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Study of the variability and evolution of *Orobanche cumana* populations infesting sunflower in different European countries

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Abstract The parasitic plant *Orobanche cumana* Wallr. has become a limiting factor for sunflower crops in infested countries. Over the past few years the progression of this parasitic plant, its introduction into new countries, and the development of new and more virulent races have all been observed. Consequently, the survey and understanding of broomrape population evolution is now crucial for the establishment of efficient breeding programmes. With this in prospect, the genetic variability *of O. cumana* populations from infested European countries, Bulgaria, Romania, Turkey and Spain, was studied using RAPD markers. Eight populations with a total of 180 plants were analysed. Twenty three primers were used to obtain 133 reproducible bands which led to a binary matrix. This matrix was subjected to various complementary analyses including pairwise distances computed with the Nei and Li coefficient, AMOVA, Nei's genetic diversity statistics, and an estimation of gene flow among populations with the infinite-island formula. The results gave consistent conclusions whatever the method used for data treatment. We show that this parasitic plant is probably self-pollinated, that there is little intra-population variability, and very little gene exchange appears to occur between different geographic regions. Populations were well structured and organized into two distinct groups (one group corresponding to the East European countries, Bulgaria, Romania and Turkey, and the other group corresponding to Spanish populations) and could have a monophyletic origin. These results are discussed in relation to the applied uses of RAPD markers in the determination of true *O. cumana* races instead of populations.

Key words RAPD · *Orobanche cumana* · *Helianthus annuus* · Genetic diversity

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Introduction

Broomrape (*Orobanche cumana* Wallr.) is a holoparasitic angiosperm which infects sunflower (*Helianthus annuus* L.) roots. This parasitic plant originated in the Mediterranean region and has now colonized Eastern Europe, South West Asia and Western Australia (Musselman 1986). Strikingly, this species of broomrape is not found in France in spite of a landborder with Spain where it is frequent. Broomrape can cause considerable losses in the sunflower crop and is an important problem because of the large area sown to sunflowers in infested zones (Joël 1988). Mijatovic and Stojanovic (1973) noted that in Yougoslavia broomrape decreased sunflower yield by 33% and consequently areas under sunflower declined by about 37%. Breeding programmes for the incorporation of resistance genes to broomrape in sunflower were started many years ago in Eastern Europe. At present, the disease is controlled mainly by the use of resistant cultivars. However, this control is overcome by the

development of new races. As a consequence, the survey and understanding of *Orobanche* population evolution is crucial for sunflower breeders.

Broomrape belongs to genus of the *Orobanche* and to section Osproleon, and is characterized by an unbranched stem. In this section, the two species known as *Orobanche cernua* Loefl. and *O. cumana* are closely related and were considered for a long time as the same species. More recently, *O. cumana* has been regarded as a variant or sub-species of *O. cernua* (e.g. Chater and Webb 1972; Feinbrun-Dothan 1978) and only very recently as a separate species based on both morphological and behavioral characters (Joel 1988). The host range of *O. cernua* comprises several Solanaceae species while the *O. cumana* host range seems to be limited to sunflower, although it has been also reported on tomato and *Arthemisia maritima* L. (Katzir et al. 1996; e.g. Chater and Webb 1972). Molecular studies have shown that *O. cernua* and *O. cumana* could be easily distinguished (Katzir et al. 1996; Paran et al. 1997).

Various biotypes of *O. cumana* have been recorded differing in their virulence to sunflower cultivars. Vranceanu et al. (1986) described five biotypes (races) in Romania (A–E). According to Shindrova (1993) these five biotypes were also found in Bulgaria. In Spain, *O. cumana* was first detected in 1958 in the province of Toledo on confectionery sunflowers by Diaz-Celayeta (1974) and on oil sunflowers by Sackston (1978). Gonzalez-Carrascosa (1992) reported the presence of three races in Spain. These races are probably different to those described in Eastern Europe (Melero-Vara et al. 1989). In Spain, morphological differences were found within and between *Orobanche* populations infesting sunflowers, but such differences in the morphology bore no relation to the host cultivar they parasitized (Gonzales-Torres et al. 1982). Moreover, molecular studies on *O. crenata* and *O. aegyptiaca* in Israël have also shown that populations variability was not due to the host (Paran et al. 1997). The isozyme patterns of sunflower parasite populations indicated that the more complex isozyme systems of the parasite population are related to the duration and severity of the field infestation as a result of growing susceptible crops for several years (Casteljon-Munoz et al. 1991a). The present study used the RAPD technique (Williams et al. 1990), Katzir et al. (1996) and Paran et al. (1997) having shown that RAPD markers can serve as simple and reliable tools in the taxonomic analysis of the genus *Orobanche*.

