

Genetic Diversity of *Orobanche cumana* Populations in Serbia

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In this study, we report genetic characterization of *Orobanche cumana*, the causal agent of sunflower wilting in Serbia. The genetic diversity of this parasitic plant in Serbia was not studied before. Random amplified polymorphic DNA (RAPD) markers and partial *rbcL* gene sequences analysis were used to characterize the *O. cumana* populations at the molecular level. While phylogenetic analyses of RAPD-PCR amplicons were performed using unweighted pair-group Method analyses, *rbcL* gene sequences were analyzed using neighbor joining method and minimum spanning tree. Molecular analyses of RAPD-PCR analysis revealed high genetic diversity of *O. cumana* populations which indicated high adaptive potential of this parasitic weed in Serbia. Further analyses of *rbcL* gene using minimum spanning tree revealed clear differences among diverse sections of *Orobanche* genus. Although this molecular marker lacked the resolution to display intrapopulation diversity it could be a useful tool for understanding the evolution of this parasitic plant. Our results suggested that *O. cumana* has great genetic potential which can lead to differentiation of more virulent races which is

important for determining crop breeding strategies for their control.

Keywords : intrapopulation variation, phylogeny, RAPD, *rbcL*, sunflower broomrape

Orobanche cumana Wallr. (broomrape) is a holoparasitic plant found as the main parasitic plant on sunflower roots in many countries, causing severe yield losses in sunflower crops (Fernandez-Aparicio et al., 2016; Pineda-Martos et al., 2013). Successful infection of *O. cumana* suppress host defense responses and turned host roots to a source for efficient nutrient for the parasite (Yang et al., 2020). They are native primarily in the Mediterranean region (i.e., North Africa, the Middle East, and southern Europe), as well as western Asia, where they cause significant crop damage (Parker and Riches, 1993). *O. cumana* has seriously devastated sunflower producing countries in central and eastern Europe as well as Mediterranean countries causing great economic losses (Calderón-González et al., 2019). In Serbia, broomrape was first discovered in 1951 by Aćimović (1977) and has appeared almost every year with varying intensity since then (Maširević and Medić-Pap, 2009).

In Serbia, *O. cumana* can be found in the different areas where sunflower is intensively cultivated, primarily in Bačka and Banat regions (Maširević and Medić-Pap, 2009). Due to drastic reduction in morphological characters commonly used in taxonomic identification, difficulties in species identification within the genus *Orobanche* are recognized (Manen et al., 2004). Recently a new race of *O. cumana* was discovered with no difference with other *O. cumana* races, but with ability to parasitize beside sunflower and with host-range expanding to Solanaceae crops (Dor et al., 2020). In order to get better identification of *O. cumana*

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species molecular identification is necessary.

To improve species identification and phylogenetic studies molecular analyses of several plastid genes were conducted (Manen et al., 2004; Wolfe and dePamphilis, 1997; Young et al., 1999). Molecular studies on genetic diversity within and among populations of *O. cumana* were performed in Spain (Castejón-Muñoz et al., 1991; Malek et al., 2017; Martín-Sanz, 2016; Pineda-Martos et al., 2013), as well as between population from Romania, Bulgaria, and Turkey (Gagne et al., 1998). Genetic studies of *O. cumana* diversity and race composition were performed in Tunisia, Turkey, and Moldova (Bilgen et al., 2019; Duca et al., 2017, 2019; Jebri et al., 2017). The random amplified polymorphic DNA (RAPD) markers are often used to detect variability within an *Orobanche* population (Atanasova et al., 2005; Katzir et al., 1996; Paran et al., 1997; Williams et al., 1990). Polymorphic amplification products were found useful in detection of small genetic differences among individuals and in distinguishing populations (Gagne et al., 1998; Joel et al., 1998; Roman and Rubiales, 1999). The genus *Orobanche* consists of 170 species, grouped in four sections: sect. *Orobanche* (=sect. *Osproleon* Wallr.), sect. *Trionychon* Wallr., sect. *Gymnocaulis* Nutt., and sect. *Myzorrhiza* (Philippi) Beck. (Manen et al., 2004). *Orobanche* species were also investigated using the *rbcL* nucleotide sequence as a molecular marker for identification and better understanding of phylogenetic relations (Benharrat et al., 2000; Manen et al., 2004; Wolfe and de Pamphilis, 1997).

To study variability within and between populations of *O. cumana*, genetic DNA variations among individuals were investigated, using RAPD markers and *rbcL* gene sequences. The tested hypotheses were that (1) there is a genetic diversity in the *O. cumana* population from Serbia since growing areas of sunflower are located in diverse geographical regions and (2) analysis of partial *rbcL* gene sequences could provide better understanding of genetic relatedness among *O. cumana* collected in Serbia and with different *Orobanche* species.

