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The usage of the SCoT Molecular Markers for determining the genetical purity of the commercial hybrids of *Hellianthus annuus*

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Abstract
Genetic purity is an important factor in the production of hybrid seeds, as it is necessary to conserve and maintain its agronomic qualities. Poor purity leads to degradation over time in sunflower hybrids, particularly in inbred lines from breeding programs. In the present study, we investigated the use of two types of SCoT (Start Codon Targeted Polymorphism) primers for identifying genetic purity. The study included the SCoT34 and SCoT7 primers to assess the genetic purity of hybrid AH1023. Statistical calculations showed a higher polymorphism identity for the SCoT34 primer (73%) compared to the SCoT7 primer (50%). Furthermore, the number of alleles generated was 11 for the SCoT34 primer and only 4 alleles for the SCoT7 primer. Analysis of the Jaccard coefficient of hybrid AH1023 identified 10 genotypes with common similarity and 5 genotypes that differed substantially from the group genotypically, resulting in a genetic purity of 67% for hybrid AH1023. Thus, the SCoT primers successfully identified the initial degradation of genetic purity and provided insights into the genetic profile of hybrid AH1023.

Keywords: Sunflower Hybrids, Genetic Purity, Molecular Markers, PCR, SCoT primers;

Introduction

Sunflower (*Helianthus annuus L.,* 2n=34) is an oilseed crop plant of great importance to agriculture, industry, and the economy. Its seeds are harvested to extract oils used for everyday food consumption as well as industrial purposes. The seeds are also used as animal feed, for human consumption in raw form, and as sources of fatty acids like oleic acid (omega-9), linoleic acid (omega-6), palmitic acid, and stearic acid [1], which are utilized in the food, pharmaceutical [2], cosmetic [18], and chemical industries [3].

The issue of genetic purity in creating sunflower hybrids is an important topic in plant genetics and agricultural sciences because it influences the quality, productivity, and characteristics of crop plants. The creation of sunflower hybrids aims to combine valuable hereditary traits, such as high yield, resistance to diseases, pests, and stress caused by soil and climatic conditions [9, 23].

Maintaining the genetic purity in sunflowers involves spatial and reproductive isolation to prevent unintended cross-pollination from other varieties that could lead to genetic contamination [5], preserving the purity of parental lines (inbred lines) to ensure uniformity and stability in maintaining genetic traits, controlling pollination by using androsterile maternal lines and fertile paternal lines as parents, testing genetic purity through genetic analyses with molecular markers or by assessing phenotypic uniformity through observation of physical traits [6, 24].

Genetic purity can be established by identifying genetic polymorphisms through biotechnology and molecular markers, which allow for the identification of DNA sequences associated with the desired traits [7]. Molecular markers have proven efficient in identifying genetic polymorphism, providing information on variability and genome mapping [10]. They are classified based on their inheritance pattern (dominant or codominant markers) and the method used for detection (SSR, ISSR, RAPD, SCoT, etc.) [11]. Dominant markers do not allow differentiation between heterozygous and homozygous individuals. They enable the observation of a DNA fragment but cannot determine if it comes from one or both alleles. Examples include RAPD, ISSR, and SCoT [12]. Codominant markers allow for the identification of both alleles and distinguish between homozygous and heterozygous individuals, making them more precise and useful in detailed genetic analyses. Examples include SSR and SNP [13].

The results of the application of molecular markers in sunflower breeding obtained in the Institute of Field and Vegetable Crops are reviewed by Saftic-Pankovic proving that the results on genetic distance (GD=7-75%) between sunflower inbred lines obtained with RAPD and SSR markers indicate large variability and provide important information for the selection of parental lines for future. The successful hybridization between H. rigidus and H. annuus was confirmed with RAPD markers, and the variability between F1 and BC1F1 plants is discussed. CAPS markers for resistance to downy mildew, that can be used in marker-assisted selection, are presented [14, 15].

Validated markers that are available for diverse monogenic traits including disease resistance, Orobanche resistance, herbicide tolerance, high oleic acid, high tocopherol content, and fertility restorers were identified [16].

