SSR Analysis of Nuclear DNA of Annual and Perennial Sunflower Species (Helianthus L.)

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Abstract: Genotyping of 29 species of genus Helianthus L., including 5 annual and 24 perennial species from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources has occurred, for this purpose were selected 52 SSR markers, with localization on all the 17 linkage groups of the sunflower genome. All the studied sunflower samples had unique SSR loci banding patterns. The mean PIC value varied was 0.72, which indicates the high resolution of this SSR based system for sunflower nuclear genome investigations. The discriminatory power of the marker system allowed us to classify all the sunflower species and provide the molecular barcoding. The UPGMA dendrogram, reflecting the genetic differences between 29 species of the genus Helianthus L., was constructed. Allele distribution data of the studied sunflower samples is a database that can be used to determine the levels of genetic variability, provide molecular barcoding and control the genetic integrity of collection sunflower samples.

Keywords: SSR Markers, Polymorphism, UPGMA, Sunflower

Introduction

The investigations of plant genomes variability is an up today issue with both fundamental and applied interest. The application of genomic data enables the determination of the genetic diversity and relationships of various plant species, as well as the development of barcoding systems for agricultural and collection samples. To study the variability of the sunflower genome, a number of different criteria were used: morphological (Schilling and Heiser, 1981), chemical (Spring and Schilling, 1989; 1990), isoenzyme (Carrera and Poverene, 1995; Cronn et al., 1997), RFLP (Gentzbittel et al., 1994; Berry et al., 1994; Schilling, 1997), DNA sequence data (Vischi et al., 2006; Timme et al., 2007), transcriptomes (Baute et al., 2015; Smith et al., 2018), as well as variations in the number of retrotransposons copies (Mascagni et al., 2015; 2018). However, even nowadays, for the identification of annual and perennial wild-growing sunflowers species, the Heizer's classification (Heiser et al., 1969), based on visual assessment of the morphological characteristics, is still predominantly used. Nevertheless, such classification has some disadvantages and limitations, for instance, the morphological characters are not always clearly expressed at different stages. Thus the application of DNA markers revealed new possibilities for studying genetic diversity and relationships at the intraspecific and generic levels (Knapp et al., 2001). The most effective and simplest molecular methods for assessing genetic polymorphism are PCR-based techniques. Among such techniques, the Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers are widely used, allowing rapid detection of the variability of a large number of genome loci (Sivolap and Solodenko, 1998; Sossey-Alaoui et al., 1998; 1999; Markin et al., 2016; Suresha et al., 2017; Yang et al., 2018; Uma et al., 2018). The spectra of DNA fragments, obtained as a result of their amplification, can be used as genetic markers for species identification and barcoding, as well as for determining taxonomic differences between species.

The aim of the current investigation is genotyping of annual and perennial sunflower species from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) based on the polymorphism of microsatellite markers.
Materials and Methods

The objects of the study were samples of 5 annual and 24 perennial species of sunflower from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (Table 1).

Using sunflower map from NCBI database (https://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=4232&query=Helianthus), we selected 52 SSR markers, with localization on all the 17 linkage groups (chromosomes) of the sunflower genome (Table 2).

**Table 1:** The list of sunflower samples from the VIR collection according to the Schilling and Heiser (1981) species classification

