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Genetic diversity of broomrape (*Orobanche cumana* Wallr.) populations from different geographical origins assessed by ISSR markers

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Abstract: The present study was focused on the assessment of genetic diversity in twenty-three populations of *Orobanche cumana* parasitizing on sunflower in Bulgaria, Turkey, Moldova and Romania using 13 ISSR markers. The obtained results on the genetic diversity parameters showed that the broomrape populations were characterised by a significant level of the intrapopulation diversity. In addition, descriptive population genetic statistics revealed that Turkish populations had a higher level of genetic diversity indices than populations from several areas of the northeast and east of the Balkan Peninsula included in Eastern Europe. The analysis of molecular variance showed that 38 % of the genetic variability was due to differences within populations, 34 % was due to differences among populations and the lowest molecular variation was among countries (28 %). According to clustering and PCA methods, Moldavian, Bulgarian and Romanian broomrapes shared more genetic traits with each other than with Turkish populations within a main gene pool. As a whole, all results of this study showed that there is a high intrapopulation diversity of the *O. cumana* gene pool in the Black Sea basin. From the clustering and PCA analyses, it can be concluded that the grouping of broomrape populations is partly determined by their geographical origin, as well as by the genetic differences and similarities accumulated over time, and is not related to virulence. The information obtained from this study may be highly relevant in contributing to the development of sustainable control strategies of the pathogen and breeding programmes for sunflower resistance to broomrape.

Keywords: genetic diversity; ISSR markers; *Orobanche cumana*; populations; origin; sunflower

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1 Introduction

The widespread intensification of sunflower cultivation as a highly profitable crop in recent decades has led to the emergence and rapid spread of highly virulent broomrape biotypes in many countries, which have overcome the immunity of resistant cultivars and hybrids.

The comprehensive study of *Orobanche cumana* evolution and population genetic structure in order to understand the mechanism of its development in different countries, and as a result – the development of effective sunflower breeding programmes for broomrape resistance, as well as durable pathogen control strategies, it is an important task in improving sunflower yield and oil quality (Calderón-González 2021; Cvejić et al. 2020; Duca et al. 2019; Fernández-Martínez et al. 2008; Kaya and Evci 2009; Wright 1978). Therefore, the issues related to the study of the racial structure of the parasite populations and the characterisation of new virulent biotypes have now become extremely relevant, for the solution of which the modern and quite effective molecular methods are increasingly used (Benharrat et al. 2002; Calderón-González et al. 2019; Ciuca et al. 2004; Duca et al. 2017; Duca et al. 2021; Guchetl et al. 2014b; Jebri et al. 2017; Pineda-Martos et al. 2013).

Molecular marker systems possess a high level of polymorphism, therefore they are already widely applied in the development of a genetic linkage map, estimation of gene flow, study of genetic diversity, analysis of phylogenetic relationships and genetic structure of *O. cumana* populations in different countries (Benharrat et al. 2002; Calderón-González et al. 2019; Clapco et al. 2020; Duca et al. 2019; Guchetl et al. 2014b; Molinero-Ruiz et al. 2015; Pineda-Martos et al. 2014a; Pineda-Martos et al. 2014b).

The aim of this study was to investigate the molecular genetic diversity of 23 broomrape populations from different geographical origins using ISSR molecular markers.

2 Materials and methods

2.1 Plant materials

23 broomrape populations were collected from infected sunflower (*Helianthus annuus* L.) fields in different geographical regions of Bulgaria, Turkey, Moldova and Romania (Table 1). Seeds of 10 broomrape populations were kindly provided by the research centers in Bulgaria, Turkey and Romania. All other populations were collected during the different expeditions in the Republic of Moldova. *O. cumana* seeds were germinated on sunflower roots in the laboratory greenhouse. Two broomrape populations analysed were classified as race E, five were identified as race G and the

Table 1: Origin and racial status of 23 *O. cumana* populations studied.