Materials and Methods

Plant material. *O. cumana* plants were collected from infested sunflowers in the main agricultural sunflower-growing areas in four districts and 11 locations in Serbia (Table 1). Three fields were sampled per location, and for each field from 50 to 300 mature *O. cumana* plants were collected at equal distances across a diagonal transect of the field. Plants collected in one or three fields in the same locality were assigned as population. Samples were placed

Table 1. Area of origin and codes of *Orobanche cumana* populations used in study

Area of origin	Districts	Location	Code
Eastern Serbia	Bor District	Negotin	OC-3
Northern Serbia	South Banat	Vršac	OC-2
Northern Serbia	North Bačka	Subotica	OC-4
Northern Serbia	North Bačka	Subotica	OC-7
Northern Serbia	North Bačka	Subotica	OC-10
Northern Serbia	North Bačka	Feketić	OC-6
Northern Serbia	North Bačka	Feketić	OC-9
Northern Serbia	North Bačka	Feketić	OC-12
Northern Serbia	West Bačka	Kula	OC-5
Northern Serbia	West Bačka	Kula	OC-8
Northern Serbia	West Bačka	Kula	OC-11

in separate paper bags and stored in a field refrigerator at 4°C until returning to the lab. Seeds were separated from the plants and stored in glass jars at 4°C in the dark.

DNA extraction, amplification, and electrophoresis.

Genomic DNA was extracted from plant dried material using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer protocol. Most *rbcL* sequences were amplified with primers RH1 and 1352R (Wolfe and dePamphilis, 1997). PCR reactions were performed in a volume of 25 µl PCR reaction mixture for *rbcL* consisted of 0.64 µM of each primer, 1 U *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCL pH 9.0, 0.1% Triton X-100; Promega, Madison, WI, USA), 2.0 mM dNTPs, 2.0 mM MgCl₂, and 0.0125 units of *Taq* DNA polymerase (Promega) and 30 ng of genomic DNA. PCR conditions were set according to the protocol described by Wolfe and dePamphilis (1997).

The resulting PCR products were separated by electrophoresis on 1% agarose gels in 0.5× TAE buffer for 2 h at 5 V/cm and the amplicons were purified (purification kit, Qiagen) for sequencing (Macrogen, Seoul, Korea). Sequences were compared with other related sequences of *O. cumana* representatives using BLAST program of NCBI for primary identification. *O. cumana* sequences were deposited into NCBI GenBank. The list of *O. cumana* DNA accession numbers and list of different *rbcL* gene sequences obtained from the NCBI database, including 30 *Orobanche* species are provided in Table 2.

RAPD analysis. PCR reactions were performed in 25 µl reaction volume containing 30 ng of genomic DNA, 0.2 mM of the dNTPs (Fermentas, Lithuania), 0.5 µM of primer, 50 mM KCl, 10 mM Tris, 1.5 mM MgCl₂, and 1

Table 2. *Orobanch*e specimens used in this study their geographic origin, reference and GenBank accession numbers

Species names	Area of origin	Reference	GenBank accession no.
<i>O. cumana</i> (OC – 2)	Vršac	This study	KP222282
<i>O. cumana</i> (OC – 3)	Negotin	This study	KP222283
<i>O. cumana</i> (OC – 4)	Subotica	This study	KP222284
<i>O. cumana</i> (OC – 5)	Kula	This study	KP222285
<i>O. cumana</i> (OC – 6)	Feketić	This study	KP222286
<i>O. cumana</i> (OC – 7)	Subotica	This study	KP222287
<i>O. cumana</i> (OC – 8)	Kula	This study	KP222288
<i>O. cumana</i> (OC – 9)	Feketić	This study	KP222289
<i>O. cumana</i> (OC – 10)	Subotica	This study	KP222290
<i>O. cumana</i> (OC – 11)	Kula	This study	KP222291
<i>O. cumana</i> (OC – 12)	Feketić	This study	KP222292
<i>Orobanche cernua</i>	-	Wolfe and dePaphilis (1997)	U73968
<i>Orobanche cumana</i>	France	Delavault and Thalouran (2002)	AF090349
<i>Orobanche rigens</i>	France	Manen et al. (2004)	AY582259
<i>Orobanche rapum-genistae</i>	France	Manen et al. (2004)	AY582255
<i>Orobanche</i> cf. <i>gracilis</i>	Italy	Manen et al. (2004)	AY582199
<i>Orobanche cumana</i>	France	Delavault and Thalouran (2002)	AF090350
<i>Orobanche coerulescens</i>	Austria	Manen et al. (2004)	AY582190
<i>Orobanche aconiti-lycoctoni</i>	Spain	Manen et al. (2004)	AY582181
<i>Orobanche uniflora</i>	USA	Manen et al. (2004)	AY582180
<i>Orobanche fasciculata</i>	-	Wolfe and dePaphilis (1997)	U73970
<i>Orobanche californica</i>	USA	Manen et al. (2004)	AY582178
<i>Orobanche corymbosa</i>	-	Wolfe and dePaphilis (1997)	U73969
<i>Orobanche alsatica</i>	Austria	Manen et al. (2004)	AY582182
<i>Orobanche amethystea</i>	Spain	Manen et al. (2004)	AY582183
<i>Orobanche artemisiae-campestris</i>	Switzerland	Manen et al. (2004)	AY582184
<i>Orobanche bartlingii</i>	Croatia	Manen et al. (2004)	AY582186
<i>Orobanche caryophyllacea</i>	Georgia	Manen et al. (2004)	AY582187
<i>Orobanche cernua</i> var. <i>cernua</i>	Spain	Manen et al. (2004)	AY582189
<i>Orobanche cernua</i> var. <i>australiana</i>	Australia	Manen et al. (2004)	AY582188
<i>Orobanche crenata</i>	Greece	Manen et al. (2004)	AY582196
<i>Orobanche gracilis</i>	Spain	Manen et al. (2004)	AY582197
<i>Orobanche hederæ</i>	France	Manen et al. (2004)	AY582201
<i>Orobanche lutea</i>	Georgia	Manen et al. (2004)	AY582206
<i>Orobanche minor</i>	Greece	Manen et al. (2004)	AY582208
<i>Orobanche pubescens</i>	Greece	Manen et al. (2004)	AY582275
<i>Orobanche teucrii</i>	France	Manen et al. (2004)	AY582269
<i>Orobanche transcaucasica</i>	Georgia	Manen et al. (2004)	AY582272
<i>Orobanche mutelii</i>	France	Manen et al. (2004)	AY582247
<i>Orobanche nana</i>	France	Manen et al. (2004)	AY582248
<i>Orobanche ramosa</i>	Spain	Manen et al. (2004)	AY582251
<i>Orobanche tunetana</i>	Spain	Manen et al. (2004)	AY582273

