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1 **Pathogenic and molecular diversity in highly virulent populations of the parasitic**
2 **weed *Orobanche cumana* (sunflower broomrape) from Europe**

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12 **Short title**: Diversity in highly virulent *Orobanche cumana* from Europe

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Código de campo cambiado

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Summary

The parasitic weed *Orobanche cumana* (sunflower broomrape) constrains sunflower production in eastern and southern Europe and in the Middle East. Although genetic resistance is the most effective control method, new parasite races evolve overcoming sunflower resistance. In this work, highly virulent populations of *O. cumana* were analysed for pathogenicity and genetic diversity. The virulence of 11 populations from Hungary, Romania, Spain and Turkey was assessed and compared after infection of sunflower inbred lines to differentiate races of the parasite under glasshouse conditions. Molecular diversity among and within 27 parasite populations was studied by RAPD-PCR, UPGMA and AMOVA analyses. Highly virulent race F was identified in Hungary, Spain and Turkey. The most virulent race (G) was also found in Turkey. The molecular analysis among highly virulent populations of *O. cuman*a identified four molecular clusters, respectively grouping populations from Central Spain, Hungary, South Spain and Turkey. The genetic homogeneity within parasite populations was confirmed, since no molecular divergences were found within them. This work constitutes the first geographical study of *O. cumana* together with pathogenicity and molecular traits inherent to each geographical group, and provides useful information for possible phylogenetic analyses of *O. cumana*. In addition, molecular markers associated with geographic origin could be developed and used as diagnostic tools to track new broomrape introductions into areas free of virulent races where they might represent a threat to sunflower production.

Keywords: AMOVA analysis, genetic resistance, genetic diversity, *Helianthus annuus,* molecular characterisation, pathogenicity, RAPD-PCR analysis.

1 **Introduction**

2 Sunflower (*Helianthus annuus* L.) is the most important annual oilseed crop in southern 3 Europe and the Black Sea area. The holoparasitic angiosperm *Orobanche cumana* Wallr. 4 (sunflower broomrape) is regarded as one of the most important constraints for crop 5 production in most countries of the Middle East and eastern and southern Europe, 6 including Hungary, Romania, Spain and Turkey. *Orobanche cumana* infects sunflower 7 roots and depletes the plant of water and nutrients causing up to 50% of yield losses in 8 susceptible cultivars (Dominguez, 1996). Soil fumigation and solarization are not 9 economically feasible (Foy *et al*., 1989; Jacobson *et al*., 1980) and imidazolinone 10 herbicides can be very effective, but they must be applied to imidazolinone-resistant 11 sunflowers (Clearfield production system) (Tan *et al*., 2005). Genetic resistance is still the 12 most frequent, feasible and reliable control method against sunflower *O. cumana*.

13 Within *Orobanche* spp., *O. cumana* is the only species that exhibits a clear race 14 structure with respect to sunflower genotypes. Single major genes (Or_1 to Or_5) were 15 reported to confer resistance to races A to E of *O. cumana* (Vrânceanu *et al*., 1986). Race 16 F, which overcomes the resistance gene Or_5, was identified in Spain (Saavedra del Río *et* 17 *al*., 1994) and also in Romania (Pacureanu-Joita *et al*., 2008) in the mid 1990's. Parasite 18 populations infecting sunflower genotypes that carry Or_5 were later reported in Turkey 19 (Kaya *et al*., 2004). In Hungary, populations of *O. cumana* have been pathogenically 20 characterised as low to moderately virulent (races A to D) (Zoltán, 2001).

21 Different aggressiveness of populations of *O. cumana* race F have been reported 22 (Molinero-Ruiz *et al.*, 2009) and genetic heterogeneity within some of these populations 23 has been suggested (Molinero-Ruiz *et al.*, 2008). In fact, the L86 line, which was 24 registered as resistant to race F (Fernández-Martínez *et al.*, 2004), showed up to 65% 25 incidence of very low infections, as compared with the susceptible control (3 and 23 *O.* 26 *cumana* stems per sunflower plant respectively) after inoculation with some race F

populations (Molinero-Ruiz *et al.*, 2009). This suggested that these infections might be due to highly virulent components ("new races") within the parasite population. Therefore, genetic diversity among and within populations of *O. cumana* race F might be responsible for a) different aggressiveness on Or_5, and/or b) consistent infections in sunflower inbred lines with resistance to race F, respectively.

The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique shows extensive divergence among, but little variation within, species. Therefore, it is extensively used to distinguish biotypes and/or germplasm accessions within weed species and crops (Volenberg *et al*., 2007; Zivkovic *et al*., 2012), as well as to differentiate races or isolates of plant pathogens (Gómez-Lama Cabanás *et al*., 2012; Villarino *et al*., 2012). Genetic diversity based on RAPD markers has been also assessed among *Orobanche* spp. (Paran *et al*., 1997; Roman *et al*., 2003; Atanasova *et al*., 2005), including *O. cumana* (Gagne *et al*., 1998). However, no molecular analyses have been carried out to compare races or the infection ability of *O. cumana* populations on sunflower genotypes carrying resistance genes.