Nr.	Population code	Collection locations	Race	Number of accessions
		BULGARIA		
1.	B1	Debovo/Pleven/Nikopol	G	12
2.	B2	Selanovtsi/Vratsa/Oryahovo	G	12
3.	B3	Radnevo/Stara Zagora/Radnevo	H	12
4.	B4	Rosenova/Dobrichka/Dobrich	H	12
		TURKEY		
5.	T1	Keşan/Edirne/Thrace	H	9
6.	T2	Adana	H	7
7.	T3	Merkez/Edirne/Thrace	H	7
8.	T4	Lüleburgaz/Kirklareli/Thrace	H	7
9.	T5	Trakia	G	11
		REPUBLIC OF MOLDOVA		
10.	RM1	Izbiște/Criuleni	H	12
11.	RM2	Svetlii/Comrat	H	12
12.	RM3	Taraclia	H	11
13.	RM4	Soroca	H	12
14.	RM5	Alexander field/Cahul	H	8
15.	RM6	Bălți I	H	12
16.	RM7	Bălți II	H	11
17.	RM8	Prepelița/Sîngerei	H	12
18.	RM9	Grigorievca/Căușeni	H	12
19.	RM10	Popeasca/Stefan Voda	G	21
20.	RM11	Chisinau	G	22
21.	RM12	Căzănești/Telenești	E	11
22.	RM13	Congaz/Comrat	E	12
		ROMANIA		
23.	R1	Brăila	H	12

remaining 16 belonged to race H (Table 1). Fresh tissue samples were collected from each population and stored at −70 °C until DNA extraction. In total, 269 accessions were sampled as experimental material for the genetic diversity study.

2.2 DNA extraction, purification and quantification

Total genomic DNA was extracted from frozen plants of broomrape using Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol

(Thermo Fisher Scientific, USA). The extracted DNA was further purified by means of 12 M solution of lithium chloride with reducing its final concentration in a solution to 4 M. Quantity and quality of isolated DNA were determined by spectrophotometer (T60 UV-VIS, PG Instruments Limited, England) and also checked by 1 % agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate, pH 8.0; 1 mM EDTA) at 2.5 V/cm (Sambrook and Russell 2001).

2.3 ISSR amplification

A set of thirteen ISSR primers ranging from 14 to 18 bases in length, unanchored or anchored at the 3′ end by 1–2 nucleotides (NN) and having di- (BC 807, BC 810, BC 835, BC 841, BC 857, $(CA)_6RG$, $(CT)_8TC$, $(CA)_6AC$, $(AG)_8YA$ - 69.23 %), tri- ($(CAA)_5$, $(CTC)_4RC$, $(CAG)_5$–23.08 %) and tetra repeats ($(GACA)_4$–7.69 %), was used to amplify of DNA. The 15 µL reaction solution contained 60 ng ADN, 200 µM dNTP mixture (dATP, dCTP, dGTP, dTTP), 2.5 mM $MgCl_2$, 0.4 µM each primer, 1 U/µL Dream Taq Green DNA polymerase and nuclease-free H_2O (Thermo Scientific, USA). The polymerase chain reactions (PCRs) were performed in the thermocycler Genset 9700 “Applied Biosystems” according to the standard procedure (Sambrook and Russell 2001). The amplification was programmed for 35 cycles, which were carried out according to the following steps: the first denaturation, 5 min at 95 °C; for 35 consecutive cycles, denaturation at 95 °C for 30 s, annealing at 45 °C for 45 s, extension at 72 °C for 2 min; and one extension in the last cycle at 72 °C for 5 min.

2.4 PCR fragment separation, visualisation and documentation

The PCR products were subjected to 2 % agarose gels electrophoresis, stained with ethidium bromide and visualised on a transilluminator under UV radiation (wavelength λ = 305 nm). The molecular size of the amplicons was estimated using GeneRuler Express DNA Ladder, ready-to-use SM1553 (Thermo Fisher Scientific, USA). The molecular analysis results were documented using Doc-Print VX2 gel documentation system (SXT-F20.M, France).

2.5 Data analysis

Identification and analysis of DNA amplified fragments was performed using the Photo-Capt V.15.02 program. The presence or absence of each ISSR fragment was treated as a binary character (coded as 1 or 0) and then used to construct the original binary data matrix.

Parameters of descriptive population genetic statistics (observed number of alleles, effective number of alleles, Nei’s gene diversity, Shannon’s Information index, number of polymorphic loci and percentage of polymorphic loci) were calculated to evaluate genetic diversity using the POPGENE V.1.32 software.

