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Two genetic groups of *Orobanche cumana* Wallr. in Moldova show contrasting regional affinities

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Summary. Broomrape (*Orobanche cumana* Wallr.) is a parasitic plant of sunflower which is racially evolving and geographically expanding across most sunflower-growing regions. In the Republic of Moldova, broomrape has been present since 1937. Genetic diversity and structure of seven Moldovan broomrape populations, and reference populations from neighbouring Türkiye, Bulgaria, Romania and Serbia, were analysed using a set of 90 single nucleotide polymorphism (SNP) markers. Genetic diversity was moderate but highly variable among Moldovan populations, with appreciable unbiased expected heterozygosity and Shannon's index values in several populations. Observed heterozygosity was consistently low relative to expected heterozygosity, a pattern consistent with the predominantly selfing broomrape mating system. Population structure analyses, based on STRUCTURE, principal coordinates analysis (PCoA) and Neighbor-Joining clustering, consistently revealed the presence of two main genetic groups within Moldova. These groups had different affinities with populations from neighbouring countries, with genetic group G1 close to Romanian populations and genetic group G2 close to populations from Türkiye and Bulgaria, indicating a geographical component underlying the observed genetic differentiation. Analysis of molecular variance (AMOVA) indicated that most of the genetic variation was explained by differences among populations, confirming strong broomrape population structuring within Moldova.

Keywords. Genetic diversity, parasitic weed, SNP markers, virulence, population structure.

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INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an important oilseed crop, but its production is severely constrained by the root holoparasitic weed *Orobanche cumana* Wallr. The parasite has rapidly expanded its distribution in recent decades, particularly in Eastern Europe and the Mediterranean basin, where the intensification of sunflower cultivation has favoured emergence and spread of highly virulent populations able to overcome

resistance genes deployed in commercial sunflower hybrids (Cvejić *et al*., 2020).

Understanding the genetic structure and diversity of *O. cumana* populations is essential for interpreting evolutionary dynamics of the parasite, and its capacity to adapt to resistant sunflower genotypes. High levels of genetic diversity may enhance the adaptive potential of the species, facilitating occurrence of new virulence profiles, and breakdowns of host resistance. Although *O. cumana* is a predominantly self-pollinating species, several studies have reported substantial levels of cross-fertilization and gene flow between populations (Rodríguez-Ojeda *et al*., 2013a; Pineda-Martos *et al*., 2014). The existence of cross-fertilization, together with efficient seed dispersal that may lead to multiple introductions into particular areas (Nabloussi *et al*., 2023), and admixture of populations (Fernández-Melero *et al*., 2023), may contribute to increasing genetic diversity within populations, and to reduced geographic differentiation among populations compared with expectations for a predominantly autogamous species (Duca and Bivol, 2023).

Several studies have analysed population diversity of *O. cumana* using different types of molecular markers, including RAPD, ISSR, AFLP, SSR, and, more recently, SNPs (Gagne *et al*., 2000; Pineda Martos *et al*., 2013; Guchetl *et al*., 2014; Molinero-Ruiz *et al*., 2014; Martin-Sanz *et al*., 2016; Malek *et al*., 2017; Jebri *et al*., 2017; Ari *et al*., 2022; Duca and Bivol, 2023; Fernández-Melero *et al*., 2023; Nabloussi *et al*., 2023; Dedić *et al*., 2026). In the Black Sea basin, which is a main centre of *O. cumana* distribution, several studies have described the genetic relationships among populations from countries such as Bulgaria, Romania, Türkiye and Moldova. Analyses based on ISSR and SSR markers have generally shown substantial genetic differentiation among populations, although with weak geographic structuring and a tendency for Eastern European populations to share broadly related genetic backgrounds (Guchetl *et al*., 2014; Duca *et al*., 2019; Duca and Bivol, 2023; Duca *et al*., 2024).

Moldova is a relevant but still poorly characterised region for *O. cumana* population genetics. Previous studies evaluated Moldovan populations using limited numbers of ISSR or SSR markers (Duca *et al*., 2019; Duca *et al*., 2022; Duca and Bivol, 2023). Although these studies detected genetic differentiation among populations, they did not clearly resolve whether Moldovan populations shared one regional genetic background, or comprised differentiated genetic groups with distinct affinities to populations from neighbouring areas of the Black Sea region. Consequently, the internal subdivision of Moldovan populations and their regional genetic relationships remain only partially understood. The resolution of ISSR and SSR markers for detailed analyses of inter- and intrapopulation genetic diversity is limited and may be insufficient to detect fine scale population structure or recent divergence processes (Dossa *et al*., 2025). In contrast, single nucleotide polymorphism (SNP) markers provide increased resolution for detecting genetic variation, population structure, and admixture patterns (Morin *et al*., 2004; Helyar *et al*., 2011). Therefore, the use of a denser panel of locus-specific SNP markers may help to clarify the fine scale genetic structure of *O. cumana* populations within Moldova, and their affinities with populations from neighbouring regions.

