 (RM202). The observed heterozygosity (H_0) ranged between 0.00 and 1.0, with an average H_0 of 0.0485. Only one marker RM35 exhibited zero value of H_0 and rest markers showed H_0 level more than zero. The mean gene diversity or expected heterozygosis (He) was observed to be 0.379 bracketed within the value of 0.0 to 0.531. Shah *et al.* (2012) studied genetic diversity among 22 genotypes representing restorer, maintainer and male sterile lines by using SSR markers linked to *Rf* genes as well as few non-linked markers. They reported very high genetic diversity among the genotypes with PIC value ranging from 0.72 to 0.94 showing the diverse nature of the genotypes taken. The low genetic diversity observed in the present investigation may be due to the use of less number of linked markers. Therefore, more number of *Rf* linked markers should be used to better categorize the parental lines.

The clustering analysis showed that both the male sterile lines CRMS32A and CRMS31A along with the genotypes Tapaswini MAS, Karjat-3 and Tapaswini formed a separate group from the rest of the genotypes under study (Figure 3). The genotypes Sonasali, CR3696-3-2-2-1-2, Ranbir basmati, SwarnaSub-1, IR64, Pusa 1592-6, Samba Mahsuri, CR2690-3-2-1-1-1, CRL32R, Saket4, Naveen, BAS-366, IR 84898-B-168-24-1-1-1, Krishnabhog, Lalat MAS, IET 22731, Padmanava, Sampad and IET 21287 formed a single cluster, whereas the genotypes Annapurna, Parijata, Lalitgiri, Panindra, MTU1071, IR84894-B-140-6-1-1-1, IR36 and Satabdi formed another cluster. Similarly, the genotypes Vandana, MAS-946, PTB 10, 11,

Table 3. Genetic diversity parameters of 46 genotypes based on the Rf-linked SSR markers

Marker	Min-Max mol.wt.	No.of alleles	Major Allele Frequency	Gene Diversity (H_e)	Hetero-zygosity (H_0)	PIC
RM17	155-190	3	0.6875	0.4503	0.1000	0.3773
RM18	150-165	3	0.6111	0.4837	0.0667	0.3770
RM35	205-205	1	1.0000	0.0000	0.0000	0.0000
RM202	165-200	3	0.6154	0.5312	0.0513	0.4613
RM228	110-155	3	0.7195	0.4340	0.0244	0.3835
Mean	-	2.6	0.7267	0.3798	0.0485	0.3198

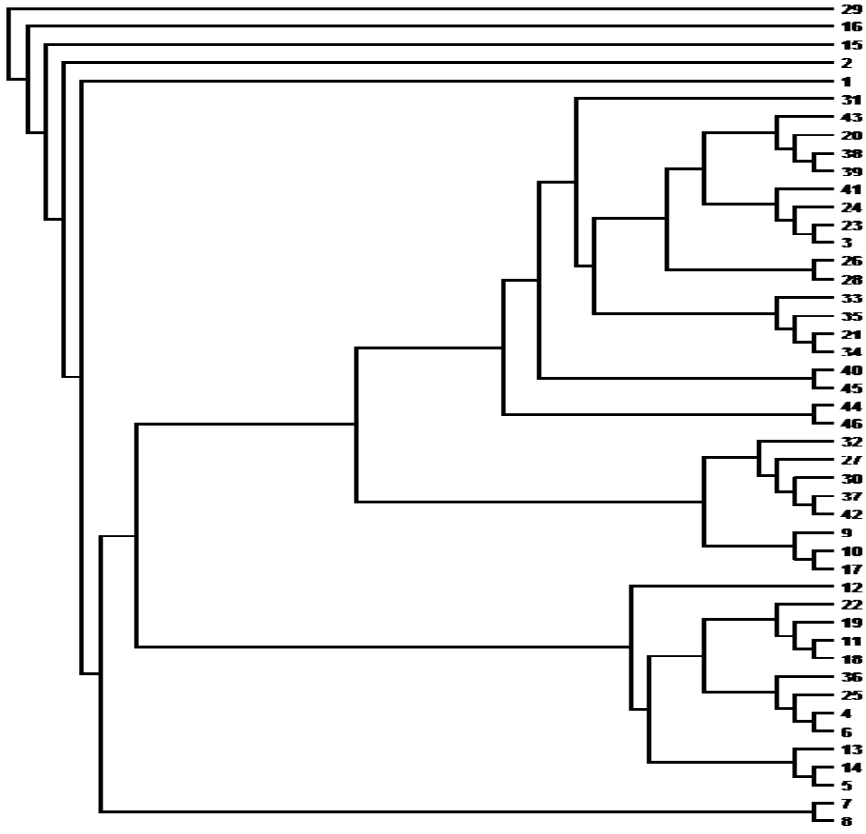

Fig. 3. Cladogram generated with unweighted pair group method arithmatic average (UPGMA) algorithm based on Jaccard's coefficients. The numbers denote the genotypes presented in Table 1.

IR84899-B-179-16-1-1, IET 22704, Chandan, CR Dhan 601, IR84887-B-156-17-1-1-1, CO-41, Pyari and CR Dhan 300 were grouped together. The genotypes VLDhan-82 and IR84899-B-183-6-1-1-4 together formed one cluster. Shah *et al.* (2012) could group the genotypes into different clusters by using Rf-linked markers. Singh *et al.* (2016) studied 36 genotypes to identify their ability for fertility restoration and maintenance of WA type CMS lines using linked SSR markers for *Rf*3 and *Rf*4 genes.

The phenotypic confirmation of fertility restoration is a tedious and time taking job, whereas selecting the lines for presence of fertility restoration genes is easy to detect in the laboratory. Hence, prior selection of the restorer lines by using linked markers for different *Rf* genes from a large set of genotypes and then characterising only those selected lines in the field will be comparatively easy and reliable approach. The present study showed that the genotypes Krishnabhog, CR2690-3-2-1-1-1, Samba Mahsuri, Chandan, Saket4, Naveen, Lalat MAS, IR 84898-B-168-24-1-1-1, IET 22704 and Pusa 1592-6 were positive for q*Rf*4, q*Rf*11 and q*Rf*10-2. Certain genotypes like IR84887-B-156-17-1-1-1, Vandana, CO-41, Tapaswini MAS, Karjat-3, Tapaswini, Sonasali and BAS-366 observed to possess q*Rf*4 and q*Rf*10-2., whereas the genotypes Parijata, Annapurna, IR 84898-B-168-24-1-1-1, IR64 and CR3696-3-2-2-1-2 possessed q*Rf*11 and q*Rf*10-2. These lines, after further phenotypic confirmation for fertility restoration of WA, BT and DA type CMS lines for future development of hybrid rice.

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