Australian Journal of Crop Science AJCS
AJCS 5(3):251-261 (2011) ISSN:1835-2707

Estimating the genetic purity in cytoplasmic male sterile (CMS) lines of Egyptian rice

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Abstract

Cytoplasmic male sterile (CMS) lines often get contaminated with cognate isonuclear maintainer lines during multiplication. The fingerprinting of rice hybrids and their respective parental lines and testing genetic purity of rice hybrids using genetics markers are tested in the present study. To develop a reliable polymerase chain reaction (PCR) assay for distinguishing CMS and maintainer lines, a recommended primer pair called *drrcms* was used. PCR was performed with *drrcms* marker using the template DNA from a CMS line (IR 70368A), its cognate isonuclear maintainer line (IR 70368B), and the hybrid. This marker could unambiguously distinguish CMS (or the hybrid) from maintainer line. The PCR-based DNA marker *drrcms* described helps to detect contamination of maintainer and other male fertile lines in seed lots of WA-CMS lines. Up to our knowledge, this is the first study reported *drrcms* marker used to amply distinct fragments in Egyptian CMS and their cognate isonuclear maintainer lines.

Keywords: Cytoplasmic male sterile; *drrcms* marker; genetic purity; isozymes; *Oryza sativa*; PCR; rice hybrids.
Abbreviation: CMS_ Cytoplasmic male sterile; CTAB_ Cetyl trimethyl ammonium bromide; DUS_ Distinctness, uniformity and stability; PCR_ Polymerase chain reaction; WA_ wild abortive.

Introduction

Rice (*Oryza sativa* L.) is the well-known holder of two important titles of one of the most important food crop in the world and a model cereal species. At present the most effective and economic way is to develop and extend super rice varieties or hybrids with wide adaptation and super high yielding potential, which is also a fundamental solution to food security problem and an important way to maintain social stability (Li-yun et al., 2007). In 1970, Chinese researchers discovered a male sterile rice plant growing naturally within a population of wild rice (*Oryza sativa f. spontanea*) on Hainan Island. This plant was called wild rice with abortive pollen (WA) and had a particular cytoplasm. The cytoplasm induces the cytoplasmic male sterility (CMS) through interaction with the cell nucleus (Virmani, 1981). Reliable CMS systems can eliminate labor-intensive steps of emasculation and hand pollination in F_1 seed production and breeding programs (Newton, 1988). Since rice is strictly a self-pollinated crop, hybrid seed production must be based on male sterility systems. Currently, the most popular male-sterility system in rice is the three-line method is based on cytoplasmic genic male sterility and the fertility restoration system. This method involves three lines—the CMS line (A), cognate iso-nuclear maintainer line (B), and restorer line (R)—for the commercial production of rice hybrids. The seed of the male sterile line is multiplied by crossing A and B lines in an isolation plot. Hybrid seed is produced by crossing the A line with an R line in isolation in another plot. The majority of the rice hybrids that are currently under commercial cultivation in the world derive their cytoplasm from the WA source (Yuan, 1995). However, one of big problems is that there might be some parental seeds in commercial hybrids causing low level of genetic purity of hybrids. Any impurities in the hybrids would reduce the expected yield. It has been estimated that every 1% mixture of female line seed in the hybrid seed results in yield reduction of 100 kg per hectare (Mao et al., 1996). The Indian seed act prescribes that, for hybrid rice, the purity should be 98% (Verma, 1996); while in China it is mandated that the purity of hybrid rice should be at least 96% (Yan, 2000). To ensure the required levels of purity in hybrid seed, the parental lines that are utilized in hybrid seed production should have a very high level of purity (*ca.* 99%). One of the common admixtures that observed during hybrid seed production is that of maintainer lines with those of the CMS lines. Because these are isonuclear, it is not possible to distinguish between them until they flower (Yashitola et al., 2004). The fingerprinting of rice hybrids and identification of their genetic relationships are very important for plant improvement, variety registration system, DUS (distinctness, uniformity and stability) testing, seed purity testing and the protection of plant variety and breeders' rights. Accordingly, clear-cut identification of elite crop varieties and hybrids is essential for protection and

Table 1. Mean performance values of the CMC line IR 70368A for some growth yield and its components characters

Season	Entries	Populations	Pollen test %	Populations	Spikelet bagged	Populations	Days to heading	Populations	Plant height	Populations	No. of panicles Plant^{-1}
2006	IR70368A-H	44	38.00	44	42.60	36	102.80	28	100.20	33	19.60
	IR70368A-L	36	16.80	36	30.00	11	95.40	49	78.80	8	8.20
	IR70368A-S	18	22.60	18	35.20	3	97.80	3	89.80	3	14.60
	IR70368A-S	23	35.20	23	38.70	12	98.40	17	91.80	12	13.80
	IR70368A-S	28	24.00	28	33.80	31	98.20	31	92.60	13	14.00
	IR70368A-H	35	30.00	35	41.20	36	102.40	24	96.40	50	19.00
2007	IR70368A-L	22	24.60	22	35.60	38	95.40	6	79.40	17	10.20
	IR70368A-S	26	0.00	26	0.00	26	96.80	2	90.20	2	14.20
	IR70368A-S	47	0.00	47	0.00	47	98.40	26	88.60	18	14.20
	IR70368A-S	49	0.00	49	0.00	49	97.00	47	89.60	26	13.80

