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Molecular Characterization of Broomrape Populations from Republic of Moldova using SSR Markers

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Abstract: The genetic diversity study of 39 broomrape populations from the Republic of Moldova was performed using 12 highly polymorphic SSR primer pairs, which shown the high level of polymorphism (average PIC value 0.57). We found that some of the SSR primers (Ocum-59 and Ocum-108) produced polymorphic bands suitable for discrimination between the studied populations. The diversity analysis within broomrape populations revealed a higher number of detected alleles and heterozygous loci in the accessions from the Southern region when compared to the Northern and Central ones. The average PIC values for the Northern, Central and Southern accessions ranged from 0.43, 0.48 to 0.56, respectively. Some populations from the Southern region (especially, Carabetovca, Alexanderfeld, Stefan-Voda and Slobozia Mare) have shown the major differences in the profiles obtained and presented the high level of genetic variability. The dendrogram based on genetic distance divided the 39 broomrape accessions into twelve clusters. High variability of *O. cumana* populations at molecular level was observed, which could be useful for further genetic studies and resistance-breeding programs.

Keywords: broomrape, genetic study, intrapopulational variability, molecular characterization, Orobanche cumana, SSR markers

Introduction

The Orobanchaceae family includes 15 genera. The largest one is genus *Orobanche*, which contains more than 200 species, without chlorophyll and

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parasitizing roots on a wide range of host plants (Pusch and Gunther, 2009). The highest diversity of *Orobanche* species is observed in the Northern hemisphere and especially around the Mediterranean area due to its warmer climate (Schneider *et al*., 2016).

The first studies regarding inter- and intraspecific variability of holoparasitic plants were focused on morphological characteristics. Pollen (Piwowarczyk *et al*., 2015; Zare *et al*., 2014) and seed fenotype (Piwowarczyk, 2015; Plaza *et al*., 2004; Domina and Colombo, 2005; Duca *et al*., 2014) for example, are widely used for differentiation within and between *Orobanche* spp. However, the recent studies on *O. cumana* seed micromorphology did not reveal any intraspecific differences and did not provide any significant criteria for geographical distribution or race identification of pathogen (Krupp *et al*., 2014).

The protein markers (Verkleij *et al*., 1986, 1991) represent another approach to evaluate the diversity of *Orobanche* spp .(*O. crenata* and *Phelipanche aegyptiaca*). Nevertheless, protein markers have a series of drawbacks and limitations, such as the influence of environmental conditions and development stage on variations at the protein level (Román, 2013).

The methods based on PCR, such as RAPD, SSR ISSR, etc. are more informative for the inter- and intraspecific genetic variability determination of broomrape populations.

RAPD markers were applied for interspecific discrimination between the representatives of genus *Orobanche* (Atanassova *et al*., 2005) and intraspecific polymorphism determination. Thus, using 173 RAPD primers and different populations of *O. cumana* from Bulgaria, Romania, Turkey and Spain, two distinct genetic groups were revealed (Spanish and Eastern Europe). These have high genetic similarity and presumably monophyletic origin (Gagne *et al*., 1998). In case of broomrape populations from the Republic of Moldova, Ukraine and Romania clear correlation was also established between the molecular pattern and geographical distribution (Glijin *et al*., 2014). Meanwhile, the analysis of genetic polymorphism of *O. cumana* from Romania, Spain and former Yugoslavia using RAPD markers revealed a low polymorphism of populations studied and no correlation with regard to the geographical distribution (Cuica *et al*., 2004).

Some studies support the idea that ISSR markers are valuable tool in the identification of closely related *Orobanche* species, such as *O. cumana* and *O. cernua* (Benharrat *et al*., 2002) or several distant *Orobanche* species, such as *O. ramosa* (Buschmann *et al*., 2005), *O. crenata* (Román *et al*., 2002) and representatives of genus *Orobanche* subsection Minores (Stoyanov *et al*., 2012; Hristova *et al*., 2011), as well as for estimation of intraspecific variability.