U of *Taq* DNA polymerase (Kapa Biosystems, Woburn, MA, USA). The primers used were as follows: OPA 02 (5'-TGCCGAGCTG-3'); OPA 01 (5'-CAGGCCCTTC-3'); OPA 13 (5'-CAGCACCCAC-3'); OPB 01 (5'-GTTTC-GCTCC-3'); OPB 10 (5'-CTGCTGGGAC-3') (Moretti et

al., 2004). Amplification was carried out in the thermal cycler (Eppendorf, Hamburg, Germany) under the following condition: initial denaturation at 94°C for 5 min, followed by 45 cycles at 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. PCR

products were separated in a 1% agarose gel in TAE buffer (Tris-acetate 0.04 M, EDTA 0.001 M) at 70 V for 4 h, visualized under UV light after staining with ethidium bromide and photographed.

Data analysis. The positions of the bands were assessed visually and each amplification band was treated as a unit character and was scored as present (1) or absent (0). The NEIGHBOR software of the PHYLIP computer package (Felsenstein, 1993) was used to create a phylogenetic tree. Distance matrix was constructed by using the mathematical equation proposed by Nei and Li (1979) based on the proportion of shared DNA fragments and the unweighted pair-group method (UPGMA) with arithmetic means was used for clustering.

To evaluate the phylogenetic relationship between the *O. cumana* population in Serbia and different *Orobanchae* species, the phylogenetic tree was constructed. Neighbor joining (NJ) analyses were performed with nucleotide sequences of partial *rbcL* gene sequences which were assembled and edited using FINCHTV v.1.4.0 (<http://www.geospiza.com>). Multiple alignments and comparisons of sequences of different *Orobanchae* species were performed using CLUSTALW integrated into MEGA6 software (Tamura et al., 2013). A bootstrap analysis with 1,000 replications was performed by the same software.

The minimum spanning tree (MST) was generated in SplitsTree software (Huson and Bryant, 2006) by using our



Fig. 1. Map of Serbia showing localities in which *Orobanchae cumana* populations were collected.

sequences and sequences obtained from NCBI database that represents *Orobanchae* species diverse sections. Generated sequences served as the tree nodes. The method estimated a probability of ancestry for each individual species. Individuals were assigned to one cluster or jointly to two or more clusters if their genotypes indicated that they were

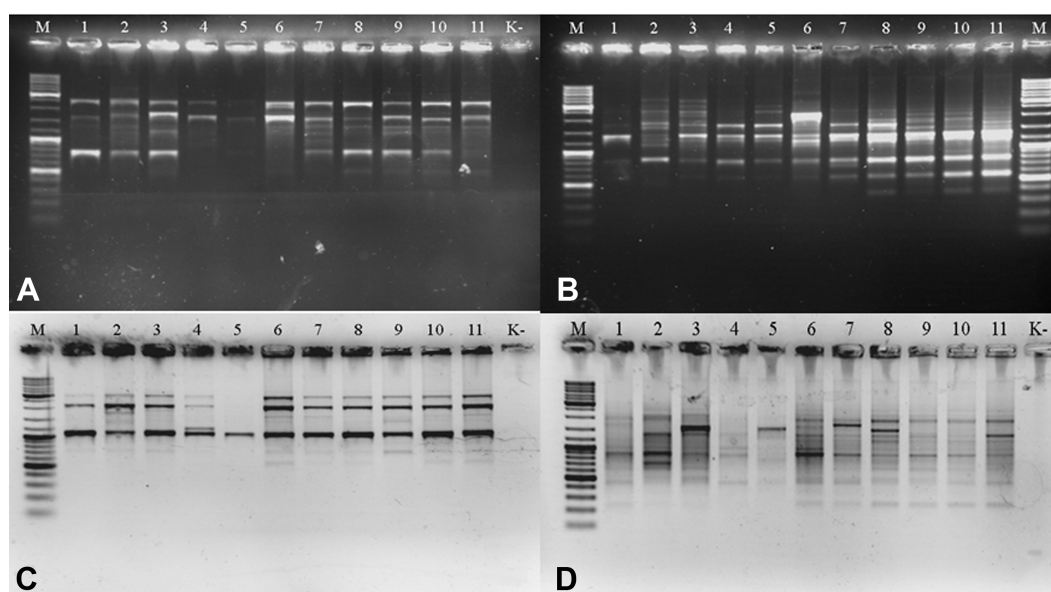


Fig. 2. Agarose gel electrophoresis of random amplified polymorphic DNA-based polymerase chain reaction: (A) OPA01, (B) OPA02, (C) OPB01, (D) OPA13, fingerprint patterns obtained from 11 *Orobanchae cumana* populations from Serbia. Lane M, DNA molecular size marker (GeneRuler DNA LadderMix); lane 1, OC-2; lane 2, OC-3; lane 3, OC-4; lane 4, OC-5; lane 5, OC-6; lane 6, OC-7; lane 7, OC-8; lane 8, OC-9; lane 9, OC-10; lane 10, OC-11; lane 11, OC-12.

closely related.