Season	Entries	Populations	Panicle exsertion (%)	Populations	Seed set (%)	Populations	Panicle weight	Populations	100-grain weight	Populations	Grain yield plant^{-1}
2006	IR70368A-H	23	76.10	18	42.30	44	2.18	5	2.68	36	27.20
	IR70368A-L	24	43.40	25	19.70	33	1.20	11	2.60	14	11.80
	IR70368A-S	12	67.90	3	31.70	3	1.92	3	2.65	13	21.80
	IR70368A-S	13	65.40	12	28.80	17	1.63	13	2.67	17	19.20
	IR70368A-S	31	64.20	31	30.00	13	1.75	31	2.64	31	20.00
	IR70368A-H	22	75.40	31	39.60	35	2.10	13	2.69	35	23.60
2007	IR70368A-L	30	52.50	10	18.70	21	1.22	21	2.60	3	10.40
	IR70368A-S	18	67.50	26	29.30	2	1.68	2	2.65	26	22.00
	IR70368A-S	26	64.00	47	28.60	18	1.58	47	2.68	47	20.60
	IR70368A-S	47	67.80	49	31.20	49	1.56	49	2.65	49	18.00

prevention of unauthorized commercial use (Nandakumar et al., 2004). On the other hand, purity of hybrid seeds supplied to farmers must surpass 96% (Ichii et al., 2003). Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environmental condition and inefficient. In contrast, DNA-based markers are highly heritable, available in high numbers, and exhibit enough polymerphism, hence they can be used to discriminate closely related genotypes of a plant (Kumar, 1999; Yashitola et al., 2002; Wang et al., 2005). For this reasons, DNA fingerprinting for cultivar or varietals identification has become an important tool for genetic identification in plant breeding and germplasm management (McGregor et al., 2000). Furthermore, Isozymes have been used in genetics for defining systematic phylogentic relationships and to assess the genetic divergence between taxa (Tanksley and Orton, 1983; Bonnin et al., 1996; Yang et al., 1996). The complementary enzyme bands may be used as one of the biochemical indicators for predicting the genetic purity of CMS line. As the esterase isozyme of the progeny has complementary enzyme bands, which differ in its parents, this characteristic has been used in China to do preliminary evaluation of the purity of hybrid seeds. Many scientists studied the correlation of the esterase isozyme with purity in female parent on the bases of the number of complementary enzyme bands with high activity (Devanand et al., 2000)**.** The present study was carried out for estimation the genetic purity of a CMS line (IR 70368A) with its maintainer (IR 70368B) utilizing morphological, biochemical and molecular characterization including isozymes and PCR techniques.

Materials and methods

Plant material and growth conditions

The best time for rice planting is the periods between April 10th and May 10th. The June is the worse cultivation date and reduces all plant properties and consequently grain yield (Abou khalifa, 2009). Egypt has a Mediterranean climate with a typical seasonal rhythm strongly marked with respect to temperature, precipitation and weather in general, hot summers from mid-May to mid-September and rainy, rather changeable, winters from November to mid-March. The plant materials were sown at the experimental farm and Biotechnology Laboratory of the Rice Research and Training Center, Sakha, Kafrelsheikh, Egypt. The cultivation was carried in a clay soil type along summer season (May of 2005, 2006 and 2007) under conditions of no rainfall and humidity 70 – 80%. Three cytoplasmic male sterile lines wild abortive type (WA); IR 58025A, IR 69625A and IR 70368A were tested with their maintainers to determine their genetic purity. These particular lines were chosen based on studies of heterosis and combining ability of 10 CMS lines and 5 Egyptian testers (restorer) to get useful information for hybrid rice program in Egypt. Among the ten CMS lines, IR 58025A, IR 69625A and IR 70368A were the best general combiners for grain yield. During 2005 season, three periodical sowing dates were applied with 15 days intervals to overcome the differences of heading date among the parental lines. Each line was planted in 4 rows, 5 m length and 20 cm apart between plants and rows under isolation plots. A total of 50 single crosses were made and harvested separately for each CMS line. In seasons 2006 and 2007, about 50 populations for each line under isolation plot were sown in the nursery for identification and after 21 days for multiplication. Five replications were grown in randomized complete block design, each replication consisted of one row for the maintainer (1-50) and one row of $F_{1\ (1\text{-}50)}$ crosses (A / B). Each row was 5 m long and contained 25 individual plants. Seedlings were carefully pulled from the nursery after 30 days from seeding and transferred to the permanent field. Seedlings were handling transplanted in hills at the rate of 1-2 Seedlings/hill.