SSR markers are considered to be the most useful for intraspecific variability studies. Among 18 broomrape populations from different geographical locations and host plants, 79 SSR out of 228 primer pairs developed, have generated reproducible, high quality profiles with amplicons of expected size. Moreover, this study has shown the transferability of these markers to other *Orobanche* species, such as *O. cernua* (Pineda-Martos *et al.*, 2014). Subsequently, the investigation of a larger set of 50 populations from Spain with 15 highly polymorphic SSR markers has demonstrated differences between two distinct groups of population from two geographical regions: Cuenca province and Guadalquivir Valley (Pineda-Martos *et al.*, 2013).

The aim of the current study is to estimate the genetic diversity and genetic structure of different broomrape populations in order to reveal new information about races and evolution of this pathogen, which could potentially be useful for breeders and sunflower seed producers to improve their control strategies.

Materials and methods

Biological material

In present work the genetic variability of 39 broomrape populations gathered on the sunflower agricultural fields from Republic of Moldova were evaluated (Table 1). The seed of these populations (150 mg of broomrape per 1 kg of soil in 1 liter plastic pots) were used in the greenhouse experiment with sunflower susceptible genotype LMD1 offered by Limagrain company (Acciu, 2016). Aerial shoots from each population were frozen in liquid nitrogen and stored at −80 °C.

Table 1: Geographical origin and year of collection of 39 broomrape populations.

Northern part	Central part	Southern part
2009 (Balti) 2012 (Donduseni) 2014 (Soroca, Prepelita)	2011 (Chisinau) 2012 (Singera, Bacioi) 2014 (Verejeni, Cazanesti, Brinzenii Noi Costuleni, Ciocilteni, Rassvet, Frasinesti, Izbiste, Holercani, Floreni, Buteni, Sarata Mereseni, Fundul Galbenei)	2006 (Stefan-Voda, Ciadir-Lunga) 2008 (Cimislia) 2009 (Taraclia) 2014 (Cazangic, Gura Galbenei, Grigorievca, Ermoclia, Talmaza, Congaz, Chirsova, Besalma, Svetlii Carabetovca, Corten, Alexanderfeld, Manta, Slobozia-Mare, Crihana Veche)

DNA extraction and SSR amplification

DNA isolation and purification was performed using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. For estimation of genetic diversity of populations studied, 15 highly polymorphic SSR primer pairs (Ocum-52, -59, -70, -74, -75, -81, -87, -108, -122, -141, -160, -174, -196, -197 and -206) previously reported in literature (Pineda-Martos *et al.*, 2013) were used. PCR was realized with the following concentrations of components in the reaction mixture: 200 µM dNTP, 2.0 mM $MgCl_2$, 1.0 unit of DreamTaq Green DNA Polymerase (Thermo Scientific), 0.4 µM of each primer and 50 ng of extracted DNA. Reaction volume was 15 µl. PCR was performed in Veriti Termocycler (Applied Biosystems) with the cycling conditions: 95 °C – 3 min; 35 cycles of 95 °C – 30 s, 57 °C – 45 s, 72 °C – 1 min; 72 °C – 5 min. The results were visualized in 8 % polyacrylamide non-denaturing gels with TBE buffer.

Polymorphic Information Content (PIC) estimation and clustering analysis

The polymorphism level for each primer pair was established using PIC determination, calculated according to Anderson *et al.* (1993): $PIC = 1 - \sum_{i=1}^{n} p_i^2$, where p_i is the frequency of the i-th band and n is the total number of detected bands. Based on the data obtained, the binary matrix was created, where the symbol "[1]" indicated the presence and "[0]" – absence of the band. Genetic similarity and genetic distance were calculated based on Jaccard similarity index using D-UPGMA free software (Garcia-Vallve *et al.*, 1999). The same software was applied for UPGMA (Unweighted Pairwise Group Method with Arithmetic Mean) clustering.

Results and discussions

Characterization of markers used in study

SSR markers used for the genetic diversity assessment of local broomrape populations were perfect dinucleotide, trinucleotide, tetranucleotide and hexanucleotide repeats (Table 2). The primers for tri-, tetra- and hexanucleotide

Table 2: Polymorphism of studied loci in investigated genotypes.