The objectives of the present study were to determine whether *O. cumana* populations from the Republic of Moldova comprise differentiated genetic groups, and to examine their relationships with reference populations from neighbouring countries, using SNP markers.

MATERIALS AND METHODS

Sunflower broomrape populations

The study focused on seven Moldovan broomrape populations that were previously characterized using ISSR and SSR markers (Duca *et al*., 2022; Duca and Bivol, 2023). These populations were selected because archived DNA of sufficient quality was available for several individuals, whereas DNA from the remaining populations was too degraded for reliable SNP genotyping. The selected populations were from a broad geographical range within Moldova, although they should not be considered an exhaustive representation of the genetic diversity present in this country (Figure 1). They included: RM4 (Soroca), RM6 and RM7 (Bălți) in northern Moldova; RM12 (Căzănești) in the central part of the country; RM9 (Grigorievca) in south-eastern Moldova; and RM2 (Svetlii) and RM13 (Congaz) in southern Moldova.

The reference populations were selected to place the genetic variation observed in Moldova within a broader regional context. The assessments included most of the previously analysed Bulgarian (B2 to B4), Turkish (T1, T2, T3, T5), and Romanian (R1) populations, together with the Moldovan populations analysed by Duca and Bivol (2023), allowing direct comparison with the earlier ISSR-based study. Two additional previously characterized populations, and representatives of well-known gene pools, were also included as reference populations, including OCCS010 from Braila, Romania and OCCS009, from North Bačka County in Vojvodina, Serbia (Dedić *et al*., 2026). Selection was therefore based primarily on geographical relevance, continuity with previous studies, and available information on popula-

