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Genetic Relationships among *Orobanche* Species as Revealed by RAPD Analysis

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RAPD markers were used to study variation among 20 taxa in the genus *Orobanche*: *O. alba*, *O. amethystea*, *O. arenaria*, *O. ballotae*, *O. cernua*, *O. clausonis*, *O. cumana*, *O. crenata*, *O. densiflora*, *O. foetida*, *O. foetida* var. *broteri*, *O. gracilis*, *O. haenseleri*, *O. hederae*, *O. latisquama*, *O. mutelii*, *O. nana*, *O. ramosa*, *O. rapum-genistae* and *O. santolinae*. A total of 202 amplification products generated with five arbitrary RAPD primers was obtained and species-specific markers were identified. The estimated Jaccard's differences between the species varied between 0 and 0·864. The pattern of interspecific variation obtained is in general agreement with previous taxonomic studies based on morphology, and the partition into two different sections (*Trionychon* and *Orobanche*) is generally clear. However, the position in the dendrogram of *O. clausonis* did not fit this classification since it clustered with members of section *Trionychon*. Within this section, *O. arenaria* was relatively isolated from the other members of the section: *O. mutelii*, *O. nana* and *O. ramosa*. Within section *Orobanche*, all *O. ramosa* populations showed a similar amplification pattern, whereas differences among *O. crenata* populations growing on different hosts were found. *Orobanche foetida* and *O. densiflora* clustered together, supporting the morphological and cytological similarities and the host preferences of these species.
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Key words: *Orobanche*, RAPD, genetic relationships, genetic diversity.

INTRODUCTION

Orobanche species are achlorophyllous annual or perennial plants that parasitize roots of different plant species. Parasitism has led to a simplification in their morphology and therefore to a reduction in features used to distinguish species. The intrinsic taxonomic difficulties in *Orobanche* are further compounded by the fact that important differential characters can be observed only with difficulty, or not at all, in dried specimens, and the features used to distinguish species are poorly defined (Musselman, 1986). Polyploidy, interspecific crosses, hybridization among different ploidy levels, parthenogenesis, chaotic meiosis and mitotic abnormalities also contribute to the taxonomic difficulties in the group (Cubero, 1996).

Beck von Mannagetta (1930) proposed a subgeneric classification of the genus in four sections: *Gymnocaulis* Nutt., *Myzorrhiza* (Phil.) Beck, *Trionychon* Wallr. and *Osproleon* Wallr. Section *Osproleon* is now treated as a synonym of section *Orobanche* according to the rules of the International Code of Botanical Nomenclature (Greuter *et al*., 2000). The species of the greatest agronomic importance are found in sections *Orobanche* and *Trionychon.* These are distinguished by characters of the bracts, placentation, inflorescence type, cytology and distribution. Relationships among *Orobanche* spp. have mainly been investigated by means of morphological studies, and infrageneric systematics has been the subject of disagreement. Holub (1990) suggested that the genus be split into four genera, and Teryokhin (1991) placed some Russian members of *Trionychon* in the genus *Phelipanche* Pomel.

Recently the use of more accurate methods has contributed to a better understanding of the systematic relationships within the genus. Light and scanning electron microscopy have been used to study pollen morphology and seed micromorphology (Abu Sbaih *et al*., 1994), and chemotaxonomic techniques have been used to measure phenolic compounds (Andary, 1994; Georguieva and Edreva, 1994) and the fatty acid and tocochromanol composition of *Orobanche* seeds (Velasco *et al*., 2000). Unfortunately, these attempts have not been completely successful, and distinction between some *Orobanche* subsections is still not possible.

