RAPD MARKERS LINKED TO INDUCED GLAUCOUSNESS IN BREAD WHEAT

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Plants have various morphological and physiological characteristics that enable them to grow and reproduce in low-rainfall environments (Johnson *et al.* 1983). Glaucousness has been previously reported as an adaptive trait for drought tolerance in bread wheat (Johnson *et al.* 1983; Fischer and Wood, 1979), in durum wheat (Clarke *et al.,* 1994), in barley (Baenziger *et al.,* 1983), in oat (Bengtson, *et al.,* 1978), and in sorghum (Jordan *et al.,* 1983). An important function of leaf epicuticular waxes (glaucousness) is to increase the efficiency of stomatal control of water loss by reducing the cuticular conductance to water vapor (Jordan *et al.,* 1984). This advantage of glaucousness may permit more efficient use of soil water in dryland situations, and supports the finding that the ratio of net carbon exchange to transpiration is higher for glaucous genotypes (Richards *et al.,* 1986).

The inheritance of glaucousness in wheat has been studied on the conventional level (Al-Bakry, 2009). Chromosomes 2B, 2D, and 3A were found to bear three different glaucousness genes in wheat (Stuckey, 1972). These three genes are responsible for the production of glaucousness of leaf, spike and peduncle.

Molecular biology techniques in plant include genetic identification or fingerprinting of molecular markers. The technique of analyzing molecular markers is based on the detection of the DNA sequences or combinations that are unique to the individual plant under study (Henry, 1997). Molecular markers are powerful tools that can be used for marker-assisted selection (Horvath *et al*., 1995) and as landmarks for map-based cloning of resistance genes (Michelmore, 1995; Bai *et al*., 1999; Liu *et al*., 2001). In wheat, RFLPs, AFLPs, SSRs, and RAPD markers have been used to study wheat resistance genes (Liu *et al*., 2001; Abdel-Tawab *et al.*, 2003). Thus, molecular marker technology may provide new tools for the investigation of molecular markers linked to glaucousness in wheat.

RAPD have been proposed by several groups as efficient tools for identification of DNA markers associated with agronomically important traits (Banerjee *et al.,* 1999). Linkage between a polymorphic marker and the target locus is confirmed and quantified by using the segregating population from which the bulks

were generated. Probes or primers for loci that are polymorphic and absolutely linked to the gene or region used to distinguish the individuals comprising the bulks will detect clear differences between the bulks. Bulked segregant analysis does not reveal novel types of variation but rather allows the rapid screening of many loci and therefore the identification of segregating markers in the target region (Michelmore *et al.*, 1991).

The present study involved glaucous wheat mutant line (GWM6), nonglaucous wheat cultivar Giza 164, and three F_2 bulks resulting from the crossing between them. Bulk 1 contains glaucous type of F_2 plants; bulk 2 contains moderately glaucous type of F_2 plants; and bulk 3 contains nonglaucous type of F_2 plants. This study was conducted to identify some RAPD markers linked to glaucousness using the bulked segregant analysis (BSA).

MATERIALS AND METHODS

Plant materials

The present study was carried out at Plant Res., Dept., Nuclear Res., Center, Atomic Energy Authority, Anshas, Egypt; and Seed Technology Dept., Agricultural Res., Center, Giza, Egypt

Mapping population: GWM6 X Giza 164, developed by crossing glaucous wheat mutant line 6 (GWM 6) to nonglaucous wheat cultivar (Giza 164) was used for the genetic analysis of glaucousness on leaf blade and spike of wheat plants (Al-Bakry, 2009). GWM6 was developed by wheat breeding program at Atomic Energy Authority, Egypt (Al-Bakry, 2004). It is characterized by has high epicuticular wax content on leaf sheath, leaf blade, peduncle and spike. Giza164 wheat cultivar was obtained from Wheat Dept., Crop Res., Institute, Agric. Res., Center, Egypt. Leaf blades and spikes of Giza164 cultivar are totaly nonglaucous. F_1 plants of this cross between GWM 6 and Giza 164 was moderately glaucous for both leaf blade and spike. The ratio of segregated wheat plants in F_2 was, 1 glaucous: 2 moderately glaucous: 1 nonglaucous. The occurrence of glaucous plants for leaf blade but nonglaucous for spike in F_2-segregated plants might refer to the separate inheritance of these two traits.