Morphological analysis

The morphological characterizations were conducted by using 5 replicates for all 50 populations of the CMS line. Analysis was reordered for days to heading (measured as days from date of sowing to the date of the first panicle exsertion), plant height (measured as centimeter from soil surface to the tip of the panicle). Yield and yield component characters were measured according to Donald and Humblin (1976); and Yoshida (1981) including, panicle weight (measured as weight (g) of the main panicle after drying), panicle length (measured as the number of centimeters from the panicle neck to the panicle tip-excluding the awn), grain yield (measured as weight (g) of the grain of the each of individual plant), 100-grain weight (recorded as the weight (g) of 100 random filled grains), seed set (%) (seed set = No. of failed grains/No. of total grains x 100), spikelet fertility (measured as number of grains per bagged panicle). The method given by Singh and Haque (1999) was used for determination of pollen viability.

Biochemical and molecular analysis

Isozyme electrophoresis

Fresh leaves of 21 days old seedling (plumules) were used for isozyme analysis (esterase and peroxidase), 200 mg samples were used for the extraction with 1 mL chilled extraction buffer (50 mM Tris, pH 6.8, 3.0 mM EDTA and 20% (w/v) sucrose). Each sample was vortexed for 15 sec and the homogenates were centrifuged at 15000 ×*g* for 5 min at 4 °C, then the resultant supernatants were used as sources for esterase and peroxidase, using two different substrates, α-naphthyl acetate and hydrogen peroxide, respectively. The supernatants were then separated in 8% native-PAGE according to Davis, (1964). After electrophoresis, the gels were individually stained according to the enzyme type using the appropriate substrate and chemical solution (Scarndalios, 1969; Tu et al., 1968) then incubated at 37 °C in a dark room for complete staining. Gels were scanned using Gel Doc-2000 Bio-Rad system.

DNA extraction

The *drrcms* marker desgned by Rajendrakumar et al., (2007) was used in the PCR assay. This marker flanks a repeat motif that is polymorphic between WA-CMS lines and their cognate maintainers. Total genomic DNA was extracted using CTAB (Cetyl trimethyl ammonium bromide) method according to Murray and Thompson (1980). One g leaf tissue of parents as

Table 2. Mean performance values of maintainer line IR 70368B for some morphological, yield and its component characters

Season	Entries	Populations	Days to heading	Populations	Plant height	Populations	No. of panicles plant^{-1}	Populations	Panicle length
2006	IR70368B-H	20	98.40	29	110.40	38	18.80	15	24.00
	IR70368B-L	32	93.00	16	101.00	45	9.80	39	18.60
	IR70368B-S	26	96.00	26	108.40	26	14.40	26	23.20
	IR70368B-S	34	97.60	34	105.40	34	16.20	41	22.40
	IR70368B-S	50	95.80	50	107.00	50	15.40	50	21.40
2007	IR70368B-H	17	97.40	45	110.00	9	19.20	35	24.40
	IR70368B-L	3	93.40	35	102.20	22	11.00	8	18.80
	IR70368B-S	20	97.00	20	108.20	13	16.00	20	22.40
	IR70368B-S	21	96.80	21	106.60	21	17.40	13	22.60
	IR70368B-S	33	95.40	33	109.80	33	15.40	21	22.00

Season	Entries	Populations	Seed set %	Populations	Panicle weight	Populations	100-grain weight	Populations	Grain yield plant^{-1}
2006	IR70368B-H	50	97.80	38	3.96	34	2.69	7	48.80
	IR70368B-L	18	88.50	19	2.50	31	2.51	19	26.80
	IR70368B-S	15	95.90	26	3.38	41	2.63	26	44.00
	IR70368B-S	41	95.30	34	3.44	28	2.60	34	43.20
	IR70368B-S	34	96.40	50	3.34	50	2.64	50	40.60
2007	IR70368B –H	42	97.80	17	4.08	25	2.69	14	48.20
	IR70368B-L	25	89.80	40	2.34	41	2.52	30	28.80
	IR70368B-S	20	96.60	20	3.56	13	2.64	20	46.40
	IR70368B-S	21	95.40	13	3.40	21	2.60	21	46.00
	IR70368B-S	33	97.40	33	3.16	38	2.65	33	40.80

well as F_1 hybrids were ground to fine powder in liquid nitrogen using sterile, pre-cooled mortars and pestles. The obtained fine powder was further homogenized in 10 mL of pre warmed extraction buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA; 5M NaCl; 2.0% CTAB; 0.2% β-Mercaptoethanol) and incubated at 65°C for 1 h with intermittent swirling for cell lysis. After incubation, volume of chloroform: isoamyl alcohol (24:1) was added to the samples, mixed by hand for 5 min, and then centrifuged at 15,000 ×*g* for 10 min at 4°C.

