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Phylogenetic Relationships and Genetic Diversity among *Orobanche cumana* Wallr. and *O. cernua* L. (Orobanchaceae) Populations in the Iberian Peninsula

Abstract: *Orobanche cumana* is found in the Iberian Peninsula as an allochthonous species parasitizing exclusively sunflower, in contrast to the closely related species *Orobanche cernua*, which is an autochthonous species that only parasitizes wild Asteraceae hosts. Ten *O. cumana* populations were collected in the two traditional areas of sunflower broomrape occurrence, the Guadalquivir Valley, Southern Spain (six populations) and Cuenca province, Central Spain (four populations). Twelve *O. cernua* populations were collected on wild hosts across its natural distribution area in Southeastern Spain. Genetic relationships within and between both sets of populations were studied using a set of 50 robust and co-dominant SSR markers from *O. cumana*. The results supported the taxonomic separation of the two species and the existence of two distant genetic groups for *O. cumana*, one in Guadalquivir Valley and another one in Cuenca province. The inter- and intra-population variability was extremely low for *O. cumana*, whereas the overall genetic diversity was much higher for *O. cernua*. The genetic structure of *O. cumana* populations probably reflects a founder

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effect, with the two genetically distant groups deriving from separate introduction events. The high degree of genetic differentiation observed in *O. cernua* is mainly explained on the basis of restricted gene flow due to ecological barriers together with the occurrence of a predominantly self-pollinating mating system. Complementary diversity studies on both species in its current distribution area are required for understanding global genetic variability and evolutionary characteristics of the parasitism.

Keywords: genetic diversity, *Helianthus annuus*, microsatellite markers, *Orobanche cernua*, *Orobanche cumana*, sunflower broomrape

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Introduction

Orobanche cumana Wallr. (sunflower broomrape) is a holoparasitic plant that parasitizes sunflower roots. It is present in sunflower crops in many countries around the world, especially in Central and Eastern Europe, Spain, Turkey, Israel, Iran, Kazakhstan, China (Ŝkorić *et al.*, 2010), and more recently in new areas such as France (Jouffret and Lecomte, 2010). *O. cumana* was first described in the Iberian Peninsula parasitizing confectionary sunflower (*Helianthus annuus* L.) crops in 1958 in Toledo province (Díaz-Celayeta, 1974). The presence of *O. cumana* in oilseed sunflower fields was observed later in wide areas of Cuenca province in Castilla-La Mancha region (Central Spain) and the Guadalquivir Valley in Andalucía (Southern Spain) (González-Torres *et al.*, 1982). Since then, *O. cumana* has spread over the whole sunflower cultivation regions, comprising new and traditional areas of Castilla-León (Northern Spain), Castilla-La Mancha, and Andalucía, causing severe yield losses in sunflower crops (Alonso *et al.*, 1996; Fernández-Martínez *et al.*, 2012).

The closely related species *Orobanche cernua* L. was observed for the first time near Aranjuez (Central Spain) (Loefling, 1758). The species is mainly distributed in the North- and the South-East of the Iberian Peninsula and is only found in the wild, in arid areas of degraded, xerothermic scrub, parasitizing different species of the Asteraceae, being most frequently found on plants of the genus *Artemisia* (Pujadas-Salvà and Velasco, 2000). *O. cumana* and *O. cernua* have been traditionally considered as very closely related taxa (Pujadas-Salvà and Velasco, 2000). Several studies based on different molecular

marker systems, such as RAPDs (Random Amplified Polymorphic DNAs) (Katzir *et al.*, 1996; Paran *et al.*, 1997; Román *et al.*, 2003) or ISSRs (Inter Simple Sequence Repeats) (Benharrat *et al.*, 2002), as well as those based on ecological, morphological, and biochemical data (Pujadas-Salvà and Velasco, 2000) or seed morphology analysis (Plaza *et al.*, 2004), clearly support the taxonomic separation of *O. cumana* and *O. cernua* and the treatment of both taxa as different species.

