Open Access

Saida Guchetl*, Tatiana S. Antonova
and Tatiana Tchelustnikova

Interpopulation Genetic Differentiation *Orobanche cumana* Wallr. from Russia, Kazakhstan and Romania Using Molecular Genetic Markers

Abstract: Broomrape (*Orobanche cumana* Wallr.) is an obligate parasite of higher plants, which affects sunflower in many countries, cultivating this crop. For the past decades, it is noted the formation of new highly virulent biotypes of broomrape and their spreading to other areas. In our work we studied the molecular genetic diversity of broomrape populations of *O.cumana*, parasitizing on sunflower in Russia, Romania, and Kazakhstan, by using codominant microsatellite markers. During cluster analysis, the broomrape populations are divided into two clusters, regardless of their racial composition. One cluster grouped 19 samples from Russia and Kazakhstan, and the other – 5 populations from Romania. The genetic distance between clusters according to Nei was 0.137. The analysis of molecular variance (AMOVA) revealed that 22% of genetic variability was due to differences among the gene pools and 78% was due to differences within the gene pools. Pairwise comparisons made using Wright's statistics showed that the differences between these two gene pools are sufficient (Fst = 0.219) to state the existence of a small genetic differentiation between them. Descriptive population genetic statistics for each of the two pools showed that the broomrape populations from the former Soviet Union countries are characterized by a higher level of intrapopulation diversity than the populations from Romania. Molecular genetic differences between broomrape populations parasitizing on sunflower on the post-Soviet territory and in

***Corresponding author: Saida Guchetl,** Laboratory of Immunity and Electrophoresis, All-Russia Research Institute of Oil Crops by the name of V.S. Pustovoit, 17 Filatova str., Krasnodar, 350038, Russia, E-mail: antonova-ts@mail.ru
Tatiana S. Antonova, Tatiana Tchelustnikova, Laboratory of Immunity and Electrophoresis, All-Russia Research Institute of Oil Crops by the name of V.S. Pustovoit, 17 Filatova str., Krasnodar, 350038, Russia

Romania were insignificant. Possible reasons for these results are being discussed.

Keywords: genetic diversity, molecular characterization, *Orobanche cumana*, SSR markers, sunflower

DOI 10.1515/helia-2014-0020
Received July 24, 2014; accepted September 18, 2014

Introduction

Broomrape (*Orobanche cumana* Wallr.) is an obligate parasite of higher plants parasitizing on the roots of sunflower. In severe cases of strong broomrape infestation yield losses of sunflower can reach up to 100%. In Russia, as well as in the other countries, widespread intensification of sunflower cultivation as a high-yielding crop in the last decade led to the formation of highly virulent biotypes of broomrape, overcoming the resistance of cultivated assortment (Antonova *et al.*, 2012).

The researches on the evolution of the parasite and the population genetic structure are important for understanding the mechanism of its development in different countries and for developing a long-term strategy of control over it (Fernandez-Martinez *et al.*, 2012; Škorić *et al.*, 2010).

The molecular genetic differences between the populations of *O. cumana* from different countries have already been studied (Ciuca *et al.*, 2004; Atanasova *et al.*, 2005; Benharrat *et al.*, 2002; Gagne *et al.*, 1998; Pineda-Martos *et al.*, 2013; Molinero-Ruiz *et al.*, 2013). By using RAPD PCR technology, it has been shown that the populations of *O. cumana* from Bulgaria, Spain, Romania, and Turkey have low intrapopulation variability, and very little gene exchange appears to occur between different geographic regions (Gagne *et al.*, 1998). The weak polymorphism of the populations of *O. cumana* from Spain, Yugoslavia and Romania is shown (Ciuca *et al.*, 2004). The existence of two distant gene pools in two provinces in Spain is revealed, one is in Cuenca province and another one in Guadalquivir Valley. Within each gene pool, both inter- and intrapopulation variability were extremely low (Pineda-Martos *et al.*, 2013). The molecular analysis among highly virulent populations of *O. cumana* identified four clusters, respectively, grouping populations from Central Spain, Hungary, South Spain and Turkey. The genetic homogeneity within parasite populations was confirmed, since no molecular differences were found within them (Molinero-Ruiz *et al.*, 2013).

