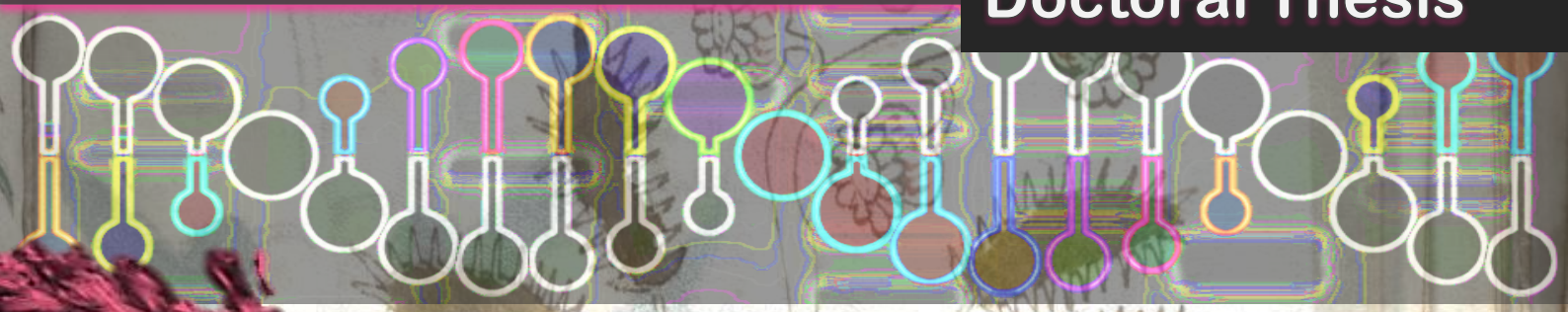
Molecular Studies on Sunflower Broomrape (*Orobanche cumana* Wallr.)

Estudios Moleculares en Jopo de Girasol (*Orobanche cumana* Wallr.)

Doctoral Thesis



Rocío Pineda Martos

Plant Breeding Department
Institute for Sustainable Agriculture
(IAS-CSIC)

TITULO: *Estudios Moleculares en Jopo de girasol (Orobanche cumana Wallr.)/ Molecular Studies on Sunflower Broomrape (Orobanche cumana Wallr.)*

AUTOR: *Rocío Pineda Martos*

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School of Agricultural and Forestry Engineering (ETSIAM)

Departamento de Genética
Department of Genetics

TESIS DOCTORAL
POR COMPENDIO DE PUBLICACIONES Y CON MENCIÓN INTERNACIONAL

DOCTORAL THESIS
BY COMPENDIUM OF PUBLICATIONS AND WITH INTERNATIONAL MENTION

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MEMORIA DE TESIS DOCTORAL presentada por ROCÍO PINEDA MARTOS,
Ingeniera Agrónoma (Biotecnología Agroforestal), para optar al grado de DOCTORA

DOCTORAL THESIS presented by ROCÍO PINEDA MARTOS,
Agricultural Engineer (Agroforestry Biotechnology), for the degree of DOCTOR

Los directores,
The supervisors,

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Departamento de Mejora Genética Vegetal / Department of Plant Breeding
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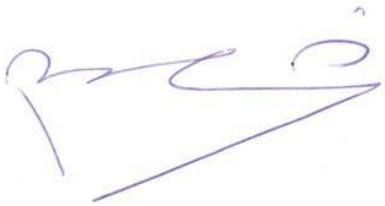
INFORMA:

Que **ROCÍO PINEDA MARTOS**, Ingeniera Agrónoma (Especialidad: Biotecnología Agroforestal), ha realizado bajo mi dirección en el Departamento de Mejora Genética Vegetal del Instituto de Agricultura Sostenible (IAS-CSIC), el trabajo titulado: "Estudios Moleculares en Jopo de Girasol (*Orobanche cumana* Wallr.) / Molecular Studies on Sunflower Broomrape (*Orobanche cumana* Wallr.)", y que a mi criterio dicho trabajo reúne los méritos suficientes para optar al Grado de Doctora Ingeniera Agrónoma.

Y para que así conste y surta los efectos oportunos, firmo el presente informe en Córdoba a 30 de Julio de 2014.



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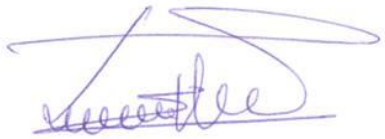

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DOCTORANDO/A: Rocío Pineda Martos

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La Tesis Doctoral que se presenta tiene como objetivo global el desarrollo de herramientas moleculares en la planta parásita *Orobanche cumana* y su aplicación a estudios de diversidad genética y estructura de poblaciones en esta especie. Para ello, se han realizado tres trabajos de investigación centrados en 1) Desarrollo y caracterización de marcadores microsatélites (SSRs) específicos de *O. cumana*; 2) Estudio de la diversidad genética de poblaciones españolas de *O. cumana* empleando los marcadores SSR desarrollados; y 3) Estudio de la diversidad genética, estructura de poblaciones, y virulencia sobre girasol de poblaciones de *O. cumana* que parasitan especies silvestres en su área natural de distribución en la región del mar Negro de Bulgaria. El desarrollo experimental de la Tesis Doctoral ha requerido la formación en la realización de ensayos de inoculación artificial en invernadero, una amplia gama de técnicas de laboratorio, así como numerosos programas informáticos para estudios de marcadores moleculares, diversidad genética, y estructura de poblaciones. Este trabajo experimental se ha desarrollado de forma ejemplar por parte de la Doctoranda, lo que ha conducido a unos resultados a nuestro juicio excepcionales que han contribuido de forma importante a profundizar en el conocimiento de la diversidad genética y estructura de poblaciones de *O. cumana*, tanto sobre girasol como sobre especies silvestres. Asimismo, se han desarrollado y caracterizado por primera vez marcadores moleculares específicos de esta especie que ya están siendo empleados por otros grupos de investigación que trabajan sobre esta especie. Los tres trabajos mencionados han dado lugar a artículos ya publicados o en prensa en prestigiosas revistas internacionales como son Weed Research y The Scientific World Journal. Los Directores consideramos que se han cumplido con creces los objetivos propuestos y que el desarrollo de Tesis ha sido plenamente satisfactorio.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de Julio de 2014

Firma del/de los director/es

Fdo.: Dra. Begoña Pérez Vich Fdo.: Dr. Leonardo Velasco Varo

TÍTULO DE LA TESIS: Estudios moleculares en jopo de girasol (*Orobanche cumana* Wallr.) / Molecular studies on sunflower broomrape (*Orobanche cumana* Wallr.)

DOCTORANDO/A: Rocío Pineda Martos

ESCRITO RAZONADO DEL RESPONSABLE DE LA LÍNEA DE INVESTIGACIÓN

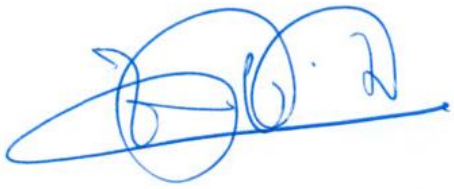
(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca a la Universidad de Córdoba).

Como tutor y responsable de la línea de investigación 'Mejora Genética Vegetal' ratifico el informe presentado por los directores de la tesis.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 11 de julio de 2014

Firma del responsable de línea de investigación



Fdo.: Juan Gil Ligero

A mis padres y a mi hermana,
con cariño y gratitud.

Agradecimientos
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Mi reconocimiento al Consejo Superior de Investigaciones Científicas (CSIC), al Instituto de Agricultura Sostenible (IAS) y al Fondo Social Europeo, por considerarme beneficiaria de una de las ayudas predoctorales del Programa "Junta para la Ampliación de Estudios" (Programa JAE Predoctoral, JAEPre_08_00370), que ha permitido mi formación investigadora en una de las mayores instituciones de investigación de la Unión Europea.

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Un gracias muy especial para el Prof. Dr. José María Fernández Martínez, con su talento y amorosa dedicación inspiradora.

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My gratefulness to the International Parasitic Plant Society (IPPS) for giving me one of its grants (Student Subsidies WCPP, 2009) to attend the Tenth World Congress on Parasitic Plants (Kusadasi, Turkey).

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Index

01 Resumen

> > >

Los jopos (*Orobanche* spp. y *Phelipanche* spp.) son un grupo de aproximadamente 170 especies de plantas holoparásitas distribuidas principalmente en el Hemisferio Norte. A pesar de que la mayor parte de *Orobanche* spp. sólo parasitan plantas silvestres, algunas de ellas se han convertido en malas hierbas nocivas en un rango variable de cultivos. *Orobanche cumana* Wallr. se distribuye de forma natural desde Asia Central hasta el sudeste de Europa, dónde parasita especies silvestres de la familia Asteraceae. La costa del Mar Negro en el este de Bulgaria es una de las principales áreas naturales de distribución de *O. cumana*, dónde esta especie se encuentra principalmente parasitando *Artemisia maritima* L. Esta especie, conocida como jopo del girasol (*Helianthus annuus* L.), es también una importante mala hierba parásita de este cultivo, dónde fue observada por primera vez parasitando girasol en Rusia en la década de 1890. *Orobanche cumana* está presente en los cultivos de girasol en muchos países de todo el mundo, especialmente en el centro y este de Europa, España, Turquía, Israel, Rusia, Ucrania, Irán, Kazajstán, China, Francia y Túnez. En la mayoría de estas áreas, *O. cumana* causa severas pérdidas de rendimiento en los cultivos de girasol. *Orobanche cumana* se encuentra en España como una especie alóctona que parasita al girasol cultivado exclusivamente, a diferencia de la especie estrechamente relacionada *O. cernua* L., que es una especie autóctona que sólo parasita huéspedes silvestres de la familia Asteraceae, principalmente *Artemisia* spp. Durante muchos años, *O. cumana* se distribuyó en el Valle del Guadalquivir (Andalucía, Sur de España) y en la provincia de Cuenca (Catilla-La Mancha, Centro de España), pero en los últimos años, se ha extendido a otras áreas de cultivo de ambas zonas y en nuevos campos de otras regiones como Castilla y León (Norte de España). La información sobre la diversidad genética, la dinámica de poblaciones, el sistema de reproducción, el flujo genético y la genética de la virulencia en *O. cumana* es escasa, especialmente en relación con el análisis molecular entre y dentro de las poblaciones, debido a la falta de marcadores moleculares adecuados para este tipo de estudios. Sólo unos pocos estudios moleculares se han llevado a cabo en *O. cumana*, limitados a la evaluación de la diversidad genética a través de isoenzimas o marcadores RAPD. Además, sólo algunos estudios se han descrito sobre las interacciones genéticas entre formas silvestres y de malas hierbas de especies de plantas parásitas. La aplicación de estos estudios es importante para el desarrollo de estrategias de mejora para el control a largo plazo que implica la resistencia genética en girasol. Por otra parte, el conocimiento de las interacciones entre los genotipos silvestres y de malas hierbas de las plantas parásitas es importante porque la vegetación silvestre puede jugar un papel como reservorio de diversidad genética para la superación de los mecanismos de resistencia genética en los cultivos huéspedes. Pero, por otro lado, la evolución de la virulencia en las poblaciones de malas hierbas parásitas también puede tener un impacto sobre la distribución de las especies en la naturaleza. Unido a esto, se necesitan marcadores alternativos como marcadores microsatélites (SSRs) que son reproducibles, no influenciados por el ambiente, multialélicos y codominantes, para permitir análisis más efectivos en *O. cumana*. Dado que los estudios sobre la variabilidad genética de poblaciones de *O. cumana* se han basado en un número restringido de poblaciones recolectadas en girasol y tipos de marcadores, una evaluación a mayor escala podría contribuir a una mejor comprensión de la estructura genética y dinámica de la especie. Tampoco hay información sobre la estructura genética de poblaciones de *O. cumana* que parasitan especies silvestres y su relación con las poblaciones de malas hierbas parásitas en zonas en las que conviven, y su virulencia en girasol. Como los recursos disponibles para estudios moleculares con

marcadores SSR son limitados en *O. cumana* y en otras *Orobanche* spp., hemos desarrollado y caracterizado un conjunto de pares de cebadores microsatélites para jopo de girasol. Cuatro mil doscientas secuencias candidatas para microsatélites se obtuvieron de *O. cumana* usando Secuenciación de Nueva Generación (454 GS-FLX Titanium), de las que 298 pares de cebadores SSR fueron diseñados y 217 de ellos utilizados para validación. Setenta y nueve cebadores SSR fueron reproducibles con amplificaciones de alta calidad y del tamaño esperado siendo polimórficos entre 18 poblaciones de *O. cumana* de diferentes lugares geográficos (España, Turquía, Bulgaria) y huéspedes (girasol, huéspedes silvestres de la familia Asteraceae). El número de alelos por locus varió de dos a diez, con un valor medio de contenido de información polimórfica (PIC) de 0,37. Los marcadores SSR de *O. cumana* fueron altamente transferibles a la especie estrechamente relacionada *O. cernua*. Los marcadores SSR mostraron un alto poder resolutivo; el análisis de agrupamiento UPGMA permitió la clasificación adecuada de las muestras de *Orobanche* spp. en especies (*O. cumana* y *O. cernua*), origen geográfico y planta huésped. La colección funcional de marcadores SSR adquiridos en la presente Tesis Doctoral constituye una valiosa herramienta para los análisis genéticos en *O. cumana* y las especies relacionadas y contribuirá en los conocimientos sobre la biología y la genética de esta mala hierba parásita.

Se utilizó un conjunto de marcadores SSR de esta colección para evaluar la diversidad genética en un grupo elevado de poblaciones de *O. cumana* de España. El análisis clúster sobre el conjunto de 50 poblaciones usando 15 marcadores microsatélites reveló la existencia de dos grupos genéticos separados, uno en la provincia de Cuenca y otro en el Valle del Guadalquivir. Dentro de cada grupo genético, la variabilidad inter- e intra-poblacional fue extremadamente baja. Esta estructura poblacional probablemente refleja un efecto fundador, con los dos grupos de poblaciones genéticamente diferentes derivados de eventos de origen separados. Diferentes razas se encuentran dentro de un mismo grupo genético, lo que sugiere que las razas actuales podrían haber evolucionado a través de mutación a partir de un fondo genético común. La mayor parte de las poblaciones de las nuevas áreas eran idénticas a las poblaciones del Valle del Guadalquivir. Sólo unas pocas poblaciones mostraron mayor variación intrapoblacional. En estos casos, nuestros resultados sugieren la coexistencia de ambos grupos genéticos dentro de la misma población, así como la aparición de recombinación genética entre ellos.

La diversidad genética, la estructura poblacional, y la capacidad de parasitar girasol de poblaciones de *O. cumana* que crecen en plantas silvestres en la costa del Mar Negro de Bulgaria, así como su relación con las poblaciones de malas hierbas parásitas de girasol también se investigó con el mismo conjunto de SSRs. Tejido fresco de ocho poblaciones de *O. cumana* y semillas maduras de cuatro de estas poblaciones fueron recogidas *in situ* sobre huéspedes silvestres. Se identificaron dos principales grupos genéticos en las poblaciones Búlgaras, con la mayor parte de las poblaciones con características intermedias. Experimentos de inoculación cruzada revelaron que las poblaciones de *O. cumana* recogidas en especies silvestres poseían capacidad similar para parasitar girasol que las recogidas sobre este cultivo. La ocurrencia de flujo genético bidireccional entre poblaciones de *O. cumana* silvestres y de malas hierbas en el este de Bulgaria, puede tener un impacto sobre las poblaciones silvestres ya que nuevas razas fisiológicas emergen permanentemente en las poblaciones de malas hierbas parásitas. Además, la variabilidad genética de las poblaciones silvestres puede favorecer la capacidad de las poblaciones de malas hierbas para superar los mecanismos de resistencia en girasol.

02 Abstract

Broomrapes (*Orobanche* spp. and *Phelipanche* spp.) are a group of around 170 holoparasitic plant species mainly distributed in the Northern Hemisphere. Even though most of the *Orobanche* spp. only parasitize wild plants, some of them have become noxious weeds on a variable range of cultivated hosts. *Orobanche cumana* Wallr. is naturally distributed from Central Asia to Southeastern Europe, where it parasitizes wild Asteraceae species. The Black Sea coast in Eastern Bulgaria is one of the main natural distribution areas for *O. cumana*, where this species is mainly found parasitizing *Artemisia maritima* L. This species, known as sunflower broomrape, is also an important parasitic weed of sunflower (*Helianthus annuus* L.), where it was first observed parasitizing this crop in Russia in the 1890s. *Orobanche cumana* is present in sunflower crops in many countries around the world, especially in Central and Eastern Europe, Spain, Turkey, Israel, Russia, Ukraine, Iran, Kazakhstan, China, France and Tunisia. In most of these areas, *O. cumana* causes severe yield losses in sunflower crops. *Orobanche cumana* is found in Spain as an allochthonous species parasitizing exclusively cultivated sunflower, in contrast to the closely related species *O. cernua* L., which is an autochthonous species that only parasitizes wild Asteraceae hosts, mainly *Artemisia* spp. For many years, *O. cumana* was distributed in the Guadalquivir Valley (Andalucía, Southern Spain) and Cuenca province (Castilla-La Mancha, Central Spain), but in recent years, it has spread to other areas of these both cultivation regions and new fields in other regions such as Castilla y León (Northern Spain). Information on genetic diversity, population dynamics, mating system, gene flow and virulence genetics in *O. cumana* is scarce, particularly concerning molecular analysis among and within populations, due to the lack of suitable molecular markers for such studies. Only a few molecular studies have been conducted in *O. cumana*, restricted to evaluating genetic diversity through isoenzymes or RAPD markers. Furthermore, only some studies have been described on genetic interactions between wild and weedy forms of parasitic plant species. The application of these studies is important for the development of long-term breeding strategies for control involving genetic resistance in sunflower. Moreover, knowledge about interactions between wild and weedy genotypes of parasites is significant because wild vegetation may play a role as reservoir of genetic diversity for overcoming genetic resistance mechanisms in the host crops. But on the other hand, evolution of virulence in weedy populations may also have an impact on the distribution of the species in the wild. 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 analyses in *O. cumana*. Since studies on genetic variability of *O. cumana* populations have been based on restricted numbers of weedy populations collected on sunflower and marker types, a larger-scale evaluation would contribute to a better understanding of genetic structure and dynamics of this species. There is also no information on the population structure of *O. cumana* populations parasitizing wild species and their genetic relationship with weedy populations in areas where they co-exist, and their virulence on sunflower. As very limited SSR resources are available for molecular research in *O. cumana* and other *Orobanche* spp., we developed and characterized a collection of SSR primer pairs for sunflower broomrape. Four thousand two hundred SSR-containing candidate sequences were obtained from *O. cumana* using Next Generation Sequencing (454 GS-FLX Titanium), from which 298 SSR primer pairs were designed and 217 of them used for validation. Seventy nine SSR primers produced reproducible, high quality amplicons of the expected size that were polymorphic among 18 *O.*

cumana populations from different geographical locations (Spain, Turkey, Bulgaria) and hosts (sunflower, wild hosts from the Asteraceae family). The number of alleles per locus ranged from two to ten, with an average Polymorphism Information Content (PIC) value of 0.37. The *O. cumana* SSR markers were highly transferable to the closely related species *O. cernua*. SSR markers showed high resolving power; UPGMA cluster analysis allowed proper classification of *Orobanche* spp. samples into species (*O. cumana* and *O. cernua*), geographical origin and host. The functional collection of SSR markers reported in the present Doctoral Thesis constitutes a valuable tool for genetic analyses in *O. cumana* and related species and will contribute insights into the biology and genetics of this parasitic weed.

