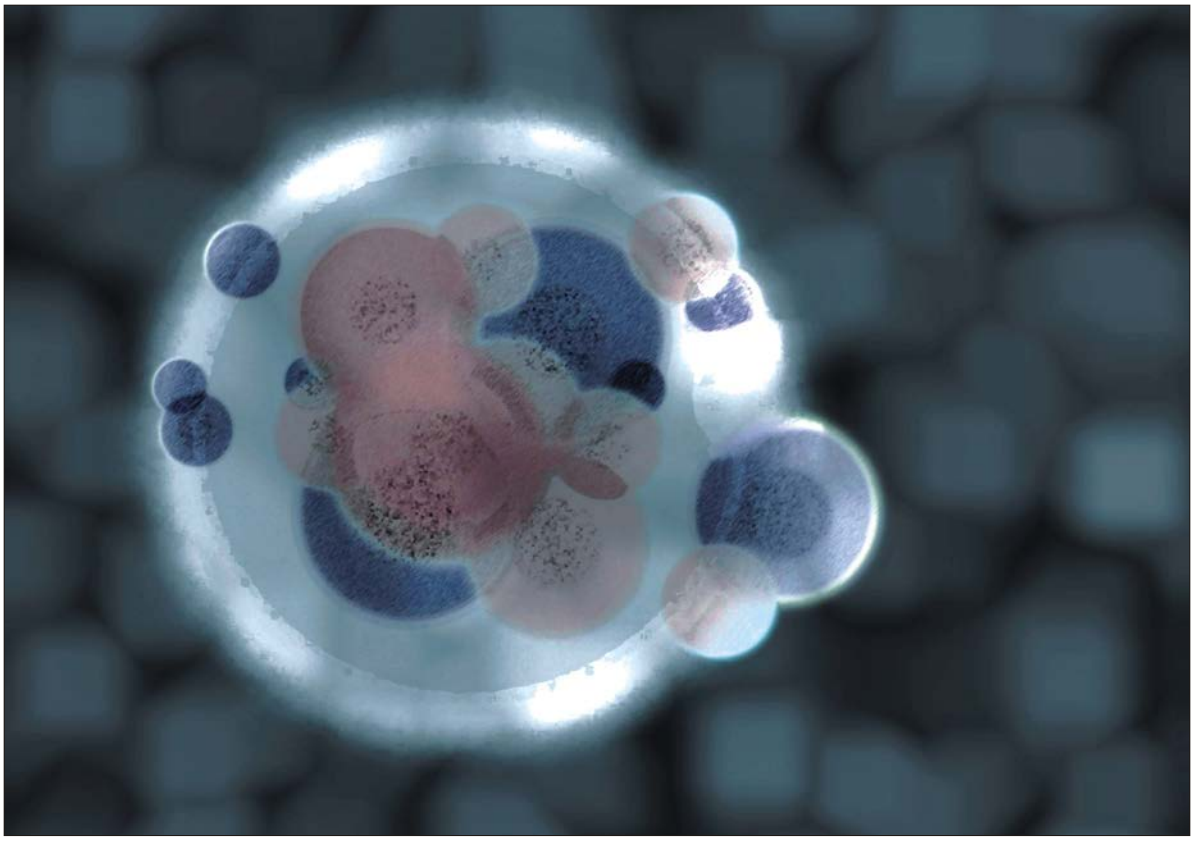

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 12 (3): 154-160, 2012
ISSN 1727-3048 / DOI: 10.3923/jbs.2012.154.160

Molecular Characterization of Different Cytoplasmic Male Sterile Lines Using Mitochondrial DNA Specific Markers in Rice