Molecular genetic differences of the populations of *O. cumana*, affecting sunflower in different regions of Russia and Kazakhstan have not yet been

studied. The purpose of our research is to analyze the molecular genetic diversity between broomrape populations, spread on the territory of Russia, and to compare them with the populations from Romania and Kazakhstan by using codominant microsatellite markers.

Material and methods

The seeds of *O. cumana* from 24 populations were collected in 2012 and 2013 on sunflower fields of the Krasnodar, Stavropol, Rostov, Volgograd, and Saratov regions of Russia, as well as Romania and Kazakhstan, and were stored at a temperature of −18°C (Table 1).

Table 1: The characteristics of seed sample of *O. cumana* used for the analysis

No	Place of collecting of broomrape seeds	Country	Collection year	Races, prevailing in seeds sample
V1	Krasnodar region, Krylovskiy district	Russia	2012	G
V2	Krasnodar region, Yeyskiy district	Russia	2013	F,G
V3	Stavropol region, Trunovskiy district	Russia	2012	F,G
V4	Rostov region, Azovskiy district, DOS VNIIMK	Russia	2012	E,F,G
V5	Rostov region, Milyutinskiy district	Russia	2012	G
V6	Rostov region, Azovskiy district, v. Krugloe	Russia	2012	G
V7	Rostov region, Bokovskiy district	Russia	2012	F,G
V8	Rostov region, Zernogradskiy district	Russia	2012	F,G
V9	Rostov region, Matveevo-Kurganskiy district	Russia	2012	F,G
V10	Rostov region, Matveevo-Kurganskiy district	Russia	2012	G
V11	Rostov region, Bokovskiy district	Russia	2012	F,G
V12	Rostov region, Bokovskiy district	Russia	2012	F,G
V13	Rostov region, Zernogradskiy district	Russia	2012	F
V14	Rostov region, Zernogradskiy district, stud farm	Russia	2012	F,G
V15	Rostov region, Kagalnitskiy district	Russia	2012	G
V16	Saratov region, Sovetskiy district	Russia	2013	E,F,G
V17	Volgograd region, Alexeevskiy district	Russia	2013	F,G
V18	Volgograd region, Novoanninskiy district	Russia	2013	F,G
V19	Shemonaikhinskiy district, Ust Kamenogorsk	Kazakhstan	2012	D
V20	Medgidia	Romania	2013	G
V21	Calarasi	Romania	2013	G
V22	Tulcea	Romania	2013	G
V23	Urziceni	Romania	2013	D
V24	Lunca	Romania	2013	G

For artificial inoculation with broomrape, sunflower plants of VNIIMK 8883 variety were cultivated in a growth chamber in plastic boxes with a capacity of 10 kg of a mixture of soil with river sand in the ratio 3:1. This variety has never been bred for resistance to broomrape. Broomrape seeds were applied to soil mixture at the rate of 100 mg per 1 kg. Twenty sunflower seeds were sown per each box. Watering was carried out at the drying up of topsoil. The operating regime of chamber: 16-hour photoperiod at a temperature of 25–27°C at daytime and 20°C – at night. 50 days after the emergence of seedlings, the plants were dug out of the pots, the root system was washed with water, and tubercles, stems and inflorescences of broomrape were collected for analysis. Fresh-cut tissue was stored at a temperature of –80°C.

DNA was extracted from frozen tissues by the method of Doyle and Doyle (1987) with our modifications. A mixture of 5–10 individual plants of equal weight was taken from each population for DNA extraction of broomrape.

PCR-amplifications were used with 10 ng of genomic DNA in 25 μL reactions. Each 25 μL of reaction volume contained 67 mM Tris–HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 1.5–3.0 mM $MgCl_2$; 0.01 % Tween 20; 0.2 mM deoxynucleoside triphosphates, 10 μM primer and 1.0 unit Tag DNA polymerase (Moscow, Sibenzim). DNA amplification was performed in a thermal cycler S1000тм (BioRad, USA). The amplification conditions: initial denaturation at a temperature of 96°C for 2 minutes followed by 30 cycles in accordance with temperature-time mode: annealing at a temperature of 60°C for 40 second, elongation at 70°C for 1 minute, denaturation at 94°C for 30 second, and final elongation for 2 minutes. 15 SSR (simple sequence repeat) primers were used that were selected in work (Pineda-Martos *et al.*, 2013).