Locus	Motif	Allele no.	Detected alleles		PIC*			
			Common alleles	Uncommon alleles	N	C	S	M
Ocum-52	$(AG)_{10}$	4	122/129	106/132	0.44	0.62	0.63	0.60
Ocum-59	$(TC)_{11}$	4	87/89/93	97	0.00	0.69	0.70	0.68
Ocum-70	$(TG)_{11}$	5	104/109	100/110/115	0.69	0.00	0.55	0.55
Ocum-74	$(GA)_{12}$	5	113/119	97/115/122	0.75	0.65	0.57	0.63
Ocum-81	$(CA)_{13}$	8	87/91	75/89/94/106/109	0.49	0.69	0.63	0.65
Ocum-87	$(TTC)_{13}$	3	105/124	131	0.59	0.00	0.52	0.52
Ocum-108	$(GTAT)_6$	4	81/140	107/129	0.50	0.46	0.66	0.60
Ocum-122	$(AGTGTG)_6$	3	230	241/341	0.00	0.36	0.19	0.27
Ocum-141	$(CTT)_6$	2	182/192	–	0.50	0.40	0.35	0.41
Ocum-174	$(AAG)_7$	4	187/202/213	190	0.50	0.64	0.55	0.61
Ocum-197	$(GA)_7$	5	105/111/116/121	96	0.72	0.76	0.76	0.77
Ocum-206	$(TG)_8$	3	120/127	116	0.00	0.50	0.55	0.53
MEAN		**4.17**	–	–	**0.43**	**0.48**	**0.56**	**0.57**

Notes: N – PIC for Northern region, C– PIC for Central region, S – PIC for Southern region populations.

repeats generated clearer electrophoresis profiles, with greater allele size differences, than those for dinucleotides, similarly reported in the other studies (Weising *et al.*, 2005). Three primers Ocum-75, Ocum-160 and Ocum-196 were excluded from this study due to the stuttering and overloaded profiles difficult to analyze.

The studied primers showed different level of polymorphism and produced a total number of 50 alleles among *O. cumana* populations with the average 4.17 (Table 2; Figure 1). The number of detected alleles per locus in various accessions ranged from two (Ocum-141) to eight (Ocum-81). Thus, SSR markers revealed a higher number of alleles per locus, whereas in other similar studies evaluating populations from Spain (Pineda-Martos *et al.*, 2013) or Russia, Kazakhstan and Romania (Guchetl *et al.*, 2014), the reported primers have produced lower average alleles number values (2.2 and 2.3, respectively).

Each SSR primer pair produced alleles/bands with high and low frequency (Table 2). The microsatellites investigated have exhibited one allele (105 bp for Ocum-87) that was common among all the broomrape accessions.

The uncommon alleles are of great interest for distinguishing between the populations studied (Table 2). Two alleles, 106 bp and 109 bp, generated by Ocum-81 were specific for population from Svetlii. Also, allele of 131 bp

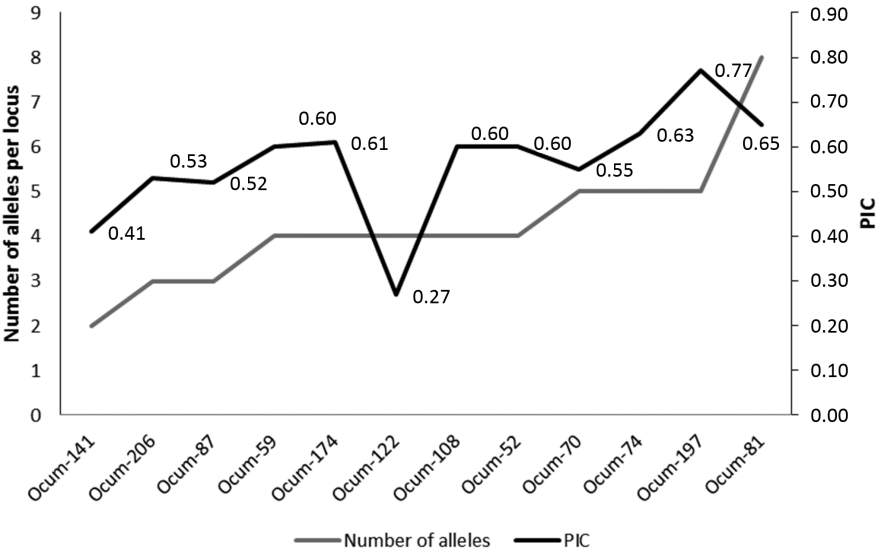

Figure 1: Correlation between PIC value and number of alleles per locus.

exhibited by Ocum-87 were specific only for populations from Prepelita and Slobozia Mare. Population from Alexanderfeld showed 100 bp specific allele generated by Ocum-70.

