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GENETIC DIVERSITY ANALYSIS OF SUNFLOWER BROOMRAPE POPULATIONS FROM REPUBLIC OF MOLDOVA USING ISSR MARKERS

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ABSTRACT

The holoparasitic *Orobanche cumana* Wallr. has become a limiting factor for sunflower yield in Eastern Europe. Better knowledge of broomrape biology, including genetic diversity, is important in any attempt to develop resistance-breeding programs. There is little information available on the diversity of broomrape in the Republic of Moldova.

The aim of this study was to evaluate the interpopulation variability of the *Orobanche cumana* Wallr. using Inter Simple Sequence Repeats (ISSR) markers.

Data from the present study revealed that ISSR marker system could be used for estimating genetic diversity, since it had high percent polymorphism. All of the genetic parameters indicated that the populations from Center are genetically different from those collected in the South and North regions of the Republic of Moldova. The results of genetic polymorphism showed that the application of 14 ISSR markers made it possible to group the studied 39 broomrape populations into different clusters. The association with geographic origin was established.

Keywords: genetic polymorphism, Inter Simple Sequence Repeats (ISSR) markers, *Orobanche cumana* Wallr., Unweighted Pair Group Method with Arithmetic Mean (UPGMA), variability.

INTRODUCTION

The *Orobanchaceae* family contains approx. 2000 species and is the largest family of parasitic flowering plants, accompanied by partial or complete loss of photosynthetic ability, with a specialized lifestyle, including tolerance to drought and parasitism (Bennett et al., 2006). The *Orobanche* genus, compared to other genera, is characterized by a complexity and large genetic diversity of species (Parker, 2009), containing *Orobanche cumana* Wallr., *O. cernua* Loefl., *O. crenata* Forsk. and *O. reticulata* Wallr., known as parasites of sunflower and other crops (Piednoël et al., 2012).

For the analysis of diversity and systematization of *Orobanche* species a variety of techniques were used, including morphological (Musselman, 1986) and biochemical methods (Verkleij et al., 1991), electron microscopy of pollen grains (Abu Sbaih et al., 1994; Piwowarczyk et al., 2015; Zare et al., 2014) or seeds (Plaza et al., 2004; Domina et al, 2005), chemotaxonomic methods for measuring the content of phenolic compounds (Andary, 1994; Georguieva, 1994) and fatty acids (Velasco et al., 2000). The pattern and distribution of genetic variation in this important genus is little known (Katzir et al., 1996).

The application of molecular marker techniques in combination with classical taxonomic approaches can greatly enhance our understanding of broomrape diversity in the region, including the description and resurrection of new and neglected taxa with local or regional distribution (Hristova et al., 2011).

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Based on these considerations, the aim of the paper was to evaluate the variability among *Orobanche cumana* Wallr. populations from the Republic of Moldova, using the Inter Simple Sequence Repeat (ISSR) markers.

MATERIAL AND METHODS

Plant material

The study encompassed a collection of 39 populations of *O. cumana* Wallr., collected from 3 different geographic regions of the Republic of Moldova as following: 4 – from the North, 16 – from the Center and 19 – from the Southern part (Table 1).

According to our previous study, the populations belong to different races (Duca, 2017b) and demonstrate a great genetic diversity (Duca, 2017a).

DNA isolation

Broomrape aerial shoots stored in liquid nitrogen at -80°C were used for molecular analysis. A mixture of 10 individual broomrape plants of equal weight was taken from each population for the DNA extraction. DNA isolation and purification was performed from 50 mg of frozen material, using the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific), in accordance to the manufacturer's instructions.

Table 1. List of broomrape populations included in the study

BROOMRAPE RACE			
≤ E	**F**	**G**	**H**
NORTH			
Donduşeni	Prepeliţa	Soroca	Bălţi
CENTER			
Sîngera, Băcioi, Căzăneşti, Brînzenii Noi, Izbişte, Floreni, Holercani, Buţeni, Chişinău, Fundul Galbenei	Rassvet	Vejereni, Costuleni	Sărata Mereşeni, Frăsîneşti, Ciocîlteni
SOUTH			
Cimişlia, Crihana Veche, Cazangic	Carabetovca, Slobozia-Mare, Ştefan-Vodă, Grigorievca	Gura Galbenei, Talmaza, Ermoclia, Manta, Chirsova, Beşalma, Corten, Ciadîr Lunga	Congaz, Svetlîi, Taraclia, Alexanderfeld