Plastid and nuclear DNA analysis represents an important tool for phylogenetic and diversity studies of parasitic flowering plants. The plastid genome of holoparasites often appears to have evolved under relaxed selection and has thus accumulated mutations that can be used to detect differences among species. Analysis of the plastid genome has been used to establish the relationships of four *Orobanche* spp. (Wolfe and dePamphilis, 1997) and to identify four species of subsection *Minores* (Benharrat *et al*., 2000). In the case of nuclear DNA,

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TABLE 1. *Sections, number of individuals and number of populations analysed in each species*

Species	Section	No. of samples	No. of populations
O. alba Stephan ex Willd.	*Orobanche*	4	1
O. amethystea Thuill.	*Orobanche*	37	6
O. arenaria Borkh.	*Trionychon*	12	5
O. ballotae A. Pujadas	*Orobanche*	17	2
O. cernua L.	*Orobanche*	17	2
O. clausonis Pomel.	*Orobanche*	10	2
O. crenata Forssk.	*Orobanche*	45	5
O. cumana Wallr.	*Orobanche*	18	2
O. densiflora Reut.	*Orobanche*	12	2
O. foetida Poir.	*Orobanche*	9	1
O. foetida var. *broteri* (J.A. Guim.) Merino	*Orobanche*	12	2
O. gracilis Sm.	*Orobanche*	28	4
O. haenseleri Reut.	*Orobanche*	16	4
O. hederae Duby	*Orobanche*	11	2
O. latisquama (F.W. Schultz) Batt.	*Orobanche*	3	1
O. mutelii F.W. Schultz	*Trionychon*	12	1
O. nana (Reut.) Beck.	*Trionychon*	9	1
O. ramosa L.	*Trionychon*	44	5
O. rapum genistae Thuill.	*Orobanche*	7	1
O. santolinae Loscos & J. Pardo	*Orobanche*	16	2

molecular taxonomy has been studied using RAPD markers in parasites of economically important crops such as *O. crenata* Forsk., *O. cumana* Wallr. and *O. ramosa* L. and their relatives (*O. cernua* L. and *O. mutelii* F.W. Schultz) (Katzir *et al*., 1996; Paran *et al*., 1997). The RAPD technique (Williams *et al*., 1990) based on the use of short primers of arbitrary nucleotide sequence in the polymerase chain reaction has been shown to be useful for a wide range of applications (Williams *et al*., 1993) and offers advantages in speed, technical simplicity and identification of polymorphisms. Although most economically important *Orobanche* spp. have been analysed with DNA markers, little information is available regarding the genetic relationships of species that parasitize wild hosts. Comparative studies with *Orobanche* spp. in natural habitats are of great importance since they can clarify the evolutionary path from wild parasitic plants into aggressive parasitic weeds (Verkleij and Pieterse, 1994). For instance, the outcrossing behaviour of *O. crenata* might produce interspecific crosses and, as a consequence, a change in the host that could be monitored by molecular markers. Consequently, an accurate taxonomic catalogue of *Orobanche* spp. would be of great interest since any of them could eventually evolve into a crop parasite. This has already been reported in *O. foetida* on faba bean (*Vicia faba* L.) and winter chickpea (*Cicer arietinum* L.) in North Africa (Kharrat *et al*., 1992).

The objectives of this study were to determine the genetic relationships among *Orobanche* spp. from Spain which are mainly parasites of wild hosts, to evaluate the usefulness of the RAPD technique in generating DNA markers for genetic and taxonomic studies, and to relate morphological to molecular data, in order to obtain a better taxonomic classification of *Orobanche*.

MATERIALS AND METHODS

Plant material

A total of 347 specimens from 52 populations belonging to 19 *Orobanche* spp. (including two varieties of *O. foetida*) was collected from naturally parasitized hosts across Spain during spring in 1999 and 2000. The number of populations per species and number of plants per population are shown in Table 1. Of the 20 taxa analysed, 17 were collected on wild hosts: *O. alba*, *O. amethystea*, *O. ballotae*, *O. cernua*, *O clausonis*, *O. densiflora*, *O. foetida*, *O. foetida* var. *broteri*, *O. gracilis*, *O. haenseleri*, *O. hederae*, *O. latisquama*, *O. rapum-genistae* and *O. santolinae* (section *Orobanche*), and *O. arenaria*, *O. mutelii* and *O. nana* (section *Trionychon*). Two were found only on crops; *O. cumana* (section *Orobanche*) and *O. ramosa* (section *Trionychon*); and one was found on wild and cultivated hosts: *O. crenata* (section *Orobanche*). Specimens of all accessions are deposited at the herbarium COA of the Departamento de Ciencias y Recursos Agrícolas y Forestales of the University of Córdoba, Spain.

