Genetic diversity of *Orobanche cumana* and *Orobanche cernua* populations as revealed by variability of Internal Transcribed Spacers1/2 of ribosomal cistron and ribulose-bisphosphate carboxylase pseugene

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ABSTRACT

The sunflower broomrape - *Orobanche cumana* (Wallr) parasitizes on roots of sunflower plants and is a serious constraint on sunflower production, causing yield losses of up to 60%. The variability of Internal Transcribed Spacers1/2 of ribosomal cistron (ITS1/2) and ribulose-bisphosphate carboxylase pseugene (RbcL) in 32 samples of *O. cumana* and 4 samples *O. crenata* collected from different European locations were studied. The results showed that *O. cumana* can be differentiated from *O. cernua*, by single C/T transition located in ITS2 (rel. position 423). Rubisco large subunit in *O cumana* differs from *O. cernua* with two transversion: T/G (rel. position 15) and A/C (rel. position 84). The genetic diversity observed in *O. cumana* was lower than in *O. cernua*. When comparing the ITS and rbcL sequences isolated from *O. cernua* two single SNPs were found that can discriminate different origins. Both ITS1/2 and rbcL sequences isolated from *O. cumana* however were completely homogeneous, despite the fact that samples were collected form very distant locations: from Volgograd, Russia to the East to Spain to the West. This observation is in favor of hypothesis that the move of *O. cumana* from wild hosts on sunflower was a single act that occurred once and all invasive races are descendants from ancient Caucasus population. Probably genes related to *O. cumana* aggressiveness should be identified and used for molecular markers to determine genetic relationships within and among *O. cumana* populations.

Key words: Broomrapes – molecular markers – molecular phylogeny – *Orobanche cernua* – *Orobanche cumana*

INTRODUCTION

The sunflower broomrape - *Orobanche cumana* (Wallr.) parasitizes on roots of sunflower plants and is a serious constraint on sunflower production, causing yield losses of up to 60% (Parker, 2009). *O. cumana*. originates from wild habitats in Southern Russia and around the Black Sea coast: Romania, Turkey and Bulgaria (Parker and Riches, 1993). It forms there isolated wild population parasitizing mainly Artemisia spp. (Venkov and Bozoukov, 1994). The first reports of *O. cumana* parasitizing sunflower came from Saratov region in Russia in 1890 (Morozov, 1947). The research on the problem started more than 100 years ago (Parker and Riches, 1993) and the breeding of resistant varieties has resulted in the first sunflower varieties resistant to a race A of *O. cumana,* developed by Plachek in 1918 (Morozov, 1947). A few years later Ždanov (in 1926) identified a new *O. cumana* race - B in Rostov area and developed a number of sunflower varieties resistant to it (Morozov, 1947). Sunflower selection for broomrape resistance so far produced some significant results. Dominant genes for resistance against *O. cumana* races A, B, C, D, E, and F have been found and incorporated into cultivated sunflower genotypes. In the last few years, however new broomrape populations have been discovered in several different countries (Romania, Russia, Turkey, Spain and Ukraine). None of the existing commercial hybrids resistant to races A, B, C, D, E, and F have proven resistant to these new populations of the *O. cumana* (Parker, 2009).

The taxonomy status of *O. cumana* is also subject of debate. According to some authors *O. cumana* is conspecific with *Orobanche cernua* (Teryokhin, 1997). However other authors argued that *O. cumana* and *O. cernua* are closely related, but yet distinct species (Katzir et al., 1996; Pujadas-Salvà and Velasco,

2000). Molecular analyses can provide information about taxonomy status and intraspecific genetic variations of *O. cumana*. Other important question is: "what the races are?": physiological adaptations or there is a genetic background determining the races existence. Recently Pineda-Martos and co-authors (2013 a, b) found 78 SSR primers that produced reproducible results and allowed them to distinguish *O. cumana* from *O. cernua.* In addition they were able to discriminate among *O. cumana* isolates by geographic origin and host. Molecular diagnostics however, works better when data from random amplification of DNA targets can be compared with sequence data from specific DNA targets. In this sense, we used sequences of two genetic markers: the first one is a fragment of ribosomal cistron including 3' end of gene encoding 18S rRNA - Internal Transcribed Spacer1 (ITS1) – gene for 5,8S rRNA – ITS2 – and 5' end of gene encoding 26S rRNA. The second is fragment of plastid pseudogene encoding ribulose-bisphosphate carboxylase (RbcL). Both sequences have been used widely before for molecular taxonomy studies of Orobanchaceae representatives (Schneeweiss et al., 2004; Weiss-Schneeweiss, 2006; Wolfe et al., 1992; Benharrat et al., 2000). Here we present our study on variability of ITS1/2 and RbcL in 32 samples of *O. cumana* and 4 samples of *O. crenata* collected from different European locations.