The aim of the present study was to characterize molecular markers to determine the genetic variability of *O. cumana* populations originating from highly infested countries, such as Bulgaria, Spain, Romania and Turkey, in order to gain an insight into the fundamental genetic evolution of this parasitic weed, in addition to a practical survey of these populations.

Materials and methods

Plant materials

Orobanche was collected on infested sunflowers in several geographic origins. Three Bulgarian (populations 1, 2 and 3), one Turkish (population 4), one Romanian (population 5), and three Spanish populations (populations 6, 7 and 8) were studied. Seeds from a minimum of 300 *Orobanche* plants were collected in one or a few fields in the same area. When known, the characteristics of sunflowers on which the *Orobanche* was growing were noted (Table 1). Seeds from each of the eight regions were mixed with soil in pots in which susceptible sunflower genotypes were grown, AD66 for East European populations or RPGL 6 for Spanish populations. Ten to twenty seven *Orobanche* shoots per region, and called populations, were kept and two flower buds per shoot were collected and freeze dried (East European populations) or else frozen (Spanish populations) for DNA extraction. A total of 180 *Orobanche* plants was studied and the number of plants per population is shown in Table 1.

Genomic DNA isolation

Two flower buds were mixed with sand and 500 µl of CTAB 2% buffer (Tris/HCl 10 mM, EDTA 100 mM, NaCl 1.4 mM, CTAB 2% w/v, PVP 40,000 1% w/v, pH 8.0). The rest of the procedure was similar to that of Rogers and Bendich (1985).

Table 1 Origin of the *O. cumana* populations used in this study

Population	Origin	Characteristics of the sunflower host	Number of plants	Year of harvest
1	Bulgaria, South Bulgaria near Burgass town	Different unknown genotypes	24	1995
2	Bulgaria, 40 km to North-West of Dobrich town	Peredovik cultivar	22	1995
3	Bulgaria, 45 km to North-East of Dobrich, near the Romanian frontier, 10 km to Black Sea	Peredovik and another susceptible genotype	27	1995
4	Turkey, 50 km to Bulgarian border	Unknown	20	1994
5	Romania near Bucarest	Unknown	25	1995
6	Spain, Ecija, near Sevilla	Cultivars resistant to population 7	27	1994
7	Spain, El Coronil, near Sevilla	Different confectionary susceptible sunflowers	25	1990
8	Spain, Ecija	Sunflowers resistant to population 6	10	1996

RAPD analysis

PCR reactions were performed in a volume of 25 µl combining 30 ng of genomic DNA, 200 µM of the dNTPs (Pharmacia, Saint-Quentin, France), 0.2 µM of primer (Operon Technologies Inc., Alameda, USA), 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, Triton X100 0.1%, gelatin 0.2% and 1.5 u of *Taq* DNA polymerase (Appligene, Illkirch, France). Amplification was performed in a 9600 Perkin Elmer Cetus thermal cycler as follows: initial denaturation for 5 min at 91°C, then 40 cycles of 1 min at 91°C, 1 min at 36°C and 2 min at 72°C. The 40 cycles were followed by a final extension for 5 min at 72°C. PCR products were separated on a 1.4% agarose gel in TAE buffer (Tris-acetate 0.04 M, EDTA 0.001 M) at 70 V constant for 4 h, stained with ethidium bromide and photographed in UV light.

