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Studies on fertility restoration of A_4 cytoplasm in pigeonpea

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Abstract Fertility restoration of CMS-based hybrids is an integral part of breeding hybrids and the development of new hybrid parents with desirable agronomic and market preferred traits on regular intervals is essential for sustainability of such programs. This paper reports identification of 25 male-sterility maintainers and 179 fertility restorers of A_4 cytoplasm in pigeonpea (*Cajanus cajan* (L.) Millsp.). Multi-location evaluation of hybrids exhibited high stability for fertility restoration across diverse environments. The diversity study showed a large variation for important traits both at phenotypic as well as genetic levels. The potential use of this information in hybrid pigeonpea breeding has been discussed.

Keywords Pigeonpea · Cytoplasmic nuclear male-sterility (CMS) · Fertility restorers · Male-sterility maintainers · Hybrid breeding

Introduction

Hybrid technology has played an unparalleled role in global food security and in the last few decades its power has been demonstrated in various field, vegetable, and other crops with several fold increases in their productivity. The availability of diverse male-sterile lines and their fertility restoration have played an important role in exploiting hybrid vigor at commercial scale. Dominant fertility restoring nuclear genes are transmitted from male parent which allow seed set on the hybrid plants. However, the expression of fertility restoration among testers may vary from 0 (complete male-sterility) to 100 % (full fertility). In certain cases environment also plays an important role in the expression of pollen fertility (Kaul 1988). Therefore, for the success of any commercial hybrid breeding program, it is essential to identify/breed male parents which not only provide stability to the hybrids but also produce high yields. The CMS-based hybrid technology in pigeonpea (*Cajanus cajan* (L.) Millsp.) is new and it is based on A_4 CMS-system (Saxena and Kumar 2013). To develop a long-lasting broad based hybrid breeding program, it is essential that a number of diverse fertility restorers are available. In the present study data on fertility restoration of A_4 CMS lines, generated over 4 years, have been summarized and promising maintainers and restorers have been identified.

Materials and methods

The male-sterility system used in hybrid pigeonpea breeding program was developed by transferring nuclear genome of a cultivated line into the cytoplasm of a wild species *C. cajanifolius* (Saxena et al. 2005);

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and it was designated as A_4 CMS system. This wild relative of pigeonpea was first described by Haines (1920) and it is a natural habitat of central India, where it grows wild in the forests of southern Odisha and Baster region of Madhya Pradesh. The local tribal community identifies *C. cajanifolius* as 'Ban Arhar', meaning 'wild pigeonpea'. Morphologically, this species resembles with cultivated species in a number of traits. Recently, Mallikarjuna et al. (2012) concluded that only 5–6 major genes differentiate *C. cajanifolius* from the cultivated type.

To identify potential fertility restorers, a total of 502 diverse germplasm and advanced breeding lines were crossed as testers with A_4 CMS line during 2008–2011. Each tester was crossed manually with the male-sterile line and the resultant seeds were sown in the subsequent year in field. A minimum of 15 plants from each cross were grown under irrigated conditions on ridges, spaced 75 cm apart. Important morphological traits such as days to flower, days to mature, plant height, 100-seed weight and seed colour were recorded on three randomly selected plants. The fertility status of each F_1 plant in each cross was determined, first visually and then the suspected male-sterile plants were further examined in laboratory to confirm their male-sterility status. For this purpose, five fully grown unopened buds were harvested randomly from each plant and their anthers were squashed on a glass slide and drenched with 1 % aceto-carmine solution. In each slide 3–5 microscopic fields were examined with 10× magnification and counts were made for stained (fertile) and empty/unstained (sterile) pollen grains. Plants with ≥80 % pollen fertility were classified as fertile and those with ≤5 % pollen fertility as male-sterile. The pollen fertility of the restorers was studied at four different locations Patancheru, Parbhani, Aurangabad and Phaltan in 3 years. A field technique developed earlier at ICRISAT (Reddy et al. 1990) for simultaneous screening of wilt and sterility mosaic resistance was used to record the disease incidence. The test materials were sown at the onset of rainy season and the disease build-up was monitored by sowing one row each of a susceptible and a resistant control after every 10 test rows. The susceptible (completely or partially dead) and resistant (disease free) plants were counted when most of the resistant plants reached maturity (180–190 days). To study the stability of pollen fertility restoration, hybrids involving 35 promising restorers were evaluated in multi-location trials for 2–3 years in 3–4 locations. These trials were conducted according to local cultural practices and the fertility restoration was studied by examining all the plants of each hybrid visually.