MATERIALS AND METHODS

Plant materials: *O. cumana* samples used in this investigation originate from different regions in Europe (table 1). Fourteen samples were supplied as isolated total DNA from Instituto de Agricultura Sostenible (IAS-CSIC), Cordoba, Spain, twelve samples were provided from seeds collections of the University of Novi Sad, Serbia, eight from seeds collections of Agrobioinstitute, Sofia and two samples from seed collection of Agricultural Research & Development Institute Fundulea, Fundulea, Romania.

Primers design: The annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231) were processed by Vector NTI 10.0 software for multiple allayment and finding conservative regions and next consensus sequences were used for design of primers by and Primer 3 plus software.

We used similar approach to design primers for *Orobanche cernua* / *O. cumana* Rubisco large subunit (rbcL) pseudogene (Accession: U73968, AY582189, AY582188, AF090349). Primers were purchased Metabion AG, Germany. Upon arrival they were dissolved in DNAse free water up to 100 µmol concentration and stored at -20 ºC until use. Before use 10 µmol aliquots were prepared.

Isolation of total (genomic and chloroplast) DNA: Seeds came as pools by locations. About 50 mg of each seeds sample were grind to fine powder in pre-cooled by liquid nitrogen mortar and pestle. The powder was quickly transferred in pre-cooled microcentrifuge tube and the DNA was isolated by Analytic Yena kit following the standard protocol.

PCR reaction conditions: Approximately 150 ng DNA template was taken from each sample and mixed in 200 µL PCR tube with 1 µL of each primer (10 $mmol.L^{-1}$ concentration), 25 µL PCR master mix (Fermentas, Cat № K0171) and 21 µL DNAse-free water (supplied with the master mix kit). The PCR tubes were places TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried-out by using the following program: initial DNA melting at 94 ºC – 5min; next 35 cycles of 94 ºC – 45 s; 57 ºC –45 s; 72 ºC – 2 min 30 s and final extension at 72 ºC for 10 min.

Purification of the PCR products: Initially PCR products were separated by agarose gel electrophoresis. For this purpose each sample was mixed with 5 mL of loading dye (Fermentas, Cat № R0611), loaded onto 1% agarose gel containing 0.5 mg/mL ethidium bromide (final concentration) covered with 0,5 X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler Cat № SM0311). The PCR products were visualized by UV light and documented by BIO-VISION+3026.WL system (Vilber Lourmat). Next the band containing separated PCR the products were sliced out of the gels by clear surgical blades and isolated from the agarose by QIAquick Gel extraction kit (Qiagen, Cat № 28704) according to the original protocol. Purified PCR products were sent for sequencing to GATC – Biotech AG, Cologne, Germany.

Data analysis: Initially the sequences were subjected to online analyses in NCBI database to confirm that the isolated sequences are indeed those of interest using the nblast algorithm of Altschul et al. (1997). The multiple alignments of obtained sequences, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).

RESULTS

Primer design

The universal ITS1 primers available elsewhere had poor performance during our initial experiments and therefore we decided to design our own primers based on annotated in NCBI sequences for *O. cumana* and *O. cernua*. Multiple sequence alignments provided information about conservative regions in annotated sequences. The multiple sequence alignment of *Orobanche cernua* / *O. cumana* ITS1/5,8S/ITS2/26 S region in presented as illustration on Fig. 1.

