

## Analysis of genetic relationships between broomrape populations from different countries using ISSR markers

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### Abstract

*Orobanche cumana*, commonly known as sunflower broomrape, poses a significant risk to sunflower cultivation in areas located along the Black Sea and across Europe. A study was conducted to analyze the genetic diversity and differentiation among populations of this parasitic plant originating from Bulgaria, Turkey, Republic of Moldova, and Romania. A total of 269 individuals from 23 populations were genotyped using 13 ISSR markers. The frequency distribution of alleles in the broomrape populations from Romania, Turkey and Bulgaria is more homogeneous than that from Moldavian populations. However, according to genetic diversity parameters *O. cumana* populations from Moldova and Turkey were more variable (total gene diversity  $H_t=0.188$ ;  $0.214$ ), than those from Bulgaria and Romania ( $H_t=0.112$ ;  $0.067$ ). The highest values of gene diversity within populations ( $H_s = 0.138$ ) were found in Turkish broomrape. The value of  $G_{st}$  ranged between  $0.359-0.516$ , indicating a very high level of genetic differentiation among populations. These results are consistent with low  $N_m$  values ( $0.468-0.895$ ). Pairwise differentiation index (PhiPT) and Nei's unbiased measures of genetic distance (GD) showed similar patterns, indicating a maximum (PhiPT=0.500; GD=0.261) and, respectively, minimum (PhiPT=0.238; GD=0.079) values between broomrape from Turkey and Romania, respectively, Romania and Moldova. Dendrogram constructed using the UPMGA method based on Nei's genetic distances and Pearson's dissimilarity clustered together Moldavian and Romanian broomrape and grouped in two separate clusters populations belonging to Bulgaria and Turkey. The PCA analysis confirmed the results of UPGMA clusterization. Overall, both methods suggest that the groupings of broomrape are partly determined by its geographical origin, as well as by the genetic differences and similarities accumulated over time, and are not related to virulence.

**Keywords:** diversity; gene flow; genetic differentiation; genetic structure; ISSR markers; *O. cumana*; phylogenetic relationship

### Introduction

Genetic population structure has a pivotal role in facilitating the broader natural distribution, heightened environmental adaptability and increased survivability of a species, unveiling its evolutionary

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potential under changing environmental conditions. The genetic diversity and structure of population are determined by the dynamic interplay of various processes, including gene flow, genetic drift, natural selection and the plant reproduction system.

In the exploration of genetic diversity of populations, various mono- and multilocus molecular markers, such as RAPD (Bivol and Barbacar, 2013; Dwivedi *et al.*, 2018; Moghadam *et al.*, 2021), SSR (Ali *et al.*, 2017; Ghețea *et al.*, 2018; Chen *et al.*, 2021) and ISSR (Chen *et al.*, 2016; Luz *et al.*, 2020) have been employed. The Inter-Simple Sequence Repeats (ISSRs) marker system discerns nucleotide sequence diversity in microsatellite regions dispersed throughout the genome, particularly in non-coding regions of the nuclear genome characterized by di- and trinucleotide repeats (Amiteye, 2021). ISSRs, as dominant markers, have proven valuable for detecting multilocus polymorphism within a population (Reddy *et al.*, 2002; Kim *et al.*, 2021). This technique is cost-effective, simple, rapid, and efficient, requiring minimal template DNA (5-50 ng per reaction) without the need for prior DNA sequence data, and exhibits high reproducibility (92-99%) (Joshi *et al.*, 1999; Sarwat, 2012). ISSR markers have been used to assess the genetic diversity, taxonomic and phylogenetic relationships within various species of *Orobanche* and closely related species from the same section. They have been employed to distinguish between different populations of *O. bederae*, *O. cernua*, or *O. cumana* (Benharrat *et al.*, 2002), as well as to detect inter- and intrapopulation polymorphism in *O. cumana* from different geographical regions, including Bulgaria (Stoyanov *et al.*, 2012; Duca and Bivol, 2022), Spain (Román *et al.*, 2002), Republic of Moldova (Duca *et al.*, 2020; Clapco *et al.*, 2020), China (Shi *et al.*, 2019), Romania (Benharrat *et al.*, 2002), Turkey (Duca and Bivol, 2023), Egypt (Sharawy and Karakish, 2015; Abdalla *et al.*, 2016), Russia, Ukraine and others.