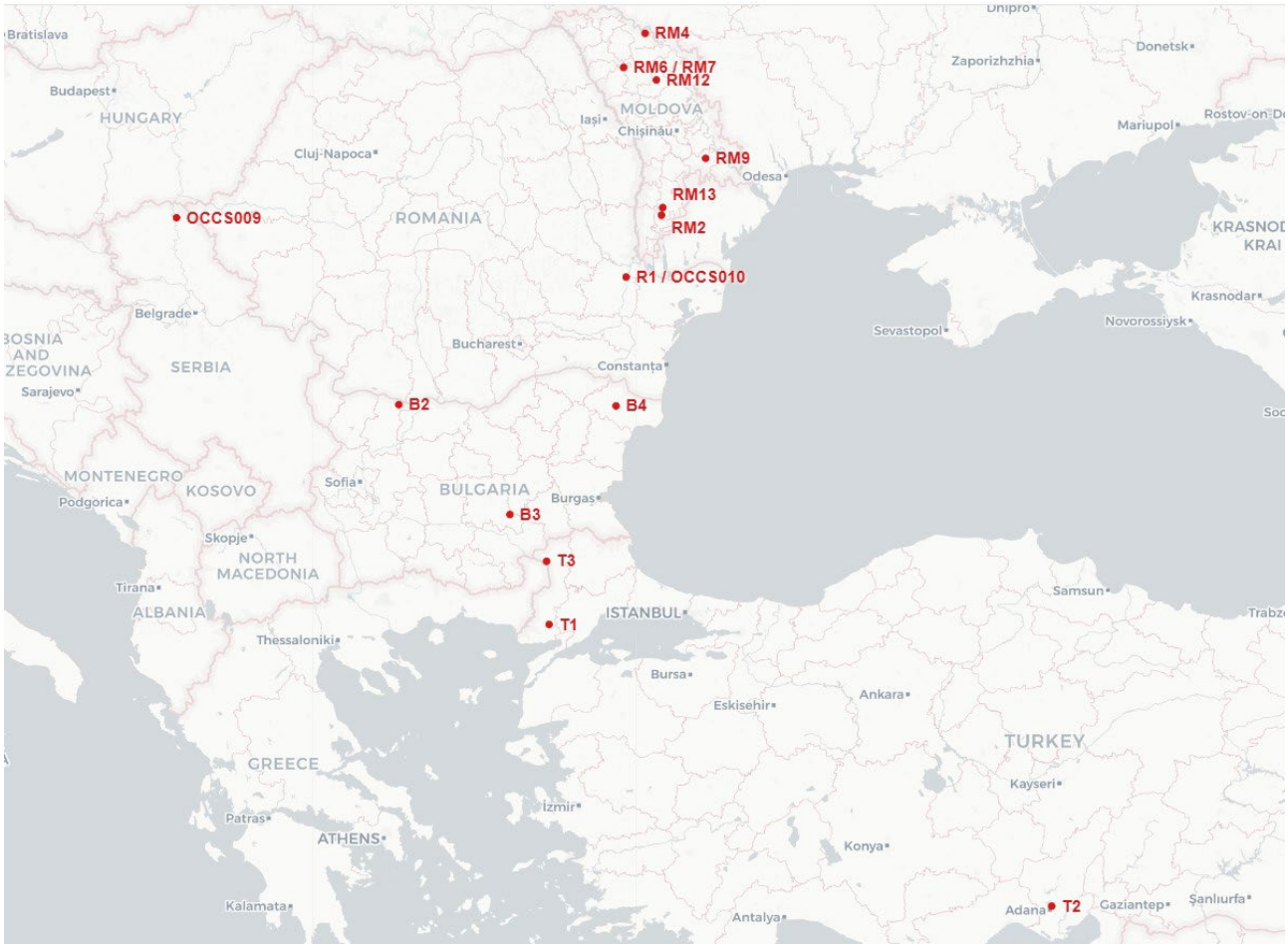

Figure 1. Geographic origins of the Moldovan and reference populations of *Orobanche cumana* analysed in this study. Population codes correspond to those used in the text and tables of this paper.

tion genetic relationships, rather than on virulence classification.

The numbers of individuals per population are provided in Table 1 for the Moldovan populations. For the reference populations, the numbers of individuals ranged from five in T3 to 14 in OCCS009 and OCCS010. Because the study was based on archived biological material, the numbers of successfully genotyped individuals were small and unequal among populations. Consequently, estimates of allele frequencies and intrapopulation diversity, particularly for populations represented by four to six individuals, should be interpreted cautiously.

Plant genotyping

DNA of individual plants, and in some cases SNP genotyping data, were available from the previous studies mentioned above. Genotyping of individual broomrape plants was carried out using 96 *O. cumana* SNP markers selected from the set reported and mapped by Calderón-González *et al.* (2019). After removing the markers with more than 10% missing data, the assessments were carried out with 90 SNP markers (Supplementary Table S1).

Genetic diversity and population structure analyses

Genetic diversity within populations was assessed using GenAlEx v.6.5 (Peakall and Smouse, 2012). For each population, intrapopulation diversity was described by estimating the percentage of polymorphic loci (P), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), the fixation index (F), and Shannon's diversity index (I). Mean values and standard errors were calculated across loci. Because of the small and unequal

Table 1. Intrapopulation genetic diversity parameters of seven *Orobanche cumana* populations from Moldova, including the number of individuals analysed (N), percentage of polymorphic loci (P), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), fixation index (F), and Shannon's diversity index (I).

Population	No.	P (%)	Ho (± SE)	He (± SE)	uHe (± SE)	F (± SE)	I (± SE)
RM2	6	66.67	0.11 ± 0.02	0.25 ± 0.02	0.27 ± 0.02	0.47 ± 0.05	0.37 ± 0.03
RM4	4	56.67	0.02 ± 0.01	0.23 ± 0.02	0.27 ± 0.03	0.94 ± 0.04	0.34 ± 0.03
RM6	9	74.44	0.09 ± 0.01	0.26 ± 0.02	0.27 ± 0.02	0.56 ± 0.05	0.38 ± 0.03
RM7	8	22.22	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.09 ± 0.04	0.06 ± 0.01
RM9	10	58.89	0.01 ± 0.01	0.20 ± 0.02	0.21 ± 0.02	0.96 ± 0.03	0.30 ± 0.03
RM12	7	1.11	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	—[a]	0.01 ± 0.01
RM13	5	56.67	0.00 ± 0.00	0.27 ± 0.03	0.29 ± 0.03	0.98 ± 0.02	0.37 ± 0.04

[a]The fixation index (F) was not reported for RM12, because it was based on a single polymorphic locus and was therefore not considered informative at population level.

population sample sizes, differences among population-level diversity estimates were interpreted descriptively, but were not subjected to formal significance tests. Nei's unbiased genetic distance was also computed between pairs of populations. The resulting distance matrix was used to construct a Neighbor-Joining (NJ) tree. The analysis was carried out using MEGA12 software (Kumar *et al*., 2024), with default parameters. The NJ tree was used to visualize patterns of genetic similarity among populations.