ISSR analysis

A total of 14 ISSR primers, including those reported by Benharat et al. (2002) were used to analyze genetic diversity among broomrape populations (Table 2). The reaction mixture had a final volume of 25 µl/tube: 30 ng of DNA; 265.6 µM dNTP; 7.5 pmol primer (0.4 µM); 1.3 unit of DNA Polymerase. The amplification reactions were performed by GeneAmp® PCR system 9700 thermocycler (Applied Biosystems) with the following program: initial denaturation at 94°C for 7 minutes, followed by 35 denaturation cycles at 94°C for 30 seconds, alignment at 45°C for 45 seconds, extension at 72°C for 2 minutes and the final extension at 72°C for 2 minutes.

The amplification products were separated by electrophoresis on a 1.5% agarose gel with TBE 1x buffer for 60 minutes at 160V. After electrophoresis, DNA was stained with ethidium bromide and visualized by a UV- transilluminator.

Statistical and bioinformatic analysis

The bands were detected using the Photo Capt Software. Only the fragments with a high luminous intensity were considered in the statistical analysis.

Table 2. List of the ISSR primers and their characteristics

No.	Primer	Sequence (5'-3')	Nr. nitrogen bases
1.	807	AGAGAGAGAGAGAGAGT	17
2.	810	GAGAGAGAGAGAGAGAT	17
3.	835	AGAGAGAGAGAGAGAGYC	18
4.	841	GAGAGAGAGAGAGAGAYC	18
5.	857	ACACACACACACACACYG	18
6.	(CAA)5	CAACAACAACAACAA	15
7.	(GACA)4	GACAGACAGACAGACA	16
8.	(GATA)4	GATAGATAGATAGATA	16
9.	(CA)6RG	CACACACACACARG	14
10.	(CTC)4RC	CTCCTCCTCCTCRC	14
11.	(CAG)5	CAGCAGCAGCAGCAG	15
12.	(CT)8TC	CTCTCTCTCTCTCTCTTC	18
13.	(CA)6AC	CACACACACACAAC	14
14.	(AG)8YA	AGAGAGAGAGAGAGAGYA	18

PIC value was calculated for each of the ISSR primers according to Anderson et al. (1993).

The data obtained with the ISSR primers was processed into a binary matrix, the presence and the absence of the band was marked as "1" and "0", respectively. The binary matrix was then used to calculate genetic distances (Jaccard coefficient) and the construction of dendrograms by Unweighted Pair Group *Method* with Arithmetic Mean (Garcia-Vallve, 1999) with DendroUPGMA program (http://genomes.urv.cat/UPGMA/).

RESULTS AND DISCUSSION

Genetic studies of *Orobanche* populations are of great importance, for the reason that understanding the variability within and between the populations of these pathogens is essential if selection programs tend to develop resistance sources. Some efforts have been made so far to analyze genetic diversity among Moldovan broomrape populations using RAPD (Duca et al., 2013) and SSR markers (Duca et al., 2017c).

ISSR analysis

In the aforementioned research, the genetic variability of 39 broomrape populations was investigated using 14 ISSR primers. All of the primers generated 132 clear and reproducible bands (mean = 9.4/primer), 74.7% of which were polymorphic (Table 3). Electrophoretic analysis revealed that primer 807 generated the largest number of loci (20), being the most informative, whereas primer $(CA)_6AC$ produced the minimum number of fragments (5).

To assess the differences between markers, the percentage of polymorphic bands and the PIC index was calculated. Different primers showed a different level of polymorphism (P). Thus, polymorphism had values between 22% for primer 810 and 100%, in case of primers 807, $(GACA)_4$, $(CTC)_4RC$, $(CAG)_5$, $(CT)_8TC$, values, which according to the method of data analysis (Calderini et al., 1999), can be considered statistically significant.

The value of the PIC index ranged from 0.32 for primer $(CA)_6AC$ to 0.46 for 807, average being 0.40 (Table 3 and Figure 1). The comparative analysis of generated loci number and the value of the PIC index revealed a strong correlation (Spearman correlation coefficient value of 0.84) between these two indices (Figure 1).