Six maintainers and 69 randomly identified fertility restorers were used for molecular characterization. The methods described for molecular characterization earlier by Saxena et al. (2010) were also used in this study. In the present study, the PIC value of markers was estimated using the following formula (Anderson et al. 1993):

$$PIC = 1 - \sum_{i=1}^{k} Pi^2$$

where k is the total number of alleles detected for a given marker locus and Pi is the frequency of the ith allele in the lines analyzed.

Results and discussion

Frequency of fertility restorers and maintainers

Restoration of fertile pollen production on the male-sterility based hybrid plants is the key factor in exploiting hybrid vigor in sexually reproducing crop species. This generally happens when dominant fertility restoring nuclear gene(s) present in the male-parent are transmitted to the hybrid plants. Such genes repair the damage caused by mitochondrial DNA aberrations in the male-sterile plants. Recent inheritance studies by Saxena et al. (2011a) and Sawargaonkar et al. (2012) revealed that restoration of pollen fertility in A_4 CMS system of pigeonpea was controlled by either single dominant or two duplicate dominant genes. Saxena et al. (2011a) further reported that for stability of fertility restoration across diverse

Table 1 Frequency of fertility restorers and male-sterility maintainers of A_4 cytoplasm in different maturity groups

Group	Early	Medium	Late	Total (%)
Maintainers	8	15	2	25 (5.0)
Restorers	35	113	31	179 (35.7)
Segregating	65	205	28	298 (59.4)
Total	108	333	61	502

environments, presence of both the dominant genes was essential.

In the present study, of the 502 genotypes crossed with male-sterile line, 179 (35.7 %) restored male fertility in the hybrid plants. In contrast, the frequency of male-sterility maintainers was quite low and only 25 (5.0 %) lines maintained male-sterility (Table 1). The remaining 298 (59.4 %) crosses had variable proportions of male-sterile and fertile plants. Also, some hybrids produced plants with partial fertility

Table 2 List of male-sterility maintainers and their important traits

S. No.	Genotype	Days to flower	Days to maturity	Plant height (cm)	100-seed weight (g)	Wilt (%)	Sterility mosaic (%)	Seed colour
Early maturing								
1	ICPL 11335	53	95	115	8	NA	NA	Brown
2	ICP 14425	76	127	150	9.8	86	64	Brown
3	ICP 14857	80	115	90	9.1	71	7	Brown
4	ICP 16172	73	138	110	10.4	NA	NA	Brown
5	ICP 14849	66	107	70	8.9	100	9	Brown
6	ICP 10915	68	115	75	5.4	79	14	Brown
7	ICP 10907	68	110	50	6.4	88	13	Brown
8	ICPL 98011	66	112	145	8.7	81	38	Brown
	Mean	68.6	114.9	100.6	8.3			
	Sem (±)	2.9	4.6	12.7	0.6			
Medium maturing								
9	ICP 28	81	128	127	10.2	68	32	Brown
10	ICPL 20282	98	148	185	10.3	42	50	Brown
11	ICPL 20286	98	145	174	10.5	86	7	White
12	ICPL 20288	102	158	185	11.2	67	17	White
13	ICPL 20287	105	158	170	10.7	12	16	White
14	ICPL 99050	123	175	225	11.1	0	0	Brown
15	ICPL 20093	127	183	283	12	8	0	Brown
16	ICPL 20099	127	184	292	14.7	5	0	Brown
17	ICPL 20094	129	185	280	10.6	0	0	Brown
18	ICPL 20176	114	162	198	10	0	0	Brown
19	ICPL 99052	123	178	235	11.9	0	0	Brown
20	ICPL 118	103	146	132	13.7	0	2.2	Brown
21	ICPL 96058	120	177	220	10.5	0	0	Brown
22	ICP 5529	104	158	190	8.4	91	36	Brown
23	ICPL 96053	128	184	198	10.5	0	4	White
	Mean	112.1	164.6	206.3	11.1			
	Sem (±)	3.7	4.6	13.0	0.4			
Late maturing								
24	ICP 14085	142	193	190	13.2	20	20	Brown
25	ICPL 20092	148	198	140	9.6	23	0	White
	Mean	145.0	195.5	165.0	11.4			
	SE	3.0	2.5	25.0	1.8			
	Total mean	100.9	151.2	169.2	10.2			
	Combined sem (±)	5.4	6.2	13.2	0.4			