To identify the DNA markers that are linked to glaucousness, the bulked segregant analysis (BSA) was used. Three bulks were constructed from the F_2 mapping population derived from the previous cross. Bulk 1 contains 10 F_2 plants of glaucous type; bulk 2 contains 10 F_2 plants of moderately glaucous type; and bulk 3 contains 10 F_2 plants of the nonglaucous type for both leaf blade and spike.

Molecular markers

Genomic DNA extraction

DNeasy plant minikit (Quigen Inc., Cat.no.69104, USA) was used for DNA extraction from the GWM6 glaucous wheat mutant and nonglaucous wheat cultivar (Giza164) and the three bulked F_2 segregating populations.

RAPD- PCR analysis

Forty oligonucleotide primers were screened for polymorphism, and among them, eleven primers showed clear polymorphic patterns. These primers with the 5′ → 3′ sequences are shown in Table (1).

The reaction conditions were optimized and mixtures were prepared (30 µl total volume) consisting of the following, DNTPs 2.4 µl, $MgCl_2$ 3.0 µl, 10 x buffer 3.0 µl, Primer (10 um) 2.0 µl, Taq (5 u/µl) 0.2 µl, Template DNA (50 ng/µl)2.0 ul, H_2O (dd) 17.4 ul. Amplification was carried out in a PTC- 200 thermal cycler (MJ Research, Watertown , USA) programmed as follows: Denaturation, 94°C for 2 minutes, followed 40 cycles. Each cycle consisted of 1 minute at 94°C, 1 minute at 37°C, 2 minutes and 30 second at 72°C, followed by a final extension time of 12 minutes at 72°C and 4°C (infinitive).

Gel electrophoresis

Gel electrophoresis was applied according to Sambrook *et al.* (1989). Agarose (1.2%) was used for resolving the PCR products. The run was performed for one hour at 80 volt in pharmacia submarine (20 x 20 cm). Bands were detected on UV-transilluminator and photographed by Gel documentation 2000, Bio-Rad.

Data analysis

Bands were detected on UV–transilluminator and photographed by Gel documentation 2000, Bio- Rad. The similarity matrices were done using gel works 1D advanced software UVP-England program. The relationships among the parents and the three bulks under study as revealed by dendrogram were done using SPSS windows (version10) program.

RESULTS AND DISCUSSION

The two parents used in the present study have sufficient genetic variation regarding induced glaucousness trait. The bulked segregant analysis provides a rapid and technically simple alternative for identifying markers linked to specific genes; and the only prerequisite is the existence of a population resulting from a cross that segregates for the gene of interest. In addition, the success of this approach depends on the genetic divergence between the parents in the target region (Michelmore *et al.*, 1991). Thus, the mapping population resulting from the crossing between these two contrasting parents under study is suitable for identifying molecular markers associated with induced glaucousness.

Polymorphism detected by RAPD analysis

In the present study, RAPD analysis was used to determine the genetic variation and molecular markers associated with induced glaucousness genes. Eleven arbitrary primers were used for PCR amplification of the genomic DNAs of GWM6 (P_1), Giza 164 (P_2), F_2 glaucous bulk, F_2 moderately bulk and F_2 nonglaucous bulk of the wheat cross GWM6 x Giza164. The total number of amplified

fragments produced by the 11 arbitrary primers is presented in Table (2). Among the 11 arbitrary primers, amplification products of five primers (45.45%) generated polymorphic RAPD markers linked to induced glaucousness (Fig. 1). However, amplification products of six primers (55.55%) generated monomorphic RAPD fragments (Fig. 2). The number of amplified fragments produced per primer varied from 10 (OP-B14, OP-C06, OP-C11 and 10) to 46 (OP-B03). A total of 51 DNA amplicons, ranging from 200 to 2700 bp, were generated for the tested genotypes (Table 3). Out of these amplified DNA amplicons, 34 were conserved (monomorphic) among all genotypes, while 17 were polymorphic. The number of RAPD amplicons produced by each primer varied from 2 (primers 0P-B14, 0P-C06, 0P-C11, 0P-C20) to 11 (primer 0P-B03) with an average of 5 per primer. The highest numbers of polymorphic DNA fragments were 5 (0P-B13), while the lowest number was 0 (primers 0P-A10, 0P-B14, 0P-C06, 0P-C11 and 0P-C20).

The total number of monomorphic amplicons was 34. The number of polymorphic amplicons was 17. The number of amplified polymorphic DNA amplicons across the 5 primers ranged from 1 to 5 amplicons. The maximum number of amplicons was amplified with the primer 0P-B03, while the primer 0P-A09 amplified the minimum number. However, the percentage of polymorphism ranged from 25%was shown by 0P-A09 and 0P-C08 primers to 71% shown by 0P-B13 primer.