Amplification products were resolved by electrophoresis in 8% polyacrylamide gel in 1xTBE buffer at 230 V constant, using VE-20 (Helicon, Russia) vertical camera. Subsequent staining was performed with ethidium bromide. Visualization of electrophoresis results in UV and their documentation was provided by using the system of digital video documentation BIO-PRINT (Vilber Lourmat, France). A 100-bp DNA ladder (Thermo Fisher Sciencific Inc, Lithuania) was used as a standard molecular weight marker to get an approximate size of DNA fragments. Calculation of fragments size after electrophoresis was scored manually with the aid of Bio-Capture software (Vilber Lourmat, France).

To determine the differences between the broomrape samples, data of PCR analysis were processed by Ward's method. To do this, amplified fragments were scored for the presence (1) or absence (0) of homologous bands. Data were compiled into a binary data matrix. Cluster analysis was performed using a program package (Statistica 6.0). Geometric distances were calculated using

Euclidean distance. Descriptive population genetic statistics (number of alleles per locus *Na*, effective number of alleles *Ne*, polymorphism *P*, observed *Ho* and expected *He* heterozygosity, Shannon's Information Index *I*), as well as AMOVA, *F* statistics, Nei Genetic Distance and Nei Genetic Identity were calculated by using Gen AlEx 6.5 program (Peakall and Smouse, 2012). Probability P for Fst, Fis and Fit is based on 999 permutations across the full data set. AMOVA procedure follows the methods of Excoffier *et al.* (1992), calculation of Nei's standard genetic distance between pairs of populations was performed by Nei (1972).

Results and discussion

Among the 15 primers initially assayed, 9 were selected since they produced consistent polymorphisms. As a result of amplification of 9 microsatellite loci 21 alleles were detected from 24 broomrape populations, from 2 to 4 alleles per locus (Table 2).

Table 2: The characteristics of microsatellite loci, used for evaluating genetic diversity in 24 *O. cumana* populations

Locus	Size	Alleles
Ocum-52	114, 131	2
Ocum-59	90, 100	2
Ocum-70	127, 120,130	3
Ocum-81	72, 90	2
Ocum-87	132, 136, 134,138	4
Ocum-108	144, 152	2
Ocum-141	186, 191	2
Ocum-196	192, 197	2
Ocum-197	104, 113	2

The dendrogram resulting from the Ward analysis of the SSR data set distinguished two well-differentiated clusters among the 24 samples of *O. cumana* (Figure 1). Cluster I grouped the 19 samples, collected on the territory of Russia and Kazakhstan, regardless of racial composition. Cluster II combined broomrape populations, collected in different regions of Romania.

Cluster I was divided into 2 subclusters with a union distance of 5 units. Subcluster Ia combined the populations originating from Krasnodar and

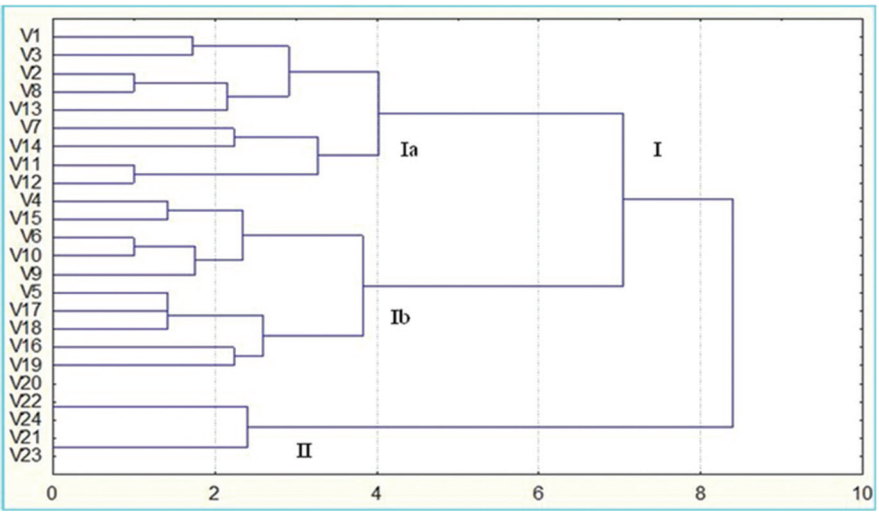

Figure 1: Ward's method dendrogram based on Euclidean distances between 24 populations of *Orobanche cumana* (see Table 1), collected in different regions of Russia, Kazakhstan and Romania using 9 SSR markers. I – Pop 1; II – Pop 2

