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# RAPD markers for identification of cytoplasmic genic male sterile, maintainer and restorer lines of pigeonpea

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## ABSTRACT

**The cytoplasmic-genetic male sterility (CMS) system is considered to be the feasible approach to develop hybrids in pigeonpea. Identification of CMS lines, their maintainers and putative restorers using molecular markers in the early stage of growth is important and economical in the long duration pigeonpea. Random amplified polymorphic DNA (RAPD) markers were used to identify CMS lines derived from crosses between wild (*Cajanus scarabaeoides* and *C. sericeus*) and cultivated (*C. cajan*) pigeonpea. Of the 108 RAPD primers screened, 80 were found to be polymorphic. A set of RAPD primers were identified that could distinguish the CMS systems of GT 288 A/B and 67 A/B. Moreover, specific primers differentiating the CMS lines (GT 288A/67A), maintainers (GT 288B/67B) and putative restorers (ICP 41 and DPPA 85-7) were identified. The use of these primers in heterosis breeding is discussed.**

Key words : *C. cajan*, *C. scarabaeoides*, *C. sericeus*, CMS system, Marker, Pigeonpea, RAPD

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a highly nutritious and very important grain legume of the tropical and sub-tropical regions of the world. The production and productivity of this crop has remained static over the years and the research efforts for enhancing the productivity through pure line breeding have not been very successful (Saxena *et al.* 2005). Unlike other legumes, pigeonpea is an often cross pollinated crop exhibiting 25-70% natural out crossing across locations (Saxena *et al.* 1990). Therefore, in order to break the yield plateau of this crop, serious efforts were made for the development of hybrids in pigeonpea by taking advantage of the pollination behaviour and potentials of heterosis breeding after discovery of genetic male sterility (GMS) (Reddy *et al.* 1978). These efforts resulted in the development of six genetic male sterility based hybrids in India *viz.*, ICPH 8, PPH 4, COPH 1, COPH 2, AKPH 4101 and AKPH 2022. But the GMS system proved failure because of labour intensiveness in seed production and seed purity concerns. Concerted research efforts resulted in the development of cytoplasmic genetic male sterility system using cytoplasm of wild species like *C. sericeus* (Ariyanayagam *et al.* 1995; Saxena *et al.* 1996) and *C. scarabaeoides* (Tikka *et al.* 1997). In the development of CMS based hybrids, male sterile (A), maintainer (B) and restorer (R) lines are required. Unambiguous characterization of the parental lines not only reduces the cost, labour, and time but also leads the hybrid development in the proper perspective.

DNA based markers are the best option to characterize the parental lines involved in the development of hybrids as they are numerous, independent of environmental effects and can detect the plants of interest at an early growth stage. The review of literature shows very few reports on the utilization of molecular markers in pigeonpea like assessment of genetic diversity using RAPD (Ratnaparkhe *et al.* 1995; Parani *et al.* 2000) and AFLP (Panguluri *et al.* 2006), mapping and tagging of *Fusarium* wilt resistance locus (Kotresh *et al.* 2006) and characterization of CMS lines (Souframanien *et al.* 2003). Molecular markers have also been used for characterization of CMS lines in crops like rice (Ichii *et al.* 2003, Tar'an B *et al.* 2005), maize (Gabay-Laughnan *et al.* 2004), pearl millet (Rajeshwari *et al.* 1994), sorghum (Pring and Tang 2004) and sunflower ( Kusterer *et al.* 2004). In the present study, RAPD analysis was used to study the genomic DNA variation among two male sterile lines (GT 288A and 67A), their corresponding maintainer lines (GT 288B and 67B) and putative restorer lines (ICP 41 and DPPA 85-7).

## MATERIALS AND METHODS

*Plant materials:* The plant material consisted of CMS lines (GT 288A and 67A with S-rr genome derived from *C. scarabaeoides* and *C. sericeus*, respectively), their corresponding maintainers (GT 288B and 67B with F-rr genome) and fertility restorer lines (ICP 41 and DPPA 85-7 with F-RR genome) (Table 1).

