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ISSR fingerprinting of genetic diversity within and among *Orobanche cumana Wallr.* populations from different countries

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Abstract. Molecular genetic diversity was analysed for 23 *Orobanche cumana* populations from four countries using 13 ISSR markers. Descriptive population genetic statistics and AMOVA analysis revealed a marked degree of intrapopulation differentiation. PCA and UPGMA clustering showed a clear division into two main regional groups, i.e. the first group was more closely related to the Middle East and the second group belonged to Eastern Europe. The results of this research would suggest that the Moldavian, Bulgarian, and Romanian populations, which have many molecular markers in common, may share many genetic traits that indicate their monophyletic origin.
Keywords: *O. cumana*, markers, genetic diversity, populations, differentiation.

Amprentarea ISSR a diversităţii genetice în cadrul şi între populaţiile de *Orobanche cumana Wallr.* din diferite țări

Rezumat. Diversitatea genetică moleculară a fost analizată la 23 de populații de *Orobanche cumana* din patru țări folosind 13 markeri ISSR. Statistica genetică descriptivă și analiza AMOVA a datelor obținute au relevat un grad ridicat de diferențiere intrapopulațională. Gruparea PCA și UPGMA a evidențiat o divizare clară în două grupuri regionale principale, respectiv primul grup a fost mai strâns legat genetic de Orientul Mijlociu, iar al doilea grup a aparținut Europei de Est. Rezultatele acestor cercetări ar sugera că populațiile din Moldova, Bulgaria și România cu un număr mare de markeri moleculari în comun, pot împărtăși multe trăsături genetice care ar indica originea lor monofiletică.
Cuvinte-cheie: *O. cumana*, markeri, diversitate genetică, populații, diferențierea.

1. Introduction

Broomrape (*Orobanche cumana Wallr.)* sunflower infestation is a significant reason for its yield decrease and gross seed harvests in many regions worldwide [1]. On average, sunflower seed losses due to broomrape can be more than 50% when susceptible hybrids are grown and as high as 100% in heavily infested fields [2]. The degree of negative effects of broomrape parasitism depends critically on the host resistance, the parasite's aggressiveness, the sunflower's developmental stage at the time of infection, the level of

contamination, and environmental factors [3]. Intensive sunflower cultivation in violation of crop rotation, import of foreign breeding seeds with susceptibility to the local broomrape, rapid parasite evolution, climate changes favorable for broomrape expansion, and other causes have led to the emergence of sudden genetic changes in broomrape populations and the active spread of new highly virulent races of the parasite in almost all sunflower producing countries [4]. A species' genetic relatedness and population structure are key determinants of its natural distribution, environmental adaptability, survival, and evolutionary potential under changing conditions [5]. The genetic structure of a species is formed by the influences of both internal (size, density, distribution of the species' population, gene flow, genetic drift, natural selection, and plant mating system) and external factors (changes in the boundaries of the species' distribution) determined by past and present evolutionary processes. Changes in climate and habitat often accelerate the expansion or contraction of a species' range. Studies of the genetic structure and diversity of the parasitic species *O. cumana* are important for understanding the underlying genomic, evolutionary, and demographic processes occurring in populations, which is of great importance for the development of effective control strategies of the pathogen and breeding programs for resistance to broomrape in sunflower.

This study aimed to investigate the genetic diversity and differentiation of 23 Black Sea broomrape populations of different origins using ISSR molecular markers.

2. Materials and Methods

269 plants of 23 sunflower broomrape populations from different regions of Bulgaria (notation keys B1-B4), Turkey (T1-T5), Republic of Moldova (RM1-RM13) and Romania (R1), belonging to 3 virulent races (race E- populations RM12, RM13; race G -B1, B2, T5, RM10, RM11; and race H- B3, B4, T1-T4, RM1-RM9, R1), were used in this study.