The supernatant was transferred to a fresh tube and extracted another time with 0.6 volume of cold isopropanol and then incubated for 15 min at 4°C to precipitate the DNA. The DNA precipitate was then collected by a brief spinning at 10000 × *g* for 10 min. The resulting pellet was washed with 70% cold ethanol, dried for 30 min in air and resuspended in 500 µl TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0). The samples were then incubated for 2 h at 37°C in the presence of 5 µl/g leaf tissue of RNase A (Sigma, 10 mg/mL) leaf tissue) to eliminate RNA. The quality of DNA was checked by 0.8% agarose gel electrophoresis in 0.5X TAE buffer (0.045 M Tris-borate, 0.001 M EDTA (pH 8.0) using a standard containing 100 ng per µl genomic λ DNA., stained with ethidium bromide (0.5 µg/mL) and observed under UV transilluminator.

PCR amplification

PCR was performed according to Williams et al. (1990) by using Perkin-Elmer (2400) DNA thermocycler and *drrcms* primers (Forward: 5' ACCTTTGGGCGATGGTT 3'; Reverse 5' GGGTTTAGAGTCGCCAC 3'). Primers were designed at Metabion international AG, D-82152 Martisried/Deutschland. PCR was carried out in 20 µl reaction mixture containing 1.0 µl of template DNA (50 ng/µl), 0.2 µl dNTPs (10 mM),1.6 µl Mg Cl_2 (25 mM), 2.0 µl 10X buffer (10 mM tris, pH 8.0, 50 mM KCl and 50 mM ammonium sulphate), 4.0 µl primer (15 pmole), 0.1 µl taq DNA polymerase (10 U/µl). PCR conditions were 95ºC for 7 min (initial denaturation), followed by 45 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 2 min, and a final extension of 5 min at 72ºC followed by storage at 4°C.

Statistical analysis

The data were analyzed statistically by using Statistical Package for the Social Sciences (SPSS) V10. One factor analysis of variance (ANOVA) in a completely randomized design in each self-group using standard statistical procedure was followed (Panse and Sukhatme, 1954). The percentage and ratio data were subjected to arcsine transformation prior to statistical analysis.

Results and discussion

The development of hybrid rice technology and adoptions of hybrid rice cultivars to Egyptian environments offer one approach to the problem of matching food supply to expected demand. Tables 1-4 show the main morphological traits and basics statistics of CMS lines IR 70368A and IR 70368B.

Morphological traits and basics statistics of CMS lines

Pollen and spikelet bagged testes

During identification and multiplication experiments of the CMS line IR 70368A, the pollen and spikelet bagged traits are very important to reject the undesirable populations before heading. In 2006, from 50 manually crosses planted of IR 70368A, only 5 populations exhibited partial fertility to pollen grains and seed set in spikelet bagged with percentage of purity 90%. According to Nishiyama (1984), all stages of reproductive phase ranging from pollen formation to fertilization are vulnerable to climatic fluctuations; therefore, occurrence of some fertile plants in perfect male sterile lines could be due to fluctuations in temperature and day length. In the second experimental year, 2007, the genetic purity percentage increased to 96%, since only a couple of populations exhibited partial fertility to pollen grains (Table 1). Therefore, the recurrent selection for the CMS line with the manual crosses method is the best method to maintain and increase the genetic purity for IR 70368A line. The selection was done on the basis of pollen test and spikelet bagged for the populations which have complete sterility. High pollen sterilities (99.00%-99.9%) were also observed in CMS lines breed by Hossain and Li (2002); Pradhan and Jachuck, (1993); Kumar et al., (1996) and Pandey et al., (2010).

Days to heading

Heading time is an important agronomic character which determines the regional and seasonal adaptability of rice varieties. Development of early maturing and high yielding varieties is one of the important objectives in rice breeding (Peng et al., 1995). Analysis of variance indicated that the general mean values of the IR 70368A were 98.92 and 98.46 days and the average days to heading ranged from 102.80 to 95.40 and 102.40 to 95.40 in seasons 2006 and 2007, respectively (Table 1.). According to the mean values for IR 70368A, the promising populations were recorded for populations No.3, 12, 31 in 2006 and 26, 47, 49 in 2007 with average days to heading of 95 days. Differences in days to heading were observed among the CMS maintainer line IR 70368B indicating variability among them for these traits (Table 2. and 4.). The CMS line IR 70368B had shorter days to heading with mean values 95.71 and 95.16 in 2006 and 2007, respectively. The average days to heading for promising population were 96 days (Table 2.).

Plant height

Analysis of variance indicated that, in 2006 the average plant height of IR 70368A ranged from 78.80 to 100.20 cm with a general mean value of 93.02 cm. However, in 2007 a general mean value was 89.64 cm with average plant height ranged from 79.40 to 96.40 cm. In 2006, the superior mean values were recorded with populations' No. 3, 17 and 31 but in 2007 were populations 2, 26 and 47. The nucleus seeds of CMS line were available with average plant height of 90 cm.

