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FINDING AN INFORMATIVE MULTILOCUS POLYMORPHISM IN SUNFLOWER BROOMRAPE POPULATIONS

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Summary. Multilocus polymorphic DNA analysis was performed using 13 ISSR markers for 14 broomrape populations of Serbian and Moldovan origin. 279 ISSR fragments were identified for the whole sample, of which 265 (94.98%) were polymorphic. The seven groups of broomrape studied (whole sample, by country separately - Serbia and Moldova, and by race depending on the country - races ≤E serb, ≤E mold, H mold, H+ mold) showed different levels of genetic diversity. Based on these studies' results, broomrape's multilocus polymorphism was highly correlated with geographical origin, racial classification, and genetic background.

Keywords: broomrape, ISSR markers, DNA polymorphism, genetic diversity, races, genetic structure of populations.

Introduction

Global climate change has the most significant impact on many aspects of life on Earth, including ecosystems, which in exchange influence the evolution, distribution, and aggressiveness of various species, including parasites such as *Orobanche cumana* Wallr. (sunflower broomrape) [1]. For parasites, climate change may lead to the reorganisation of habitat, changes in the duration of the active period, and accelerated evolution and adaptation. Changes in temperature and precipitation patterns will lead to changes in the habitat boundaries for many species, including parasites. Many parasites may spread to new regions where they have not previously been found, which will be made possible by climate warming. Parasites may have longer periods of activity throughout the year as the climate warms. This can increase the number and frequency of infestations. Accelerated climate change may stimulate rapid adaptive changes in parasites, increasing their virulence and ability to infect new host species.

The rate of formation of new races of broomrape has now been greatly accelerated, not only due to climate change, but also by the import into sunflower-producing countries of seed material of foreign selection, susceptible to local broomrape, and by the intensification of crop cultivation with the reduction of scientifically based crop rotation to 1-3 years, and, as a consequence, the emergence of conjugate parasite-host evolution and the emergence of new races of the parasite capable of overcoming the immunity of resistant varieties and hybrids, adapting to various stress factors. Since the beginning of the new millennium, new races of the sunflower broomrape have appeared every 4-5 years, and the racial structure of their populations is a rapid leveling towards the dominance of the most virulent race. It has been found that different climatic zones and countries where sunflower is cultivated have their racial composition of broomrape, which differs in virulence to the varietal diversity of the host plant.

There are currently eight races of broomrape identified worldwide A, B, C, D, E, F, G, and H [2; 3]. Today, *O. cumana* is present in all the countries of southern Europe and in the areas around the Black Sea where sunflowers are grown, North Africa, Israel, and China [4]. The new highly virulent broomrape races (F, G, and H), which overcome the effect of the known sunflower resistance genes Or5, Or6, and Or7, have been identified and described since the mid-1990s in the most infected areas of Spain [5], Russia [6], Kazakhstan [7], Moldova [8], Romania [9], Turkey [10], Bulgaria [2; 11], Serbia [12], Ukraine [13], China [14] and others.

To address these challenges to agriculture, scientists and farmers have taken a variety of measures: develop crop varieties that are resistant to broomrape; use agricultural technologies and biological control; apply chemical pesticides; implement educational programs for farmers and agricultural workers; involve monitoring and forecasting to track habitats and population dynamics to develop preventive measures. These measures help to minimise the damage caused by broomrape to the economy and food security, and to adapt to changing climatic conditions, ensuring that agriculture is resilient to global climate change.

To develop adaptive strategies to meet the economic needs of society, an in-depth knowledge of the constantly changing state of broomrape gene pools, as assessed by the levels of intra- and interpopulation gene diversity, is required. Quantitative assessments of population genetic parameters using molecular genetic markers are needed to monitor the gene pools according to the current state of scientific development.

Our work aimed to analyse the genetic variability of two broomrape gene pools of Serbian and Moldavian origin at different taxonomic levels. Based on the results obtained in this work and by summarising previously obtained results, the level of genetic diversity as a function of geographical origin and racial background was determined, which is an integral part of population genetic monitoring of broomrape gene pools using molecular genetic markers.

