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Identification of sunflower (*Helianthus annuus*, Asteraceae) hybrids using simple-sequence repeat markers

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ABSTRACT. Hybrid identification of 16 sunflower hybrids was confirmed using simple-sequence repeat methodology. Of 20 specific simple-sequence repeat primers, 18 authenticated the purity of these hybrids; the remaining two specific primer pairs gave ambiguous DNA fragments. The results indicate that simple-sequence repeat analysis for the identification of hybrids derived from the crossing of different inbred sunflower lines can improve the accuracy of selection, save time and reduce cost.

Key words: Diversity; RAPD marker; Sunflower

INTRODUCTION

Sunflower is of great importance because its seed has high oil contents ranging from 40-50% (Skoric and Marinkovic, 1986). Only 30% of edible oil requirements of Pakistan are met through local production, and the other 70% of the country's requirements are met through importation, costing huge amounts of foreign exchange. The imported edible oils are mainly palm and soybean oil. However, the shortage of edible oil still persists. The situation of oil production in Pakistan is better than before, but due to increasing demand by the ever-increasing human population, continuous improvement in its productivity is highly desirable. For the last few years, yield improvement in sunflower varieties has not been substantial. The narrow genetic base and pure local hybrids of the germplasm used have been considered major drawbacks in the development of an ideal high-yielding local hybrid. The identification of sunflower hybrids is normally performed through morphological markers. The information about genetic diversity and hybrid identification in the available germplasm and among elite breeding material is essential in plant breeding (Mosges and Friedtu, 1994). The future breeding program depends on the availability of a pure local hybrid to increase productivity. Traditionally, the assessment of hybrid purification has been based on the differences in morphological traits. RFLP (restriction fragment length polymorphism) markers have been used for hybrid studies and genetic mapping of different crops (Hernández et al., 2000). Polymerase chain reaction (PCR)-based DNA marker techniques seem to provide the means for generating useful information for hybrid purification, genetic relatedness and diversity. PCR-based simple-sequence repeat (SSR) markers are co-dominant markers and extensively used in hybrid identification (Chalmers et al., 2001), and the identification of markers is linked to different traits (Bai et al., 2003). SSR methodology has been used for pure hybrid analyses in several crops (Li and Nelson et al., 2001). SSR analysis was carried out in 16 genotypes of sunflower for hybrid identification and to determine purity among them, and to compare these 16 genotypes with their 8 parents. The use of these markers frequently results in the exclusion of hybrid seedling from segregating populations, which is considered highly undesirable in breeding program.

MATERIAL AND METHODS

This study included 8 parents of sunflower and their 16 hybrids. The sunflower parents were CM-612, HA-27, B-SIN-82, HA-314, RL-54, RL-51, R-SIN-82, and RL-46, and their 16 hybrids were designated hybrid-1, hybrid-2 ….hybrid-16. The genotypes were grown in plastic containers. The temperature during germination was 35°C in the growth chamber and light was supplied for 16 h. Leaf tissues, 0.2-0.3 g, were obtained from the 6-day-old leaves of the sunflower genotypes. The weighed leaves were then immediately transferred to plastic zipper bags containing 1.5 mL CTAB (Khan et al., 2004). Finally, the concentration of DNA was measured at 260 nm in a spectrophotometer (CECIL CE 2021 2000 Series). The quality of DNA was checked by running 5 µL DNA on a 1.2% agarose gel prepared in 0.5X TBE buffer. The DNA samples giving a smear on the gel were rejected.

PCR amplification

DNA concentration in the working solution of approximately 15 ng/µL in d3H_2O was

confirmed by spectrophotometry. For SSR analysis (Williams et al., 1990), the concentrations of genomic DNA, 10X PCR buffer with $(NH_4)2SO_4$, $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), 10-mer random primer and Taq DNA polymerase (Fermentas) were optimized. DNA amplification reactions were performed in a thermal cycler (Eppendorf AG, Hunberg). The PCR profile was as follows: one cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and a final extension for 10 min at 72°C. The SSR fragments were separated by electrophoresis on 1.2% agarose gels with ethidium bromide (10 ng/100 mL of agarose solution in TBE). A 100-bp ladder was also loaded along with the DNA to check the size of the DNA fragments of the parents and the hybrid (Figure 1). All 16 hybrids showed an exact relationship with their parents. Only two SSR primers showed ambiguous bands, which may be due to the undesired annealing temperature or due to the non-optimal concentration of $MgCl_2$.

