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Genetic diversity in *Orobanche crenata* populations from southern *Spain*

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Abstract The pattern of genetic variation within and among natural populations of broomrape (*Orobanche crenata* Forsk.) from southern Spain was analysed by RAPD markers. Hierarchical analysis of phenotypic diversity using AMOVA was performed to analyse the partitioning of the variation among populations and among individuals. Although most of the genetic diversity was attributable to differences among individuals within a population (94.29%), significant ϕ_{st} values among populations suggested the existence of phenotypic differentiation. Moreover, corresponding HOMOVA analysis revealed that molecular variances were significantly heterogeneous among populations although no clear grouping pattern could be established. These results are to be expected considering the predominant outcrossing behaviour of *O. crenata*.

Keywords *Orobanche crenata* · Genetic diversity · AMOVA · Population structure · Parasitic plants

Introduction

Broomrape (*Orobanche crenata* Forsk.) is a holoparasitic weed that seriously attacks legume crops, such as faba bean, lentils, peas, chickpea and vetch, but also a large number of wild legume species (Cubero 1983), being a major constraint for legume production in Mediterranean countries. Several control methods have been proposed such as hand weeding, chemical or biological control, delayed sowing and crop rotation, but all of them with uncertain success. The extraordinary high number of tiny seeds, their prolonged viability in the soil and its broad host range make its control particularly difficult.

The study of population genetic variability of crop pathogens is of great importance since the understanding of the variability within and between pathogenic populations is essential if selection programmes need to target sources of resistance at different areas and suitable breeding strategies need to be developed.

Over the years detection of genetic variation has progressed gradually from morphological or physiological analysis to electrophoretic assays of biochemical and molecular DNA variation among individuals. Although morphological markers have been widely used in diversity studies of a large number of species, their use in *Orobanche* has been quite difficult (Musselman 1994). Some of the disadvantages are related with: (1) the inherent morphological variation within populations reflected in biological aspects like chromosomal aberrations (Cubero and Moreno 1991) or reproduction mechanisms, (2) the reduction of available characters in diversity studies that holoparasitism represents, since *Orobanche* species do not have chlorophyll or leaves and only develop false roots, and (3) the ability to infect different hosts, which can promote changes in plant morphology (Musselman and Parker 1982). Since morphological markers also vary with environmental changes and are subjected to estimation errors, alternative strategies that overcome these difficulties are needed.

Isozymes were the first markers used in diversity studies in this genus. Verkleij et al. (1986) found isoenzymatic differences between *Orobanche aegyptiaca* and *O. crenata*. Isozyme studies have also been used in intraspecific variation among *Orobanche cumana* (Castejón-Muñoz et al. 1991) and *O. crenata* populations (Verkleij et al. 1989, 1991). However, the number of isozyme

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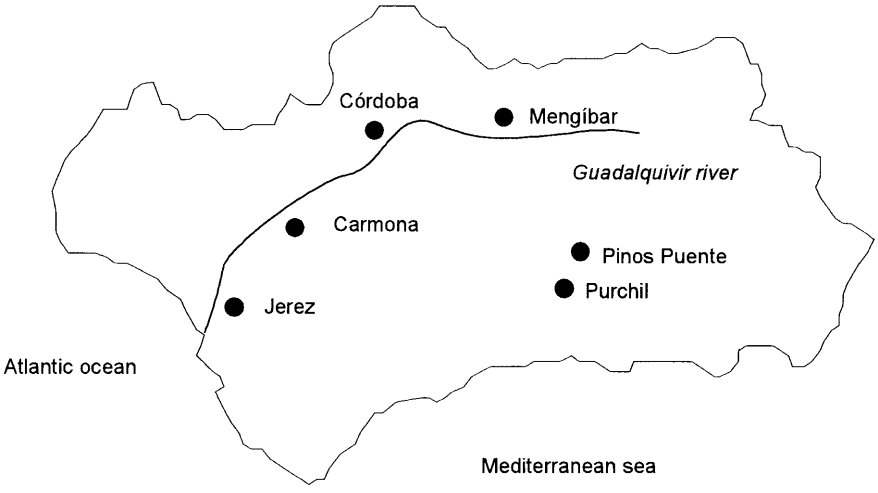

Fig. 1 Six *O. crenata* populations sampled from naturally infected faba bean plants from different locations on southern Spain (Andalucía)

Table 1 Sequences of RAPD primers used in the analysis of *O. crenata* populations