Population relationships were further explored by principal coordinates analysis (PCoA), carried out in GenAlEx v.6.5 using a matrix of pairwise codominant genotypic distances among individuals, and calculated across the 90 SNP loci. Graphical representation of PCoA results was produced using OriginPro 2023b (OriginLab Corporation).

Population structure was inferred with STRUCTURE v.2.3.4 (Pritchard *et al*., 2000), using an admixture model with correlated allele frequencies (Falush *et al*., 2003). The number of genetic clusters (K) tested ranged from 1 to 10, with ten independent runs performed for each K value. Each run consisted of 100,000 Markov chain Monte Carlo (MCMC) iterations, following a burn-in period of 10,000 iterations. The most likely number of clusters was determined using Structure Selector (https://lmme.ac.cn/StructureSelector/). Cluster alignments across runs were carried out with CLUMPP v.1.1.2b (Jakobsson and Rosenberg, 2007), using the FullSearch algorithm. Following the STRUCTURE analysis, individuals were assigned to clusters based on membership coefficients, and the two principal coordinates of PCoA were replotted, with the individuals from Moldova separated into those assigned to the genetic groups 1 (G1) and 2 (G2), based on more that 95% membership, and those admixed (A), having less than 95% membership to both genetic groups.

Analyses of molecular variance (AMOVA) were carried out using GenAlEx v.6.5 (Peakall and Smouse, 2012) to partition genetic variation among and within populations. This analysis was restricted to the Moldovan populations. Statistical significance of variance components were assessed using permutation tests implemented in the software.

RESULTS

Genetic diversity within Moldovan broomrape populations showed considerable variation among populations (Table 1). Percentages of polymorphic loci (P) ranged widely, from 1.11% in RM12 to 74.44% in RM6. Most populations exhibited moderate levels of polymorphism, with values greater than 50%, whereas RM7 (22.22%) and particularly RM12 (1.11%) showed low polymorphism. Observed heterozygosity (Ho) was consistently low across all the populations, ranging from 0.00 in RM13 to 0.11 in RM2. Expected heterozygosity (He) and unbiased expected heterozygosity (uHe) followed similar patterns. RM7 and RM12 had very low uHe values of, respectively, 0.03 and 0.01, consistent with their low P values, while the remaining populations had values between 0.21 and 0.29 (Table 1). The fixation index (F) was positive in all populations for which it had been reported, ranging from 0.09 in RM7 to 0.98 in RM13. Particularly high values were observed in RM4 (0.94), RM9 (0.96) and RM13 (0.98), indicating marked deficits of heterozygotes. Shannon's diversity indices (I) followed a similar pattern to the expected heterozygosity, with the lowest values in RM12 (I = 0.01) and RM7 (I = 0.03), while the remaining populations had values ranging from 0.30 to 0.38.

Analysis of molecular variance (AMOVA) showed that most of the genetic variation was explained by dif-

Table 2. Analysis of molecular variance (AMOVA) for *Orobanche cumana* populations from Moldova, based on SNP markers, showing the partitioning of genetic variation among populations, among individuals within populations, and within individuals.

Source	df	SS	MS	Estimated Variance	%
Among Populations	6	971.753	161.959	10.508	53
Among Individuals	42	707.696	16.850	7.547	38
Within Individuals	49	86.000	1.755	1.755	9
Total	97	1765.449		19.810	100

ferences among populations (53%), followed by variation among individuals within populations (38%), whereas variation within individuals was low (9%) (Table 2).

Figure 2 shows the biplots of the three principal coordinates obtained from the PCoA. The first three principal coordinates accounted for 53.5% of the total genetic variation (respectively, 23.6, 18.6 and 11.3%). Therefore, the biplots provide a partial representation of the multilocus variation, and were interpreted together with the STRUCTURE, Neighbor-Joining, and AMOVA results. In the plot of PCo1 *vs.* PCo2, some Moldovan individuals were located close to individuals from Romania, whereas the remaining individuals were positioned close to those from Türkiye and Bulgaria. A similar pattern was observed in the PCo1 *vs.* PCo3 plot. The plot of PCo2 *vs.* PCo3 did not provide additional information.