Table 1. List of the studied samples

№	Acronym	Species	Geographic origin, year collected, (host if different from sunflower)
1.	IAS cum 1	*O. cumana*	Andalucía, Córdoba, Spain, 2008
2.	IAS cum 2	*O. cumana*	Andalucía, Sevilla, Ecija, Spain, 2008
3.	IAS cum 4	*O. cumana*	Castilla-La Mancha, Cuenca, Villarejo de Fuentes, Spain, 2008
4.	SE 01	*O. cumana*	Andalucía, Sevilla, El Coronil, Spain 1989
5.	Co 06	*O. cumana*	Andalucía, Cordoba, La Carlota, Spain 2001
6.	Cu 05	*O. cumana*	Castilla-La Mancha, Cuenca, La Almarcha, Spain, 1996
7.	Cu 07	*O. cumana*	Castilla-La Mancha, Cuenca, Carrascosa del Campo, Spain, 1996
8.	Cu 12	*O. cumana*	Castilla-La Mancha, Cuenca, Palomares del Campo, Spain, 2008
9.	Boco 34	*O. cumana*	Balchik, Bulgaria, 2006 (*Artemisia maritima*)
10.	Boco 35	*O. cumana*	Gorun-Tyulenovo, Bulgaria, 2006 (*Anthemis arvensis*)
11.	Boro 37	*O. cernua*	Andalucía, Almeria, Nijar-Lucainena, Spain, 2006 (*Launaea lanifera*)
12.	Boro 38	*O. cernua*	Andalucía, Almeria, Venta de Los Yesos, Spain, 2006 (*A. barrelieri*)
13.	Boro 43	*O. cernua*	Andalucía, Jaen, Jodar, Spain, 2006 (*A. barrelieri*)
14.	Boro 44	*O. cernua*	Andalucía, Jaen, Cabra Santo Cristo, Spain, 2006 (*A. barrelieri*)
15.	Barolo	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
16.	Barolo - Ro	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
17.	Beluga	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
18.	Or5	*O. cumana*	Sombor, Vojvodina, Serbia, 2012
19.	Or5+	*O. cumana*	Sombor, Vojvodina, Serbia, 2012
20.	24/11	*O. cumana*	Sivac, Vojvodina, Serbia, 2011
21.	31/11	*O. cumana*	Sombor, Vojvodina, Serbia, 2011
22.	32/11	*O. cumana*	Sombor, Vojvodina, Serbia, 2011
23.	Negotin	*O. cumana*	Negotin, Bor District, Serbia, 2011
24.	Norh-34	*O. cumana*	Novi Sad, Vojvodina, Serbia, 2011
25.	VRSAC	*O. cumana*	Novi Sad, Vojvodina, Serbia, 2009
26.	Sunfl-2010	*O. cumana*	Backa topola, Vojvodina, Serbia, 2010
27.	Volgograd	*O. cumana*	Volgograd, Volgograd District, Russia, 2002
28.	Krapets	*O. cumana*	Krapets, Dobrich District, Bulgaria, 2002
29.	Vratza	*O. cumana*	Vratza, Bulgaria, 2001
30.	Edirne	*O. cumana*	Edirne, Eastern Thrace, Turkey, 2003
31.	Zaporozhye	*O. cumana*	Zaporozhye, Southeastern Ukraine, 1998
32.	Gen. Toshevo	*O. cumana*	General Toshevo, Dobrich District, Bulgaria, 2008
33.	Izmir	*O. cumana*	İzmir, Anatolia District, Turkey
34.	Sadovo	*O. cumana*	Sadovo, Upper Thrace, Bulgaria
35.	PF 100 (Rom)	*O. cumana*	Fundulea, Romania, 2012
36.	1238/2 (Rom)	*O. cumana*	Fundulea, Romania, 2012

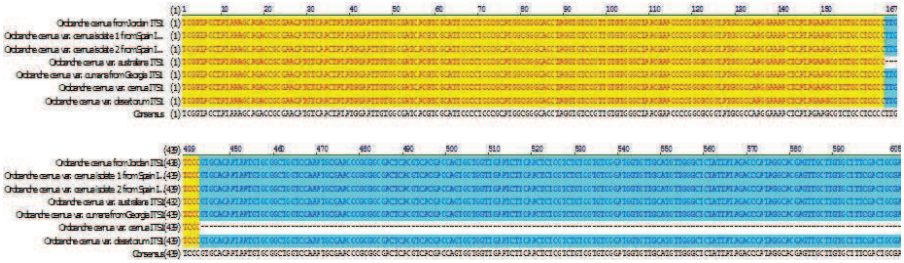

Fig. 1. Multiple alignment of annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231.

We used the consensus sequences to designed two sets of specific primers for each sequence by Primer3 plus (Table 2). The external sets of primers were used to isolate sequences, while the internal were used for control PCRs and sequencing.

Table 2. List of specific primers designed for isolation of ITS1/2 and RbcL sequences. Primers Fw1/Rev 1 are external toward Fw2/Rev2.