Statistical analysis

Only the presence (1) or absence (0) of major and reproducible bands was scored, as were the amplification products that were systematically obtained only in repeat experiments. The identification of monomorphic and polymorphic bands led to the construction of a data matrix which was analysed for the variability of all populations. Pairwise distances were computed with the Nei and Li coefficient (1979). The distance matrix was subjected to principal component analysis (PCA) with UNIWIN Plus version 2 distributed by UNIWARE. The distance matrix was also subjected to an analysis of molecular variance, AMOVA (Excoffier et al. 1992), to estimate variance components for RAPD phenotypes, partitioning the variation within populations, between populations and between regions or groups (East European countries: Bulgaria, Romania and Turkey vs Spain). The resulting test statistics, Φ-statistics, are analogous to Wright's (1951, 1965) statistics. The significance levels for variance components and Φ-statistics estimates were computed by permutational procedures with Splus Software (Becker et al. 1992).

Assuming that each RAPD band corresponds to a biallelic locus, and that all individuals were homozygous, Nei's (1973) genetic diversity statistics were used to measure the total gene diversity (Htm) as well as the intra-population (Hs) and intra-group (Hg) differences. Hsm and Hgm were respectively Hs mean and Hg mean. The genetic differentiation between populations, Gst, was calculated as Gst = (Htm − Hsm)/Htm, which is equivalent to Wright's Fst for biallelic loci, as well as Gct = (Htm − Hgm)/Htm (genetic differentiation among groups) and Gsc = (Hgm − Hsm)/Hgm (genetic differentiation among populations within a group). Empirical standard deviations were obtained with 200 bootstrap resamplings, with ten individuals per population which were randomly chosen in order to compare all populations among themselves.

Gst was calculated also for all population pairs with both polymorphic and monomorphic loci. With the matrix obtained, a minimum spanning tree was constructed. To estimate the gene flow among populations, the number of migrants exchanged per generation, Nm, was calculated indirectly from Gst values by applying Wright's (1931) infinite-island formula: Nm = (1 − Gst)/4Gst, where N is the effective population size and m is the proportion of migrants exchanged per generation. These calculations were carried out with Splus Software (Becker et al. 1992).

Results

RAPD analysis

One hundred and seventy seven arbitrary decamer primers were screened for their suitability for PCR reactions using DNA from one plant from each population. Among 173 primers giving DNA amplification, 23 were selected for their reproducibility. These 23 primers generated a total of 133 bands: 57 polymorphic bands and 76 monomorphic bands. Each primer revealed an average of six bands per plant. For all 23 primers, very similar amplification patterns were obtained although the geographic origins and the sunflower genotypes were different.

Population structure

Nei and Li (1979) distances were calculated from the 0–1 matrix obtained from 133 RAPD bands and 180 *Orobanche* plants. The lowest percentage similarity (80%) was found between two plants from the Bulgarian (population 1) and Spanish (population 6) populations. Figure 1 shows the first PCA analysis of

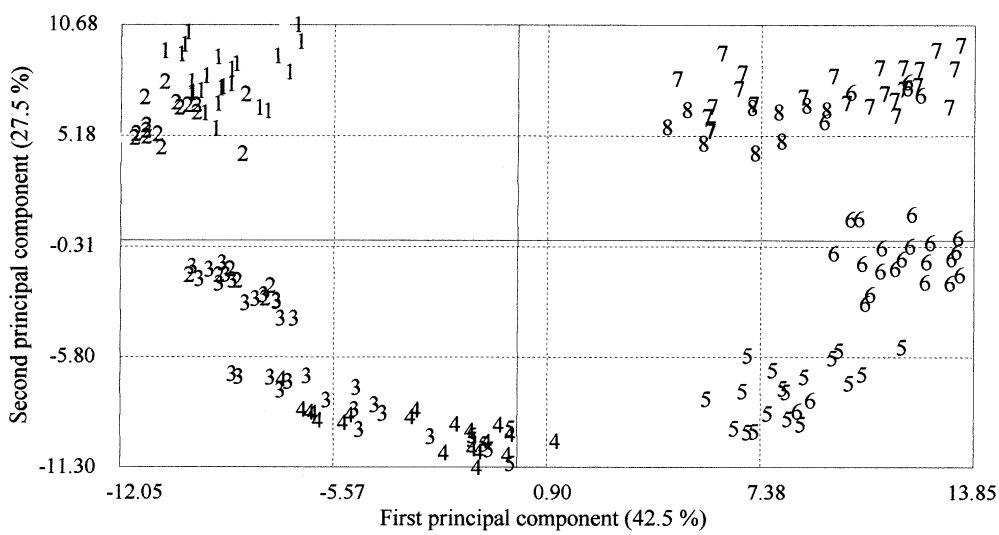

Fig. 1 Diagram resulting from the principal component analysis of the distance matrix of 180 orobanche (*O. cumana*) plants representing all the populations. The pairwise distances were computed with the Nei and Li coefficient (1979). The two principal components explain 42.5% and 27.5% respectively. Each plant is represented by the number of the population to which it belongs, given in Table 1