SCoT markers (Start Codon Targeted Polymorphism) are a type of dominant marker used for genetic analysis through genome mapping based on sequences surrounding the start codon (ATG) of genes. They are effective in detecting gene-related polymorphisms and may be particularly relevant for genetic studies of functional traits, such as disease resistance, resilience to soil and climatic stress, or high yield. The target DNA sequences are amplified through Polymerase Chain Reaction (PCR) using SCoT primers to recognize conserved regions around the start codon [17, 18]. SCoT markers have proven effective in genetic studies and plant and animal breeding, as well as evolutionary biology, due to their high polymorphism rate, providing valuable information about genetic diversity within populations and varieties [19]. Other species tested with SCoT markers are Brassica napus [20], Petroselinum crispum [21], Sesamum indicum [22].

In this experiment, we aimed to study SCoT molecular markers to determine genetic purity in commercial sunflower hybrids and to demonstrate a statistical method for assessing genetic purity based on the presence or absence of bands, using the correlation coefficient between plants of the same hybrid.

Material and Method

Germplasm

To determine genetic purity of the pre-comercial hybrid AH102. For the experiment, it was collected from the growth chamber of the Genetical Engineering department from U.S.V. “King Mihai I” from Timisoara, 100-150 mg of leaf tissue was collected from 15 plants of the hybrid. AH1023 is a mid-late, medium height, big calatidium, long-ovoid shaped seeds, oil content of 50-51%, *imidazoline* resistant, drought and heat tolerant, potential yield of 4000-4200 kg/ha.

DNA Extraction

To reveal the sunflower genetic diversity, molecular analyses were performed in the Molecular Biology Laboratory, U.S.V. “King Mihai I” from Timisoara. Genetic diversity was determined through molecular tools such as Start Codon Targeted (SCoT) markers (**Table 1**).

For each population, samples of 2 different plants and the DNA were extracted from young leaves in early stages based on the Doyle and Doyle method. DNA concentration and quality were evaluated with the Nanodrop 8000 (ThermoFisher Scientific, Waltham, Massachusetts, USA). The concentration was very high; therefore, the samples were diluted to 100 ng/µL. Then, the DNA samples were amplified using SCoT primer [23, 24].

The amplification mixture contained DNA 100 ng/µL, GoTaq® Green Master Mix (Promega, Madison, WI, USA) 2x—12.5 µL, primer 20 µM—1 µL, $MgCl_2$ 10 µM—1 µL and sterile distilled water up to 25 µL. The amplification products were separated by electrophoresis on a 1.8% agarose gel and visualized under U.V. radiation in the presence of ethidium bromide.

Table 1. Primers used for the study

Primer	Primer Sequences 5′–3′
SCoT 34	CAATGCACGAATCGTGTC
SCoT 7	ACATGGCAAGCAGGTCGCA

The size of the amplified fragments was evaluated relative to the BenchTop 100 bp DNA ladder. Then, band scoring was performed, with presence denoted as 1 and absence as 0. The results obtained were statistically interpreted

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Statistical interpretations

For the statistical analyses of the 15 plants associated with the two SCoT primers (35 and 7), mathematical and statistical methods were employed to interpret the results. Following the analyses performed with various primers, only distinct bands were recorded as present (1), while those marked as absent (0) were considered unresolved. These bands were then translated into a binary matrix. To assess the potential of SCoT primers for determining genetic purity, the following parameters were considered:

Total polymorphism generated by a specific primer (PIC):

$$PIC = 1 - \sum_{i=1}^{n} P_{ij}^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Discrimination index (PI):

$$PI = \sum PIC$$

Marker index (MI):

$$MI = EMR * PIC$$

The genetic purity among the analyzed alfalfa varieties was determined using the Jaccard coefficient based on data obtained from the two SCoT primers. A multivariate heatmap illustrating the genetic purity of the 15 plants analyzed with the SCoT 35 and SCoT 7 primers was generated using the heatmap module in RStudio v4.4.1.

Genetic purity control assessment

To determine the genetic purity of the hybrids the number of other plants is "similar" based on the Jaccard coefficient more >0.75 was counted. Each plant was then evaluated to see if it meets the majority.
The genetic purity of the hybrid was then calculated as:

$$\text{Genetic purity coefficient (\%)} = \frac{\text{Number of plants that meets the majority similiarty coeffcient}}{\text{Total number of plants}}$$

Results and Discussion

The agarose gel electrophoresis results for the hybrid analyzed with the SCoT 34 primer highlight key metrics for polymorphic capacity (**Figure 1** and **Table 2**). SCoT 34 amplified 11 bands with a size range of 301–1893 bp, of which 8 were polymorphic (73%).