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Abstract: With the objective of identifying mitochondrial DNA based markers that can distinguish cytoplasmic sources and restorer lines, 7 male sterile and fertile counterparts of Cytoplasmic Male Sterile (CMS) lines and 4 restorers (R) lines were characterized, using 20 universal mitochondrial DNA specific markers. Diverse CMS lines, other than the Wild Abortive (WA) one have been developed at International Rice Research Institute (IRRI), Philippines (IR66707A with cytoplasm of *Oryza perennis*), Central Rice Research Institute (CRRI), Cuttack (CRMS-32A with ''Kalinga'' cytoplasm from 'Dunghansali') and at Directorate of Rice Research (DRR), Hyderabad (DMS 3A with cytoplasm of *Oryza nivara* and DMS 4A with cytoplasm of *Oryza rufipogon*). Traditionally, the differences between the cytoplasmic sources were studied by differential response of the Cytoplasmic Male Sterile (CMS) lines when test crossed with a set of known maintainers and restorers. However, these methods could not distinguish the genetic variation at molecular level, since, CMS trait has been found to be associated to mitochondrial DNA aberration. In the present study, seven potential primers were identified, viz., cox1B, nad4ex1, nad5D, nad1, alp, nad4ex2 and rpS14 which showed polymorphism. The results suggested effective utilization of mitochondrial specific primer pairs in hybrid purity testing and marker aided heterosis breeding in rice.

Key words: Hybrid rice, cytoplasmic male sterility, mitochondria, maintainer, restorer, polymerase chain reaction

INTRODUCTION

To meet the demand of increasing population and maintain self sufficiency the present rice production needs to be increased by 30% by the year 2020 (Singh *et al*., 2012). The task is quite challenging and the options available are very limited in view of plateauing trend of yield in high productivity areas, decreasing and degrading land and scarcity of water and labour (Santhanalakshmi *et al*., 2010). During the past decade, hybrid rice technology has emerged as one of the most practical and acceptable approaches to achieve this target (Tiwari *et al*., 2011). It has not only contributed to food security but also benefited the environment (Sreedhar *et al*., 2011). Rice is one of the major crop species in which hybrids are used commercially (Selvaraj *et al*., 2011).

In India IR58025A is the most widely used Cytoplasmic Male Sterile line (CMS) in hybrid rice production and contains WA type cytoplasm. This cytoplasmic uniformity of a single source of CMS may lead to genetic vulnerability towards disease and insects as in the case of maize (Ullstrup, 1972), pearl millet (Kumar *et al*., 1983) and more recently, rice (Biswas, 1999; Sharma *et al*., 1999). Hence, there is an urgent need for diversifying our present rice CMS genotypes. Diversified CMS lines, other than WA cytoplasm have been developed at China, IRRI, India and various other countries. Fothermor over 20 CMS sources have been developed (Fujii *et al*., 2010) from various accessions of cultivated rice and wild species.

Mitochondria, with a genome size of around 490 kb, are the energy generating apparatus that control electron transport in plants (Notsu *et al*., 2002). The mitochondrial genome of rice is constituted of five circles, wherein some part of each circle shares homologous sequences with one or other circles (Tian *et al*., 2006). This complex structure of mitochondrial (mt) genome is

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thought to be the result of intra and inter-molecular recombinations that not only alter gene organization but also affect morphological traits such as CMS (Lonsdale *et al*., 1984).

Keeping these in views, in the present study, a strategy based on mitochondrial DNA specific primers, was employed to characterize different sources of cytoplasmic male sterile lines, their maintainers and restorers. The study was based on the universal mitochondrial specific primers. The DNA marker profiles of these universal primers for 18 rice genotypes were analyzed for studying the putative polymorphism.

MATERIALS AND METHODS

Plant material: In all, 18 genotypes comprising of 7 pairs of CMS and their maintainers and 4 restorer lines were used (Table 1). The male sterility sources included five different CMS sources, viz., Wild Abortive (WA), 'Kalinga', *O. nivara*, *O. rufipogon* and *O. perennis*. The lines were made available by the International Rice Research Institute (IRRI), Los Baños, Philippines; Barwale Foundation (BF) and Directorate of Rice Research (DRR), Hyderabad, India.

Primers: A total of 20 different universal mitochondrial (mt) DNA specific primer pairs (Table 2) synthesized by Sigma Aldrich, Bangalore, were used in the study. These universal mtDNA primers were designed based on consensus mtDNA (Petit *et al*., 1998; Wu *et al*., 1998) derived from various mitochondrial genes. The details of the amplified region are given in Table 3.