**Table 1. Pigeonpea CMS lines, their maintainers and restorers used in the present study**

| Genotype | Origin | Features |
|---|---|---|
| GT 288A | *Cajanus scarabaeoides* | Erect, compact, indeterminate and resistant to sterility mosaic |
| GT 288B | GT 288 | Same as 'A' line |
| 67A | *Cajanus sericeus* | Semi-spreading, determinate and resistant to sterility mosaic |
| 67B | GT 67 | Same as 'A' line |
| ICP 41 | Selection from cultivated germplasm line | Semi-spreading, indeterminate, resistant to sterility mosaic and wilt |
| DPPA 85-7 | Selection from cultivated germplasm line | Same as ICP 41 |

*DNA extraction:* Isolation of DNA from the leaf tissues of each line was done based as per the modified protocol (Guillemant and Laurence 1992) without liquid nitrogen. Pooled 1 g leaf samples of 3-4 weeks old seedlings were ground to a fine paste with the extraction buffer [100 mM sodium acetate, pH 4.8; 500 mM Nacl; 50 mM EDTA, pH 8.0; 50 mM Tris, pH 8.0; 2% PVP (MW 10000); 1.4% SDS], and transferred to centrifuge tubes. The tubes were incubated at 65°C for 30 minutes, added 0.6 volume of 10 M ammonium acetate into each of the tubes and kept for another 15 minutes at 65°C followed by centrifugation at 10,000 rpm for 10 minutes. The supernatants were treated with 0.6 volume of chilled iso-propyl alcohol and incubated at -20°C for 60 minutes. After centrifugation, DNA pellets were washed twice with 70% ethanol and dissolved in TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0). DNA solutions were extracted with phenol: chloroform: iso-amyl alcohol (25:24:1), treated with RNAse (4 il/ml of DNA solution from stock of 10mg/ml) and incubated at 37 °C for 1 hr. The DNAs were then extracted twice with chloroform: iso-amyl alcohol (24:1). The DNAs were re-precipitated using chilled ethanol and the pellets were dissolved in TE buffer. The extracted DNAs were quantified by agarose gel (0.8%) electrophoresis using uncut lambda (ë) DNA as standard marker (300 ng/il). Dilution of the DNA solutions were done using TE buffer to a concentration of approximately 12.5ng/ìl for use in polymerase chain reaction (PCR).

*PCR and RAPD analysis:* PCR amplification was carried out in 0.2 ml thin-wall PCR tubes using a PTC thermal cycler (MJ Research). A total of 108 random decamer primers (OPA, OPAQ, OPAZ, OPBA, OPBB, OPH and OPP series from Operon Technologies, Alameda, CA, USA) were initially screened. Of them, 94 primers that produced unambiguous and repeatable DNA profiles were selected for further study. PCR reactions were performed in 25 ìl volume containing 25 ng of template DNA, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 ìM of decamer primer, 10 X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 25 mM of each dNTPs (dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, USA) in final 25 ìl volume. Amplification conditions were maintained at 94°C for 3 min, and 44 cycles at 94°C for 1 min (denaturation), 37°C for 1 min (annealing), 72°C for 2 min (elongation) followed by final extension at 72°C for 7 min. The PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (8 ìl from 1mg/ml stock in 100 ml agarose solution) using 1X TBE (Tris base, Boric acid, EDTA) buffer (pH 8.0) and visualized by illumination under UV light. The sizes of amplification products were determined in comparison to O'Gene Ruler ™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, USA).