| I/n | Species | Chromosome number, 2n | Introduction number in the VIR collection | Section | Subsectio |
|-----|---------|----------------------|------------------------------------------|---------|-----------|
| Annual |         |                      |                                          |         |           |
| 1.  | *H. annuus* L. | 34 | 598276 | Helianthi | - |
| 2.  | *H. annuus* L. | 34 | 545522 |         |         |
| 3.  | *H. annuus* L. | 34 | 441098 |         |         |
| 4.  | *H. annuus* L. | 34 | 549513 |         |         |
| 5.  | *H. annuus* L. | 34 | 506067 |         |         |
| 6.  | *H. annuus* L. | 34 | 545563 |         |         |
| 7.  | *H. annuus* L. | 34 | 545616 |         |         |
| 8.  | *H. annuus* L. | 34 | 545736 |         |         |
| 9.  | *H. annuus* L. | 34 | 436863 |         |         |
| 10. | *H. annuus* L. | 34 | 545500 |         |         |
| 11. | *H. annuus* L. | 34 | 545598 |         |         |
| 12. | *H. annuus* breeding form VIR 119 | 34 | - |         |         |
| 13. | *H. debilis* Nutt. | 34 | 560395 | Ciliares | Ciliares |
| 14. | *H. praecox* Engelm. and A. Gray | 34 | 560400 |         |         |
| 15. | *H. agropophys* Torr. and A. Gray | 34 | 1000 | Corona-solis |         |
| 16. | *H. petiolaris* Nutt. | 34 | 503232 |         |         |
| Perennial |         |                      |                                          |         |           |
| 17. | *H. ciliaris* DC. | 68, 102 | - | Ciliares | Ciliares |
| 18. | *H. californicus* DC. | 102 | 530447 |         |         |
| 19. | *H. decapetalus* L. | 34, 68 | 440439 |         |         |
| 20. | *H. tracheliolius* Mill. | 34, 68 | - |         |         |
| 21. | *H. divaricatus* L. | 34 | 2099 |         |         |
| 22. | *H. eggertii* Small | 102 | - |         |         |
| 23. | *H. giganteus* L. | 34 | 489235 |         |         |
| 24. | *H. grosseserratus* M. Martens | 34 | 545698 |         |         |
| 25. | *H. hirsutus* Raf. | 68 | 560389 |         |         |
| 26. | *H. maximilianii* Schrad. | 34 | 2099 |         |         |
| 27. | *H. mollis* Lam. | 34 | 2102 |         |         |
| 28. | *H. nutallii* Torr. and A. Gray | 34 | - |         |         |
| 29. | *H. salicifolius* A. Dietr. | 34 | 440074 | Divaricati |         |
| 30. | *H. strumosus* L. | 68, 102 | 440679 |         |         |
| 31. | *H. tomentosus* Michx. | 102 | 2107 |         |         |
| 32. | *H. tuberosus* L. | 102 | 2111 |         |         |
| 33. | *H. laevigatus* Torr. and A. Gray | 68 | - | Microcephali |         |
| 34. | *H. microcephalus* Torr. and A. Gray | 34 | - |         |         |
| 35. | *H. smithii* Heiser | 68 | - |         |         |
| 36. | *H. occidentalis* Riddel subsp. plantagineus (Torr. and A.Gray) Heiser | 34 | 441062 | Atrorubente |         |
| 37. | *H. rigidos* Desf. | 102 | 545658 |         |         |
| 38. | *H. angustifolius* L. | 34 | 1889 |         |         |
| 39. | *H. floridanus* A. Gray ex Chapm. | 34 | - | Angustifolii |         |
| 40. | *H. simulans* E. Watson | 34 | 545659 |         |         |
Table 2: SSR markers used for genotyping of sunflower