In this work, the pathogenicity and molecular biology of highly virulent populations of *O. cumana* from Hungary, Romania, Spain and Turkey were studied. The objectives were: (i) to assess and compare the race or infection ability of *O. cumana* populations on sunflower inbred lines carrying resistance to the parasite and (ii) to investigate the genetic diversity among and within the most virulent populations of *O. cumana* using RAPD analysis.

Materials and methods

Infectivity of highly virulent populations of O. cumana *on resistant sunflower*

Eleven populations of *O. cumana* were compared by their infectivity in three sunflower inbred lines. High virulence was expected because they were collected from fields cropped

1 with sunflower allegedly carrying resistance to race E in Hungary, Romania, Spain and
2 Turkey (Table 1). In order to avoid previous cross pollination among them (Rodriguez-
3 Ojeda *et al*., 2013), only those infested fields that were at least 5 km apart were considered
4 for the study. *Orobanche cumana* stems were collected at full maturity and seeds
5 recovered and kept until used, as previously described (Molinero-Ruiz *et al.*, 2009). The
6 high virulence of the parasite populations was confirmed by inoculation on the sunflower
7 inbred line NR5 (Or_5). NR5 is a tester line to differentiate races of *O. cumana* (differential)
8 that shows resistance to races A to E and susceptibility to populations of higher virulence
9 than E (Molinero-Ruiz *et al.*, 2006). Populations of *O. cumana* were also inoculated onto
10 the differential sunflower inbred lines L86 and P96. Line L86 carries resistance to race F
11 and susceptibility to races E and G; P96 is resistant to races E and F but susceptible to race
12 G (Molinero-Ruiz *et al.*, 2008) (Table 2).

13 Eight sunflower seedlings (replications) of each differential were inoculated with
14 each of the populations of *O. cumana*. Sunflower seeds were surface-sterilised by
15 immersing them in 10% sodium hypochlorite for 10 minutes, then thoroughly rinsed in
16 deionised water and incubated in the dark at saturation humidity in a germinator at 26±2ºC
17 until radicles were 2 to 5 mm long. Individual sunflower seedlings were transplanted into
18 pots with 175 g of soil mixture SS [sand:silt, 1:1, vol amended with 1 g l^{-1} of 16:7:15 (:2),
19 N:P:K (:Mg)] uniformly infested with 18 mg of parasite seeds and grown under conditions
20 conducive for infection (20 to 25ºC and photoperiod of 14 hours day^{-1}). Fourteen-day-old
21 sunflower plants were transplanted, along with the infested soil, into 5 L pots containing
22 SS mixture and grown in the glasshouse at 10 to 32ºC for 10 additional weeks until
23 physiological maturity. Plants were watered as needed.

24 The degree of attack in sunflower genotypes (DA, i.e. the number of emerged *O.*
25 *cumana* stems per sunflower plant) was assessed weekly, from the emergence of the first
26 parasite stem until sunflower senescence. A standardised area under the DA progress curve

(SAUDAPC) was calculated by trapezoidal integration method, standardised by the duration of parasite emergence in weeks (Campbell & Madden, 1990) and transformed [sqrt (SAUDAPC+0.5)] prior to analysis of variance. When significant effects were obtained, Fisher's protected LSD tests (P = 0.05) were used for comparisons of inbred lines, populations and their interaction. The experiment was performed twice and was set up as a factorial on a completely randomised design. As no significant differences between the two experiment replications were found for SAUDAPC (McIntosh, 1983), data were pooled.

Molecular characterisation of highly virulent populations of O. cumana

To examine the molecular diversity among race F populations of *O. cumana*, stems of 27 populations from Hungary, Spain and Turkey, grown on sunflower NR5 in the phenotypical characterisation described above and in previous studies (Molinero-Ruiz *et al*., 2006; 2008; 2009), were collected. In order to assess the molecular diversity within populations, stems of eight and four out of the 27 populations were also independently collected from L86 and P96 respectively, and included in the molecular analyses. Therefore, a final set of 39 samples of *O. cumana* was analysed using the RAPD-PCR technique (Table 3). All stems were collected just before flowering and 2 to 3 cm long apices were lyophilised until used.

Each sample consisted of total genomic DNA from three stem apices of each population. DNA was purified using the Speedtools Plant DNA Extraction Kit (Biotools, Madrid, Spain) according to the manufacturer's instructions. Quality and concentration of DNA samples were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Wilmington DE, USA). Finally, DNA samples were adjusted to a final concentration of 25 ng/ µL and stored at –20°C until required for RAPD analysis.