Results

Plants of *O. cumana* were collected from sunflower fields located in four districts including North Bačka (Subotica, Feketić), West Bačka (Kula), South Banat (Vršac), and Bor District (Negotin) (Fig. 1). DNA was extracted from obtained seeds and was used for evaluation of genetic diversity of population. For molecular analysis RAPD profiles were generated with OPA01, OPA02, OPA13, and OPB01 primers.

For each of the four primers, a polymorphic banding pattern was observed. RAPD analyses of these populations are shown in Fig. 2. Differences among populations were assessed visually based on migration patterns of the PCR products. The bands were clearly differentiated by agarose gel electrophoresis.

RAPD profiles obtained with OPA01 primer generated PCR products ranged in size from approximately 500 bp to over 3,000 bp. Similar DNA patterns were observed for all tested populations, with two characteristic bands on 1,600 and 2,500 bp. The third band with size of 750 bp was amplified for populations OC-2 (Vršac), OC-3 (Negotin), OC-4 (Subotica), OC-8 (Kula), OC-9 (Feketić), OC-10 (Subotica), and OC-11 (Kula), except for populations OC-5 (Kula) and OC-6 (Feketić), OC-7 (Subotica) and OC-12 (Feketić).

RAPD patterns obtained with OPB01 primer for all investigated populations showed very similar profiles indicating that this DNA marker is not very useful in determining genetic diversity. OPB01 primer gave reproducible genomic PCR profiles with bands of approximately 500 bp to over 3,000 bp. The OPB01 primer provided amplification patterns that included three specific polymorphic bands (550, 2,100, and 3,000 bp). Only the populations OC-5 and OC-6 obtained from Kula and Feketić, respectively (Fig. 2C, lanes 4 and 5) were found different from the other populations. Population OC-5 had unique bands on position 600 bp and population OC-6 on position 550 bp, but no bands were observed on 2,100 bp and 3,000 bp which were characteristic for other populations.

RAPD DNA profiles generated with OPA02 primer were complex and varied significantly among the isolates (Fig. 2B) ranging from 400 bp to over 6,000 bp. Visual analysis of OPA02 DNA profiles differentiated 11 DNA pattern profiles.

OPA13 primer sets gave reproducible RAPD DNA profiles with bands ranging from approximately 200 bp to over 2,500 bp. Visual analysis of RAPD DNA profiles

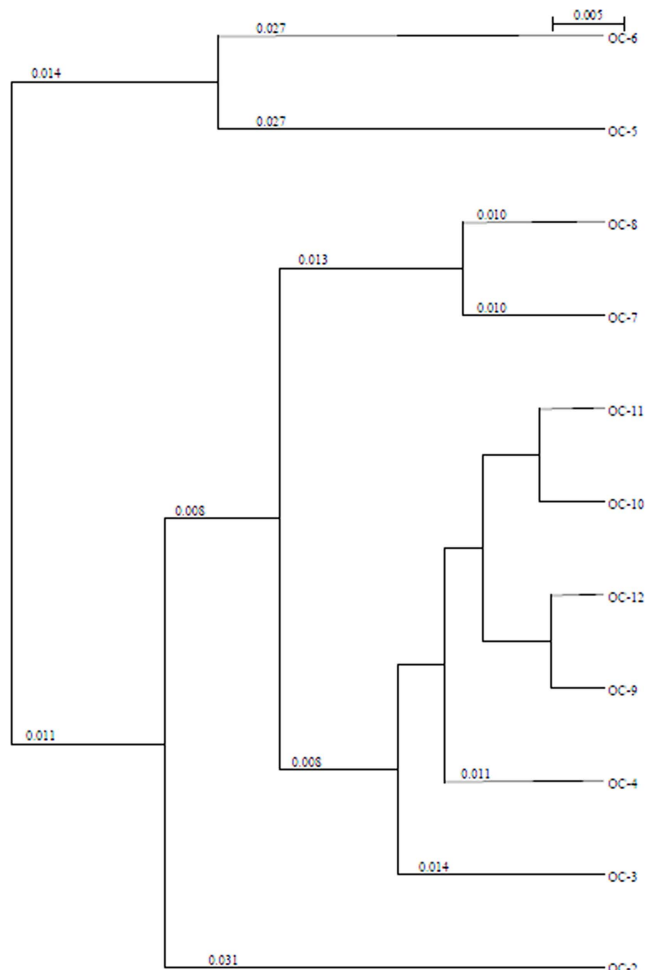


Fig. 3. Dendrogram of distances between *Orobanche cumana* populations tested for random amplified polymorphic DNA profiles. Bar represents the estimated relative distance between isolates.

obtained with OPA13 primer clearly differentiated seven DNA pattern profiles. A dendrogram produced by UP-GMA clustering showed three different clusters (Fig. 3). The first group consisted of *O. cumana* populations OC-5 and OC-6 obtained from Kula and Feketić, respectively. The second group consisted of populations OC-7 and OC-8 from Subotica and Kula, and the third group consisted of populations OC-9, OC-10, obtained from Feketić and Subotica, and population OC-11 and OC-12 from Kula and Feketić. Negotin OC-3 and Subotica OC-4 populations were also included in this cluster. DNA profiles generated from *O. cumana* population OC-2 originating from Vršac did not group in any of the mentioned clusters.