However, molecular genetic differences among *O. cumana* populations from different regions of the Black Sea basin remain insufficiently studied. This research aims to analyse various broomrape populations collected in four countries: Republic of Moldova, Turkey, Bulgaria, and Romania, that are characterized by a high density of broomrape attack in sunflower fields and a rapid evolution of broomrape races. To achieve this goal, genetic structure of 23 *O. cumana* populations was investigated using 13 microsatellite loci. Obtained data were used to construct dendrograms elucidating the genetic divergence and phylogenetic relationships between sunflower broomrape.

## Materials and Methods

### *Plant materials*

Sunflower broomrape populations kindly provided by our colleagues from the research centres in Bulgaria, Turkey and Romania have been used in this study (Figure 1). *O. cumana* seeds were germinated on sunflower roots in the laboratory greenhouse. Fresh tissue samples were collected from each population and stored at -70 °C until DNA extraction. A total of 269 broomrape accessions were included in a comparative study of genetic diversity and population genetic structure.



**Figure 1.** Location of collection sites of 23 *O. cumana* populations

#### *DNA Isolation*

Total genomic DNA was extracted from frozen broomrape plants using the *Thermo Scientific* GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol (*Thermo Fisher Scientific*, USA). The extracted DNA was purified with a 12 M lithium chloride solution. The quantity and quality of DNA was determined by spectrophotometer (T60 UV-VIS, PG Instruments Limited, England) and checked by 1% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate, pH 8.0; 1mM EDTA) at 2.5 V/cm (Sambrook and Russell, 2001).

#### *PCR procedure*

The PCR solution (15  $\mu$ l) contained: 60 ng ADN; 200  $\mu$ M dNTP mixture (dATP, dCTP, dGTP, dTTP); 0.4  $\mu$ M concentration of each primer; 1 U/ $\mu$ L DreamTaq Green DNA Polymerase in buffer solution (1x); ultrapure water; and 2.5 mM MgCl<sub>2</sub>.

Amplification was performed in a Genset 9700 thermocycler (Applied Biosystems, USA) according to the standard polymerase chain reaction procedure (Sambrook and Russell, 2001). The PCR protocol consisted of several steps, namely an initial denaturation at 95 °C (5 min), followed by 35 cycles at 95 °C (30 s), 45 °C (45 s), 72 °C (2 min) and a final extension at 72 °C (5 min). The DNA amplified fragments were subjected to electrophoresis on 2% agarose gels in 1xTAE buffer, stained with ethidium bromide (0.5  $\mu$ g/ml) The molecular size of the amplicons was estimated using GeneRuler Express DNA Ladder, ready-to-use SM1553 (*Thermo Fisher Scientific*, USA). The results of the molecular analysis were documented using the Doc-Print VX2 gel documentation system, model SXT-F20.M (Vilber Lourmat, France).

#### *ISSR primers*

Thirteen of the most effective ISSR primers from the 14 readily available primers representing di-, tri- and tetra-repeats (Table 1), reported by Benharrat *et al.* (2002) in the analysis of genetic diversity among broomrape populations, were used to amplify the DNA.

#### *Statistical analysis*

Phylogenetic relationships between populations were estimated using microsatellite allele frequencies. Nei's genetic distance and Pearson dissimilarity matrices based on the UPGMA method were used to construct

dendrograms. *Principal Component Analysis* (PCA) was used to reveal the significance of traits in overall variation and understanding the independent and balanced weighting of molecular data to analyse the genetic diversity and genetic structure of the broomrape populations. The software packages used were POPGENE V.1.32 and XLSTAT 2014 V.2014.5.03. AMOVA test was applied in the GenAlex 6.501 software.

## Results and Discussion

Assessment of genetic diversity within parasitic species like *O. cumana* is as a crucial tool in characterizing the unique features of different populations (Duca and Bivol, 2023), thus contributing to our understanding of population dynamics (Gagne *et al.*, 1998) and trends in the emergence of new populations (Ivanović *et al.*, 2021). This information has a great importance for the development of sustainable control strategies against the pathogen and for breeding programs aimed to enhance sunflower resistance to broomrape.