1 Optimised PCR assays were carried out in a final volume of 25 µL containing: 0.6
2 µM primer, 200 µM dNTPs, 2.5 µl of 10× PCR buffer (800 mM tris- HCl, pH 8.3 to 8.4 at
3 25°C, 0.2% Tween 20 wt vol^{-1}), 1.5 U of Ultratools Polymerase (Biotools, Madrid, Spain),
4 3.0 mM $MgCl_2$, and 50 ng of parasite DNA. Amplification conditions were: 4 min
5 denaturation at 94°C; followed by 45 cycles of 1 min denaturation at 94°C, 1 min of
6 annealing at 37°C, and 2 min of extension at 72°C; and a final extension step of 6 min at
7 72°C. The temperature always varied at a rate of 5°C s^{-1}. All reactions were done in a T1
8 Thermocycler (Whatman- Biometra, Goettingen, Germany). Amplification products were
9 separated by horizontal electrophoresis in 1.5% agarose gels containing 0.05 µl/ml
10 SafeView Nucleic Acid Stain (NBS Biologicals Ltd, Huntingdon, England) and visualised
11 over a UV light source. A 100- to 2,000-bp ladder (Dominion MBL, Córdoba, Spain) was
12 included in the electrophoresis.
13 RAPD analyses were carried out using 155 10-mer oligonucleotide primers
14 (Invitrogen Corporation, San Diego, CA, USA). Reactions yielding clear polymorphic
15 bands for all the samples were done at least twice and negative controls (no template
16 DNA) were included in each assay. Only primers producing consistent reproducible
17 polymorphism were considered.
18 A binary matrix based on presence (1) or absence (0) of amplicons of the same
19 molecular weight was generated for 18 selected primers. Genetic similarities among
20 isolates were calculated according to Jaccard's similarity coefficient (Jaccard, 1908) and
21 dendrograms constructed from the distance matrices using the UPGMA method. The
22 robustness of each node of the dendrogram was calculated by generation of 1000 bootstrap
23 replications of the data (Nei & Kumar, 2000). Computations were performed using
24 Fingerprinting II Informatix Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
25 The genetic structure of the populations was analysed by AMOVA (Analysis of Molecular
26 Variance) using Arlequin Software (Excoffier *et al.*, 2005). Population structure proposed

1 was checked by Fixation index (FST) calculation and the corresponding significance *P*
2 value. In addition, pairwise FSTs were also calculated by pairs of populations using
3 genetic distance method as implemented in Arlequin Software.
4
5 **Results**

Results

6
7 *Infectivity of highly virulent populations of* O. cumana *on resistant sunflower*
8 Both factors, population of *O. cumana* and sunflower inbred line, had a significant effect
9 ($P < 0.001$) on SAUDAPC. Population mean values of SAUDAPC ranged from 2.6
10 (average for LRB1603, OCR1, CU1102, CU200 and OCT3) to 13.6 (average for OCT4
11 and OCT2). When averaged across inbred lines, SAUDAPC values ranged from 2.4 in P96
12 to 10.3 in NR5 (Fig. 1). Remarkably, the SAUDAPC in each sunflower inbred line
13 depended on the population of *O. cumana*, as indicated by the significance ($P < 0.001$) of
14 the population x inbred line interaction.
15 There were populations identified as race E (OCR1, CU200 and CU1102), since
16 SAUDAPC was zero in NR5 and P96, and L86 showed 8.2 SAUDAPC (averaged across
17 populations) (Fig. 1). Race F was identified for populations OCH4, LRB1603 and OCT3,
18 since P96 showed a resistant reaction and high infection was observed in NR5 (12.3
19 SAUDAPC averaged across populations) (Fig. 1). Similar (population OCT3) or
20 significantly lower (populations OCH4 and LRB1603) infection occurred in L86 as
21 compared with NR5 (Fig. 1). When the inbred lines were inoculated with PA101 or OCT5,
22 a high infection occurred in NR5, L86 displayed a high (PA101) or moderate (OCT5)
23 reaction, and low infections levels were observed in P96 (Fig. 1). Finally, populations
24 OCT2, OCT4 and OCT6 from Turkey clearly overcame the resistance of the three inbred
25 lines and therefore they were identified as race G. The highest infection by these
26 populations occurred in NR5, and the infection in P96 was similar (OCT4 and OCT6) or