Specific and joint studies on genetic diversity and phylogenetic relationships between both *O. cumana* and *O. cernua* species growing in the Iberian Peninsula based on a larger number of populations could be of interest to clarify the relationships between the two species. Coupled with this, alternative markers such as simple sequence repeat (SSR) markers, which are reproducible, neutrally evolving, multiallelic, and co-dominant, are needed to enable more powerful genetic analyses in the genus *Orobanche*. A recently developed collection of SSR markers is available for molecular research in *O. cumana*, which proved to be highly transferable to *O. cernua* (Pineda-Martos *et al.*, 2014). Accordingly, the objective of this research was to study genetic diversity in a large set of *O. cumana* and *O. cernua* populations from the Iberian Peninsula using a subset of the newly SSR markers reported.

Materials and methods

Plant material

Ten *O. cumana* populations were collected from 1989 to 2008 in different sunflower fields located across the main traditional distribution areas of sunflower broomrape in Spain – Cuenca province in Central Spain and Guadalquivir Valley in Southern Spain (Table 1). The populations (seed or plant tissue, as indicated in Table 1) were collected by the authors with the exception of populations SE01 and CO06 from Southern Spain, and populations CU12, CU05, and CU07 from Central Spain, which were kindly provided by Dr J. Fernández-Escobar (Koipesol Semillas S.A., Sevilla, Spain). Those populations in which only seeds were collected were multiplied as described in Pineda-Martos *et al.* (2013). In addition, 12 populations (plant tissue) of *O. cernua* were collected during the years 2000–2006 in their natural distribution area in Southeastern Spain, parasitizing *Artemisia barrelieri* Besser, *Artemisia glutinosa* J. Gay ex Besser, and *Launaea lanifera* Pau (Asteraceae). Fresh tissue samples from individual broomrape plants of each population were frozen at −80°C, lyophilized, and ground individually.

Table 1: Identification and collection details of the *O. cumana* and *O. cernua* populations from the Iberian Peninsula used in this study

Population	*Orobanche* spp.	Collecting site and method employed[†]	Year	Host	*n*
O. cumana populations from Southern Spain (Guadalquivir Valley)				Sunflower hosts	
IASCum-1	*O. cumana*	Andalucía, Córdoba, Córdoba. PT	2008	Confectionary	15
IASCum-2	*O. cumana*	Andalucía, Sevilla, Écija. PT	2008	Oilseed	15
IASCum-3	*O. cumana*	Andalucía, Sevilla, Osuna. PT	2008	Oilseed	15
Boro-13	*O. cumana*	Andalucía, Sevilla, Écija. S	2002	Oilseed	15
SE01	*O. cumana*	Andalucía, Sevilla, El Coronil. S	1989	Confectionary	21
CO06	*O. cumana*	Andalucía, Córdoba, La Carlota. S	2001	Oilseed	20
O. cumana populations from Central Spain				Sunflower hosts	
IASCum-4	*O. cumana*	Castilla-La Mancha, Cuenca, Villarejo de Fuentes. PT	2008	Oilseed	15
CU12	*O. cumana*	Castilla-La Mancha, Cuenca, Palomares del Campo. S	2008	Oilseed	20
CU05	*O. cumana*	Castilla-La Mancha, Cuenca, La Almarcha. S	1996	Oilseed	20
CU07	*O. cumana*	Castilla-La Mancha, Cuenca, Carrascosa del Campo. S	1996	Oilseed	20
O. cernua populations from Southern Spain				Wild hosts	
Boro-37	*O. cernua*	Andalucía, Almería, Níjar, Lucainena. PT	2006	*L. lanifera*	9
Boro-38	*O. cernua*	Andalucía, Almería, Tabernas, Venta los Yesos. PT	2006	*A. barrelieri*	15
Boro-39	*O. cernua*	Andalucía, Almería, Níjar, Lucainena. PT	2006	*A. barrelieri*	5
Boro-40	*O. cernua*	Andalucía, Almería, Níjar, Huebro. PT	2006	*L. lanifera*	2
Boro-41	*O. cernua*	Andalucía, Almería, Albox, El Saliente Alto. PT	2006	*A. glutinosa*	1
Boro-42	*O. cernua*	Andalucía, Almería, Cabo de Gata, Vela Blanca. PT	2006	*L. lanifera*	7
Boro-43	*O. cernua*	Andalucía, Jaén, Jódar. PT	2006	*A. barrelieri*	7
Boro-44	*O. cernua*	Andalucía, Jaén, Cabra del Santo Cristo. PT	2006	*A. barrelieri*	8
Boro-45	*O. cernua*	Andalucía, Jaén, Cabra del Santo Cristo. PT	2000	*A. barrelieri*	10
Boro-46	*O. cernua*	Andalucía, Granada, Sierra de Parapanda. PT	2000	ND	1
Boro-47	*O. cernua*	Andalucía, Granada, Sierra de Parapanda. PT	2000	ND	6
Boro-48	*O. cernua*	Andalucía, Granada, Salobreña. PT	2003	*A. barrelieri*	8