DNA extraction and PCR amplification: Genomic DNA was extracted from freshly harvested young leaves of all the lines using the protocol described by Shanti *et al*. (2010). PCR was carried out using these markers and the products were resolved on 2.0% agarose (Sigma, Molecular Biology Grade) gel electrophoresis followed by ethidium bromide staining or 6% Polyacrylamide Gel Electrophoresis (PAGE) and silver staining (Nassiri *et al*., 2007). The fragment size of the amplicons were calculated using the software Utility Alphaease® (Alphainnotech, USA) using Gene Ruler 100 bp plus DNA ladder (MBI Fermentas, Lithuania) as size standard.

Table 1: List of rice genotypes used in the study

Genotype	Characteristics	Reference
IR58025A	Male sterile (MS), Wild abortive (WA) -cytoplasm	Khera *et al*. (2009)
IR58025B	Maintainer-WA	Khera *et al*. (2009)
IR68888A	MS; WA-cytoplasm	Sheeba *et al*. (2009)
IR68888B	Maintainer-WA	Sheeba *et al*. (2009)
Pusa 6A	MS; WA-cytoplasm	Sheeba *et al*. (2009)
Pusa 6B	Maintainer-WA	Sheeba *et al*. (2009)
CRMS 32A	MS; Kalinga-cytoplasm	Pradhan *et al*. (1990)
CRMS 32B	Maintainer-Kalinga	Pradhan *et al*. (1990)
DMS 3A	MS; *O. nivara*-cytoplasm	Ramesha *et al*. (1999)
DMS 3B	Maintainer-*O. nivara*	Ramesha *et al*. (1999)
DMS 4A	MS; *O. rufipogon*-cytoplasm	Ramesha *et al*. (1999)
DMS 4B	Maintainer-*O. rufipogon*	Ramesha *et al*. (1999)
IR66707A	MS; *O. perinnes*-cytoplasm	Dalmacio *et al*. (1995)
IR66707B	Maintainer-*O. perennis*	Dalmacio *et al*. (1995)
IR10198	Restorer of WA	Sheeba *et al*. (2009)
IR40750	Restorer of WA	Sheeba *et al*. (2009)
KMR3	Restorer of WA	Shanti *et al*. (2010)
PRR78	Restorer of WA	Shanti *et al*. (2010)

Table 2: List of universal mitochondrial DNA specific primers used in the study

	Sequence (5'>3')			
Primer name	Forward sequence	Reverse sequence	Annealing temp. (°C)	Amplicon size (bp)
Nad1	GCATTACGATCTGCAGCTCA	GGAGCTCGATTAGTTTCTGC	55	1600
Nad4ex1	CAGTGGGTTGGTCTGGTATG	TCATATGGGCTACTGAGGAG	55	1900
Nad4ex2	TGTTTCCCGAAGCGACACTT	AACCAGTCCATGACTTAACA	60	2000-4000
rpS14	CACGGGTCGCCCTCGTTCCG	GTGTGGAGGATATAGGTTGT	55	100-1400
coxII	AATCCAATCCCGCAAAGGATT	AGAAGATGATCCAGAATTGGG	55	NA
rRNA	GTGTTGCTGAGACATGCGCC	ATATGGCGCAAGACGATTCC	55	800
alp	TTTGCCAGCGGTGTIAAAGG	CTTCGCCATATTGTGCCAATTC	55	1100
Atp6	GGAGGIGGAAAITCAGTICCAA	TAGCATCATTCAAGTAAATACA	45	600
Cob	AGTTATTGGTGGGGGTTCGG	CCCCAAAAGCTCATCTGACCCC	55	350
cox1A	GGTGCCATTGCIGGAGTGATGG	TGGAAGTTCTTAAAAGTATG	52	1500
cox1B	GGCTITTCTCCACIAACCACAA	GGAGGGCTTTGTACCAICCATTC	55	1500
cox2	GATGCIGCIGAACCITGGCA	TCCGATACCATTGATGTCC	55	340
cox3	GTAGATCCAAGTCCATGGCCT	GCAGCTGCTTCAAAGCC	55	700
Nad1A	CTAGCTGAACGTAAAGTAATGGC	CCAACCIGCTATAATIATTCC	55	1550
Nad3	AATTGTCGGCCTACGAATCTG	TTCATAGAGAAATCCAATCGT	55	220
Nad3A	AATTGTCGGCCTAGGAATGTG	GCTCGIGTACGGTCIGTGGG	55	370
Nad4A	ATACGATTGATTCGGTCTGTG	TGAACTGGTACCATAGGCACTTT	55	NA
Nad5A	GAAATCTTTGATGCTTCTTGGG	ACCAACATTGGCATAAAAAAAGT	55	1000
Nad5D	ATAAGTCAACTTCAAAGTGGA	CATTGCAAAGGCATAATGAT	55	1000
Rps14	ATACGAGATCACAAACGTAGA	CCAAGACGATTTITTTATGCC	55	NA