A set of SSR markers from this collection was used to evaluate the genetic diversity in a large set of *O. cumana* populations from Spain. Cluster analysis on a set of 50 populations using 15 microsatellite markers revealed the existence of two distant gene pools, one in Cuenca province and another one in the Guadalquivir Valley. Within each gene pool, both inter- and intra-population variability were extremely low. This population structure probably reflects a founder effect, with the two genetically distant gene pools deriving from separate introduction events. Different races occurred within the same gene pool, suggesting that current races might have evolved through mutation from a common genetic background. Most of the populations from new areas were identical to the populations from the Guadalquivir Valley. Only a few populations showed larger intrapopulation variation. In these cases, our results suggested the co-existence of both gene pools within the same population, as well as the occurrence of genetic recombination between them.

The genetic diversity, population structure, and ability to parasitize sunflower of *O. cumana* populations growing on wild plants in the Black Sea coast of Bulgaria, as well as their relationship with weedy populations parasitizing sunflower was also investigated with the same set of SSRs. Fresh tissue of eight *O. cumana* populations and mature seeds of four of them were collected *in situ* on wild hosts. Two main gene pools were identified in Bulgarian populations, with most of the populations having intermediate characteristics. Cross inoculation experiments revealed that *O. cumana* populations collected on wild species possessed similar ability to parasitize sunflower to those collected on sunflower. An effective genetic exchange between populations parasitizing sunflower crops and those parasitizing wild species in Eastern Bulgaria was observed. The occurrence of bidirectional gene flow between wild and weedy *O. cumana* populations in Eastern Bulgaria, may have an impact on wild populations; as new physiological races continuously emerge in weedy populations. Also, genetic variability of wild populations may favor the ability of weedy populations to overcome sunflower resistance mechanisms.

03 Introduction

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*Sunflower (*Helianthus annuus *L.) is the major annual oilseed crop in Spain, being the sunflower broomrape (*Orobanche cumana *Wallr.) an important limitation of crop yield.* Orobanche cumana *is a holoparasitic angiosperm plant, devoid of chlorophyll and photosynthetic activity, which penetrates the vascular tissues of sunflower roots, getting both water and nutrients needed to complete its life cycle. Breeding programs for broomrape resistance led to the development of resistant hybrids but also to new* O. cumana *races that surpassed this resistance. Races D and E were predominant till the middle 1990s, and they were satisfactorily controlled by the resistance gene* Or5*, widely used in commercial hybrids. Sunflower broomrape populations overcoming* Or5 *resistance were detected in 1995 in Spain and shortly after in Romania, Turkey, and several other countries. Currently, reports on races named as F, G, and H are common in several countries and they are rapidly spreading to new areas. It is unknown whether the rapid emergence of more virulent populations of* O. cumana *is due to high genetic diversity that responds to a strong selection pressure, high frequency of mutation in relatively homogeneous populations, or introduction of new races by importing and planting sunflower seeds contaminated by broomrape. A comprehensive knowledge of population and racial structure of the parasite at phenotypic and molecular levels is necessary for the design of new and sustainable control strategies.*

3.1. Origin and Production of Cultivated Sunflower

Cultivated sunflower (*H. annuus*) is a member of the family Compositae (Asteraceae). Prior to the arrival of the European explorers to the New World, the progenitor of cultivated sunflower, the wild *H. annuus*, was restricted to the Southern United States (Heiser, 1978). It was extended across North America and Mexico accompanying other well-established crops such as corn (*Zea mays* L.), beans (*Phaseolus vulgaris* L.) and squash (*Cucurbita pepo* L.) (Putt, 1997). Wild *H. annuus* was used for food by the Native American Indians tribes (Kindscher, 1987) and, due to its association with humans, it became a camp-following weed that was introduced into the central part of the U.S., where it was domesticated and carried to the east and southwest (Harter *et al*., 2004; Heiser *et al*., 1969). The domesticated sunflower was introduced from North America into Europe by the early Spanish explorers in 1510 (Putt, 1977, 1978), where it initially gained popularity as a garden ornamental plant. The agronomic development of sunflower as an oilseed crop and for use as edible achenes (confectionary types) took place in Russia, where a number of landraces had been developed by the late 1800s. Initial selection emphasis was given to early maturity, disease and pest resistance, and high seed oil content. Sunflower was reintroduced from Russia to North America in the latter part of the 19th century (Putt, 1997; Rieseberg and Seiler, 1990).

Sunflower seed is the fourth most important source of vegetable oil in world trade at present – after soybean, oil-palm and rapeseed/canola –, with an average annual production around 36 million tonnes, and cultivated acreage over 25 million hectares, mainly concentrated in the Russian Federation, Ukraine, India, and Argentina, which totalize approximately 75% of sunflower world acreage [FAO, 2013 (FAO Statistical Yearbook, 2013; FAO Trade and Markets Division, 2013)]. Rapeseed is the most widely grown oilseed in the European Union, accounting for over 60% of the total area of oilseeds and over 70% of total production. Globally, the EU is the third largest producer of sunflower behind Ukraine and Russia (MAGRAMA, 2013). Major European Union producers of sunflower seed are France, Romania, Bulgaria, Hungary and Spain. Production is generally vulnerable to drought, especially since larger producer countries/regions are in Southern Europe. However, better genetics and newer production technologies are helping to stabilize yields. The EU is a net importer of sunflower oil, mainly used for food purposes, and not for biodiesel. The largest EU exporters of sunflower oil are Spain, Hungary, Bulgaria and Romania. These countries are also the largest crushers in the EU (USDA, 2013). The sunflower is the main annual oilseed crop in Spain, with a percentage higher that 90%, followed by rapeseed which in recent years has experienced a significant increase and soybean with an average of fewer than 500 hectares. Cultivated sunflower area in 2013/2014 has increased by 14% compared to the previous season – around 849 thousands of hectares (2013) compared to 761 thousands of hectares (2012) –, while the increase in production has been above 60% (about 1029.3 thousands tonnes) because yield was not affected by drought as the previous season. Sunflower acreage in Spain during the last five seasons was approximately 778 thousands of hectares with and average production of around 860 thousands tonnes and mean yield value of about 1.11 tonnes/hectare.

3.2. Parasitic Angiosperms: Impact of Parasitism in Agricultural Ecosystems

Plants are also parasitized by other plants. Indeed, parasitism among plants seems to have evolved many times during angiosperm evolution (Walters, 2010). It has been estimated that about 1% of angiosperms, nearly 3000 species in total, are parasitic on other plants (Parker and Riches, 1993). Families – recently revised based on molecular studies – are treated as *sensu lato* (s.l.) (Nickrent, 2010). Parasitic angiosperm families are arranged in about 12 orders – indicating that parasitism has evolved independently several times (Heide-Jørgensen, 2013) –, and living in diverse habitats ranging from tropical forest to artic islands (Musselman and Press, 1995; Nickrent, 2002b). The parasitic plants have suffered a drastic adaptation as some lack leaves, stems, roots and the capacity for photosynthesis (Young *et al*., 1999).

Among the parasitic angiosperms, there are species that parasitize crop plants and can be devastating for the agricultural systems (Nickrent, 2002b; Walters, 2010). Parasitic plants of commercial importance include six genera belonging to three families: the genus *Cuscuta* within the Convolvulaceae family, genera *Orobanche*, *Phelipanche* and *Striga* belonging to the Orobanchaceae family, and genera *Arceuthobium* and *Viscum*, both of the Viscaceae family (Nickrent, 2002b; Schneeweiss, 2013). Parasitic plants can exert a considerable impact on plant communities (Press and Phoenix, 2005), and the assortment of host resources to parasitic plants can have large effects on host growth and reproductive output (Walters, 2010). Some of the most damaging parasitic plants belong to the genera *Striga* (witchweeds) and *Orobanche/Phelipanche* (broomrapes), both of them of the Orobanchaceae family (Musselman, 1980). The genus *Arceuthobium* causes significant losses in forest species in North America and the genus *Striga* mainly affects maize, millets [*Pennisetum glaucum* (L.) R. Br.], and sorghum [*Sorghum bicolor* (L.) Moench] in Central Africa and Asia. Yield losses in cereals parasitized by *Striga* can reach 100%, and fields can be so heavily invaded that they are abandoned by farmers (Berner *et al*., 1995). In the Mediterranean area, the genera *Orobanche*, *Cuscuta* and *Viscum* are the most significant, especially the first two (Parker and Riches, 1993).

3.2.1. Host Recognition by Parasitic Plants and Modes of Parasitic Nutrition

The identification of appropriate host roots is most critical for obligate parasites like *Orobanche* and *Striga* and host recognition systems are most advanced in these genera (Yoder, 1999). Parasitism can be a great lifestyle in adapted organisms (Mach, 2010). It is a gradual process that begins with the seed germination of parasitic plant and continues with the attachment and root penetration of the host plant establishing vascular connections. These events involve the recognition of signs of development and coordination of genetic processes between the parasite and the host (Estabrook and Yoder, 1998; Joel *et al*., 1995; Tomilov *et al*., 2006). Parasitic angiosperms live in intimate associations with their host plants, and by their very definition fulfill at least some of their nutritional requirements by directly invading other plants for water and nutrients (Kuijt, 1969). In some species, host-plant identification and invasion is coordinated through chemical signaling between the host and parasite. Most especially, parasitic species of Orobanchaceae use molecules made by the host root to trigger various developmental procedures including seed germination, host attachment and invasion, and the establishment of physiological conduits through which nutrients are transferred from the host to the parasite (Press and Graves, 1995).

Parasitic plants obtain some or all their nutritional requirements from a host plant. They develop a special organ known as the haustorium, which invades host plant

tissues and serves to withdraw water and nutrients (Joel, 2013). There is considerable diversity in the extent to which parasitic angiosperms rely on the host for growth. Some have functional roots and can therefore take up inorganic nutrients from the soil, while others, have nothing that neither resembles a root nor functions as one. There is also considerable variation in the extent to which parasitic plants rely on the host for photoassimilates. Thus, some parasitic plants are able to photosynthesize and can grow with a carbon supply from the host, while others, lack or possess a very low photosynthetic capacity and are unable to grow without a carbohydrate supply from the host (Hibberd and Jeschke, 2001).

According to their mode of nutrition, parasitic plants can be holoparasitic or hemiparasitic. The first ones, are those completely dependent on their hosts because of their lack of chlorophyll, therefore are unable to carry out any photosynthetic activity, so they cannot synthesize their nutrients (Kuijt, 1969; Mohamed *et al.*, 2006; Nickrent, 2002b; Stewart and Press, 1990). The haustoria of holoparasitic plants are attached to the sieve tubes of the phloem – tissue that carries assimilated products –, and are the most extreme manifestation of parasitism (Nickrent, 2002b; Sosa and Tressens, 2000). In contrast, the hemiparasitic plants have chlorophyll at least during some stage of their life cycle, and partially synthesize the elements necessary for nutrition (Mohamed *et al*., 2006). The hemiparasitic plants can be facultative or obligate parasites. Facultative hemiparasites do not require a host to complete their life cycle and flower and fruit in preparasitic stage, in the absence of host plants. Obligate hemiparasites should be attached to the host plant to complete their cycle. The hemiparasitic plants are usually set to the xylem tissue that carries water and inorganic nutrients (Nickrent, 2002b; Sosa and Tressens, 2000). The genera *Arceuthobium*, *Striga* and *Viscum* are clear representatives of the hemiparasitism, while the genera *Cuscuta* and *Orobanche* are considered strictly holoparasitic (García, 2002).

3.2.2. The Haustorium as the Key Organ of Absorption in Parasitic Plants

Host contact triggers the development of a specialized invasive feeding structure called the haustorium, as mentioned previously. It is a characteristic organ in parasitic plants – both morphologically and physiologically –, playing a role as a bridge between the parasite and the host (Joel, 2013; Kuijt, 1969). Host roots can be sensed either by direct contact or also by perception of diffusible phenolic compounds, specifically quinones, from the host. The haustorium-inducing factors (HIFs) (Thorogood and Hiscock, 2010) sensed by the parasite include phenolic compounds derived from lignin, such as the quinone 2,6 dimethoxy-1,4-benzoquinone (DMBQ) (Mach, 2010; Westwood, 2010). Investigations on haustorium development have been greatly facilitated by the early observations that these structures can be induced *in vitro* by applying host root exudates

to the roots of aseptically grown parasites (Riopel and Musselman, 1979). In the first stage of growth, it is presented as prominences called 'sterile haustoria' which penetrate into the host plant to reach the conducting tissues. Two main types of haustoria are recognized (Heide-Jørgensen, 2013; Kuijt, 1969). The haustorium is primary or terminal when it originates from the radicle and develops directly from the apex of the primary root, and secondary or lateral when is formed from other tissues and develops laterally on young lateral or adventitious roots (Heide-Jørgensen, 2013; Nickrent, 2002a). Most species from the Orobanchaceae family have numerous lateral haustoria and many hosts, but some advanced species such as *Striga hermonthica* (Del.) Benth. and some holoparasites have only a terminal haustorium (Dörr, 1997; Heide-Jørgensen, 2013; Kuijt, 1969).

Through haustorium, nutrients and water are transferred from the host plant to the parasite, and also facilitates all hormonal interactions between the two organisms (Press and Graves, 1995). The haustorium penetrates the epidermis of the host root or stem, growing between the cells to reach the xylem and/or phloem vessels. In the case of hemiparasites, xylem vessels are entered via the pits within their walls; cells in the center of the haustorium then differentiate into xylem vessels to ensure a continuous connection between host and parasite. Haustoria cells typically show high metabolic activity, with numerous mitochondria, which is thought to help water and nutrient uptake (Wood, 2013). Although it has been suggested that haustorium cells mechanically force the cells of the host tissues, Losner-Goshen *et al*. (1998) studied the possible involvement of enzymes in the process of penetration of invading cells from *Orobanche* species. The enzyme pectin methyl esterase (PME) was detected in the cytoplasm and cell membranes of the invading cells of *O. cumana* and *Phelipanche aegyptiaca* (Pers.) Pomel, as well as in the apoplast of sunflower and tomato (*Lycopersicon esculentum* Mill.), respectively revealing the presence and activity of pectolytic enzymes in the area of haustorium infection.

3.3. Orobanchaceae, the Parasitic Broomrape and Witchweed Family

Orobanchaceae is by far the largest family of parasitic plants after inclusion of the hemiparasitic root parasites, which were earlier placed in Scrophulariaceae (Bennett and Mathews, 2006; Olmstead *et al*., 2001; Young *et al*., 1999). The Orobanchaceae family has been regarded traditionally as a related group to Scrophulariaceae. Numerous authors as Boeshore (1920), Cronquist (1981), Hutchinson (1969), Kuijt (1969), Rix and Webb (1972) and Weber (1981), emphasized the close relationship between hemiparasitic and holoparasitic Scrophulariaceae and holoparasitic Orobanchaceae; based on the continuity of their morphological characters and arguing that they form part of a linear evolutionary series which starts in the hemiparasitism of

Scrophulariaceae, passes through holoparasitism of Scrophulariaceae, and culminates in the holoparasitism of Orobanchaceae. Subsequent phylogenetic studies support a reclassification of the Orobanchaceae and Scrophulariaceae families. Thus, Young *et al*. (1999) noted that the Orobanchaceae, in the traditional sense, are monophyletic but not the holoparasitic taxa of both families. That study did not support the hypothesis of a single linear evolution from hemiparasitism to holoparasitism, but the acquisition of this condition from several independent sources. Young *et al*. (1999) mentioned a new systematic proposal that affect these two traditional families and comprise at least five new or redefined families (Olmstead *et al*., 2001).

Orobanchaceae contains several genera, such as *Striga*, *Alectra*, and *Orobanche*, which are some of the most serious agricultural parasites (Gevezova *et al*., 2012; Riches and Parker, 1995; Rubiales and Heide-Jørgensen, 2011). Currently 89 genera, containing ca. 2061 species, are recognized in Orobanchaceae (Gevezova *et al*., 2012; Nickrent, 2006). The family is represented in all climatic zones and on all continents except Antarctica. Orobanchaceae, as redefined by Young *et al*. (1999), is a morphologically diverse family of predominantly herbaceous, parasitic plants. All species are annual or perennial herbs. In general, parasitic plants of crops behave as annual while species that parasitize wild plants are generally perennial (Pujadas-Salvà, 2002; Schneeweiss, 2007). Flowers are bilaterally symmetrical and most pollinated by insects. Some are self-fertilizing or facultative selfers (Heide-Jørgensen, 2013; Satovic *et al*., 2009; Teryokhin *et al*., 1993). *Orobanche* spp. have been traditionally classified into two main groups according to their matting system, grouped as species pollinated by insects – particularly bumblebees and bees –, or self-pollinating species (Musselman *et al*., 1981). Self-parasitism, occurring when haustoria form between different parts of the same plant, is also known, mainly in *Cuscuta*, *Cassytha*, and the Orobanchaceae.

3.3.1. *Orobanche* L. *sensu lato*, the Genus of the Broomrapes

Orobanche L. s.l. (including *Phelipanche* Pomel) is the largest genus amongst the holoparasitic members of Orobanchaceae and comprises approximately 170 species (Gevezova *et al*., 2012). It is mainly distributed throughout the subtropical and temperate regions of the Northern Hemisphere, with the Mediterranean region being one of the most important centers of its diversity (Mohamed *et al*., 2006; Piwowarczyk, 2012; Plaza *et al*., 2004). Taxonomy of the genus *Orobanche* is difficult and more problematic than that of most other taxa. This is due to the typical morphological diversity within populations and – because of holoparasitism – the number of characters of taxonomic utility is reduced; no development of photosynthetic function, leaves are bracts and small, and have a root system reduced to a mass of short and thick roots. Besides, the changing of diagnostic characters on dried plant material and lack of adequate field notes on herbarium sheets are among other causes such as the distinct

colors of the *Orobanche* species in the field turn into a uniform brown after pressing (Zare and Dönmez, 2013). Moreover, according to authors such as Musselman (1994), the host plant can influence the morphology of the parasitic plant. Problematic identification keys in the floras and insufficient distribution data have led to a high number of misidentifications. However, the identification of the species of the genus *Orobanche* is particularly desirable because it is important to determine the types of host plants mainly crops, which they may parasitize. Morphological characteristics (Musselman, 1986) such as of seeds – to be observed under the microscope due to their tiny size –, were used in most taxonomic studies conducted on the genus *Orobanche* (Abu Sbaih and Jury, 1994; Ashworth, 1976; Joel, 1987a; Plaza *et al*., 2004; Teryokhin *et al*., 1993). The morphological characteristics of the seed make it easy to distinguish sections within the genus (Abu Sbaih and Jury, 1994; Plaza *et al*., 2004). However, the classification of species within each section is more complex (Portnoy *et al*., 1997).