Notes: [†]PT: plant tissue; S: seed; ND: not determined; *n*: number of individuals analyzed.

SSR analyses

The ten *O. cumana* populations were genotyped in a previous study (Pineda-Martos *et al.*, 2014) with a set of 50 *O. cumana* SSR markers (Table 2) showing high quality. The same set of 50 *O. cumana* markers was used to genotype the 12 *O. cernua* populations, following the procedures described in Pineda-Martos *et al.* (2014). Despite the samples were pooled for each population, no complex banding patterns were observed and SSR amplification products for each population consisted in one single band (allele) in *O. cumana* and one single band or two bands in *O. cernua*. Accordingly, the bands were scored as homozygous or heterozygous patterns, although this did not represent individual genotypes, but homogeneity or heterogeneity among the individuals bulked within each population. Marker informative values, such as the total number of alleles (NA) and polymorphism information content (PIC), were calculated as implemented in PowerMarker version 3.25 software package (Liu and Muse, 2005) (Table 2).

Table 2: *O. cumana* SSR markers and its diversity parameters in the study of 22 *Orobanche* spp. populations collected in the Iberian Peninsula

SSR marker†	*O. cumana* populations from Southern and Central Spain collected on sunflower ($N = 10$)		*O. cernua* populations from Southern Spain collected on wild Asteraceae ($N = 12$)	
	NA†	PIC†	NA	PIC
Ocum-002	1	0.0000	2	0.2533
Ocum-004	1	0.0000	4	0.5665
Ocum-005	1	0.0000	3	0.4342
Ocum-006	1	0.0000	4	0.6713
Ocum-009	0	–	3	0.4992
Ocum-012	1	0.0000	4	0.6992
Ocum-014	1	0.0000	5	0.7036
Ocum-015	1	0.0000	5	0.4828
Ocum-028	1	0.0000	5	0.6539
Ocum-037	2	0.3648	5	0.5590
Ocum-042	1	0.0000	6	0.7087
Ocum-045	1	0.0000	5	0.6192
Ocum-046	1	0.0000	4	0.6218
Ocum-052	2	0.3648	7	0.8278
Ocum-063	2	0.3648	3	0.4992
Ocum-066	1	0.0000	3	0.4186
Ocum-067	1	0.0000	3	0.3633

(*continued*)

Table 2: (*Continued*)