DNA extraction, amplification and electrophoresis

Floral buds from 347 specimens were used for DNA extraction using the method reported by Lassner *et al*. (1989), modified by Torres *et al*. (1993). We followed the template-mixing strategy where equal amounts of working solution DNAs from each group of individuals of the same species were pooled as the ‘species-template DNA’ prior to the PCR reaction. DNA was extracted from single plants and each population was represented by the bulk of a variable number of individuals, depending on the availability of samples after collection (Table 1). A preliminary amplification of each sample was carried out to detect

TABLE 2. *Sequences of RAPD primers used in the analysis of* Orobanche *species*

Primer	Sequence (5′–3′)	No. of fragments scored	Approx. fragment size range (bp)
OPB03	CATCCCCCTG	32	440–2590
OPJ01	CCCGGCATAA	38	328–2273
OPE12	AGACCCAGAG	29	629–2095
OPE17	CTACTGCCGT	50	430–2332
OPU11	AGACCCAGAG	54	343–2301

'off-type' specimens. For RAPD analysis, approx. 20 ng 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). Products were amplified in an Applied Biosystems GeneAmp 9700 thermocycler.

RAPD from five primers (ten bases long) were analysed (Table 2). Primers were purchased in commercially available kits from OPERON Technologies (Alameda, CA, USA). The selection was made from a pool of primers that gave clear and strong amplifications in previous studies with *O. crenata* populations (Román *et al.*, 2001). Amplified products were separated on 1 % agarose, 1 % Nu-Sieve agarose, 1 × TBE gels, and visualized by ethidium bromide staining. Bands were scored manually using the Kodak Digital Science 1D Software program.

Statistical analysis

The banding patterns obtained with the DNA mixing strategy were analysed to estimate genetic relationships among the 19 *Orobanche* spp. Amplified samples were scored for the presence (1) or absence (0) of homologous bands to create a binary matrix of the different RAPD genotypes. 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 unweighted pair group method with arithmetical averages (UPGMA). 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 randomization procedure as implemented in TFPGA (Tools for Population Genetic Analyses) (Miller, 1998) software package included 1000 permutations.

RESULTS AND DISCUSSION

Our results indicate that RAPD markers are particularly valuable in the study of *Orobanche*, where extensive genetic characterization of the nuclear genome is lacking. A primary object of this research was to characterize the genetic diversity in 19 *Orobanche* spp. This study examines a substantially larger number of populations and loci than examined in previous studies and allows a thorough overview of RAPD diversity across a wide range of *Orobanche* spp. parasitic on wild hosts. DNA mixing procedures were appropriate strategies to generate banding patterns representative of each of the studied species.

The five RAPD primers generated a total of 202 reliable fragments from the template-mixing patterns of the 19 *Orobanche* spp. The approximate size of the fragments ranged from 300 to 2600 bp (Table 2). The total number of amplified bands per primer varied from 29 (OPE 12) to 54 (OPU 11) with an average of 40·8 fragments per primer. The total number of markers and proportion of unique and invariant markers are valuable parameters in determining intraspecific variability and genetic relationships among species. We did not find any monomorphic markers shared across all the species. Some of the markers used in this study were only amplified in one species: bands OPJ01-1269 and OPU11-1416 (*O. cumana*); OPE12-2095 and OPE17-442 (*O. haenseleri*); OPE17-2276 and OPE17-756 (*O. ramosa*); OPJ01-2116 (*O. arenaria*); OPU11-728 (*O. densiflora*); OPU11-1313 (*O. hederae*); OPU11-980 (*O. latisquama*); OPU11-384 (*O. rapum-genistae*); OPU11-516 (*O. santolinae*); and OPU11-1380 (*O. foetida* and *O. foetida* var. *broteri*). These bands could be potential species-specific markers after checking that every individual from that species shows the marker in question. These markers could be used to detect instances of natural interspecific gene introgression. Nevertheless, further analysis with more primers would be required to establish fully the specificity of loci to particular taxa and subsequent interspecific flow in *Orobanche*. Direct sequencing of amplified bands may also be useful for future phylogenetic studies.