Table 2 Analysis of molecular variance (AMOVA) of 180 *Orobanche* plants, representing eight populations using 133 RAPD markers. The total data set contains individuals from two arbitrary regions or groups: Spain (represented by three populations) and East European countries (represented by five populations from Bulgaria, Romania and Turkey). The statistics include sums of squared deviations (SSD), mean squared deviations (MSD), variance component estimates (compV), the percentages of the total variance (comp%) contributed by each component, Φ-statistics with Φct, Φst and Φsc representing differentiation among groups, among populations within the whole population, and among populations within groups respectively, the probability (*P*) of obtaining a more extreme component estimate by chance alone, obtained with 200 bootstrap resamplings over loci

Source of variation	SSD	*df*	MSD	Comp V	Comp%	Φ-Statistics	*P*-value
Spain vs East countries	1.644	1	1.644	0.010	15.025	Φct = 0.15	< 0.01
Among populations/group	3.407	6	0.568	0.033	47.042	Φsc = 0.56	< 0.01
Within populations/group	4.52	172	0.026	0.026	37.933	Φst = 0.62	< 0.01

Table 3 Nei's genetic diversity estimates for each population of *O. cumana*. Htm = total genetic diversity; Hs = within-population genetic diversity, Hsm = Hs mean; Hg = between populations within group genetic diversity, Hgm = Hg mean. Gst, Gsc and Gct represent the differentiation among populations within the whole population, among populations within groups, and among groups respectively. Numbers in the first column refer to population number in Table 1

Group	Populations	Hs			Hg		
		Lower bound	Mean	Higher bound	Lower bound	Mean	Higher bound
East country populations	1	0.051	0.075	0.100			
	2	0.044	0.066	0.087			
	3	0.071	0.082	0.094	0.154	0.162	0.171
	4	0.073	0.087	0.101			
	5	0.073	0.090	0.108			
Spanish populations	6	0.060	0.087	0.114			
	7	0.067	0.085	0.104	0.109	0.119	0.128
	8	0.028	0.037	0.045			
	Hsm	0.070	0.076	0.082			
	Hgm	0.134	0.141	0.147			
	Htm	0.174	0.180	0.186			
	Gst	0.539	0.577	0.615			
	Gsg	0.315	0.357	0.399			
	Ggt	0.197	0.220	0.242			

the distance matrix, which accounts for 70% of the inertia. We observed that populations from the East European countries could be separated from the Spanish populations, although a few individuals from the population 6 appeared to be similar to population 5.

Molecular variance analysis

An AMOVA was carried out on the distance matrix, demonstrating significant genetic differences between groups, as well as between populations within groups and between individuals within populations. Of the total genetic diversity, 15% was attributable to divergence between groups, 47% to population differentiation within groups, and 37.9% to individual differences within populations. Φst, Φct and Φsc values are shown in Table 2.

Nei's statistics

The mean of intra-population genetic diversity Hsm is low, but not negligible (0.076), compared with the total genetic diversity Htm (0.18). Hs for Spanish population 8 (0.037) is not significantly different from that of Bulgarian population 2 (0.066) but is significantly different from all the Hs of the other populations. Within-group genetic diversities (Hg) are significantly different : 0.162 for the East European group and 0.119 for the Spanish group. Gst, Gsc and Gct values are shown in Table 3.