Genetic relationship among genotypes studied

Bands generated from the 11 RAPD primers, were utilized to calculate the genetic similarity index (RAPD-GS) among GWM6, Giza164 cultivar and the three bulked F_2 segregated populations (Table 4). Genetic similarity (GD) among wheat genotypes ranged from 0.82 to 0.95, with an average of 0.89. The smallest genetic similarity (82%) was observed between glaucous parent and nonglaucous F_2 bulk, while the largest similarity (95%) was between glaucous parent and glaucous F_2 bulk followed by 93% between nonglaucous parent and nonglaucous F_2 bulk.

The dendrogram based on cluster analysis of RAPD data is presented in Fig.. (3). The genotypes under study were separated into two distinct groups, the first group is separated into two subgroups the first subgroup included glaucous parent and glaucous F_2 bulk, while, the moderately glaucous was in a separate subgroup. The second group was composed of nonglaucous parent and nonglaucous F_2 bulk.

RAPD markers associated with induced glaucousness

Out of the eleven primer pairs tested in this study, five primers gave polymorphism and developed molecular markers associated with glaucousness (Table 5). These molecular markers were based on polymorphism between the DNA of glaucous and nonglaucous genotypes.

Two molecular markers, appeared in the glaucous parent and glaucous F_2 bulk, were detected by the primers 0P-B03 and 0P-B13 at molecular sizes of 527 bp and 1312 bp, respectively, while there were two bands with molecular sizes of 1453, 456 bp of primer 0P-C08. On the other hand, three molecular markers were detected in the glaucous parent, glaucous F_2 bulk and moderately glaucous F_2 bulk. These three markers were detected by primers 0P-B13, 0P-B15 and 0P-C05, respectively, at 634 bp, 888 bp, 622 bp.

In wheat no molecular markers associated with glaucousness related genes were reported before in a study conducted by Liu *et al.* (2007). They investigated the genetic control of non-glaucousness in synthetic Line 3672 and to map the gene(s) using molecular markers. They found that non-glaucousness in that Line was controlled by a single dominant gene, temporarily designated *Iw*3672. Five SSR markers linked to *Iw*3672 gene were mapped on chromosome 2DS. In addition, two EST-derived markers and a SNP marker were developed and were also linked to *Iw*3672 gene.

The conventional plant breeding is primarily based on the selection of superior individuals among segregating progenies of sexual matings. In addition, selection for plant improvement has largely been carried out on the whole-plant or phenotype, which is the result of genotype and environmental effects. Thus, conventional plant breeding is often hampered by difficulties in selecting for agronomically important traits, especially when they are considerably influenced by the environment. Moreover, testing procedures may be difficult, unreliable, or expensive, due to the nature of the target traits or the target environment. For these reasons, selection through molecular markers might be an efficient complementary breeding tool, especially when selection is done under unfavorable conditions (Ribaut *et al*., 2001). The associated molecular markers with induced glaucousness identified in the present study can greatly enhance the efficiency of incorporating glaucousness into new varieties. This favorable allele can be introgressed in new target germplasm through backcrossing, or can be selected in a segregated population i.e., the seven RAPD markers associated with induced glaucousness detected in the present study can be used in marker-assisted selection.

SUMMARY

The present study was conducted to identify some DNA markers that are linked to glaucousness. RAPD analysis was used to determine the genetic variation and molecular markers linked to induced glaucousness genes. Eleven arbitrary primers were used for PCR amplification of the genomic DNAs of GWM6 (P1), Giza 164 (P2), F_2 glaucous bulk, F_2 moderately bulk and F_2 nonglaucous bulk of the wheat cross GWM6 x Giza164. Among the 11 arbitrary primers, amplification products of 5 primers (45.45%) generated polymorphic RAPD markers linked to glaucousness. However, amplifi-

cation products of 6 primers generated monomorphic RAPD bands. The number of amplified bands produced per primer varied from 10 to 46. A total of 51 DNA amplicons, ranging from 200 to 2700 bp, were generated for the tested genotypes. Out of these amplified DNA amplicons, 34 were common (monomorphic) among all genotypes, while 17 were polymorphic. The number of RAPD amplicons produced by each primer varied from 2 to 11 with an average of 5 per primer. Out of the eleven primer pairs tested in this study, five primers gave polymorphism and developed molecular markers associated with glaucousness. Two molecular markers, appeared in the glaucous parent and glaucous F_2 bulk, were detected by the primers 0P-B03 and 0P-B13 at molecular sizes of 527 bp and 1312bp respectively, while two molecular markers were observed with molecular sizes of 1453 and 456 bp of primer 0P-C08. On the other hand, three molecular markers were exhibited in the glaucous parent, glaucous F_2 bulk and moderately glaucous F_2 bulk by primers 0P-B13, 0P-B15 and 0P-C05, respectively, at 634 bp, 888 bp and 622 bp.

