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To cite this article: A O Alfalahi *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **388** 012043

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Molecular discrimination of maize CMS type and genetic relationship using RAMs markers

A O Alfalahi^{1*}, R M Theer¹, M A Mohammed², M H Abdullah¹, O M Dhannoon¹, Z T Hussein¹ and M M Drej¹

¹Field Crops Department, College of Agriculture, University of Anbar, Iraq.

²Horticulture Department, College of Agriculture, University of Anbar, Iraq.

*Email: ag.ayoob.obaid@uoanbar.edu.iq

Abstract. Molecular characterization of CMS is an environmentally-independent tool and represents an alternative way to consider genetic variation within and between the different maize populations. The current study which consisted of six cms lines of maize in the 8th generation (S8) revealed that all of these lines were C-type. The adopted six specific markers (forward and reverse) in detecting the three cms types produced a fragment with a molecular size of ~350 bp. The used RAMs markers (UBC-810, UBC-812, UBC-821, UBC-848, UBC-854 and UBC-855) produced a total of 36 fragments across the lines genomes, 25 out of them were polymorphic scoring a polymorphism percentage of 69.4%. The PIC values indicated that UBC-821 was the most discriminative primer with a PIC value of 0.40 compared to UBC-854, and UBC-855 primers which had the humble capability to distinguish between the studied inbreds with a PIC value of 0.33. The results of cluster analysis based on the molecular discrimination confirmed that A1 has the lowest values of genetic similarity, hence it was the most genetically divergent against the other cms inbred lines, especially A6 (0.42). On the other hand, A2 and A3 lines found to be genetically related by scoring the maximum genetic similarity (0.92). Molecular investigations have several advantages over the traditional methods and will be helpful in the real evaluation of any genotype.

1. Introduction

Cytoplasmic Male Sterility "CMS" become a widespread phenomenon in plant populations and it was used extensively and still in the commercial production of F1 hybrids. The phenomenon results from novel and complex mitochondrial DNA rearrangements, giving rise to chimeric genes [1;2]. CMS is often associated with abnormal open reading frames (ORFs) found in mitochondrial genomes, hence such ORF's in turn will produce novel protein responsible for unfunctional pollens [3;4]. The expression of CMS mitochondrial genes are associated with nuclear partners termed restorers of fertility *rf*, that can suppress ORF's effect and reinstate viable pollen production [5].

Depending on *rf* system, cms can be classified into four types, N (Normal), T (Texas) S (USDA) and C (Charrua) [6]. There are two different types of fertility restoration system in maize sporophytic which take place in the sporophytic tissues resulting in complete absence of viable pollens like in T-CMS, meanwhile



gametophytic cms, is the other type highly related to gametes and resulting in partial absence of viable pollen like in S-CMS [7].

Unfortunately, traditional test crossing which is the most conclusive method of characterizing maize cytoplasms, seems to be time consuming and labor intensive process. This represents a significant motive to many specialists to propose specific molecular markers to distinguish between the four major types of maize cytoplasm [8].

These primers are designed to target specific sequences in the mitochondrial DNA found to be discriminative for each type of cytoplasm [1].

The level of genetic diversity among maize CMS populations will provide priceless information for successful improvement programs in future. On the other hand, it represents a valuable source for genetic diversity that may widened the genetic base, hence minimizing the risks of genetic vulnerability [9].

Molecular biology made a huge development during the past three decades leads to create new ways for drawing genetics relationship among different plant genotypes and species. Most of plant genetic studies make their conclusions depending on the morphological performance which is significantly affected by environment fluctuations resulting in bias evaluation [10]. Therefore, molecular characterization which is environmentally independent represents an alternative approach to consider the genetic variation existed within and among different maize populations [1]. Random Amplified Microsatellite markers (RAMs), by their dominant nature and multiallelic found to be efficient in detecting and estimating the genetic variation at the DNA level, hence drawbacks of morphological evaluation will be avoided. RAMs, ISSR (Inter Simple Sequence Repeats) are different expressions to describe simple nucleotide repeats serving in genetic diversity measurement rather than constructing genetic maps [11].

Recently, several studies involving the use of DNA based RAM markers which definite various sizes of genetic variation within and/or between the studied genotypes. ISSR markers were adopted to reveal the genetic variation of 50 accessions [12]. The used markers (17 primers) amplified a total of 108 fragments, 83 out of them found to be polymorphic scoring 75.2% percentage of polymorphism. The estimated genetic diversity was about 0.26.