SSR marker†	*O. cumana* populations from Southern and Central Spain collected on sunflower ($N = 10$)		*O. cernua* populations from Southern Spain collected on wild Asteraceae ($N = 12$)	
	NA†	PIC†	NA	PIC
Ocum-069	1	0.0000	3	0.5669
Ocum-070	2	0.3648	4	0.5593
Ocum-075	2	0.3648	6	0.6085
Ocum-076	1	0.0000	3	0.5045
Ocum-080	1	0.0000	4	0.4760
Ocum-085	2	0.3648	6	0.6955
Ocum-087	2	0.3648	4	0.5350
Ocum-089	1	0.0000	6	0.7879
Ocum-092	2	0.3648	3	0.3680
Ocum-094	2	0.3648	5	0.6890
Ocum-123	1	0.0000	3	0.4491
Ocum-124	1	0.0000	4	0.5182
Ocum-129	1	0.0000	2	0.2533
Ocum-140	1	0.0000	2	0.2392
Ocum-141	2	0.3648	3	0.4491
Ocum-144	1	0.0000	2	0.3047
Ocum-149	1	0.0000	1	0.0000
Ocum-163	1	0.0000	2	0.3457
Ocum-167	1	0.0000	3	0.4102
Ocum-168	1	0.0000	6	0.7456
Ocum-174	2	0.3648	3	0.4491
Ocum-176	1	0.0000	5	0.7560
Ocum-180	1	0.0000	3	0.3633
Ocum-185	1	0.0000	2	0.2392
Ocum-187	1	0.0000	2	0.2392
Ocum-192	1	0.0000	1	0.0000
Ocum-196	2	0.3648	3	0.4491
Ocum-197	2	0.3648	3	0.4361
Ocum-198	1	0.0000	3	0.4102
Ocum-205	1	0.0000	7	0.7209
Ocum-206	2	0.3648	3	0.4491
Ocum-215	1	0.0000	3	0.4442
Ocum-216	2	0.3648	8	0.7983
Mean	1.28	0.1117	3.78	0.4968

Notes: †SSR characteristics (primer sequences, annealing temperatures, and product length) and its amplification quality are reported in Pineda-Martos *et al*. (2014); *N*: number of populations within the group; NA: number of alleles; PIC: polymorphism information content.

Analysis of bands was done following the shared-alleles method. Bands with the same mobility were considered identical, scored as present (1) or absent (0), and compiled into a binary data matrix. Cluster analysis based on the similarity matrix and Dice index was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method of NTSYSpc, Numerical Taxonomy System, version 2.21r (Exeter Software, Setauket, NY, USA). Monomorphic markers were excluded from the analysis. The cophenetic correlation coefficient was computed, and Mantel's test was performed.

Results and discussion

O. cumana and *O. cernua* have traditionally been considered closely related species. Putt (1978) suggested the possibility that *O. cumana* developed from a single population of *O. cernua* after the sunflower crop began to have economic importance in Russia in the nineteenth century. Although *O. cumana* has often been regarded as a variant of *O. cernua*, Joel (1987) and Jacobsohn *et al.* (1991) clearly differentiated the two species based on morphological differences and host. Subsequent molecular studies clearly supported the distinction between *O. cumana* and *O. cernua* (Katzir *et al.*, 1996; Paran *et al.*, 1997; Benharrat *et al.*, 2002; Román *et al.*, 2003). The results reported by Katzir *et al.* (1996) revealed identical diagnostic markers in *O. cumana* samples, supporting the hypothesis that these populations were different from those of *O. cernua* collected from the wild. These results suggest that the two species are genetically different, which has been supported by Pujadas-Salvà and Thalouran (1998) and by studies of morphological, phenological, ecological, and biochemical characters performed in both species by Pujadas-Salvà and Velasco (2000).

SSRs are currently considered the markers of choice in many areas of molecular genetics, due to their co-dominance and high level of polymorphism, even between closely related species. A valuable set of 217 SSR markers has been isolated from *O. cumana* and characterized in diverse populations of this species and its closely relative *O. cernua* (Pineda-Martos *et al.*, 2014). In the present research, a subset of 50 of these *O. cumana* SSR markers was used to evaluate the evolutionary relationships and genetic characteristics in the genetic makeup of these *Orobanche* species. SSR-cluster analysis resulted in a dendrogram with a high cophenetic value ($r = 0.9952$, $p < 0.001$) that separated the populations of both species into two main clusters, corresponding with the two species analyzed (Figure 1). *O. cumana* populations clustered together at similarity values of 0.68 or higher, while *O. cernua* populations clustered together at similarity