1 lower than the one in L86 (Fig. 1). In order to analyse the most virulent populations, stems
2 of OCH4, PA101, OCT2, OCT5 and OCT6 were collected from inbred line NR5. Also,
3 stems of populations PA101, OCT2, OCT5 and OCT6 were collected from lines L86 and
4 P96. All the collected stems were included in the subsequent molecular analysis, together
5 with stems collected in previous work by our research group (Table 3).
6
7 *Molecular characterisation of highly virulent populations of* O. cumana
8 Among the 155 primers initially assayed, 18 were selected since they produced
9 consistent polymorphisms. A total of 100 reproducible polymorphic bands were amplified
10 from the 39 assessed samples, the size ranging from 25 to 2,213 bp (N05 and G14
11 respectively). Amplicons generated by each primer varied between two (C16) and nine
12 (G03) (Table 4).
13 The dendrogram resulting from the UPGMA analysis of the RAPD data set
14 distinguished four well-differentiated clusters among the 39 samples of *O. cumana* (Fig.
15 2). Cluster I grouped the 24 samples collected in the south of Spain, which shared about
16 89% similarity. Two sub-clusters were identified within cluster I: one of them (Ia) grouped
17 exclusively N- samples of 16 populations, and the other (Ib) grouped N- and L- samples
18 from the remaining four populations from South Spain. The unique sample of *O. cumana*
19 from Hungary was grouped in cluster II, genetically closer to cluster I than to the rest of
20 the clusters. All parasite samples from Turkey shared 86% similarity and were grouped in
21 cluster III, irrespective of the sunflower inbred line they infected. Finally, the four samples
22 from populations collected in Central Spain grouped in cluster IV (80% similarity) (Fig.
23 2).
24 An AMOVA analysis among populations was carried out attending to geographic
25 origin of *O. cumana* (i.e. Spain *vs* Turkey *vs* Hungary). The analysis revealed that 60% of
26 genetic variability was due to differences among countries; however, up to 40% of the

genetic variability was due to differences among populations within the same country (Table 5, analysis 1). A similar analysis but considering the South and the Centre of Spain as different groups yielded the results showed in Table 5, analysis 2. The latter checked the variability among the groups obtained in the UPGMA dendrogram (clusters I to IV, including the isolate from Hungary as another cluster). In this analysis, percentage of variation within groups was decreased down to 21%, presumably indicating that genetic differences among Spanish groups (South *vs* Centre) accounted for 19% of the genetic variability identified among populations of *O. cumana* in analysis 1. When pairwise differences among populations were checked, there was almost as much divergence between populations from South Spain and Turkey (78.3% of molecular variation) and Centre Spain and Turkey (79.5%) as that between the Spanish geographical groups (82.2%) (Table 6).

Concerning the molecular analysis within populations of *O. cumana* using AMOVA, we found that genetic distance supported sub-clusters Ia and Ib from southern Spain as genetically differentiated (Table 5, analysis 3). In contrast, differentiation between samples of sub-cluster Ib obtained from lines NR5 (N-) and L86 (L-) was not statistically supported (Table 5, analysis 4). In cluster III, we used AMOVA to analyse whether samples obtained from P96 line (P-) could genetically be differentiated from N- and L- samples, and whether N- and L- samples differed between them. The analyses showed no significant differentiation (Table 5, analyses 5 and 6).

Finally, both presence and absence of bands were identified as markers for the molecular groups in the UPGMA clustering, even for sub-groups Ia and Ib in cluster I (Table 7). Neither the presence nor the absence of any polymorphic band was associated with genotypes NR5, L86 or P96, irrespective of the population of *O. cumana* (data not shown).

Discussion

Concerning the pathogenic characterisation of populations of *O. cumana*, some of them were identified as race E, since they did not overcome *Or*$_5$ gene in NR5 and were pathogenic to L86 (Molinero-Ruiz *et al*., 2006; 2008). This was the case of OCR1 from Romania and CU200 and CU1102 from Central Spain. Race F was identified in Hungary (OCH4) for the first time, as well as in Turkey (OCT3 and OCT5), and confirmed in Spain (PA101 and LRB1603). Populations of *O. cumana* causing the significantly highest infections in P96 were all collected in Turkey (OCT2, OCT4 and OCT6). In accordance with Kaya *et al*. (2004), who reported on the occurrence of one parasite race more virulent than F in Turkey, our results allow the characterisation of OCT2, OCT4 and OCT6 as race G.

The molecular analysis grouped *O. cumana* populations according to their geographical origin. Moreover, parasite populations within close geographical areas could be clearly differentiated as well (i.e. populations from Southern Spain *vs.* Central Spain). This is in agreement with Pineda-Martos *et al*. (2013), who recently reported on two clusters of *O. cumana* populations from Spain, one in Cuenca province (Central Spain) and the other in the Guadalquivir Valley (Southern Spain). Cluster analyses using RAPD markers also grouped, according to geographical regions, populations of crop species such as *Jatropha curcas* (Rafii *et al.*, 2012) and isolates of plant pathogens such as *Verticillium tricorpus* (Usami *et al.*, 2011) and *Fusarium oxyporum* f.sp. *dianthi* (Gómez-Lama Cabanás *et al.*, 2012). Gagne *et al*. (1998) previously studied eight populations of an unknown race of *O. cumana* by RAPD analysis. They identified one cluster that grouped populations from Bulgaria, Romania and Turkey, and another one corresponding to populations from Spain. Our results confirm the molecular differentiation of populations from Spain as compared with those from other countries in Europe. Moreover, populations from Turkey were molecularly close to those from South Spain.

