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Research Article

Phenotypic and molecular characterization of diversified cytoplasmic male sterile lines of rice (*Oryza sativa* L.)

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Abstract
Genetic diversity in cytoplasmic male sterile (CMS) lines of rice incorporating attributes of locally preferred varieties is essential to help reduce genetic vulnerability and produce desirable heterotic combinations. Seventeen diverse CMS lines were developed by backcrossing five CMS sources with six Indian rice varieties. Phenotypic evaluation of the CMS lines at seven locations exhibited superior agronomic and floral attributes. Genetic diversity study based on 45 polymorphic microsatellite markers exhibited high genetic dissimilarity (0.65) indicating diversification of CMS genotypes. The UPGMA based dendrogram showed distinct clusters indicating wide genetic diversity. Principle component analysis supported distinct clustering. CMS line specific SSR markers were detected which can help to monitor genetic purity. New CMS lines will enrich the genetic resource and allow selection of appropriate CMS lines for developing high yielding hybrids with desirable traits and reduce the risk associated with genetic uniformity.

Key words:
Rice, Cytoplasmic male sterile, Diversity

Introduction:

Rice is grown worldwide on about 154mha annually with a production of 600mt. To meet with the demand by 2025, 800mt of rice will be needed and hence rice varieties with higher yield potential and greater yield stability will need to be bred (Khush, 2005). Hybrid rice technology through heterosis has a potential to break the current yield ceiling. Availability of stable cytoplasmic male sterility (CMS) and fertility restoration system is vital for commercial exploitation of heterosis. With the discovery of the 'wild abortive' (WA) male sterility inducing cytoplasm from *Oryza sativa* f. *spontanea* and subsequent development of three-line hybrids made a breakthrough in exploitation of heterosis in rice (Lin and Yuan, 1980). Being a single widely used source of cytoplasmic sterility, it poses grave problem of increased genetic homogeneity among the hybrid lines (Virmani and Wan, 1986). Among the various approaches to develop new CMS sources, inter-sub-specific and inter-specific crosses involving cultivated and wild species of rice have been used (Hoan *et al.*, 1997). However, WA source remains the dominating feature of hybrid technology due to lack of useful alternatives. Thus there is an urgent need to diversify CMS lines of rice. Parental line improvement program needs to be carried out using recombination breeding and backcross assisted introgression of CMS trait from different CMS lines into different rice genetic backgrounds. Genetic purity of parental lines is an important prerequisite in hybrid rice production. DNA fingerprinting of rice CMS lines will help to monitor seed purity. Simple sequence repeat (SSR) markers are efficient, cost-effective and highly polymorphic. These are also highly reproducible, multi-allelic, codominant, abundant and cover whole genome in rice (Wu and Tanksley, 1993). They are ideal for genetic diversity studies and intensive genetic mapping. In this paper, a study of morphological and floral traits of new, diversified CMS lines, their assessment for genomic diversity and characterization using microsatellite based DNA markers is reported.

Material and methods

Seventeen diverse CMS lines developed at Agriculture Research Station, Ratnagiri, Maharashtra, India, using repeated backcrossing using three diverse CMS sources in the background of six elite diverse Indian varieties which included salt tolerant line Panvel2, export quality aromatic rice Pusa Basmati1 and high yielding scented variety Indrayani, locally adapted high yielding varieties HMT Sona, Ratnagiri24 and Ratnagiri60, were used in this study. Three diverse male sterile sources used were WA type CMS lines such as IR54755A, IR68885A, IR58025A; Dissi type CMS from China such as D297A and Gambiaca type CMS from China such as G46A . Anthers from 15 spikelets each of 10-12 plants were stained with 1% Iodine-Potassium Iodide (I_2-KI) solution and checked microscopically for pollen sterility (Xue *et al.*, 1995).