STRUCTURE analyses identified two main genetic groups (G1 and G2) within the Moldovan broomrape populations. The ΔK statistic showed a pronounced maximum at K = 2, providing strong support for this subdivision, whereas the secondary peak at K = 5 was considerably smaller (Figure 3). Most populations showed predominant assignment to one of the two groups, although admixed individuals were detected in several cases (Table 3). Populations RM2, RM4, RM7 and RM13 were mainly associated with G1, showing high mean membership coefficients and considerable proportions of individuals with assignment values above 95%. In contrast, RM9 and RM12 were clearly assigned to G2, with most individuals showing high membership to this group. Population RM6 displayed a more heterogeneous pattern, with individuals assigned to both groups, and a relatively high proportion of admixed genotypes. The Neighbor-Joining tree based on Nei's genetic distance (Figure 4) supported the genetic structure inferred by STRUCTURE. The Moldovan populations were separated into two main clusters, largely consistent with their assignment to G1 and G2. Populations associated with G1 tended to cluster together, and showed close relationships with reference populations from Romania (R1 and OCCS010), whereas those associated

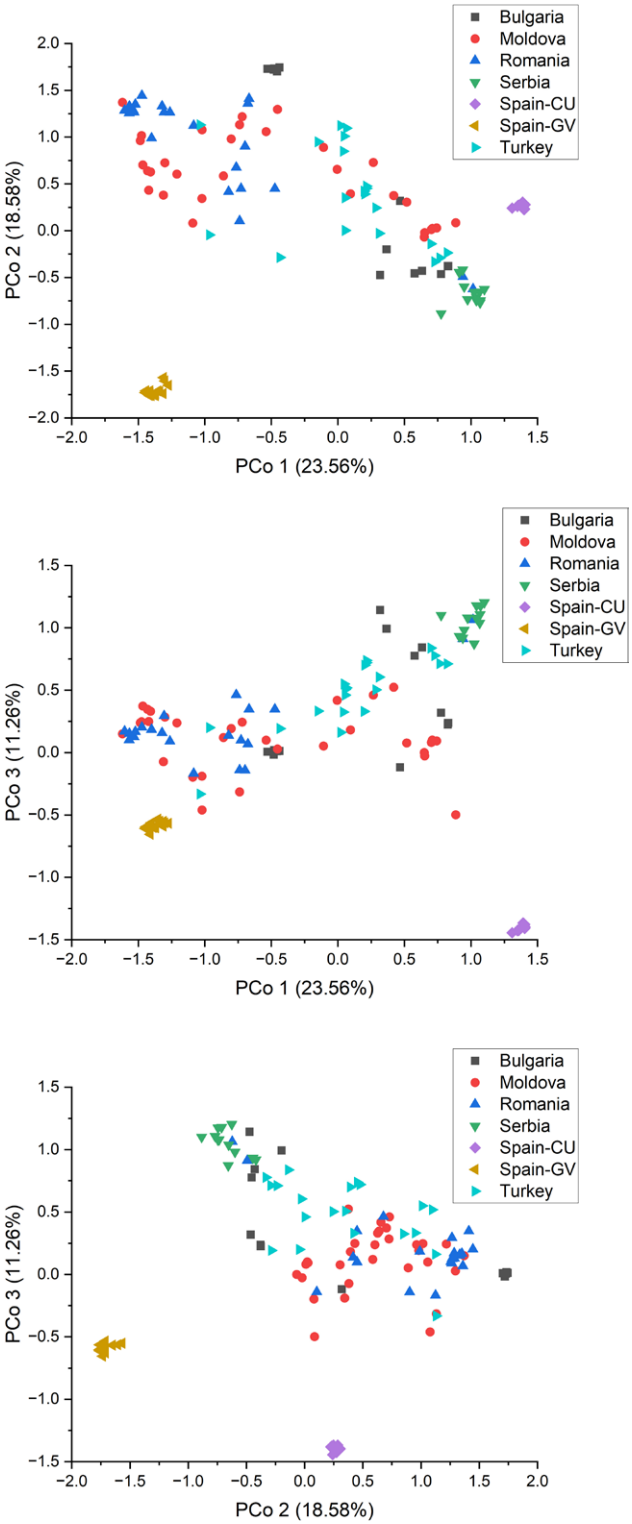

Figure 2. Principal coordinates analysis of 49 *Orobanche cumana* individuals from seven populations from the Republic of Moldova, together with 147 individuals from Bulgaria, Türkiye, Romania and Serbia used as references. Biplots with axes one and two, one and three, and two and three are shown. The percentage of variation explained by each principal coordinate is indicated in the axis titles.