Materials and methods

Altogether, 128 accessions from 14 Serbian and Moldavian broomrape populations, belonging to races ≤E, H, and H+, were selected as objects for comparative analysis of the genetic diversity and structure of the species (Table 1). Individual broomrape plants were collected and stored at -80°C until DNA extraction.

Table 1

List of the broomrape populations used in this study and their origin

Population	Code	Region	Race	Population	Code	Region	Race
SERBIA				**REPUBLIC OF MOLDOVA**			
ORSR 04	S1	Novi Sad	≤E	Căzănești	**M1**	Telenești	≤E
ORSR 07	S2			Congaz	**M2**	Comrat	
ORSR 11	S3			Grigorievca	**M3**	Căușeni	H
ORSR 14	S4			Prepelița	**M4**	Sîngerei	
ORSR 25	S5			Izbiște	**M5**	Criuleni	H+
ORSR 26	S6			Alexanderfield	**M6**	Cahul	
ORSR 43	S7			Svetlîi	**M7**	Comrat	

Total genomic DNA was isolated and purified from frozen material using the GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's instructions (Thermo Fisher Scientific, USA).

The concentration and quality of the obtained DNA were determined using a spectrophotometer (T60 UV-VIS, PG Instruments Limited, England) and assessed on a 1% agarose gel in 1xTAE buffer at 2.5 V/cm [15]. 13 inter-satellite ISSR-PCR markers were used for allele identification and genetic differentiation. Amplification was performed on a thermocycler Genset 9700 (Applied Biosystems) according to the standard program for the ISSR-PCR method: denaturation 95.0 °C for 5 min; 35 cycles in the following repetition: 95.0 °C for 30 sec, 45.0 °C for 45 sec, 72.0 °C for 2 min; then a final elongation cycle at 72.0 °C for 5 min.

Amplified DNA fragments were detected by electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 μg/ml) in 1xTAE buffer, visualised and documented under UV radiation (wavelength λ=305 nm) using a UV transilluminator with Gel documentation system Doc-Print VX2 (SXT-F20.M, Vilber Lourmat, France). The size of the PCR fragments was determined

using the GeneRuler Express DNA Ladder (ready-to-use, product #SM1553) as a standard marker (Thermo Fisher Scientific, USA).

A binary data matrix based on the presence (1) or absence (0) of the amplicons for each primer was generated by analysing the clear electrophoretic spectra in Photo-Capt V.15.02. The matrix was processed using POPGENE V.1.32 and Excel 2010 software to obtain descriptive statistical parameters (observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), total genetic diversity (Ht), Shannon's Information index (I), gene diversity among populations (Dst), coefficient of genetic differentiation (Gst) and gene flow among populations (Nm), total number of ISSR loci, number of loci with frequency ≥5%, number of rare loci with frequency ≤5%).

Results and discussions

PCR with broomrape genomic DNA individually analysed each of the 14 ISSR primers. As a result, 13 effective ISSR primers were selected for this species, identifying the largest number of clearly amplified and stably reproduced DNA fragments. Of these, 1 primer was tetranucleotide ($(GACA)_4$), 3 primers were trinucleotide ($(CAA)_5$, $(CTC)_4RC$, $(CAG)_5$) and 9 primers were dinucleotide (BC807, BC810, BC835, BC841, BC857, $(CT)_8TC$, $(CA)_6AC$, $(AG)_8YA$, $(CA)_6RG$). It should be noted that of all the primers used, 1 trinucleotide and 8 dinucleotide primers contained single and double anchor nucleotides.

In total, 279 ISSR-PCR markers were detected in 14 *O. cumana* populations, of which 265 were polymorphic (94.98%) and 14 were specific (5.02%). On average, one ISSR primer initiated the synthesis of 21.46 DNA fragments in broomrape (Fig. 1). The proportion of polymorphic loci was higher in Moldavian populations (70.61%), Serbian race ≤E (65.59%) and Moldavian race H+ (55.2%), and the rest of the studied groups had polymorphic loci less than 50%.