All 17 CMS lines and IR58025A were grown at seven diverse agro-climatic Indian locations (Jabalpur, Faizabad, Karjat, Pantnagar, Coimbatore, Ratnagiri and Hyderabad) in two replicates. Data were recorded on five randomly sampled plants in each replicate and mean was calculated. Different agronomic and floral traits were recorded (Table 1, 2). Five panicles per plant

were covered with butter paper bags before anthesis to assess seed setting per cent. Out crossing percentage (OC%) was calculated using the following formula.

$$OC\% = \frac{\text{(Total no. of spikelets in panicle - No.of unfilled spikelets in panicle)}}{\text{Total number of spikelets in panicle}} \times 100$$

Analysis of variance was carried out using IRRISTAT Windows 4.01 program.

Primer selection and PCR amplification**:** Forty five SSR primer pairs (Table 3) were used for PCR amplification. DNA was extracted from the 17 CMS lines (Nalini *et al.,* 2004). PCR was conducted in a reaction volume of 15µl. The reaction mixture contained 10X PCR buffer 1.5µl (Bangalore Genei), dNTP's (2.5mM) 1.5µl, Taq DNA polymerase (3U/µl) 0.17µl, forward and reverse primer (10.5µM/µl) 1µl each and DNA 25ng. PCR conditions were as follows: initial denaturation for 3min. at 94°C, 35 cycles of 1 min. at 94°C, 1 min. at 55°C (annealing temperature) and 1 min. at 72°C (Kumar and Bhagwat, 2012). Annealing temperature was varied between 55-64°C depending upon the specific requirements of the SSR primer pairs. The PCR amplified products were electrophoresed in 4% Metaphor agarose gels at 100V for 2-3h and stained in ethidium bromide.

Evaluation of polymorphism and data analysis:
The amplified SSR bands were scored for presence (1) or absence (0) of an allele. Data were entered in binary data matrix as discrete variables. Genetic relationships among the new CMS lines were estimated using Nei's unbiased genetic distance coefficient. Data were analyzed for dissimilarity using DARwin 5.0 software provided by ICRISAT. Pair wise genetic similarity was estimated using Jaccard's similarity co-efficient. Unweighted pair group method using arithmetic averages (UPGMA) was used for cluster analysis to generate dendrogram in radial format. Principle component analysis (PCA) was conducted based on SSR data using NTSYS PC-2.2 program using Euclidean distance matrix. CMS line specific bands were identified which could differentiate it from the rest of lines.

Results and Discussion

Phenotypic diversity among the CMS lines:
Differentiation based on pollen staining: The new CMS lines were found to be 100% male sterile. Three different kinds of sterile pollens were noticed. Pollen with irregular shape and no staining with I_2-KI were Unstained Withered Sterile (UWS) type, spherical pollen with no staining were Unstained Spherical Sterile (USS) type and pollen with spherical shape with light stain were Stained Round Sterile (SRS) type (Figure 1). UWS type of pollen was prevalent in most of the CMS lines. UWS and USS types of pollen were found in RTN1A, 2A, 11A, 14A and 20A. USS and SRS type of pollen were also found in RTN5A, 16A and IR58025A.

Phenotypic diversity**:** All 17 CMS lines exhibited significant variability for different agronomic traits. The mean plant height ranged from 74.0 (RTN13A) to 102.8 (RTN19A) with an average height of 87.3cm (Table 1). CMS lines were semi-dwarf and thus expected to be responsive to nitrogen input and tolerant to lodging. A good CMS line should be 16-29cm shorter than maintainer or restorer line for effective transfer of pollen (Azzini *et al.,* 1982). Number of productive tillers (NPT, which includes basal, secondary and tertiary tillers) contributes to yield increase and breeding aims at increasing NPT. CMS lines exhibited wide range for productive tillers ranging from 27.0 (RTN19A) to 59.0 (RTN1A) with a mean of 41.1. RTN8A and RTN10A were found to be earliest in maturity which could help in developing early maturing hybrids and other lines were mid-early like IR58025A. Variation was observed for panicle traits such as panicle length, number of spikelets per panicle etc.. Maximum panicle length (25.2cm) was recorded in RTN12A and RTN5A. The range for panicle length was 23.1-25.2cm and the differences among the CMS lines were significant. Total number of spikelet per panicle has direct influence on the yield potential of CMS lines and in turn the hybrid rice. It ranged from 204 (RTN12A) to 445 (RTN5A) with a mean of 300.6. The seeds of new CMS lines were long slender type like IR58025A, with test weight significantly lower indicating finer grains which will help in developing quality rice hybrids. RTN 16A, 17A and 18A were aromatic and may be used for developing aromatic rice hybrids.