1 Populations of race F from South Spain were clustered irrespective of their
2 previously reported aggressiveness. Indeed, some populations identified as low, moderate
3 or highly virulent on NR5 (Molinero-Ruiz *et al*., 2009) were not genetically differentiated.
4 Only the highly aggressive CR202 and PG502 populations (Molinero-Ruiz *et al.*, 2009),
5 grouped in the same sub-cluster (Ia). Qualitative resistance to *O. cumana* races A to E,
6 controlled by major genes and associated with complete absence of infection has been
7 reported in sunflower. In contrast, the genetic control of the resistance to race F into
8 sunflower germplasm has been described as both qualitative and quantitative (Fernández-
9 Martínez *et al.*, 2004; Pérez-Vich *et al.*, 2006). In our molecular analysis within
10 populations, differences were found neither among N- and L- samples from CN503,
11 CO101, CT1503 and LR100 (sub-cluster Ia), nor among N-, L- and P- samples of OCT2,
12 OCT5 and OCT6 (cluster III) and PA101 (cluster IV), indicating that these populations
13 were genetically homogeneous. Therefore, the low infection levels found in L86 and P96
14 (Molinero-Ruiz *et al.*, 2006; 2008) are not due to genetic heterogeneity within parasite
15 populations, but to the quantitative resistance of the inbred lines (Pérez-Vich *et al.*, 2006;
16 Molinero-Ruiz *et al.*, 2009).
17 On the other hand, race F populations could not be identified as a genetically
18 differentiated group, since they were present in all clusters after UPGMA analysis. These
19 results point to a polyphyletic origin for race F, arising independently at different
20 geographical origins, as has recently been suggested (Pineda-Martos *et al.,* 2013). In
21 contrast, populations of race G were located only in cluster III, suggesting a monophyletic
22 origin for race G which should be further investigated.
23 Overall, and in spite of the disadvantages of the RAPD technique such as poor
24 reproducibility, lack of true homology between bands of the same length or dominant
25 inheritance (Mercado-Blanco *et al.*, 2008), this molecular approach yielded very useful
26 results for our study, which was undertaken to search for polymorphisms among

taxonomically very close DNA samples [(i.e. polymorphisms within a race (F) of a weed species (*O. cumana*)].

This work constitutes the first geographical differentiation of *O. cumana* together with pathogenicity and molecular traits inherent to each geographical group. Our results indicate that molecularly homogeneous groups are clearly identified in highly virulent populations of *O. cumana*, and that they are first related to geographical origin in Europe. Then, some molecular groups relate only to one pathogenic group (i.e. race F from Southern Spain) while others include different pathogenic groups from the same geographical origin (i.e. races F and G from Turkey). Further research, based on more robust molecular markers, might unravel the phylogeny of *O. cumana*. *Orobanche cumana* and *O. cernua* are reported as one lineage of *Orobanche* species from the Old World (Manen *et al.*, 2004). In addition, our results provide useful information for the development of molecular markers associated with geographical origin of *O. cumana*, which could be used as a diagnostic tool for new parasite introductions into growing areas where they might represent a threat to sunflower production.

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1 **Table 1** Geographical origin (country, location and region) and year of collection of 11
2 populations of *Orobanche cumana* whose race or infectivity in genetically resistant
3 sunflower genotypes was characterised in this work
4

Population	Location			
	Country	Location, region	Year	References *
OCH4	Hungary	Bátonyterenye, Nógrád	2007	
OCR1	Romania	Braila, Braila	2005	
CU200	Spain	Segóbriga, Cuenca	2000	Molinero-Ruiz *et al*., 2008
CU1102	Spain	Montalbo, Cuenca	2002	Molinero-Ruiz *et al*., 2008
PA101	Spain	Palomares del Campo, Cuenca	2001	
LRB1603	Spain	La Rambla, Córdoba	2003	Molinero-Ruiz *et al*., 2008; 2009
OCT2	Turkey	Cesmekolu, Kirklareli	2003	
OCT3	Turkey	Hayrabolu, Tekirdag	2003	
OCT4	Turkey	Malkara, Tekirdag	2003	
OCT5	Turkey	Muratli, Tekirdag	2003	
OCT6	Turkey	Babaeski, Kirklareli	2003	

5 * References to previous works in which some populations were studied
6

1 **Table 2** Reaction of differential sunflower inbred lines to races of *Orobanche cumana* [*]
2

Differential line	Race of *O. cumana*			
	E	F		G
		Spain C	Spain S	
NR5	R [†]	S	S	S
L86	S	S	R [‡]	S
P96	R	R	R	S

3 [*] Based on the results by Molinero-Ruiz *et al*. 2006 and 2008.

4 [†] R, resistant; S, susceptible.