SE standard error of mean

restoration with sparse pollen production and all such testers were classified as partial or incomplete restorers. This situation may arise due to heterogeneity for fertility restoring genes within testers (Saxena et al. 2011a), genetic background of the genotype or effect of micro environment (Kaul 1988). Out of 179 restorers identified, 35 were of early maturing group, 113 were of medium maturity, and 31 represented late maturity group. Similarly out of 25 maintainers, eight represented early, 15 medium, and two late maturities.

In pigeonpea the fertility restoring genes are sporophytic in nature (Dalvi et al. 2008) and hence both homozygote and heterozygote hybrid plants produce fully fertile pollen grains. According to Singh and Gopalkrishnan (2013) the frequency of fertility restorers among the cultivated types for an alloplasmic CMS system is generally low due to negative association of genetic diversity of the parents with the fertility restoration of F_1 hybrid. On the contrary in the present alloplasmic CMS system, the frequency of fertility restorers was reasonably high (35.7 %) and this situation may arise due to genetic closeness of *C. cajanifolius* with *C. cajan* (De 1974; van der Maesen 1990; Mallikarjuna et al. 2012). Therefore, it can be assumed that the mitochondrial defects in *C. cajanifolius* caused by insertion of *C. cajan* genome were not of serious nature and these can be repaired easily by fertility restoring genes present in the primary gene pool of genus *Cajanus*.

Maintainers

A maintainer line is defined as a genotype which maintains the fertility of the male-sterile lines. In the present study 25 maintainers were identified. These included eight early, two late, and 15 medium maturing types. The plant and grain characteristics of the maintainers are given in Table 2. Plant maturity among the early types ranged between 95 and 127 days; and only ICP 16172 and ICP 14425, had large seeds. There was no resistance to fusarium wilt in this group and only ICP 14857 and ICP 14849 exhibited resistance to sterility mosaic virus. All the early maturing maintainers had brown seeds. In the medium maturing group, resistance to fusarium wilt and sterility mosaic diseases is of prime importance (Reddy et al. 1990). The data recorded in the disease screening nursery revealed that 10 out of 15 maintainers had resistance to both the diseases (Table 2). Four testers (ICPLs 20286, 20287, 20288, and 96053) had white seeds and ICPL 20099 had the largest seeds. ICPL 118 was determinate in growth habit, while the rest were non-determinate. One of the medium maturing maintainers, ICP 5529 had a special leaf marker, identified as "obcordate" (Fig. 1). This trait is controlled by a pair of recessive alleles (Saxena et al. 2011b) and it is expressed within 25–30 days from sowing. This trait can be used to maintain genetic purity of male-sterile lines and hybrids with minimum efforts. In the late maturity group, only two male-sterility maintainers were identified. ICP 14085 matured in 193 days and had good seed size. It was tolerant to wilt and sterility mosaic diseases, each recording 20 % incidence. The other maintainer in this group was ICPL 20092. It is a white seeded line with tolerance to wilt and resistance to sterility mosaic virus. These two maintainers can easily be purified for disease resistance with careful selection of resistant male-sterile plants in the disease-sick nursery and backcrossing them with resistant single plants of the recurrent parents.