Table 3. Results of analysis of variance (mean square) of the IR70368A populations for some of morphological, yield and its component characters

Source of variation	df	Pollen test		Spikelet bagged		Days to heading		Plant height		No. of panicles plant $^{-1}$	
		2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
Replications	4	0.000076^{ns}	0.00002^{ns}	0.00024^{ns}	0.00002^{ns}	0.984^{ns}	0.410^{ns}	8.594^{ns}	2.134^{ns}	0.33^{ns}	0.274^{ns}
Population	49	0.0431**	0.0253**	0.2114**	0.0668**	27.295**	18.9898**	114.005**	103.549**	28.1653**	27.0011**
Error	196	0.00012	0.000025	0.00016	0.000013	0.8513	0.6225	3.894	4.7524	1.3953	0.8311
Hbs	-	99.54	99.83	99.81	99.95	93.70	94.84	90.13	93.76	94.23	96.07
S	-	0.2075	0.1591	0.4598	0.2584	5.2245	4.3577	10.6773	10.1759	5.3071	5.1963
CV	-	7.2259	11.5305	6.8792	9.4739	0.0528	0.0443	0.1148	0.1135	0.4014	0.3828
Source of variation	df	Panicle exsertion		Seed set %		Panicle weight		100-grain weight		Grain yield plant $^{-1}$	
		2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
Replications	4	0.0021^{ns}	0.0007^{ns}	0.0007^{ns}	0.0002^{ns}	0.0110^{ns}	0.0009^{ns}	0.0001^{ns}	0.0001^{ns}	0.274^{ns}	1.334^{ns}
Population	49	0.0282**	0.0106**	0.0164**	0.0134**	0.3035**	0.2861**	0.0028**	0.0027**	27.0011**	58.679**
Error	196	0.0007	0.0002	0.0006	0.0005	0.0177	0.0164	0.0001	0.00007	0.8311	2.7177
Hbs	-	90.90	92.08	92.70	94.98	91.37	94.29	92.71	93.16	96.07	93.54
S	-	0.1678	0.1028	0.1283	0.1156	0.5509	0.5349	0.0529	0.0522	5.1963	7.6626
CV	-	0.2681	0.1609	0.4620	0.4055	0.3468	0.3365	0.0199	0.01976	0.3828	0.4479

ns = non significant.; ** = probability level, ns = non significant.; ** = probability level

Table 4. Results of analysis of variance (mean square) of maintainer line, IR70368B for some of morphological, yield and its component characters

Source of variation	Degree of freedom	Days to heading		Plant height		No. of panicles plant $^{-1}$		Panicle length (cm)	
		2006	2007	2006	2007	2006	2007	2006	2007
Replications	4	0.316^{ns}	0.27^{ns}	1.206^{ns}	0.17^{ns}	1.084^{ns}	0.526^{ns}	0.394^{ns}	0.106^{ns}
Population	49	9.1198**	6.8490**	28.995**	23.787**	23.894**	22.008**	10.184**	7.702**
Error	196	0.5976	0.3006	1.2652	1.1802	0.9758	0.9280	0.3511	0.2958
Hbs	-	90.89	92.31	92.15	94.63	92.06	93.80	93.18	95.04
S	-	3.0199	2.6171	5.3848	4.8773	4.8882	4.6913	3.1914	2.7754
CV	-	0.03156	0.0275	0.0511	0.0459	0.3375	0.3052	0.1466	0.1265
Source of variation	df	Seed set (%)		Panicle weigh		100-grain weight		Grain yield plant $^{-1}$	
		2006	2007	2006	2007	2006	2007	2006	2007
Replications	4	0.0008^{ns}	0.00007^{ns}	0.0005^{ns}	0.0503^{ns}	0.0005^{ns}	0.0002^{ns}	10.094^{ns}	3.966^{ns}
Population	49	0.0023**	0.0016**	0.0114**	0.8076**	0.0114**	0.0108**	178.126**	152.894**
Error	196	0.0001	0.00007	0.0002	0.0391	0.0002	0.0003	4.6246	5.9150
Hbs	-	91.38	92.11	94.29	93.14	94.29	95.93	92.37	93.93
S	-	0.0478	0.0395	0.1068	0.8987	0.1068	0.10399	13.346	12.365
CV	-	0.0506	0.0416	0.04091	0.2755	0.04091	0.0398	0.3260	0.2996

On the other hand, the CMS maintainer line IR 70368B had higher general mean values of 105.39 and 106.16 cm in 2006 and 2007, respectively. The produced populations also recorded higher average of plant height of 107 cm (Table 2. and 4.). Yu et al. (2005) have also recorded relatively higher plant height in the CMS maintainer line. Also Ali et al. (2000) have observed relatively greater range in plant height than the other characters. Plant height in rice is a complex character and is the end product of several genetically controlled factors called internodes (Cheema et al., 1987).