Diversity for floral traits**:** High pollen sterility (~100%) was observed in CMS lines (Table 2) which is desirable for commercial exploitation of heterosis (Virmani, 1994). The CMS lines with complete pollen sterility showed no seed set in bagged panicles. Selection for floral traits that increased cross pollination such as carpel length and the proportion of extruded stigma improves hybrid seed production (Cheng *et al.,* 2007). Most of the CMS lines were superior to IR58025A (42.5%) for stigma exsertion except RTN14A (38.3%) and RTN16A (41%). In most cases carpel length was at par with IR58025A (3.54mm) except RTN2A (4.08mm) and 8A (4.04mm) which showed longer carpel which is good for hybrid seed production.

Most of the CMS lines exhibited wider angle of floret opening (20.47-41.93˚) rendering them promising for hybrid seed production. Better panicle exsertion results in better seed set thus

increasing the efficiency of hybrid seed production. All CMS lines showed better panicle exsertion compared to IR58025A (82.3%). Out crossing percentage (OC %) is an important trait which decides the economic viability of hybrid seed production in rice. Highest OC% was recorded in RTN12A and RTN10A (38.6%) followed by RTN19A (34.6%) and RTN3A (34.3%) which may make them suitable as hybrid CMS lines (Xu and Li, 1998).

Microsatellite polymorphism and genetic variability analysis among new CMS lines:

Success of a hybrid rice programme largely depends on extent of genetic diversity in the CMS and restorer lines which could be assessed by microsatellite markers. Microsatellite diversity analysis of 17 CMS lines with 45 polymorphic SSR primer pairs indicated a high level of genetic diversity (0.65) and higher average number of alleles per primer (3.47) (Table 3). The PIC values; a reflection of allelic diversity and frequency among the lines, ranged from 0.22 for RM484 to 0.73 for RM162 with an average of 0.51 indicating hyper variable nature and higher polymorphism estimate for these loci. Dissimilarity matrices, based on shared allele analyses, revealed that the average genetic dissimilarity between genotypes was 0.65 with SSR markers. Maximum dissimilarity was observed between RTN17A and 20A (0.9647) and minimum between RTN1A and RTN3A (0.1632). An important application of molecular diversity study is to identify a marker which can differentiate a desirable genotype from the rest. In the present study, unique alleles were identified for the 17 CMS lines (Table 4). The potential of SSR markers in discriminating rice cultivars is well documented (Zhu *et al.,* 2012). Evaluation of morphological and molecular variation of the CMS lines will help in their identification and utilization in future for the development of commercial rice hybrids.

Genetic relationship among the CMS lines:

In order to understand the genetic relationships among different CMS lines used in this study, the genetic distance index was calculated. Nei's UPGMA dendrogram based on the genetic distance was constructed (Figure 2). Since the new CMS lines were in the backgrounds of popular, well adapted genotypes, genotype specific clustering was observed. The dendrogram revealed four distinct clusters; cluster I consisted of six CMS lines i.e., RTN1A to 6A, clusters II and III consisted of four CMS lines with two sub-clusters each. In Cluster II, RTN19A and 20A and RTN8A and RTN10A were grouped together in different sub-clusters as they were developed in the background of Ratnagiri varieties (RTN60 and RTN24 respectively). RTN11A to 14A were clubbed into cluster III as they have a common parent cv. HMT Sona. Cluster IV consisted of three CMS lines having two sub-clusters with RTN17A and 18A forming distinct sub-cluster. Cluster IV was widely separated from the other three clusters since the CMS lines were developed with aromatic rice varieties viz., Pusa Basmati1 and Indrayani. Diverse clustering among the new CMS assures sufficient genetic diversity among the clusters which will be helpful in exploiting heterosis and provide wider adaptability in hybrids. To better understand the relationships among rice CMS lines, PCA was carried out using the genetic similarities data (Figure 3). The first three eigenvectors accounted for about 83% of the variation. The groupings identified by PCA were very similar to those identified by the UPGMA cluster analysis. The PC1, PC2 and PC3 explained 39.3, 27.3 and 16.2% of the total genotypic variability respectively. The principal component analysis and cluster analysis were effective in discriminating since analysis was based on higher number of SSR markers (Cao *et al.,* 2006). The newly generated CMS lines, their inter-relationships and molecular characterization will be helpful in the development of new hybrid rice varieties.

In conclusion, diversification in CMS lines is the key to development of hybrid rice varieties and to avoid the use of single genetic source. CMS trait from three different sources was introgressed into six popular Indian varieties varying in different agro-climatic requirements and agronomic traits. The CMS lines showed a wide array of agronomic and floral attributes rendering them suitable for hybrid programs. Microsatellite markers were effective in diversity analysis and differentiation among the new CMS lines. Marker-based differentiation could be helpful to preserve the purity of CMS lines.

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References

Azzini, L. E. and Rutger, N. 1982. Amount of out crossing on different CMS lines of Rice. *Crop Sci.*, **22** : 905-906.

Cao, Q., Lu, B., Xia, H., Rong, J., Sala, F. , Spada, A. and Grassi, F. 2006. Genetic Diversity and Origin of Weedy Rice (*Oryza sativa* f. *spontanea*) Populations Found in North-eastern China Revealed by Simple Sequence Repeat (SSR) Markers. *Ann. Bot.*, **98** : 1241-1252.

Cheng S. H., Zhuang, J. Y., Fan, Y. Y., Du, J. H. and Cao, L. Y. 2007. Progress in research and development on hybrid rice, a super-domesticate in China. *Ann. Bot.*, **100** : 959-996.

Hoan, N. T., Sarma, N. P. and Siddiq, E. A.. 1997. Identification and characterization of new

sources of cytoplasmic male-sterility in rice. *Plant Breed.*, **116 :** 547-551.

Khush, G. S. 2005. What it will take to feed 5.0 billion rice consumers in 2030. *Plant Mol. Bio.*, **59**: 1-6.

Kumar, V. and Bhagwat, S. G.. 2012. Microsatellite (SSR) based assessment of genetic diversity among the semi-dwarf mutants of elite rice variety WL112. *Int. J. Plant Breed. Genet.*, **6** : 195-205.

Lin, S. C. and Yuan, L. P. 1980. Hybrid rice breeding in china. In: Innovative Approaches to Rice Breeding. International Rice Research Institute Press, Manila, Philippines, p. 35-51.

Nalini, E., Bhagwat, S. G. and Jawali, N. 2004. A simple method for isolation of DNA from plants suitable for long-term storage and DNA marker analysis. *BARC Newsl.*, **249** : 208-214.

Virmani, S. S. and Wan, S. H. 1986. Development of CMS lines in hybrid rice breeding. In: Hybrid rice. International Rice Research Institute Press, Manila, Philippines, p. 103-114.

Virmani, S. S. 1994. Outcrossing Mechanisms and Hybrid Seed Production Practices in Rice. In: Heterosis and Hybrid Rice Breeding. *Theor. Appl. Genet.*, **22**: 76-106.

Wu, K. S. and Tanksley, S. D. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol. Gen. Genet.*, **241** : 225–235.

Xu, S. and Li, B. 1998. Managing hybrid rice seed production. In : Hybrid rice. International Rice Research Institute Press, Manila, Philippines, p. 157-163.