For additional determination of genetic diversity of *O. cumana* populations, a molecular analysis of the *rbcL* gene from *O. cumana* plants collected in Serbia was performed

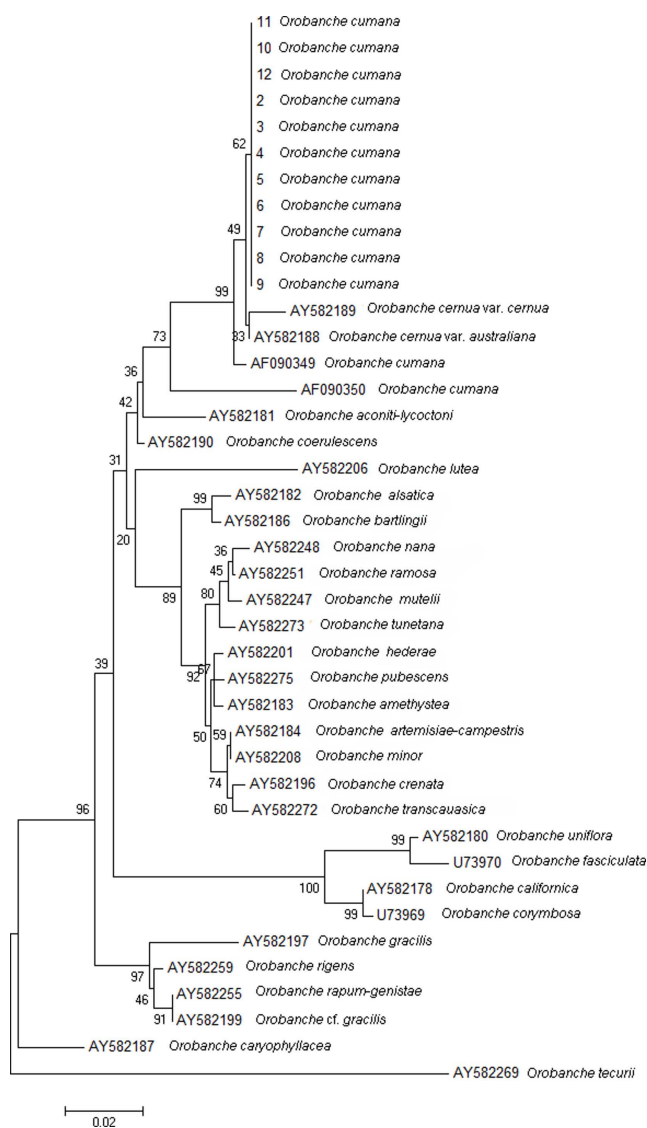


Fig. 4. Neighbor-joining tree showing the phylogenetic relationship of 11 populations of *Orobanche cumana* from Serbia and those of reference strains present in the NCBI databank, based on partial sequences of genes *rbcL*. Bootstrap values (expressed as percentages of 1,000 replications) are given at the nodes. Scale bar = estimated nucleotide substitutions per site is 0.005.

and sequences are available in GenBank database under accession numbers KP222282-KP222292 (Table 1). The sequences of the *rbcL* gene of *O. cumana* plants from Serbia did not match 100% with any of the published *O. cumana* *rbcL* gene sequences in the NCBI database, they were grouped as a monophyletic cluster (Fig. 4). Partial *rbcL* gene sequences from this study were compared with other *Orobanche* species representatives of diverse *Orobanche* genus sections obtained from NCBI database, and showed that sequences of representatives of populations from Ser-