### *Microsatellite fragments distribution*

Investigation of the polymorphism based on microsatellite fragments is important in elucidating genetic diversity within and among populations, providing valuable data for subsequent analysis of species evolution, genetic relationships between populations and adaptation to changing environmental conditions.

In the current study (Table 1), a total of 331 amplicons, generated by 13 microsatellite primers, were revealed. Notably, 312 (94.26%) of these amplicons were polymorphic, suggesting a high genetic diversity within studied populations. A such level of genetic diversity can provide a greater chance for specie's survival and adaptation to environmental fluctuations.

The size of the amplified fragments ranged from 230 to 5353 base pairs (bp), elucidated a high genetic variation among individuals within populations and among populations, that could be due by high mutation rate resulting into change in allelic array length.

**Table 1.** The polymorphism level of the ISSR primers

Primer code	Repeat Sequence (5'→ 3')	NBN	GC, %	Fragment size range, bp	Number of amplicons			P (%)
					Total	Poly-morphic	Mono-morphic	
BC807	ag ag ag ag ag ag ag ag t	17	47	300-4358	29	26	3	89.66
BC810	ga ga ga ga ga ga ga ga t	17	47	430-5353	28	26	2	92.86
BC835	ag ag ag ag ag ag ag ag yc	18	56	444-4577	25	25	0	100.00
BC841	ga ga ga ga ga ga ga ga yc	18	56	280-5000	29	28	1	96.55
BC857	ac ac ac ac ac ac ac ac yg	18	56	385-3000	23	22	1	95.65
(CA) <sub>6</sub> AC	caa caa caa caa caa	15	33	395-3759	23	21	2	91.30
(CA) <sub>6</sub> RG	gaca gaca gaca gaca	16	50	454-2476	16	15	1	93.75
(CT) <sub>8</sub> TC	ca ca ca ca ca ca rg	14	57	689-5333	27	26	1	96.30
(AG) <sub>8</sub> YA	ctc ctc ctc ctc rc	14	71	230-4699	36	35	1	97.22
(CAA) <sub>5</sub>	cag cag cag cag cag	15	67	429-4254	24	21	3	87.50
(CAG) <sub>5</sub>	ct ct ct ct ct ct ct tc	18	50	620-5000	28	27	1	96.43
(CTC) <sub>4</sub> RC	ca ca ca ca ca ac	14	50	418-5353	28	27	1	96.43
(GACA) <sub>4</sub>	ag ag ag ag ag ag ag ya	18	50	388-3902	15	13	2	86.67
<b>Total</b>				<b>230-5353</b>	<b>331</b>	<b>312</b>	<b>19</b>	<b>94.26</b>
<b>Mean</b>					<b>25.46</b>	<b>24.00</b>	<b>1.46</b>	<b>93.87</b>

\*Notes: NBN – number of nitrogen bases; GC (%) – percentage content of cytosine (C) and guanine (G) nucleotides in the primer; bp – base pairs; P – level of polymorphism (%); R (A, G); Y (C, T).

The number of fragments produced by each primer varied between 16 to 29 with an average of 25.46 bands per primer, including 24 polymorphic and 1.46 monomorphic fragments, which indicate a performance of the employed marker system.

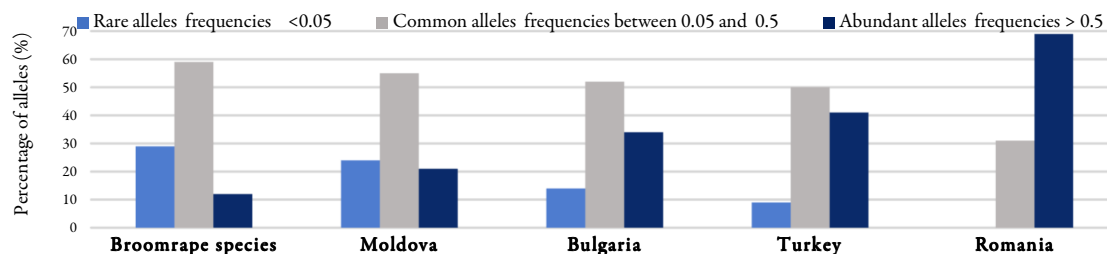
The average of polymorphism (93.87%) indicates the efficiency of ISSR primers in the estimation of genetic diversity within broomrape populations.