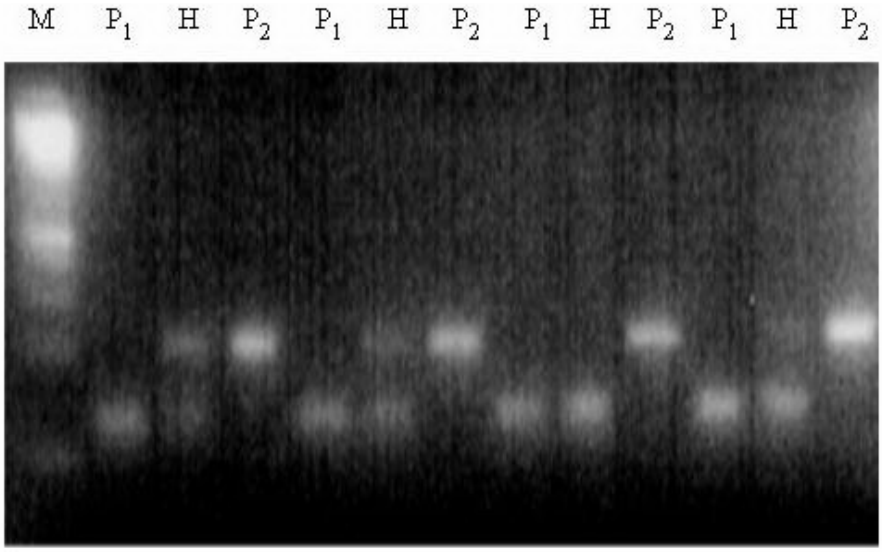

Figure 1. Hybrid identification of sunflower using SSR primer ORS 324. M = molecular marker; P_1 and P_2 = _____.

RESULTS AND DISCUSSION

DNA of 8 sunflower parents and their 16 hybrids was amplified with 20 different specific primer pairs (Table 1). The number and size of the DNA fragments were strictly dependent on the sequence of the primer. Reactions were repeated two to three times to check the consistency of the amplified products, and only easily resolved and bright DNA bands were counted. All F1 genotypes showed an authentic confirmation with their parents.

These results suggest that SSR markers provide information for the identification of sunflower hybrid genotypes (Lawson et al., 1994). The reproducibility of the SSR technique can be influenced by various factors, such as sequence of a primer, template quality and quantity, the type of thermocycler and polymerase concentration (Hernández et al., 1999). However, the use of a standardized SSR protocol can ensure a reproducible SSR pattern.

Table 1. List of SSR primers and their sequences.

S. No.	Primer name	Forward sequence	Reverse sequence
1	ORS 296	CCTTGCACTTAGCCCA	GCATTCACAACAAACATCATCA
2	ORS 290	TCTTTACTTCCACGGTGCACTA	GCATTCACAACAAACATCATCA
3	ORS 287	CGGATTCACTGCTTTCCAAT	GCATAGTTGCCCATCAGAGTAA
4	ORS 300	GAATGCGGAGACAAAGGCT	ATAAGTGTGGCGGTGGAAGA
5	ORS 301	CGTGACCTGTGAAACACCAA	CGATAACCGTGTGAAATCGTG
6	ORS 309	CATTTGGATGGAGCCACTTT	GATGAAGATGGGGAATTTGTG
7	ORS 310	AATTCCCACGCAAACTTCAA	GGGTAAATGGGGCAACCTAT
8	ORS 311	TCCCGAATTAGCCAAAGAAC	GGTGTGGGTGTTGCAGCTAT
9	ORS 315	GCCGTGAATAATGGGATTGA	GATTGGGTCAGCTTGTGTGA
10	ORS 316	TGGCGTCTTCATAGCATCAG	GAGATTTGAGCTTCGTGTTGC
11	ORS 318	TCCATGAGTTGGTCGTATGC	CCGCATATTGAAACTGCATC
12	ORS 319	TCATCAATCCAAGCACCAAA	TTGGGCCGTAAACCCTTAAC
13	ORS 321	TGTCGAAGAGTTGTCGGAAC	GGGAAGGTGAAACCCTAACC
14	ORS 322	TGCACCACTTGGAACTTGAC	GCATTCATCCATAGTCATCAAGA
15	ORS 323	CGGGAAACTAGGATCAGAGG	GCCGGAGGATTAGAGGAGTT
16	ORS 324	CACTTCTACTCCATCTTCTTCATCAA	ATGATGCTCCGCAACAGTTT
17	ORS 332	GACCAGCCGCATATTTCAA	AAACCGGCCTCTTATTTGGT
18	ORS 333	CGGTTAAGATGGTTCAGTTGG	ATATTAAGTTTTGGTTTTAGCCAGAA
19	ORS 337	TTGGTTCATTCATCCTTGGTC	GGGTTGGTGGTTAATTCGTC
20	ORS 339	CCCTCTTCCTCTCCCTTACTTT	AAATCCGCACTCCAATATGC