Primer name	*Sequence 5' → 3'*
18 S Fw1	ATAAAGCAGACCGYGAACATG
18 S Fw2	CGACTATATGGAAYTGTGGCG
26 S Rev1	AGAGCCCAACATGCAACACC
26 S Rev2	CGCAGTCGAAAGCACAAGTAG
RbcL Fw 1	CCTGCGTGATCTATGTCTGG
RbcL Fw 2	GCTCCATGGTATTCAAGTTTAAAGAG
RbcL Rev 1	TGCATTACGCTAAGGATGTCC
RbcL Rev 2	TCATTACGAGCTTGTACACATGC

Isolation of marker sequences

Newly designed primers for ribosomal gene cluster produces single reproducible bands for each sample with expected size:580 bp (Fig. 2). Isolation of fragments from Rubisco large subunit (rbcL) pseudogene was much more difficult probably due to the fact that we used as plant material dry seeds. The amount of chloroplast DNA in seeds of any species is negligible. Nevertheless we managed to isolate RbcL products with expected size from 31 out of 36 samples.

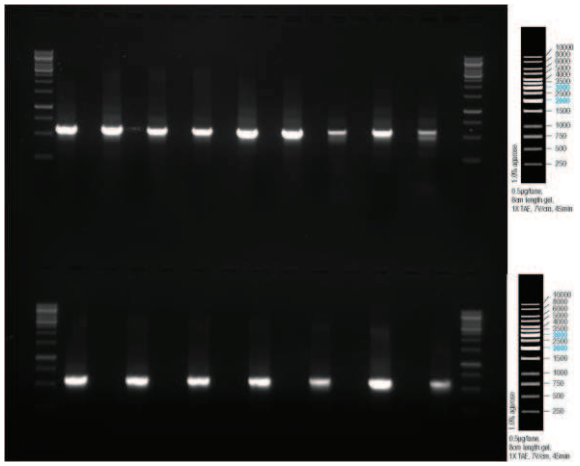

Fig. 2. The ITS1/2 products amplified by primers 18S Fw1 and 26S Rev1 were separated on 1% agarose gel containing 0.5 mg/mL ethidium bromide and visualized by UV light. The PCR products size was determined using 1 Kb Fermentas GeneRuler (Cat. № SM0311)

Sequences comparison and phylogenic analyses.

Initially we compared annotated by other authors sequences of ribosomal gene cluster. The sequences revealed clear separation of *O. cumana* from *O. cernua* by SNP located at relative position 423 (Fig. 3).

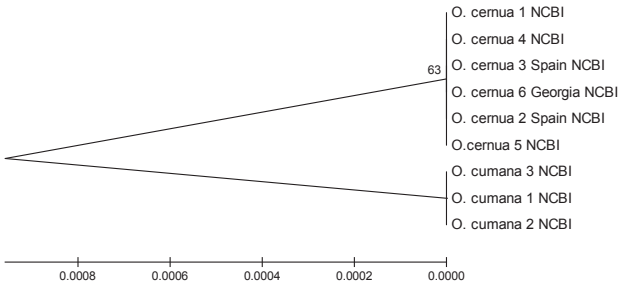

Fig. 3. Phylogenic tree build by annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231). Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 replications.

Next step was to compare our samples and officially annotated. The same algorithm was used. The final phylogenic is presented on Fig. 4. Similar approach was adopted also for comparison of annotated in NCBI and obtained by us sequences of Rubisco large subunit (rbcL) pseudogene. Because the annotated sequences were not sufficient for building separated tree, only one joint tree was produced (Fig. 5)

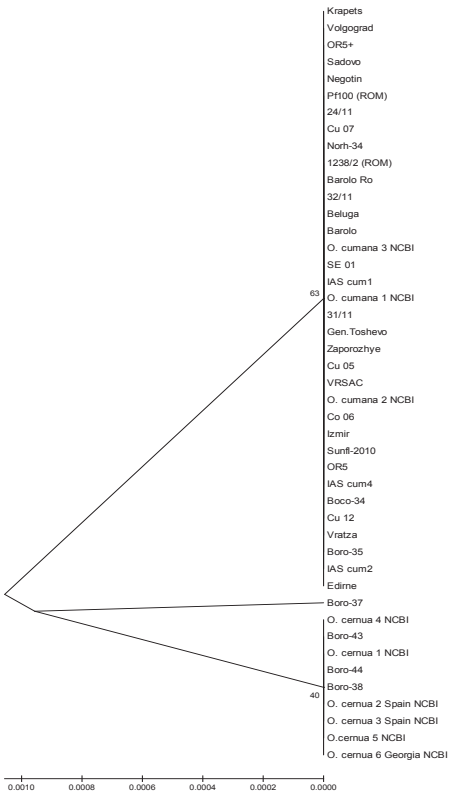

Fig. 4. Phylogenic tree build by experimental and annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region. Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 Replications.