Adapted from Petit *et al*. (1998) and Wu *et al*. (1998), I: Inosine, NA: No amplification

Table 3: List of universal mitochondrial primers used in the study with the regions they amplified

Primer name	Region amplified
nad1	NADH dehydrogenase subunit 1
nad4ex1	NADH dehydrogenase subunit 4 (exon 1 to exon 2)
nad4ex2	NADH dehydrogenase subunit 4 (exon 2 to exon 3)
rpS14	Ribosomal protein subunit 14 to apocytochrome b gene
coxII	Cytochrome C oxidase subunit 2 gene
rRNA	18S ribosomal RNA to 5S ribosomal RNA
alp	Adenosine triphosphate subunit 1 gene
atp6	Adenosine triphosphate subunit 6 gene
cob	Apocytochrome b gene
cox1A	Cytochrome C oxidase subunit 1 gene
cox1B	Cytochrome C oxidase subunit 1 gene
cox2	Cytochrome C oxidase subunit 2 gene (exon 1)
cox3	Cytochrome C oxidase subunit 3 gene
nad1A	NADH dehydrogenase subunit 1 (exon 1)
nad3	NADH dehydrogenase subunit 3
nad3A	NADH dehydrogenase subunit 3 to Ribosomal protein subunit 12
nad4A	NADH dehydrogenase subunit 4 (intron 1)
nad5A	NADH dehydrogenase subunit 5 (intron 1)
nad5D	NADH dehydrogenase subunit 5 (intron 4)
rps14	Ribosomal protein subunit 14

Adapted from Petit *et al.* (1998) and Wu *et al.* (1998)

Data analyses: The presence/absence matrices were analyzed for genetic relatedness in terms of the Dice similarity coefficient (Dice, 1945). The clustering was carried out using the SAHN/UPGMA algorithm through the software NTSYS-pc version 2.21 (Rohlf, 2000). The reliability, goodness of fit and robustness of the phenetic trees were tested by deriving the bootstrapping values (Felsenstein, 1985) carried out by employing the software WINBOOT (Yap and Nelson, 1996).

RESULTS

Out of the 20 primer pairs used, only 17 showed amplification (Table 2). The DNA profiles of ten primers, viz., rRNA, atp6, cob, cox1A, cox2, cox3, nad1A, nad3, nad3A and nad5A were monomorphic. Four primer pairs cob, cox2, nad3A and nad3 which produced amplicons sizes less than 350 bp (base pair) were resolved on PAGE, but failed to manifest polymorphism for these primer pairs.

Seven primer pairs including cox1B, nad4ex1, nad5D, nadl, alp, rpS14 and nad4ex2 exhibited conspicuous polymorphism. The four primer pairs cox1B, nad4ex1, nad5D and nadl produced a single fragment in all the lines except DMS-4A. A representative pattern for primer cox1B is depicted in Fig. 1. Further, the primer pair alp produced a single amplicon of size 1100 bp in all the lines barring DMS-3A (Fig. 1). PCR was performed twice on all the five primers above in order to rule out the possibility of amplification failure. The primer pair nad4ex2 produced amplicons of 3 kb, 2.5 kb in all the lines studied (Fig. 1). Interestingly, a 2 kb size fragment was observed only in IR58025A, Pusa 6A, IR66707A, IR66707B, IR10198 and KMR3.