The genus *Orobanche* was initially subdivided into four sections – *Gymnocaulis*, *Myzorrhiza*, *Trionychon* and *Orobanche* (*Osproleon*) (Beck-von-Mannagetta, 1930; Gevezova *et al*., 2012) –, of which sections *Trionychon* and *Orobanche* include the species of major economic importance (Pieterse, 1979). Beck-von-Mannagetta (1930) distinguished two sections – *Orobanche* or *Osproleon* and *Trionychon* Wallr. – among the *Orobanche* from the Old World. Some of them are damaging parasitic plants in agricultural systems on a more or less restricted spectrum of hosts causing yield losses close to 100% under severe infestations (Bernhard *et al*., 1998; Bülbül *et al*., 2009). An important number of *Orobanche* species showing a selective parasitism to parasitize only one species – *O. cumana* parasitizes exclusively sunflower crops, thus commonly named as sunflower broomrape – whilst others parasitize species belonging to a single genus – *O. latisquama* (F.W. Schultz) Batt. parasitizes rosemary (Lamiaceae) and *O. cernua* L. parasitizes different species of *Artemisia* and *Launea* –. Also, there are parasitic plants that have a broad host range belonging to several families, such as *O. minor* Smith., which is parasite of various legume species of the genera *Lotus*, *Trifolium* and *Vicia*; Umbel plants of the genera *Daucus* and *Foeniculum*; the genus *Plantago*, and Aster plants of the genera *Gazania*, *Hyoseris*, *Hypochoeris* and *Picris* (Pujadas-Salvà, 2002). The two sections *Trionychon* and *Orobanche* have representatives in the Iberian Peninsula and Balearic Islands and are two well-defined taxonomic sets. The differences amongst the two sections are branched stems, bracteolate flowers, whole and campanulate calyx, corolla blue or purple, white anthers and stigma preferably white of the *Trionychon* section compared with simple stems, flowers not bracteolate, calyx divided into two lateral segments, white, yellow, brown, amethyst or red corolla, yellow, brown or gray anthers, and yellow, orange, red or purple stigma color commonly found in the *Orobanche* section (Pujadas-Salvà, 2002). According to Joel (1987a) and Musselman (1986), the most important species of parasitic plants that attack crops are *P. aegyptiaca* and *P. ramosa* (L.) Pomel (*Trionychon* Wallr.), and *O.*

cernua, *O. crenata* Forsk., and *O. cumana* (*Osproleon* Wallr.). *Phelipanche aegyptiaca* possesses ability to parasitize a broad spectrum of hosts and *P. ramosa* is common on rapeseed (*Brassica napus* L.) plantations and many Solanaceae crops. *Orobanche cernua* is mainly found on carrot (*Daucus carota* L.), tomato, and tobacco (*Nicotiana tabacum* L.) while *O. crenata* mainly parasitizes legume crops and safflower (*Carthamus tinctorius* L.) (Mohamed *et al*., 2006; Parker, 2013; Pujadas-Salvà, 2002).

3.4. Sunflower Broomrape (*O. cumana*)

Sunflower broomrape is found in the wild parasitizing wild Asteraceae species, mainly *Artemisia* spp., from Central Asia to Southeastern Europe (Pujadas-Salvà and Velasco, 2000; Venkov and Bozoukov, 1994). According to Morozov (1947) the first reports of broomrape in sunflower hailed from Saratov Oblast in Russia dating back to the 1890s, and referenced that the first sunflower resistant cultivars were developed by Plachek (1918) at the Saratov breeding station (Škorić *et al*., 2010). Contrasting the other *Orobanche* and *Phelipanche* species parasites of crops – for which genetic resistance in the host is of quantitative nature or horizontal –, genetic resistance to *O. cumana* in sunflower is in most cases qualitative or vertical (Pérez-Vich *et al*., 2013). For this reason, *O. cumana* populations are usually classified into physiological races (Vrânceanu *et al*., 1980), which is not the case for the other *Orobanche* and *Phelipanche* spp. (Fernández-Martínez *et al*., 2014). *Orobanche cumana* has been traditionally classed within the self-pollinating species, mainly due to its flower morphology, with bent tubular corollas having small lower lips that do not facilitate pollinator landing (Satovic *et al*., 2009), as well as to the existence of low intrapopulation genetic diversity (Gagne *et al*., 1998). However, Rodríguez-Ojeda *et al*. (2013) suggested that the species is not strictly self-pollinated and some extent of cross-pollination should be expected.

3.4.1. The Treatment of *O. cumana* and its Closely Related *O. cernua* as Separate Species

For many years, there has been some confusion in the literature about the scientific name of sunflower broomrape, with *O. cumana* and *O. cernua* being used indistinctly. The reason for such confusion can be traced back to several classical taxonomic studies in which *O. cumana* was treated as an intraspecific taxon of *O. cernua* (Beck-von-Mannagetta, 1930; Rechinger, 1943). *Orobanche cernua* was described by Carl Linnaeus from materials collected by Pehr Löfling near Aranjuez, Central Spain (Loefling, 1758). It parasitizes different species of the Asteraceae family, being frequently found in plants of the genus *Artemisia*. This species is distributed mainly from the Mediterranean region to Central Asia (Beck-von-Mannagetta, 1930; Reuter,

1847). Pujadas-Salvà and Velasco (2000), following the International Code of Botanical Nomenclature (Greuter, 1988), named the parasitic plant as *O. cernua* L. instead of *O. cernua* Loefl., following López-González (1990). The description of *O. cumana* was made by Wallroth (1825) in plants collected in the desert regions of Southwestern Asia and Southeastern Europe. Beck-von-Mannagetta (1930) considered this species as a variety of *O. cernua* and proposed Central Asia and Southern Europe as the main distribution areas. *Orobanche cumana* is treated by some authors as a variant of *O. cernua* parasitizing cultivated plants, especially *H. annuus* (Chater and Webb, 1972). This taxon differs from *O. cernua* by its larger size and elongated inflorescence, less dense and with flowers somewhat lighter colored, sometimes whitish. Although the few morphological differences mentioned – that could be related to diversity in hosts –, suggest that is very closely related to *O. cernua*, the distinction between both species based on molecular and comparative studies (Benharrat *et al*., 2002; Katzir *et al*., 1996; Kirilova *et al*., 2014; Paran *et al*., 1997; Pujadas and Thalouran, 1998; Pujadas-Salvà and Velasco, 2000; Román *et al*., 2003), supports their treatment as different species.

3.4.2. Broomrape Consequences in Sunflower Production Areas around the World

Orobanche cumana represents one of the most serious production restraints in many sunflower-producing countries around the world, specifically in Central and Eastern Europe, Spain, Turkey, Russia, Ukraine, Israel, Iran, Kazakhstan, and China (Kaya, 2014a; Škorić *et al*., 2010). Moreover, the sunflower broomrape has spread to additional new regions in most recent years. New infestations are continuously being observed in countries such as Russia (Antonova, 2014; Antonova *et al*., 2013) and Spain (Fernández-Escobar *et al*., 2009), where the parasite has been present for many years, and also in countries where the parasite has not been observed before. The observation of broomrape attacks in France, one of the leading sunflower producing countries in Europe, has been recently reported (Jestin *et al*., 2014; Jouffret and Lecomte, 2010). Furthermore, it has also been lately detected in Tunisia (Amri *et al*., 2012). However, sunflower broomrape is not present in the center of origin of sunflower nor in the sunflower crop areas of North America; and it is also absent in the extended sunflower crop area of Argentina in South America (Cantamutto *et al*., 2012; Miladinović *et al*., 2012). The yield losses caused in sunflower crops by broomrape occurrence differ according to the intensity of the parasite incidence and climatic conditions; a slight manifestation can reduce production between 5 and 20%, a medium degree between 20 and 50% (Domínguez, 1996a), and a strong level of parasitism over 90% (Melero-Vara and Alonso-Arnedo, 1988). In the former Yugoslavia, *O. cumana* caused a decrease in sunflower yield of 33% and, consequently, growing fields declined by about 37% (Mijatović and Stojanović, 1973). In Turkey, due to the problem of broomrape,

sunflower production was reduced by about 50% during the period between 1956 and 1982 (Bülbül *et al*., 1991). In Spain, *O. cumana* is an allochthonous species exclusively found on cultivated sunflower, in contrast to its closely related species *O. cernua*, which is an autochthonous species that only parasitizes wild Compositae hosts, mainly *Artemisia* spp. (Pujadas-Salvà and Velasco, 2000). *Orobanche cumana* was first described in the Iberian Peninsula parasitizing confectionary sunflower 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 y León (Northern Spain), Castilla-La Mancha and Andalucía, causing severe yield losses in sunflower crops (Alonso *et al*., 1996; Castejón-Muñoz *et al*., 1989; Domínguez *et al*., 1996; Fernández-Escobar *et al*., 2009; Fernández-Martínez *et al*., 2012; Molinero-Ruiz *et al*., 2006).

3.4.3. Broomrape – Sunflower Host – Parasite Interactions

The fruit of *O. cumana* is a capsule loculicidally dehiscent in two valves – rarely three – and contains numerous small seeds, alveolate-crested, darkness in color, with fleshy endosperm and tiny embryo (Plaza *et al*., 2004; Pujadas-Salvà, 2002). Each fruitful capsule contains tiny seeds which are of near-microscopic size, from 250 to 380 µm long and from 150 to 240 µm wide (Joel, 1987a,b). Every seed weighs about 1.0 to 2.5 µg and is composed of only 200-300 cells (Castejón *et al*., 1991; Joel, 1987a,b; Škorić, 2012). The capsules where seeds are formed, are characterized for having some fractures that open when ripe, releasing around 1200 to 1500 seeds per capsule. Thus, individual broomrape plants can produce an impressive number of seeds – from 50000 to 500000 –, leading to a speedy increase in the soil seed bank (Gevezova *et al*., 2012; Yang-han, 1981). Most broomrape seeds are buried in the soil to a depth of about 5-10 cm, and can remain dormant and viable for a period of up to 20 years if the existing crop is not a suitable host plant (Melero-Vara and Alonso-Arnedo, 1988; Škorić, 2012; Yang-han, 1981).

Seed germination is dependent on compounds secreted by the host plant, as broomrape seedlings cannot persist in absence of a host root (Louarn *et al*., 2012). It has been widely reported that *Orobanche* spp. germination stimulation requires a conditioning period of several days under adequate environments of suitable temperature and humidity (Chae *et al*., 2003; Louarn *et al*., 2012; Maširević *et al*., 2012; Pieterse, 1979; Press *et al*., 1990). Optimal soil temperature for *Orobanche* spp. germination is between 20 and 25 ºC (Maširević *et al*., 2012; Škorić, 2012). In most *Orobanche* spp., the main host compounds that stimulate germination are strigolactones

(Yoneyama *et al*., 2010). *Orobanche cumana* seeds in the soil germinate in response to natural and specific chemical stimulants exuded from sunflower roots (Matúšová and Bouwmeester, 2006), such as indole-3-acetic acid (IAA) and abscisic acid (ABA) (Slavov *et al*., 2004), as well as dehydrocostus lactone, a guaianolide sesquiterpene lactone (Joel *et al*., 2011; Pérez-de-Luque *et al*., 2000).

Broomrape seeds produce a thin radicle that upon contact with the host root make bigger and stick to it. After germination, the radicle grows towards the host root and forms an attachment structure known as appresorium. Afterwards, the haustorium of the broomrape penetrates the cortex and reaches the vascular cylinder, where it draws water and nutrients necessary for broomrape survival (Parker and Riches, 1993; Rispail *et al*., 2007). Sunflower cambium cells respond by intensifying the division and the attacked part of the root thickens. Then connections between the vascular bundles of sunflower roots and broomrape haustorium are formed, and the parasitic plant is integrated into the physiology of its host, taking nutrients and water (Melero-Vara and Alonso-Arnedo, 1988). The rapid thickening of the nodule involves an accumulation of reserves necessary for broomrape development, which in turn develops into shoot that emerges from the soil about 4-5 weeks after sunflower. Sunflower broomrape develops from one to three shoots per tubercle (Antonova *et al*., 2012). After the growth of the broomrape plant, it blooms and matures – at about the same time as the sunflower –, so the seed produced is available for upcoming years starting a new cycle (Melero-Vara and Alonso-Arnedo, 1988; Rispail *et al*., 2007).

Sunflower broomrape has great capacity of dispersion and mutation. The production from individual broomrape plants of a high number of seeds that are easily dispersed by humans, animals, machinery, wind, irrigation water and through attachment to sunflower seeds (Castejón-Múñoz *et al*., 1989, 1991a; Păcureanu-Joiţa *et al*., 2009b), along with their longevity, contributes to the accumulation of broomrape populations in cropping systems. Furthermore, broomrape seeds can travel long distances due to their dimpled surface that allows float on water (Yang-han, 1981). On the other hand, high seed production appears to be an adaptation designed to increase the chances of finding an appropriate host (Pujadas-Salvà, 2002).

3.4.4. World Race Evolution in Sunflower Broomrape

The *H. annuus* – *O. cumana* parasitic system generally follows the gene-for-gene model (Fernández-Martínez *et al*., 2012, 2014), in which resistance reactions are governed by the interaction of host genes for resistance (*Or* genes) and the corresponding parasite genes for avirulence (*Avr* genes) (Flor, 1971; Pérez-Vich *et al*., 2013). This genetic system determines the occurrence of physiological races of broomrape that are controlled by dominant resistant genes of sunflower (Fernández-Martínez *et al*., 2012, 2014). As a vertical resistance controlled by major genes, the virulence of a given *O.*

cumana population is commonly measured by evaluating susceptibility or resistance reactions on a set of sunflower differential lines, in which the observed traits are incidence and degree of attack, that is, number of emerged shoots per sunflower plant (Fernández-Martínez *et al*., 2012, 2014; Vrânceanu *et al*., 1980). Following this approach, Vrânceanu *et al*. (1980) studied sunflower broomrape populations from Brăila in Romania. They identified five races of *O. cumana*, designated as A to E, with a set of five sunflower differential lines carrying the dominant resistance genes *Or1* to *Or5*, respectively, that provide accumulative resistance to the races.

The first sunflower cultivars resistant to the local broomrape, named as race A, were achieved in Russia in the early twentieth century (Melero-Vara and Alonso-Arnedo, 1988; Vrânceanu *et al*., 1977). Subsequently, those initial cultivars – *Or1* gene carriers – were susceptible to a new more virulent race B, which caused significant crop losses (Škorić, 2012). In the 30s, novel cultivars with the resistance gene *Or2* incorporated were developed. In the mid-1960s, a new race C was identified in Ukraine and Moldova, which was able to parasitize cultivars resistant to race B. Resistance to this new race was developed around ten years later (Alonso, 1998). The occurrence of a race C, probably different than that reported in Moldova and Ukraine, overcoming both race A and race B cultivars, was observed in Bulgaria in 1970 (Škorić, 2012). Races of *O. cumana* capable of parasitizing Peredovik cultivar – which is resistant to races A and B –, also appeared in Spain. Sunflower lines derived from interspecific crosses of *H. annuus* and *H. tuberosus* L., were obtained in 1976 in the former USSR, and were resistant to the new race C. Later studies confirmed the prevalence of races D and E in several countries such as Bulgaria (Shindrova, 1994, 2006), Serbia (Maširević and Medić-Pap, 2009), and Turkey (Bülbül *et al*., 1991). Until the 90s, several races of *O. cumana* were identified in Spain, being the races D and E the most virulent (González-Torres *et al*., 1982; Melero-Vara *et al*., 1989; Refoyo and Fernández, 1994; Saavedra-del-Río *et al*., 1994a,b). New races appeared from the middle 1990s onward in several countries. Initially, all of them were named as race F, although the relationship among the different races of F has not been studied. They have been reported in Bulgaria (Shindrova, 2006), Romania (Păcureanu-Joiţa *et al*., 2004; Raranciuc *et al*., 2006), Turkey (Kaya *et al*., 2004b), Russia (Antonova *et al*., 2013), and Ukraine (Burlov and Burlov, 2010). In 2004, some populations of *O. cumana* collected in Cuenca province in Spain exceeded the resistance of the line P96 – resistant to race F –, suggesting the presence of populations more virulent than race F (Molinero-Ruiz and Melero-Vara, 2004). Populations overcoming resistance sources to race F, named as races G and H, have been identified in most of the above mentioned countries (Antonova, 2014; Antonova *et al*., 2013; Kaya, 2014b; Kaya *et al*., 2009; Păcureanu-Joiţa, 2014; Păcureanu-Joiţa *et al*., 2009a; Raranciuc *et al*., 2006; Shindrova and Penchev, 2012). As mentioned for race F, no comparative studies have been conducted between races G and H reported in different countries (Fernández-Martínez *et al*., 2014).

3.4.5. Sunflower Breeding as a Control Strategy for Broomrape Management

The control of sunflower broomrape – as for other species of *Orobanche* – remains challenging, mainly due to the extraordinary number of seeds produced by the parasitic plant, their longevity and easy dispersion, and the ability of the parasite to overcome resistant mechanisms in the host (Goldwasser and Rodenburg, 2013; Molinero-Ruiz *et al*., 2008). Breeding strategies for broomrape resistance largely depend upon the degree of host resistance and its genetic control (Pérez-Vich *et al*., 2013). Host plant resistance is most effective at protecting yield if it acts early to counter the parasitic association (Frost *et al*., 1997). Resistance to *Orobanche* occurs at both the pre- and post-attachment stages. The traditional method for broomrape management is genetic control by seeking and incorporating resistance to the parasitic plant, thus achieving an improvement in crop productivity. The occurrence of highly virulent races of *Orobanche* is evident in the case of sunflower. Results of evaluation of sunflower germplasm for resistance to new virulent races of *O. cumana*, have shown that wild *Helianthus* species constitute the major reservoir of resistance genes (Christov *et al*., 2009), although resistance has been also found in accessions of cultivated material (Fernández-Martínez *et al*., 2000). As stated above, genetic resistance in sunflower is generally monogenic and dominant. Transfer of such a qualitative or vertical resistance is a routine procedure for plant breeders, as it can be easily incorporated into elite cultivars.

A critical difficulty attendant with the use of major gene resistance is the usual manifestation of new races of the parasite that overcome the existing resistance genes, establishing the necessity in sunflower breeding programs for identifying additional resistance genes to be introgressed into high yielding cultivars (Fernández-Martínez *et al*., 2009, 2014; Kroschel, 2002). Sources of horizontal resistance to *O. cumana* have been identified in sunflower as well, and it should be possible to combine vertical and horizontal mechanisms of resistance toward the development of a more durable resistance to *O. cumana* in this crop (Pérez-Vich *et al*., 2006). Breeding for broomrape resistance is considered a priority breeding trait in sunflower areas prone to broomrape parasitization, being among the most sustainable means of control (Fernández-Martínez *et al*., 2009). Alternatively, sunflower broomrape can be controlled by post-emergence application of imidazolinone (IMI) herbicides on non-GMO, IMI-tolerant hybrids, derived from IMI-resistant alleles identified in wild populations (Al-Khatib *et al*., 1998; Kaya and Evci, 2007; Kaya *et al*., 2004a, 2012) or developed by mutagenesis (Sala *et al*., 2008).