For the same purpose, Muhammad et al. [13] used inter simple sequence repeat (ISSR) markers to probe the genetic variability between 21 maize genotypes. The applied primers succeeded in amplifying 190 fragments, all were polymorphic. The detected genetic similarity that ranged between 0.88 and 0.11 was enough to group the tested maize lines into two main groups in cluster analysis.

2. Materials and Methods

2.1. Genomic DNA Extraction

Total genomic DNA was extracted from grinded seed samples using Wizard Genomic DNA kit (Promega – USA) according to the supplier protocol. The DNA purity was checked with spectrophotometer based on the absorbance at 260/280 nm wave length [14]. Samples were diluted with double distilled water to final working concentration of 50 ng/μl to be amplified by PCR.

2.2. Primers and PCR Conditions

Three primer sets (Table 1) were specifically designed to detect the three types of maize CMS; CMS-T, CMS-S and CMS-C [8], in addition to pair of specific primers adopted in characterizing Zein gene [15]. All the implemented primers were synthesized by Alpha DNA – Canada in lyophilized form, then diluted with deionized distilled water to 10 pmol/μl.

Table 1. Primer details used to discriminate the three maize CMS types (CMS-T, CMS-S and CMS-C) and Zein gene.

Primer	Sequence 5'-3'	Amplicon Size bp	Gene specificity	Reference
CMST-F	CATGAAATGGGTGAAGTCTCTTTC	~450	CMS-T	[8]
CMST-R	AAGAGAAAGGGAGACTTTGGTCCC			
CMSS-F	CAACTTATTACGAGGCTGATGC	~799	CMS-S	[8]
CMSS-R	AGTTCGTCCCATATACCCGTAC			
CMSC-F	ATGCTAATGGTGTCCGATTCC	~350	CMS-C	[8]
CMSC-R	AGCATCATCCACATTCGCTAG			
Ze-F	AGTGCGACCCATATCCAG	~277	Zein	[15]
Ze-R	GACATTGTGGCATCATCATTT			

DNA amplification was carried out in the Varieties Identification and Fingerprinting Laboratories – General State for Seed Test and Certification using Eppendorf Mastercycler. PCR reactions were 25 µl in volume containing 12.5 µl of MasterMix (2X PCR buffer; pH=8.5), dNTPs (400µM) and Taq polymerase (5 U), 50 ng total genomic DNA, 3 µl of each specific primer or 2 µl of RAM primer. The reaction volume was completed to 25 µl with nuclease water. The applied amplification profile was as follows: Initial denaturation and denaturation steps were at 94°C for 2 and 1 min., respectively. Annealing was at 55°C for 1 min., followed by extension and final extension steps at 72°C for 1.5 min. for 40 cycles. Then denaturation was set at 95°C for 5 min. and 30 sec. respectively, annealing was at 56°C for 30 sec., and extension was at 72°C for 1 min. for 23 cycle. Final extension was at 60°C for 30 sec. for one cycle.

The same thermal profile was subjected to amplify Zein gene with a little modification involved annealing at 60°C. The established amplification program for RAM consisted of an initial denaturation at 95°C for 5 min., followed by 34 cycles of 94°C for 1 min., then annealing was at 55°C for 1 min., extension and a final extension steps were at 72°C for 1 and 7 min., respectively.

2.3. Electrophoresis and Data Analysis

The amplified products were analyzed on 1.5% agarose gel along with 1 kb DNA ladder. The amplified fragments represented in binary data (0-1) and statistically analyzed according to Jaccard's coefficient method [16] to estimate the genetic similarity.

The phylogenetic tree was constructed with aid of MVSP software using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). PIC value was estimated according to the following formula; $PIC = 1 - \sum P_i^2$; Where P_i is the allele frequency [17].

3. Results and Discussion

3.1. Discrimination of maize CMS type

To ensure the DNA validity a pair of specific universal primers was designed to amplify the zein region. The PCR product was about 277 bp (Figure 1) as described in the previous literatures. The using of the three pairs of specific primers designed for amplifying the CMS region responsible for cytoplasmic sterility in the six tested inbred lines of maize resulted in amplifying a fragment with a molecular size of ~350 bp (Figure 1) which approved the C-type of cytoplasmic male sterility in the six maize inbred lines.

Based on the morphological characterization there was a doubtful belief that the detected CMS is T-type, although the instability of normal anther development has created strong misgivings about this early conclusion and thinking in a more precise way resolves it. However, the environmental independency of

molecular markers confirmed the suspicious about the morphological characterization and approved the C-type of maize CMS.