Table 3. Nucleotide sequence of selected primers with the number of amplified products and fragment size range (bp)

No.	Primer name	Nr. of loci generated	The range of length of the amplified bands (min. max., bp)	PIC	P, %
1.	807	20	516-2736	0.46	100
2.	810	9	607-2680	0.23	22.0
3.	835	6	807-3000	0.37	83.3
4.	841	10	354-2467	0.39	60.0
5.	857	9	375-3900	0.44	88.8
6.	$(CAA)_5$	6	888-3000	0,39	83.3
7.	$(GACA)_4$	9	932-4437	0.41	100
8.	$(GATA)_4$	9	835-3195	0.48	66.6
9.	$(CA)_6RG$	10	750-5160	0.44	70.0
10.	$(CTC)_4RC$	14	403-4200	0.48	100
11.	$(CAG)_5$	7	1039-2686	0.46	100
12.	$(CT)_8TC$	6	1240-4566	0.44	100
13.	$(CA)_6AC$	5	913-1979	0.32	40.0
14.	$(AG)_8YA$	12	392-3394	0.28	58.3
Total		132	-	-	-
Average				0.40	74.7

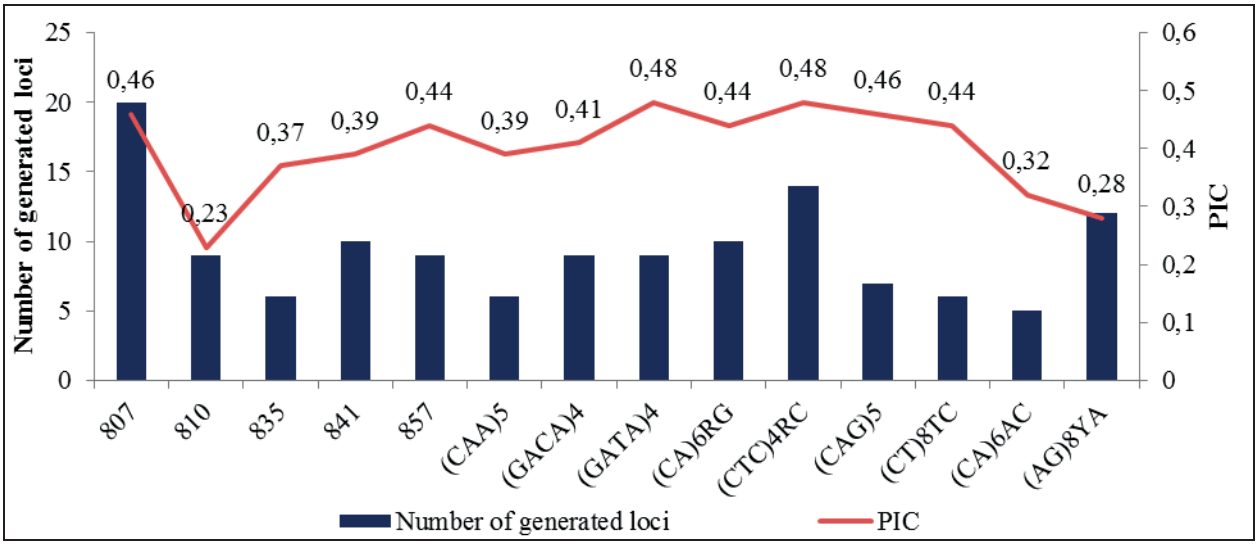

Figure 1. The correlation between the PIC value and the number of loci detected per primer

Interpopulation genetic diversity

The populations used in this study demonstrated different levels of genetic variability, evidenced by the application of one or the other primer. A pronounced (from 58% to 100%) level of genetic polymorphism between populations was established. Molecular genotyping with selected primers allowed the detection of common, polymorphic and specific DNA fragments (Table 4, Figure 2).

Thus, the presence of 7 common DNA fragments for all populations, where obtained with primers 841 (*926, 1170, 1432 bp*), $(GATA)_4$ (*835 bp*), $(CA)_6RG$ (*750, 1584 bp*), and $(CA)_6AC$ (*1062 bp*). These PCR results show the existence of conserved regions in the broomrape genome of most or all studied populations.

Each ISSR primer, with the exception of primer 807, identified both specific (present in a small number of populations) and unique loci (present only in one accession).