Knowledge of changes in *allele frequencies* is crucial for understanding the genetic relationships between populations and gaining insights into the genetic and molecular basis of evolutionary change (Franks *et al.*, 2018).

The proportion of abundant (frequency >0.5), common ( $\geq 0.05 \leq 0.5$ ) and rare (<0.05) alleles in populations varied depending on their origin (Figure 2). The presence of common alleles in the genomes of broomrape plants from different geographical region/countries suggests that they inherited from a common ancestor.

The percentage of common alleles was approximately the same in Moldavian (55%), Bulgarian (52%) and Turkish (50%) broomrape. The lowest number of common alleles was established in the genome of Romanian broomrape (31% from the total number of identified alleles).

The highest number of abundant alleles were found in the genomes of Romanian broomrape (69%) followed by those from Turkey (41%), Bulgaria (34%) and Moldova (21%). Rare alleles were identified in Turkish (9%), Bulgarian (14%) and Moldavian (24%) broomrape, but were not found in those from Romania (Figure 2).



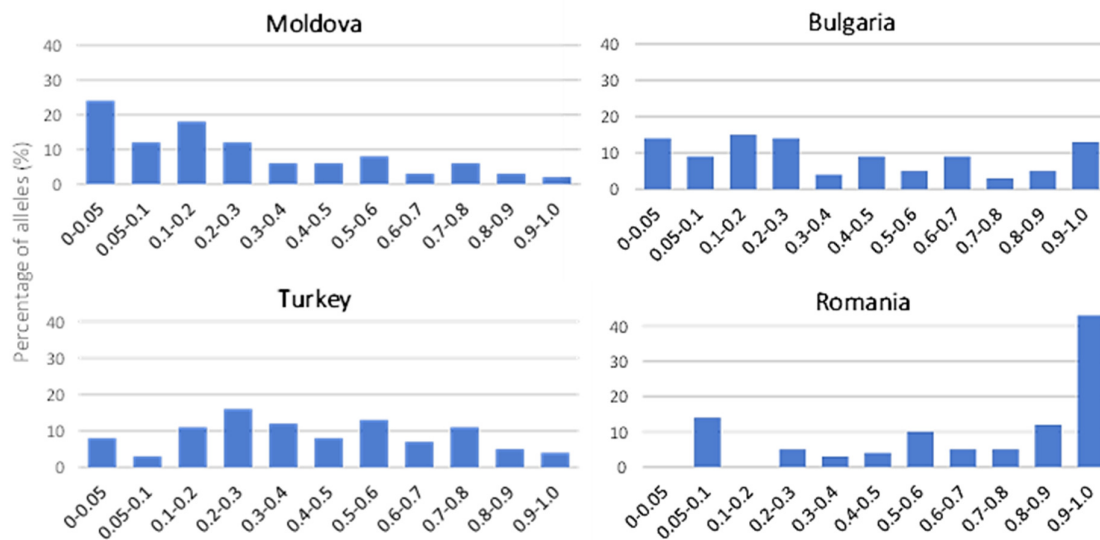
**Figure 2.** Frequency distribution of all 331 alleles at 13 loci for broomrape from 4 countries

The excess of common alleles can be explained by the population bottlenecks, absence of population subdivision or purifying (negative) selection, characterized by elimination of alleles that are deleterious from a population (Linck and Battey, 2019). Purifying selection reduces genetic diversity and play an important role in shaping genomic diversity in natural populations (Cvijović *et al.*, 2018). On the other hand, a population bottleneck causes the preferential loss of low-frequency variants, producing an excess of intermediate-frequency variants (Harris and Meyer, 2006).

By contrast, high proportion of rare alleles provide evidence of population expansion, mutation rates, gene flow and reveal geographically localized population subdivision (Gompert *et al.*, 2014; Linck and Battey, 2019). The abundant alleles in the genome indicate on the stabilizing selection in populations under constant environmental conditions, resulting in the maintaining of individuals with an average value of the trait. It is known that it favours a more uniform population and decreasing of genetic diversity (National Research Council, 2013).