The estimated Jaccard's differences between the *Orobanche* populations varied between 0 (in the case of two populations of *O. hederae* and two populations of *O. ramosa*) and 0·864 (between *O. rapum-genistae* and the rest of the populations considered) (Fig. 1). The UPGMA method showed a good fit to the matrix on which it was based since the Mantel test revealed a high and significant cophenetic correlation coefficient ($r = 0{\cdot}95458$, $P < 0{\cdot}001$).

The pattern of interspecific variation revealed in the dendrogram using the 202 RAPD markers is largely in agreement with previous taxonomic studies of *Orobanche*. The differences between sections *Trionychon* and *Orobanche* observed in this study in general corroborate the taxonomic classification established by Beck von Mannagetta (1930) on the basis of morphological traits. Micromorphological studies of seed and pollen (Andary, 1994) have also confirmed the division of *Orobanche* into two sections: seed testa cells with circular homogeneous perforations and spherical non-aperturate pollen in section *Orobanche*, and seed testa cells with a large perforation and tricolpate or triaperturate pollen in *Trionychon*. According to Abu Sbaih *et al.* (1994) and Abu Sbaih and Jury (1994), members of *Trionychon* can easily be separated from members of section *Orobanche* by their exine and seed coat sculpturing. The composition of caffeic glycoside esters (CGEs) was also found to differ between the two sections (Andary, 1994). The present molecular study shows that, although all the species belonging to section *Trionychon* clustered together, *O. clausonis* (sect *Orobanche*) is also included in the same cluster and is positioned closer to some