Gst values were calculated between all population pairs (Table 4), The highest Gst was obtained between the Spanish (8) and Bulgarian (1) populations whereas the lowest Gst was between the Bulgarian (3) and Turkish (4) populations. The minimum spanning tree was calculated from the Gst matrix (Fig. 2) and shows populations 3 and 4 as being the closest. The two Bulgarian populations 1 and 2 appear well separated

Table 4 Genetic differentiation (Gst) calculated between all pairs of *O. cumana* populations. Numbers refer to population numbers in Table 1

Number	1	2	3	4	5	6	7	8
1	–							
2	0.29	–						
3	0.36	0.26	–					
4	0.41	0.38	0.16	–				
5	0.47	0.44	0.31	0.21	–			
6	0.46	0.47	0.39	0.34	0.27	–		
7	0.44	0.44	0.40	0.42	0.36	0.25	–	
8	0.57	0.55	0.47	0.53	0.48	0.40	0.25	–

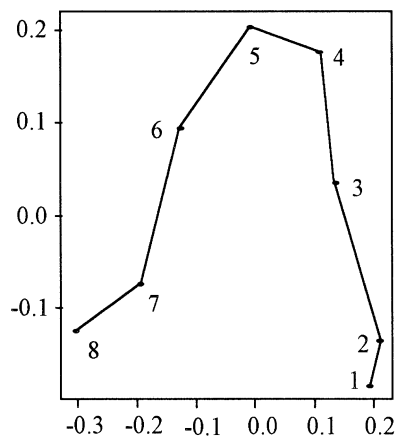

Fig. 2 Principal component analysis of the pairwise Gst matrix and minimum spanning tree among the eight populations of *O. cumana*

from the Spanish populations. Nm estimates between populations were calculated from Gst data (Table 5) and ranged from 0.19 (between populations 1 and 8) to 1.31 (between populations 3 and 4).

Discussion

Because of the increased occurrence of *O. cumana* on sunflower crops and the appearance of new races not controlled by many resistance genes, a precise knowledge of this parasite has become important. The only published study of variability of broomrape populations infesting sunflower was based on isozyme analysis, thus concerning a limited number of loci, and was restricted to Spain (Casteljon-Munoz et al. 1991a), although this species was first reported at the begining of the century in Eastern Europe (Cubero 1989) and was only observed recently in Spain (Diaz-Celayeta 1974). The present study was therefore undertaken to analyse and compare the genetic variability of broomrape populations from Bulgaria, Romania, Turkey and Spain, using the RAPD technique which has been shown to be useful for estimating of genetic relationships within and between species (Landry et al. 1994; Thorman et al. 1994; Katzir et al. 1996; Paran et al. 1997).

Table 5 Estimation of gene flow among populations of *O. cumana*. Nm (number of migrants exchanged per generation) was calculated from Gst values by applying Wright's (1931) infinite-island formula: $Nm = (1 - Gst)/4Gst$. N is the effective population size and m is the proportion of migrants exchanged per generation

Normal	1	2	3	4	5	6	7	8
1	–							
2	0.62	–						
3	0.45	0.70	–					
4	0.35	0.42	1.31	–				
5	0.28	0.32	0.57	0.96	–			
6	0.30	0.28	0.39	0.48	0.67	–		
7	0.31	0.32	0.37	0.35	0.45	0.75	–	
8	0.19	0.20	0.28	0.22	0.27	0.37	0.77	–

The breeding system of *O. cumana*

The breeding system of *O. cumana* is not well known. Some authors have reported this species as being self-pollinated, like *O. cernua* (Pieterse and Verkleij 1991). Hamrick and Godt (1989) reviewed the plant allozyme literature concerning variation among populations and have shown that self-pollinated species were characterized by a relatively high Ht values, low Hs values and high Gst values. Our results are consistent with this observation, the values of Htm, Hsm, and Gst obtained being respectively 0.180, 0.076 and 0.577 (Table 3). In particular, our Gst value is very similar to that given by Hamrick and Godt (1989) for self-pollinated species (0.510). However, it should be noted that allelic frequencies were estimated assuming homozygosity of plants, i.e. selfing, this being justified by most of the literature on *Orobanche*. Nevertheless, we also estimated allelic frequencies under panmixia assuming that the '0' allele was recessive, and found that the magnitude of Nei statistics remained unchanged (data not shown), which again supports the selfing hypothesis. In addition, our analysis of molecular variance showed that the inter-population component was large: 0.47 (Table 2). In cross-pollinated species such a high inter-population component is never encountered: for example, Huff et al. (1993) found only a 9.7% difference between populations of buffalograss within regions and a 58.4% regional divergence. In the present study the regional divergence value was only 15%. Overall, these results suggest that *O. cumana* populations are self-pollinated, whatever their geographic origins.