| №  | SSR marker | Linkage group | Amplicon size, bp | PIC  |
|----|------------|---------------|------------------|------|
| 1  | ORS 610    | 1             | 144              | 0.59 |
| 2  | ORS 509    |               | 198              | 0.56 |
| 3  | ORS 552    | 2             | 200, 246, 500    | 0.68 |
| 4  | ORS 1194   | 2             | 180, 217, 280, 300, 380 | 0.82 |
| 5  | ORS 1045   |               | 155              | 0.83 |
| 6  | ORS 653    | 3             | 312, 500         | 0.97 |
| 7  | ORS 545    | 3             | 100, 180         | 0.61 |
| 8  | ORS 1021   |               | 280, 309         | 0.58 |
| 9  | ORS 488    |               | 179              | 0.53 |
| 10 | ORS 963    | 4             | 100, 300, 340, 600 | 0.93 |
| 11 | ORS 785    |               | 100, 161, 200    | 0.75 |
| 12 | ORS 1217   |               | 300, 431         | 0.68 |
| 13 | ORS 1024   | 5             | 224, 250         | 0.97 |
| 14 | ORS 1159   |               | 200, 255, 400    | 0.99 |
| 15 | ORS 1120   |               | 250, 300, 321, 400, 600 | 0.95 |
| 16 | ORS 650    | 6             | 100, 412         | 0.99 |
| 17 | ORS 381    |               | 100, 216, 550    | 0.96 |
| 18 | ORS 1256   |               | 150, 180, 210    | 0.68 |
| 19 | ORS 426    | 7             | 334              | 0.91 |
| 20 | ORS 966    |               | 372              | 0.31 |
| 21 | ORS 901    |               | 407              | 0.32 |
| 22 | ORS 1043   | 8             | 204              | 0.86 |
| 23 | ORS 243    |               | 170              | 0.94 |
| 24 | ORS 894    |               | 150, 252, 350    | 0.90 |
| 25 | ORS 1265   | 9             | 222, 250         | 0.63 |
| 26 | ORS 887    |               | 252              | 0.88 |
| 27 | ORS 1220   |               | 257              | 0.68 |
| 28 | ORS 878    | 10            | 203, 320         | 0.58 |
| 29 | ORS 437    |               | 342              | 0.59 |
| 30 | ORS 691    |               | 200, 447         | 0.66 |
| 31 | ORS 625    | 11            | 204, 300         | 0.61 |
| 32 | ORS 1214   |               | 369              | 0.35 |
| 33 | ORS 697    |               | 238, 450         | 0.66 |
| 34 | ORS 502    | 12            | 120              | 0.59 |
| 35 | ORS 946    |               | 191              | 0.37 |
| 36 | ORS 810    |               | 398              | 0.97 |
| 37 | ORS 707    | 13            | 100, 160         | 0.52 |
| 38 | ORS 1179   |               | 315              | 0.99 |
| 39 | ORS 799    |               | 143              | 0.76 |
| 40 | ORS 578    | 14            | 238, 300, 600, 800 | 0.98 |
| 41 | ORS 398    |               | 298              | 0.89 |
| 42 | ORS 1086   |               | 140              | 0.68 |
| 43 | ORS 151    | 15            | 180, 220, 454    | 0.73 |
| 44 | ORS 687    |               | 168              | 0.71 |
| 45 | ORS 857    |               | 212              | 0.68 |
| 46 | ORS 899    | 16            | 323              | 0.98 |
| 47 | ORS 656    |               | 196              | 0.80 |
| 48 | ORS 788    |               | 263              | 0.94 |
| 49 | ORS 996    |               | 150, 292, 700    | 0.79 |
| 50 | ORS 297    | 17            | 225              | 0.73 |
| 51 | ORS 727    |               | 192, 210, 250, 280, 300 | 0.66 |
| 52 | ORS 1097   |               | 130, 161         | 0.68 |

Genomic DNA was isolated from sunflower leaf tissue, with our modifications (Markin et al., 2016). PCR The PCR was carried out in 25 μL reaction mixture of the following composition: 67 mM Tris-HCl buffer, pH 8.8, 16 mM (NH₄)₂SO₄, 2.5 mM MgSO₄, 0.1 mM mercaptoethanol, 0.25 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 400 nM primers, 2.5 units of Taq polymerase and 15 ng of DNA template. Amplification was performed in the thermocycler Palm Cycler (Corbett Research, Australia). Thermal regime of the reaction was chosen individually for each pair of primers on the basis of their sequences. For majority of
reactions the optimal thermal regime was as follows: (1) denaturation at 94°C for 4 min, (2) 35 cycles at the following thermal and time regime: denaturation 94°C - 20 sec, annealing 58°C - 20 sec, elongation 72°C - 60 sec (3) final elongation at 72°C for 10 min. The primer sequences of the SSR markers are taken from the GenBank NCBI.

The amplicons were separated by electrophoresis in 3% agarose gel supplemented with ethidium bromide in Tris-Borate buffer. The obtained gels were analyzed with the Gel-Documenting System (GelDoc 2000, BioRad, United States). 100+ bp DNA Ladder (Evrogen, Russia) was used as a molecular weight marker. All the procedures were performed in 3-5 replicates.