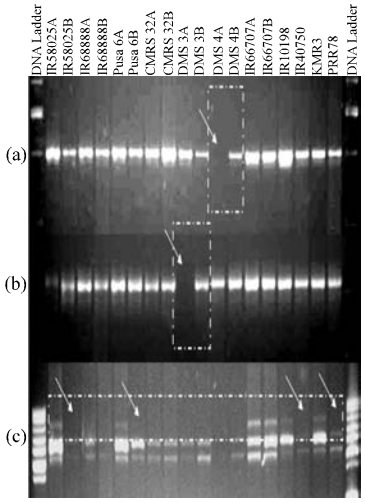

Fig. 1: Electrophoretic banding pattern on 2% agarose (a) Pattern from amplification of DNA from primer pair cox1B, (b) Pattern from amplification of DNA from primer pair alp, (c) Pattern from amplification of DNA from primer pair nad4ex2, Arrows indicate putative polymorphism and dashed blocks indicates putative pattern of polymorphism

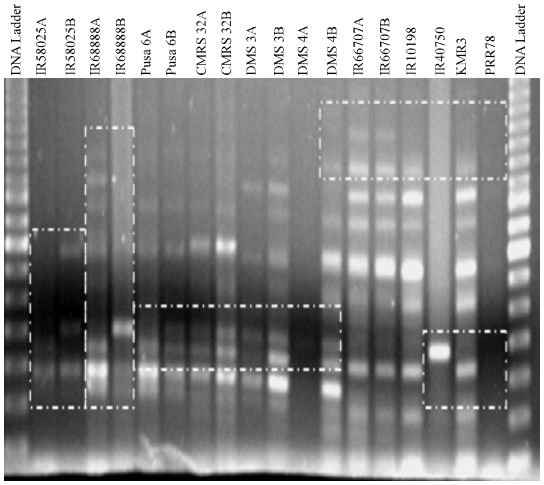

Fig. 2: Electrophoretic banding pattern on 2% agarose produced from amplification of DNA from primer pair rpS14, Dashed blocks indicates putative pattern of polymorphism

The primer pair rpS14: The primer pair rpS14 produced encouraging results (Fig. 2). The CMS line, IR58025A produced 2 amplicons of 180 and 230 bp, out of which the 230 bp amplicon was conspicuously absent in its maintainer, however, the maintainer line possessed two additional bands of 280 and 550 bp. IR68888A displayed

8 amplicons (1000, 850, 750, 550, 450, 230, 180 and 100 bp) all of which were polymorphic between its maintainer line except 550, 180 and 100 bp. Interestingly, its maintainer line had a unique amplicon of size 280 bp. With regard to Pusa-6A and its maintainer Pusa-6B, six amplicons of sizes 1000, 750, 550, 230, 180 and 100 bp were present in both the lines; again a fragment of 280 bp was amplified only in the maintainer line.

The CMS line of 'Kalinga' background CRMS-32A and its maintainer line CRMS-32B, amplified seven amplicons (1000, 750, 550, 280, 230, 180 and 100 bp), all being monomorphic. For DMS-3A (with *Oryza nivara* cytoplasm) and DMS-3B, both produced five common amplicons (850, 550, 280, 180 and 100 bp). However, three amplicons of size 100, 750 and 230 bp were present only in DMS-3B. The CMS line DMS-4A (with *Oryza rufipogon* cytoplasm) had no amplicons, while the corresponding maintainer yielded nine additional polymorphic amplicons.

The CMS line IR66707A of *Oryza perennis* background and its corresponding maintainer line IR66707B, produced seven monomorphic amplicons (1000, 850, 750, 550, 450, 230 and 100 bp) and an additional band of 1400 bp was present only in the maintainer line IR66707B.