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Table (1): Random primer names and their sequences used for RAPD- PCR analysis.

Primer name	Sequence	Primer name	Sequence
0P-A09	5′ GGGTAACGCC 3′	0P-C05	5′ GATGACCGCC 3′
0P-A10	5′ GTGATCGCAG 3′	0P-C06	5′ GAACGGACTC 3′
0P-B03	5′ CAT CCCCCTG 3′	0P-C08	5′ TGGACCGGTG 3′
0P-B13	5′ TTCCCCCGCT 3′	0P-C11	5′ AAAGCTGCGG 3′
0P-B14	5′ TCCGCTCTGG 3′	0P-C20	5′ ACTTCGCCAC 3′
0P-B15	5′ GGAGGGTGTT 3′		

Table (2): Number of amplified DNA fragments scored for GWM6, Giza164, F_2 glaucous bulk, F_2 moderately bulk and F_2 nonglaucous bulk of the wheat cross GWM 6 x Giza 164.

Primer	Number of amplified DNA bands					Total	Mean
	GWM 6	Giza164	F_2 glaucous bulk	F_2 moderately bulk	F_2 nonglaucous bulk		
OP-A09	3	3	4	4	4	18	3.6
OP-A10	4	4	4	4	4	20	4.0
OP-B03	10	9	10	8	9	46	9.2
OP-B13	6	3	5	4	3	21	4.2
OP-B14	2	2	2	2	2	10	2.0
OP-B15	4	2	3	4	2	15	3.0
OP-C05	5	4	5	5	2	21	4.2
OP-C06	2	2	2	2	2	10	2.0
OP-C08	8	6	8	6	6	34	6.8
OP-C11	2	2	2	2	2	10	2.0
OP-C20	2	2	2	2	2	10	2.0
Total	48	39	47	43	38	215	

Table (3): Levels of polymorphism based on RAPD analysis scored for GWM6, Giza164, F_2 glaucous bulk, F_2 moderately bulk and F_2 nonglaucous bulk of the wheat cross GWM6 x Giza164.

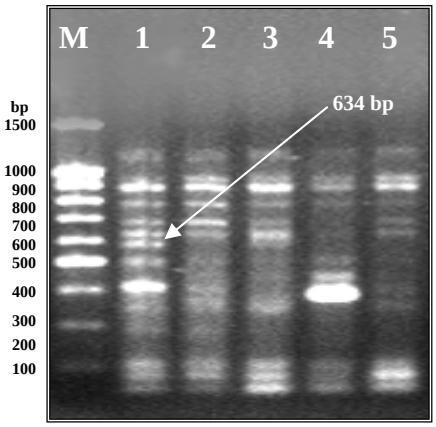
Primer	Number of amplicons	Polymorphic amplicons	Monomorphic amplicons	Polymorphism %
0P-A09	4	1	3	25%
0P-A10	4	0	4	0%
0P-B03	11	4	7	36%
0P-B13	7	5	2	71%
0P-B14	2	0	2	0%
0P-B15	4	2	2	50%
0P-C05	5	3	2	60%
0P-C06	2	0	2	0%
0P-C08	8	2	6	25%
0P-C11	2	0	2	0%
0P-C20	2	0	2	0%
Total	51	17	34	

Table (4): RAPD-based genetic similarity (GS) matrices among the GWM6 (P_1), Giza164 (P_2), F_2 glaucous bulk, F_2 moderately glaucous bulk, and F_2 nonglaucous bulk of the wheat cross GWM6 x Giza164 based on RAPD analysis.

Genotype	GWM6	Giza164	F_2-glaucous bulk	F_2-moderately glaucous bulk
Giza164	0.884			
F_2-glaucous bulk	0.957	0.907		
F_2-moderately glaucous bulk	0.889	0.902	0.911	
F_2-nonglaucous bulk	0.824	0.935	0.871	0.864

Table (5): RAPD markers and their molecular sizes (bp) associated with induced glaucousness based on DNA of GWM6, F_2 glaucous bulk and F_2 moderately glaucous bulk of the wheat cross GWM6 x Giza164.