Total genomic DNA was isolated from frozen stem material, using the Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit #K0791 (Thermo Fisher Scientific, USA). The quantitative and qualitative content of isolated DNA was assessed using a spectrophotometer (T60 UV-VIS, PG Instruments Limited, England), and verified by 1% agarose gel electrophoresis [6]. Thirteen ISSR primers, representing di-, tri-, and tetra- repeats that produced clear and reproducible bands, previously reported by Benharrat [7], were selected to investigate genetic diversity. DNA amplification was carried out on a Genset 9700 thermocycler (Applied Biosystems, USA), using the following parameters: initial denaturation - 5 min at 95°C (1 cycle); denaturation - 30 s at 95°C, annealing - 45 s at 45°C, extension - 2 min at 72°C (35 cycles); and final extension - 5 min at 72°C (1 cycle). PCR products were analysed by electrophoresis on a 2% agarose

gel containing 0.5 μg/ml ethidium bromide in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0; 1 mM EDTA) and visualised under UV light (wavelength λ=305 nm). The ready-to-use GeneRuler Express DNA Ladder, SM1553, (Thermo Fisher Scientific, USA) was used as the standard molecular weight marker. Photographs were taken, using the Doc-Print VX2 gel documentation system, model SXT-F20.M (Vilber Lourmt, France).

The binary data matrix of amplification profiles for all samples, obtained by the Photo-Capt V.15.02 program, was used to calculate descriptive population genetic statistics parameters, total genetic diversity, genetic structure, and genetic relationships of the broomrape populations, using the POPGENE V.1.32, GenAlex 6.503, and XLSTAT V.2016.02.28451 software packages.

3. Results and Discussions

13 ISSR primers were used to assess the genetic diversity of a group of *O. cumana* populations from several countries in the Black Sea basin. Of the 23 populations sampled, population RM11 had the highest observed number of alleles (Na = 1.53), but population B2 had the lowest (Na = 1.17), the maximum value of the effective number of alleles was recorded in population T2 (Ne = 1.30) and the minimum in population B2 (Ne = 1.08) (Fig. 1). The Nei's gene diversity index (H) for all populations was estimated to be between 0.17 (RM11 and T2) and 0.05 (B2). Shannon's information index (I) has varied between 0.26 (RM11) and 0.08 (B2). The number of polymorphic loci (NPL) and the percentage of polymorphic loci (PPL) ranged from 175 (RM11) to 55 (B2) and 52.87 (RM11) to 16.62 (B2), respectively.

The results, obtained on the average value of the indices of genetic diversity among 23 studied broomrape populations, showed that the highest observed number of alleles (Na = 1.37) and the effective number of alleles (Ne = 1.24), the highest value of Nei's gene diversity (H = 0.14) and Shannon's Information index (I = 0.21), as well as the largest number of polymorphic loci (NPL = 123) and the highest percentage of polymorphic loci (PPL = 37.16) were found for populations from Turkey, followed by Moldova and Romania (Fig. 1). The lowest mean values were observed for populations from Bulgaria (1.19, 1.09, 0.06, 0.09, 62.75 and 18.96, respectively). Moreover, it was revealed that the range of variation of Na, Ne, H, I, NPL, and PPL values was much wider for Moldavian populations (1.21-1.53, 1.11-1.28, 0.07-0.17, 0.10-0.26, 69-175, 20.85-52.87, respectively) than for the Turkish ones (1.29-1.43, 1.15-1.29, 0.09-0.17, 0.14-0.25, 95-143, 28.70-43.20, respectively) (Fig. 1). The Bulgarian population, on the other hand, had a relatively