Nei’s and Euclidean genetic distances were estimated using the software packages POPGENE V.1.32 and XLSTAT 2014 V.2014.5.03 to differentiate populations for similar and dissimilar traits.

Analysis of Molecular Variance (AMOVA) and Principal Component Analysis (PCA) were conducted using GenAlex 6.501 software to assess the genetic structure and genetic diversity within and among populations.

3 Results

3.1 Genetic diversity and differentiation

The ISSR profile data of 23 *O. cumana* populations with 269 accessions were used to analyse the level of genetic diversity and the relationship among populations under investigation.

According to the data of single-population descriptive statistics for the studied broomrape populations, the highest mean values of observed number of alleles (Na = 1.372), effective number of alleles (Ne = 1.236), Nei's gene diversity (H = 0.138), Shannon's Information index (I = 0.205), number of polymorphic loci (NPL = 123) and percentage of polymorphic loci (PPL = 37.16 %) were found in populations from Turkey, followed by Moldova (1.284, 1.150, 0.091, 0.139, 93.85, 28.35 %, respectively) and Romania (1.227, 1.109, 0.067, 0.105, 75, 22.66 %, respectively) (Table 2). The lowest mean values were observed in populations from Bulgaria (1.190, 1.090, 0.057, 0.088, 63 and 18.96 %, respectively). The results of the analyses showed that the range of variation of Na, Ne, H, I, NPL and PPL values was much wider for Moldavian populations (1.209–1.529, 1.112–1.283, 0.068–0.168, 0.104–0.255, 69–175, 20.85–52.87, respectively) than for Turkish populations (1.287–1.432, 1.150–1.293, 0.092–0.168, 0.141–0.248, 95–143, 28.70–43.20, respectively) (Table 2). The Bulgarian populations, on the other hand, had a relatively small range of variation (1.1662–1.2326, 1.0764–1.1145, 0.0483–0.0720, 0.0757–0.1117, 55–77, 16.62–23.26, respectively) compared with all populations studied. The genetic diversity indices (Na, Ne, H, I, NPL and PPL) for all 23 populations combined (4 Bulgarian, 5 Turkish, 13 Moldovan and 1 Romanian) were 1.284, 1.157, 0.094, 0.143, 93.96 and 28.39, respectively (Table 2).

Analysis of Molecular Variation (AMOVA) revealed genetic differences in the partitioning of variation among/within populations and countries achieved by the ISSR marker system (Table 3). The results showed that differences within all populations accounted for a higher proportion of variation (38 %) than variation among populations (34 %) and among countries (28 %). The total genetic differentiation within all populations was very large (PhiPT = 0.622), whereas among population within region was slightly less (PhiPR = 0.474), with statistical significance at $p < 0.001$.

PhiPT (differentiation index) between all broomrape populations ranged from 0.077 (T1 and T2) to 0.754 (B2 and T3) (Figure 1). The results of the pairwise PhiPT analysis, as interpreted by Wright (1978), indicate moderate (0.05–0.15), large (0.15–0.25) and very large (>0.25) genetic differentiation in the broomrape populations. Thus, 97 % of the very large (between 23 populations), 2 % of the large (4 Moldavian and 3 Turkish populations) and 1 % of the moderate (2 Moldavian and 3 Turkish populations) genetic differentiation was detected from the total variation.

Nei's unbiased measures of genetic distance (GD) among *O. cumana* populations ranged from 0.016 to 0.406 (Figure 1). Populations RM3 and RM12 had the highest

Table 2: Descriptive statistical analysis of ISSR markers in 23 *O. cumana* populations.