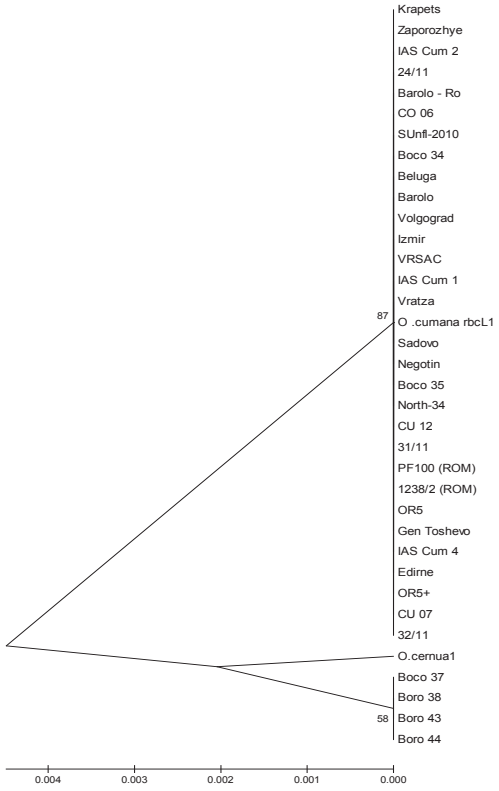

Fig. 5. Phylogenic tree build by experimental and annotated in NCBI sequences of *Orobanche cernua* / *cumana* partial Rubisco large subunit (rbcL) pseudogene (Accession: U73968, AY582189, AY582188, AF090349). Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 Replications.

DISCUSSION

The ITS1/2 regions of the ribosomal cistron are one of the most popular sequences for phylogenetic analyses at the generic and infrageneric levels in plants. The main feature that makes this region so widely used in molecular phylogeny and evolution studies is the combination of highly conservative (rRNA genes) and highly variable (ITS) regions (Moller and Cronk, 2001; Cruichshank, 2002). Other advantages of the use of ITS sequences are:

• The ITS1/2 regions is relatively short (500-800 nt).It is easily amplified by PCR using primers complementary to the conservative 18S and 26 S regions ITS-flanking
• ITS 1/2 regions are easily amplified even from diluted or degraded DNA samples.
• ITS 1/2 regions are highly variable – they could accumulate differences between closely related species and even between populations within a species.

As the ITS regions are not functional, the evolution of these sequences seem to occur according to the neutral model of Kimura, in which the genetic drift is the major driving force. Natural selection cannot operate on non-functional sequences because they don't have adaptive meaning for the organism, so the changes occurring in such sequences are random and accumulate mutations uniformly with the time (molecular clock). This makes neutral sequences useful markers for phylogeny analyses because the mutation rate reflects the divergence time between populations/species. (Kimura, 1968, 1985). Similar to the ITS 1/2 regions in non photosynthetically active plastids of broomrapes, Rubisco large subunit (rbcL)

is relaxed from evolutionary pressure and is a pseudogene. So we can expect both nuclear and plastid sequences to accumulate uniformly mutations.

Our experimental as well as those officially annotated in NCBI data demonstrated that *O. cumana* can be differentiated from *O. cernua*, by single C/T transition located in ITS2 (rel. position 423). Rubisco large subunit in *O cumana* differs from *O. cernua* with two transversion: T/G (rel. position 15) and A/C (rel. position 84). The genetic diversity observed in *O. cumana* was lower than in *O. cernua*. When comparing the ITS and rbcL sequences isolated from *O. cernua* two single SNPs were found that can discriminate different origins. Both ITS1/2 and rbcL sequences isolated from *O. cumana* however were completely homogeneous, despite the fact that samples were collected form very distant locations: from Volgograd, Russia to the East to Spain to the West. This observation is with agreement of earlier by study of Gagne et al. (1998). The authors also found *O. cumana* populations from different geographical origins to be genetically very similar, pointing to a possible monophyletic origin Gagne et al. (1998). Such assumption supports the hypothesis that the move of *O. cumana* from wild hosts on sunflower was a single act that occurred once and all invasive races are descendants from ancient Caucasus population. The high sequence similarity between *O. cumana* and *O. cernua* could mean that the separation of the two groups is a recent event. It is not possible however at this stage to draw conclusion whether they are specie or subspecies. Various molecular processes can impact ITS and RbcL sequences. Among the most prevalent complications is the existence in many plant genomes of extensive sequence variation, arising from ancient or recent array of duplication events, presence of pseudogenes at various states of decay, and/or incomplete intra- or interarray homogenization (Álvarez and Wendel, 2003). This is the reason why in their extensive review, Álvarez and Wendel (2003) recommend together with non functional sequences other single-copy functional nuclear genes to be used. Probably genes related to *O. cumana* aggressiveness should be identified and used for molecular markers. We assume the reported by Pineda-Martos et al. (2013a,b) SSRs flank such genetic regions and their sequencing can provide molecular tools to determine genetic relationships within and among *O. cumana* populations.