3.4.5.1. Sources of Resistance: Search and Transfer of Resistance to Cultivated Sunflower

The genus *Helianthus* includes diploid ($2n = 2x = 34$), tetraploid ($2n = 4x = 68$) and hexaploid ($2n = 6x = 102$) species, all with a basic chromosome number of $n = 17$. The closest relative genera appear to be *Tithonia*, *Viguiera* and *Phoebanthus* (Heiser *et al*., 1969). The genus *Helianthus* includes both annual species – including the diploid species *H. annuus* – as well as perennial species (Jan and Seiler, 2007; Seiler and Rieseberg, 1997). The common sunflower (*H. annuus*) is the most important species grown commercially, although other species are also cultivated, e.g. *H. tuberosus*, which is grown for production of edible tubers, and several other species grown as ornamental.

Sources of genetic variability from cultivated germplasm and wild species are available in germplasm collections to be used in breeding programs. Selection and development of sunflower resistance to broomrape was common in the early breeding programs in the former USSR, via methods of individual selection (Gorbachenko *et al*., 2011; Pustovoit, 1966). The first varieties resistant to broomrape were developed in 1915 by the researchers of the Saratov experimental station, E. M. Plachek and A. I. Steboot (Gorbachenko *et al*., 2011). Between the years 1918 and 1925, cultivars resistant to the race A such as Saratovsky 169, Saratovsky 206 (Plachek and Steboot, 1915), Saratovsky 420, Kruglik A-41 (Pustovoit, 1926), Zelenka 76 and Fuksinka 10, were developed in the former USSR (Melero-Vara and Alonso-Arnedo, 1988). Saratovsky 169 was grown for several years at a plot that exceeded one million hectares (Škorić, 2012). After the appearance of race B, several experimental stations in Russia were involved in the development of parental germplasm material of sunflower resistant to the new race. The first broomrape-resistant sunflower plants were developed by L. A. Žhdanov in 1926 in Rostov Oblast, specifically cultivars as Zhdanovsky 6432, Zhdanovsky 8281 and Stepnyak, which were widely cultivated in the following years (Gorbachenko *et al*., 2011). Other resistant cultivars with the resistance gene *Or2* incorporated were Zhdanovsky 6393, Zhdanovsky 8884 and Zhdanovsky 8885 (Pustovoit, 1966). Žhdanov was a pioneer in the use of wild species of *Helianthus* spp., particularly *H. tuberosus*, for sunflower breeding for broomrape resistance (Pustovoit and Gubin, 1974). A number of sources of resistance to race B – including other wild sunflower species such as *H. maximiliani* Schrad. and *H. mollis* Lam. –, were developed in the subsequent years in the former USSR (Melero-Vara and Alonso-Arnedo, 1988; Škorić *et al*., 2010). Soon after, in Krasnodar (Russia), Pustovoit combined resistance to race B and high productivity and developed, among others, cultivars VNIIMK 1646 and VNIIMK 6540. Since resistance genes are usually identified in germplasm of wild species, the recovery of good agronomic characteristics after the introgression of the new resistance gene is a difficult task. Between 1937 and 1950, this research developed

several cultivars resistant to race B, highly productive and with great oil content, such as VNIIMK 8883, VNIIMK 8931, Peredovik and Smena, which have come to be known worldwide. In the same period, within the Armavirsk station, the resistant cultivars to race B, Armavirsky 3497, Armavirsky 9343 and Armavirsky 9345 were produced. All of them have been widely employed in breeding programs to obtain sunflower hybrids resistant to broomrape (Melero-Vara and Alonso-Arnedo, 1988). In 1978 in Krasnodar, the development of the sunflower cultivars Progress, Yuvileyniy 60, Oktobar and Novinka – resistant to the latest race C – was reported (Melero-Vara and Alonso-Arnedo, 1988). In the late 1970s, the most valuable sources of resistance in Romania were cultivars derived from resistant germplasm developed in the former USSR, as well as populations derived from interspecific crosses between *H. annuus* and *H. tuberosus* (Vrânceanu *et al*., 1980).

Evaluation of cultivated germplasm has been extensively used though wild species represent the most diverse source of genetic variability in sunflower breeding. Resistance is mainly found in perennial *Helianthus* spp., though resistant annual species have been identified as well. The main problem using wild species as sources of variability is that many of them do not cross readily with the cultivated sunflower. The cultivated sunflower crosses without difficulty with most other annual diploid species and less easily with perennials. Introgressing broomrape resistance genes from wild annual *Helianthus* species into elite germplasm can be accomplished rather easily with conventional crossing and backcrossing (Fernández-Martínez *et al*., 2009), although embryo rescue may be required in early generations, and accurate testing for broomrape resistance is required at each generation (Velasco *et al*., 2012). Regarding perennials, different number of chromosomes and the genetic divergence makes it difficult crossing them with the cultivated sunflower. This is mainly due to early hybrid embryo abortion as well as high levels of sterility in the F_1 and BCF_1 generations (Georgieva-Todorova, 1984; Jan, 1997; Kräuter *et al*., 1991). These complications can be prevented with the employment of the embryo culture technique (Chandler and Beard, 1978, 1983; Kräuter *et al*., 1991) and subsequent chromosome doubling of the F_1 (Jan, 1988; Korell *et al*., 1996a). In 1998, Sukno *et al*. studied the reproductive behavior of interspecific hybrids from reciprocal crosses between cultivated sunflower and five wild perennial species with different levels of polyploidy, including *H. resinosus* Small, *H. pauciflorus* Nutt., *H. laevigatus* Torr. & A. Gray, *H. nuttallii* spp. *nuttallii* Torr. & A. Gray, and *H. giganteus* L. Furthermore, they evaluated the transmission and expression of resistance to *O. cumana* race E in the F_1 generations and the backcrosses. Conventional crosses in all species studied were successful, except for the diploid species *H. giganteus*, in which the use of the technique of embryo rescue was necessary. Pollen viability and seed production were higher in F_1 hybrids with hexaploid species and lower with the diploid *H. giganteus*. In addition, wild species and interspecific F_1 hybrids were resistant to broomrape attack, except for *H. nuttallii*, in which segregation was observed, indicating

that resistance was in most cases a dominant character. Successful development of resistant germplasm incorporating genes from wild *Helianthus* spp. has been reported both for annual (Hladni *et al*., 2009; Velasco *et al*., 2012) and perennial species (Cvejić *et al*., 2012; Christov *et al*., 2009; Jan *et al*., 2002; Korell *et al*., 1996b; Petcu and Păcureanu, 2011).

As mentioned above, the transfer of resistance to broomrape in sunflower is possible starting from cultivated material. Such is the case of restorer inbreed line R-41 resistant to race E, obtained from a F_6 generation through polycrosses among 15 sunflower hybrids of different origins (Domínguez, 1996b). Subsequently, seven sunflower lines R-185, R-188, R-190, R-201, R-202, R-206 and R-207 also resistant to broomrape and equally derived from *H. annuus* were developed (Miller and Domínguez, 2000). Regarding the transfer of resistance to *O. cumana* race E from wild species, Ruso *et al*. (1996) assessed the resistance of 26 perennial and 18 annual species of *Helianthus*, as well as 29 lines derived from perennial species against three populations of *O. cumana* race E. Resistance was found in a high proportion of them, including most of the perennial and two annual species, *H. anomalus* S.F. Blake and *H. debilis* Nutt.

Evaluation of sunflower germplasm for resistance to race F of *O. cumana* was conducted by Fernández-Martínez *et al*. (2000). Artificial inoculations were performed with seeds of *O. cumana* in 54 wild species – annual and perennial – of *Helianthus*, and 55 lines of *H. annuus*. Most perennial wild *Helianthus* species were completely resistant to race F, except *H. divaricatus* L., *H. maximiliani* and *H. pauciflorus* subsp. *pauciflorus* which showed different proportions of susceptible plants, with an incidence of parasitism that ranged from 10 to 80%. The accessions of the annual wild species *H. anomalus* and *H. agrestis* Pollard were identified as completely resistant. Amphiploids of the wild species *H. gracilentus* A. Gray, *H. hirsutus* Raf., *H. strumosus* L., *H. maximiliani*, *H. nuttallii* and *H. grosseserratus* M. Martens, among others, have been produced and used as a bridge to transfer resistance to broomrape race F (Jan and Fernández-Martínez, 2002). The development of four genetic stocks of sunflower, BR1 to BR4, resistant to *O. cumana* race F, was made possible by the introgression of resistance genes from the three wild perennial species *H. maximiliani, H. grosseserratus*, and *H. divaricatus* (Jan *et al*., 2002). The sunflower line LR1, resistant to race F, was developed from the cross between *H. annuus* and *H. debilis* subsp. *debilis* (Labrousse *et al*., 2004). In 2004, new genes for resistance to race F of broomrape were identified in germplasm of cultivated sunflower, resulting in the resistant lines K-96, L-86, P-96 and R-96 (Fernández-Martínez *et al*., 2004). Sunflower germplasm with resistance to race F of *O. cumana* has been expanded in 2006 with the development of the AM-1, AM-2 and AM-3 lines, with quantitative resistance (Pérez- Vich *et al*., 2006). Extensive evaluations of wild *Helianthus* spp. for resistance to the latest broomrape races have been conducted in recent years by several research groups

(Antonova *et al*., 2011; Christov *et al*., 2009; Petcu and Păcureanu, 2011; Terzić *et al*., 2010). Velasco *et al*. (2012) identified an accession of *H. debilis* subsp. *tardiflorus* (PI 468691) which exhibited complete resistance to the sunflower broomrape population OC-17, classified as race G because it parasitized plants of several race F-resistant lines (Fernández-Martínez *et al*., 2004; Jan *et al*., 2014).

3.4.5.2. Genetics and Inheritance of Resistance to Sunflower Broomrape

Low levels of resistance to *Orobanche* spp. have been identified in most crop plants, generally being under polygenic, non-race-specific genetic control (Fernández-Martínez *et al*., 2014; Linke, 2002). This is the case for a number of legumes such as faba bean (*Vicia faba* L.) (Fernández-Aparicio *et al*., 2012; Hernández *et al*., 1984; Sillero *et al*., 2010), common vetch (*Vicia sativa* L.) (Fernández-Aparicio *et al*., 2009; Gil *et al*., 1984, 1986), and pea (*Cicer arietinum* L.) (Rubiales *et al*., 2006), as well as other crops such as tomato (Abdeev and Sherbinin, 1978; Qasem and Kasrawi, 1995), tobacco (Buschmann *et al*., 2005), rapeseed (Zehhar *et al*., 2003), or parsley [*Petroselinum crispum* (Miller) A.W. Hill.] (Goldwasser and Kleifeld, 2002). Nevertheless, only in a minority group of crops like carrot (Zehhar *et al*., 2003) and tomato (Dor *et al*., 2010) have been identified genotypes exhibiting a high degree of resistance. In such cases, resistance is controlled by alleles at a single locus determining a strigolactones deficiency (Koltai *et al*., 2010; Dor *et al*., 2011). Sunflower is a significant exception where resistance to *O. cumana* has been found in most cases to be monogenic, dominant, and race specific (Fernández- Martínez *et al*., 2008; Pérez-Vich *et al*., 2013). The genetic control of broomrape resistance by a single dominant gene was first reported by Pogorletsky and Geshele (1976). Shortly after, Vrânceanu *et al*. (1980) found that each dominant gene conferred resistance to the corresponding *O. cumana* race and also to the previous one.

To efficiently use the available sources of resistance to *O. cumana* in breeding programs, it is necessary to determine the inheritance of resistance and possible relationships among resistance genes. Pustovoit (1966) reported quantitative resistance. Several studies confirmed monogenic dominant resistance to the early identified *O. cumana* race E (Ish-Shalom-Gordon *et al*., 1993; Lu *et al*., 2000; Pérez- Vich *et al*., 2004b; Saavedra-del-Río *et al*., 1994b; Sukno, 1997; Sukno *et al*., 1999; Vrânceanu *et al*., 1986). Pérez-Vich *et al*. (2004b) suggested that resistance to broomrape race E in sunflower is controlled by a combination of qualitative resistant – specific of each race and responsible for the presence or absence of broomrape plants –, and quantitative resistant – race nonspecific and related to the number of broomrape plants –. However, other studies reported genetic control by one recessive gene (Ramaiah, 1987), two

independent dominant genes, considering the *Or5* gene and other non-allelic gene with similar effect (Domínguez, 1996b), and two complementary genes (Krokhin, 1983).

Similarly, several different results have been reported for races overcoming *Or5* resistance, including one dominant gene (Păcureanu-Joiţa *et al*., 2004; Velasco *et al*., 2012), two independent dominant genes (Păcureanu-Joiţa *et al*., 2008), one dominant and one modifying gene (Pérez-Vich *et al*., 2004a; Velasco *et al*., 2007), and two recessive genes (Akhtouch *et al*., 2002; Rodríguez-Ojeda *et al*., 2001). It has also been described the existence of epistatic interactions and annulment of dominance in crosses of susceptible lines with the same source of resistance (Pérez-Vich *et al*., 2002), modifier genes (Velasco *et al*., 2007), and quantitative factors (Pérez-Vich *et al*., 2004b). Labrousse *et al*. (2004) studied the resistance to race F of *O. cumana* based on induction of germination of broomrape seeds, parasitic plant necrosis, and its growth or stages of development. The diversity observed for these characters suggested polygenic resistance.

3.5. Molecular and Diversity Studies on the Genera *Orobanche*/*Phelipanche* and *Sriga*

The thoughtfulness on knowledge about the genetic diversity among and within *Orobanche*/*Phelipanche* and *Striga* populations is required if breeding programs are to target the sources of host resistance to parasite populations in different ecological and geographic areas. Comparative studies of the genetic diversity of parasitic biotypes in natural and agricultural habitats are also significant for understanding the evolutionary path from wild plants to aggressive parasitic weeds attacking crops. This can facilitate assessing the risk of the appearance of new parasite genotypes and races that are capable of parasitizing a non-host crop or overcome resistance developed in the host plant (Román, 2013). Diversity studies among and within populations of weedy parasites were first based on the parasite morphology. Host range and host preference have also been used to differentiate populations of weedy parasites and races (Cubero and Moreno, 1979; Joel *et al*., 2000; Radwan *et al*., 1988). Comparative studies between parasitic plant populations attacking wild species and those growing on crops from the same region may clarify host specialization. However, the question whether observed variations are genetically determined or induced by environmental conditions is best answered using molecular techniques (Román, 2013).

The most important biochemical or protein markers are isoenzymes, variants of an enzyme and therefore a direct expression of an allelic series. Isoenzymes were the first molecular markers used for diversity studies. These are detected when a protein extract of a tissue such as leaf or seed is subjected to electrophoresis and stained with a specific substrate for the relevant enzyme activity. Stained bands appear at different heights of the gel if there is a change in the amino acid composition or protein structure.

These markers are expressed as a co-dominant, which, coupled with their relatively cost of analysis, have become an ideal technique for a long time. However, protein markers have a series of disadvantages; they may be affected by environmental conditions and different stages of development of the organism under study. Furthermore, there are a limited number of loci susceptible to study; for that reason, discrimination of genotypes is not always possible. Verkleij *et al*. (1986, 1991) used protein markers to study the diversity of populations of *O. crenata* and *P. aegyptiaca* in Syria (Verkleij *et al*., 1986) and *O. crenata* in Spain (Verkleij *et al*., 1991). The isoenzyme markers revealed high consistency among *Orobanche*/*Phelipanche* populations of Syria, while the diversity of *Orobanche* populations from Spain was greater. This difference in results was attributed to the different environmental country conditions which the analyzed broomrape plants were obtained. Enzyme electrophoresis was used by Bharathalakshmi and Musselman (1990) to investigate genetic diversity in three populations of *S. hermonthica* – one sorghum-adapted population from Sudan and two populations (sorghum-adapted and millet-adapted, respectively) from Burkina Faso –, and to determine the level of differentiation among host-specialized populations. Levels of polymorphism were similarly high in all three populations and a slight to moderate level of genetic differentiation among the populations was detected. The two populations from Burkina Faso were more closely related to each other than to the population from Sudan, suggesting that geographic separation is more important than host specialization in contributing to population differentiation.

Molecular markers based on DNA have largely overcome the handicaps of isoenzyme markers, they are not so influenced by environmental conditions and do not rely on transcribed DNA. The use of short arbitrary primers – in order to obtain molecular markers in any genome applying the Polymerase Chain Reaction (PCR) technique –, was presented independently by two research teams in 1990. Welsh and McClelland (1990) called the method as Arbitrarily Primed PCR (AP-PCR). No prior knowledge of the molecular biology of the organisms to be investigated is required. Williams *et al*. (1990) called the method as Random Amplified Polymorphic DNA (RAPD). The process is based on amplification of genomic DNA with primers of arbitrary nucleotide sequence (Xena-de-Enrech, 2000). Williams *et al*. (1990) proposed the use of primers of nine or ten nucleotides in length, and G+C composition between 50% and 80%. In the PCR reaction, each primer amplifies several DNA segments which are detected by agarose gel electrophoresis and, in many of them, polymorphisms appear among the species analyzed. RAPD markers are dominant; it is not possible to distinguish when a segment of DNA has been amplified from a heterozygous locus (one copy) or homozygous (two copies). The method is simple and inexpensive, does not require radioactive markers and uses minimal amounts of DNA. However, RAPD markers have limitations such as marker allele dominance and poor reproducibility.

Despite the dominant and low reproducible nature of RAPDs, they were used in several early genetic studies in *Orobanche*, *Phelipanche* and *Striga* spp. Katzir *et al*. (1996) studied five species of *Orobanche*/*Phelipanche* from Israel fields, *P. aegyptiaca*, *P. ramosa*, *O. cernua*, *O. cumana* and *O. crenata*, using the RAPD technique. The study revealed high significant genetic differentiation among the species. Polymorphisms found in *O. crenata* and *O. cumana* were confirmed by the study of plants of the same species collected in Spain. The use of 86 RAPD markers allowed the creation of a dendrogram in which the five *Orobanche* species studied were clearly distinguished. Additionally, *Orobanche* species belonging to *Trionychon* section (*P. aegyptiaca* and *P. ramosa*) and the *Orobanche* section (*O. cernua*, *O. cumana* and *O. crenata*), could be separated. That study also distinguished between *O. cumana* and *O. cernua* as well as between *P. aegyptiaca* and *P. ramosa*. In 1997, Paran *et al*. studied the diversity among and within natural populations of *Orobanche*/*Phelipanche* using also RAPD markers. The study was conducted on the species *P. aegyptiaca*, *P. mutelii* F.W. Schultz, *O. cernua*, *O. cumana* and *O. crenata*. The pattern of interspecific diversity and genetic distances observed in that study were consistent with previous taxonomic characterization based on morphological characteristics among species (Musselman, 1986). Intraspecific variation was determined for *P. aegyptiaca* and *O. crenata*; while 99% of the fragments amplified were polymorphic among species, only 23% and 21%, respectively, were polymorphic within *P. aegyptiaca* and *O. crenata*. Román *et al*. (2001) analyzed the pattern of genetic variation among and within natural populations of *O. crenata* from Southern Spain using RAPD markers. Although molecular variances were significantly heterogeneous among populations, most of the genetic diversity was attributable to differences among individuals within a population. No clear grouping pattern was established, supporting the predominant outcrossing behavior of *O. crenata*. Five RAPD markers were used by Román *et al*. (2003) to study the diversity among 20 species of the genus *Orobanche*/*Phelipanche* from the Iberian Peninsula: *O. alba* Stephan ex Willd., *O. amethystea* Thuill., *P. arenaria* (Borkh.) Pomel, *O. ballotae* A. Pujadas, *O. cernua*, *O. clausonis* Pomel., *O. crenata*, *O. cumana*, *O. densiflora* Reut., *O. foetida* Poir., *O. foetida* var. *broteri* (J.A. Guim.) Merino, *O. gracilis* Sm., *O. haenseleri* Reut., *O. hederae* Duby, *O. latisquama* (F.W. Schultz) Batt., *P. mutelii*, *O. nana* (Reut.) Beck., *P. ramosa*, *O. rapum genistae* Thuill., and *O. santolinae* Loscos & J. Pardo. They obtained a total of 202 amplification products. The pattern of interspecific diversity corresponded with previous taxonomic and morphological studies (Beck-von-Mannagetta, 1930), and the difference between *Trionychon* and *Orobanche* sections was confirmed. However, *O. clausonis* was grouped with members of *Trionychon* section. Within this section, *P. arenaria* was farthest from other species of the section as *P. mutelii*, *O. nana* and *P. ramosa*. Within the *Orobanche* and *Trionychon* sections, all *P. ramosa* populations showed similar patterns of amplification, while the populations of *O. crenata* presented differences depending on the parasitized host.