The PIC ranged from 0.27 (Ocum-122) to 0.77 (Ocum-197) with a mean value 0.57 (Table 2). These values were different from those reported by Pineda-Martos *et al.* (2014) – from 0.33 to 0.37, which may be due to the different origin of broomrape populations and detection system used in the study (Pineda-Martos *et al.*, 2014). However, Liu *et al.* (2011) argued that the PIC value for any SSR locus is not constant and only presents a reference for the relative ability of that marker to attest genetic diversity (Liu *et al.*, 2011).

The medium correlation between PIC value and the number of alleles per locus (Spearman`s rank correlation coefficient 0.47) was observed. The results obtained could be explained by the presence of such cases as Ocum-197 (five alleles with the highest PIC 0.77) and Ocum-122 (the lowest PIC 0.27 with three alleles) (Figure 1).

It should be mentioned that primer pairs Ocum-59 and Ocum-108 which generated low number of bands for each population but were highly polymorphic, are more useful for the discrimination between populations than primers with overloaded profile (2-3 major bands and a number of minor bands) such as Ocum-52 and Ocum-74 (Figure 2).

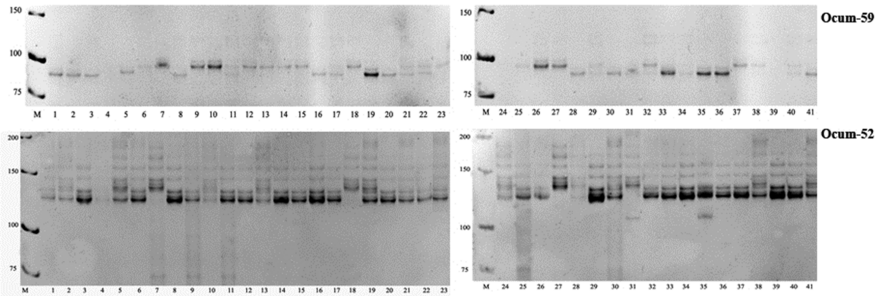

Figure 2: Results of amplification with Ocum-59 and Ocum-52 primer pairs.

Genetic diversity

The populations used in this study have demonstrated the different level of genetic variability, highlighted by application of one or another primer. The analysis of the electrophoretic profiles obtained has clearly elucidated that some populations (mainly, from Carabetovca, Alexanderfeld, Stefan-Voda and Slobozia Mare) showed the distinct profile, different from that of the other populations in case of several primer pairs (3-5 primers from 13). All populations, which revealed the most pronounced differences in SSR profile, were collected from the Southern region.

Generally, the total number of generated bands per population has varied from 17 (Congaz and Gura Galbenei) to 24 (Singera and Carabetovca). Comparing the results obtained in the gel electrophoresis for populations from the Northern region of the Republic of Moldova with those from the Central and Southern parts, it was observed that most of populations from the Central part (68.75%) were characterized by the presence of more than 20 bands. This indicates the higher genetic diversity of populations from this geographical region, as compared to the populations from the other ones. For example, all the populations collected from the North of RM have demonstrated a total number of bands lower or equal to 20. In contrast to *O. cumana* collected from the Center and the North, among 19 populations from Southern part of RM 52.6% showed total number of bands lower or equal to 20 and 47.4% presence of more than 20 bands. Thereby, this group of populations has an intermediate position between another two groups (Figure 3).