## RESULTS AND DISCUSSION

Out of 108 RAPD primers used in the present study to discern banding pattern, differences between the genomic DNAs of A, B and R lines, 94 were found to generate clear and unambiguous banding patterns. Eighty primers were polymorphic. A total of 512 bands were amplified of which 364 were found polymorphic, showing an average of 4.55 polymorphic bands/primer. However, the average number of amplified bands per primer was 5.45. The number of amplified loci varied from one (OPBB 13 and OPH 09) to 14 (OPBA 03) while the size of amplified bands was in the range of 250 bp (OPAQ 07) to 3100 bp (OPA 04). The primers OPAZ 01 and OPBA 13 amplified two loci each (OPAZ 01 $_{1900\&2100}$ and OPBA 13 $_{900\&1000}$) exclusively in 288 A/B system while 5 loci amplified by 4 primers (OPAZ 05 $_{1050\&1030}$; OPP08 $_{1500}$; OPP 09 $_{1500}$ and OPH 03 $_{1600}$) were 67 A/B system specific (Table 2). Irrespective of the CMS system, A and B lines could be differentiated by 4 primers amplifying 8 and 4 loci, respectively. Seven bands amplified by 6 primers were restorer specific (Table 2). The photographs showing amplification of 'R' and 'B' specific bands using the primers OPA 10 and OPH 04 are depicted in Fig. 1 (a, b).

**Table 2. RAPD markers differentiating GT 288 A/B and 67 A/B CMS systems and its individual components in pigeonpea**

| Primer | Mol.wt | 67A | 67B | 288A | 288B | ICP 41 | DPPA 85-7 |
|---|---|---|---|---|---|---|---|
| **CMS system specific markers** | | | | | | | |
| OPAZ 01 | 2100 | - | - | + | + | - | - |
| OPAZ 01 | 1900 | - | - | + | + | - | - |
| OPBA 13 | 1000 | - | - | + | + | - | - |
| OPBA 13 | 900 | - | - | + | + | - | - |
| OPAZ 05 | 3050 | + | + | - | - | - | - |
| OPAZ 05 | 1031 | + | + | - | - | - | - |
| OPP 08 | 1500 | + | + | - | - | - | - |
| OPP 09 | 1500 | + | + | - | - | - | - |
| OPH 03 | 1600 | + | + | - | - | - | - |
| **CMS, maintainer and restorer line specific markers** | | | | | | | |
| OPBB03 | 2000 | + | - | + | - | - | - |
| OPAQ07 | 1750 | + | - | + | - | - | - |
| OPAQ07 | 1200 | + | - | + | - | - | - |
| OPAQ07 | 900 | + | - | + | - | - | - |
| OPAQ07 | 400 | + | - | + | - | - | - |
| OPAQ07 | 250 | + | - | + | - | - | - |
| OPP14 | 650 | + | - | + | - | - | - |
| OPP08 | 1220 | + | - | + | - | - | - |
| OPP10 | 600 | - | + | - | + | - | - |
| OPH04 | 800 | - | + | - | + | - | - |
| OPBB06 | 800 | - | + | - | + | - | - |
| OPBA19 | 310 | - | + | - | + | - | - |
| OPBA19 | 280 | - | - | - | - | + | + |
| OPAZ04 | 3000 | - | - | - | - | + | + |
| OPAZ04 | 2000 | - | - | - | - | + | + |
| OPA10 | 500 | - | - | - | - | + | + |
| OPA02 | 500 | - | - | - | - | + | + |
| OPH19 | 1200 | - | - | - | - | + | + |
| OPAQ04 | 3000 | - | - | - | - | + | + |