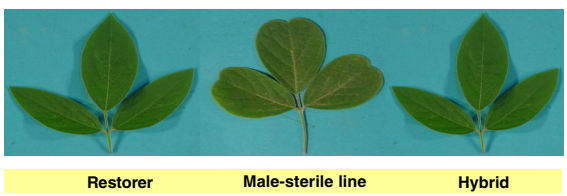

Fig. 1 A male-sterile line with recessive obcordate leaf marker (*center*), normal trifoliate restorer (*left*) and hybrid trifoliate (*right*)

Table 3 Variation for important traits among fertility restorers of early, medium, and late maturity groups recorded at Patancheru

Trait	Early ($n = 35$)	Medium ($n = 113$)	Late ($n = 31$)
Days to flower	50–85	90–130	131–158
Days to mature	101–141	138–200	186–241
Plant height (cm)	70–165	90–228	135–260
100-seed weight (g)	6.2–12.1	6.8–17.3	7.7–18.1
Wilt (%)	52–100	0–100	0–100
Sterility mosaic (%)	3–67	0–100	0–100

Table 4 Mean pollen fertility of promising restorers recorded in multi-location trials, 2008–2010

S.No	Restorers ICP/ICPL No.	2010 Locations (4)	2009 Locations (3)	2008 Locations (3)	Mean
1	ICPL 87119	96	85	97	93
2	ICPL 20093	100	93	81	91
3	ICPL 20096	89	97	95	94
4	ICPL 20098	92	88	96	92
5	ICPL 20104	99	99	93	97
6	ICPL 20106	96	91	97	95
7	ICPL 20107	95	88	91	91
8	ICPL 20108	91	100	97	96
9	ICPL 20111	95	93	95	94
10	ICPL 20112	92	89	94	92
11	ICPL 20116	98	91	87	92
12	ICPL 20120	87	78	98	88
13	ICPL 20123	100	96	95	97
14	ICPL 20125	96	96	94	95
15	ICPL 20127	95	96	88	93
16	ICPL 20128	94	95	97	95
17	ICPL 20129	95	95	81	90
18	ICPL 20136	93	98	97	96
19	ICPL 20186	76	96	91	88
20	ICPL 20205	100	97	99	99
21	ICPL 20118	91	80	–	85.5
22	ICPL 20126	97	87	–	92
23	ICPL 20137	100	100	–	100
24	ICP 7086	100	98	–	99
25	ICPL 20117	96	93	–	94.5
26	ICPL 20176	93	98	–	95.5
27	ICPL 20177	95	100	–	97.5
28	ICPL 20201	98	100	–	99
29	ICP 10650	92	98	–	95
30	ICP 8094	95	100	–	97.5
31	ICPL 99044	100	100	–	100
32	MA 3	99	100	–	99.5
33	MA 6	99	98	–	98.5
34	MA15	100	91	–	95.5
35	ICP 11376	95	91	–	93

2010 locations: Patancheru, Aurangabad, Phaltan, Parbhani

2009 locations: Patancheru, Phaltan, Parbhani

2008 locations: Patancheru, Aurangabad, Parbhani

10–15 plants were studied at each location

Fertility restorers

A fertility restorer line is defined as a genotype which restores the fertility of the progeny. Hanson and Bentolila (2004) and Wang et al. (2006) reported that CMS is a function of certain unusual open reading frames coding for a polypeptide chain. Male fertility in such genotypes can be restored by specific nuclear

Table 5 List of elite medium maturing fertility restorers and their important traits