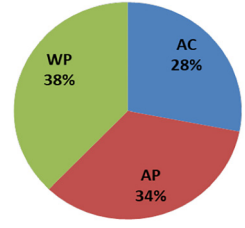
Origin	Population code	Na	Ne	H	I	NPL	PPL, %
Bulgarian populations	B1	1.233 ± 0.423	1.115 ± 0.244	0.072 ± 0.143	0.112 ± 0.214	77	23.26
	B2	1.166 ± 0.373	1.076 ± 0.206	0.048 ± 0.120	0.076 ± 0.181	55	16.62
	B3	1.184 ± 0.388	1.088 ± 0.218	0.055 ± 0.128	0.086 ± 0.192	61	18.43
	B4	1.175 ± 0.381	1.082 ± 0.222	0.051 ± 0.125	0.079 ± 0.186	58	17.52
	Mean ± SD	1.190 ± 0.391	1.090 ± 0.223	0.057 ± 0.129	0.088 ± 0.193	62.75	18.96
Turkish populations	T1	1.384 ± 0.487	1.243 ± 0.356	0.141 ± 0.193	0.210 ± 0.279	127	38.37
	T2	1.432 ± 0.496	1.293 ± 0.376	0.168 ± 0.204	0.248 ± 0.294	143	43.20
	T3	1.351 ± 0.478	1.227 ± 0.342	0.133 ± 0.190	0.197 ± 0.276	116	35.05
	T4	1.405 ± 0.492	1.265 ± 0.363	0.154 ± 0.198	0.228 ± 0.286	134	40.48
	T5	1.287 ± 0.453	1.150 ± 0.281	0.092 ± 0.160	0.141 ± 0.236	95	28.70
	Mean ± SD	1.372 ± 0.488	1.236 ± 0.344	0.138 ± 0.189	0.205 ± 0.274	123	37.16
Moldavian populations	RM1	1.245 ± 0.431	1.127 ± 0.265	0.078 ± 0.151	0.119 ± 0.223	81	24.47
	RM2	1.218 ± 0.413	1.097 ± 0.226	0.062 ± 0.131	0.097 ± 0.198	72	21.75
	RM3	1.257 ± 0.438	1.131 ± 0.280	0.078 ± 0.153	0.121 ± 0.223	85	25.68
	RM4	1.215 ± 0.411	1.123 ± 0.276	0.072 ± 0.152	0.109 ± 0.222	71	21.45
	RM5	1.221 ± 0.415	1.120 ± 0.263	0.073 ± 0.148	0.112 ± 0.219	73	22.05
	RM6	1.227 ± 0.419	1.113 ± 0.247	0.071 ± 0.143	0.109 ± 0.213	75	22.66
	RM7	1.287 ± 0.453	1.171 ± 0.316	0.100 ± 0.173	0.150 ± 0.251	95	28.70
	RM8	1.326 ± 0.470	1.200 ± 0.336	0.116 ± 0.183	0.173 ± 0.264	108	32.63
	RM9	1.209 ± 0.407	1.112 ± 0.253	0.068 ± 0.144	0.104 ± 0.214	69	20.85
	RM10	1.447 ± 0.498	1.215 ± 0.328	0.130 ± 0.178	0.201 ± 0.258	148	44.71
	RM11	1.529 ± 0.500	1.283 ± 0.357	0.168 ± 0.192	0.255 ± 0.275	175	52.87
	RM12	1.227 ± 0.419	1.116 ± 0.249	0.072 ± 0.145	0.111 ± 0.216	75	22.66
	RM13	1.281 ± 0.450	1.147 ± 0.272	0.091 ± 0.158	0.140 ± 0.235	93	28.10
	Mean ± SD	1.284 ± 0.440	1.150 ± 0.282	0.091 ± 0.158	0.139 ± 0.232	93.85	28.35

Table 2: (continued)

Origin	Population code	Na	Ne	H	I	NPL	PPL, %
Romanian populations	R1	1.227 ± 0.419	1.109 ± 0.250	0.067 ± 0.141	0.105 ± 0.208	75	22.66
	Mean ± SD	1.227 ± 0.4193	1.109 ± 0.2499	0.067 ± 0.1405	0.105 ± 0.2084	75	22.66
23 populations	Mean ± SD	1.284 ± 0.020	1.157 ± 0.014	0.094 ± 0.008	0.143 ± 0.011	93.96	28.39

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; ±SD, standard deviation.

Table 3: AMOVA results showing genetic differentiation among broomrape populations using ISSR data.