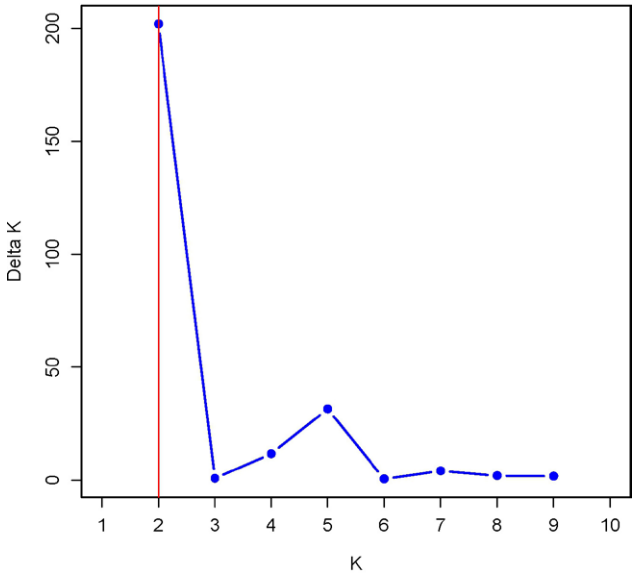

Figure 3. Delta K for values of K in the analysis of genetic structure of the *Orobanche cumana* populations from Moldova evaluated in this study.

Table 3. Genetic structures of sunflower broomrape populations from Moldova, showing the mean membership coefficients (q) in genetic groups G1 and G2, the number of individuals assigned to each group, and the number of admixed individuals. Individuals with q > 0.95 for either group were assigned to that group, whereas those with q ≤ 0.95 for both groups were classified as admixed.

Population	Mean q, G1	Mean q, G2	G1 (n)	G2 (n)	Admixed
RM2	0.82	0.18	2	0	4
RM4	0.87	0.13	2	0	2
RM6	0.63	0.37	4	1	4
RM7	0.98	0.02	7	0	1
RM9	0.07	0.93	0	8	2
RM12	0.00	1.00	0	7	0
RM13	0.85	0.15	3	0	2

with G2 were more closely related to populations from Türkiye (T1–T3, and T5), Bulgaria (B2–B4), and Serbia (OCCS009).

Figure 5 shows the PCoA (axes 1 and 2), replotted according to the STRUCTURE-based classification of Moldovan individuals. A clear correspondence was apparent between the distance-based PCoA and STRUCTURE analysis, with individuals assigned to each genetic group generally located at opposite extremes of the distribution of Moldovan samples, and admixed individuals occupying intermediate positions.

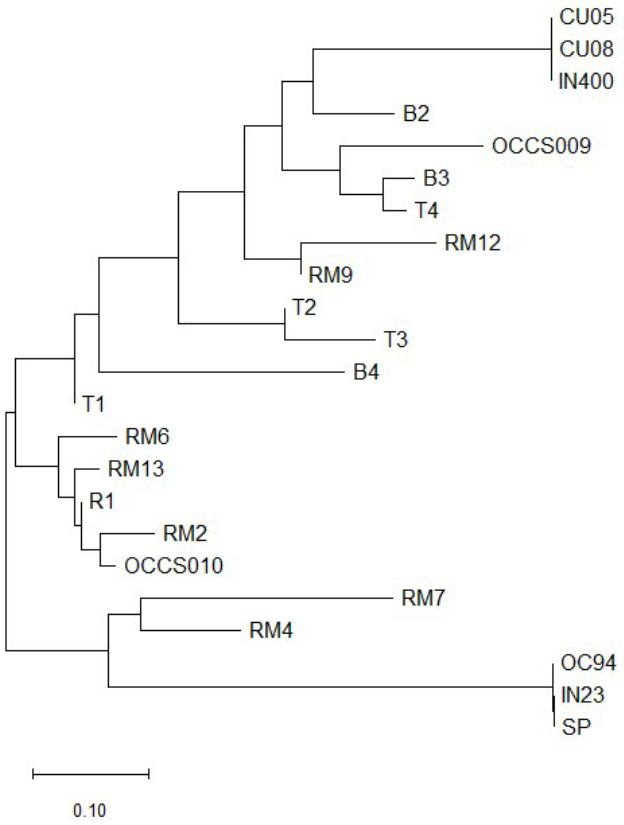

Figure 4. Neighbor-Joining (NJ) tree showing genetic relationships among *Orobanche cumana* populations based on Nei's unbiased genetic distance. The tree illustrates the genetic similarity among populations from Moldova and with reference populations from other countries. The scale bar represents genetic distance.