This may be due to both the relatively low lot of samples and their large heterogeneity.

Table 4. Common and specific DNA fragments generated by the ISSR primer set

No.	Name primer	Common loci	*O. cumana* populations	Specific Loci	*O. cumana* populations
1.	807	-		-	
2.	810	**846** **1092** **1762** **2193**	All, except pop. Chirsova, Beşalma, Alexanderfeld	**2680**	Svetlîi, Alexanderfeld, Manta, Slobozia Mare, Crihana Veche
3.	835	**1289** **1548**	All, except pop. Alexanderfeld All, except pop. Grigorievca	**1195**	Sîngera, Svetlîi, Gura Galbenei, Cimişlia, Ermoclia, Slobozia Mare
4.	841	**926** **1170** **1432**	All All All	**345** **814**	Fundul Galbenei, Gura Galbenei, Congaz, Ciadîr Lunga, Crihana Veche Fundul Galbenei, Gura Galbenei, Congaz
5.	857	-	-	**375** **672** **2000**	Donduşeni Donduşeni Crihana Veche, Alexanderfeld
6.	$(CAA)_5$	-	-	**1450**	Bălţi
7.	$(GACA)_4$	-	-	**1301** **1480** **1772** **2155** **2774**	Chişinău Donduşeni Donduşeni Svetlîi Donduşeni, Sîngera, Chişinău, Cimişlia, Ermoclia
8.	$(GATA)_4$	**835** **1126** **1593**	All All, except pop. Grigorievca All, except pop. Grigorievca	**2567**	Chişinău, Sărata Mereşeni, Cimişlia, Ermoclia
9.	$(CA)_6RG$	**750** **1584** **1978**	All All All, except pop. Beşalma, Carabetovca, Prepeliţa, Alexanderfeld	**885** **1417**	Sîngera, Gura Galbenei Rassvet, Holercani
10.	$(CTC)_4RC$	-	-	**403** **1065** **1191** **2815**	Soroca Donduşeni, Soroca, Căzăneşti Donduşeni, Căzăneşti, Alexanderfeld Căzăneşti
11.	$(CAG)_5$	-	-	**1039** **1583**	Svetlîi, Manta, Slobozia Mare, Crihana Veche, Manta, Slobozia Mare, Crihana Veche
12.	$(CT)_8TC$	-	-	**4566**	Donduşeni
13.	$(CA)_6AC$	**913** **1062** **1163**	All, except pop. Donduşeni All All, except pop. Beşalma, Alexanderfeld	**1625**	Costuleni, Ciocîlteni, Rassvet, Frăsîneşti, Izbişte
14.	$(AG)_8YA$	**778** **940**	All, except pop. Congaz, Carabetovca	**392**	Donduşeni, Bălţi, Frăsîneşti