Number of panicles plant^{-1}

In rice, the manipulation of panicles number is important for grain yield, but the physiological basis of the regulation of tiller growth remains unclear (Mohapatra and Kariali, 2008). Results of CMS line of IR 70368A and its maintainer IR 70368B showed a wide range of differences (Table 1. and 2.). The general mean values of IR 70368A for this trait were 13.22 and 13.58 with average number of panicles plant $^{-1}$ ranged from 8.20 to 19.60 and 10.20 to 19.00. However, its maintainer IR 70368B had general mean values of 14.48 and 15.37 with average number 9.80 to 18.80 and 11.00 to 19.20 in 2006 and 2007, respectively. Moreover, the best mean values were obtained by populations showed number of panicle plant $^{-1}$ with average of 14 and 16 panicles/plant of CMS line of IR 70368A and its maintainer IR 70368B, respectively (Table 1. to 4.). These results are in accordance with Sidharthan et al. (2007) and Pandey et al. (2010). They reported a wide range of differences for panicles number ranged from 5.2 to 18.6 and 9.2 to 22.5, respectively.

Panicle exsertion

Panicle exsertion, the distance between flag and panicle, is an importantly morphological trait of rice, which seriously affects the production of seeds in hybrid rice (Yuan et al., 1988). Higher panicle exsertion is favorable for high outcrossing (Sidharthan et al., 2007). In our study, the general mean values of IR 70368A were similar in 2006 and 2007 (62.58 and 63.76, respectively). However, the averaged panicle exsertion (%) ranged from 43.40 to 76.10 and from 52.50 to 75.40 in 2006 and 2007, respectively, with promising mean values for populations' No. 12, 13, 31 and No. 18, 26, and 47. Plants produced from these populations gave panicle exsertion with average of 66 % without spray growth regulation. The results suggested that, recurrent selection for these populations would improve the panicle exsertion which increases the outcrossing rate. Panicle exsertion of 30-70 % or more was observed in CMS lines developed by Sidharthan et al. (2007); Abraham et al. (1998) and Azzini and Rudger (1982).

Seed set (%)

Crossablitiy between two different species is the percent seed set after crossing which is good measure of genetic affinity among and between the genotypes of the respective crops. Overall seed set percentage varies in the genotypes which is remarkable (Hoque et al., 2007). In CMS line of IR 70368A seed set percentage ranged from 19.70 to 42.30 and 18.70 to 39.60, in 2006 and 2007, respectively. Among populations, the best mean values were recorded in populations' No. 3, 12 and 31 in 2006 while in 2007 were recorded for populations' No. 26, 47 and 49 with average seed set percentage of 30% (Table 3.). Chu et al. (1969) found seed set rate 39 to 42% in inter specific hybridization. Similar results were also reported by Wang et al. (2002). On the other hand, the CMS maintainer line gave higher range of seed set percentage with general mean values of 94.46% and 95.08% in 2006 and 2007, respectively. The results of seed set percentage ranged from 88.50 to 97.80 % in 2006 while in 2007 were from 89.80 to 97.80% (Table 3.). Thus, the average of seed set percentage for the produced populations was 96%. Sawant et al. (2003) found similar results.

Panicle weight

The larger number of filled grains per panicle, regarded as a component of the number of spikelelets per panicle, mainly contributed to the higher grain yield per plant (Virmani et al., 1981; Kabaki, 1993; Murayama and Sarker, 2002). The CMS line IR 70368A exhibited high range of panicle weight with low coefficient of variation of 0.3468 and 0.3365. The average ranged from 1.20 to 2.18 and from 1.22 to 2.10 in 2006 and 2007, respectively. In 2006, the desirable mean values for IR 70368A were recorded in populations' No. 23, 17and 13 while in 2007 were populations' No. 2, 18 and 49 with average panicle weight of 1.6 g. On the other hand, the CMS maintainer line IR 70368B displayed a wide range of panicle weight differences among its populations (Table 2.). The panicle weight ranged from 2.50 to 3.96 in 2006 and 2.34 to 4.08 in 2007. The obtained populations of IR 70368B$_{1\text{-}50}$ gave the highest average of panicle weight of 3.4 g compared to IR 70368A$_{1\text{-}50}$.

100-grain weight

Among the yield attributes, the most important contributing characters in rice that are taken into consideration for drawing up an indication of grain yielding potentialities are number of effective tillers, number of grains per panicle and weight of 100 grains (Nagai, 1959; Abbasi et al., 1995). Both CMS line and its maintainer differed statistically with each other in respect of grain weight. The analysis of variance resolved that the 100-grain weight trait of the CMS line IR 70368A has a similar mean value of 2.64 in both seasons (2006 and 2007) with a slight difference in the average of 100-grain weight (Table 1. and 3.). Seeds obtained from these populations were usable with average of 2.65g. The mean values of IR 70368B in 2006 and 2007 was also similar with 2.61g and 2.62 g, respectively. Meanwhile, the average of 100-grain weight for the produced populations reached to 2.65g. This supports the well-known statement that 100-grain weight is a stable genetics character in rice (Yoshida, 1981)