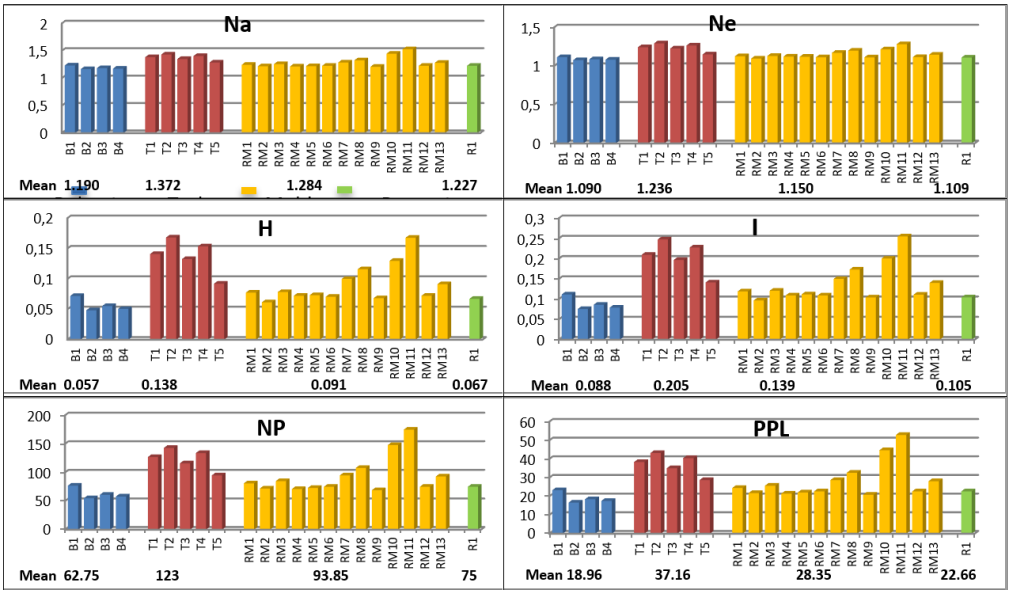

Figure 1. Genetic diversity indices for each separate population *O. cumana* revealed by ISSR markers: *Na – observed number of alleles, Ne – effective number of alleles, H – Nei's gene diversity, I – Shannon's Information index, NPL – number of polymorphic loci, PPL – percentage of polymorphic loci.*

small range of variation (1.17-1.23, 1.08-1.12, 0.05-0.07, 0.08-0.11, 55-77, 16.62-23.26, respectively) compared to all the populations studied.

At each polymorphic locus, the total allelic diversity is known to be represented by the expression Ht = Hs + Dst, where Hs is the mean within-population allelic diversity and Dst is the among-population allelic diversity. These quantities are, in turn, related to the fraction of total allelic diversity found between populations (Gst) by the expression Dst/Ht [8]. According to the analysis of the population genetic structure for all loci of *O. cumana* species, the genetic diversity at the species level (Na = 2.00, Ne = 1.38, H = 0.23, I = 0.37, Ht = 0.24, Hs = 0.18, NPL = 331, PPL = 100%) was higher compared to the genetic diversity at the population level (Fig. 1, Table 1). However, when the expected proportion of heterozygous genotypes per total sample (Ht = 0.24) was compared with the expected proportion of heterozygous genotypes within populations for all loci (Hs = 0.18), the low population heterozygosity among populations (Dst = 0.07) was noted, suggesting that the genetic variation of the broomrape studied is mainly within populations (Table 1). Based on the analysis of genetic diversity parameters, it can be concluded that Turkish broomrape exhibits the highest genetic diversity among the broomrape populations compared. It is

also clear that Turkish broomrape is more distantly related to broomrape from European countries. However, Moldavian populations showed a wider range of variation in genetic diversity indices. Thus, the frequency and abundance of common alleles in the broomrape gene pool in the Black Sea basin have changed, and there is a high probability that new virulent genes will emerge over time.

Table 1. Genetic diversity parameters and differentiation of *O. cumana* for all loci.

	Sample size	Na	Ne	H	I	Ht	Hs	Dst	Gst	Nm (Gst)	NPL	PPL
Mean	269	2.00	1.38	0.23	0.37	0.24	0.18	0.07	0.28	1.30	331	100
SD	-	0.00	0.32	0.17	0.22	0.03	0.02	-	-	-	-	-

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, NPL – number of polymorphic loci, PPL – percentage of polymorphic loci, SD – standard deviation

Table 2. Distribution of genetic diversity in *O. cumana* populations by AMOVA.

Variation source	DF	SS	MS	Est. Var.	Phi-statistics	Variance percentage, %
AC	3	2461.45	820.48	12.69	PhiRT=0.28	28
AP	19	3760.41	197.92	15.44	PhiPR=0.47	34
WP	246	4209.91	17.11	17.11	PhiPT=0.62	38
TOTAL	268	10431.8	-	45.25	-	100

AC – among countries, AP – among populations, WP – within populations, DF – degree of freedom, SS – sum of squares, MS – mean squares, Est. Var. – estimate of variance component, PhiRT – among region variation, PhiPR – among population variation within region, PhiPT – total variation within all populations.