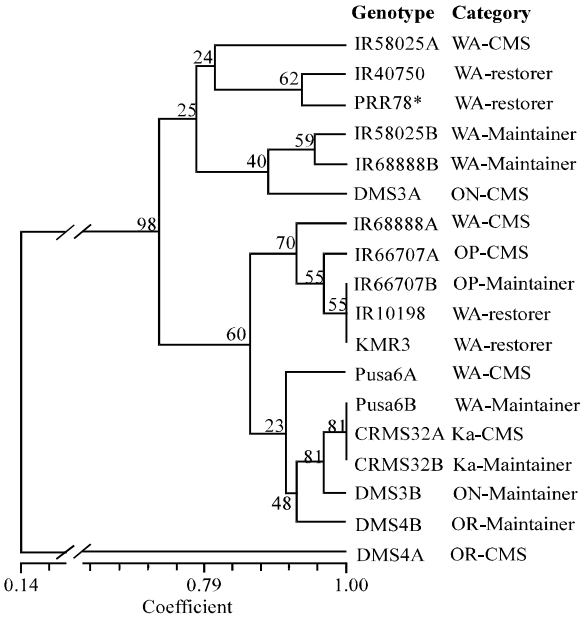

Fig. 3: SAHN/UPGMA clustering of CMS, maintainer and restorer lines (Digits at the forks represent the bootstrap values). WA: Wild Abortive, ON: *Oryza nivara*, OP: *Oryza perinnes*, Ka: Kalinga and OR: *Oryza rufipogon*. *The cytoplasm of PRR78 is sterile WA type

Among the restorer lines, IR40750R produced only one amplicon of 280 bp size whereas, PRR78 did not show any amplicon. The remaining two restorer line IR10198R and KMR3 produced monomorphic amplification pattern, both having seven fragments (1000, 850, 750, 550, 450, 230 and 100 bp).

UPGMA clustering: Cluster analysis as revealed by SAHN/UPGMA resulted in three major clusters (Fig. 3). These clusters did not demarcate any specific group. Cluster 1 was further divided into two sub-clusters; the subcluster 1 contained the genotypes IR58025A, IR40750 and PRR78; sub-cluster 2 contained IR58025B, IR68888B and DMS-3A. Cluster 2 was also subdivided into two sub-clusters; sub-cluster 1 containing IR68888A, IR66707A, IR66707B, IR10198 and KMR3; sub-cluster 2 contained Pusa 6A, Pusa 6B, CRMS 32A, CRMS 32B, DMS-3B and DMS-4B. The genotype DMS-4A; which has cytoplasm from *Oryza rufipogon* fell apart as a separate third cluster.

DISCUSSION

Majority of primers analyzed were monomorphic in nature. This might be attributed to the conservative nature of mt DNA (Fujii *et al*., 2010). The polymorphism shown for DMS-4A and DMS-4B, with respect to four primer pairs cox1B (which amplified the region of Cytochrome C oxidase subunit 1 gene), nad4ex1 (NADH dehydrogenase subunit 4-exon 1 to exon 2), nad5D (NADH dehydrogenase subunit 5-intron 4) and nad1 (NADH dehydrogenase subunit 1) can be utilized in monitoring maintenance of the DMS-4A CMS line. Similarly, the polymorphism perceived for DMS-3A and DMA-3B from primer alp (Adenosine triphosphate subunit 1 gene) could be used for seed purity testing of DMS-3A CMS line. It is worthwhile to mention here that no restorer line for these CMS lines has yet been discovered or developed (Ramesha *et al*., 1999). The presently detected polymorphism would be of practical use only when such restorer line would be available.

The primer pair nad4ex2 which amplified an NADH dehydrogenase subunit 4-exon 2 to exon 3 gene was also quite informative and evidently potential in marker aided heterosis breeding. Furthermore, the 2 kb size fragment present in IR58025A, Pusa 6A, IR66707A, IR66707B, IR10198 and KMR3 genotypes should be subjected to critical analysis via sequencing.