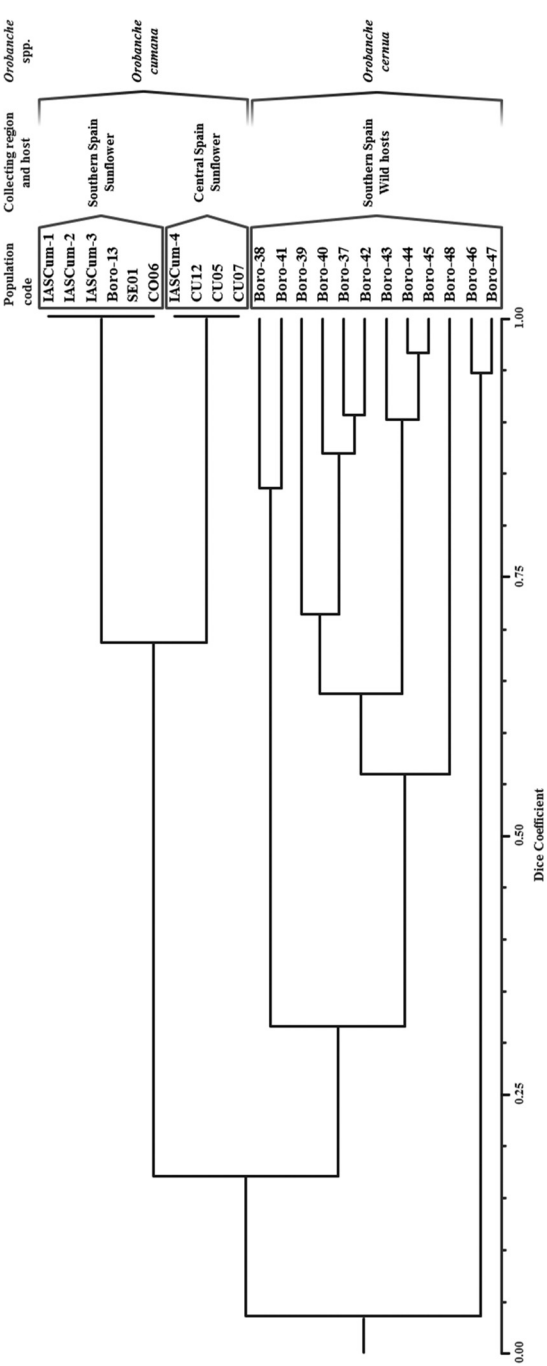

Figure 1: UPGMA dendrogram based on Dice similarity matrix between 10 populations of *O. cumana* and 12 populations of *O. cernua* collected in the Iberian Peninsula obtained with 48 SSR polymorphic markers (see Table 1 for additional population details)

values of 0.32, with the exception of populations Boro-46 and Boro-47. *O. cumana* populations were grouped into two main groups: one group contained all the populations from Southern Spain (provinces of Córdoba and Sevilla), and a second one contained all the populations from Central Spain (province of Cuenca) (Figure 1). *O. cernua* cluster was separated into four different groups. These groups, listed in decreasing similarity order, comprised four populations from the south-west of Almería province (Boro-37, Boro-39, Boro-40, and Boro-42), three populations from the south of Jaén province (Boro-43, Boro-44, and Boro-45), one population from the south of Granada province (Boro-48), and two populations from the central area of Almería province (Boro-38 and Boro-41) (Figure 1). The two populations from the central area of Granada province (Boro-46 and Boro-47) (Figure 1), not included in the main *O. cernua* cluster, would require a new sampling determining their host and a re-evaluation for establishing a more accurate classification.

In the Iberian Peninsula, *O. cumana* is not found in the wild, but exclusively within sunflower fields (Pujadas-Salvà and Velasco, 2000). The great genetic separation between populations of Cuenca and the Guadalquivir Valley suggests that they may derive from seed introductions from different areas. It is also interesting to note that genetic diversity observed in *O. cumana* was considerably lower than in *O. cernua*, despite the geographically proximal populations used in the *O. cernua* set. Gagne *et al.* (1998) concluded that *O. cumana* populations from different geographical origins were genetically very similar, pointing to a monophyletic origin. The high genetic differentiation observed among the groups of *O. cernua* populations suggested the presence of effective ecological barriers preventing gene flow between the populations together with the occurrence of a predominantly self-pollinating mating system.

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