Primer	Sequence (5´–3´)	Primer	Sequence (5´–3´)	Primer	Sequence (5´–3´)
OPB03	CATCCCCCTG	OPAH04	CTCCCCAGAC	OPAA07	CTACGCTCAC
OPE17	CTACTGCCGT	OPAG04	GGAGCGTACT	OPAB07	GTAAACCGCC
OPI16	TCTCCGCCCT	OPD02	GGACCCAACC	OPAH13	TGAGTCCGCA
OPG13	CCACACTACC	OPG07	GAACCTGCGG	MER02	GTTAGGTCGT
OPP09	GTGGTCCGCA	OPJ01	CCCGGCATAA	MER04	GTCCCGTTAC
OPU09	CCACATCGGT	OPJ20	AAGCGGCCTC	MER06	GGTGATGTCC
OPV09	TGTACCCGTC	OPS04	CACCCCCTTG	MER07	GGGTTGCCGT
OPAB04	GGCACGCGTT	OPU11	AGACCCAGAG		

studies of genetic variability in parasitic plants is still reduced when compared with the large amount of data in other species (Hamrick and Godt 1989). As isozymes are gene products they vary depending on the tissue, the plant developmental stage or the environmental conditions. Furthermore, the number of resolved loci is limited and some genetic differences can not be detected.

Some of these problems have been resolved with DNA markers that present great power to resolve different classes of mutational changes. The development of *random amplified polymorphic DNA* (RAPD) markers has provided a powerful tool for the investigation of genetic diversity. These markers are simple and rapid and do not require prior knowledge of the genome because they rely on universal sets of primers. RAPD markers have been successfully used to describe the genetic structure of plant populations (Huff et al. 1993; Shah et al. 1994; Karihaloo et al. 1995; Le Corre et al. 1997). In *Orobanche*, molecular markers have been used in inter-specific diversity studies (Katzir et al. 1996; Paran et al. 1997; Zeid et al. 1997) and population studies in *O. cumana* (Gagne et al. 1998) and *O. aegyptiaca* (Joel et al. 1998). Wolfe and dePamphilis (1997) used the photosynthetic *rbc*L gene of the plastid genome in evolutionary studies with four species of the genus.

In the Mediterranean area *O. crenata* causes large losses in legume crops, being especially harmful in Andalucía (southern Spain). However, information concerning the parasitic population structure and the processes affecting its change is lacking in *O. crenata*. The aim of this work is to determine the genetic relationship among populations of *O. crenata* collected from faba bean fields in Andalucía using RAPD markers.

Materials and methods

Materials

Plant material

Six *O. crenata* Forsk. populations sampled from naturally infected faba bean plants from different locations in southern Spain (Andalucía) were used in the study. The sampled locations were: Córdoba (north-western Andalucía), Mengíbar (province of Jaén, north-eastern Andalucía), Carmona (province of Sevilla, western Andalucía), Jerez (province of Cádiz, south-western Andalucía), Pinos Puente and Purchil (province of Granada, south-eastern Andalucía). Each population consisted of ten *O. crenata* mature plants (Fig. 1).

Methods

RAPD analysis

Floral buds were used for DNA extraction using the method proposed by Lassner et al. (1989), modified by Torres et al. (1993). For RAPD analysis, approximately 20 ng of genomic DNA was used as a template in a 25-µl volume per PCR reaction. Mixture composition and reaction conditions were as described by Williams et al. (1990) with slight modifications (Torres et al. 1993). Reaction mixtures were covered with a drop of mineral oil. Products were amplified in a Termocycler Perkin Elmer Cetus 480 (Perkin Elmer Cetus, Calif., USA). A total of 23 RAPD primers (Table 1) were analysed. Nineteen of them named OP were pur-

Table 2 Phenotypic diversity revealed by 121 RAPD bands in six *O. crenata* populations from Andalucía (Spain). Abbreviations: H_t, total diversity; H_p, intra-population diversity; D_{pt}, phenotypic differentiation among populations; N: number of individuals per population

Source of variation	N	Proportion of polymorphic loci	Shannon's index of phenotypic diversity
Population			
Jerez	10	0.774	0.636
Carmona	10	0.661	0.515
Mengíbar	10	0.790	0.603
Pinos Puente	10	0.806	0.636
Purchil	10	0.677	0.396
Córdoba	10	0.661	0.411
H_t			0.692
H_p			0.533
D_{pt}			0.230