REFERENCES

Álvarez, I., and J.F. Wendel. 2003 Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29:417-434.

Benharrat, H., P. Delavault, C. Theodet, C. Figureau, and P. Thalouarn. 2000. RbcL Plastid pseudogene as a tool for *Orobanche* (Subsection Minores) identification, Plant Biol. 2:34-39.

Cruichshank, R. 2002. Molecular markers for the phylogenetics of mites and ticks. Syst. Appl. Acarol. 7:3-14.

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

Katzir, N, V. Portnoy, G. Tzuri, M. Castejón-Muñoz, and D.M. Joel. 1996. Use of random amplified polymorphic DNA (RAPD) markers in the study of the parasitic weed *Orobanche*. Theor. Appl. Genet. 93:367-372.

Kimura, M. 1968. Evolutionary rate at the molecular level. Nature 217:624-6266.

Kimura, M. 1985. The Neutral Theory of Molecular Evolution, Cambridge University Press, 384 pp.

Möller, M., and Q.C.B. Cronk. 2001. Evolution of morphological novelty: A phylogenetic analysis of growth patterns in *Streptocarpus* (Gesneriaceae). Evolution 55:918-929.

Morozov, V.K. 1947. Sunflower breeding in USSR. Pishchepromizdat, Moscow, p. 1-274 (In Russian).

Parker, C., and C.R. Riches. 1993. Parasitic Weeds of the World: Biology and Control. CAB International, Wallingford, UK.

Parker, C. 2009. Observations on the current status of *Orobanche* and *Striga* problems worldwide. Pest Manag. Sci. 65:453-459.

Pineda-Martos, R., L. Velasco, J. Fernández-Escobar, J.M. Fernández-Martínez, and B. Pérez-Vich. 2013. Genetic diversity of *Orobanche cumana* populations from Spain assessed using SSR markers. Weed Res. 53:279-289.

Pineda-Martos, R., L. Velasco, and B. Pérez-Vich. 2014. Identification, characterisation and discriminatory power of microsatellite markers in the parasitic weed *Orobanche cumana,* Weed Res. 54:120-132.

Pujadas-Salvà, A.J., and L. Velasco. 2000. Comparative studies on *Orobanche cernua* L. and *O. cumana* Wallr. (Orobanchaceae) in the Iberian Peninsula, Bot. J. Linn. Soc. 134:513-527.

Schneeweiss, G.M., A. Colwell, J.M. Park, C.G. Jang, and T.F. Stuessy. 2004. Phylogeny of holoparasitic *Orobanche* (Orobanchaceae) inferred from nuclear ITS sequences. Mol. Phylogen. Evol. 30:465-478.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.

Teryokhin, E. 1997. Weed Broomrapes – Systematics, Ontogenesis, Biology, Evolution. Aufstieg Verlag, Landshut, Germany, 243 pp.

Venkov, V, and H. Bozoukov. 1994. Influence of different species of *Orobanche* on cultivated sunflower and tobacco. p. 349–350. In: A.H. Pieterse, J.A.C. Verkleij, and S.J. ter borg (eds.), Biology and Management of *Orobanche*: Proc. 3rd Int. Workshop on *Orobanche* and related *Striga* Research. Amsterdam, The Netherlands.

Weiss-Schneeweiss, H., J. Greilhuber, and G.M. Schneeweiss. 2006. Genome size evolution in holoparasitic *Orobanche* (Orobanchaceae) and related genera. Am. J. Bot. 93:148-156.

Wolfe, K., C. Morden, and J. Palmer. 1992. Small single-copy region of plastid DNA in the non-photosynthetic angiosperm *Epifagus virginiana* contains only two genes. Differences among dicots, monocots and bryophytes in gene organization at a non-bioenergetic locus. J. Mol. Biol. 223:95-104.