S. No.	Genotype	Days to flower	Days to mature	Plant height (cm)	100-seed weight (g)	Wilt (%)	Sterility mosaic (%)	Seed colour
1	ICPL 87119	122	172	228	10.6	0	0	Brown
2	ICPL 20093	123	180	190	12.6	8	0	Brown
3	ICPL 20096	120	176	155	10.9	0	0	Brown
4	ICPL 20098	122	177	180	13.0	0	0	White
5	ICPL 20104	120	178	190	12.9	7	0	Brown
6	ICPL 20106	122	179	185	12.5	9	0	White
7	ICPL 20107	119	173	162	8.6	20	0	Brown
8	ICPL 20108	119	177	192	10.9	0	0	White
9	ICPL 20111	122	181	192	10.4	15	0	Brown
10	ICPL 20112	120	178	188	8.8	14	0	White
11	ICPL 20116	116	175	148	10.8	0	0	Brown
12	ICPL 20120	139	199	185	9.8	3	1	Brown
13	ICPL 20123	121	182	170	11.6	0	0	Brown
14	ICPL 20125	120	181	170	10.4	20	20	Brown
15	ICPL 20127	125	184	162	10.4	85	15	Brown
16	ICPL 20128	122	181	198	11.3	0	0	Brown
17	ICPL 20129	129	192	195	12.1	13	0	Brown
18	ICPL 20136	118	177	170	11.2	0	0	Brown
19	ICPL 20186	121	178	209	9.4	28	6	Brown
20	ICPL 20205	128	189	220	10.2	0	0	Brown
21	ICPL 20118	120	182	165	10.5	8	0	White
22	ICPL 20126	119	180	172	12.2	0	0	Brown
23	ICPL 20137	130	187	195	11.4	0	0	White
24	ICP 7086	135	198	220	9.8	35	0	White
25	ICPL 20117	130	191	198	10.3	0	0	Brown
26	ICPL 20176	107	180	182	10.9	0	0	Brown
27	ICPL 20177	121	181	190	8.4	9	0	White
28	ICPL 20201	126	185	245	9.7	56	78	Brown
29	ICP 10650	134	186	105	9.3	35	6	Brown
30	ICP 8094	153	200	228	7.7	15	10	White
31	ICPL 99044	131	185	185	10.5	0	0	White
32	MA 3	137	190	198	9.2	22	0	Brown
33	MA 6	154	204	220	9.3	13	0	Brown
34	MA15	150	196	210	11.1	29	0	Brown
35	ICP11376	136	204	170	8.5	0	0	Purple

genes which encode fertility restorer genes through the production of pentatricopeptide. In the early maturing group 35 fertility restorers were identified. The flowering and maturity periods among the early maturing restorers varied from 50 to 85 and 101 to 141 days, respectively (Table 3). ICP 3868, ICPL 90012, and ICPL 90051 had seed size of 10 g/100 seeds or more. Like maintainers among the restorers also, resistance to diseases in this maturity group was limiting and only ICPL 89032 had resistance to fusarium wilt, while ICP 14057, ICPL 5, ICPL 92043, and 93107 were resistant to sterility mosaic virus. Six restorers viz., ICPLs 89, 90030, 90036, 90048, 93103, and 93107 had white seeds. Medium

maturity group is very important from adaptation point of view and hence, a large number of crosses were attempted and 113 fertility restorers were identified. These represented a fairly good genetic variation with respect to key plant characters (Table 3). In this group 72 restorers were resistant to both wilt and sterility mosaic diseases. The variation for maturity was from 138 to 200 days. Sixty-nine testers had seed size of ≥10 g/100 seeds. In the late maturing group 31 restorers were identified (Table 3). These included 11 from Africa and 16 from India. In this group 18 testers were resistant to sterility mosaic virus; while only five exhibited resistance to fusarium wilt. ICPL 20103, MA 16, ICPL 20120, ICP 11376, ICP 13092, and ICP 14282 were found resistant to both the diseases. Plant maturity in this material ranged from 186 to 241 days. Two testers ICP 13379 and ICP 8051 had seed size of 18 g/100 seeds; while seven recorded seed size of >15 g/100 seeds.