5 [‡] Inbred line L86, which was registered as R to race F, showed high incidences of very
6 few broomrape stems per sunflower plant when inoculated with populations of *O.*
7 *cumana* race F from the south of Spain, suggesting its quantitative resistance to the
8 parasite (Molinero-Ruiz *et al.*, 2009).

9

1 **Table 3** List of 39 samples of *Orobanche cumana* included in the molecular analysis of 2 populations of the parasite from Spain, Turkey and Hungary, and sunflower inbred lines 3 they were collected from

Sample	Population of *Orobanche cumana*, country *	Genotype of sunflower	References †
N-OCH4	OCH4, Hungary	NR5	
N-MO197	MO197, Spain (C)	NR5	Molinero-Ruiz *et al*., 2008
N-PA101	PA101, Spain (C)	NR5	
L-PA101	PA101, Spain (C)	L86	
P-PA101	PA101, Spain (C)	P96	
N-AA702	AA702, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-CN503	CN503, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
L-CN503	CN503, Spain (S)	L86	Molinero-Ruiz *et al*., 2009
N-CO101	CO101, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
L-CO101	CO101, Spain (S)	L86	Molinero-Ruiz *et al*., 2009
N-CO1002	CO1002, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-CP203	CP203, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-CR202	CR202, Spain (S)	NR5	Molinero-Ruiz *et al*., 2006; 2009
N-CT803	CT803, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-CT1503	CT1503, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
L-CT1503	CT1503, Spain (S)	L86	Molinero-Ruiz *et al*., 2009
N-EC403	EC403, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-HE200	HE200, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-IN115	IN115, Spain (S)	NR5	
N-JC302	JC302, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-LC299	LC299, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-LG602	LG602, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-LR100	LR100, Spain (S)	NR5	Molinero-Ruiz *et al*., 2006; 2009
L-LR100	LR100, Spain (S)	L86	Molinero-Ruiz *et al*., 2006; 2009
N-MN802	MN802, Spain (S)	NR5	
N-PG502	PG502, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-SE500	SE500, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-SP102	SP102, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-TP402	TP402, Spain (S)	NR5	Molinero-Ruiz *et al*., 2009
N-OCT2	OCT2, Turkey	NR5	
L- OCT2	OCT2, Turkey	L86	
P- OCT2	OCT2, Turkey	P96	
N-OCT3	OCT3, Turkey	NR5	
N-OCT5	OCT5, Turkey	NR5	
L-OCT5	OCT5, Turkey	L86	
P-OCT5	OCT5, Turkey	P96	
N- OCT6	OCT6, Turkey	NR5	
L- OCT6	OCT6, Turkey	L86	

P- OCT6	OCT6, Turkey	P96

[*] The geographical origin Centre (C) or South (S) is indicated for samples from Spain.

[†] References to previous work in which some populations were studied.

1 **Table 4** Consistent random amplified polymorphic DNA (RAPD) fragments generated for

2 39 samples of *Orobanche cumana* obtained from the sunflower inbred lines NR5, L86 or

3 P96 and screened with 18 DNA primers [*]

4

Primer code	Sequence (5'-3')	No. of amplicons scored [†]	Size of amplicons (bp)
A17	GACCGCTTGT	5	526-1414
B08	GTCCACACGG	3	697-962
B19	ACCCCCGAAG	7	38-83
C11	AAAGCTGCGG	8	336-1112
C16	CACACTCCAG	2	305-644
G02	GGCACTGAGG	6	454-1057
G03	GAGCCCTCCA	9	331-1045
G04	AGCGTGTCTG	4	616-1189
G10	AGGGCCGTCT	4	522-1006
G13	CTCTCCGCCA	7	525-1416
G14	GGATGAGACC	8	400-2213
G15	ACTGGGACTC	6	495-850
N05	ACTGAACGCC	3	25-51
N09	TGCCGGCTTG	5	631-1161
N12	CACAGACACC	4	437-964
N16	AAGCGACCTG	7	473-1184
N17	CATTGGGGAG	5	528-1191
V15	CAGTGCCGGT	7	27-79

5 [*] Primers were tested twice on each of the 39 samples of *O. cumana*.

6 [†] Only intense and reproducible bands were scored.

7

1 **Table 5** Genetic structure of 39 accessions of *Orobanche cumana* analysed using Analysis

2 of Molecular Variance (AMOVA)

Analysis [*]	Source of variation	d.f. [†]	Sum of squares	Variance components	Percentage of variation	Φ Statistic	Probability (P)
1	Among groups	2	151.927	8.614 Va	60.08	0.60084	$P < 0.001$
	Within groups	36	206.021	5.722 Vb	39.92	-	-
2	Among groups	3	250.499	11.365 Va	78.73	0.78732	$P < 0.001$
	Within groups	35	107.450	3.070 Vb	21.27	-	-
3	Among groups	1	25.125	2.179 Va	53.68	0.53676	$P < 0.001$
	Within groups	22	41.375	1.881 Vb	46.32	-	-
4	Among groups	1	0.75	0.000 Va	0.00	0.00000	$P = 1$
	Within groups	6	4.5	0.750 Vb	100.00	-	-
5	Among groups	1	5.581	0.626 Va	17.49	0.1749	$P = 0.094$
	Within groups	8	23.619	2.952 Vb	82.51	-	-
6	Among groups	1	4.702	0.462 Va	12.92	0.1292	$P = 0.1594$
	Within groups	5	15.583	3.117 Vb	87.08	-	-