Table 2. Polymorphic capacity summary for Scot34 and Scot7 Primers

Summary	Scot34	Scot7
Band Size Range	301-1893	1024-2650
Total Bands (n)	11	4
Polymorphic Bands (np)	8	2
Polymorphic Bands Percentage (Pb%)	73 %	50 %
Polymorphic Information Content (PIC)	0.11	0.06
Marker Index (MI)	0.92	0.13

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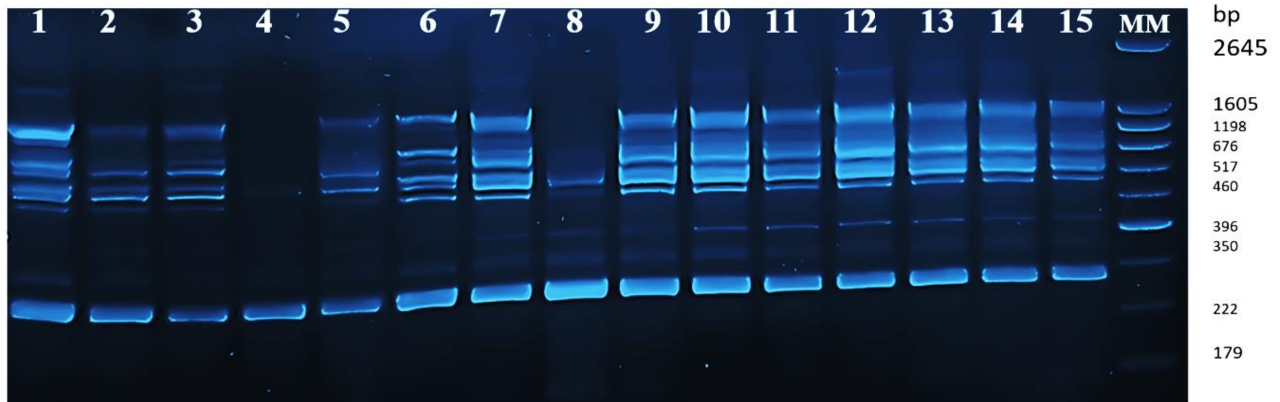

Figure 1. Agarose gel-electrophoresis results for the *SCoT 34: CAATGCACGAATCGTGTC*

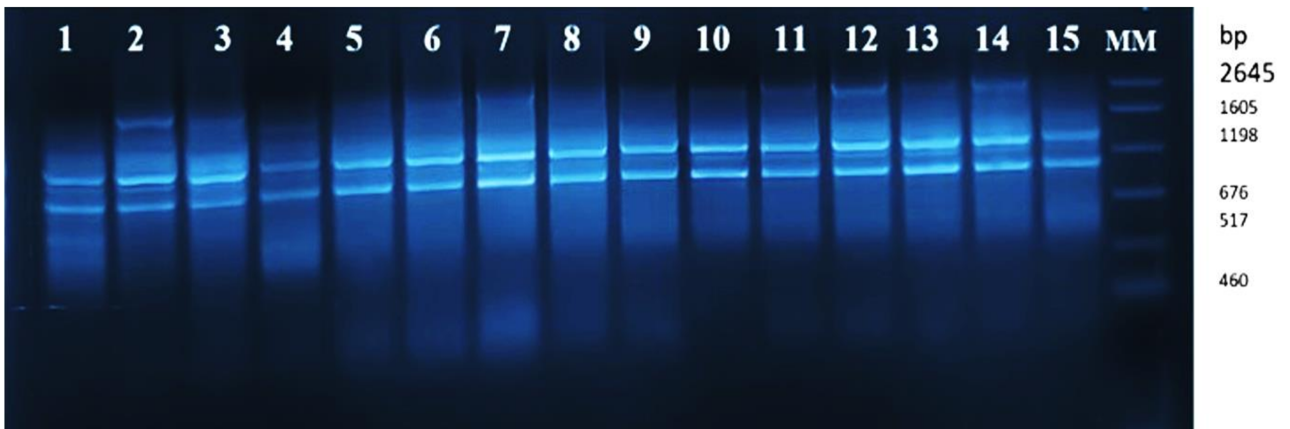

Figure 2. Agarose gel-electrophoresis results for the *SCoT 7: ACATGGCAAGCAGGTCGCA*