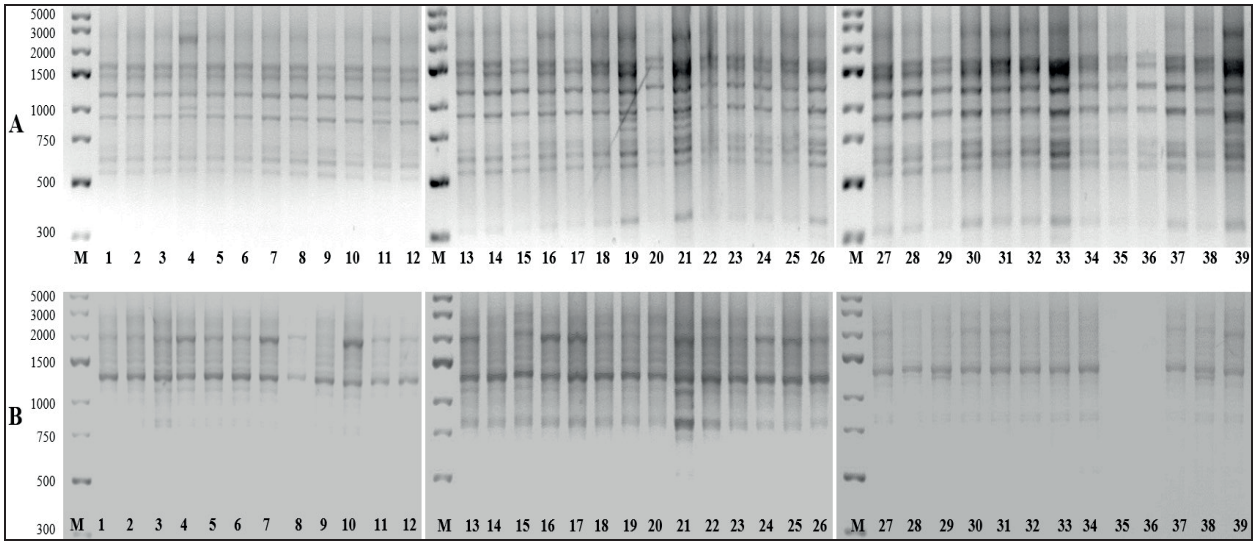

Figure 2. Electrophoretic analysis of DNA fragments amplified of *O. cumana* populations with 841 (A) and 835 (B) ISSR primers
1-Donduşeni; 2-Soroca; 3-Bălţi; 4-Verejeni; 5-Căzăneşti; 6-Brînzenii Noi; 7-Costuleni; 8-Ciocîlteni; 9-Rassvet; 10-Frăsîneşti; 11-Izbişte; 12-Holercani; 13-Sîngera; 14-Chişinău; 15-Băcioi; 16-Floreni; 17-Buţeni; 18-Sărata Mereşeni; 19-Fundul Galbenei; 20-Cazangic; 21-Gura Galbenei; 22-Cimişlia; 23-Ermoclia; 24- Talmaza; 25-Ştefan-Vodă; 26-Congaz; 27-Chirsova; 28-Beşalma; 29-Svetlîi; 30-Carabetovca; 31-Prepelița; 32-Corten; 33-Ciadîr-Lunga; 34-Taraclia; 35-Grigorievca; 36-Alexanderfeld; 37-Manta; 38-Slobozia-Mare; 39-Crihana Veche.

Five of the investigated primers generated 10 unique amplicons for certain broomrape accesses, including 857 *(375 bp* and *672 bp)* at population from Donduşeni, $(CAA)_5$ *(1450 bp)* at population from Bălţi, $(GACA)_4$ at populations from Chişinău *(1301 bp)*, Donduşeni *(1480 bp* and *1772 bp)* and Svetlîi *(2155 bp)*, $(CTC)_4RC$ at populations from Soroca *(403 bp)* and Căzăneşti *(2815 bp)*, $(CT)_8TC$ at population Donduşeni *(4566 bp)*. The majority (5) unique amplified fragments belong to the population collected from Donduşeni, in the northern region of Moldova. These unique sites represent a particular interest and can be used as a molecular tool in genotyping and differentiation of populations.

The analysis of the electrophoretic profiles clearly revealed that some populations (especially Donduşeni, Svetlîi, Gura Galbenei, Slobozia Mare, Crihana Veche, Ermoclia, Cimişlia, Alexanderfeld) showed distinct profiles. All of the populations, which revealed the most pronounced differences in ISSR profiles, were collected from the southern region (Figure 3).

The maximum number of bands (88) was identified in the population of *O. cumana* from Gura Galbenei. A considerably low number of amplicons (35) was confirmed, following the PCR reaction, in the Beşalma geographic population of broomrape.

Comparing results obtained in electrophoretic spectrum it was observed that most populations of the central region (68.75%) were characterized by the presence of more than 70 bands. This indicates a greater genetic diversity of populations in this region compared to other studied regions. In contrast to the accession of *O. cumana* collected from the central region of the country, 42.1% of the populations of the southern part confirmed the presence of more than 70 bands and 57.9% indicated a total less than or equal to 70 bands (Figure 3).

Thus, data from ISSR analysis widely confirmed previous observations based on SSR markers (Duca et al., 2017c).