Stavropol regions, and Bokovskiy and Zernogradskiy districts of the Rostov region. Subcluster Ib grouped the populations from Kazakhstan, Saratov and Volgograd regions, and Matveevo-Kurganskiy, Azovskiy, Kagalnitskiy and Milyutinskiy districts of the Rostov region. Subclusters combination did not depend on the geographical origin or the level of virulence of the population, since the populations with different virulence have been grouped together (Table 1, Figure 1). For example, broomrape populations from Bokovskiy and Zernogradskiy districts of the Rostov region combined in subcluster Ia are quite far from each other (about 200 km). Broomrape from Kazakhstan, the most geographically distant from the populations of Russian group (a distance over 2,000 km and different soil and climatic conditions), is located in the one subcluster with the populations from Rostov, Volgograd, and Saratov regions. We suppose that distribution of populations on subclusters doesn't indicate true relationship between populations, showing insignificance of distinctions between them. Grouping into clusters more correspond to the actual genetic relationships between the populations. The level of genetic differentiation between the main clusters was quantified by calculating Nei genetic distances (Nei, 1972). Nei Genetic Distance and Genetic Identity Values were 0.137 and 0.872, respectively (Figure 1).

All 5 broomrape samples from Romania were virtually identical. Percentage of polymorphic loci was 11.11. The populations originating from Russia and Kazakhstan were more polymorphic, with percentage of polymorphic loci of 100.00. For example, allelic variation of the five populations from Romania and of the five populations from Russia at the Ocum-87 SSR locus is shown in Figure 2.

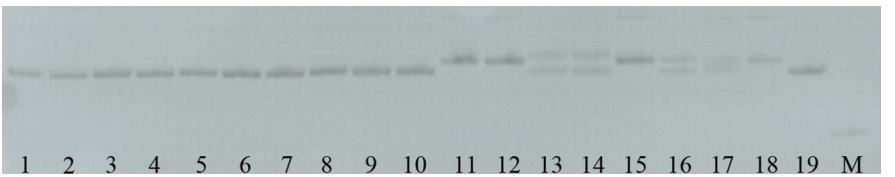

Figure 2: Allelic variation of the Ocum-87 SSR locus of ten *Orobanche cumana* populations. 1,2 – V20 (Medgidia, Romania); 3,4 – V21 (Calarasi, Romania); 5,6 – V22 (Tulcea, Romania); 7,8 – V23 (Urziceni, Romania); 9,10 – V24 (Lunca, Romania); 11,12 – V16 (Saratov region, Sovetskiy district, Russia); 13,14 – V2 (Krasnodar region, Yeyskiy district, Russia); 15,16 – V17 (Volgograd region, Alexeevskiy district, Russia); 17,18 – V18 (Volgograd region, Novoanninskiy district, Russia); 19 – V6 (Rostov region, Azovskiy district, v. Krugloe, Russia); M – DNA fragment 100 bp long

Low level of genetic variability of parasite populations from Romania was revealed also by means of RAPD DNA technique in research of Ciuca *et al.* (2004). The most different source was Calarasi-Romania source. According to our data, also in populations of Calarasi (V21) and Urziceni (V23) from Romania was detected a small intrapopulation polymorphism in locus Ocum 52 (data not shown). A study on genetic diversity in eight *O. cumana* populations from Spain, Bulgaria, Romania and Turkey reported low intrapopulation variation (Gagne *et al.*, 1998).

To determine mean descriptive population genetic statistics, the analysis of molecular variance AMOVA and *F* statistics of populations of *O. cumana* were combined by geographical origin. Next, the parasite populations originating from Russia and Kazakhstan are indicated as Pop1, and the populations from Romania are indicated as Pop2. Descriptive diversity statistics for populations from the two main gene pools showed significant variation for number of alleles per locus, number of alleles with frequencies pi > 5%, effective number of alleles, observed heterozygosis and expected heterozygosis (Table 3). The average number of alleles per locus ranged from 1.11 in Pop2 to 2.33 in Pop1. The effective number of alleles ranged from 1.05 in Pop2 to 1.77 in Pop 1. Pop1 showed the highest heterozygosis value (0.44), whereas Pop2 showed

heterozygosis value of 0.05. The values for the mean expected heterozygosis ranged from 0.03 in Pop2 to 0.41 in Pop1. Diversity analysis within the genetic pools using Shannon's Information Index revealed the highest diversity values in Pop1 (0.63) and the lowest in Pop2 (0.05) (Table 3).