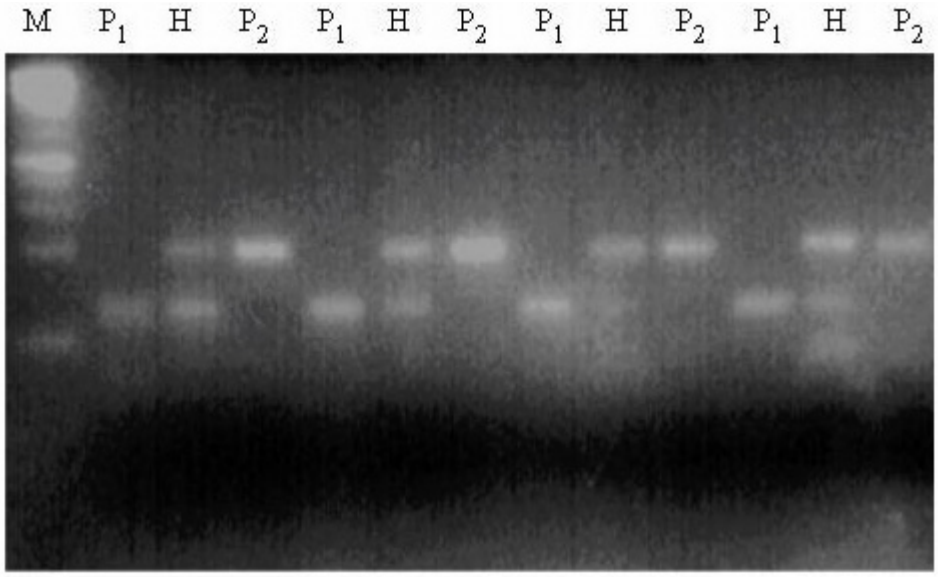

Figure 2. Hybrid identification of sunflower using SSR primer ORS 316. M = molecular markes; P_1 and P_2 = _____; H = _____.

Different concentrations of $MgCl_2$, Taq DNA polymerase and concentration of template DNA were optimized for PCR conditions. DNA concentrations of 5, 10, 15, 20, and 25 ng/25 µL in each reaction were studied. A concentration of 10 ng/25 µL was found to produce the most consistent and reproducible banding patterns. Murray et al. (1980) used the SSR technique to evaluate some mutants and found that a 3 mM concentration was optimal for

better amplification. In this study, 3 mM $MgCl_2$ was found to be optimal for consistent results. More than 3 mM $MgCl_2$ produced nonspecific amplification. Similarly, one unit concentration of Taq DNA polymerase was found to be optimal for better amplification of genomic DNA. Other reaction conditions were also kept constant, and the results were found to be consistent and reproducible. All amplified bands were identical in each repetition. The SSR technique used here was found to be quite effective in determining the genetic authenticity among sunflower parents and hybrids. By knowing about the authenticity of sunflower hybrid lines, a plant breeder can use this for further breeding programs.

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