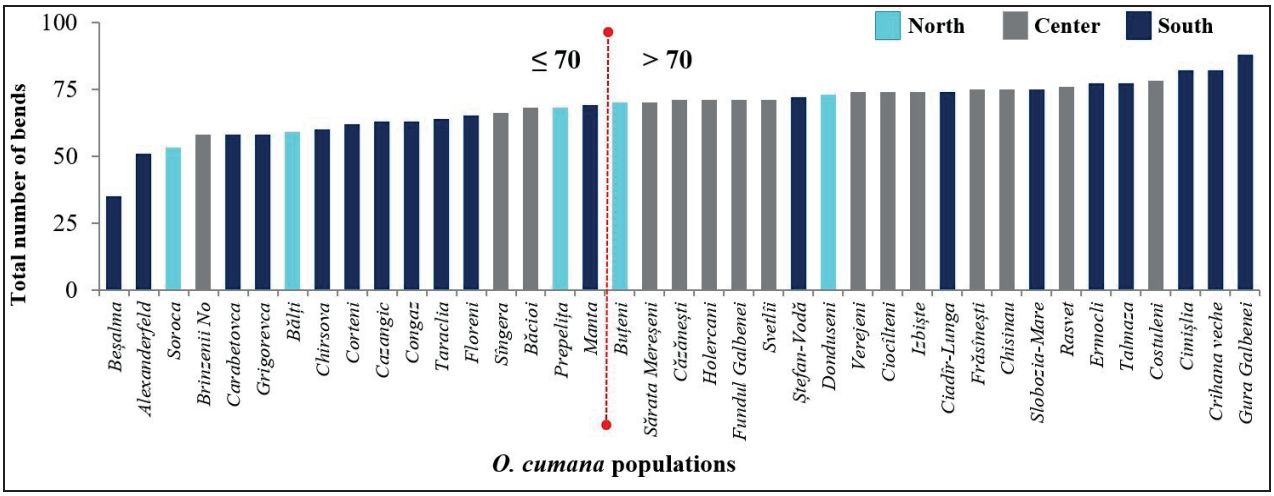

Figure 3. Total number of fragments generated by ISSR markers in *O. cumana* populations

Analysis of genetic distances and UPGMA clustering

The genetic similarity matrix was estimated by the Jaccard coefficient. Based on this matrix, the populations were grouped using the UPGMA method. The cophenetic correlation coefficient was high (CP = 0.86) and significant.

Generally, the genetic distance between the broomrape accessions ranged from 0.17-0.81. Data shows that the lowest value of the genetic distance was found between the populations of Ermoclia (South) and Buţeni (Center) and the highest in the populations of Beşalma (South) and Bălţi (North).

The dendrogram revealed a separated genetic group of *O. cumana* populations from the Republic of Moldova according to the genetic distance (Figure 4). Thus, 39 populations were predominantly grouped into 11 small subclusters, containing two populations: Ciadîr Lunga with Taraclia (GD=0.25); Corteni with Prepeliţa (GD=0.17); Manta with Crihana Veche (GD=0.22); Sărata Mereşeni with Fundul Galbenei (GD=0.22); Gura Galbenei with Cimişlia (GD=0.27); Buţeni with Ermoclia (GD=0.19); Băcioi with Floreni (GD=0.25); Rassvet with Frăsîneşti (GD=0.30); Costuleni with Ciocîlteni (GD=0.23); Vejereni with Căzăneşti (GD=0.23); Bălţi with Izbişte (GD=0.30).

However, the dendrogram highlights the existence of two big clusters A and B which include 34 of the 39 studied populations. Four populations (Beşalma, Alexanderfeld, Soroca and Congaz) where clearly isolated, demonstrating their genetic divergence from the majority of accessions by counting allelic substitutions per locus that occurred within each population (Figure 4).

In other studies (Ciucă et al., 2004) the dendrogram did not reveal a clear association of populations with the geographic location. However, in our study certain regularities could be observed in respect to the aforementioned. We can establish a differentiation of populations from the Central and Southern regions in previously mentioned two clusters, which allows us to draw conclusions about population differentiation by geographic region.

So, cluster A includes 11 populations collected from South region, except population from Prepeliţa (North). Cluster B includes 24 *O. cumana* accesses (2 - North, 16 - Center and 6 - South), grouped in many subclusters. A similar result appears in subcluster B_1, which includes 9 populations from central and 1 population from North region (Bălţi) and subcluster B_2 with 5 populations collected from South and 1 (Buţeni) – from Central region of the Republic of Moldova.