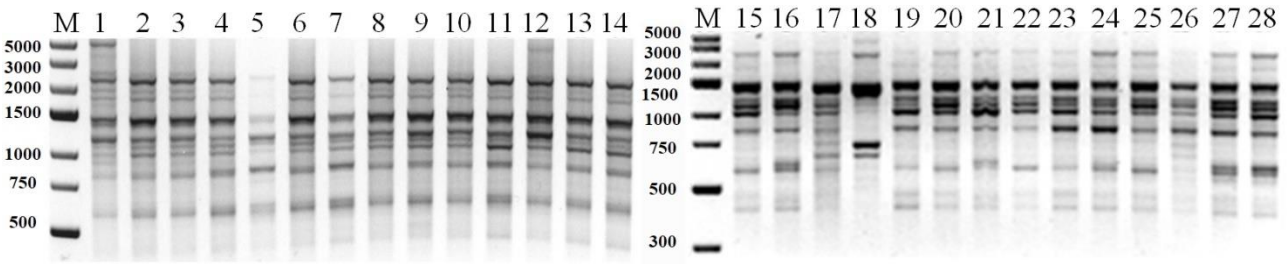


Figure 1. ISSR fingerprints of 28 samples from Serbian and Moldavian broomrape populations using primer BC810: M – molecular weight marker GeneRuler Express DNA Ladder (ready-to-use, product #SM1553), 1-2 – accessions from S4 population, 3-9 – accessions from S5, 10-14 – accessions from S6, 15-28 – accessions from M3.

The observed number of alleles (Na) for the whole species was 2, and was the highest in the Moldavian populations (1.71), among the races the race ≤E serb (1.66) stood out for this indicator (Fig. 2). The effective number of alleles (Ne) was highest in the Serbian populations and race ≤E serb (1.39). Nei's gene diversity (H) and Shannon's Information index (I) were also highest for the Serbian populations and the race ≤E serb (0.23 and 0.34, respectively).

The most common measure of genetic variation in a population is heterozygosity. The total gene diversity (Ht) for the whole species was 0.31, the expected proportion of heterozygous genotypes in a separate population for all loci (Hs) was 0.21, and the gene diversity among populations (Dst) was 0.10 (Fig. 2). These indicators were the highest in Serbian populations (Ht=0.23, Hs=0.15) compared to Moldavian populations (Ht =0.20, Hs=0.10), which cannot be said for the gene diversity among populations (Dst=0.08, Dst=0.11, respectively). Among the racial groups, the highest values were found in the race ≤E serb (Ht=0.23, Hs=0.15) and the lowest in the race ≤E mold (Ht=0.14, Hs=0.10). Average genetic diversity values were obtained for the races H

mold (Ht=0.158, Hs=0.109) and H+ mold (Ht=0.17, Hs=0.08). Concerning the high values of gene diversity among populations, the races H+ mold (Dst=0.09) and ≤E serb (Dst=0.08) stood out.

The coefficient of gene differentiation among populations (Gst) of the species shows that the interpopulation component accounts for 31.24% of the total genetic diversity based on this gene flow among populations was higher than 1 (Nm=1.10) (Fig. 2). The highest differentiation was found among the Moldavian populations (52.75%), which is explained by the low value of gene flow among populations (Nm=0.45), in the Serbian populations it is slightly underestimated to 36.16% and gene flow is higher (Nm=0.88). Among the races, the H+ mold race stands out with the highest percentage of differentiation (51.23%).