Furthermore, *O. foetida* and *O. densiflora* were grouped into the same cluster, supporting the morphological and cytological similarity and host preference of these species. *Phelipanche ramosa* and *O. cumana* have been studied by Atanasova *et al*. (2005) using six RAPD markers and samples of seeds from different agricultural regions of South Eastern Europe and the Middle East. Populations of *O. cumana* were collected in Bulgaria, Romania, Turkey, Spain, Russia and Ukraine, and *P. ramosa* populations were from Bulgaria and Israel. With one of the primers, an intense band of 660 bp in length was specifically detected for *O. cumana*. The absence of this band was perceived in two of the 21 *O. cumana* populations of the study from both of Kirovgrad (Russia) and Suvorovo (Bulgaria), respectively. Two bands of 780 bp and 1250 bp, specific for the 21 *O. cumana* populations, were also observed. Another fragment of 1600 bp was not observed on the original population of Volgograd (Russia), and one of 1350 bp in populations from Kirovgrad and Suvorovo. A band of 750 bp was observed in all *P. ramosa* populations except for one population from Sadovo (Bulgaria). With the other five primers, high genetic diversity among populations from different geographical areas could also be observed.

Orobanche foetida was typically found parasitizing wild plants in the Western Mediterranean area but have also been described as an agricultural problem in legume crops in Tunisia (Kharrat *et al*., 1992; Rubiales *et al*., 2005). The model of genetic diversity between and within two *O. foetida* populations growing on chickpea and faba bean, separately, was analyzed by ten RAPD markers (Román *et al*., 2007a). The *O. foetida* populations parasitizing chickpea and those growing on faba bean in Tunisia revealed significant divergence at the molecular level, suggesting a host-differentiation process. The specialization process seems to be the consequence of the strong selection pressure by the different crops. The pattern of genetic variation among populations of two *O. gracilis* taxa (var. *gracilis* and var. *deludens* (Beck) A. Pujadas) from Northern and Southern Spain growing on different hosts was analyzed by Román *et al*. (2007b) using a set of 15 RAPD markers. The diversity analysis within populations revealed a higher level of diversity in the populations from the North when compared to the Southern ones. The results clearly established the separation of populations according to the taxonomical variety and the geographical origin. The diversity among three French *P. ramosa* populations in terms of virulence was investigated by cross inoculations using three host species *B. napus, N. tabacum,* and *Cannabis sativa* L. (hemp) (Brault *et al*., 2007). Differences in virulence of populations and greater affinity between the host species and the parasite were observed. Analysis of genetic variability of the three *P. ramosa* populations by PCR of 24 RAPD markers confirmed that they may be considered as three distinct races (Brault *et al*. 2007). Aigbokhan *et al*. (2000) studied the variability in *S. aspera* (Willd.) Benth., *S. hermonthica* and their reciprocal F_1 hybrids from different locations in Nigeria using morphological characters and RAPD markers. Analyses revealed that *S. aspera* and *S. hermonthica* were genetically and

morphological distinct. Comparative morphological analysis of wild and hand-pollinated populations showed some samples from the wild clustered with the hybrids, suggesting that hybrids may exist in nature.

Other markers that have been used in the molecular analysis of the genera *Orobanche*/*Phelipanche* and *Striga* are Inter-simple Sequence Repeat markers (ISSRs), Amplified Fragment Length Polymorphism markers (AFLPs), and diagnostic markers from the plastid genome or plastome (cpDNA). In the first case, ISSRs are dominant markers and considered more reliable and robust than RAPDs. The primers used are longer and derived from genomic regions of simple repeat sequences. Benharrat *et al*. (2002) applied five ISSR primers to study genetic diversity among four species of *Orobanche*; *O. hederae* and *O. amethystea* – morphologically distinct –, and *O. cernua* and *O. cumana* – closely related –. All primers detected polymorphisms between *O. hederae* and *O. amethystea*. Also, a greater diversity of three populations of *O. hederae* was detected, which did not correspond to their geographical location. Polymorphisms were also detected in five populations of *O. cernua* and *O. cumana* collected in different countries, allowing the differentiation of the two species. Román *et al*. (2002) used ISSR markers to assay the variation among and within populations of the parasitic weed *O. crenata* from Spain and Israel. Only 24% of the total ISSR marker diversity of the outcrossing *O. crenata* was attributable to divergence between Spain and Israel, despite the 71% of within-population diversity (Román *et al*., 2002). ISSR analysis of five *O. minor* populations in the U.S. revealed a low level of polymorphism, with individuals within populations having nearly all SSR fragments in common (Westwood and Fagg, 2004). The reason for this low diversity may be that the populations originated from just a few founder plants. Two clearly different groups of populations were detected in the U.S., implying that the populations originated from two separate introduction events. In two studies reported by Thorogood *et al*. (2008, 2009), ISSR markers provided evidence of host-driven divergence of the coastal clade *O. minor* ssp. *maritima* parasitizing sea carrot [*D. carota* ssp. *gummifer* (Syme) Hook. fil.] from the host-generalist lineage *O. minor* var. *minor* growing on clover (*Trifolium pratense* L.). The virulence of eight *P. ramosa* populations collected in France against their potential host plants (*Buddleja linleyama* F., *B. napus*, *C. sativa*, *Fagopyrum esculetum* Moench, and *N. tabacum*), was investigated by Benharrat *et al*. (2005), resulting in significant variation in their aggressiveness. From the same study, the identification of two *P. ramosa* pathovars was studied by ISSR markers and compared with the eight populations collected from different host plants, allowing the characterization of two groups of populations which each corresponded to a different pathovar (A or B). Genetic diversity studies with ISSR markers also allowed characterization of two *P. ramosa* populations with different levels of virulence parasitizing nine tobacco cultivars grown in Europe (Buschmann *et al*., 2005). Stoyanov and Denev (2008) studied five species of *Phelipanche* (*P. purpurea* (Jacq.) Sojak, *P. arenaria*, *P. mutelii*, *P. oxyloba* (Reut.) Sojak, and *P.*

ramosa) in Bulgaria using ISSR markers. A specific grouping of species and sections was observed. Five ISSR primers were used by Stoyanov and Denev (2010) to evaluate molecular-taxonomic relationships of *Orobanche* subsect. *Glandulosae* representatives (*O. alba*, *O. reticulata* Wallr. subsp. *pallidiflora*, *O. serbica* Beck., and *O. pancicii* Beck & Petrovic) from different regions in Bulgaria. The grouping of the known species was confirmed, with the group of *O. alba* showing some levels of high diversity. A collection of one hundred ISSR primers were screened by Hristova *et al*. (2011) to identify a polymorphic set for study Orobanchaceae taxonomic and population diversity in Bulgaria. Sixteen ISSRs were selected by produce polymorphic bands suitable to distinguish the known sections, genera and probably, higher taxonomy ranks. Stoyanov *et al*. (2012) identified a useful set of ten different ISSR markers for characterize the biodiversity and the phylogenetic relationships of six species of genus *Orobanche* subsection *Minores* in Bulgaria. The representatives of subsection *Minores*, *O. minor*, *O. amethystea*, *O. esulae* Pančić var. *bulgarica* T. Georgiev, *O. pubescens* d'Urv., *O. loricata* Rchb., and *O. crenata*, were examined. The grouping classification was displayed by species and by geographic populations, supporting that the assignment of the small-flowered species to agg. *O. minor* is not genetically justified, and evidenced the independent taxonomic status of the Balkan endemic species *O. esulae*.

AFLPs show a higher capability of discriminating a large number of reproducible loci. The genetic diversity of an *O. foetida* population growing on cultivated vetch in Morocco was compared, using AFLP markers, to the diversity of four populations attacking wild *Scorpiurus muricatus* L. and *Ornithopus sativus* Brot. in the same region. The vetch-parasitizing *O. foetida* population was closer to native populations parasitizing *S. muricatus*, whereas the population collected on *O. sativus* was the most divergent one, suggesting that it was not a new introduction to the region and that the wild population of *O. foetida* attacking *S. muricatus* gave rise to a new population that was able to parasitize the crop (Vaz Patto *et al*., 2008). The genetic structure and host-parasite interaction of four different populations of *S. gesnerioides* (Willd.) Vatke parasitizing *Indigofera hirsuta* L. from Central Florida (United States) was analyzed using AFLP markers. Cluster analysis grouped all four *S. gesnerioides* populations from Florida into a single group, differentiating them from a separate group of *S. gesnerioides* populations attacking *I. hirsuta* and cowpea [*Vigna unguiculata* (L.) Walp.] from West Africa. The very high level of genetic uniformity observed among and within the Florida populations suggested that there was probably a strong host-driven selection for genetic uniformity, in addition to inbreeding (Botanga and Timko, 2005). A relatively uniform low level of genetic diversity was found among 17 populations of *S. asiatica* (L.) Kuntze, and 24 populations of *S. hermonthica* studied in Kenya with AFLP markers, and there was no evidence of isolation by distance in any populations of the two species (Gethi *et al*., 2005). It was also possible to detect differences among *S. asiatica* populations that are separated by only small distances,

using AFLP markers (Botanga *et al*., 2002). This technique was used to estimate genetic variability among and within 14 populations of *S. asiatica* collected from different regions in the Republic of Benin on maize and sorghum. The *Striga* populations, which were more adapted to maize than sorghum, exhibited different degree of virulence on susceptible host plants and high degree of host-specialization (Botanga *et al*., 2002).

Román *et al*. (2007c) identified potential cpDNA diagnostic markers which were applied to differentiate the most important *Orobanche* species attacking crops in Andalucía. They amplified a non-coding region of the plastome and studied sequences differences among the amplified fragments. Those fragments of the same length were digested with restriction enzymes. Amplification of the chloroplast region *trnD-trnT* allowed differentiating *P. ramosa* and *O. cumana* from the rest of the species studied. For *O. crenata* and *O. minor*, segments of the same length were amplified and the sequence of the two amplified bands was determined. Digestion with the enzyme *Msel*, allowed the identification of a new diagnostic band of 219 bp in *O. crenata* whereby it differed from *O. minor*. The variability of Internal Transcribed Spacers1/2 of ribosomal cistron (ITS1/2) and ribulose-bisphosphate carboxylase pseugene (RbcL) in 32 samples of *O. cumana* and four samples of *O. cernua* collected from different European locations were studied by Kirilova *et al*. (2014). The genetic diversity observed in *O. cumana* was lower than in *O. cernua*, and the results clearly supported the differentiation between both species (Kirilova *et al*., 2014).

One of the most powerful approaches to characterize genetic diversity employs Simple Sequence Repeats Markers (SSRs, microsatellites), which have high polymorphism indices, and are robust, reproducible, neutrally evolving and co-dominant markers. This technique is clearly needed for more accurate population genetic studies on weedy Orobanchaceae. In a recent study, Le-Corre *et al*. (2014) developed 13 microsatellite markers for *P. ramosa* using next-generation sequencing data. Microsatellite characterization was assessed in populations collected in France within six fields cultivated with tobacco, hemp or oilseed rape. Genetic diversity within each cultivated field was very low although strong differentiation was observed among individuals collected on oilseed rape from those collected on hemp or tobacco, suggesting an evidence for host-associated genetic divergence in *P. ramosa*. Estep *et al*. (2011) used a set of 12 microsatellite primers pairs to describe the diversity of 11 *S. hermonthica* populations collected across a broad swath of environments in four different agricultural zones in Mali. Extensive genetic diversity was observed with high number of heterozygous plants for most markers. The *Striga* populations were characterized by broadly distributed allelic diversity across populations with little genetic differentiation and large amount of gene flow. Indistinguishable witchweed populations were identified in nearby fields of pearl millet and sorghum. Although some population structure was apparent, it was also observed that population structure did not correlate with local environment or host (sorghum and pearl millet), suggesting that

seed transportation or other human-driven variables act to differentiate Central from Southern *S. hermonthica* populations in Mali.

3.5.1. Genetics and Population Dynamics of Sunflower Broomrape

The consideration of *Orobanche* genetics is of great importance given that understanding the genetic diversity of broomrape populations is essential for the development of sustainable and long-term breeding strategies for control involving genetic resistance in sunflower (Kaya, 2003). Information on genetic diversity, population dynamics, mating system, gene flow and virulence genetics in *O. cumana* is scarce, particularly concerning molecular analysis. A limited number of molecular studies on genetic diversity within and among populations of sunflower broomrape have been reported, and were based on restricted numbers of populations and marker types.

Castejón-Múñoz *et al*. (1991b) studied intrapopulation diversity in five *O. cumana* populations from Southern Spain using isoenzymes markers. They found low genetic diversity in four populations, but greater genetic diversity in the fifth population. A founder effect was suggested as the reason for the differences in variability within the five populations of *O. cumana* in Spain (Castejón-Múñoz *et al*., 1991b). Founder events are to be expected following the introduction of *Orobanche* populations into new areas without previously broomrape occurrence. In the study reported by Castejón-Múñoz *et al*. (1991b), it was argued that the higher diversity of the *O. cumana* populations parasitizing confectionary sunflower – which is highly susceptible – compared to the diversity of the other four populations could be attributed to the number of years that the susceptible sunflower crop grew in that area and consequently to the very high severity broomrape seed bank. The lower variability, which was mainly manifested by the loss and fixation of some alleles in the other four populations, was due to a more recent origin from only few individuals that were transferred on sunflower achenes (Castejón-Múñoz *et al*., 1991b).

The genetic diversity in eight *O. cumana* populations from several European countries – three from Bulgaria, one from Turkey, one from Romania, and three from Southern Spain –, was studied by Gagne *et al*. (1998) using RAPD markers. They identified low intrapopulation and large interpopulation genetic variation, concluding the existence of two main genetic pools, one comprising the populations from Eastern Europe and another one including the populations from Southern Spain. Pineda-Martos (2007) analyzed 43 DNA samples from 33 *O. cumana* populations collected on four sunflower cultivars (NR5, B117, L86 and P96) using RAPD markers. Populations originally come from Spain – 26 populations from the Guadalquivir Valley and nine populations from Cuenca – and Turkey (eight populations), and the sunflower cultivars possess resistance against *O. cumana* races E and F acquired from different sources. Populations were classified into three distinct groups according to their geographical

origin, and no trend was observed in the grouping of the populations according to the sunflower cultivar. Molinero-Ruiz *et al*. (2013) confirmed the manifestation of great genetic distance between populations of Southern and Central Spain using RAPD markers, though greater genetic diversity within each genetic pool was detected. The occurrence of low genetic diversity in most sunflower broomrape populations suggested the monophyletic origin of the genetic pools identified thus far (Gagne *et al*., 1998). Pineda-Martos (2007) found that populations from Turkey were closer to populations from Southern Spain than to populations from Central Spain. The results reported by Molinero-Ruiz *et al*. (2013) supported the previous ones, since they found that one population from Hungary and four populations from Turkey were closer to populations from Southern Spain than to populations from Central Spain.

Gagne *et al*. (2000) used AFLP markers to study two populations of *O. cumana* from Spain and Bulgaria, respectively. In this research, they proved the applicability of AFLP markers in the study of genetic diversity among and within populations of sunflower broomrape. The results were compared with those obtained with RAPD markers. The main difference between both marker techniques was the high resolution provided by RFLP markers to analyze closely related germplasm compared with RAPD markers.

04 Aims & Scope

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The present work aims to investigate the genetic diversity and population structure of the sunflower parasitic weed O. cumana *through the use of robust, reproducible, neutrally evolving, and co-dominant molecular markers. For this, in a first phase, it was necessary to develop such powerful approach and tools, which were not available at the beginning of this research work. In a second stage, the molecular genetic diversity of weedy* O. cumana *populations was studied in Spain, where this species is found as an allochthonous species parasitizing exclusively sunflower. Finally, genetic diversity, population structure, and virulence on sunflower were investigated on* O. cumana *populations parasitizing wild plants from the Black Sea coast in Eastern Bulgaria, a country for which this species is autochthonous and where wild and weedy populations of* O. cumana *co-exist.*

Thus, the Doctoral Thesis has the following hypotheses and objectives.

1. To identify and characterize Simple Sequence Repeats (SSR) markers for *O. cumana*

Information on genetic diversity, population dynamics, mating system, gene flow and virulence genetics in *O. cumana* is scarce – particularly concerning molecular analysis and due to the lack of suitable markers resources for such studies –, despite of the importance of the development of long-term breeding strategies of control involving genetic resistance in sunflower. At the beginning of this work, only a few molecular studies had been conducted in *O. cumana*, restricted to evaluating genetic diversity through isoenzymes (Castejón-Múñoz *et al*., 1991b) or RAPD markers (Gagne *et al*., 1998). SSR markers have proven to be useful tools for genetic diversity studies and for determining gene flow and mating system in several other parasitic species of the Orobanchaceae (Crichton *et al*., 2012; Ducarme and Wesselingh, 2013; Estep *et al*., 2011; Rodrígues *et al*., 2012; Yoshida *et al*., 2010).

Our hypothesis is linked with the fact that alternative markers such as SSRs, which are reproducible, neutrally evolving, multiallelic and co-dominant, are needed to enable more powerful genetic analyses in *O. cumana*. Therefore, we hypothesize that functional SSR markers should constitute a valuable tool for genetic analyses in *O. cumana* and related species and should contribute insights into the biology and genetics of this parasitic weed.

2. To validate SSR markers isolated from the parasitic weed *O. cumana* on its close relative *O. cernua*

Molecular markers systems, such as RAPDs (Katzir *et al*., 1996; Paran *et al*., 1997; Román *et al*., 2003) or ISSRs (Benharrat *et al*., 2002), as well as those based on ecological, morphological and biochemical data (Kirilova *et al*., 2014; Pujadas and Thalouran, 1998; Pujadas-Salvà and Velasco, 2000), clearly support the separation between *O. cumana* and *O. cernua* and the treatment of both taxa as different species.

Our hypothesis is that validating the usefulness of microsatellite markers in terms of quality of amplification, polymorphism and reproducibility for studies of genetic diversity in *O. cumana*, and identifying those highly transferable markers for their use in *O. cernua*, will allow comparative studies between both taxa and clarify their differentiation as closely related species.