Stability of fertility restoration

A total of 35 restorers were used to study stability of pollen fertility in hybrid combinations at diverse locations in different years. Of these, 20 were evaluated at 10 environments for 3 years (Table 4). Their mean pollen fertility ranged from 88 to 99 %. The remaining 15 hybrids were evaluated in seven environments for 2 years and their pollen fertility ranged from 85.5 to 100 %. The results of these multi-location trials showed that the testers were highly stable in their ability to restore fertility across diverse environments. The plant and grain characteristics of 35 elite restorers (Table 5) showed a considerable variation for important agronomic traits and this provides options to breeders for selecting desired hybrid parents. The flowering and days to maturity among the restorers ranged from 107 to 154 and 172 to 204 days, respectively. The restorers ICPL 20098 and ICPL 20104 had the largest seed size. Twenty-one restorers were found resistant to both wilt and sterility mosaic diseases.

Molecular diversity among maintainers and restorers

In the present study a set of six maintainers and 69 restorers was characterized at molecular level using 24 SSR markers. All the markers were found to be polymorphic across the maintainers and restorers. These markers amplified a total of 224 alleles with an average of 9.3 alleles per marker (Table 6). The number of alleles in different lines ranged from 3 (CcM2409, CcM2505) to 24 (CcM1011). The polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a given germplasm, depending on the number of detectable alleles and the distribution of their frequency. The PIC value of these markers ranged from 0.19 (CcM1373) to 0.92 (CcM1011) with an average of 0.62. In order to assess the genetic diversity, marker genotyping data of maintainers and restorers were used to generate UPGMA based tree and the dendrogram revealed six distinct clusters (Fig. 2). Study of genotypic diversity revealed that six maintainers clustered along with restorers in different groups. The cluster I comprised of four

Table 6 Marker polymorphism across restorer lines using 24 SSR markers

SSR marker	Number of alleles	PIC
CcM2818	9	0.57
CcM0988	9	0.71
CcM2409	3	0.36
CcM2505	3	0.36
CcM2221	4	0.51
CcM2697	11	0.74
CcM2379	5	0.64
CcM1109	10	0.75
CcM1207	14	0.79
CcM2871	14	0.75
CcM1373	4	0.19
CcM1366	20	0.89
CcM0673	4	0.62
CcM0962	5	0.44
CcM2710	11	0.75
CcM2895	22	0.87
CcM0443	19	0.88
CcM1011	24	0.92
CcM0785	4	0.51
CcM2241	4	0.59
CcM1982	8	0.73
CcM1079	6	0.42
CcM2332	7	0.71
Range	24-Mar	0.19–0.92
Mean	9.3	0.62