Variation level	Source of variation	Degree of freedom, df	Sum of squares, SS	Mean squares, MS	Estimate of variance component, Est. Var.	Differentiation, (Phi), $P < 0.001$	Molecular dispersion distribution diagram
Countries and populations	Among countries	3	2461.451	820.484	12.694	PhiRT = 0.281	
	Among populations	19	3760.405	197.916	15.443	PhiPR = 0.474	
	Within populations	246	4209.907	17.113	17.113	PhiPT = 0.622	
	TOTAL	268	10,431.8	–	45.251	–	

PhiRT- among region variation; PhiPR- among population variation within region; PhiPT- total variation within all populations.

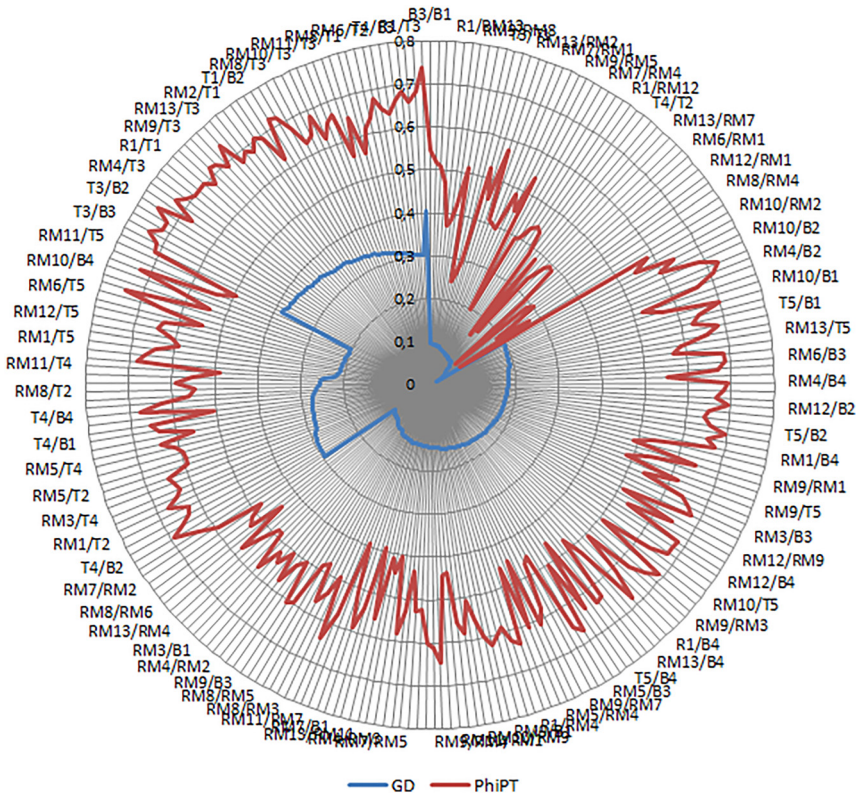


Figure 1: The values of pairwise PhiPT and Nei's unbiased measures of genetic distance in 23 *O. cumana* populations.

similarity (GD = 0.016) and therefore low variability, in contrast to populations T3 and R1, which had the highest variability and lower identity (GD = 0.406). The results obtained from the GD values showed that most of the broomrape populations studied had very small (about 65 %) and small ranges of genetic differentiation (17 %), indicating that they were genetically identical, and only some populations (about 18 %) had moderate genetic differentiation, testifying their more distant relationship and the greatest diversity.

3.2 Genetic relationships and divergence patterns

A molecular phylogenetic tree was constructed to understand the evolutionary relationships and divergence patterns of broomrape populations. The dendrogram