3 [*] 1 = Spain *vs* Turkey *vs* Hungary, 2 = South Spain *vs* Centre Spain *vs* Turkey *vs* Hungary, 3

4 = South Spain Ia *vs* South Spain Ib, 4 = South Spain Ib N *vs* South Spain Ib L, 5 = Turkey

5 NL *vs* Turkey P, 6 = Turkey N *vs* Turkey L.

6 [†] d.f. = degrees of freedom.

7

Table 6 Comparisons of pairs of *Orobanche cumana* populations (FST indexes) as a measure of population differentiation due to genetic structure

	FST [*]	*P* [†]
Comparisons according to geographic origin		
Centre Spain *vs* South Spain	0.82240	<0.001
Centre Spain *vs* Turkey	0.79519	<0.001
South Spain *vs* Turkey	0.78309	<0.001
Comparisons according to infectivity on sunflower genotypes		
South Spain N (Ia) *vs* South Spain NL (Ib)	0.53676	<0.001
South Spain N (Ib) *vs* South Spain L (Ib)	0.00000	0.991
Turkey NL vs Turkey P	0.17490	0.171
Turkey N vs Turkey L	0.12922	0.135

[*] Population pairwise FST index calculated using pairwise differences as distance method as implemented in Arlequin software.

[†] Significance level of FST *P* values < 0.001.

1 **Table 7** Markers of four molecular groups identified within 39 DNA samples of
2 *Orobanche cumana* after the molecular analysis of the band patterns obtained with
3 PCR amplification using 18 10-mer primers

Marker (bp)	Molecular groups				
	I		II	III	IV
	Ia	Ib			
27	-	-	-	-	+
32	+	+	+	+	-
37	-	-	-	-	+
41	-	-	+	-	+
48	+	+	+	+	-
51	+	+	+	+	-
57	-	-	-	+	+
62	-	-	+	+	+
73	+	+	-	+	-
79	-	-	+	-	-
331	-	-	-	+	-
336	-	+	-	-	-
400	-	+	-	-	-
458	-	+	-	-	-
463	-	-	-	+	-
525	-	-	-	+	-
526	+	+	+	+	-
616	-	-	-	+	+
631	+	+	+	+	-
697	+	+	+	-	+
715	-	-	-	-	+
754	-	-	-	+	-
823	+	+	-	-	+
953	-	-	-	-	+
962	+	+	+	-	+
1092	-	-	-	-	+
1112	+	+	+	-	-
1145	+	+	+		+

1161	-	-	-	-	+
1184	+	-	-	-	-
1416	-	-	-	+	-

1

2

1 **Captions for Figures**

2

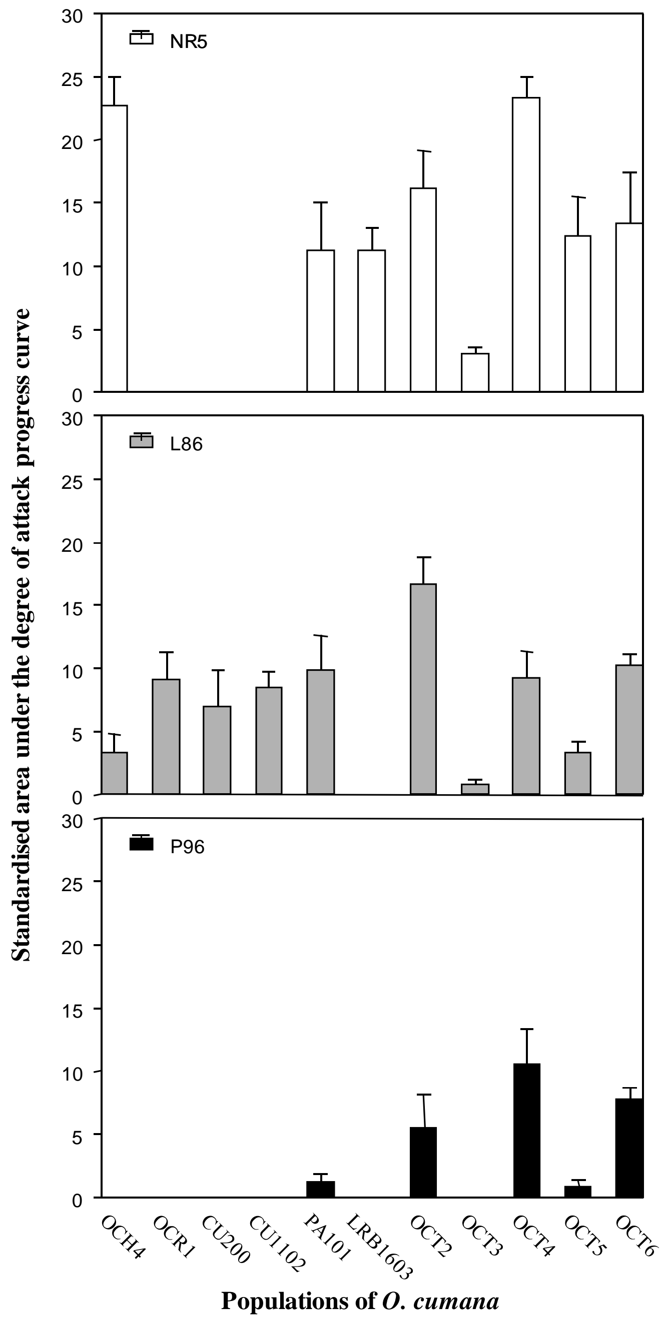
3 **Fig. 1** Reaction, expressed as the standardised area under the degree of attack progress
4 curve, of three sunflower inbred lines (NR5, L86 and P96), differentials for races of
5 *Orobanche cumana*, after inoculation with 11 populations of the parasite collected in
6 Hungary, Romania, Spain and Turkey and growth under shadehouse conditions (critical
7 least significant difference value = 5.15).