In spite of this, among 50 alleles analyzed in this study, 45 alleles were detected in 19 populations from the South, 37 alleles in 16 populations from the Center and 31 alleles in 4 populations from the North. According to this data it can be concluded that populations from the Southern region showed the greater genetic diversity than other ones. Also, this idea is supported by the average of

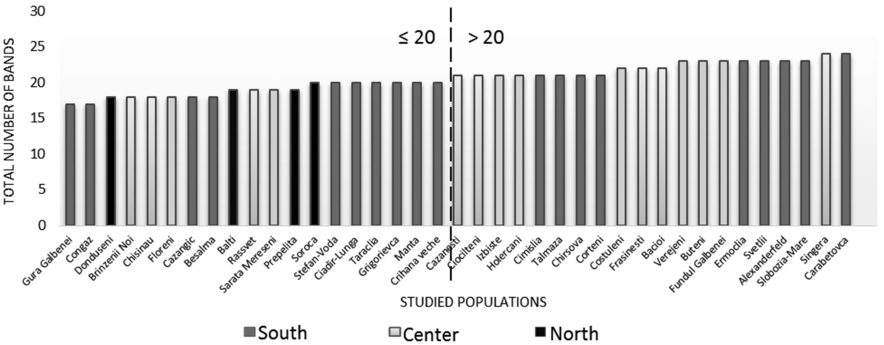

Figure 3: Total number of bands in studied broomrape populations.

PIC values, which were equal to 0.43, 0.48 and 0.56, for the Northern, Central and Southern accessions respectively (Table 2).

These data indicate that broomrape populations from the Northern region had relatively low level of genetic polymorphism comparatively with the accessions from two other regions. The observed higher genetic diversity of populations from the Southern regions of Moldova is in line with the previously obtained data regarding the increased pathogenicity and variability of populations from the South of RM (Duca, 2015; Duca *et al.*, 2015). Also, our previous data have shown that infection with broomrape often preferentially appears in the Central and Southern regions of Moldova, which explains the differences in the number of collected populations used in our study (5 from the Northern part, 17 from the Center and 19 from the Southern part of RM). Such trend in geographical distribution of broomrape in Moldova was observed till the 1970s (Sharova, 1969; 1977).

The populations from Verejeni, Cazanesti, Ciocilteni, Frasinesti, Izbiste, Singera, Bacioi, Buteni, Fundul Galbenei, Cimislia, Ermoclia, Talmaza, Chirsova, Carabetovca, Corteni, Alexanderfeld, Manta and Slobozia Mare showed predominantly heterozygous state of the analyzed loci and only 2-3 loci in homozygous state, whereas those from Donduseni, Soroca, Balti, Brinzenii Noi, Rassvet, Chisinau, Floreni, Cazangic, Gura Galbenei, Congaz and Besalma contain an approximately equal number of homo- and heterozygous loci. Generally, 57% from 468 of analyzed cases were assigned to the heterozygous state of locus, 35% to homozygous and 8% represent atypical profile (more than two bands). The predominance of the heterozygous state of the studied loci indicates a higher level of population genetic variability. The atypical pattern of the locus detected e. g., at Carabetovca (Ocum-70, -74 -108), Svetlii (Ocum-81, -108), Alexanderfeld (Ocum-70, -108), presumably, is due to the intrapopulational heterogeneity (bulked DNA samples) or of the duplicated loci.

The heterozygous loci, in almost all the cases, were highlighted by primers Ocum-52, -70, -74, -81, -87, -197, -206. The primers, Ocum-59, -108, -122, -141, -174, predominantly showed the homozygous state of the identified loci. Ocum-87 is of special interest primer because it has generated a heterozygous profile of locus for all the studied populations.

Clustering analysis

The clustering analysis based on the molecular pattern of 39 broomrape populations has resulted in the dendrogram with high cophenetic correlation coefficient (CP = 0.75).

In contrast to the results of other authors, which have shown the clear correlation between the cluster and geographical distribution of analyzed populations (Pineda-Martos *et al.*, 2013), our study did not reveal any direct relationship between these two parameters. The 39 broomrape populations from RM clustered predominantly *in* small groups of two or single-population.