Objectives 1 and 2 are addressed in the Research Article Pineda-Martos, R.; Velasco, L.; Pérez-Vich, B. Identification, Characterisation and Discriminatory Power of Microsatellite Markers in the Parasitic Weed Orobanche cumana. Weed Research ***2014***, 54(2), *120–132.*

3. To study genetic diversity in a large set of *O. cumana* populations from Spain using robust, neutrally evolving and co-dominant SSR markers

In Spain, *O. cumana* is an allochthonous species exclusively found on cultivated sunflower, in contrast to the closely related species *O. cernua* which is an autochthonous species that only parasitizes wild Compositae hosts, mainly *Artemisia* spp. (Pujadas-Salvà and Velasco, 2000). Therefore, Spanish populations of *O. cumana* originated from seed introductions from other areas. For many years, *O. cumana* was distributed in the Guadalquivir Valley in Andalucía and Cuenca province in Castilla-La Mancha region, but in recent years, it has spread to new areas of Andalucía, Castilla-La Mancha and Castilla y León (Fernández-Escobar *et al*., 2009). Molecular studies on genetic diversity among and within populations of *O. cumana* are scarce (Castejón-Múñoz *et al*., 1991b; Gagne *et al*., 1998). Studies on genetic variability of *O. cumana* populations from Spain have been based on restricted numbers of populations and marker types.

Our hypothesis is that a larger-scale evaluation with co-dominant molecular markers would contribute to a better understanding of genetic structure and dynamics of *O. cumana* populations and will promote the establishment of improved crop breeding and management strategies for *O. cumana* control.

Objective 3 is attended in the Research Article Pineda-Martos, R.; Velasco, L.; Fernández-Escobar, J.; Fernández-Martínez, J. M.; Pérez-Vich, B. Genetic Diversity of Orobanche cumana *Populations from Spain Assessed Using SSR Markers.* Weed Research ***2013***, 53(4), *279–289.*

4. To investigate genetic diversity and population structure of *O. cumana* populations parasitizing wild plants and comparative studies with weedy populations in Eastern Bulgaria using SSR markers to examine a possible host specialization

Comparative studies between parasitic plant populations attacking wild species and those growing on crops from the same region may clarify host specialization (Román, 2013). Wild and cultivated host plants represent different habitats for parasitic plants, especially when cultivated plants carry qualitative resistance genes, as is the case of the sunflower – *O. cumana* system. There are few studies on genetic interactions between wild and weedy forms of parasitic plant species. Studies on genetic diversity among and within *O. cumana* populations are scarce and focused on weedy populations collected on sunflower (Gagne *et al*., 1998). There is no information on the population structure of *O. cumana* populations parasitizing wild species and their genetic relationship with weedy populations in areas where they co-exist.

Our hypothesis is that knowledge about interactions between wild and weedy forms of parasitic plant species is important because wild vegetation may play a role as reservoir of genetic diversity for overcoming genetic resistance mechanisms in the host crops. On the other hand, an effective genetic exchange can occur between populations parasitizing sunflower crops and those parasitizing wild species. Genetic variability of wild populations of *O. cumana* may favor the ability of weedy populations to overcome sunflower resistance mechanisms.

5. To investigate the ability to parasitize sunflower of native *O. cumana* populations growing on wild plants in the Black Sea coast of Bulgaria

It is vital to the lasting success of resistance breeding to predict and monitor virulence in the parasite populations (Pérez-Vich *et al*., 2013). There is no information on wild *O. cumana* populations virulence on sunflower. It is unknown whether *O. cumana* possesses natural ability to parasitize sunflower or this ability arose in particular genotypes following mutation. The occurrence of bidirectional gene flow may have an impact on wild populations, as new physiological races continuously emerge in weedy populations.

Our hypothesis is based on the possibility of an effective gene transfer from weedy to wild *O. cumana* populations, and that evolution of virulence in weedy populations may also have an impact on the distribution of the species in the wild.

6. To investigate the mating system of native populations of *O. cumana* parasitizing wild plants in their natural habitat in Eastern Bulgaria

Mating systems – varying from strict inbreeding to obligate outcrossing – affect the amount and partitioning of genetic diversity among and within populations (Román, 2013). Molecular studies have verified the relation between genetic diversity of weedy parasites and their mating system. *Orobanche cumana* has been considered to be primarily a self-pollinated species by low rate of outcrossing (Ivanov *et al*., 1998), on the basis of the occurrence of high genetic differentiation among populations with low intrapopulation variability (Gagne *et al*., 1998) and flower morphology (Satovic *et al*., 2009). However, a recent study using a gene that determines absence of anthocyanin pigmentation estimated the occurrence of a certain rate of cross pollination in *O. cumana* plants (Rodríguez-Ojeda *et al*., 2011).

Our hypothesis is that the study of wild *O. cumana* populations in their natural habitat may provide new data about its mating system.

Objectives 4, 5 and 6 are developed in the Research Article Pineda-Martos, R.; Pujadas-Salvà, A. J.; Fernández-Martínez, J. M.; Stoyanov, K.; Velasco, J.; Pérez-Vich, B. The Genetic Structure of Wild Orobanche cumana *Wallr. (Orobanchaceae) Populations in Eastern Bulgaria Reflects Introgressions from Weedy Populations.* The Scientific World Journal ***2014***, vol. 2014, *Article ID 150432, 15 pages. doi:10.1155/2014/150432.*

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06 Publications

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Scientific Publication #1

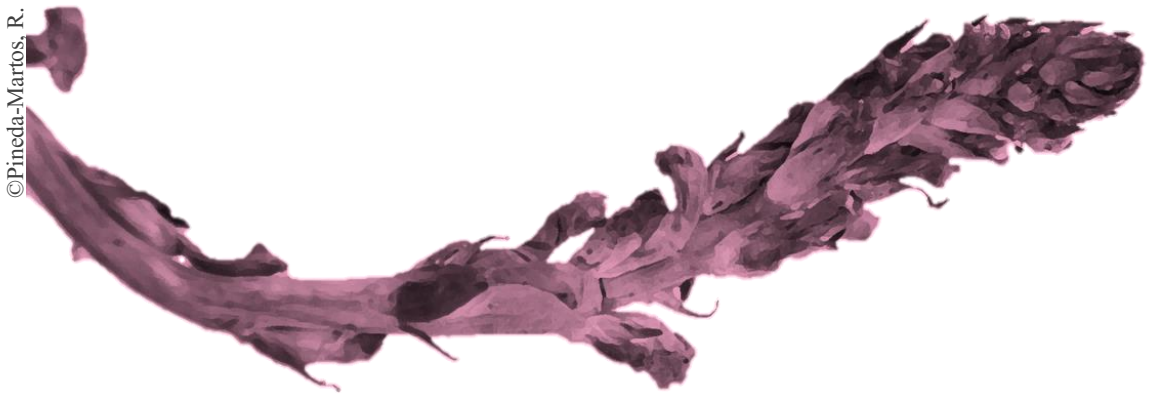
Pineda-Martos, R.; Velasco, L.; Pérez-Vich, B. Identification, Characterisation and Discriminatory Power of Microsatellite Markers in the Parasitic Weed *Orobanche cumana*. *Weed Research* **2014**, *54(2)*, 120–132.

Scientific Publication #2

Pineda-Martos, R.; Velasco, L.; Fernández-Escobar, J.; Fernández-Martínez, J. M.; Pérez-Vich, B. Genetic Diversity of *Orobanche cumana* Populations from Spain Assessed Using SSR Markers. *Weed Research* **2013**, *53(4)*, 279–289.

Scientific Publication #3

Pineda-Martos, R.; Pujadas-Salvà, A. J.; Fernández-Martínez, J. M.; Stoyanov, K.; Velasco, J.; Pérez-Vich, B. The Genetic Structure of Wild *Orobanche cumana* Wallr. (Orobanchaceae) Populations in Eastern Bulgaria Reflects Introgressions from Weedy Populations. *The Scientific World Journal* **2014**, *vol. 2014*, Article ID 150432, 15 pages. doi:10.1155/2014/150432.



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Pineda-Martos, R.; Velasco, L.; Pérez-Vich, B. Identification, Characterisation and Discriminatory Power of Microsatellite Markers in the Parasitic Weed *Orobanche cumana*. *Weed Research* **2014**, *54(2)*, 120–132.

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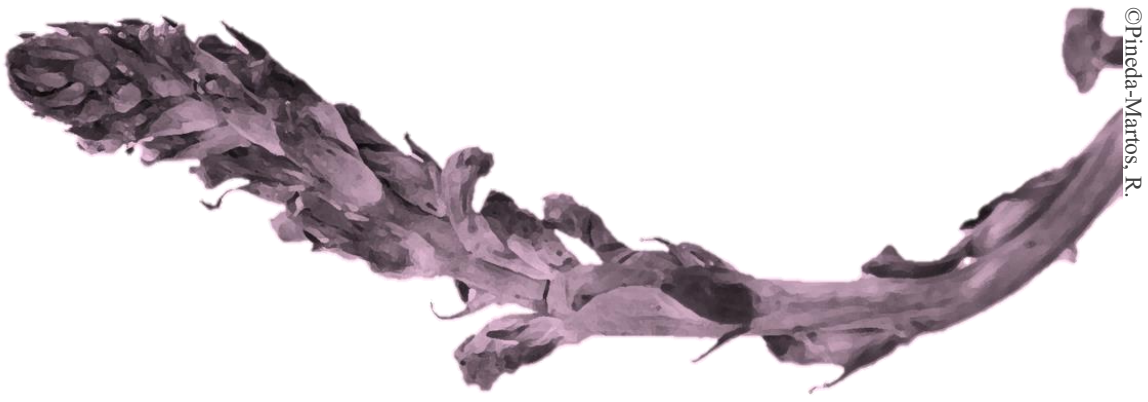

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METHODS

Identification, characterisation and discriminatory power of microsatellite markers in the parasitic weed *Orobanche cumana*

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Summary

Orobanche cumana is an obligate root parasite of sunflower. It represents a major agricultural problem in many countries of southern and eastern Europe. Information on *O. cumana* population genetics, structure and dynamics is scarce, particularly due to the lack of suitable molecular markers for such studies. The objective of this study was to identify and characterise simple sequence repeat (SSR) markers for *O. cumana*. Four thousand two hundred SSR-containing candidate sequences were obtained from *O. cumana* using next-generation sequencing, from which 298 SSR primer pairs were designed and 217 of them used for validation. Seventy nine SSR primers produced reproducible, high quality amplicons of the expected size that were polymorphic among 18 *O. cumana* populations from different geographical locations and hosts (sunflower, wild hosts from the Compositae family). The number of alleles per locus ranged from 2 to 10, with an average polymorphism information content value of 0.37. The *O. cumana* SSR markers were highly transferable to the closely related species *Orobanche cernua*. SSR markers showed high resolving power; UPGMA cluster analysis allowed proper classification of *Orobanche* spp. samples into species (*O. cumana* and *O. cernua*), geographical origin and host. The functional SSR markers reported in this study constitute a valuable tool for genetic analyses in *O. cumana* and related species and will contribute insights into the biology and genetics of this parasitic weed.

Keywords: genetic diversity, marker development, microsatellites, molecular markers, *Orobanche cernua*, simple sequence repeat, sunflower broomrape.

PINEDA-MARTOS R, VELASCO L & PÉREZ-VICH B (2014). Identification, characterisation and discriminatory power of microsatellite markers in the parasitic weed *Orobanche cumana*. *Weed Research* **54**, 120–132.

Introduction

Orobanche cumana Wallr. (Orobanchaceae; sunflower broomrape) is an obligate root parasite of sunflower. The species is naturally distributed from central Asia to south-eastern Europe parasitising *Artemisia* spp. (Pujadas-Salvà & Velasco, 2000; Parker, 2013). It was first observed parasitising sunflower (*Helianthus annuus* L.) in Russia in the 1890s (Škorić, 2012). Since then, *O. cumana* has spread over a vast area of sunflower cultivation becoming a serious constraint on sunflower production, causing yield losses of up to 60% (Parker,

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2009). Even though vertical genetic resistance has been identified in sunflower, its actual value is limited because the parasite has shown an ability to overcome resistance mechanisms, which has resulted in a number of pathogenic races (Fernández-Martínez *et al*., 2012).

Information on genetic diversity, population dynamics, mating system, gene flow and virulence genetics in *O. cumana* is scarce, particularly concerning molecular analysis, despite the importance of such studies for the development of long-term breeding strategies for control involving genetic resistance in sunflower. Only a few molecular studies have been conducted in *O. cumana*, restricted to evaluating genetic diversity through isoenzymes (Castejón-Muñoz *et al*., 1991) or RAPD markers (Gagne *et al*., 1998). Alternative markers such as SSRs, which are reproducible, neutrally evolving, multiallelic and co-dominant (Rafalski & Tingey, 1993), are needed to enable more powerful genetic analyses in *O. cumana*. SSR markers have proven to be useful tools for genetic diversity studies and for determining gene flow and mating system in several other parasitic species of the Orobanchaceae (Yoshida *et al*., 2010; Estep *et al*., 2011; Crichton *et al*., 2012; Rodrigues *et al*., 2012; Ducarme & Wesselingh, 2013).

Very limited SSR resources are available for molecular research in *O. cumana* and other *Orobanche* spp. We have recently developed a collection of SSR markers in *O. cumana*. A set of 15 SSR markers was used to evaluate genetic diversity in *O. cumana* populations from Spain (Pineda-Martos *et al*., 2013). In this manuscript, we report the development of the whole collection of *O. cumana* SSR markers, including (i) description of the major steps in the isolation of 4200 SSR-containing sequences and in the development of 298 *O. cumana* SSR primer pairs, including full sequences and primer information publication, and (ii) report on validation and discriminatory power of 217 SSRs in *O. cumana* populations from diverse geographical origins and hosts, and on their cross-species amplification in *Orobanche cernua* L.

Materials and methods

SSRs development

Pooled genomic DNA from 49 *Orobanche cumana* populations collected from 1988 to 2008 in different sunflower fields located across the main broomrape distribution areas in Spain (Table S1) was used for SSRs development using next-generation sequencing (Genoscreen, Lille, France). Genomic DNA was isolated from lyophilised plant tissue using a modified version of the protocol described by Rogers and Bendich (1985). Microsatellite-enriched library preparation and sequencing by 454 GS-FLX Titanium (Roche Applied Science, Indianapolis, IN, USA) was carried out according to Malausa *et al*. (2011). Briefly, the pooled DNA sample was subjected to genomic DNA fragmentation, ligated to standard adapters and enriched with eight microsatellite probes (AG, AC, AAC, AAG, AGG, ACG, ACAT and ACTC). The enriched DNA was then amplified using adapter-specific primers. The resulting library was tagged with a specific multiplex identifier (MID) tag sequence and pooled together with eight other samples in a quarter of a 454 GS-FLX Titanium run for sequencing. The resulting 24061 reads were analysed with QDD version 1 (Meglécz *et al*., 2010) in two steps. In step 1, reads longer than 80 bp containing SSRs with at least four repetitions of 2- to 6-bp motifs were selected. In step 2, similar sequences were detected using the all-against-all BLAST algorithm (Altschul *et al*., 1997) within QDD (*E*-value parameter set to 1E-40) with soft-masked SSR motifs. Sequences with a maximum difference of 5% (excluding SSR motifs) were grouped in contigs. Sequences with a significant BLAST hit with other sequences with a difference above 5% (excluding SSR motifs) were removed. Since the sample used for sequencing contained pooled DNA from several *O. cumana* individuals from different populations, the aligned sequences grouped in contigs were scored manually as well as with the DNASP (DNA Sequence Polymorphism) version 5.10.1 software package (Librado & Rozas, 2009) to detect SSR polymorphisms. SSR containing unique and consensus sequences were also analysed for SSR frequency and types, and for compound SSR identification with SCIROKO 3.1 software (Kofler *et al*., 2007). Finally, candidate sequences for primer design were selected within the remaining unique and consensus sequences, on the basis of a total sequence length of at least 100 bp containing a minimum of five repetitions of the SSR motif and flanking regions of at least 30 bp in length containing no more than four monobase repeats and two di-hexabase. Primer pairs were designed automatically by Primer3 release 1.1.4 (Rozen & Skaletsky, 2000) within QDD using selection criteria detailed in Lepais and Bacles (2011). From 4200 non-redundant sequences containing microsatellites motifs (Table S2), 2322 primers were designed, of which 298 were bioinformatically validated and selected (Table S3).

SSRs characterisation

A subset of 217 SSR markers containing at least six repetitions of the microsatellite motif was selected for characterisation. Targeting loci with high number of

repeats was desirable, because these more repetitive loci tend to show greater allelic variability (Kelkar *et al.*, 2008). Initially, each primer pair was tested on DNA from four *O. cumana* samples from different geographical areas. Each sample consisted of pooled DNA extracted from 15 to 22 individuals from each of the following *O. cumana* populations: SE03 and CO03 collected in sunflower fields in Southern Spain (Table S1), CU14 collected in a sunflower field from Central Spain (Cuenca province) and Boro-32 collected on the wild host *Artemisia maritima* L. in South-Eastern Bulgaria (Table 1). For each SSR marker, the quality of the amplification [(+++): strong signal and easy to score; (++): moderate signal but able to score; (+): weak signal and difficult to score; (−): no signal], the specificity of the product and the polymorphism were recorded. PCR amplification was carried out in 30 μL reaction mixtures, consisting of 50 ng of template DNA, 0.03 U μL^{-1} of Taq DNA polymerase (FIREPol DNA Polymerase, Solis BioDyne, Tartu, Estonia), 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTP's (dNTP Set, Solis BioDyne, Tartu, Estonia) and 0.3 μM of primer. A touchdown PCR program was used on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), which consisted of an initial denaturation of 94°C for 2 min, followed by 1 cycle of 94°C for 30 s, final annealing temperature (T_A) + 10°C for 30 s, and 72°C for 30 s, nine cycles in which the annealing temperature was decreased 1°C, and 32 cycles at 94°C for 30 s, T_A for

Table 1 *Orobanche cumana* and *O. cernua* populations used for SSR characterisation