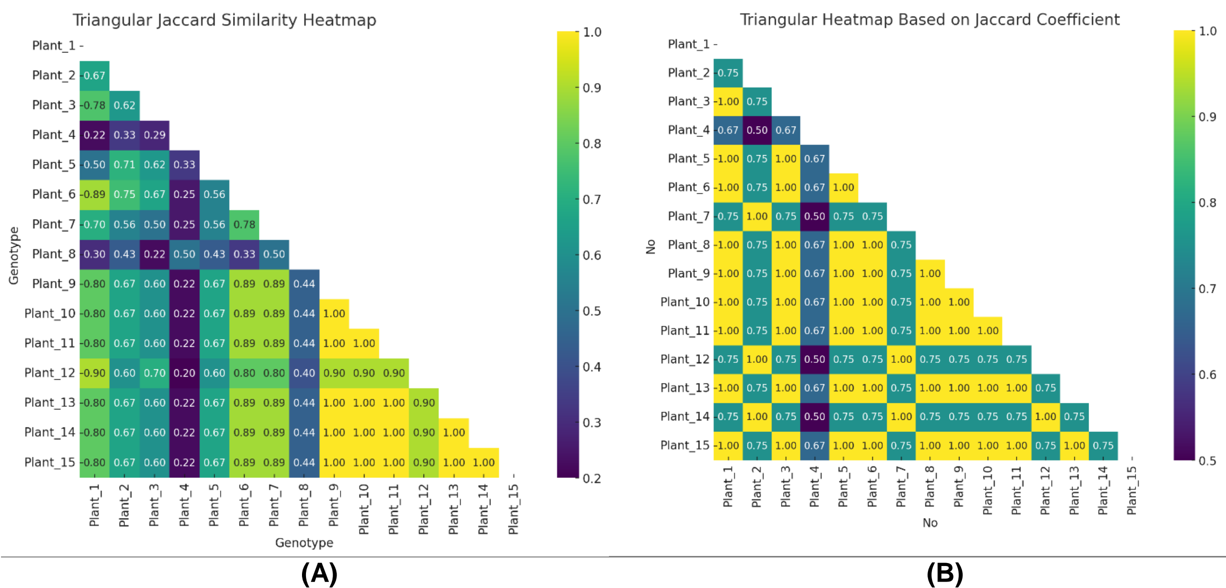

Figure 3. Jaccard coefficient analysis of the 15 plants of sunflower hybrid A1023 (A) – Primer SCoT34 and (B) – SCoT 7

Table 4. Evaluation of the genotypes that meets the similarity with the majority

Genotype	Similarities_Nr	Meets_Majority
Plant_1	9	+
Plant_2	1	-
Plant_3	1	-
Plant_4	0	-
Plant_5	0	-
Plant_6	10	+
Plant_7	8	+
Plant_8	0	-
Plant_9	9	+
Plant_10	9	+
Plant_11	9	+
Plant_12	9	+
Plant_13	9	+
Plant_14	9	+
Plant_15	9	+

According to the results obtained regarding genetic purity, 10 of the 15 plants analyzed from hybrid AH1023 (**Table 4**) show a common similarity based on Jaccard's coefficient (**Figure 3)**. The genetic purity of the hybrid is 67%. On the other hand, it is important to mention that the number of plants analyzed, and the type of germplasm used may influence the ability of the primers to assess genetic purity. With this in mind, it is proposed to include additional types of SCoT and SSR primers for molecular analysis, as well as the use of other types of germplasm.

Conclusions

The study demonstrates the effectiveness of SCoT molecular markers in assessing the genetic purity of sunflower hybrids. SCoT 34 proved to be efficient, generating a higher number of polymorphic bands, which allowed for clearer differentiation of genetic variability among plants and thus the identification of genetic purity within the AH1023 hybrid. In contrast, the SCoT 7 primer did not produce polymorphic bands in the plants identified as impure by the SCoT 34 primer.

These findings underscore the utility of SCoT markers as reliable tools for genetic analysis and hybrid purity determination, significantly contributing to the improvement of sunflower breeding programs. Looking ahead, new approaches and molecular techniques will be explored to identify genetic purity, including the use of additional SCoT primers as well as codominant SSR primers.

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