Table 3: Mean descriptive population genetic statistics for each of the two populations of *O. cumana* followed by standard deviation values

Population	Na	Na Freq. >5%	Ne	I	Ho	He
Pop1	2.33 ± 0.23	2.11 ± 0.26	1.77 ± 0.11	0.63 ± 0.07	0.44 ± 0.06	0.41 ± 0.04
Pop2	1.11 ± 0.11	1.11 ± 0.11	1.05 ± 0.05	0.05 ± 0.05	0.04 ± 0.04	0.03 ± 0.03

Na: No. of different alleles; Na (Freq >= 5%): No. of different alleles with a frequency >5%; Ne: No. of effective alleles; I: Shannon's Information Index; Ho: Observed heterozygosity; He: Expected heterozygosity

The great intrapopulation diversity of broomrape in Russia and Kazakhstan in comparison with Romanian seems natural. Here, many populations are at large distances from each other (from 200 to 2,000 km), whereas in Romania they are not enough distant geographically.

AMOVA revealed that 22% of genetic variability was due to differences among the populations and 78% was due to differences within the populations (Table 4).

Table 4: Analysis of molecular variance in two groups of populations of *O. cumana*

Source of variation	df	Sum of squares	Variance components	% Variation	*P*
Among populations	1	8.552	0.443	22	<0.001
Within populations	24	38.500	1.604	78	
Total	25	47.042	2.047	100	

When pairwise differences among populations were checked, it was confirmed that there was sufficient differentiation between Pop1 and Pop2 (Fst = 0.219; Table 5) to suggest the existence of a small genetic differentiation of these two pools. The small value of Fis (−0.019) indicates the deficit of mean heterozygosis in each population, and Fit, which characterizes the deficiency or excess of mean heterozygosis in the group of populations, has an average value (0.205). In our case, the populations from Romania have virtually no heterozygosis, thereby reducing the Fis.

Table 5: Comparisons of pair of *O. cumana* populations (*F*-Statistics) as a measure of population differentiation due to genetic structure

F-Statistics	Value	Probability (*P*)
Fst	0.219	0.001
Fis	−0.019	0.577
Fit	0.205	0.012

The gene pools of broomrape parasitizing on sunflower in the former Soviet Union countries and Romania have much in common. There are almost no unique alleles in the studied populations (except the alleles 120 and 130 bp long in locus Ocum-70 of broomrape samples from Trunovskiy district, Stavropol region, Russia), high genetic similarity (0.872) of gene pools and Fst (0.219).

Our results showed that broomrape populations from the indicated three countries are grouped into clusters according to the country of origin, regardless of their racial composition. At the same time, one of the clusters combines the parasite populations from the countries of the former Soviet Union. This fact can be explained by the following. According to our data, the broomrape from Kazakhstan is characterized by weak virulence and does not overcome the resistance gene *Or4* in sunflower. It suggests that this broomrape population is represented by seeds preserved in the soil since the USSR time. We believe that the similarity between the Kazakh and Russian broomrape populations and their intrapopulation polymorphism is based on genetic diversity and similarity of sunflower open pollinated varieties, which were cultivated in the former Soviet republics. The isolation of the USSR territories from the assortment of foreign countries for many years formed here the peculiarity of gene pool of both sunflower and its parasite. And this certain peculiarity had not yet been neutralized by a modern free exchange of seed material between countries. Years of parasitizing on open pollinated varieties can explain the fact that the gene pool of broomrape from the former Soviet Union countries is characterized by a higher level of intrapopulation diversity than the populations of Romania.

We believe that a small variability in the genome of *O. cumana*, also described by several authors (Ciuca *et al.*, 2004; Gagne *et al.*, 1998) may be due to the fact that the habitat of this parasitic plant is metabolites of sunflower plant, while the external factors (climate, soil, etc.) can affect the parasite only indirectly through its host. Considering the fact that broomrape parasitizes on sunflower for a little more than 100 years, it is too early to expect its high variability.