Intra-population genetic diversities of populations 1 to 7 were not significantly different. The only population showing a lower intra-population genetic diversity was the Spanish population 8 (Table 3). This

population is the most recent and the most virulent, and is only rarely found in Spain. It was collected on a sunflower genotype resistant to populations 6 and 7. In their study of Spanish populations, Casteljon-Munoz et al. (1991a) showed that those with most isoenzymatic variability were collected from fields in which *Orobanche* had been observed for many years and/or at high infestation levels. Our results seem in agreement with this conclusion. Furthermore, seed-dispersal methods are an additional characteristic of breeding systems influencing genetic diversity in plant species (Hamrick and Godt 1989; Hamrick et al. 1992). According to Casteljon et al. (1991b) the wind acts, at least for short distances, as an important agent of broomrape dispersal. Other studies on *O. crenata* and its host Faba bean (*Vicia faba*) have shown that the spatial dispersion of broomrape infestations took place mainly in the direction of crop rows, due to tillage and harvesting operations, while wind played a secondary role (Lopez-Granados and Garcia-Torres 1993). The genetic diversity within populations of *O. cumana* is certainly due to a combination of all these factors: breeding and seed-dispersion systems, age of population, and infestation level.

The populations organized in two distinct groups could have a monophyletic origin

We have analysed the organization of the populations using different methods (AMOVA, Nei's statistics, PCA), which all indicate the existence of two distinct groups. The minimum spanning tree (Fig. 2) shows Turkish, Romanian and Bulgarian populations in one group, with the Spanish populations in a separate group. According to Wright (1978), considerable genetic differentiation between groups implies different geographic origins for these groups. However, most of our data on intra-population genetic diversity are not significantly different, and do not suggest any obvious center for their origin.

Gene exchanges between populations were generally low (Table 5). The highest values of Nm were found between populations which appear grouped in PCA (Fig. 1); for example Nm is 0.67 between the Eastern European population 5 and Spanish population 6. Population 5 appears to link the two groups suggesting that Spanish population 6 could come from Romanian population 5, and transported by sunflower seeds as shown by Casteljon et al. (1991b) who have observed that approximately 5% of the sunflower seeds collected in infested fields were carriers of broomrape seeds.

The large genetic diversity between the populations that we have observed (Table 3) could be due to small gene exchanges resulting from the self-pollination, as discussed above. Self-pollinated species maintain as much diversity at their polymorphic loci as cross-pollinated species (Hamrick and Godt, 1989). According to Putt (1978), *O. cumana* originated from a single population of *O. cernua* which became adapted to sunflowers when this crop became important in Russia in the nineteenth century. The occurrence of *O. cumana* in Mediterranean countries followed the introduction of the sunflower in this area during the twentieth century (Katzir et al. 1996). In Israël, Joel (1988) suggested that *O. cumana* populations were introduced only recently. Katzir et al. (1996) have shown the presence of *O. cumana* with identical diagnostic markers in both Spain and Israel. Our studies with *O. cumana* populations from East European countries, Turkey and Spain indicated that these populations are all very similar whatever their geographic origin and the sunflower genotype on which they were collected. The lowest genetic similarity that we calculated between individuals from different populations was 80. Thus our data corroborate the hypothesis of a monophyletic origin for *O. cumana*.

In conclusion, our study shows *O. cumana* as a self-pollinated species, as suggested for *O. cernua*. Very little gene exchange appears to occur between different geographic regions. When the populations were selected from various origins, we did not expect such a structured pattern between populations as was shown by the PCA analysis. According to Gonzales-Torres et al. (1982) and Paran et al. (1997) the groups defined should be independant from the genotypes of the sunflower hosts, suggesting a correspondance to different race groups. In prospect, this approach may be an efficient method for the identification of pathogenicity groups of *O. cumana*. The study of the virulence of individuals from each population is in progress to determine their relationships with molecular genetic diversity. This should allow to us to characterize races instead of populations, and eventually to obtain molecular markers for these broomrape races.

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