Xu, Y., Beachell, H. and McCouch, S. R. 2004. A marker-based approach to broadening the genetic base of rice in USA. *Crop Sci.* **44** : 1947-1959.

Xue, S. Y., Xiang, S. N. and Xu, D. X. 1995. Breeding of CMS Lines Resistant to Rice Blast by Backcrossing Method. *Hybrid Rice*, **3** : 5-24.

Zhu, Y., Qin, G., Hu, J., Wang, Y., Wang, J. and Zhu, S. 2012. Fingerprinting and variety identification of rice (Oryza sativa L.) based on simple sequence repeat markers. *Plant Omics J.*, **5** : 421-426.

Table 1. Agronomic traits of different CMS lines used in the study

CMS lines	Plant Height (cm)	No. of Basal Tillers/ plant	No. of Productive Tillers/ plant	Days to 50% Flowering	Panicle Length (cm)	Total no. of Spikelets/ panicle	Test Weight (g)	Grain Type	Aroma	Awn
RTN 1A	82.2	17.5	59.0	105.6	24.5	377	1.7	LS	-	AL
RTN 2A	81.0	19.1	49.4	106.6	24.3	358	1.6	LS	-	AL
RTN 3A	77.7	16.2	45.9	106.0	24.5	381	1.8	LS	-	AL
RTN 4A	75.6	16.9	49.5	107.0	25.0	371	1.9	LS	-	AL
RTN 5A	79.8	16.7	47.2	109.5	25.2	445	1.7	LS	-	AL
RTN 6A	82.5	14.9	34.6	101.4	24.3	353	1.7	LS	-	AL
RTN 8A	96.4	16.5	46.3	94.7	23.2	311	1.8	LS	-	AL
RTN 10A	92.5	10.1	41.1	95.0	25.1	286	1.7	LS	-	AL
RTN 11A	82.5	15.7	37.9	108.3	24.0	215	1.7	LS	-	AL
RTN 12A	84.4	14.8	31.0	105.5	25.2	204	1.7	LS	-	AL
RTN 13A	74.0	17.6	35.0	106.4	24.8	332	1.8	LS	-	AL
RTN 14A	86.8	18.0	39.3	109.3	23.2	247	1.8	LS	-	AL
RTN 16A	90.2	16.8	34.3	105.3	23.9	234	1.4	LS	Present	AL
RTN 17A	96.6	16.4	36.0	112.4	24.3	267	1.6	LS	Present	PA
RTN 18A	99.2	17.2	42.3	110.7	23.1	235	1.7	LS	Present	PA
RTN 19A	102.8	17.2	27.0	100.3	23.2	261	1.8	LS	-	AL
RTN 20A	101.0	15.3	45.5	103.2	23.9	287	1.8	LS	-	AL
IR 58025A	86.5	16.7	39.0	105.2	24.9	247	2.0	LS	-	PA
Mean	87.3	16.3	41.1	105.1	24.2	300.6	1.7	-	-	-
Range	74 - 102.8	10.1 - 19.1	27 – 59	94.7 - 112.4	23.1 - 25.2	204 – 445	1.4 – 2.0	-	-	-
SE(±)	2.10	0.44	1.84	1.13	0.17	16.12	0.03	-	-	-
CD at 5%	6.35	2.14	2.87	6.64	0.42	39.82	0.09	-	-	-

LS: Long Slender, AL: Awnless, PA: Partial Awning.