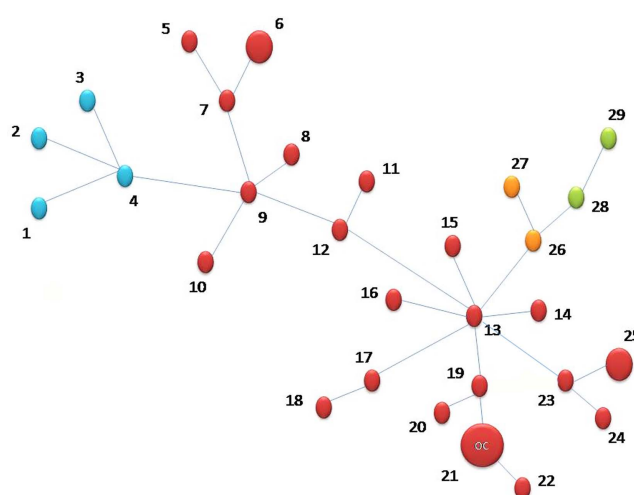


Fig. 5. Mini spanning tree splits genealogical network for representative species of four sections of *Orobanche* genus (section *Orobanche* colored in red, section *Myzorrhiza* colored in yellow, section *Gymnocaulis* colored in green and section *Trionychon* colored in blue) and populations of *O. cumana* from Serbia (designated as OC). Each strain is represented with separate node, size of a node is presented with the number of the isolates: 1, *O. mutellii* AY582247; 2, *O. tunetana* AY582273; 3, *O. nana* AY582248; 4, *O. ramosa* AY582251; 5, *O. crenata* AY582196; 6, *O. artemisiae-campestris* AY582184 and *O. minor* AY582208; 7, *O. transcaucasica* AY582272; 8, *O. hederiae* AY582201; 9, *O. amethystea* AY582183; 10, *O. pubescens* AY582275; 11, *O. alsatica* AY582182; 12, *O. bartlingii* AY582186; 13, *O. coerulescens* AY582190; 14, *O. lutea* AY582206; 15, *O. aconiti-hycoctoni* AY582181; 16, *O. cumana* AF090350; 17, *O. caryophyllacea* AY582187; 18, *O. teurcii* AY582269; 19, *O. cernua* var. *australiana* AY582188; 20, *O. cernua* var. *cernua* AY582189; 21, *O. cumana* (Serbian population); 22, *O. cumana* AF090349; 23, *O. rigens* AY582259; 24, *O. gracilis* AY582197; 25, *O. rapum-ganistae* AY582255 and *O. cf. gracilis* AY582199; 26, *O. californica* AY582178; 27, *O. corymbosa* U73969; 28, *O. fasciculata* U73970; 29, *O. uniflora* AY582180.

bia were clustered in same clade with *O. cumana* and *O. cernua* species.

An MST generated from partial DNA sequences of *rbcL* gene of *O. cumana* from Serbia and representative sequences data available in the NCBI, depicted the relationships among *Orobanche* spp. The MST constructed using statistical parsimony with split network revealed no ambiguous connections between species and clearly clustered four sections of *Orobanche* genus. Most haplotypic groups contained only one species except for haplotype 6 (*O. artemisiae-campestris* and *O. minor*), haplotype 21 (*O. cumana* OC 2-12), and haplotype 25 (*O. rapum-ganistae* and *O. cf. gracilis*), resulting in 29 distinct haplotype groups.

MST is shown in Fig. 5, where the colors represent four *Orobanche* sections, section *Orobanche* colored in red, section *Myzorrhiza* colored in yellow, section *Gymnocaulis* colored in green and section *Trionychon* colored in blue. The reconstructed MST agreed with the NJ phylogenetic tree and recovered hierarchical phylogenetic relationships between *O. cumana* and *O. cernua*. It is noticeable that sequences of *O. cumana* were located at the interior nodes of the tree and were linked with the two different *O. cernua* varieties.

Discussion

Different studies on the genetic diversity of *O. cumana* populations from various European countries have indicated that populations from eastern Europe belong to one main gene pool, while populations from Spain belong to a second main gene pool (Castejón-Muñoz et al., 1991; Gagne et al., 1998; Molinero-Ruiz et al., 2014; Pineda-Martos et al., 2013). It was also discovered that the Spanish populations were closer to the Romanian population, than the Bulgarian and Turkish populations (Gagne et al., 1998). The authors reported low intrapopulation and high interpopulation genetic variation as well as little gene exchange between geographical regions (Gagne et al., 1998). In Spain, most of the populations had low intra- and interpopulation diversity, with only a few populations with high intrapopulation genetic diversity (Pineda-Martos et al., 2013). In recent studies, Martín-Sanz et al. (2016), discovered two distant *O. cumana* gene pools, one in center and another in the south of the country. The RAPD technique was used to assess genetic diversity in Serbian populations using four different primers, and it showed high intrapopulation diversity. The OPA13 and OPA02 primers were more discriminative in differentiating the *O. cumana* populations than OPA01 and OPB01 primers. The UPGMA analyses revealed the existence of genetic variability among the populations which resulted in a phylogenetic tree where populations were grouped in four diverse clusters. Plants collected from northern Serbia (Subotica, Kula, and Feketić), showed more than one genomic pattern indicating that at least three different populations of *O. cumana* parasitize sunflower in this region. Populations from Negotin, located in eastern Serbia, suggested similar profiles as some isolates from northern Serbia.

It can be concluded that *O. cumana* plants collected from diverse agricultural regions in Serbia are members of the same population with great genetic heterogeneity. Our results differ from those obtained in other countries where high levels of variation among populations and low intra-

population variability were found (Gagne et al., 1998). This could be explained by geographic position of sunflower-growing areas which are located near the country borders and sunflower-growing areas in Romania, Bulgaria, and Hungary. The broomrape has very light seeds and can be easily spread by water, wind, animals and by human-derived agricultural and cultivation practices, as well as crop-seed trade and the use of contaminated sunflower seed stocks (Satovic et al., 2009), which can overpass spatial distances or barriers common in natural ecosystems (Pineda-Martos et al., 2014). Further, intensive gene flow among populations significantly influences genetic events and differences in all above-mentioned populations of *O. cumana*, which probably lead to high genetic heterogeneity in Serbian population of *O. cumana*. Close relation among the populations of *O. cumana* from Negotin and northern populations suggests that probably use of contaminated sunflower seed contributed to spread of specific genotypes. Genetic diversity among the populations suggests the possibility of seed introduction from different areas and genetic recombination between different populations (Pineda-Martos et al., 2013). Seed exchange among diverse districts could give possibilities for genetic recombination between distant population which could lead to the intrapopulation diversity in Serbia. All of these gene exchange mechanisms involved may be a good path for creating new genetic variability and virulence change (Pineda-Martos et al., 2013). In this study, the use of the RAPD technique to assess genetic diversity was very useful in variability examination within and between *O. cumana* populations, which is important for improvement of crop breeding strategies for *O. cumana* control.