The Gst is a measure of population differentiation and its values range from zero to one. Genetic differentiation among populations of *O. cumana* species was high (Gst = 0.28) (Table 1), meaning that approximately 72% of the total genetic variation occurred

within populations, while only 28% of the genetic variation was among populations, following the classification of Gst degree established by Buso [8]. The transfer of genetic material from one population to another can significantly influence the dynamics of gene frequencies within an entire species. This process can also promote local adaptation and co-evolution between parasites and their hosts by introducing new alleles or beneficial mutations to populations with limited genetic diversity. Populations will differentiate locally when Nm is less than 1, and there will be little differentiation among populations when Nm is greater than 1 [9]. At the species level, the gene flow value among the broomrape populations exceeded 1 (Nm=1.30) (Table 1). This indicates that there has been moderate gene flow among populations with little differentiation [10].

A dendrogram of the 23 abovementioned populations was constructed, based on Euclidean genetic distances and Pearson's dissimilarity, using UPGMA cluster analysis. The populations studied were partitioned into three main groups in both methods (Fig. 2). However, the clustering patterns generated, using Euclidean genetic distances and Pearson's dissimilarity, were unidentical and contained a slightly different composition of the groups. Based on Euclidean clustering, group I comprised the populations of Bulgaria, Moldova, and Romania. Meanwhile, the Turkish populations T1-T4 formed group II, while the T5 population fell into a separate third group. According to the Pearson clustering approach, the genetic variation among the Bulgarian (B1- B4) and Turkish (T1-T5) broomrape populations placed these populations in the first and second separate groups, respectively. The third group contained thirteen populations from Moldova (RM1-RM13) and one Romanian population (R1). These results suggest that the Turkish populations are genetically distant from the European broomrape populations according to these clustering patterns. The same cannot be said for the Bulgarian populations, which both resembled and differed from the Moldavian and Romanian broomrape populations, forming a separate group in one case and a combined group in the other. The Romanian and Moldavian populations were the most diverse of all the populations, showing many similarities and invariably forming a separate group.

The PCA analysis supported population clustering of different origin broomrape, derived from the dendrograms (Fig. 2, 3). The proportions of variation of the first and second components were 32.97 and 15.53%, respectively, of the total variability of the molecular data in 48.50%.

The results of PCA showed that the Middle East broomrape collection from Turkey was far from the other 18 Eastern European populations from Bulgaria, Moldova, and Romania and formed a separate group with two subgroups (T5 and T1-T4). Moreover, the PCA presented that both groups were distributed along two opposite coordinate

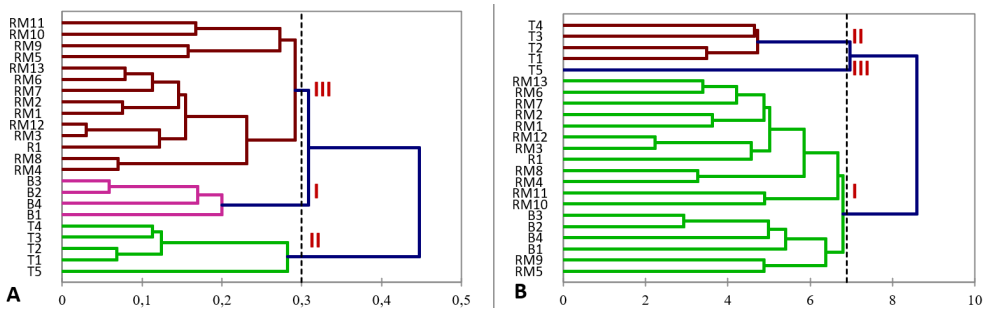

Figure 2. The relationships between 23 sunflower broomrape populations based on ISSR data using UPGMA methods with Pearson's dissimilarity (A) and Euclidean genetic distances (B).

axes. Such a distribution mainly reflects the geographical location of populations. The genetically closest populations (Moldova, Romania, and Bulgaria) were combined, while those farthest apart resulted in separate groups (the first group – Turkey, the second group – Moldova, Bulgaria, and Romania).

In the present study, the different origin broomrape populations from the Black Sea basin were selected to acquire a deeper insight into their population structure and genetic variability at the species level. As the emergence of more aggressive broomrape populations (races), which can rapidly spread to new areas, has been observed in the last 20 years in the sunflower-producing countries covered in this article (Moldova, Turkey, Romania, and Bulgaria), many breeders and geneticists are interested in the operational detection of the different interactive genetic processes (mutation, genetic drift, gene flow, etc.) within species for constant monitoring of broomrape resistance [11, 12].