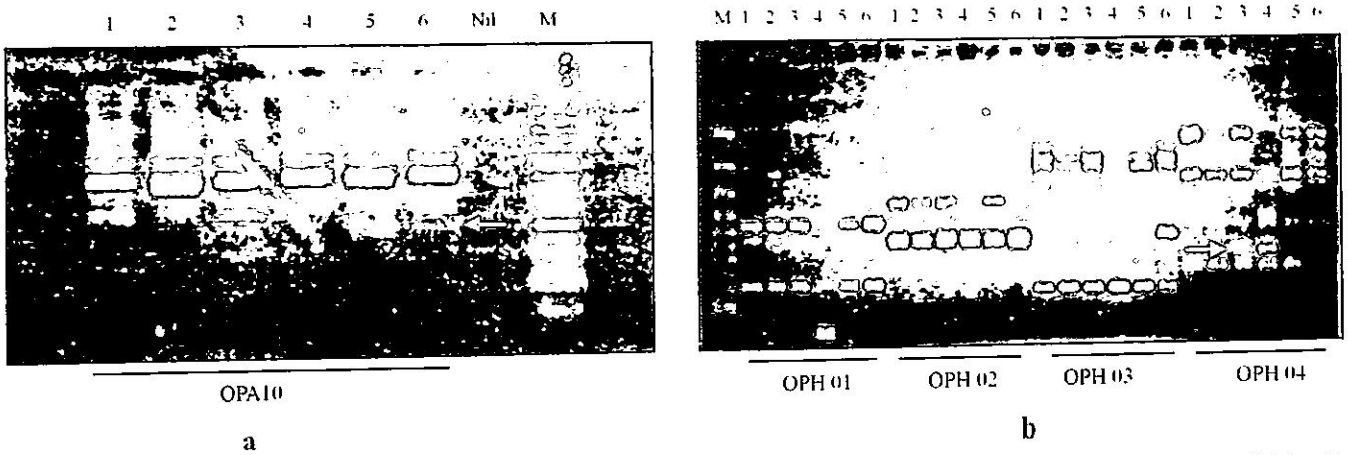


Fig.1. Gel photographs showing (a) restorer lines specific RAPD bands (OPA 10 $_{900}$) and (b) maintainer lines specific RAPD bands (OPH 04 $_{800}$). Legend for lanes: 1= 67A, 2= GT 288A, 3= 67B, 4= 288 B, 5= ICP 41 and 6= DPPA 85-7, M= 100bp DNA ladder

The development of hybrids in pigeonpea by exploiting cytoplasmic-nuclear male-sterility (CMS) system is an important approach to increase the production which otherwise has remained static for past few decades. Unambiguous characterization of the parental lines in the CMS system alleviates concerns regarding the seed purity in hybrid seed production. RAPD has been used unambiguously for identification of parental lines and their hybrids in several crops like menthe (Shasany *et al.* 2005), *Bromus* species (Yasas *et al.* 2002), tomato (Rom *et al.* 1995), rice (Wang *et al.* 1994), poplar (Rajora and Rahaman 2003) including closely related species of common bean and soybean (Martin *et al.* 1996).

In case of pigeonpea, efforts have been made (Sivaramakrishnan *et al.* 1997) to distinguish male parents from the CMS lines using RFLP markers. Souframanien *et al.* (2003) reported a set of RAPD markers that could distinguish each component of the CMS system. The set of primers identified in the present study to distinguish the CMS systems derived from *C. scarabaeoides* (288 A/B) and *C. sericeus* (67 A/B) adds to the information generated earlier by Souframanien *et al.* (2003). Differences between CMS lines and its maintainers are mainly due to the presence of sterile (S-rr) and fertile cytoplasms (F-rr). Therefore, A and B lines are assumed to be near isogenic lines except for the genomic portion related to male sterility. Further, the primers distinguishing the CMS lines from their maintainer lines may be related to the cytoplasmic genome (mt DNA), while the restorer line specific primers may be reflecting the differences related to the nuclear genome responsible for restoration in the two restorers (*viz.*, ICP 41 and DPPA 85-7) used in the present study.

The primers identified in the present study could be useful in detecting the genetic structure of the plants rapidly and easily by PCR technique *via* marker aided selection in their early stages of growth (Akagi *et al.* 1995) which is otherwise very tedious, time, land and labour intensive especially in the long duration crop like pigeonpea. In case of rice, RAPD markers unique to the male sterile lines (Souframanien *et al.* 2003) and maintainers (Wang *et al.* 1994) have been identified, while Ichii *et al.* (2003) observed a 1600 bp fragment generated by the RAPD primer OPA12 in the total nuclear and mitochondrial DNA of the CMS lines. To understand the nuclear-cytoplasm combination in restorations of fertility in sorghum, Jaiswal *et al.* (1998) applied RAPD technique and found that RAPD primers of the total DNA could distinguish the lines on the basis of their variable restoration ability. Further, RAPD analysis was also used successfully to assess the diversity in the different alloplasmic CMS lines of sorghum (Nateson *et al.* 2002), while Kumar *et al.* (2004) identified two RAPD primers (OPP13 $_{1350}$ and OPW19 $_{900}$) associated with the fertility restorer gene (*Rf*) of pepper and Zhang *et al.* (2004) found two RAPD markers (UBC 111 $_{900}$ and UBC 188 $_{500}$) associated with the fertility genes of D8 CMS lines in cotton. The primers identified in the present investigation would be useful in the identification of CMS lines, their maintainers and restores at DNA level even at early stage of their growth which, in turn, would be an alternative to grow out test of these lines, when they will be used commercially. Further, this type of diagnosis will reduce the cost and tedious nature of maintenance due to the outcrossing of CMS lines, their restorers and maintainers in pigeonpea.