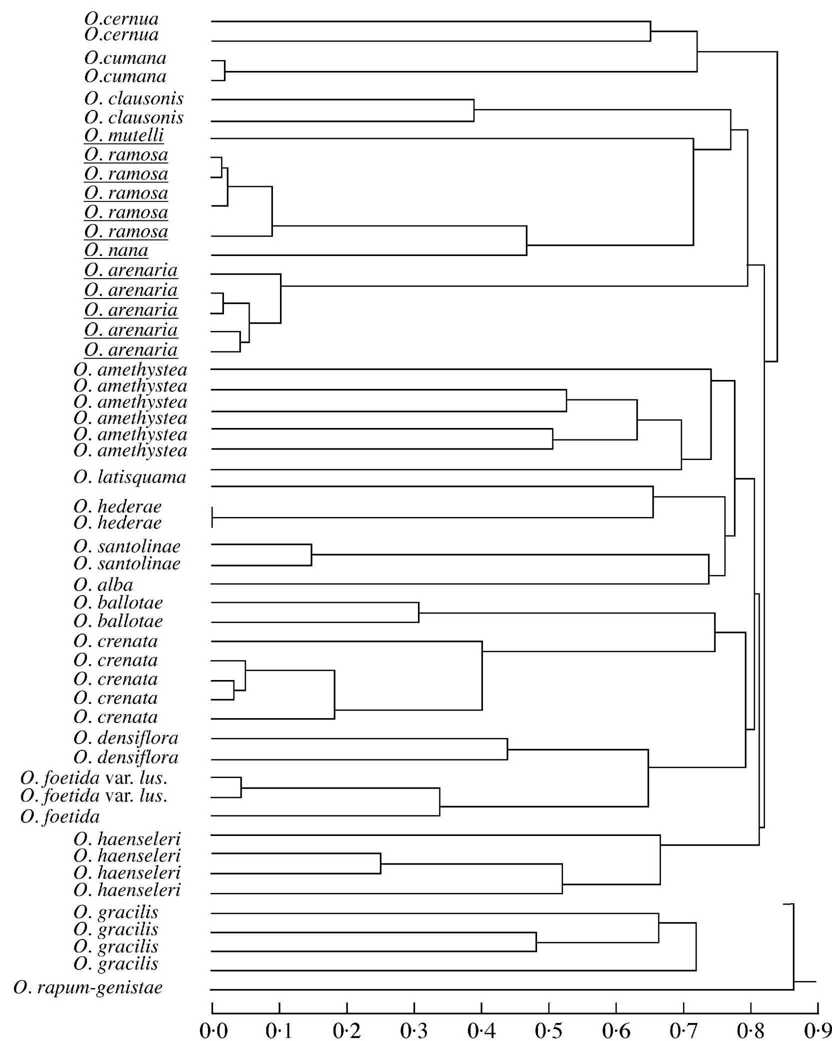

FIG. 1. UPGMA dendrogram using Jaccard's genetic distances among *Orobanche* spp. Members belonging to section *Trionychon* are underlined.

species of *Trionychon* (*O. mutelli*, *O. ramosa* and *O. nana*) than *O. arenaria* (also *Trionychon*) is to those species. Although some characters of *O. clausonis*, including the fused calyx segments, are similar to those of species in section *Trionychon*, the position of *O. clausonis* did not fit the widely accepted classification. To obtain a deeper insight into the position of this species, it will be necessary to increase both the number of *O. clausonis* populations and the number of specimens analysed.

Within section *Trionychon*, our dendrogram shows *O. arenaria* to be relatively isolated from the other members of the section analysed: *O. mutelii*, *O. nana* and *O. ramosa* (Fig. 1). Previous studies have also found the same differentiation between *O. arenaria* and other members of the section. Novopokrovsky and Tzvelev (1958) divided this section into two sections, *Holoclada* Novopokr. containing *O. arenaria* and *Pleioclada* Novopokr. including *O. mutelii*, *O. nana* and *O. ramosa*. Subsequently, Andary (1994) suggested the division of *Trionychon* into two subsections: subsection *Ramosae* (type *O. ramosa* L.) usually with branched stems, testa cell walls with large perforations (>5 μm) containing typically poliumoside and acetylpoliumoside, and subsection *Arenariae* (type *O. arenaria*) usually with simple stems, testa cell walls with reticulate perforations, typically containing phelipo-side and arenarioside. Recently Velasco *et al*. (2000), studying the fatty acid and tocochromanol pattern in *Orobanche* seeds, detected high γ-tocopherol content and a low oleic to linoleic acid ratio in *O. arenaria* as well as high δ-tocopherol and high oleic to linoleic acid ratios in *O. mutelli*, *O. nana and O. ramosa*. Schneeweiss (2001) confirmed this distinction in an analysis of the internal transcribed spacers (ITS) of nuclear ribosomal DNA.

In the present study, all *O. ramosa* populations showed a similar amplification pattern, and the genetic distance values were low, varying from 0 to 0·092 among pairs of *O. ramosa* populations. The preliminary RAPD analysis of individual samples to detect 'off-type' genotypes also revealed a similar banding pattern among *O. ramosa* specimens within populations (data not shown). This may

indicate that this species is autogamous. Isoenzyme studies of Schuchardt and Wegmann (1996) also detected uniform zymograms in an *O. ramosa* population, suggesting an influence of the autogamous breeding system in this species where mature stamens touch the stigma by the time the corolla opens (Musselman *et al*., 1982). Future AMOVA analysis of data from individual samples of these *O. ramosa* populations should lead to better understanding of the intra- and inter-population variation as obtained for other economically important weeds like *O. cumana* (Gagne *et al*., 1998) and *O. crenata* (Román *et al*., 2001).

Although the five *O. ramosa* populations analysed were quite close in the dendrogram, a slight divergence was found between the population parasitizing tomato from those on tobacco. This suggests host differentiation that should be investigated in future analyses with individual DNA samples from each population.