8

9 **Fig. 2** Dendrogram derived from random amplified polymorphic DNA (RAPD) analysis
10 of 39 DNA samples of *Orobanche cumana* infecting sunflower (see Table 3) and
11 generated using 18 10-mer primers and the unweighted paired group method with
12 arithmetic averages (UPGMA). Scale represents percentage similarity using Jaccard's
13 similarity coefficient. Bootstrap support percentages (≥75) for 1000 replicates are
14 indicated at the nodes.

15

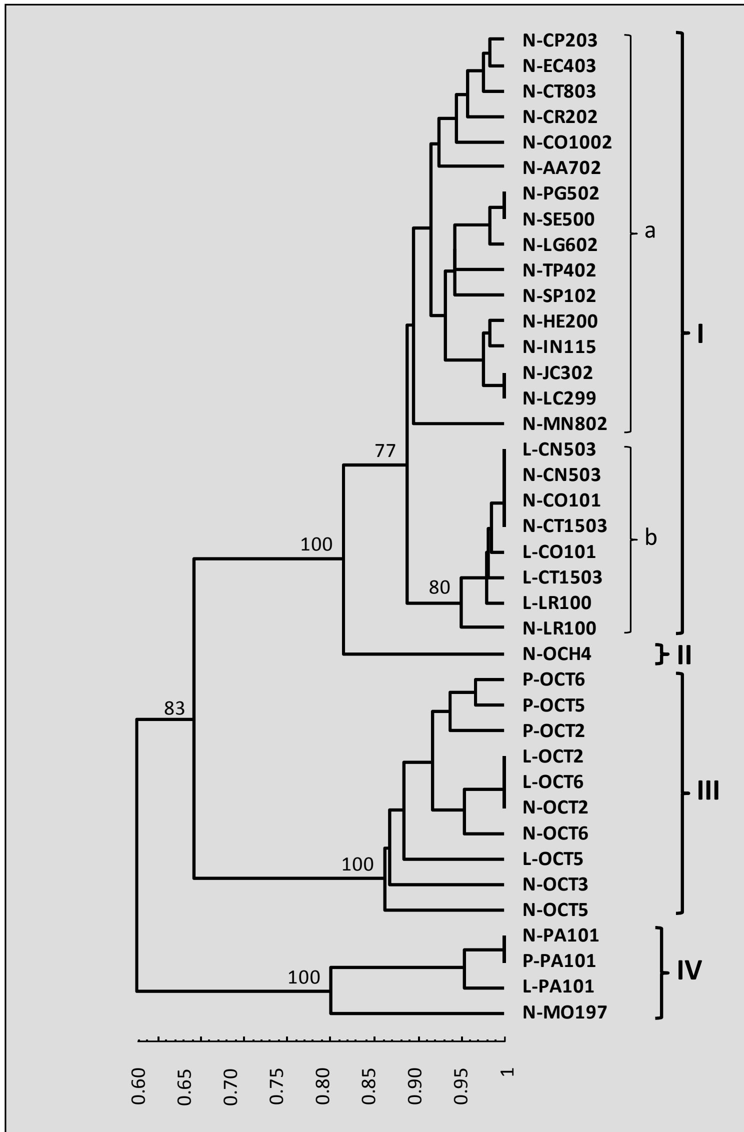
1 **Fig. 1**



2

3

1 **Fig. 2**



2