Table 2. Improved floral traits of newly developed CMS lines

CMS lines	PS (%)	CL (mm)	AFO (˚)	SE (%)	PE (%)	OC (%)
RTN 1A	100	3.09	22.91	42.69	81.47	19.3
RTN 2A	100	4.08	34.47	49.63	73.60	20.6
RTN 3A	100	3.06	23.90	50.00	82.43	34.3
RTN 4A	100	3.56	20.47	43.20	80.07	24.3
RTN 5A	100	3.51	23.53	43.20	82.37	32.0
RTN 6A	100	3.03	27.23	49.27	79.99	19.0
RTN 8A	100	4.04	34.53	49.77	82.97	25.3
RTN 10A	100	3.72	41.93	45.13	74.77	38.6
RTN 11A	100	2.45	25.80	56.47	79.57	17.6
RTN 12A	100	3.07	24.27	49.00	82.00	38.6
RTN 13A	100	2.49	22.30	52.90	81.03	20.3
RTN 14A	100	3.51	37.03	38.33	81.83	22.3
RTN 16A	100	3.56	34.43	41.00	83.30	28.6
RTN 17A	100	3.04	38.70	43.67	86.60	17.3
RTN 18A	100	2.50	35.53	52.67	93.10	29.0
RTN 19A	100	3.50	27.17	42.50	84.60	34.6
RTN 20A	100	3.50	25.20	44.70	83.40	23.3
IR 58025A	100	3.54	23.27	42.50	88.43	25.3
Mean	100	3.36	28.89	46.76	82.30	26.12
Range	-	2.45 – 4.08	20.47 – 41.93	38.30 – 56.40	73.60 – 93.10	17.3 – 38.6
SE(±)	-	0.075	0.542	0.909	0.185	0.66
CD at 5%	-	0.288	1.549	2.596	0.528	13.3

FL: Filament Length, PS: Pollen Sterility, CL: Carpel Length, AFO: Angle of Floret Opening, SE: Stigma Exsertion, PE: Panicle Exsertion, OC: Out Crossing.

Table 3. List of microsatellite primers and their PIC values used in this study

Primer	Linkage Group	Allele No.	PIC	Sl No.	Primer	Linkage Group	Allele No.	PIC
RM5	1	4	0.48	24	RM133	6	5	0.68
RM431	1	3	0.57	25	RM125	7	2	0.27
RM1	1	5	0.65	26	RM455	7	3	0.52
RM237	1	3	0.54	27	RM118	7	3	0.42
RM495	1	2	0.35	28	RM152	8	5	0.55
RM312	1	4	0.61	29	RM408	8	3	0.37
RM283	1	3	0.54	30	RM44	8	3	0.49
RM259	1	5	0.66	31	RM284	8	4	0.52
RM452	2	6	0.72	32	RM433	8	2	0.41
RM154	2	3	0.61	33	RM447	8	3	0.34
RM338	3	2	0.31	34	RM105	9	3	0.48
RM514	3	5	0.65	35	RM215	9	2	0.42
RM55	3	4	0.51	36	RM316	9	4	0.64
RM489	3	2	0.42	37	RM474	10	5	0.69
RM13	3	4	0.54	38	RM171	10	3	0.42
RM124	4	3	0.47	39	RM484	10	2	0.22
RM307	4	2	0.37	40	RM271	10	3	0.53
RM507	5	3	0.52	41	RM552	11	4	0.61
RM161	5	2	0.31	42	RM287	11	5	0.59
RM413	5	4	0.57	43	RM536	11	3	0.38
RM178	5	5	0.69	44	RM144	11	2	0.31
RM162	6	5	0.73	45	RM277	12	4	0.51
RM510	6	4	0.62			Total	156	22.81
						Average	3.47	0.51

PIC = Polymorphic Information Content.

Table 4. Presence of unique SSR bands in CMS lines

CMS lines	SSR Primer	~ Band size (bp)
RTN 1A	RM 431	245
RTN 2A	RM 283	160
RTN3A	RM 514	240
RTN 4A	RM 161	175
RTN 5A	RM 162	300
RTN 6A	RM 452	210
RTN 8A	RM 133	230
RTN 10A	RM 118	155
RTN 11A	RM 316	205
RTN 12A	RM 44	115
RTN 13A	RM 474	230
RTN 14A	RM 552	165
RTN 16A	RM 237	145
	RM 259	170
	RM 178	120
RTN 17A	RM 13	90
RTN 18A	RM 154	210
RTN 19A	RM 259	145
RTN 20A	RM 312	95