Genotype	Marker size (bp)	Primer	Total
GWM 6	527 634 1312 888 622 456 1453	0P-B03 0P-B13 0P-B13 0P-B15 0P-C05 0P-C08 0P-C08	7
F_2 glaucous bulk	527 634 1312 888 622 456 1453	0P-B03 0P-B13 0P-B13 0P-B15 0P-C05 0P-C08 0P-C08	7
F_2 moderately bulk	- 634 - 888 622 - -	- 0P-B13 - 0P-B15 0P-C05 - -	3



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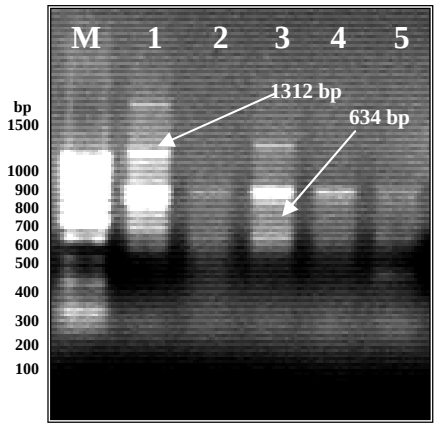

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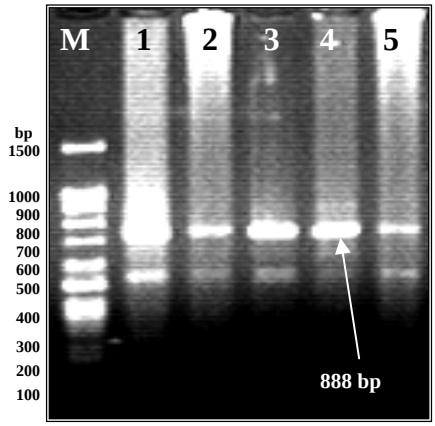

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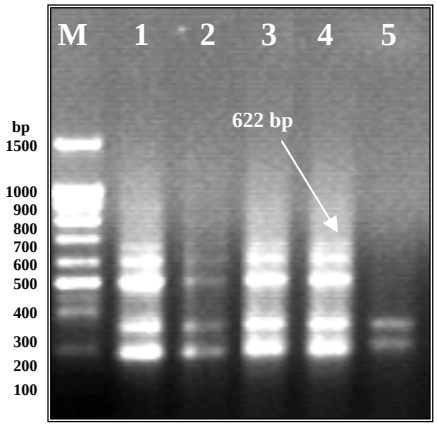

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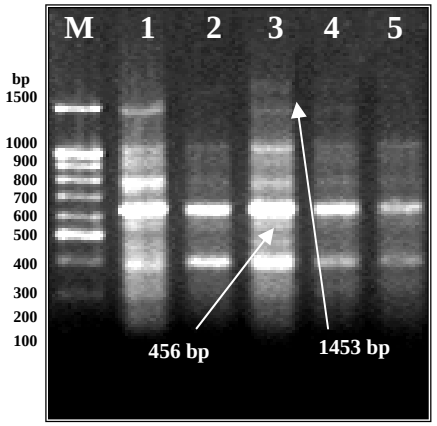

OP-C08

Fig. (1): RAPD banding patterns for 1:GWM6 (P_1), 2: Giza164 (P_2), 3: F_2-glaucous bulk (G), 4: F_2-moderately glaucous bulk (MG), and 5: F_2-nonglaucous bulk (NG) M: DNA Marker (100+1.5 kb ladder marker).