Table 3 AMOVA and HOMOVA analysis for the partitioning of RAPD variation among and within *O. crenata* populations from Andalucía

Source of variation	*df*	Variance components	% Total variance	ϕ-statistics	*p*-value	Bartlett's index	*p*-value
Among populations	5	0.54	5.71	ϕ_{st}=0.057	<0.001	B_p=0.2286	<0.001
Within populations	54	8.86	94.29				

chased in commercially available kits from OPERON Technologies (Alameda, USA). The rest named MER, were chosen because they produced intense and consistent amplification products in a previous study (Torres et al. 1993). Amplified products were electrophoresed on 1% agarose, 1% Nu-Sieve agarose, 1×TBE gels, and visualised by ethidium bromide staining.

Statistical analysis

Amplified fragments were scored for the presence (1) or absence (0) of homologous bands to create a binary matrix of the different RAPD phenotypes. Estimates of diversity within populations (H_0) were calculated using Shannon's information measure, $H_0=-\Sigma P_i \log_2 P_i$, where P_i is the phenotypic frequency (Lewontin 1972). Shannon's index of phenotypic diversity (Chalmers et al. 1992) was used to measure the total diversity (H_t) as well as the intra-population (H_p) diversity. The phenotypic differentiation among populations $D_{pt}=(H_t-H_p)/H_t$, was calculated.

The analysis of molecular variance (AMOVA) was used to partition the total phenotypic variance into within-populations and among-populations (Excoffier et al. 1992). The AMOVA was performed using the RAPD profile as a haplotype (Huff et al. 1993) with WinAMOVA ver. 1.55 software (Excoffier 1992). The distance among individuals was measured as an Euclidean metric distance that was calculated between all possible pairwise combinations of molecular genetic markers (RAPD bands) for individual plants. The variance components were tested statistically by non-parametric randomisation tests using 1,000 permutations.

A non-parametric test for the homogeneity of molecular variance (HOMOVA) based on Bartlett's statistics (Bartlett 1937) was performed to test variance homogeneity among populations (Stewart and Excoffier 1996). Bartlett's null distributions were obtained after 1,000 permutations.

Pairwise population comparisons examined with AMOVA resulted in values of ϕ_{st} that are equivalent to the proportion of the total variance that is partitioned between two populations. To obtain a distance matrix, ϕ_{st} values between each pair of populations were interpreted as the inter-population distance average between any two populations (Huff 1997; Gustine and Huff 1999). A cluster analysis based on the ϕ_{st} matrix was performed using the UPGMA method of the TFPGA 1.3 software package (Miller 1997).

The cophenetic correlation coefficient was calculated and Mantel's test (Mantel 1967) was performed to check the goodness of fit of a cluster analysis to the matrix on which it was based. The randomisation procedure as implemented in TFPGA software package included 1,000 permutations.

Jaccard's similarity coefficient (Jaccard 1908; Gower 1972) was computed using the SYSTAT 7.0 software package. A cluster analysis based on the similarity matrix was performed using the UPGMA method and the dendogram was obtained in order to visualise the relationships among single individuals.

Results and discussion

The 23 RAPD primers analysed generated 121 clear and reproducible bands that were used in the population analysis. From the primers analysed 91% were polymorphic and the number of bands per primer varied from 2 to 9 with an average of 5.26 bands/primer. Out of 121 bands, 62 were polymorphic and the number of polymorphic fragments per primer ranged from 1 to 7. No diagnostic markers were found and population discrimination was done using band frequencies. A diagnostic marker is defined as a marker with a frequency $p>0.50$ in one population, but absent in the other (Rodriguez et al. 1999). The proportion of polymorphic loci varied among populations with the highest proportion in Pinos Puente (0.806) and the lowest in Carmona and Córdoba (0.661) (Table 2). None of the populations displayed unique bands.

The diversity analysis within populations using Shannon's information measure revealed the highest intra-population diversity in Jerez and Pinos Puente (0.636). This study detects that most of the variation from the total diversity occurs within populations ($1–D_{pt}$=77%) (Table 2).