In *O. crenata*, the dendrogram shows populations collected from cultivated faba bean (*Vicia faba*) to be a closely related group, with the population collected on a wild *Vicia* sp. falling in a more isolated position. Whereas the genetic distance values among *O. crenata* populations on crops ranged from 0·034 to 0·183, the distance between the *O. crenata* populations infesting a wild host and the rest of the *O. crenata* populations was 0·403. Out of 21 bands specific to *O. crenata* populations, eight were only present in the population attacking the wild *Vicia*. Since previous molecular analysis with six *O. crenata* populations growing on cultivated *V. faba* in southern Spain did not show interpopulation differences and specific bands per population were not found (Román *et al*., 2001), our results suggest a possible host differentiation in the population growing on the wild *Vicia* sp. In future analyses it will be of great interest to consider this population as well as others parasitizing cultivated common vetch (*Vicia sativa*).

The similarity between *O. cumana* and *O. cernua* has been a matter of debate. These two species have been traditionally considered to be closely related, and the names have even been used as synonyms by some authors. In contrast, other authors have classified both taxa as separate species. However, recent molecular studies (Katzir *et al*., 1996; Paran *et al*., 1997), as well as the contrasting seed fatty acid profiles between these two species (Pujadas-Salvà and Velasco, 2000), clearly support the separation of *O. cumana* (occurring only in agricultural fields) from *O. cernua* (attacking only wild hosts). Our results also support these findings since the dendrogram clearly separated both species.

Special attention should be paid to *O. foetida* parasitizing wild hosts. Until its appearance in Tunisian crop fields (Kharrat *et al*., 1992), this species had only been reported as a parasite of indigenous plants. For this reason, one *O. foetida* and two *O. foetida* var. *broteri* populations growing on wild hosts were included in this study. The three populations clustered together with a Jaccard distance between *O. foetida* and *O. foetida* var. *broteri* of 0·34. *Orobanche densiflora* showed the highest similarity to *O. foetida* (including var. *broteri*), with a genetic distance of 0·648. *Orobanche foetida* and *O. densiflora* are both parasites of wild herbaceous members of Fabaceae and show some morphological similarities, i.e. appearance and floral characters (hair covering, corolla shape, filaments and stigma colour). Tetraploidy (basic number $2n = 38$) has also been recorded in both species (Cubero, 1996). The morphological and cytological similarities and the host preferences of these species could support this cluster in the dendrogram.

The most distinct species according to our molecular data is *O. rapum-genistae* (clustered with the rest at a distance of 0·864). The differences found in seed morphology of this species (Andary, 1994), and the higher ploidy level ($2n = 12x$) (Palomeque, 1979), support the large genetic distance between *O. rapum-genistae* and the rest of the species shown in the dendrogram.

Subsection *Minores* was defined by Beck von Mannagetta (1930) after research based largely upon herbarium studies. Specimens of *O. amethystea*, *O. hederae* and *O. santolinae* from the grex *Minores* Beck (= Series *Minores* Novopokrovsky and Tsvelev) clustered together with species not belonging to the grex, *O. latisquama* and *O. alba*. Further analyses are needed to determine the correct infrageneric taxonomic treatment of *O. clausonis* as it falls in section *Trionychon* in our analyses, contrasting with the classification of Beck von Mannagetta (1890, 1930), in which it is included in the grex *Minores*. *Orobanche ballotae*, although described and included by Pujadas-Salvà (1997) in grex *Minores*, clustered with *O. crenata*. This subsection is the largest and most complex group in the genus, and distinction between some members of the grex is still a difficult task.

This study represents a first approach in using nuclear DNA fingerprint markers as a tool to study molecular systematics in *Orobanche*. The analysis of additional populations and species, and the use of different types of nuclear molecular markers such as ITS (internal transcribed spacers) or plastid/mitochondrial DNA sequences will improve the accuracy of resolution of genetic relationships and contribute to a more accurate classification of the genus *Orobanche*.

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