3.2. RAMs Markers

To assess the genetic similarity between the six CMS maize inbred lines, six RAMs primers were applied and produced a total of 36 fragments (table 2). Most of the amplified fragments (25 fragment) were found to be polymorphic scoring a polymorphism percentage of 69.4%. In the same context, the mean PIC value of the used primers (UBC-810, UBC-812, UBC-821, UBC-848, UBC-854 and UBC-855) which was 0.36 approved the moderate ability of the used primers in distinguishing between the studied inbred lines.

The UBC-810 primer was capable of amplifying 6 fragments (Figure 2), most of it (5 fragments) were polymorphic, thus the calculated percentage of polymorphism was about 83.3% (table 2). Furthermore, based on PIC value of the previously mentioned primer (0.37) it has a moderate discriminative ability in distinguishing between the studied maize inbreds.

Same results were gained via the second RAM primer (UBC-812), as it generated 5 polymorphic fragments out of the six amplified fragments, gaining a polymorphism percentage of 83.3%. This primer showed almost the same ability in discriminating between the studied inbreds with a PIC value of 0.38.

Less fragments (5) were generated by the UBC-821 primer across the six maize genomes (figure 2), but all were polymorphic, hence, the estimated polymorphism was in its highest level (100%), in addition to the PIC value (0.40) that making the current primer the most definitive in detecting the molecular differences between the six maize CMS lines (table 2).

In contrast, UBC-848 primer showed a total of 6 fragments, half of it were polymorphic (3), therefore, the polymorphism percentage was 50%. The PIC value showed by this primer was a little lower than the previous primer (0.39) approving lower efficiency in distinguishing between the different maize lines.

Although, UBC-854 primer succeeded in amplifying the highest number of fragments (7), only 2 fragments described as polymorphic, accordingly the polymorphism percentage dropped to 28.5%. This primer has the humble ability to characterize the genetic differences on the DNA level with PIC value of 0.33.

The last primer (UBC-855) revealed the same results of the first two primers with 6 amplified fragments, and 5 polymorphic fragments recording a polymorphism percentage of 83.3% (figure 2, table 2).

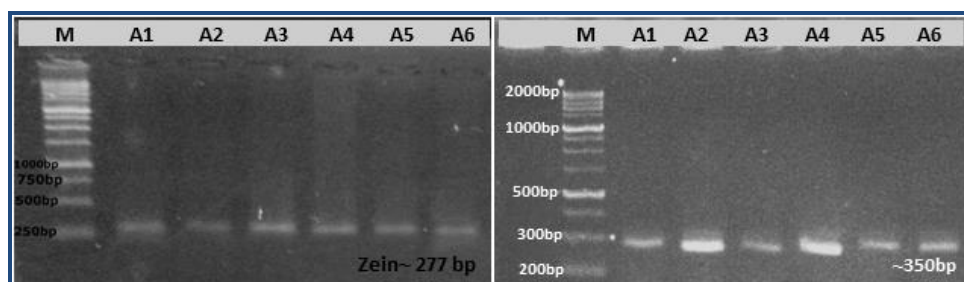


Figure 1. PCR profiles generated by Zein and the three applied CMS-specific markers between the six maize CMS lines.

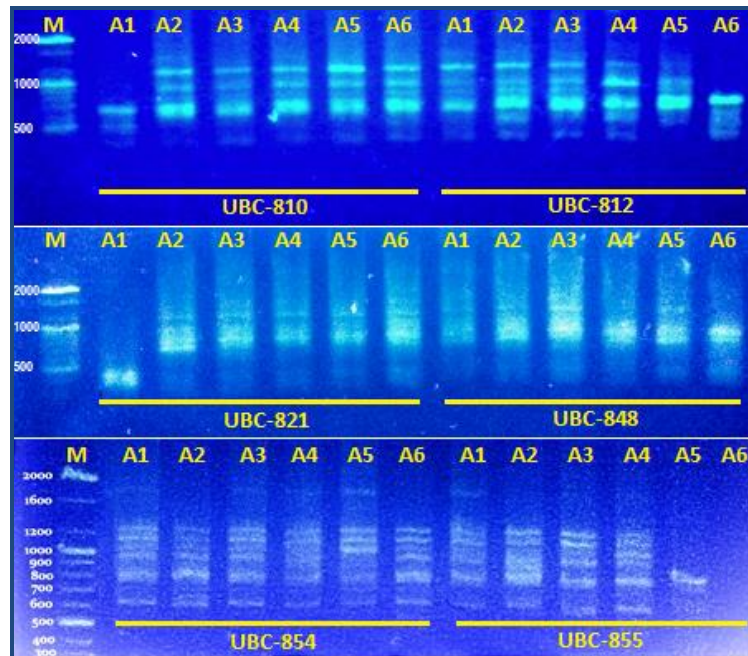


Figure 2. PCR profiles generated by the six used RAM markers between the six maize CMS lines.