The effects of evolutionary processes can result in different classes of genetic variants characterized by their relative frequency in one or another population. The distribution of ISSR amplicons in 11 groups according to allele frequencies (Figure 3) showed different tendencies of alleles distribution determined by the characteristics of adaptive evolution of broomrape in each country. The highest number of abundant alleles (60% from the total of alleles), with the frequency ranging from 0.7 to 1, were found in *O. cumana* from Romania. The presence in populations of individuals with an extreme value of a trait (either of decreasing or

increasing its value) can contribute to a further increase in variability and, consequently, to its consolidation by the driving selection of the most adapted individuals in new conditions of their existence. Therefore, it can be concluded that the frequency distribution of alleles in the broomrape populations from Romania, Turkey and Bulgaria is more homogeneous than that from Moldavian populations.



**Figure 3.** Frequency distribution of alleles of 13 loci in broomrape from 4 countries

The overall *allelic diversity* at each polymorphic locus can be expressed as the sum of two components ( $H_t = H_s + D_{st}$ ), where  $H_s$  is the mean allelic diversity within populations, and  $D_{st}$  represents the allelic diversity among populations. Furthermore, the proportion of total allelic diversity among populations ( $G_{st}$ ) is linked to these components through the ratio  $D_{st}/H_t$  (Buso *et al.*, 1998).

The heterogeneity within and among populations, as indicated by gene diversity ( $H_s$ ) and differentiation ( $D_{st}$ ) data, highlighted distinct patterns based on geographical origin (Table 2). The highest values of  $H_t$  (0.214) and  $H_s$  (0.138) and comparatively low  $G_{st}$  (0.359), respectively,  $D_{st}$  (0.076) values were observed in Turkish populations, which indicated that the genetic diversity is especially determined by the variations at the intrapopulation level. Contrary, in the Moldavian and Bulgarian broomrape populations the genetic diversity is explained both by variations within and among populations ( $H_s = 0.091$  and  $D_{st} = 0.097$ , respectively,  $H_s = 0.057$  and  $D_{st} = 0.055$ ).

**Table 2.** Genetic diversity parameters of broomrape in different countries using 13 ISSR markers

Country	Ht	Hs	Dst	Gst	Nm (Gst)
Bulgaria	0.112	0.057	0.055	0.493	0.513
Turkey	0.214	0.138	0.076	0.359	0.895
Moldova	0.188	0.091	0.097	0.516	0.468
Romania	0.067	0.067	0	-	-

\*Notes:  $H_t$  – total gene diversity;  $H_s$  – gene diversity within populations;  $D_{st}$  – gene diversity among populations;  $G_{st}$  –  $(H_t - H_s)/H_t$ , coefficient of gene differentiation among populations;  $N_m$  – gene flow among populations from  $G_{st}$ .

The value of  $G_{st}$  ranged between 0.359-0.516, indicating a very high level of genetic differentiation (Buso *et al.*, 1998) among populations from each country. Our results are consistent with previous studies in sunflower broomrape according to those *O. cumana*, due to its predominantly autogamous behaviour, is characterized by a relatively high  $H_t$  values, low  $H_s$  values and high  $G_{st}$  values (Gagne *et al.*, 1998). As for

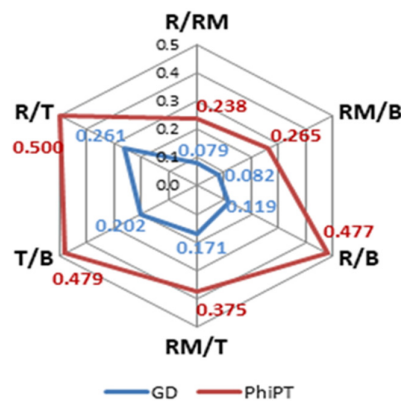
comparison, in the case of parasitic weeds with high level of outcrossing the  $G_{st}$  provided a value of 0.207, supported by the high gene flow ( $N_m = 1.912$ ) (Khamassi *et al.*, 2023). Similarly, low values of differentiation and high gene flow were reported in *Orobancha crenata* populations in Algeria (Bendaoud *et al.*, 2022).