Hierarchical analysis of phenotypic diversity using AMOVA was performed to analyse the partitioning of the variation among populations and among individuals (Table 3). Although most of the genetic diversity was attributable to differences among individuals within a population (94.29%), the significant ϕ_{st} value among

Table 4 Inter-population distance matrix ϕ_{st} for the six *O. crenata* populations. Lower matrix diagonal: ϕ_{st} value – proportion of the total variance that is partitioned between two populations. Upper matrix diagonal: corresponding *p* values

Población	Jerez	Carmona	Mengíbar	Pinos Puente	Purchil	Córdoba
Jerez		0.0000	0.2248	0.4705	0.0709	0.0000
Carmona	0.1081		0.0160	0.0000	0.0000	0.0000
Mengíbar	0.0315	0.1069		0.4625	0.0000	0.0000
Pinos Puente	0.0035	0.0994	0.0106		0.7632	0.2238
Purchil	0.0468	0.1065	0.0418	–0.0080		0.0699
Córdoba	0.0690	0.1410	0.0364	0.0258	0.0293	

Table 5 HOMOVA analysis between each pair of *O. crenata* populations. Lower matrix diagonal: Bartlett's statistic (B). Upper matrix diagonal: corresponding *p* values

Población	Jerez	Carmona	Mengíbar	Pinos Puente	Purchil	Córdoba
Jerez		0.0000	0.0569	0.6154	0.0000	0.0000
Carmona	2.0494		0.0000	0.0000	0.0559	0.0000
Mengíbar	1.2152	2.0085		0.3337	0.0000	0.0569
Pinos Puente	0.9572	1.9920	1.0317		0.5345	0.0919
Purchil	1.4331	1.9598	1.3568	0.9620		0.2727
Córdoba	1.6366	2.3353	1.2937	1.2513	1.1914	

Table 6 AMOVA analysis for three different grouping criteria for *O. crenata* populations from Andalucía

Source of variation	% Total variance and p-values depending of grouping hypotesis		
	1. Oriental and occidental Andalucía[a]	2. Geographical distances[b]	3. Guadalquivir valley[c]
Among groups	–0.74% (p=0.7423)	1.24% (p=0.2008)	–0.71% (p=0.6533)
Among populations within groups	6.17% (p<0.001)	4.70% (p=0.008)	6.10% (p<0.001)
Within populations	94.57% (p<0.001)	94.06% (p<0.001)	94.60% (p<0.001)

[a] Oriental and occidental Andalucía: 2 groups (Jerez/Carmona/Córdoba vs Mebgibar/Pinos Puente/Purchil)
[b] Geographical distances: 3 groups (Mengíbar/Córdoba vs Pinos Puente/Purchil vs Jerez/Carmona)
[c] Guadalquivir valley: 2 groups (Jerez/Carmona/Mengíbar/Córdoba vs Pinos Puente/Purchil)

populations (ϕ_{st}=0.057; p<0.001) suggested the existence of phenotypic differentiation. Moreover, corresponding HOMOVA analysis reveals that the molecular variances were significantly heterogeneous among populations (B_p=0.2286, p<0.001) (Table 3).

Between each pair of populations ϕ_{st} and HOMOVA values of molecular variances were significant in 53.3% and 46.6% of the cases respectively (Tables 4 and 5), suggesting the existence of phenotypic differentiation. Jaccard's similarity coefficient varied from 0 to 0.44 between different pairs of individuals and the UPGMA method showed a good fit to the matrix on which it was based, revealing a significant cophenetic correlation coefficient (r=0.93419; p=0.003). The dendogram obtained by the UPGMA method did not show clear separation between populations (data not shown) and further grouping of individuals into separate populations was highly inconsistent.

Considering the significant ϕ_{st} and HOMOVA values among populations we tried to define a grouping pattern performing the AMOVA by attending to different criteria: (1) oriental or occidental origin, considering two groups of three populations each (Jerez/Carmona/Córdoba vs. Mengíbar/Pinos Puente/Purchil); (2) geographical distance with three groups of two populations each (Mengíbar/Córdoba, Pinos Puente/Purchil vs. Jerez/Carmona); and (3) vicinity to the Guadalquivir river valley, comparing two groups of four and two populations (Jerez/Carmona/Mengíbar/Córdoba vs. Pinos Puente/Purchil) (Fig. 1). The two-way nested AMOVA analysis was used to partition further the total phenotypic variance into within populations, among populations within hypothetical groups, and among groups. In the three cases considered we observed that the variance among groups was not significant (Table 6). Thus, none of these grouping hypotheses was valid, preventing the detection of a definitive pattern of variation.