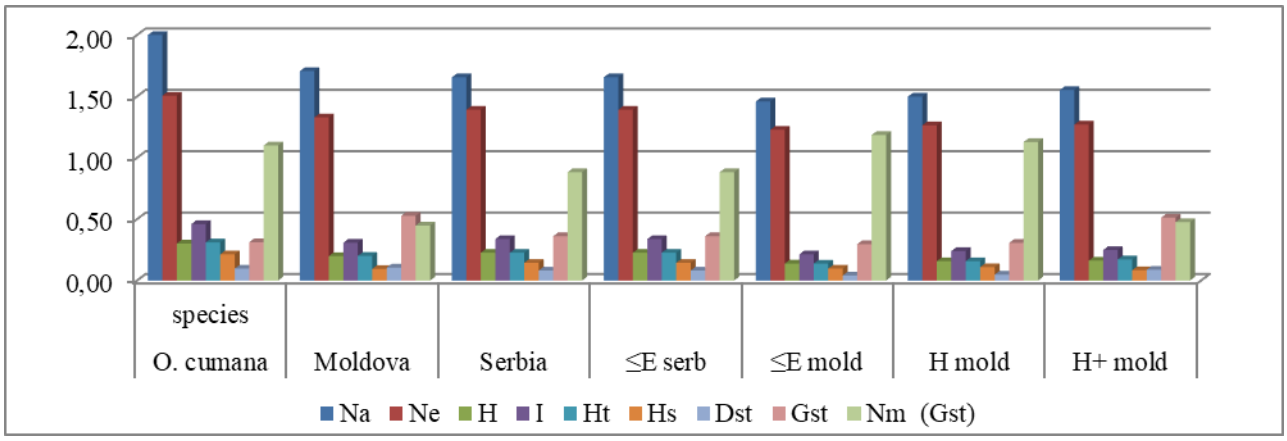


Figure 2. Genetic diversity and differentiation parameters generated in the different origin broomrape: Na – observed number of alleles, Ne – effective number of alleles, H – Nei's gene diversity, I – Shannon's Information index, Ht – total gene diversity, Hs – gene diversity within populations, Dst – gene diversity among populations, Gst=(Ht-Hs)/Ht – coefficient of gene differentiation among populations, Nm – gene flow among populations from Gst.

The analysis of genetic variability in different populations of broomrape showed various levels of multilocus polymorphism, which was closely correlated with the geographical origin and racial classification (Fig. 2). Based on the (Ne, H, I, Ht and Hs) values of total and within population gene diversity, populations from Serbia showed the highest genetic heterogeneity, not for Na values, Moldavian populations had more high values. On the other hand, high values of gene diversity (Dst) and coefficient of gene differentiation among populations (Gst) were found in Moldavian populations. According to these results, it can be assumed that high values of gene diversity (Dst) and gene differentiation (Gst) among Moldavian broomrape populations are associated with an unstable racial structure (presence of races E, and dominant races G, H, H+) [16]. In contrast to the Moldavian broomrape, the populations of *O. cumana* in Serbia were found to be stable, with no significant changes in racial composition. For more than 20 years, race E has been dominant in the broomrape-infected area in Serbia [17], and the more virulent race F has only been detected locally in the South Backa district [12]. According to Miladinović et al. [17], a chronological survey of the occurrence of *O. cumana* on sunflowers in Serbia supports the opinion of a slow evolution of the physiological races of this parasite. On the other hand, the following RAPD technique in the analysis of genetic diversity in Serbian broomrape populations has recently shown that samples collected from different agricultural regions belong to the same population with high genetic heterogeneity [18]. In addition, gene flow values (Nm=0.88) in Serbian populations indicate that there is little differentiation among populations, presumably due to self-pollination (Fig. 2). Self-pollinated species maintain as much diversity at their polymorphic loci as cross-pollinated species [19].

In plant population genetics, loci with different allele frequencies are key to understanding genetic diversity, evolution, and species adaptation. For the whole broomrape species, 81 (29.03%)

rare and 198 (70.97%) frequent ISSR-PCR markers were identified with insignificant total gene diversity (Ht=0.31) (Fig. 3). The largest number of rare alleles (65-32.99%) was found in Moldavian populations and a smaller number was identified in Serbian populations (32-17.02%). In contrast, a larger number of frequent loci and higher total gene diversity were found in the Serbian population (156-82.98%, Ht=0.23, respectively) than in the Moldavian population (132-67.01%, Ht=0.20, respectively). Among the races studied, the H mold (68-47.55%) and H+ mold (56-35.44%) races had the largest number of rare alleles, and the smallest number was found in the ≤E serb race (32-17.02%). Based on the largest number of frequent loci, the races ≤E serb (156-82.98%) were identified with the highest total gene diversity Ht=0.23 and ≤E mold (88-68.22%) with the lowest total gene diversity Ht=0.14.