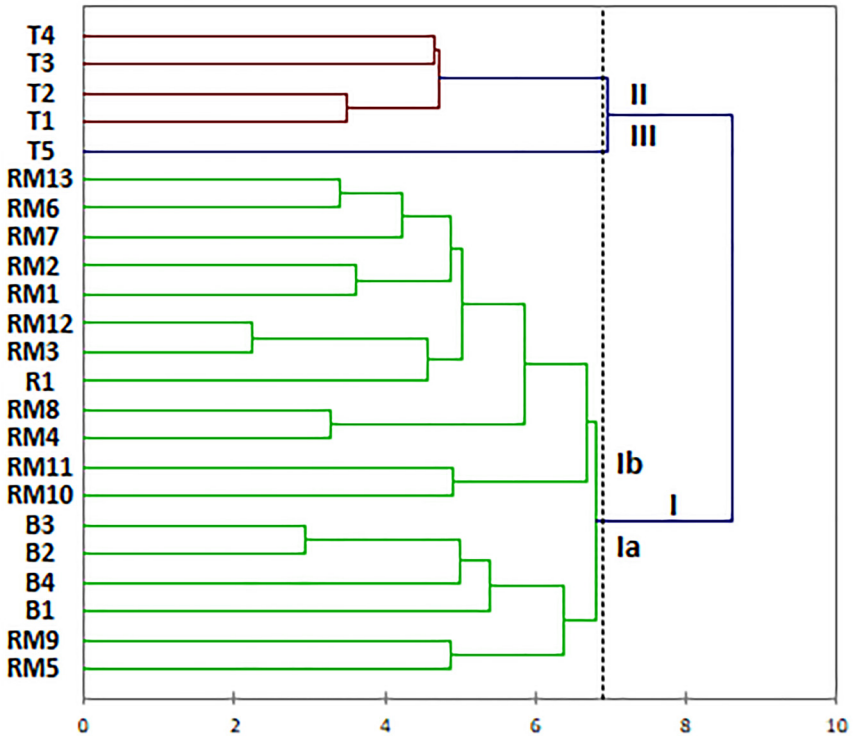


Figure 2: Dendrogram of the 23 populations of *O.cumana* based on ISSR markers using the UPGMA method and Euclidean genetic distances.

obtained from ISSR profiles based on the UPGMA method revealed three major clusters, with eighteen populations from the Eastern European region placed in the first cluster (78 %), while the remaining five populations from the Middle East were accommodated in the second (18 %) and third (4 %) clusters (Figure 2). The first cluster could be further divided into two sub-clusters: the first sub-cluster (Ia) included the populations B1, B2, B3, B4, RM5, RM9 and the second sub-cluster (Ib) comprised the populations RM1, RM2, RM3, RM4, RM6, RM7, RM8, RM10, RM11, RM12, RM13, R1.

The results of the principal component analysis (PCA) indicated to the isolated position of the Turkish populations and the overlapping position of the Eastern European populations from Bulgaria, Moldova and Romania. The first and second components explained 32.97 % and 15.53 % of the variation, respectively, of the total variability of the molecular data in 48.50 % (Figure 3).