Fig. 1 Microscopic view of three categories of pollens stained with iodine stain

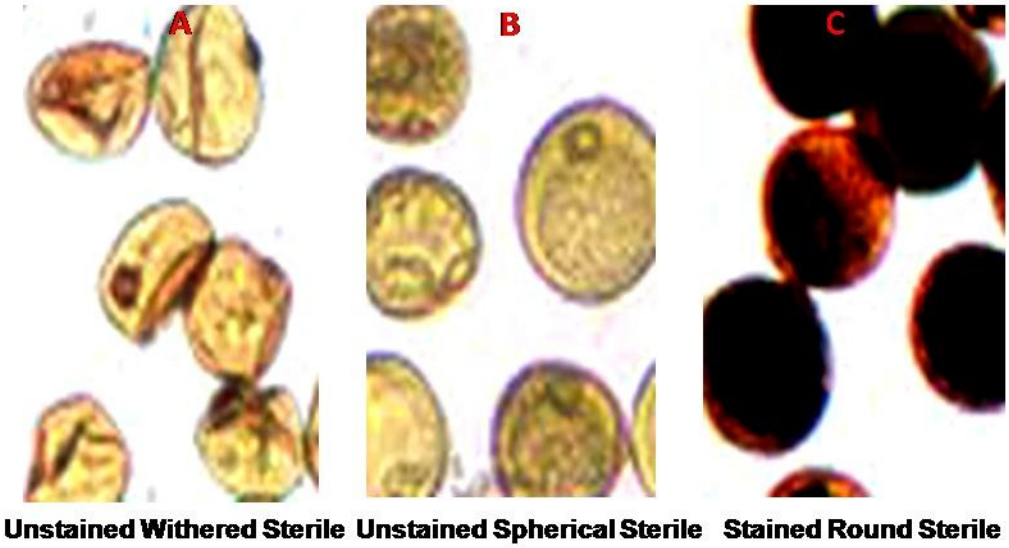

Fig. 2 Dendrogram showing genetic relationships among the 17 CMS lines based on UPGMA clustering

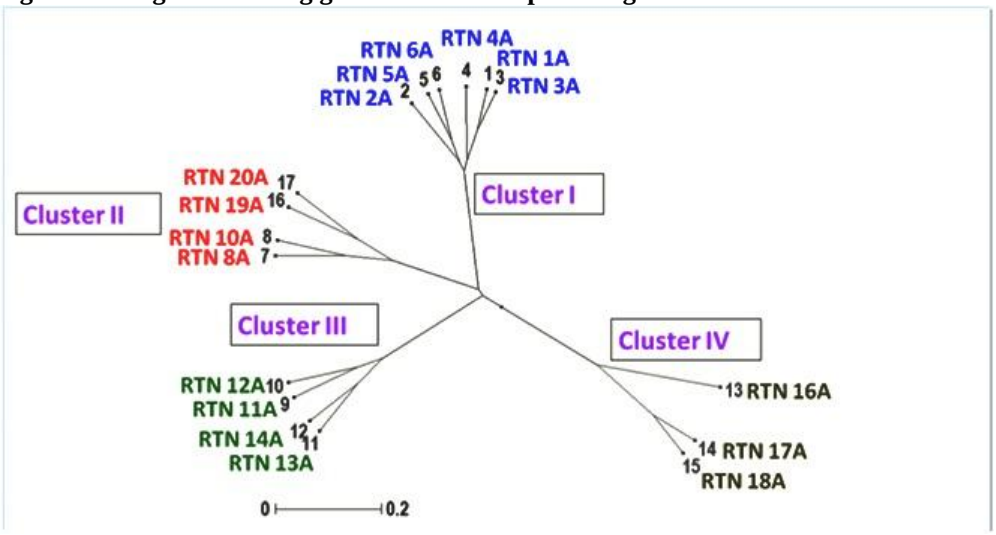

Fig. 3 Principal component analysis of 17 CGMS lines using SSR marker data