Our results are in line with those obtained in the results that have been previously reported by other authors. The researchers noted that clustering of

populations was not associated with virulence groups (Gagne *et al*., 1998; Pineda-Martos *et al*., 2013; Molinero-Ruiz *et al*., 2013). In all this studies, place of collecting of broomrape was more important. In this connection, in the research Pineda-Martos *et al*. (2013), it was assumed that the population structure probably could be attributed to the founder effect. Gagne *et al*. (1998) studied the genetic diversity in eight *O. cumana* populations from Spain, Bulgaria, Romania and Turkey concluded that well-structured populations organized in two differing groups (one group corresponding to the East European countries, Bulgaria, Romania and Turkej and other group corresponding to the Spanish populations) could be monophyletic origin, although data on the intrapopulation genetic diversity are not significantly different and did not offer the obvious center of their origin.

In conclusion, this research revealed the existence of two poorly differentiated gene pools of *O. cumana*: Russian-Kazakh and Romanian. Broomrape populations from the former Soviet Union are more polymorphic and have a larger intrapopulation genetic diversity than the populations from Romania.

Acknowledgments: The authors would like to thank the Spanish colleagues represented by Leonardo Velasco for courtesy of the nucleotide sequence of SSR-primers, Syngenta company for the given broomrape seeds, as well as Limagrain company represented by Laszlo Hargitay and Mariana Gheorghe that gave us the opportunity to collect broomrape seeds in 5 populations of Romania.

The research was financially supported by the RFBR and the Administration of the Krasnodar region, grant No. 13-04-96521.

Research funding: RFBR and the Administration of the Krasnodar region (Grant/Award Number: "13-04-96521")

References

Antonova, T.S., Strelnikov, E.A., Araslanova, N.M., Ramazanova, S.A., 2012. Adaptive specifics of broomrape's (*Orobanche cumana* Wallr.) ontogenetic on sunflower. OIL CROPS. Scientific and Technical Bulletin of VNIIMK 1(150): 110–116.

Atanasova, R., Batcharova, R., Todorovska, E., Atanassov, A., 2005. Molecular study of broomrape (*Orobanche Spp*.) by RAPD analyses. Biotechnology and Biotechnological Equipment 19(3): 51–60.

Benharrat H., Veronesi, C., Theodet, C., Thalouarn, P., 2002. *Orobanche* species and population discrimination using intersimple sequence repeat (ISSR). WEED Research 42(I. 6): 470–475.

Ciuca, M., Pacureanu, M., Iuora, M., 2004. RAPD markers for polymorphism identification in parasitic weed *Orobanche cumana* Wallr. Romanian Agriculture Research 21: 29–32.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

Excoffier, L., Peter, E., Smouse, P.E., Quattro, J.M., et al., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction sites. Genetics 131: 479–491.

Fernandez-Martinez, J.M, Velasco, L., Perez-Vich, B. 2012. Progress in research on breeding for resistance to broomrape. *In*: Proc 18th Int. Sunfl. Conf., Mar Del Plata & Balcarce, Argentina, Int. Sunfl. Assoc., Paris, France, pp. 57–62.

Gagne, G., Roeckel-Drevet, P., Grezes-Besset, B., Shindrova, P., Ivanov, P., Grand-Ravel, C., Vear, F., Tourvieille de Labrouhe, D., Charmet, G., Nicolas, P., 1998. Study of the variability and evolution of *Orobanche cumana* populations infesting sunflower in different European countries. Theoretical and Applied Genetics 96: 1216–1222.

Molinero-Ruiz, L., García-Carneros, A.B., Collado-Romero, M., Raranciuc, S., Domínguez, J., Melero-Vara, J.M., 2013. Pathogenic and molecular diversity in highly virulent populations of the parasitic weed *Orobanche cumana* (sunflower broomrape) from Europe. Weed Research. DOI: 10.1111/wre.12056.

Nei, M., 1972. Genetic distance between populations. The American Naturalist 106: 283–392.

Peakall, R., Smouse, P.E., 2012. GenAlEx 6.5: Genetic analysis in excel. Population genetic software for teaching and research-an update. Bioinformatics 1: 1–3.

Pineda-Martos, R., Velasco, L., Fernandez-Escobar, J., Fernandez-Martinez, J.M., Perez-Vich, B., 2013. Genetic diversity of *Orobanche cumana* populations from Spain assessed using SSR markers. Weed Research 53: 279–289.

Škorić, D., Păcureanu-Joiţa, M., Sava, E., 2010. Sunflower breeding for resistance to broomrape (*Orobanche cumana Wallr.*) Genetica şi ameliorarea plantelor an. I.N.C.D.A. Fundulea LXXVIII(1): 63–79.