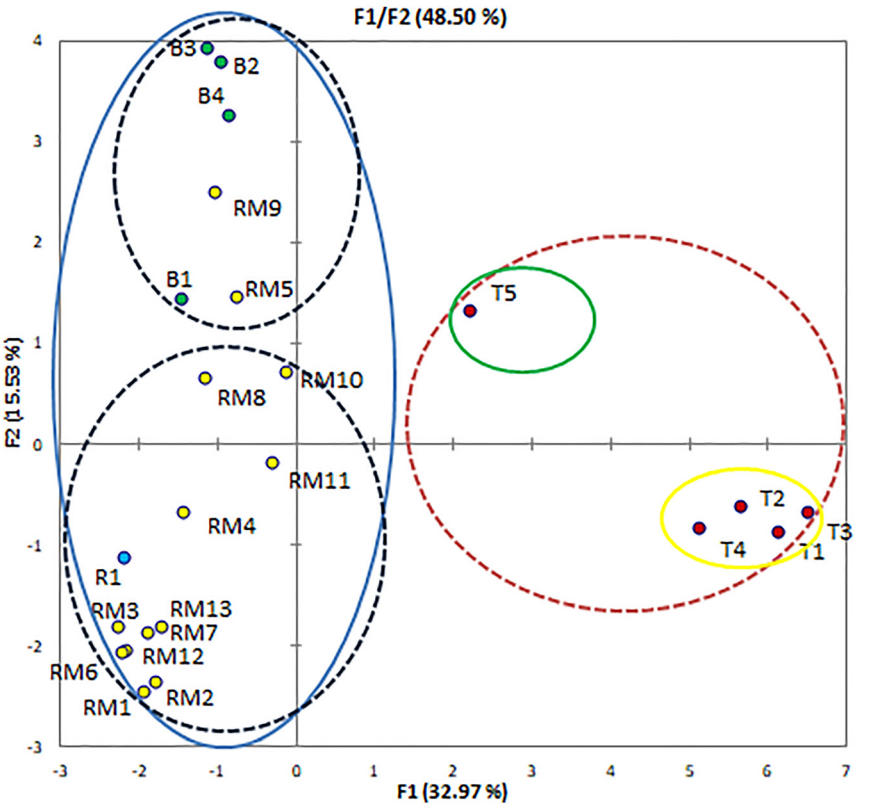


Figure 3: Two-dimensional plot obtained using principal component analysis based on ISSR data for 23 *O. cumana* populations.

4 Discussion

The genetic diversity of species is the basis for adaptation and survival during the entire period of the historical existence of species, and is formed as a result of natural selection and evolution in changing environments (Stockwell et al. 2003; Yang et al. 2014). Abundant genetic diversity can strengthen the ability of a parasite species to respond to the renewing assortment of commercial hybrids/sorts of host species in changing environments, and thereby increasing its evolutionary potential. And it is a direct threat to the profitable production of crops, in this case sunflower. In this study was analysed a large number of geographical populations of the obligate parasitic plant *O. cumana*, which affects sunflower in many countries, where this crop is grown.

Our results using ISSR markers in *O. cumana* species showed very high genetic diversity within populations and the low differentiation among populations. The integral analysis of the mean values of the genetic variability indices (Na, Ne, H, I, NPL and PPL) obtained from the ISSR data for all 23 populations revealed the moderate polymorphism of the microsatellite loci (1.284, 1.157, 0.094, 0.143, 93.96, 28.39, respectively) (Table 2). The AMOVA test showed that the higher genetic differences were due to differences within populations (38 %), the lower level was due to differences among the populations (34 %) and the lowest level was due to differences among countries (28 %) (Table 3). Based on these results, it can be concluded that there is one main gene pool in the Black Sea basin, comprising the populations from Bulgaria, Turkey, Moldova and Romania. The same results were reported by Ivanović et al. (2021) for Serbian *O. cumana* using RAPD analysis, their high intrapopulation diversity was explained by the fact that broomrape plants collected from different agricultural regions in Serbia are members of the same population with great genetic heterogeneity. Similar results about a rather high proportion of the intrapopulation genetic diversity were also obtained from studies of genetic variability in populations from Russia, Kazakhstan, Romania, as well as Tunisia, and Turkey using SSR markers (Bilgen et al. 2019; Guchetl et al. 2014a; Jebri et al. 2017).

To understand the level of genetic diversity among broomrape populations from the Black Sea basin, the ISSR dataset was used to generate a UPGMA dendrogram and a PCA plot. Cluster analysis grouped all the 23 populations into three clusters and two subclusters. Populations from Bulgaria, Romania and Moldova were arranged into one cluster because of similar molecular profiles, which may indicate a monophyletic origin, and may also be explained by geographical proximity or similar climatic conditions. Populations from Turkey were classified into two other clusters, suggesting that they're somewhat distinct from each other. The PCA analysis was consistent with the UPGMA clustering results (Figures 2 and 3). In particular, the UPGMA dendrogram and the PCA plot showed a clear pattern of clustering according to the locations from which broomrape germplasm was collected.

5 Conclusions

The study revealed that the different broomrape populations possess a significant level of genetic diversity, especially within populations, whereas little differentiation was observed among populations. Moreover, the clustering and PCA methods showed that the Moldavian, Bulgarian and Romanian broomrape populations share more genetic traits with each other than with the Turkish populations. By and large, the clustering and PCA analyses allowed come to the conclusion that the grouping of broomrape is partly determined by its geographical origin, as well as by the genetic

differences and similarities accumulated over the course of time, and is not related to virulence. In summary, it can be concluded that there is a main gene pool of *O. cumana* in the Black Sea basin, comprising populations from Bulgaria, Turkey, Moldova and Romania.

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Informed consent: Informed consent was obtained from all individuals included in this study.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
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Data availability: The data that support the findings of this study are available on request from the corresponding author, [Bivol Ina].

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