Many authors have previously suggested the use of *rbcL* gene sequences in phylogenetic research at inter- and intra-specific genetic levels (Benharrat et al., 2000; Manen et al., 2004; Wolfe and dePamphilis, 1998). In the present study, the partial sequence of the *rbcL* gene that encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase was also examined and found to be suitable for phylogenetic analysis of *Orobanche* species which is in accordance with Wolfe and dePamphilis (1998). Partial sequences of the *rbcL* gene and constructed MST tree clearly divided *Orobanche* species into four sections of *Orobanche* genus and suggested that all *O. cumana* populations from Serbia were clustered in one monophyletic subcluster together with representative sequences of *O. cumana* and *O. cernua*. Close relationship between these two species was presented with direct connection of the nodes of the tree, suggesting a strong relationship between these two species. The MST tree provided further information about the relations

among *Orobanch*e species and showed existence of different *O. cumana* haplotypes. *O. cumana* populations from Serbia represent one haplotype and differ from *O. cumana* haplotype originated from France obtained from NCBI database. Although *rbcL* gene sequences were found to be very informative at the interspecific level, it was interesting to reveal it can cluster distinct *O. cumana* haplotypes.

The *rbcL* gene as a molecular marker did not have the resolution to display intrapopulation diversity, but it could be a valuable method for studying the evolution of geographically remote populations. Since RAPD analyses revealed intraspecific variations were present among populations in Serbia, further investigations are needed to study phylogenetic relations with populations from other countries. High genetic potential revealed in this research implicates that more virulent races could appear and that determination of broomrape genetic structure is very significant for new control strategies of this parasitic plant. It is important to note that the overall efficiency and effectiveness of weed control programs and also most appropriate means of combating the weeds will be enhanced by the knowledge on genetic relatedness or diversity available within local and regional populations and varieties of *O. cumana*.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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References

- Aćimović, M. 1977. Distribution of important sunflower diseases in Yugoslavia. Institute of Field and Vegetable Crops, Novi Sad, Yugoslavia. 17 pp.
- Atanasova, R., Batchvarova, R., Todorovska, E. and Atanassov, A. 2005. Molecular study of broomrape (*Orobanch*e spp.) by RAPD analyses. *Biotechnol. Biotechnol. Equip.* 19:51-60.
- Benharrat, H., Delavault, P., Theodet, C., Figureau, C. and Thalouarn, P. 2000. *rbcL* plastid pseudogene as a tool for *Orobanch*e (subsection *Minores*) identification. *Plant Biol.* 2:34-39.
- Bilgen, B. B., Barut, A. K. and Demirbaş, S. 2019. Genetic characterization of *Orobanch*e *cumana* populations from the Thrace region of Turkey using microsatellite markers. *Turk. J. Bot.* 43:38-47.
- Calderón-González, Á., Pouilly, N., Muños, S., Grand, X., Coque, M., Velasco, L. and Pérez-Vich, B. 2019. An SSR-SNP linkage map of the parasitic weed *Orobanch*e *cumana* Wallr. including a gene for plant pigmentation. *Front. Plant Sci.* 10:797.
- Castejón-Muñoz, M., Suso, M. J., Romero-Muñoz, F. and García-Torres, L. 1991. Isoenzymatic study of broomrape (*Orobanch*e *cernua*) populations infesting sunflower (*Helianthus annuus*). In: *Proceedings of the 5th International Symposium of Parasitic Weeds*, eds. by J. K. Ransom, L. J. Musselman, A. D. Worsham and C. Parker, pp. 313-319. International Maize and Wheat Improvement Center, Nairobi, Kenya.
- Delavaault, P. and Thalouarn, P. 2002. The obligate root parasite *Orobanch*e *cumana* exhibits several *rbcL* sequences. *Gene* 297:85-92.
- Dor, E., Plakhine, D., Joel, D. M., Larose, H., Westwood, J. H., Smirnov, E., Ziadna, H. and Hershenhorn, J. 2020. A new race of sunflower broomrape (*Orobanch*e *cumana*) with a wider host range due to changes in seed response to strigolactones. *Weed Sci.* 68:134-142.
- Duca, M., Joița-Păcureanu, M., Port, A., Martea, R., Boicu, A., Rîșnovceanu, L. and Clapco, S. 2019. Genetic diversity analysis of sunflower broomrape populations from Republic of Moldova using ISSR markers. *Rom. Agric. Res.* 37:89-97.
- Duca, M., Port, A., Boicu, A. and Șestacova, T. 2017. Molecular characterization of broomrape populations from Republic of Moldova using SSR markers. *Helia* 40:47-59.
- Felsenstein, J. 1993. Phylogeny inference package version 3.5c. URL <https://csbf.stanford.edu/phylip/> [13 August 2021].
- Fernández-Aparicio, M., Reboud, X. and Gibot-Leclerc, S. 2016. Broomrape weeds. Underground mechanisms of parasitism and associated strategies for their control: a review. *Front. Plant Sci.* 7:135.
- Gagne, G., Roeckel-Drevet, P., Grezes-Besset, B., Shindrova, P., Ivanov, P., Grand-Ravel, C., Vear, F., de Labrouhe, D. T., Charmet, G. and Nicolas, P. 1998. Study of the variability and evolution of *Orobanch*e *cumana* populations infesting sunflower in different European countries. *Theor. Appl. Genet.* 96:1216-1222.
- Huson, D. H. and Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23:254-267.
- Jebri, M., Ben Khalifa, M., Fakhfakh, H., Pérez-Vich, B. and Velasco, L. 2017. Genetic diversity and race composition of sunflower broomrape populations from Tunisia. *Phytopathol. Mediterr.* 56:421-430.
- Joel, D. M., Benharrat, H., Portnoy, V. H. and Thalouarn, P. 1998. Molecular markers for *Orobanch*e species: new approaches and their potential uses. In: *Current Problems of Orobanch*e *Researchers. Proceedings of the 4th International Workshop on Orobanch*e *Research*, eds. by K. Wegmann, L. J. Musselman and D. M. Joel, pp. 115-124. Institute for Wheat and Sunflower Dobroudja, Albena, Bulgaria.