For the estimation of SSR loci polymorphism, the Polymorphism Information Content (PIC) value was used. \[ \text{PIC} = 1 - \sum p_i^2 \], where \( p_i \) is the frequency of the \( i \)-th allele among the total number of alleles (Nei, 1973).

For determination of the genetic differences in sunflower samples as well as for dendrogram construction, the TREECON program (Van de Peer and De Wachter, 1993) was applied.

**Results and Discussion**

According to molecular genetic analysis of 40 sunflower samples, it was determined that all 52 selected SSR markers provided well reproducible and informative data. The electrophoresis analysis of amplicons revealed 1-5 bands for each SSR marker. The variability in amplicon size was from 100 bp to 800 bp. In total, 99 allelic variations across all studied SSR loci were defined (Table 2). While analyzing electrophoregrams the differences between samples were observed according to amplicon size, presence/absence of amplicon and multiply banding (multiple loci). As an example, Fig. 1 show the SSR profiles of annual and perennial species of sunflower using primers for one loci ORS 610.

![Fig. 1: SSR profiles of annual (A) and perennial (B) species of sunflower using primers for one loci ORS 610. The numbers indicated index number of species (Table 1). M – molecular weight standard (100+ bp DNA Ladder (Evrogen, Russia))](image)
Fig. 2: UPGMA dendrogram of 42 sunflower samples based on 52 SSR loci. The introduction number of the studied samples is indicated in parentheses. The numbers indicate bootstrap values (only values greater than 90% are included).

All the studied sunflower samples had unique SSR loci banding patterns. Thus the current approach indicated the effectiveness of SSR genotyping and made it possible to evaluate the genetic polymorphism of the samples. PIC values varied from 0.31 to 0.99 with the mean 0.72, which indicates the high resolution of this SSR based system for sunflower nuclear genome investigations. Moreover, the discriminatory power of the marker system allowed us to classify all the sunflower species and provide the molecular barcoding. Based on the data obtained, the UPGMA dendrogram, reflecting the genetic differences between 29 species of the genus Helianthus L., was constructed. In the presented dendrogram (Fig. 2), the sunflower species are combined into two main clusters with high statistical reliability - 98% and 91% bootstrap. The first cluster includes all plants of perennial species of Helianthus L. and the second - all samples of annual sunflower. In turn, the perennial species cluster is divided into two subclusters: The first one includes only two species (H. tuberosus and H. microcephalus), while the second one - all other perennial species, in which the most divergent are H. mollis, H. salicifolius (Fig. 2). Annual species form two subclusters (subclusters 3 and 4), one is presented by H. annuus species, including the VIR 119 line and another one combines all other samples of annual species: H. praecox, H. petiolaris, H. debilis and H. agrophyllus (Fig. 2). The obtained topology of the dendrogram of perennial species differs from the taxonomy data proposed by Schilling and Heiser (1981), which was constructed according to the analysis of morphological characters. There is no relationship between clusters and subsections identified by morphological characteristics. The reticulate speciation in the Helianthus L. genus and the high level of genetic variation can explain topological incongruence between current data and the classical ideas about the systematics of perennial sunflower species (Timme et al., 2007; Mascagni et al., 2017; 2018).
The high potential of SSR markers for investigations of plant genetic diversity was established in many studies (Ahmad et al., 2017; Wang et al., 2018; Parthiban et al., 2018). Current research of sunflower nuclear polymorphism based on analysis of 52 SSR loci, allowed genotyping 29 species of the genus Helianthus L. Allele distribution data of the studied sunflower samples is a database that can be used to determine the levels of genetic variability, provide molecular barcoding, and control the genetic integrity of collection sunflower samples. Also, the dendrogram displaying the genetic relationships between the studied sunflower species was provided. Thus, the studied SSR markers are informative for assessing the level of genetic diversity of the genus Helianthus L.

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Author’s Contributions

All the five authors equally participated in the laboratory study, data analysis and the entire process of the article preparation.

Ethics

This article is original and contains unpublished material. The authors declare that there is no conflict of interest regarding publication of this paper. The authors declare that no ethical issues are going to arise after the work has been published.

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