Grain yield plant^{-1}

Grain yield plant^{-1} is another yield attributing trait for rice (Abbasi et al., 1995). In the CMS line IR 70368A, a great variability (11.80 to 27.20 g) and (10.40 to 23.60 g) was exhibited in 2006 and 2007, respectively, with general mean values of 17.29 and 18.10 g (Table 1. and 4.). Both CMS lines, IR 70368A and IR 70368B, showed different behaviors in their grain yield. IR 70368B has general mean values of 40.94 and

Table 5. Description of esterase patterns of the CMC line IR 7036A and their maintainer

No.of bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	+++	++++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	++++	+++	+++
2	++++	++++	++++	+++++	-	-	-	-	-	++	++	-	-	+++	-
3	+++	++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++	+++	+++
4	+++++	+++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++	+++++	+++++	+++++	+++++	+++++
5	+++++	++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++	+++++	+++++	+++++	+++++	+++++
6	+++	+++	+++	++	++	++	++	++	++++	++	++++	++++	++++	++++	++++
7	-	++	++	++	++	++	+	++	++	-	++	++	+	++	++
8	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
9	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Description of peroxidase patterns of the CMS line, IR70368A and their maintainer

No.of bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	+++++	+++++	+++++	+++++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
3	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	++++	+	++	++++	++	++	++	++++	++	++	++	++	++	++	++
6	++	+	+	++	+	+	+	++	+	++	+	+	+	++	+
7	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	-	+	-	+	+	-	+	+	+	+	-	+	+	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

41.37 g with an average grain yield plant^{-1} ranged from 26.80 to 48.80 g and from 28.80 to 48.20 g in 2006 and 2007, respectively. The populations with the desirable mean values for both CMS lines exhibited high difference in average grain yield plant $^{-1}$, 18 and 45.5 g, respectively. These results were generally agreed with those found by Mo et al. (2004) and Cai (2002). Variation in rice grains yield might be due to the environment (Mahapatra, 1993) or the correlation of grain yield plant^{-1} with various yield contributing characteristics like; fertility of soil, flag leaf area, grains panicle^{-1}, number of grains panicle^{-1} and grain weight and correlation with these traits. Similarly Mirza et al., (1992) reported positive correlation among number of panicle plant^{-1}, panicle length, number of grains panicle^{-1} and 1000-grain weight and grain yield plant^{-1}.

Statistical analysis

The results of ANOVA revealed highly significant differences among IR70368A CMS line and its maintainer IR70368B with its 50 populations for all tested characters (Table 3. and 4.). Populations possessed highly significant differences for all characters, indicating that the average improvement was significant in all populations. The mean square values of tested traits were highly significant among populations, but were insignificant among reps. Furthermore, all studied characters gave high values in the heritability test, indicating that the environmental effect was very low and these traits were controlled by additive and non additive genetic variances and could be selected in early generations. The results of this study were in agreement with Sun et al. (2006). All the studied traits showed lower CV values in the obtained rice populations. The CV indicates the degree of precision in which the treatments are compared and is a good index of the reliability of the experiment, it expresses the experimental error as percentage of the mean; thus, the lower CV values mean more reliability of experiment. On the basis of experimental data, a wide range of differences was found among the parental lines and their populations in the maintainer line IR 70368B. From the experiments conducted it may be concluded that IR 70368B was promising especially in 2007 and may be utilized for the development of line hybrids with superior yield and quality.

Biochemical and molecular analysis

Isozymes studies

In the present study an attempt was made to assay the variation of activity of a couple of isozyme patterns; esterase and peroxidase to determine the genetic pairing crosses into the CMS lines. The leaves crude extract was used for measuring enzyme activities since seeds is not desirable not only because F_1 seeds are limited on account of crossing barrier, but also the seeds are destroyed when used for analysis. Variations in number and activity of bands are shown in Tables 5, 6 and Fig 1. A complex of sub-patterns was observed among the various tested rice lines (Table 5. and Fig.1.A). Description of esterase isozyme patterns of the CMS line and its maintainer line with their populations showed that some bands were present in rice