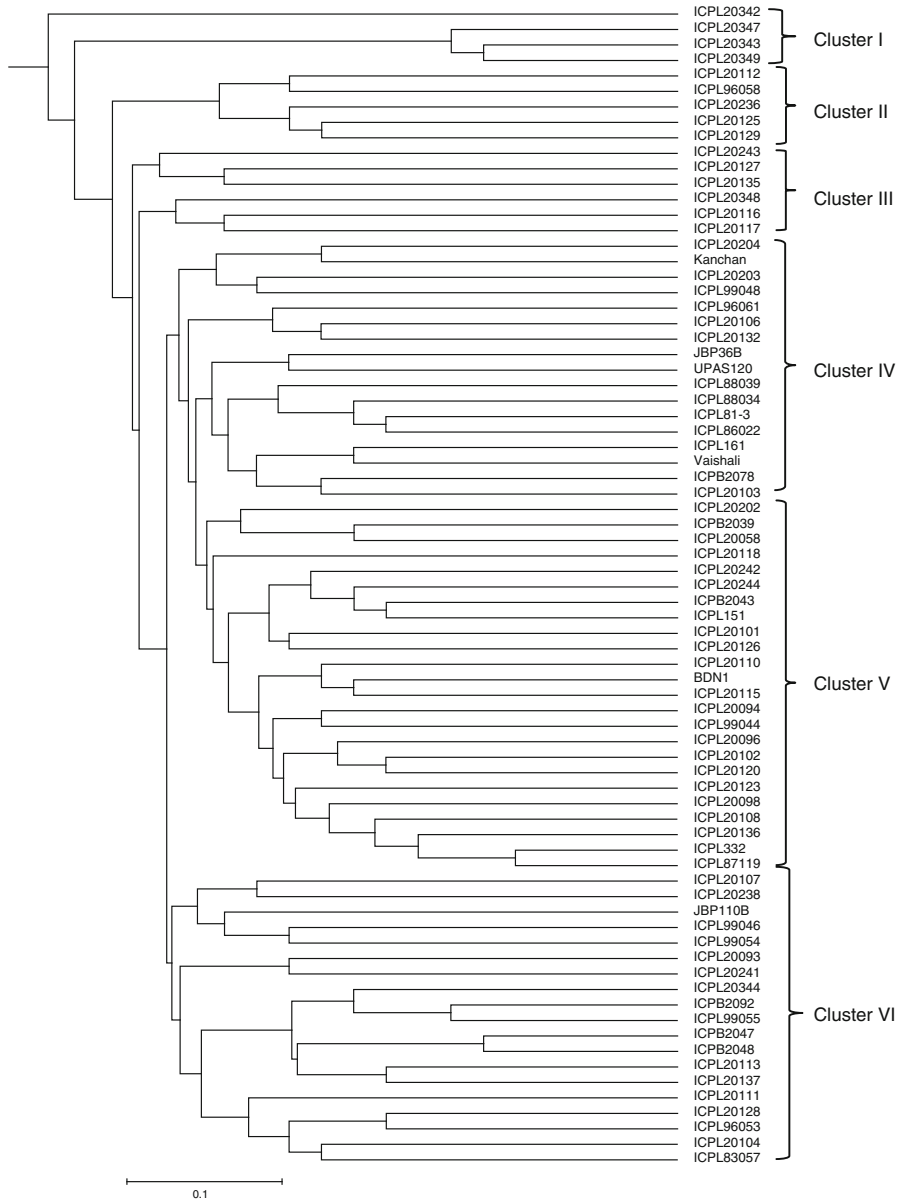

Fig. 2 Dendrogram showing genetic diversity among maintainers and restorer lines

restorers derived from wild species; ICPL 20343 and ICPL 20347 from *C. acutifolius*; ICPL 20349 from *C. platycarpus* and ICPL 20342 from *C. scarabaeoides*. These formed a separate group indicating their divergence from the cultivated type. Cluster II comprised of five restorers derived from the cultivated types. The cluster III had six restorers; while in cluster IV, 17 restorers and one maintainer (ICPB 2078) were included. In cluster V, two maintainers (ICPB 2043 and ICPB 2039) and 22 restorers were included; whereas Cluster VI contained 16 restorers and three maintainers (ICPB 2047, ICPB 2048, and ICPB 2092). These results showed a considerable genetic diversity among maintainers and restorers. This information can also be used to establish heterotic groups and breeding high yielding hybrids parents using from diverse clusters for a greater genetic advance.

Conclusions

To meet the food needs of growing population, it is essential that hybrid technology is regularly upgraded so that hybrids with high yield and adaptation are developed at regular intervals. In view of potential importance of early maturing cultivars and limited variability among hybrid parents (Saxena and Kumar 2013), it seems necessary to widen their genetic variability. In both long and medium maturity groups wilt and sterility mosaic diseases can cause severe damage to the crop (Reddy et al. 1990) and no susceptible hybrid would find acceptance among farmers. Hence, high importance should be given to diseases resistance in breeding hybrid parents. Genetic diversity is known to plays an important role in reaping the benefits of hybrid technology In this context the molecular markers (Saxena et al. 2010) can be useful in identifying diverse parents. In the present study seven inter-specific derivatives involving *C. acutifolius*, *C. platycarpus*, *C. scarabaeoides*, and *C. lineatus* restored pollen fertility of A_4 CMS system and provided additional variability for any hybrid breeding program. To breed new restorer lines, crosses among selected diverse restorers can be made to identify desirable genotypes with respect to different consumer preferred traits. Further, based on the genetic diversity, combining ability and per se performance a set of heterotic groups be developed for use in hybrid breeding programs.

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