DISCUSSION

The present study has shown that the Moldovan broomrape populations analysed did not constitute a single homogeneous regional genetic background, but comprised at least two differentiated groups with contrasting affinities within the Black Sea region. This pattern was consistently supported by STRUCTURE, PCoA, and distance-based clustering analyses, indicating that the observed structure was robust across different analytical approaches, and has consistent biological relevance. Nevertheless, because only seven populations and a limited number of individuals were available, extrapolation of this pattern to the full diversity of *O. cumana* in Moldova requires caution.

Previous studies have also reported the existence of two differentiated *O. cumana* gene pools at country levels. This was the case, for example, of countries outside the main centre of distribution of the parasite, such as in Spain (Pineda-Martos *et al.*, 2013) and Morocco

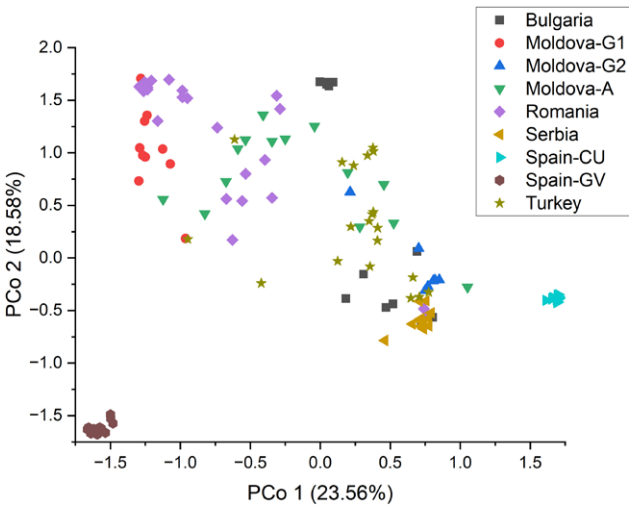

Figure 5. Principal coordinates plot (PCo 1 vs. PCo 2) of 49 *Orobanche cumana* individuals from seven populations from the Republic of Moldova, separated into those assigned to the genetic groups 1 (G1) and 2 (G2), based on more that 95% membership, and those admixed (A), having less than 95% membership to both genetic groups, together with 147 individuals used as references, from Bulgaria, Türkiye, Romania and Serbia. The percentage of variation explained by each principal coordinate is indicated in the axis titles.

(Nabloussi *et al*., 2023). However, in both of these countries, absence of genetic diversity within populations pointed to separate introductions followed by founder effects, rather than to local evolutionary processes of broomrape populations (Pineda-Martos *et al*., 2013; Nabloussi *et al*., 2023). In contrast, Dedić *et al.* (2026) identified two contrasting genetic groups in Serbia, associated with separate evolutionary histories and probably different introduction events. The pattern observed in Moldova in the present study is probably consistent with different introductions, as the two identified genetic groups have affinities with populations from neighbouring countries, Romania for genetic group G1, and Türkiye and Bulgaria for genetic group G2. This indicates that the differentiation observed in Moldovan populations may reflect historical processes of diversification and gene flow within the Black Sea region.

The contrasting affinities of G1 and G2 are compatible with the contribution of at least two historical regional sources for the present Moldovan populations. The relationship of G1 with Romanian populations may reflect connections with the Lower Danube region, whereas the affinities of G2 with Bulgarian and Turkish populations point to genetic connections with the western and southern sectors of the Black Sea basin. Movement of the very small broomrape seeds through contaminated host seed lots, in soil transported by agricultural machinery, or from other agricultural exchanges, could have contributed to these regional connections, followed by local multiplication and differentiation under predominant self-pollination. Nevertheless, the present set of reference populations is insufficient to establish direction or timing of broomrape dispersal.

AMOVA results showed that most of the genetic variation was explained by differences among populations (53%), providing quantitative support for strong population structuring in sunflower broomrape populations from Moldova. Previous studies based on ISSR and SSR markers that included some of the Moldovan populations analysed here, together with populations from other countries, also showed strong genetic structuring in *O. cumana* populations (Duca *et al*., 2022; Duca and Bivol, 2023). However, in those studies AMOVA was conducted on broader datasets than assessed in the present study, including populations from Romania, Türkiye, Serbia, Bulgaria and China, with the variance partitioned among countries, among populations within countries, and within populations. Therefore, the results are not directly comparable with those from the present study. Nevertheless, the present results demonstrate that strong population structuring is already evident when considering Moldovan populations alone. AMOVA has rarely been used to partition genetic variation among and within populations of *O. cumana* at country or regional levels. In a study based on *O. cumana* populations growing on wild hosts in the Black Sea region of Bulgaria, Pineda-Martos *et al.* (2014) reported 59.5% of variance among populations and 40.5% within populations, values consistent with those obtained in the present study. Other studies have applied AMOVA mainly to compare gene pools (Guchetl *et al*., 2014), or to assess genetic variation among countries and regions (Molinero-Ruiz *et al*., 2014).