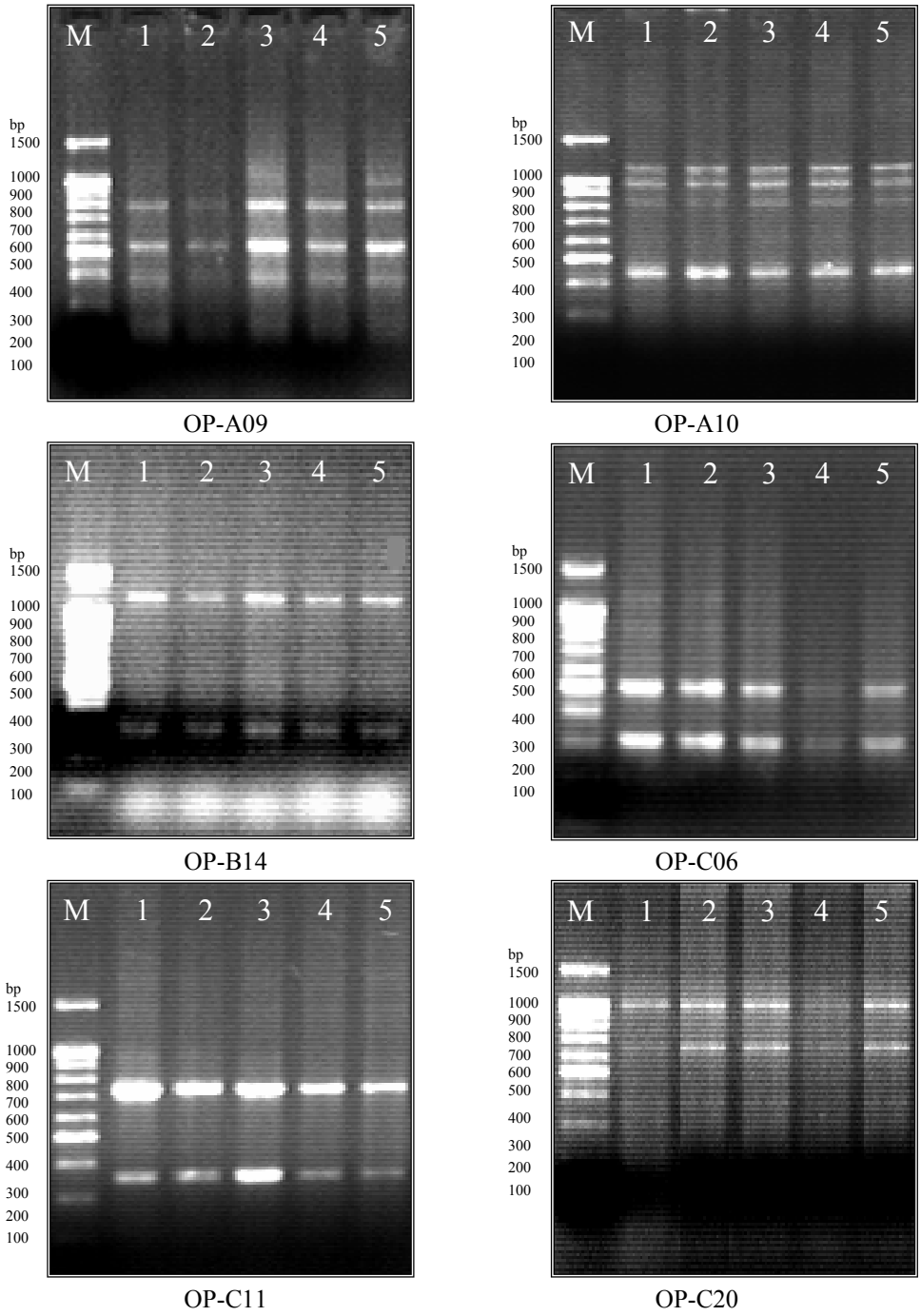

Fig. (2): RAPD banding patterns for 1:GWM6 (P_1), 2:Giza 164 (P_2), 3:F_2-glaucous bulk (G), 4:F_2-moderately glaucous bulk (MG), and 5:F_2-nonglaucous bulk (NG) M: DNA Marker (100+1.5 kb ladder marker).

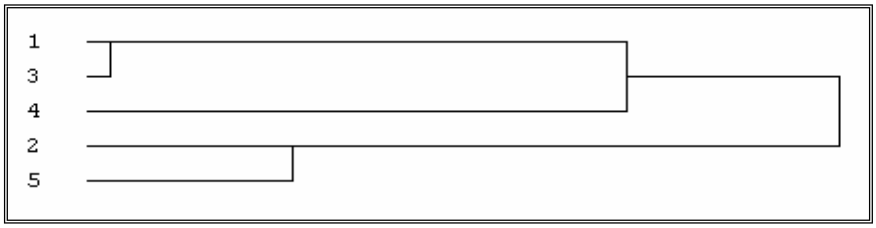

Fig. (3): Dendrogram based on cluster analysis of RAPD data.
1:GWM6 (P_1), 2:Giza 164 (P_2), 3:F_2-glaucous bulk (G), 4:F_2-moderately glaucous bulk (MG), and 5:F_2-nonglaucous bulk (NG)