Generally, it is considered that, if  $N_m < 1$ , the genetic drift will result in substantial local differentiation, and if  $N_m > 1$ , the little differentiation among populations will be observed (Wright, 1951). Gene flow is an important factor for the genetic structure of broomrape populations because it can facilitate local adaptation and co-evolution between parasites and hosts by supplying new alleles or universally favoured mutations to populations with limited genetic variance. Over time, allelic migration reduces the genetic differences between populations as a result of the homogenizing effect, thereby preventing or delaying the evolution of the populations in different geographical areas into separate parasite species.

In our study, the gene flow among broomrape populations at the individual country level were found to be less than 1 (Table 2), indicating that the sampled populations were subject to genetic drift (Slatkin, 1987). Low  $N_m$  values (0.468-0.895) are consistent with the result of high genetic differentiation. The populations from Turkey showed the highest gene flow, being less differentiated. AMOVA analysis also supported these results since 35% of the total variation is attributed to differences between countries and 65% to differences within countries.

The pairwise differentiation index (PhiPT) serves as a metric for quantifying genetic differences among subpopulations or groups, considering internal variation within populations. The pairwise highest differentiation (0.500) was observed between Turkish and Romanian broomrape populations, while the lowest (0.238) was found between Moldavian and Romanian populations (Figure 4). Substantial genetic differentiation was, also, revealed between broomrape from Turkey and Bulgaria (T/B - 0.479), Romania and Bulgaria (R/B - 0.477), Romania and Turkey (R/T - 0.375). According to Wright's interpretation (1978) these values suggest a high and very high level of genetic differentiation among studied regions.

A similar pattern of genetic differences between *O. cumana* populations was provided by genetic distances (GD), the highest (0.261) and, respectively, lowest (0.079) GD values being found between broomrape from Turkey and Romania, respectively, Romania and Moldova (Figure 4). Pairwise comparisons of GD values between individual countries indicated very small genetic distances for RM/B (0.082), R/B (0.119), RM/T (0.171) and small genetic distances for T/B (0.202), implying insignificant genetic differences.



**Figure 4.** Pairwise comparisons based on PhiPT values and Nei's unbiased measures of genetic distance between *O. cumana* from 4 countries

\*Notes: GD – Nei's genetic distance, PhiPT – total variation within all populations; RM – populations from Moldova, R – populations from Romania, B – populations from Bulgaria, T – populations from Turkey

In the majority of cases, both indexes revealed congruent patterns of genetic variation. An exception is related to R/B broomrape populations relationships. Thus, the internal structure of these populations appeared more intricate (PhiPT = 0.477) compared to the RM/T (PhiPT = 0.375). However, the genetic distance,

which specifically addresses the genetic separation between populations or individuals, exhibited a contrary trend, the GD being 0.119 for R/B and 0.171, respectively for RM/T.

*Phylogenetic relationships*

The analysis of dendrograms (Figure 5) provides insights into the phylogenetic relationships among broomrape populations. Three main clusters reflecting distinct genetic groups were generated. The first cluster included Moldavian and Romanian broomrape populations, suggesting a close genetic affinity between these neighbouring countries. This observation aligns with the geographical proximity of Moldova and Romania, indicating a common genetic heritage among broomrape populations in this cluster. The second cluster included Bulgarian broomrape, suggesting a unique genetic profile, different from the Moldavian and Romanian populations. Turkish broomrape populations are grouped together in the third cluster, indicating on a common genetic background or on possible gene flow among these samples. The clustering of Turkish broomrape separately from the Moldavian, Romanian and Bulgarian populations highlight the genetic distinctiveness of these populations.