The primer pair rpS14 (which amplified the region from ribosomal protein subunit 14 to apocytochrome b gene) emerged as an interesting candidate for identification of all CMS lines included in the study from

their marker profile. This primer could be effectively used for further utilization in marker assisted heterosis breeding including identification of CMS lines, marker aided maintenance of CMS lines, distinguishing restorer lines and most importantly, hybrid purity testing. The polymorphism present between IR58025A and its maintainer line IR58025B; IR68888A and IR68888B; Pusa 6A and Pusa 6B; DMS 3A and DMS 3B; DMS-4A and DMS-4B and IR66707A and its maintainer line IR66707B could be useful in monitoring CMS maintenance. Furthermore, it is quite interesting to note that for the three pairs of WA CMS lines and their maintainer lines, the fragment of 280 bp is unique to the maintainer line but absent in their CMS lines. This evidenced for genetic proximity of the three WA-maintainers IR58025B, IR68888B and Pusa-6B. This particular band could be subjected to critical analysis through sequencing and further development of WA-maintainer specific DNA marker. Among the restorers, though IR10198 and KMR3 showed similar pattern, the pattern was polymorphic among IR58025A, IR68888A and Pusa 6A. This could be utilized for hybrid purity test involving these WA CMS lines. Similarly, the presence of a unique band in the restorer line IR40750 could facilitate hybrid purity testing.

The unweighted paired grouping by mathematical averaging based on the Dice similarity coefficient was done to resolve finer genetic differences. The phenetic clusters obtained were supported by high bootstrap values (range from 24-98), indicating the stability of the inferred interrelationships as well as the robustness of the mtDNA marker data used for the genetic diversity analysis. The UPGMA cluster analysis provided a better resolution of the genetically highly similar rice genotypes. The 18 genotypes fell into three major clusters. The CMS, maintainer and restorer lines were not able to cluster into distinct group indicating that the cytoplasmic variability studied based on these mtDNA markers could not correlate to phenotypic expression of fertility and sterility for any genotypic group. Further, DMS-4A immerged as a unique group with only one genotype.

Researchers have long been in the quest for identifying markers for hybrid purity which could replace the tedious job of Grow-out-test (GOT). Various mitochondrial variations have been reported in the past, which help to characterize CMS sources. Yashitola *et al.* (2004) developed a Sequence Tagged Site (STS) marker which amplified 384 bp band in WA-CMS and no band in the maintainer line. This 384 bp band was found to be homologus to *rps3-rpl16-nad3-rps12* gene cluster. Similarly, Rajendran *et al.* (2007), developed a BF-STS-401 464 bp dominant STS marker specific to WA-CMS. It was found to be homologous to NADH gene subunit. Further, a co-dominant marker *drrcms* was developed by Rajendrakumar *et al.* (2007) from mitochondrial specific Simple Sequence Repeats (SSR) marker RMT 6, showing homology with nad5 subunit. More recently, Ngangkham *et al.* (2010) observed high degree of genetic differentiation of WA-cytoplasm from its normal fertile counterpart due to DNA rearrangements involving five (coxI, coxIII, cob, atp6, rps3) mitochondrial genes.

Though there are a lot of diversified sources available, the major problem lies in the lack of efficient techniques for characterizing CMS sources and non-availability of effective restorers for new CMS sources, particularly in cases where the cytoplasmic donors and the recipient parents are distantly related (Brar *et al.*, 1998). The present endeavor evidences for the potential of applying molecular approach in fingerprinting, distinguishing and utilizing CMS, maintainer and restorer lines aiming at marker aided heterosis breeding. Search for more polymorphic primer pairs would reinforce the available database. It is also imperative to discover or develop restorer line for the CMS lines with wild rice background and a way to diversify the CMS sources to circumvent the constraint of vulnerability to disease epidemics.

ACKNOWLEDGMENTS

The authors thank Mr. Dinesh C. Joshi, Executive Director and Dr. Usha B. Zehr, Director, Barwale Foundation, for providing all the facilities for the execution of this research work. Thanks to Dr. C.N. Neeraja, Principal Scientist, Dr. M.S. Ramesha, Senior Scientist, Dr. M. Illyas Ahmed, Dr. B.C. Viraktamath, Principal Scientist and Dr. B. Mishra, Project Director, Directorate of Rice Research, Hyderabad for providing valuable inputs, DNA and seeds of some of the CMS and maintainer lines for the study.

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