The predominance of among-population variation is biologically consistent with the mating system of *O. cumana*. Predominant self-pollination reduces effective gene flow and recombination among local populations, favours the maintenance of homozygous genotypes, and allows genetic drift and founder effects to generate marked differentiation among geographically separated populations (Nybom, 2004). The consistently low observed heterozygosity and the small within individual component of AMOVA support this interpretation. Local selection imposed by the sunflower genotypes cultivated in each area could further contribute to differentiation, if populations have been exposed to different resistance genes (Pérez-Vich *et al*., 2013). However, information on the historical composition of sunflower cultivars at the collection

sites assessed in the present study was not available, and the relative contribution of host-mediated selection cannot here be separated from demographic processes.

The two *O. cumana* genetic groups identified in this study should not be interpreted as distinct virulence groups. According to previously reported race classifications, G1 includes populations classified as race H (RM2, RM4, RM6 and RM7) and race E (RM13), whereas G2 includes one race H population (RM9) and one race E population (RM12) (Duca and Bivol, 2023). Therefore, the available information does not indicate that virulence is simply associated with the genetic groups revealed by the SNP analysis. This is biologically plausible, because virulence may be determined by variation at one or a few avirulence loci, and may therefore differ among populations that otherwise share similar overall genetic backgrounds (Rodríguez-Ojeda *et al.*, 2013b; Fernández-Melero *et al.*, 2023).

The overall level of genetic diversity observed within Moldovan *O. cumana* populations was moderate, but was highly variable among populations. Observed heterozygosity was consistently low compared to expected heterozygosity, and the positive fixation index recorded in all populations for which these indices were reported (Table 1) indicated a marked deficit of heterozygotes. This pattern is consistent with the predominantly self-pollinating mating system of *O. cumana* (Rodríguez-Ojeda *et al.*, 2013a), and with the low intrapopulation variability and strong genetic differentiation among populations previously reported for the species (Gagne *et al.*, 1998). The presence of populations with very low diversity, such as RM12 and to a lesser extent in RM7, may reflect founder effects or recent bottlenecks (Cvejić *et al.*, 2020).

The results of the present study provide additional insights compared with previous research in the region. Duca *et al.* (2022) and Duca and Bivol (2023), using SSR and ISSR markers on a set of populations that included those analysed in the present study, also detected substantial genetic structuring among *O. cumana* populations from Moldova and other countries. However, the increased resolution provided by SNP markers in the present study allowed a clearer identification of two differentiated genetic groups within Moldovan populations. While SSR and ISSR markers are useful for detecting overall genetic variability and population differentiation, their capacity to resolve fine scale genetic structure is limited (Özbek, 2024). Despite the reduced number of populations and individuals analysed compared with the previous studies, the use of SNP markers allowed clear resolution of genetic structure. This indicates that marker resolution may be more important than sample size for detecting fine scale population structures, although both aspects remain important. Low sampling intensity may limit detection of low frequency variants, and therefore the present results should be interpreted within this context.

The SNP panel used in this study also provided a baseline for future molecular surveillance of *O. cumana* in the Black Sea region. Comparison of newly collected populations with the G1 and G2 reference profiles could help to identify new introductions, monitor changes in admixtures, and allow tracing of regional spread of emerging populations. Molecular surveillance should be combined with standardized virulence phenotyping to assess race composition of new populations. Integration of both types of information could support selection of populations for resistance screening, early detection of changes in virulence, and coordinated regional deployment and diversification of host resistance sources.

CONCLUSIONS

Combined evidence from diversity indices, AMOVA, STRUCTURE, PCoA and distance-based analyses indicates that the studied Moldovan populations of *O. cumana* comprise at least two genetic groups with distinct regional affinities: G1 with Romanian populations, and G2 with Bulgarian and Turkish populations. These results provide a genetic baseline for monitoring the regional dispersal and admixture of sunflower broomrape populations in Moldova. Integration of SNP-based surveillance with standardized virulence phenotyping may contribute to early detection of emerging populations, and to allow informed regional deployment of resistant sunflower hybrids.

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