- Katzir, N., Portnoy, V., Tzuri, G., Joel, D. M. and Castejón-Muñoz, M. 1996. Use of random amplified polymorphic DNA (RAPD) markers in the study of the parasitic weed *Orobanchae*. *Theor. Appl. Genet.* 93:367-372.
- Malek, J., del Moral, L., Fernández-Escobar, J., Pérez-Vich, B. and Velasco, L. 2017. Racial characterization and genetic diversity of sunflower broomrape populations from northern Spain. *Phytopathol. Mediterr.* 56:70-76.
- Manen, J.-F., Habashi, C., Jeanmonod, D., Park, J.-M. and Schneeweiss, G. M. 2004. Phylogeny and intraspecific variability of holoparasitic *Orobanchae* (Orobanchaceae) inferred from plastid *rbcL* sequences. *Mol. Phylogenet. Evol.* 33:482-500.
- Martín-Sanz, A., Malek, J., Fernández-Martínez, J. M., Pérez-Vich, B. and Velasco, L. 2016. Increased virulence in sunflower broomrape (*Orobanchae cumana* Wallr.) populations from southern Spain is associated with greater genetic diversity. *Front. Plant Sci.* 7:589.
- Maširević, S. and Medić-Pap, P. 2009. Broomrape in Serbia from its occurrence till today. *Helia* 32:91-100.
- Moliner-Ruiz, L., García-Carneros, A. B., Collado-Romero, M., Raranciuc, S., Domínguez, J. and Melero-Vara, J. M. 2014. Pathogenic and molecular diversity in highly virulent populations of the parasitic weed *Orobanchae cumana* (sunflower broomrape) from Europe. *Weed Res.* 54:87-96.
- Moretti, M., Saracchi, M. and Farina, G. 2004. Morphological, physiological and genetic diversity within a small population of *Cercospora beticola* Sacc. *Ann. Microbiol.* 54:129-150.
- Nei, M. and Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.* 76:5269-5273.
- Paran, I., Gidoni, D. and Jacobsohn, R. 1997. Variation between and within broomrape (*Orobanchae*) species revealed by RAPD markers. *Heredity* 78:68-74.
- Parker, C. and Riches, C. R. 1993. Parasitic weeds of the world: biology and controls. CAB International, Wallingford, UK. 332 pp.
- Pineda-Martos, R., Velasco, L., Fernández-Escobar, J., Fernández-Martínez, J. M. and Pérez-Vich, B. 2013. Genetic diversity of *Orobanchae cumana* populations from Spain assessed using SSR markers. *Weed Res.* 53:279-289.
- Pineda-Martos, R., Velasco, L. and Pérez-Vich, B. 2014. Identification, characterisation and discriminatory power of microsatellite markers in the parasitic weed *Orobanchae cumana*. *Weed Res.* 54:120-132.
- Roman, B. and Rubiales, D. 1999. Molecular analysis of *Orobanchae crenata* populations from southern Spain. In: *Resistance to Orobanchae: the state of the art*, eds. by J. I. Cubero, M. T. Moreno, D. Rubiales and J. Sillero, pp. 99-108. Junta de Andalucía, Sevilla, Spain.
- Satovic, Z., Joel, D. M., Rubiales, D., Cubero, J. I. and Román, B. 2009. Population genetics in weedy species of *Orobanchae*. *Australas. Plant Pathol.* 38:228-234.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Yang, C., Fu, F., Zhang, N., Wang, J., Hu, L., Islam, F., Bai, Q., Yun, X. and Zhou, W. 2020. Transcriptional profiling of underground interaction of two contrasting sunflower cultivars with the root parasitic weed *Orobanchae cumana*. *Plant Soil* 450:303-321.
- Young, N. D., Steiner, K. E. and dePamphilis, C. W. 1999. The evolution of parasitism in Scrophulariaceae/Orobanchaceae: plastid gene sequences refute an evolutionary transition series. *Ann. Missouri Bot. Gard.* 86:876-893.
- Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535.
- Wolfe, A. D. and dePamphilis, C. W. 1997. Alternate paths of evolution for the photosynthetic gene *rbcL* in four nonphotosynthetic species of *Orobanchae*. *Plant Mol. Biol.* 33:965-977.
- Wolfe, A. D. and dePamphilis, C. W. 1998. The effect of relaxed functional constraints on the photosynthetic gene *rbcL* in photosynthetic and nonphotosynthetic parasitic plants. *Mol. Biol. Evol.* 15:1243-1258.