## REFERENCES

Akagi HA, Nakamura and Sawada R. 1995 Genetic diagnosis of cytoplasmic male sterility cybrid plant of rice Theoretical and Applied Genetics 90: 948-951.

Ariyanayagam RP, Rao AN and Zaveri PP 1995. Cytoplasmic-genic male sterility in interspecific matings of *Cajanus*. Crop Science 35: 981-985

Gabay-Laughnan S, Chase CD, Ortega VM and Zhao L. 2004. Molecular genetic characterization of CMS-S restorer-of-fertility alleles identified in Mexican maize and teosinte. Genetics 166: 959-970.

Guillemant P and Laurence MD. 1992. Isolation of Plant DNA: A fast, inexpensive, and reliable method. Plant Molecular Biology Reporter 10: 60-65.

Hong DL, Ichii M, Ohara Y, Taketa S and Zhao CM. 2003. Characterization of CMS and maintainer line in indica Rice (*Oryza sativa* L.) based on RAPD marker analysis. Euphytica 129: 249-252.

Ichii M, Hong DL, Ohara Y, Zhao CM and Taketa M. 2003. Characterization of CMS and maintainer line in indica Rice (*Oryza sativa* L.) based on RAPD marker analysis. Euphytica 129: 249-252.

Jaiswal P, Ranade SA, Sane AP, Nath P and Sane PV. 1998. Mitochondrial and total DNA RAPD patterns can distinguish restorers of CMS lines in sorghum. Theoretical and Applied Genetics 96: 791-796.

Kottresh H, Fakrudin B, Punnuri T, Paramesh LH and Kuruvinashetti M. 2006. Identification of two RAPD markers genetically linked to a recessive allele of a *Fusarium* wilt resistance gene in chickpea. Euphytica 149: 113-120.

Kumar S, Singh V, Singh M, Kumar S, Kalloo G and Rai M. 2004. Testing validity of fertility restorer (*Rf*) gene associated RAPD markers in newly identified restorer and maintainer lines of pepper (*Capsicum annuum* L.).New direction for a diverse planet. In: Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, 26 September -1 October 2004.

Kusterer B, Lazarescu E and Prute M. 2004. Molecular characterization of CMS sources and their fertility restoration in the genus *Helianthus*. Paper presented at the Plant and Animal Genomes XII Conference, Town and Country Convention Centre, San Diego, Ca. 10-14 January 2004.

Martin ALA, Baia GS, Filho SM, Paula TJD, Sediyama C S, Barros EGD and Moreira MA. 1996. Use of RAPD -PCR to identify true hybrid plants from crosses between closely related progenitors. Brazilian Journal of Genetics 19: 621-623.

Nateson S, Khan AKF and Shanmugasundaram P. 2002. Use of RAPD markers for the diversity analysis of new CMS lines in sorghum. Paper presented at Plant, Animal & Microbe Genome X Conference, Town & Country Convention Center, San Diago, CA, 12-16 January 2002.

Panguluri SK, Janaiah K, Govil JN, Kumar PA and Sharma PC. 2006. AFLP fingerprinting in pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives. Genetic Resources and Crop Evolution 53: 523-531.

Parani MM, Lakshmi P and Senthilkumar and Parida A. 2000. Ribosomal DNA variation and phylogenetic relationship among (*Cajanus Cajan* (L.) Millsp and its wild relative. Current Science 78: 1235-1238.