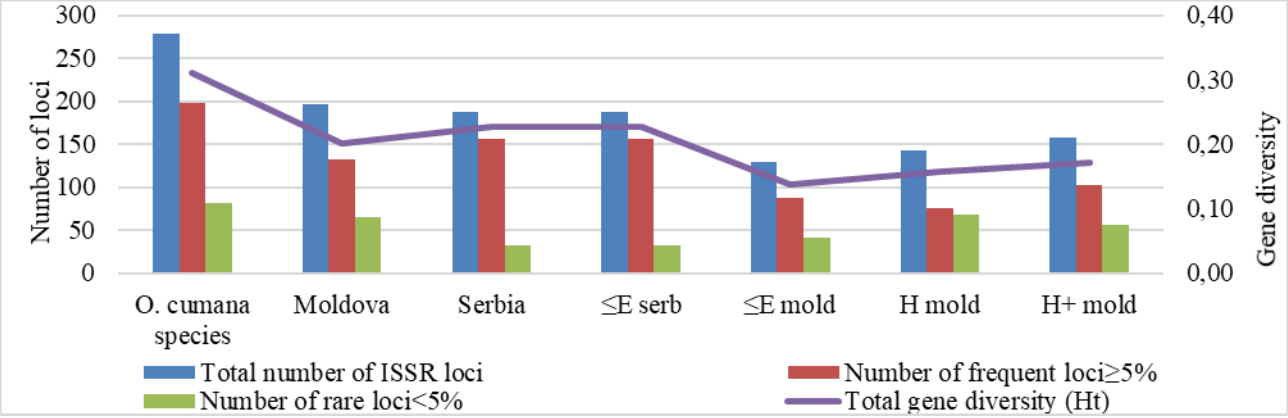


Figure 3. The ISSR markers detected, and gene diversity in the broomrape of different origins

The presence in taxonomic groups of plants of rare loci (with a frequency of less than 5%) may indicate recent mutations or specific adaptation to certain conditions. It may be neutral or even slightly harmful, but not to the extent that they are eliminated from the population. The appearance of a high number of rare loci indicates the presence of significant genetic variation and uneven branching of evolutionary processes, which may be associated with recent mutations, genetic drift, isolation of populations, or strong directional selective pressure.

The frequent loci (with a frequency of ≥5% in a population) may be either neutral or active, conferring some advantage under the conditions of a given population, indicating significant genetic diversity within a given locus. Frequently occurring alleles may be associated with important biological functions or adaptations affecting productivity, disease resistance, or stress response. According to the data results, the presence of a larger number of frequently occurring loci in the Serbian populations and race ≤E serb (156-82.98%) compared to the Moldovan populations (132-67.01%) and races indicates significant genetic diversity and adaptation to changing conditions, which is also confirmed by higher Ht values for Serbian than for Moldovan populations (0.23 and 0.20, respectively) (Fig. 3).

Conclusions

As a result of our research, it was revealed that the 14 studied populations of *O. cumana* of Serbian and Moldavian origin are characterised by different levels of multilocus polymorphism, which are highly correlated with the geographical origin, racial classification, and genetic background. Based on the obtained values of gene diversity (Ne, H, I, Ht, and Hs), it was found that the Serbian populations of broomrape have the greatest genetic heterogeneity within populations, and the Moldovan populations showed high values of gene diversity and differentiation between populations (Ds, Gst). Also, the highest number of rare alleles (65-32.99%) was detected in the Moldavian populations and the lowest in the Serbian populations (32-17.02%). This can be explained by the difference in the racial structure of Moldavian populations and the stability of

Serbian populations with a recently identified high level of intra-population genetic heterogeneity due to RAPD analysis by Ivanovic and colleagues [18].

In conclusion, it can be noted, that the high level of genetic diversity possessed by the parasite is an important contributor to its ecological plasticity, accelerating rapid adaptation to dynamically changing environmental conditions. Greater genetic diversity in broomrape populations indicates their greater microevolutionary potential.

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