Table 2. Molecular results of the used six RAM primers across genomes of six maize CMS lines.

RAM primers	Primer Sequence 5'-3'	Fragments number	Polymorphic fragments	Polymorphism percentage (%)	PIC
UBC-810	(GA)8T	6	5	83.3	0.37
UBC-812	(GA)8 A	6	5	83.3	0.38
UBC-821	(GT)8C	5	5	100	0.40
UBC-848	(CA)8AAGG	6	3	50	0.39
UBC-854	(TC)8RG	7	2	28.5	0.33
UBC-855	(AC)8YT	6	5	83.3	0.33
<i>Total</i>		36	25	69.4	0.36

3.3. Genetic Similarity and Phylogenetic Analysis

The generated fragments represented as binary data (0-1) to estimate the genetic similarity (table 3) and construct the phylogenetic tree (Figure 3).

The estimated genetic similarity between the six A-lines of maize ranged from low to high values based on the PCR results that clearly pointed out to maximum level of genetic similarity existed between A2 and A3 CMS inbred lines. This fact can be easily perceived from the phylogenetic tree (figure 3) as the previously mentioned lines occupied the same sub-cluster and being the most genetically identical lines according to the experienced RAM markers.

On the other hand, minimum genetic similarity values have been detected between some other inbred lines (table 3), indicating its unique genetic background such as A1 and A6 lines that showed the lowest values (0.42) in table 3.

The cluster analysis and the constructed phylogenetic tree (figure 4) revealed that the tested A lines divided into three main clusters, two of them were single clusters consisted of individual lines (A1 and

A6). The third main cluster enclosed A2, A3, A4 and A5 CMS lines. A5 was the most divergent line against the other lines in this cluster followed by A4 line, meanwhile A2 and A3 gained the highest genetic similarity to be located in one sub-cluster.

Table 3. Genetic similarity of the six studied maize inbred lines based on the results of six RAM primers.

CMS inbred lines	A1	A2	A3	A4	A5	A6
A1	1.00					
A2	0.69	1.00				
A3	0.72	0.92	1.00			
A4	0.56	0.80	0.83	1.00		
A5	0.62	0.77	0.80	0.78	1.00	
A6	0.42	0.61	0.69	0.71	0.63	1.00

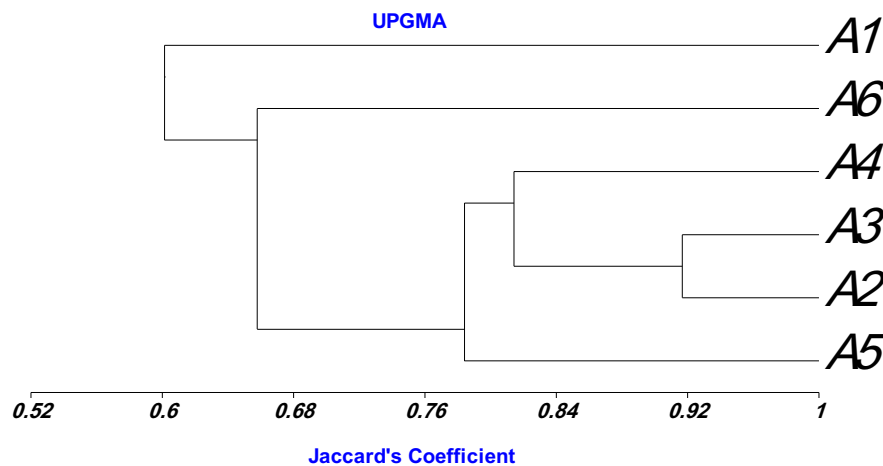


Figure 3. Phylogenetic tree of the six studied CMS maize lines using UPGMA method of six RAMs primers.

4. Conclusions

Generally, the used primers approved their high efficiency in detecting CMS type in maize, which was unpredictable and time consuming via traditionally morphological method. In the same context, all the studied lines were belonged to type C of maize cytoplasmic male sterility. Random Amplified Microsatellite (RAM) was so effective tool and accurate in drawing the genetic relationship between the subjected lines. Furthermore, the tested lines showed an acceptable magnitude of genetic diversity, thus these lines will be valuable genetic resources for producing elite genotypes in future breeding programs.

5. Acknowledgements

Authors like to express their special thanks of gratitude to Dr. Andrés Pérez-Figueroa, (University of Vigo, Spain) for his boundless help and guidance.

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