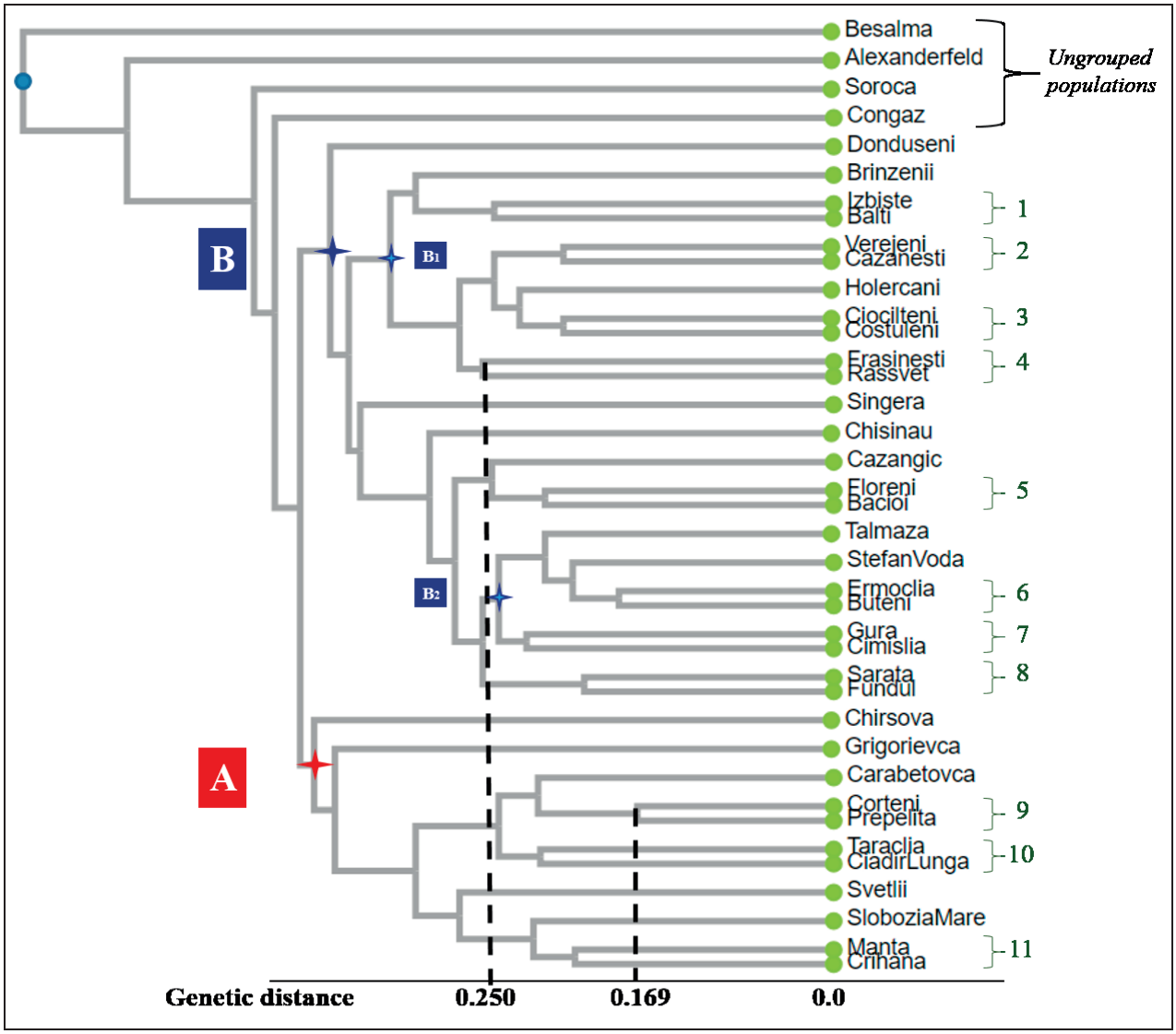

Figure 4. UPGMA dendrogram using Jaccard's genetic distances among broomrape populations from the Republic of Moldova *(CP = 0.86)*

In most cases there is a clear distribution trend according to the geographical area, which indicates that this is a distance-based isolation. The results are consistent with those obtained by several other researchers (Pineda-Martos et al., 2013; Guchetl et al., 2014), using other molecular markers.

CONCLUSIONS

The study of genetic polymorphism in 39 *Orobanche cumana* populations through the application of 14 ISSR primers made it possible to group them into different clusters in accordance with geographical origin. According to the results, the populations of *Orobanche cumana* in the Republic of Moldova have high heterogeneity and major differences at the molecular level.

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