In general it can be highlighted twelve clusters with two populations: Carabetovca with Slobozia Mare (GD = 0.433), Rassvet with Izbiste (GD = 0.182), Grigorievca with Crihana Veche (GD = 0.182), Chirsova with Corten (GD = 0.174), Floreni with Stefan Voda (GD = 0.190), Ciocilteni with Talmaza (GD = 0.091), Singera with Bacioi (GD = 0.231), Cazanesti with Fundul Galbenei (GD = 0.167), Sarata Mereseni with Taraclia (GD = 0.050), Balti with Besalma (GD = 0.150), Soroca with Cimislia (GD = 0.292) and Cazangic with Gura Galbenei (GD = 0.350). The genetic distance between such clusters was varied from 0.050 for Sarata Mereseni with Taraclia to 0.433 for Carabetovca with Slobozia Mare. Other populations were associated to this primary clusters and formed bigger clusters (Figure 4).

Similarly to other research data (Cuica *et al.*, 2004; Guchetl *et al.*, 2014), the dendrogram did not clearly discriminate the populations from Northern, Central and Southern region of RM. However, certain regularities could be observed in the populations' distribution within the clusters. Some of the populations collected from geographically close localities were clustered together (Singera and Bacioi). It should be mentioned that these localities are at a short distance (6–7 km only) and there could be the same population in both localities.

Some of the studied populations have shown the significant differences reflected by the high values of genetic distance, maximal values being attested for Brinzenii Noi and Costuleni (GD = 0.75).

Considering the results obtained for genetic distance, the cluster analysis and molecular pattern, the conclusion could be made that the populations from Republic of Moldova demonstrate major differences on the molecular level and

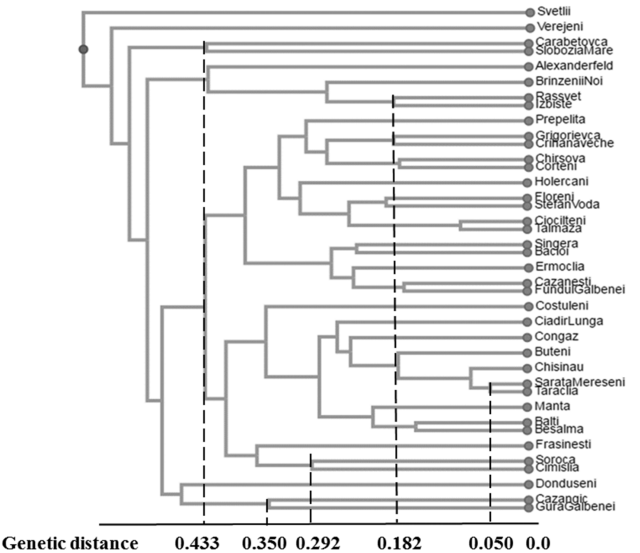

Figure 4: UPGMA dendrogram based on the genetic similarity matrix for 39 broomrape populations.

high heterogeneity. These results support the idea that the broomrape populations from the former Soviet Union countries, due to a period of isolation, have developed the specific gene pool, different from the European populations (Guchetl *et al.*, 2014).

Conclusions

The study performed for 39 broomrape populations using SSR primers, has revealed the different level of the genetic variability of studied accessions. Nevertheless, it was clearly observed that populations from the Southern region of RM, characterized by the presence of higher number of detected alleles and heterozygous loci, are more polymorphic than those from the Northern and Central regions. This information could be useful in any attempt to develop resistance-breeding programs.

The average PIC values for Northern, Central and Southern accessions were 0.43, 0.48 and 0.56, respectively. Some populations from the Southern region (especially, Carabetovca, Alexanderfeld, Stefan-Voda and Slobozia Mare) have shown the major differences in the profiles obtained and have presented the

high level of genetic variability. Another significant finding in the current study is the population-specific alleles found noticeably in the local accessions: Prepelita (North region), Rassvet and Verejeni (Central region), Slobozia-Mare (South), which demonstrate that these populations are distinct from the other. The clustering data based on the molecular patterns did not show clear distribution according to geographical region, however, some populations collected from geographically close localities (Singera and Bacioi, GD = 0.231) were clustered together.

This study of population variability of this crop pathogen is of great importance and advances the understanding of the variability between accessions in order to identify the pathogenic groups in parasite population and for comprehension of the sunflower broomrape phylogeography on the entire territory of the country.

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