Pring DR and Tang HV. 2004. Transcript profiling of male-fertile and male-sterile sorghum indicates extensive alteration in gene expression during microgametogenesis. Sexual Plant Reproduction 16: 289-297.

Rajeshwari R, Sivaramakrishnan S, Smith RL and Subrahmanyam NC. 1994. RFLP analysis of Mitochondrial DNA from cytoplasmic male sterility lines of pearl millet. Theoretical and Applied Genetics 88: 441-448.

Rajora P and Rahman H. 2003. Microsatellite DNA and RAPD fingerprinting, identification and genetic relationship of hybrid poplar cultivars. Theoretical and Applied Genetics 106: 470-477.

Ratnaparkhe MB, Gupta VS, Murthy MRV and Ranjekar V. 1995. Genetic fingerprinting of pigeonpea (*Cajanus cajan* (L.) Millsp and wild relative using RAPD markers. Theoretical and Applied Genetics 91: 893-898.

Reddy BVS, Green JM and Bisen SS. 1978. Genetic male sterility in pigeonpea. Crop Science 18: 362-364.

Rom M, Bar M, Rom A, Pilowsky M and Gidoni D. 1995. Purity control of F1 hybrid tomato cultivars by RAPD markers. Plant Breeding 114:188.

Saxena KB, Singh L and Gupta MD. 1990. Variation for natural out crossing in pigeonpea. Euphytica 46:143-148.

Saxena KB, Singh L, Kumar RV and Rao AN. 1996. Development of cytoplasmic–genic male sterility (CMS) system in pigeonpea at ICRISAT Asia centre .In: Proceedings of the Working Group on Cytoplasmic–Genic Male Sterility (CMS) in Pigeonpea, 1996, ICRISAT Centre, Patancheru, Andhra Pradesh, India, pp1-8.

Saxena KB, Srivastava DP, Chauhan YS and Ali M. 2005. Hybrid pigeonpea. In: Masood Ali and Shiv Kumar (eds). Advances in Pigeonpea Research, Indian Institute of Pulses Research, Kanpur, India, pp 96-133.

Shasany AK, Darokar MP, Dhawan S, Gupta AK, Gupta S, Shukla AK, Patra NK and Khanuja SPS. 2005. Use of RAPD and AFLP markers to identify inter- and intraspecific hybrid of mentha. Journal of Heredity 96: 542-549.

Sivaramakrishnan S, Seetha K and Rao AN. 1997. RFLP analysis of cytoplasmic male sterility lines of pigeonpea (*Cajanus cajan* (L) Millsp.) developed by interspecific crosses. Euphytica 93: 307-312.

Souframanien J, Manjaya JG, Krishna TG and Pawar SE. 2003. Random amplified polymorphic DNA analysis of cytoplasmic male sterility and male fertile pigeonpea (*Cajanus cajan* (L.) Millsp.). Euphytica 129: 293-299.

Tar'an B, Zhang C, Warkentin T, Tullu A, Vanderberg A. 2005. Genetic diversity among varieties and wild species accessions of pea (*Pisum sativum* L.) based on Molecular markers, and morphological and physiological characters. Genome 48: 257-272.

Tikka SBS, Parmar LD and Chauhan RM. 1997. First record of cytoplasmic genic male sterility system in pigeonpea (*Cajanus cajan* (L.) Millsp.) through wide hybridization. Gujarat Agriculture University Research Journal 22: 160-162.

Wang G, Castiglione S, Zhang, Fu R, Ma J, Li W, Sun Y and Sala F. 1994. Hybrid rice (*Oryza sativa* L.): identification and parentage determination by RAPD fingerprinting. Plant Cell Reports 14: 112-115.

Yasas SN, Feridinandez and Coulman BE. 2002. Evaluating genetic variation and relationship between two brome grass species and their hybrid using RAPD and AFLP markers. Euphytica 125: 281-291.

Zhang J and Stewart JM. 2004. Identification of molecular markers linked to the fertility restorer gene for CMS-D8 in cotton. Crop Science 44: 1209-1217.