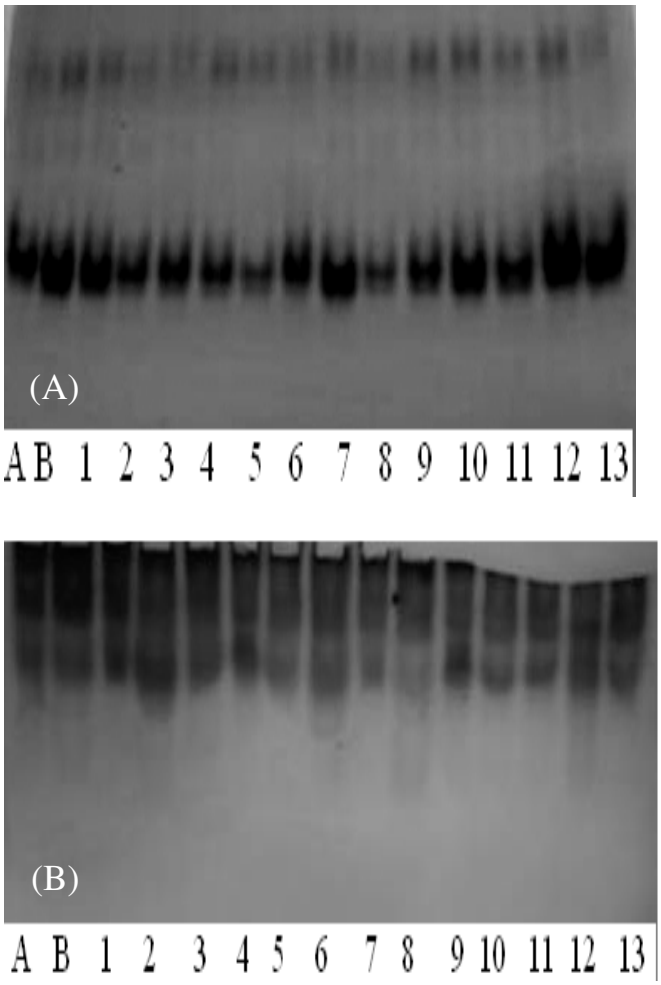

Fig 1. Zymogram of esterase (A) and peroxidase (B) isozymes of IR 70368A, IR 70368B and their derived populations in rice. Lane A, IR 70368A; lane B, IR 70368B; lanes 1 to 13, selected rice populations

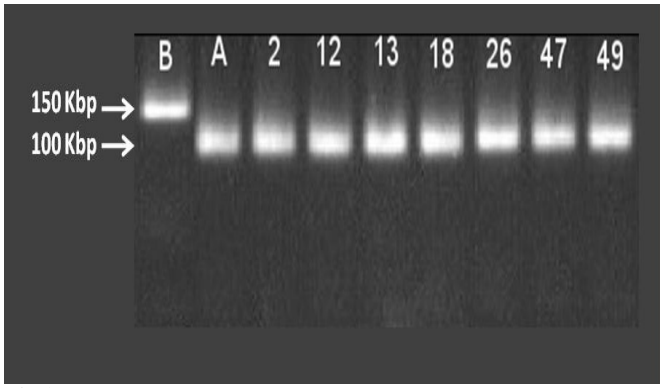

Fig 2. Purity PCR analysis using *drrcms* marker of IR 70368B, IR 70368A and their derived populations in rice. M, 50 bp DNA ladder, lane A, IR 70368B; lane B, IR 70368A; lanes 3 to 10, selected rice populations of 2, 12, 13, 18, 26, 47, 49, respectively.

lines while absent in others. An interesting finding was observed when a specific band was detected in the maintainer line as well as in all rice populations, except for population No. 10. The same band disappeared in CMS line. This band may be a result of the expression of a gene (s), that play an important genetic role in the maintaining process and could be used to identify the genetic purity of CMS line with its maintainer. Table 6 and Fig. 1.B showed the peroxidase isozyme patterns and the degree of activity for each population descendant from the crosses of CMS line, IR 70368A and its maintainer, IR 70368B. These results indicated that a common monomorphic pattern for peroxidase was appeared in this CMS line IR70368A, its maintainer line and the 13 selected genotypes. CMS line and its maintainers could be distinguished on the basis of the number of bands. Zhu and Zhang (1987) examined the correlation between heterosis and parental diversity for 8 isozymes in seedlings of hybrid rice, and found that diversity for esterase was more closely related with heterosis than diversity for the other isozymes. Chen (1996) demonstrated that the greater the difference between two parental isozymes spectra, the higher the heterosis of the F_1 hybrid. Diwakar et al. (2009) studied the identification of rice varieties through isozyme analysis and found that peroxidase and esterase isozymes were useful for identification of varieties as well as parents and hybrid and maybe served as molecular markers.

DNA marker

PCR was performed with *drrcms* primer using the template DNA from CMS line IR 70368A, its cognate isonuclear maintainer line (IR 70368B), and the hybrid. As shown in Fig. 2 this marker unambiguously distinguished CMS (or the hybrid) from maintainer line. The DNA from individual seedlings was then used for PCR amplification using the *drrcms* primer. After resolving the amplified fragments in agarose gels, IR 70368B maintainer line gave a different band with a size of 150 bp. However, IR 70368A line and the other tested poulations gave only one band with a size of 100 bp. The marker *drrcms* can distinguish WA-CMS lines from their cognate maintainers based on differential fragment sizes. Nandakumar et al. (2004) successfully employed a single restorer gene linked marker assessment for testing genetic purity of hybrid seeds that substantially reduced the time, space and labor. However, the selection of polymorphic markers is very crucial and should be done with extreme precaution as there are reports that single polymorphic marker may not always be able to distinguish all the contaminants in the commercial seed lots. In such cases, application of two markers can further improve the accuracy of the seed purity analysis (Rajendran et al., 2007). The *drrcms* marker described could be used in a PCR assay to reliably detect contamination of maintainer and other male fertile lines in seed lots of WA-CMS lines (Rajendrakumar et al., 2007).

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