This study shows that there is no clear tendency in the distribution of the genetic variability when considering geographical distances in *O. crenata* populations from Andalucía. RAPD analysis has detected low genetic differentiation among populations and considerable variation among individual broomrape plants within a population. Our results are similar to those obtained with *O. crenata* populations from Israel and Egypt by Paran et al. (1997) and Zeid et al. (1997) respectively, supporting the existence of high gene flow among populations. Studies with phytopathogenic fungi that have detected low differentiation among populations (Boeger et al. 1993; Hamelin et al. 1995) have attributed these results to a high gene flow between them or to the existence of a common ancestor (Hamelin et al. 1995). In the case of

O. crenata populations this fact is favoured by an efficient dispersal of the seeds by humans, machinery, animals or wind. The exchange of host seeds mixed with parasite seeds could also contribute to this fact. This gene flow increases the effective size of the population avoiding genetic-drift effects (Ellstrand and Elam 1993). Djè et al. (1999), attributed the low genetic differentiation among sorghum populations to a large population effective size. The huge amount of seed that a broomrape plant produces per generation could be favouring this drift restriction.

Autogamous species promote differentiation among populations, while in mixed mating or allogamous species the differences are less marked (Hamrick and Godt 1989). This study detects higher phenotypic diversity within populations than among them, an observation that is consistent with the distribution of variation in allogamous species (Schoen and Brown 1991). Intra-population variation levels in populations from Andalucía (94,29%) are similar to those described in other allogamous species such as *Buchloë dactyloides* (Huff et al. 1993), *Populus tremuloides* (Yeh et al. 1995), *Ancistrocladus koruensis* (Foster and Sork 1997) or *Bankasia* spp. (Maguire and Sedgley 1997). These results are expected considering the predominantly outcrossing behaviour of *O. crenata* (Musselman 1986). The characters that *O. crenata* shares with taxa showing high genetic diversity are its high fecundity (a single plant can produce more than 10^5 seeds (Pieterse 1979)), its allogamous mating system and its dispersal mechanisms. In the hemiparasitic allogamous plant *Striga hermonthica*, low inter-population differentiation has also been found (Bharathalakshmi et al. 1990). Our results differ from those obtained in *O. cumana* Wallr. (Gagne et al. 1998) attacking sunflower where high levels of variation among populations and low intra-population variability were found, suggesting an autogamous reproduction system in this species.

O. crenata plants collected in six different regions of Andalucía could be considered as members of the same population where gene migration forces are continuous and strong. Our results do not indicate an important geographical effect on population structure. It remains to be determined if virulence genes are also homogeneously distributed among these populations. This will require pathotyping combined with genome analysis (Burdon 1993), which is currently hampered by the lack of defined races in *O. crenata* and differential sets in *V. faba*. Cubero and Moreno (1979) and Radwan et al. (1988) found a very low level of host-parasite interaction, not supporting the existence of races in *O. crenata*. Our study confirms the lack of population diversification. However, differences in the level of aggressiveness among *O. crenata* populations have been proposed by Verkleij and Pieterse (1994). The fact that a new race of *O. crenata* attacking resistant vetches has been found in Israel (Joel 1999) may be attributed to the extensive use of a vetch variety that is resistant to the local broomrape population (Goldwasser et al. 1996).

Although great genetic variation already exists within populations of *O. crenata*, the complex inheritance and apparently broad-based moderate levels of resistance available have not effectively determined a selection for virulence in the parasite, thus not contributing to any clear evidence for the existence of races. However, the occurrence of genetic variability within populations suggests that races might develop as long as challenged by narrow-based single genes of resistance, as has happened with *O. cumana* in the sunflower where the extensive use of single genes for resistance, acting at the penetration event, have so far resulted in the rapid appearance and spread of six races.

Further studies with molecular markers in other species of the genus may contribute to an understanding of other interesting aspects which refer to the genetic variation in these parasitic plants. The outcrossing behaviour of the species may lead to the appearance of inter-specific crosses in the genus and a consequent change in the host that might be monitored by molecular markers. In fact, meiotic anomalies and variation in the basic number of chromosomes have both been observed in *O. crenata* (Moreno et al. 1979). Verkleij and Pieterse (1994) suggested that comparative studies need to be carried out with *Orobanche* biotypes in natural vegetation for a better understanding of the evolution from wild parasitic plants into aggressive parasitic weeds. The study of these relationships in Andalucía would be of great interest since different species of *Orobanche* parasitizing wild species are found (Pujadas 1999).

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