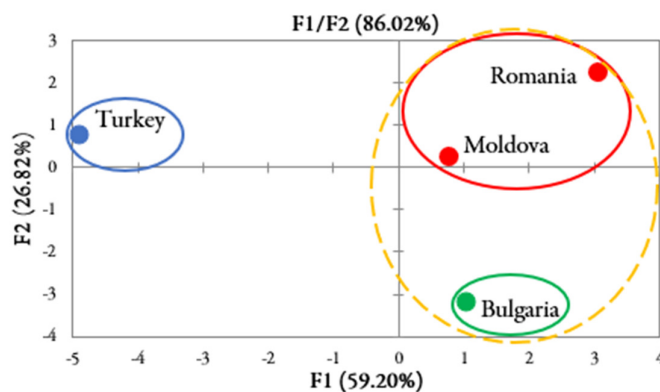


**Figure 5.** UPGMA dendrograms estimating the Nei's genetic distance (left) and Pearson dissimilarity (right) between broomrape from 4 countries using ISSR markers

Cluster analysis based on Pearson dissimilarity matrix led to similar classification results compared to that derived from clustering using Nei's genetic that confirmed the observed phylogenetic relationships. The similarities in the dendrogram structures suggest a convergence of results, further supporting the validity of the genetic distance metrics employed. The distinct clusters at the country level suggest both shared genetic ancestry and unique evolutionary trajectories among broomrape populations from Moldova, Romania, Bulgaria, and Turkey. The observed genetic differences may be influenced by historical, ecological, and environmental factors that have shaped the evolutionary dynamics of these parasitic plants in specific geographical regions.

The results of Principal Component Analysis (PCA) confirmed and complemented the findings of cluster analysis. The first principal component (F1) explained the most part of molecular variance (59.20%), emphasizing its pivotal role in capturing the major traits contributing to genetic variation. The second component (F2), contributed by 26.82%, further elucidating the intricate genetic makeup of broomrape populations (Figure 6).

According to PCA the broomrape populations were grouped in three main clusters. The first cluster included Moldova and Romania, while in the second and third clusters were grouped populations belonging to Bulgaria and, respectively, Turkey, revealing different genetic profiles.



**Figure 6.** PCA plot for 4 countries of *O. cumana* species generated from ISSR data

The congruence between and the cluster analysis confirmed the relationships between *O. cumana* populations. Corroborating the results obtained from different analytical approaches it was established that broomrape populations from Moldova, Romania, and Bulgaria showed closer genetic relationships compared to Turkish populations. These findings suggest on a potential monophyletic origin of Moldavian, Bulgarian, and Romanian broomrape populations, sharing common genetic characteristics within the main Black Sea basin *O. cumana* gene pool.

In conclusion, while shared genetic characters within the Black Sea basin *O. cumana* gene pool were evident among Moldavian, Romanian, and Bulgarian populations, the analysis also underscored the impact of geographical origin on genetic differentiation. The high genetic diversity observed at the country level was attributed to specific genetic drift processes, emphasizing the intricate interplay of factors influencing the genetic landscape of broomrape populations across different countries. Genetic analysis of broomrape from different origins showed the presence of regular gene flow among populations of the *O. cumana* distributed in the Black Sea basin, which could be attributed to the occurrence of cross-pollination and also the presence of self-pollination within a species.

## Conclusions

The results of this study demonstrate the usefulness of studied microsatellites as markers for discriminating between different broomrape populations and for reconstructing of potential/ hypothetical phylogenetic relationships, even with a limited number of analysed samples and including situations where history of populations is unclear. It has been established that the genome of broomrape populations from Turkey show a specific microsatellite allele distribution different from Bulgarian, Moldavian, and Romanian populations.

The clusterization of *O. cumana* populations into four main groups by country may be explained by the long independent evolution of these groups of broomrape populations within geographically distinct regions. From this, it can be concluded that the genetic drift, as a micro-evolutionary process taking place in the populations of the countries of the Black Sea region, can randomly cause changes in allele frequencies (complete disappearance of gene variants or fixation of rare alleles) in the next generations and that the direction of its action cannot be pre-established. The information resulting from the genetic relationship analysis of different origin broomrape may be useful for the design of further studies on the parasitic weed broomrape control and the development of breeding strategies.

### Authors' Contributions

Conceptualization: MD; Data curation: IB; Formal analysis: IB; Funding acquisition: MD, SC; Investigation: IB, AM; Methodology: MD, SC, AP; Software: IB; Writing - original draft: IB; Writing - review and editing: MD, SC, AP, AM. All authors read and approved the final manuscript.

### Ethical approval (for researches involving animals or humans)

Not applicable.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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