Genetic analysis, using ISSR markers in different broomrape populations, showed the presence of very high genetic diversity, especially within populations, whereas little differentiation was observed among populations. The level of ISSR diversity was quite high among 269 accessions studied according to the genetic diversity indices (Na = 2.00, Ne =1.38, H = 0.23, I = 0.37) (Table 1). The gene diversity values among and within broomrape populations were the indicators of total genetic polymorphism in the species. It was found that the gene diversity within all populations (Hs = 0.18) was significantly higher than the gene diversity among populations (Dst = 0.07) to the total gene diversity (Ht = 0.24), signifying the low interpopulation heterozygosity and suggesting that the genetic variation of the studied broomrape was mainly within populations. The Gst value of 0.28 confirmed that 72% of the genetic variation was within populations. Moreover, the moderate gene flow value (Nm = 1.30) also confirmed that a significant degree of gene

exchange between different populations was one of the reasons for little differentiation among populations [10]. The AMOVA test revealed the same pattern, showing high genetic differences within populations (38%), a low level among the populations (34%), and a lower level among countries (28%) (Table 2). Based on these results, it is possible to conclude that a main genetic pool exists in the Black Sea Basin, comprising populations originating from Bulgaria, Turkey, Moldova, and Romania. This study demonstrated the existence of regular gene flow among populations of the *O. cumana* gene pool distributed in the Black Sea basin, which could be attributed to the frequency of cross-pollination and self-pollination within a species. The recent study confirms the occurrence of a relatively high rate of cross-fertilization in *O. cumana* plants, ranging from 14.8% to 40.0% [13], which may be a major creative force of the race evolution in O. cumana, with reassortment of avirulence genes conferring specificity against resistance genes, as also proposed by Joel [14]. The opposite results of low intrapopulation and high interpopulation genetic variation in *O. cumana* from several countries (Bulgaria, Turkey, Romania, and Spain) using RAPD markers were reported by Gagne [15]. Pineda-Martos [16] also demonstrated extremely low intra- and interpopulation genetic variation in two main gene pools of *O. cumana* in Spain, using SSR markers, probably due to a founder effect. However, in another study, RAPD analysis revealed high intrapopulation diversity in Serbian O. cumana, which can be explained by the fact that broomrape plants collected from different agricultural regions in Serbia, belong to the same population with high genetic heterogeneity [17]. Similar results about a rather high proportion of the intrapopulation genetic diversity were obtained from the genetic variability studies in populations from Russia, Kazakhstan, Romania, Tunisia, and Turkey, using SSR markers [18, 19, 20]. Two different methods of multivariate analysis, PCA and cluster analysis were used to group the broomrape accessions in this study. The resulting UPGMA-based dendrograms divided 23 populations by region into two main groups, with some subgroups depending on which distance methods were used (Fig. 2, 3). Group I was closely related to the Middle East, while Group II, consisting of Moldavian, Bulgarian, and Romanian population collections, belonged to Eastern Europe. The second group of populations shared some of the same genetic characteristics, which could indicate their monophyletic origin. The clustering results of both UPGMA and PCA analyses support this conclusion (Fig. 2, 3). The same results were obtained in another of our studies on the genetic relationships between broomrape of different origins, based on the frequency distribution of alleles at the country level using ISSR markers. The genome of Turkish broomrape was shown to have a specific microsatellite allele distribution that differed from that of Bulgarian, Moldavian, and Romanian broomrape [21].

4. Conclusions

In conclusion, our results demonstrated the effectiveness of ISSR marker systems in elucidating species population structure and genetic variability. The investigation revealed that the different broomrape populations have a significant level of genetic diversity, especially within populations, whereas little differentiation was observed among populations. We suggest that there is a main gene pool of *O. cumana* in the Black Sea basin, comprising populations from Bulgaria, Turkey, Moldova, and Romania, supported by regular gene flow among populations. Based on the results of UPGMA and PCA analyses, Moldavian, Bulgarian, and Romanian broomrape populations were found to have more genetic similarities with each other than with Turkish populations within the main gene pool of *O. cumana* in the Black Sea basin. The groupings of broomrape were mainly influenced by its geographical origin, as well as genetic differences and similarities that have accumulated over time. Clustering and PCA analyses confirmed that these patterns were not associated with virulence.

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