Population	*Orobanche* spp.	Collecting site	Collection Year	Host	*n*	Line in Fig. 1
Populations from Southern Spain (Guadalquivir Valley) collected on sunflower (I group in Fig. 1)						
IASCum-1	*O. cumana*	Spain, Andalucía, Córdoba, Córdoba	2008	Confectionary sunflower	15	1
IASCum-2	*O. cumana*	Spain, Andalucía, Sevilla, Écija	2008	Oilseed sunflower	15	2
IASCum-3	*O. cumana*	Spain, Andalucía, Sevilla, Osuna	2008	Oilseed sunflower	15	3
Boro-13	*O. cumana*	Spain, Sevilla, Écija	2002	Oilseed sunflower	15	4
SE01	*O. cumana*	Spain, Andalucía, Sevilla, El Coronil	1989	Confectionary sunflower	21	5
CO06	*O. cumana*	Spain, Andalucía, Córdoba, La Carlota	2001	Oilseed sunflower	20	6
Populations from Central Spain collected on sunflower (II group in Fig. 1)						
IASCum-4	*O. cumana*	Spain, Castilla-La Mancha, Cuenca, Villarejo de Fuentes	2008	Oilseed sunflower	15	7
CU12	*O. cumana*	Spain, Castilla-La Mancha, Cuenca, Palomares del Campo	2008	Oilseed sunflower	20	8
CU05	*O. cumana*	Spain, Castilla-La Mancha, Cuenca, La Almarcha	1996	Oilseed sunflower	20	9
CU07	*O. cumana*	Spain, Castilla-La Mancha, Cuenca, Carrascosa del Campo	1996	Oilseed sunflower	20	10
Populations from Eastern Europe collected on sunflower (IV group in Fig. 1)						
Boro-15	*O. cumana*	Turkey, Malkara	2006	Oilseed sunflower	15	15
Boro-18	*O. cumana*	Bulgaria, central Bulgaria	2006	Oilseed sunflower	15	16
Boro-14	*O. cumana*	Turkey, Edirne	2006	Oilseed sunflower	15	17
Boro-19	*O. cumana*	Bulgaria, north-eastern Bulgaria	2006	Oilseed sunflower	15	18
Populations from Eastern Europe collected on wild Compositae hosts (III group in Fig. 1)						
Boro-31	*O. cumana*	Bulgaria, Burgas	2006	*Artemisia maritima* L.	9	11
Boro-32	*O. cumana*	Bulgaria, Pomorie-Aheloj	2006	*Artemisia maritima* L.	15	12
Boro-34	*O. cumana*	Bulgaria, Balchik	2006	*Artemisia maritima* L.	15	13
Boro-35	*O. cumana*	Bulgaria, Gorun-Tyulenovo	2006	*Anthemis arvensis* L.	15	14
Spanish *O. cernua* populations (V group in Fig. 1)						
Boro-37	*O. cernua*	Spain, Andalucía, Almería, Níjar-Lucainena	2006	*Launaea lanifera* Pau	9	19
Boro-38	*O. cernua*	Spain, Andalucía, Almería, Venta de Los Yesos	2006	*Artemisia barrelieri* Besser.	15	20
Boro-43	*O. cernua*	Spain, Andalucía, Jaén, Jódar	2006	*Artemisia barrelieri* Besser.	7	21
Boro-44	*O. cernua*	Spain, Andalucía, Jaén, Cabra Santo Cristo	2006	*Artemisia barrelieri* Besser.	8	22

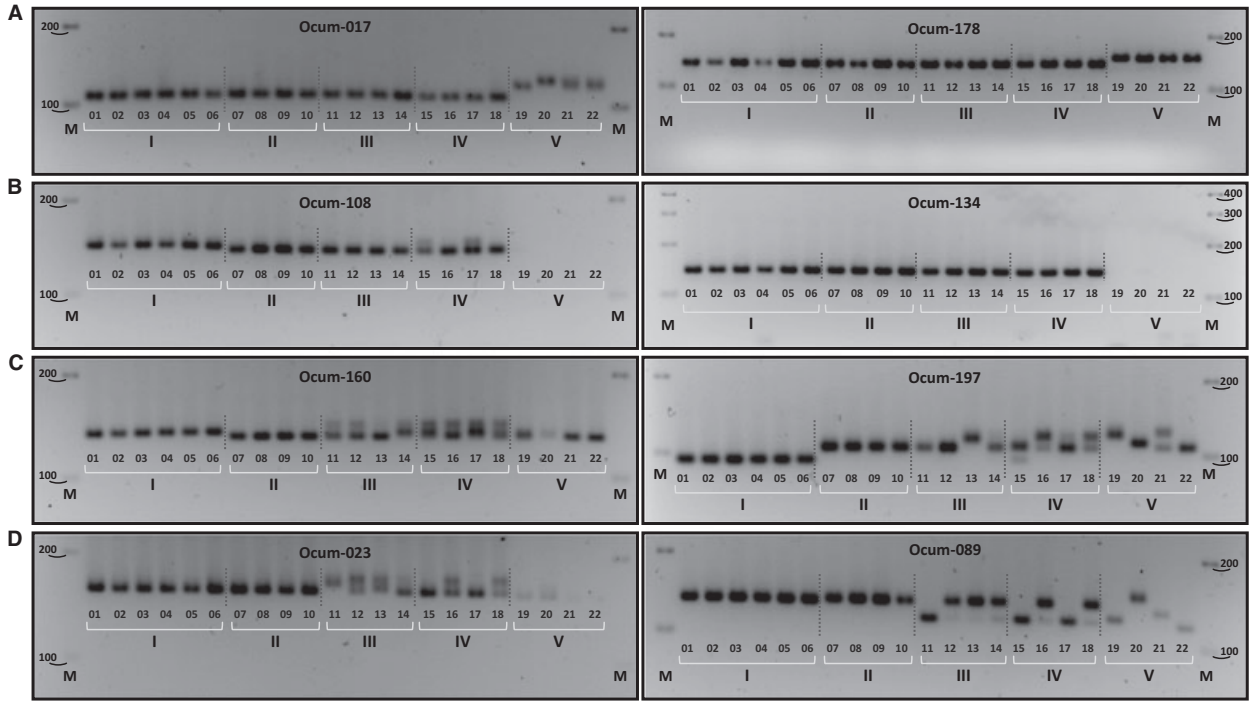

Fig. 1 Allelic variation on a 3% Metaphor agarose gel at the SSR loci: (A) Ocum-017 and Ocum-178, (B) Ocum-108 and Ocum-134 (C) Ocum-160 and Ocum-197 and (D) Ocum-023 and Ocum-089 in 18 populations of *Orobanche cumana* (lines 1 to 18; I, II, III and IV groups) and 4 populations of *O. cernua* (lines 19 to 22; V group) from different origins, as indicated in Table 1.

30 s, and 72°C for 30 s, with a final extension of 20 min at 72°C. For eighteen SSR markers, PCR amplification was optimised by adjusting reaction mixtures to varying concentrations of $MgCl_2$, primers and DNA, or using a non-touchdown PCR program. Amplified products were separated on 3% MetaPhor (BMA, Rockland, ME, USA) agarose gels in 1x TBE buffer with SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Huntingdon, UK) incorporated in the gel, in such a way that microsatellite alleles were effectively resolved with size differences between alleles by 2%. A 100 bp DNA Ladder (Solis BioDyne, Tartu, Estonia) was used as a standard molecular weight marker to get an approximate size of DNA fragments. Resultant gel images were scored manually with the aid of Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The amplification profile for each microsatellite was scored visually and independently.

Those SSR markers showing intense and consistent amplification products were selected for further characterisation in a set of 18 *O. cumana* populations collected on different hosts (sunflower and wild hosts from the Compositae family) and geographical origins, in order to capture maximum genetic variation (Table 1). The populations were collected by the authors with the exception of populations Boro-14 and Boro-15, provided by Dr. Y. Kaya (Trakya Agricultural Research Institute, Edirne, Turkey), Boro-18 and Boro-19, provided by Dr. R. Batchvarova (AgroBio Institute, Sofia, Bulgaria), and SE01, CO06, CU05, CU07, and CU12 provided by Dr. J. Fernández-Escobar (Koipesol Semillas S.A., Sevilla, Spain). Populations Boro-31, Boro-32, Boro-34 and Boro-35 were collected by the authors in collaboration with Dr. A. Pujadas-Salvà (University of Córdoba, Spain) and Dr. K. Stoyanov (Agricultural University of Plovdiv, Bulgaria). Additionally, four populations of the closely related species *O. cernua* (Table 1) collected by Dr. A. Pujadas-Salvà in Southern Spain were also used in the SSR characterisation. The plant samples were frozen at −80°C, lyophilised and ground individually. DNA was extracted from individual plants as mentioned above, and equal amounts of DNA of *Orobanche* spp. plants from each population, as indicated in Table 1, were pooled and used as a template for PCR amplification following the conditions mentioned above. Amplified products were resolved and scored as previously described, with the exception that in this case ambiguous data were re-examined in a new electrophoresis run on Novex Pre-Cast 10% or 20% Polyacrylamide TBE Gels (Life Technologies Corporation, Carlsbad, CA, USA).

Table 2 Frequency and distribution of different types of SSRs identified in the analyses of 4200 SSR-containing unique and consensus sequences, number of them found in compound SSR associated to motifs used for library enrichment (no. in compound SSR LE), and number of primers designed per motif type

SSR motif*	Number of repeat units																							Total	No. in compound SSR LE	No. of primers designed
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	>25			
AC	1357	775	435	359	306	178	122	81	62	56	48	50	33	29	22	24	12	8	9	8	7	1	15	3997		96
AG	776	324	289	390	264	170	99	83	66	53	41	45	35	34	35	33	35	19	27	19	12	13	162	3024		87
AAG	228	119	60	32	15	17	8	5	3	5	5	3	4	3	2	–	1	–	1	–	–	–	1	512		58
AT	259	63	45	33	16	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	421	173	5
AAC	117	57	19	9	6	4	–	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	214		32
ATC	44	15	12	5	6	–	1	–	2	–	–	–	–	–	1	–	1	–	–	1	–	–	2	90	0	3
AGG	41	20	10	5	5	1	2	1	1	–	–	2	–	2	–	–	–	–	–	–	–	–	–	90		5
ATAC	19	5	4	2	–	2	1	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	35		4
CG	22	9	2	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	34	22	
AGGG	16	8	7	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	34	26	
ACG	22	3	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	26	0	
ATAG	10	5	1	1	3	–	–	–	1	1	–	2	–	–	–	–	–	–	–	–	–	–	–	24	9	
ACC	13	4	2	1	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	22	6	1
AAT	16	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	20	0	1
ACT	7	3	–	–	1	–	–	–	–	1	1	1	–	–	–	–	–	–	1	–	–	–	–	15	6	3
AACTG	7	3	1	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	13	0	
AGC	3	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	0	1
AAGGAG	2	1	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	3	
CCG	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	0	
AAAC	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	3	
AAAT	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	0	
AAAG	3	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	0	
ACGC	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	3	
ACCT	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	0	
AGAGGG	1	–	–	–	–	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	3	0	
AACCT	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	–	1	–	–	–	–	–	–	3	0	
Others†																								32		2
Total																								8644		298

*Motif types are fully standardised. Those used for library enrichment are highlighted with grey lines.
†Other SSR motifs represented only once.

Data collection and analysis

Because SSR markers are co-dominant and the samples were bulked, the amplified DNA bands represented different alleles. Thus, different banding patterns were scored as different genotypes. Two measures of marker informative value, defined as the probability that a marker will distinguish between two randomly selected individuals in a population, were calculated with POWERMARKER V3.0 software package (Liu & Muse, 2005): (i) observed number of alleles and (ii) polymorphism information content (PIC).

Analysis of SSR bands was done following a 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. Dice similarity index (Dice, 1945) was calculated as follows: $S_{ij} = 2a/(2a+b+c)$, where a is the number of bands common to population i and population j, b is the total number of bands present only in population i, and c the total number of bands present only in population j. Cluster analysis based on the similarity matrix was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method of NTSYSpc ver. 2.21o (Exeter Software, Setauket, NY, USA) (Rohlf, 2010). Monomorphic markers were excluded from the analysis. The cophenetic correlation coefficient was calculated and Mantel's test (Mantel, 1967) was performed to check the goodness of fit of cluster analysis to the matrix on which it was based. The randomisation procedure included 1000 permutations.

Results

A total of 4200 unique and consensus sequences containing an SSR were identified (Table S2), which represented 17.5% of the starting number of reads, with 8644 SSR loci being detected (Table 2). The SSR average counts per million base pairs were 6931, with an average motif length of 16.32 bp. The number of compound SSRs was 2403, corresponding to 278 SSRs in compound formation per thousand SSRs. Twenty-six different core SSR sequences, represented more than once, were identified. From them, 91.4% of the SSR motifs corresponded with those used for DNA library enrichment (Table 2), which accounted for 94.6% of the 298 designed primers (Table 2). A significant number (251 of 772; Table 2) of those SSR motifs that were not used for enrichment were associated in compound SSRs with motifs employed in the library enrichment. Considering the length of the SSR motif, 7476 dinucleotide, 1000 trinucleotide, 124 tetranucleotide, 24 pentanucleotide and 20 hexanucleotide SSR motifs were identified (Table 2), with average lengths of 16.1, 16.6, 21.5, 32.2 and 34.0 bp, respectively. Following the selection criteria, all of them contained at least four repetitions of the microsatellite motif (Table 2). Based on sequence alignments in 634 contigs containing SSRs, a total of 13 *in silico* polymorphic SSRs were identified. They corresponded with AC or AG dinucleotide motifs and showed an average allele difference of 2.5 nucleotides.

From the 4200 non-redundant sequences containing microsatellites motifs, a total of 2322 primers pairs were designed, of which 298 were bioinformatically validated and selected (Table S3). From them, a subset of 217 SSR primer pairs was tested in a screening panel with four *O. cumana* samples. From 195 SSR markers that amplified successfully, most of them (76%) amplified a strong band of the expected size [quality (+++); Table S3], of which 31% showed a clear polymorphism among the four *O. cumana* samples. A total of 157 markers including all markers amplifying a very good quality product [quality (+++);Table S3], and those polymorphic markers showing moderate signal but easy to score [quality (++);Table S3] were selected for further characterisation with a set of 18 *O. cumana* populations. All SSR markers amplified one locus of the expected size, with the exception of markers Ocum-014 and Ocum-152, which amplified two loci. A total of 306 alleles were identified with a range of 1–10 alleles per locus and an average of 1.95 alleles per locus. In relation to how informative the SSR markers were, 79 of them (50.3%) were polymorphic, showing a moderate overall PIC value of 0.37, with 20 of the markers being highly informative (PIC > 0.50), 39 moderately informative (0.25 < PIC <0.50) and 20 slightly informative (PIC < 0.25) (Table 3; Table S3). No significant association was found between PIC values and number of repeats within each SSR ($r = 0.07$, $P > 0.05$) or total SSR length ($r = 0.12$, $P > 0.05$).

Marker transferability to *O. cernua* was high, with 145 (92.4%) of the 157 SSRs tested yielding fragments of moderate to high quality for at least one *O. cernua* population (Fig. 1a; Table S3). A total of 93 (64.1%) of these markers amplified alleles that were not present in *O. cumana* (Fig. 1a). It is also interesting to note that 41 of the 78 markers monomorphic in *O. cumana* showed polymorphism in *O. cernua* (Fig. 1a). A total of 8 SSRs were specific for *O. cumana*, amplifying alleles only in this species (Fig. 1b; Table S3).

Cluster analysis was used to evaluate the resolving power of the SSR markers. This analysis, based on a set of 137 SSR markers polymorphic in the *O. cumana* and *O. cernua* populations, resulted in a dendrogram with a high cophenetic value ($r = 0.997$, $P < 0.01$) that

separated the populations into two main clusters (Fig. 2), corresponding with the two species analysed, *O. cumana* and *O. cernua*. *Orobanche cumana* populations clustered together at similarity values of 0.67 or higher, while *O. cernua* populations clustered together at similarity values of 0.29. *Orobanche cumana* populations were grouped into three main groups. One cluster contained most of the populations from Eastern Europe, separated into two subgroups that corresponded with populations collected in sunflower (Boro-14 and Boro-15 from Turkey, and Boro-18 and Boro-19 from Bulgaria) and those collected on wild hosts from the Compositae family (Boro-31, Boro-32, and Boro-35 from Bulgaria) (Fig. 2). A second cluster contained all the populations from Central Spain (province of Cuenca). Finally, a third cluster showing larger genetic distance to the other two contained all the populations from Southern Spain (provinces of Córdoba and Sevilla) (Fig. 2). One population from Bulgaria collected on a wild host (Boro-34), excluded from these three main groups, was the most diverse *O. cumana* population, being separated from the rest at a similarity value of 0.67 (Fig. 2).

Diversity varied significantly within the different groups of *O. cumana* populations found in the cluster analysis. Consequently, SSR polymorphic information was analysed in detail within and between each of the main clusters (Table 4 and Table S3). Mean PIC values varied from extremely low levels within the two groups of Spanish populations, with no polymorphism found in populations from Southern Spain and only one polymorphic marker (Ocum-145; Table S3) in populations from Central Spain, to moderate levels within the group of populations from Eastern Europe, with mean PIC value of 0.37 for the three populations growing on wild hosts and 0.38 for the four populations growing on sunflower (Table 4). PIC values were also calculated for combined population groups, which ranged from 0.33 to 0.37 (Table 4 and Table S3). Thus, a number of highly polymorphic SSR markers showing polymorphism among all the population groups were detected, for example, Ocum-160 and Ocum-197 (Fig. 1c and Table S3), but also markers showing polymorphism in only specific population groups, for example, Ocum-023 and Ocum-089 showing allelic variation only in populations from Eastern Europe (Fig. 1d and Table S3).

Discussion

Molecular studies conducted so far in *O. cumana* have been based on isoenzymes (Castejón-Muñoz *et al.*, 1991) or RAPD markers (Gagne *et al.*, 1998). SSRs are currently considered the markers of choice in many areas of molecular genetics, due to their co-dominant and polymorphic nature (Madesis *et al.*, 2013). A valuable set of SSR markers has been isolated from the parasitic weed *O. cumana* and characterised in diverse populations of this species and its close relative *O. cernua*. To our knowledge, this is the first set of SSR markers publicly available thus far for these species, excepting 15 SSR markers reported by Pineda-Martos *et al.* (2013), extracted from the set developed in the present research. SSR markers have been neither reported for other *Orobanche* spp., although SSR sequences have been used as dominant inter-simple

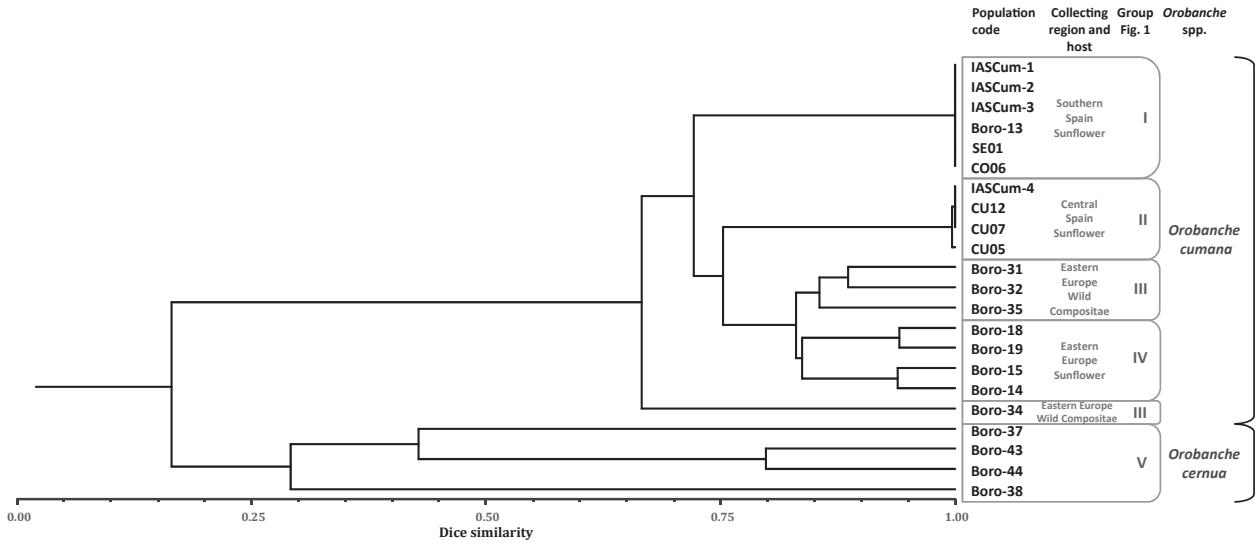

Fig. 2 UPGMA dendrogram based on Dice similarity matrix between 18 populations of *Orobanche cumana* and 4 populations of *O. cernua* obtained with 137 SSR polymorphic markers (for population details see Table 1).

Table 3 Characteristics of 79 validated *Orobanche cumana* SSR markers

Locus	Core sequence	Primer sequence (5′–3′) Forward	Primer sequence (5′–3′) Reverse	Product length (bp)	Amplification quality in *O. cumana* screening panel (n = 4)*	Informativeness in *O. cumana* characterisation panel (n = 18)	
						NA	PIC
Ocum-001	$(CTT)_8$	CGTGCACCATTTCAATTCTT	CATCACATACCGTTACAAGGGA	100	+++ (*)	2	0.362
Ocum-003	$(GAT)_8$	CTCGAACGCAAACTTTTGAA	CAAAGATGGTGGTTTTGCG	94	+++ (*)	4	0.513
Ocum-005	$(AG)_8$	TACAATATCTGCAGCCACGA	CGTGTATACATACGAAATATGTGAAGA	140	+++	2	0.099
Ocum-006	$(CT)_8$	CTTATGTATGTTGTTCTTCTCTGCC	CATACATCCAATTAACATACAAGCA	90	+++ (*)	6	0.530
Ocum-011	$(CA)_8$	GCCGTGAACTCCACTACCAC	GAGTTAGGGTCAGTCTTGCGA	274	+++ (*)	3	0.504
Ocum-013	$(TCT)_8$	TCTTGTGAAGATTATTTTGCAATC	GGAATTGTGTCTGTCTATGGTGTT	243	+++	3	0.269
Ocum-014	$(GAA)_8$	GACAGGCACTCTGAGCACAT	TTGCTTTTCATCTCCCTGCT	209	+++ (*)	3	0.294
Ocum-015	$(AG)_8$	AATGAAAGTGGTAAAGTAGTGTTGC	CATAACGATTTTGCTCTTGTACG	241	+++	2	0.105
Ocum-021	$(CTT)_9$	ACTCGTGAGGGTAACAACGTC	TTCAGAAAGTCAGTCAGGGGA	90	+++ (*)	3	0.194
Ocum-023	$(AG)_9$	CATCACCTCGAGTTTTCCGT	CGCAAGTTCACGAATTGAA	157	+++ (*)	4	0.421
Ocum-030	$(CTT)_9$	CAAGGGTTTTATCCAAATGGG	GAAACCCAGGAAGCAAACAA	106	+++	3	0.370
Ocum-031	$(AC)_9$	AGGTACAAGCACGGAAGCTG	TCTAGCCTGAACCAACTCCA	193	+++ (*)	2	0.372
Ocum-032	$(TG)_9$	CTGAGCACTTCCTTGAACCC	CCTGTAATACTAACAGAATGCCACA	156	+++ (*)	2	0.286
Ocum-033	$(CA)_9$	CTGATGAACCTAAAATATCCCCA	TGGGGTATTGAGTGACGAGA	153	+++ (*)	2	0.178
Ocum-036	$(TC)_9$	CTCTGTGGGCAGAGGCTTTA	TGTGAAGAGAAAAGGGTCGC	101	+++	2	0.321
Ocum-037	$(GA)_9$	ACAATCTCCGGTCACAATCG	CCATGCTCTGCTTGTGAAAA	121	+++ (*)	2	0.346
Ocum-040	$(ATAC)_{10}$	AACAGAATCCATCTCAGGGC	ATGTTGGCATTCTCAAGCCT	105	+++ (*)	3	0.453
Ocum-041	$(AC)_{10}$	TGAAGATGTTGAAAAGCGCA	TTTTCCTTCCTCCCACACTT	92	++ (*)	3	0.371
Ocum-043	$(AGG)_{10}$	AGGTGCACTTAACCTTGACCTT	CTGCAGGTGGTCATGCTAGA	104	++ (*)	2	0.371
Ocum-044	$(TTC)_{10}$	TTCTGGTCTAACAAACCGCA	TTTGTGAGAATTTGATGGCG	128	+++ (*)	2	0.286
Ocum-045	$(AC)_{10}$	CGTCAAGGAGCGGAGAACTA	AAGGGAAGGTTCCTACGTGAA	183	+++	2	0.178
Ocum-047	$(GT)_{10}$	CGTTTTCCGGATCCAAAGT	CCTCACACCGCAGTACAAGA	152	+++ (*)	2	0.367
Ocum-048	$(TTC)_{10}$	AGGGCAAAACGTACTCTGGA	GCCCTTTATCCTCATTCTCTATTG	127	+++	2	0.286
Ocum-052	$(AG)_{10}$	CATGTCTAAGCTTTTGGCTCG	CAAGACTTGGAACAAGCAAATC	108	+++ (*)	3	0.449
Ocum-056	$(CT)_{11}$	CACCCTGCATGTTTCAAAAG	CAAGGGTATTTTCCCTATCTCAA	111	++ (*)	3	0.398
Ocum-059	$(TC)_{11}$	TCTTGATTTGTATATGTCTGATGCAAT	ATGCTACAATAGAAATACACAACGAAC	90	++ (*)	3	0.551
Ocum-063	$(AG)_{11}$	AACCAAGTTGATGCATCCGT	TCCCTCGGCATTCAGACTTA	90	+++ (*)	3	0.568
Ocum-064	$(TCT)_{11}$	GGGCTCTTCATTGAATTTGC	CCGTGGACCCATTTCATTAT	126	+++ (*)	2	0.286
Ocum-066	$(TC)_{11}$	AGGGCTTCATTAAACTGCCT	GGGGAGGAAACATTAGGACA	193	+++ (*)	2	0.105
Ocum-067	$(AC)_{11}$	TGTCCAGTCAATTTCAGAAACG	CGCTCATTCCTGATGCAGA	241	+++	2	0.321
Ocum-069	$(AC)_{11}$	CTGTTGACAGATATCTTGAAGCGGT	AGGACAACTTAAATCGATAAGCA	240	+++	2	0.321
Ocum-070	$(TG)_{11}$	AAGCTGTAAACAATGCCTGAA	CCTCCTCCAGTACCACTAGGC	96	+++ (*)	5	0.672
Ocum-074	$(GA)_{12}$	CCTAAAATTGAAACCTTAAGGAAA	ACTTTCCGTGAGACGGAGTC	99	+++ (*)	6	0.712
Ocum-075	$(CA)_{12}$	TGTGGATAGAGTATAAGCTACCAGTTC	TTCCCGTAGCTTGGAGAATG	110	++ (*)	4	0.450
Ocum-076	$(AC)_{12}$	TTATCAACCAAAGATATGCATTTATCA	TGGCACTATAATCTTCATGCCAC	290	+++	2	0.110
Ocum-079	$(AG)_{12}$	AAATTGGTCTCAAAATCTACCCA	GATTTATAGCATATTTGTTCCACAGA	241	+++	2	0.346

Table 3. (Continued)

Locus	Core sequence	Primer sequence (5′–3′) Forward	Primer sequence (5′–3′) Reverse	Product length (bp)	Amplification quality in *O. cumana* screening panel (n = 4)*	Informativeness in *O. cumana* characterisation panel (n = 18)	
						NA	PIC
Ocum-080	$(AG)_{12}$	AGATCTCACGACCCATTTGC	TCTTCCAAGAAGAACCAAGAAA	115	+++	2	0.105
Ocum-081	$(CA)_{13}$	TTACAAGGTGAAACCACCCA	CAGCTACTGTCCGCAAGAAA	90	++ (*)	5	0.542
Ocum-083	$(AG)_{13}$	GAGAATGCGGATTTCCTGA	GCGACTAGCAACTTTAGTCCG	243	++ (*)	3	0.556
Ocum-084	$(TG)_{13}$	ATGGAATCAAGAGAATGACAACTG	TTTACTAAGACGCGCACACC	140	+++	3	0.194
Ocum-085	$(GA)_{13}$	TCACAAGGAAGTAATGCGGC	CCAGACCCAGCTCTGTGATA	137	+++	5	0.704
Ocum-087	$(TTC)_{13}$	TTCTCGACAGCTTTGGGTAAA	ATGCCAACTTCGAGTGATCC	134	+++ (*)	5	0.642
Ocum-089	$(AC)_{14}$	CCTCACTCTCCTCTGACCCA	AGCTTACGAGCTTTTGCTGC	133	+++ (*)	2	0.239
Ocum-091	$(TTC)_{15}$	CACATAGTGCACCCCTGCTA	TCCCCTTCTTCATATGCCAC	173	+++ (*)	5	0.550
Ocum-092	$(GT)_{15}$	GTCAACCTTGTTAAGGGGAGT	TGCAATCTGATGAGAGTAGAGGA	165	+++ (*)	5	0.719
Ocum-093	$(CA)_{15}$	GGAAGGAAAAAGAAGAACAGGAA	GAATCCTGGAGTGGTGATGG	105	+++	2	0.305
Ocum-094	$(GT)_{15}$	TGGGAGCTTTGTACAGACACTG	GTTTTCTATTAAACCGTAACAAACTCT	141	++ (*)	2	0.371
Ocum-097	$(CA)_{16}$	CAGATATGTTGTTTCGCGGTT	ACGTCAGGTGGGAATGCTAA	159	+++ (*)	3	0.194
Ocum-099	$(AC)_{17}$	TCCAATTCACCAACATTTCG	TACTGCGTAGGTAGTCGCGT	121	+++	3	0.204
Ocum-108	$(GTAT)_6$	TCGTTAATAAGTGGTTCACGAAAA	TGACTAAAAATAAAATGTACGGGTG	143	+++ (*)	3	0.489
Ocum-110	$(TTC)_6$	CCACTTGATTGTTCTTGTAGATTGAA	GCAACTAATATCATCAATCATGGTGTA	90	+++ (*)	2	0.362
Ocum-113	$(CT)_6$	AACCTAATCTGCAGCCGACC	TCTGCATATTTGTCTTCGGC	95	+++	2	0.337
Ocum-115	$(AGA)_6$	AAATCTCGGGAAGCTGGAAT	GCAACATTATTTGCTTTTCCG	105	+++ (*)	2	0.346
Ocum-121	$(AT)_6$	CTTAGGATCCGTTTCCGGC	GGAGTTATATTTGGGGAATCGAA	142	+++ (*)	2	0.362
Ocum-122	$(AGTGTG)_6$	GGAATACATCATTAAAGTAGTTGTCCG	GAAGGAGTCATTAAACTCCGTGA	241	+++ (*)	3	0.555
Ocum-136	(GTT)6	ACGCTGGATCATCGGATTAC	ACGACCGCTACAAGTCCAAT	126	+++	3	0.371
Ocum-141	$(CTT)_6$	CAGCAACTGTTTCTTCCATAGAG	TCCAAGAAGAGGAAAAGAAGTGA	191	+++ (*)	4	0.625
Ocum-145	$(GAA)_6$	AAGATGGCTCATTGCGGTTA	ATCTCGGGCTGAGTTTTCCT	274	+++ (*)	2	0.178
Ocum-151	$(GGA)_6$	ATCTCCGGAGGAAGAGGAAG	CCATCACTCCCCAAGAGTTC	186	+++ (*)	10	0.800
Ocum-152	$(CTT)_7$	GCAGTAGGTTTTATACTTTATTTCTCG	AACATGGGTGAAGTGTGAACAG	141	+++	2	0.329
Ocum-156	$(GA)_7$	CAACGTTTGAGTGTGCTGCT	TGAAGCTAATGGCCAACTCA	124	+++ (*)	2	0.372
Ocum-160	$(AG)_7$	TGAGGGTTTGTAAAGTGGGC	CGTACCTTATCCCTCCGTCA	136	+++ (*)	4	0.685
Ocum-163	$(AC)_7$	AAGCATTTTCCATGCGTGAT	CGGGTTTATTCAATGGGTATG	140	+++	2	0.099
Ocum-166	$(GA)_7$	TGTTGTTATCAAGAGGAAGCGA	CGAAATAAATCAATGGGCTG	94	+++	2	0.359
Ocum-167	$(ACA)_7$	CGACAAGTTGGAAGAAGTGAA	TTCATGTTAGCAGACCCCAA	139	+++	2	0.186
Ocum-174	$(AAG)_7$	CAACCAACAAACAAGTAGTGACG	TCTTGCGGCAAAACCATT	190	+++ (*)	4	0.660
Ocum-176	$(TCGTTG)_7$	GCAATTGTCGACCAAGTACG	CGCCAGATCAACATGAGGT	239	+++	2	0.099
Ocum-184	$(AG)_7$	CCGTTCAATTTTCTCTGCAA	ACGGCGTCTTCCTTGTTGTA	107	+++	2	0.099
Ocum-189	$(CA)_7$	GCACGCATGTACACATACACA	TGCTATTTTCTAGTTTCTATTCATGG	95	++ (*)	2	0.124
Ocum-190	$(TC)_7$	GCCGTTTTCTTTCCTTGTCC	CCCGAAACAAAACACCACTC	98	+++	2	0.099
Ocum-196	$(GT)_7$	GTATGTGCGCCCGTCTTG	GGGGATGACTGTGTTTCGAT	192	+++ (*)	3	0.535

Table 3. (Continued)

Locus	Core sequence	Primer sequence (5′-3′) Forward	Primer sequence (5′-3′) Reverse	Product length (bp)	Amplification quality in *O. cumana* screening panel (n = 4)*	Informativeness in *O. cumana* characterisation panel (n = 18)	
						NA	PIC
Ocum-197	$(GA)_7$	AGAGACGGCATCATCAATCA	GTGATCGTGCAGGCACCTA	95	+++ (*)	6	0.683
Ocum-199	$(TG)_7$	TTGGGTATTTGGTTTCCTGG	GTGTCTCGATCTTCACCCCT	91	+++ (*)	2	0.346
Ocum-206	$(TG)_8$	CCGATTGCTGTTTATGTTGTATT	TGTAGGAGATGCCCAGTTCA	119	+++ (*)	2	0.346
Ocum-209	$(CT)_8$	AATTTTGCATTAATCCGCGA	TTCAGGATCCCCATCTTCAG	130	+++	2	0.286
Ocum-213	$(GA)_8$	GGTACGGCATCCACTCTGAT	TTCGGGCCTCCTTACTTTCT	97	+++ (*)	2	0.321
Ocum-215	$(TG)_8$	GCTACATTGGCACATTTGTATTTC	TTCAACTCTGCTGTATTGCCA	112	+++	2	0.105
Ocum-216	$(TG)_8$	GCAATTGTTCATGTATCTTGCG	ACGCACATGACCATTACGAG	124	+++ (*)	2	0.346
Ocum-217	$(CA)_8$	TTGTTCGACTGGATGAAAGG	CACTTGTGGGGCATCATTT	120	+++ (*)	3	0.371

†Amplification quality as described in text.
(*) Polymorphism in the screening panel.
NA, Number of alleles; PIC, Polymorphism information content.

sequence repeats (ISSR) markers for diversity studies within this genus (Benharrat *et al*., 2002; Román *et al*., 2002; Thorogood *et al*., 2008; Hristova *et al*., 2011). SSR markers are being used in other parasitic species of the Orobanchaceae such as *Striga hermonthica* (Delile) Benth. (Yoshida *et al*., 2010), *Conopholis americana* (L.) Wallr. (Rodrigues *et al*., 2012), *Melampyrum sylvaticum* L. (Crichton *et al*., 2012) and *Rhinanthus* spp. (Ducarme & Wesselingh, 2013), among others.

The strategy followed in this research for microsatellite isolation was next-generation sequencing of genomic *O. cumana* DNA enriched for microsatellite motifs. This approach has been proven to be efficient when SSR discovery is the primary goal and to reduce time and cost for microsatellite marker development in non-model species (Allentoft *et al*., 2009; Zalapa *et al*., 2012; Schoebel *et al*., 2013). The percentage of raw reads that contained microsatellite motifs was 17.5%, which is similar to the value reported by Graignic *et al*. (2013) in *Acer saccharum* Marsh. using the same approach of SSR-enriched shotgun pyrosequencing as in the present research, though higher percentages have been reported in other studies (Lepais & Bacles, 2011). Inferences of microsatellite abundance in the *O. cumana* genome based on the sequence data could not be carried out, owing to the high level of biased redundancy introduced by enrichment of the library. As in previous reports (Martin *et al*., 2010; Lepais & Bacles, 2011), the enriched SSR motifs outnumbered non-enriched motifs. Nevertheless, some information on the SSR representation in the *O. cumana* genome can be extracted from the relative frequencies per motif. Thus, dinucleotide motifs are the most abundant, with the frequency of SSRs decreasing while increasing motif length. Also, the preference of (A+T)-rich repeats within tri-nucleotide SSRs was observed. These observations are in line with the results obtained in other plant genomes (Tóth *et al*., 2000; Sonah *et al*., 2011; Meglécz *et al*., 2012).

One of the advantages of SSR markers is their high level of polymorphism, even between closely related lines (Madesis *et al*., 2013). The percentage of polymorphic SSR markers obtained in this study (50.3%) was higher than that obtained with other marker systems in *O. cumana*. For example, Gagne *et al*. (1998) obtained 43% of polymorphic RAPD bands among eight *O. cumana* populations from Spain and Eastern Europe, whereas Gagne *et al*. (2000) reported 29% of AFLP polymorphism among 48 individuals from two *O. cumana* populations. These differences might be attributed to the marker system, but also to the highly diverse *O. cumana* population set used in this study, which included populations from different geographical origins and hosts.

Table 4 SSR polymorphic information within (highlighted in grey) and between the main *Orobanche cumana* clusters found in the diversity analysis

	Orobanche cumana population groups*											
	Host: Sunflower									Host: Wild Compositae		
	Southern Spain (6 pop.)			Central Spain (4 pop.)			Eastern Europe (Bulgaria, Turkey) (4 pop.)			Eastern Europe (Bulgaria) (3 pop.)		
	NA	Npm	PIC	NA	Npm	PIC	NA	Npm	PIC	NA	Npm	PIC
Host: Sunflower												
Southern Spain	156 (1)	0	–	203 (1–2)	47	0.360	232 (1–5)	58	0.332	226 (1–4)	52	0.325
Central Spain				157 (1–2)	1	–	229 (1–5)	57	0.349	218 (1–4)	49	0.336
Eastern Europe (Bulgaria, Turkey)							202 (1–4)	38	0.377	230 (1–7)	52	0.370
Host: Wild Compositae												
Eastern Europe (Bulgaria)										192 (1–3)	33	0.366

*Population groups included the *O. cumana* samples as found in the diversity analyses (main clusters in Fig. 2). For population details, see Table 1.
NA: Total number of alleles (mean and range); Npm: Number of polymorphic markers; PIC: Mean PIC value for polymorphic markers.

Transferability of SSR markers to *O. cernua* was high, which was an expected result since *O. cumana* and *O. cernua* are considered very closely related taxa (Pujadas-Salvà & Velasco, 2000). Allele specificity was also high, with 64% of the markers showing species-specific alleles, which resulted in a clear discrimination between *O. cumana* and *O. cernua* populations. These results are in agreement with those based on other molecular markers systems, such as RAPDs (Katzir *et al.*, 1996; Paran *et al.*, 1997; Román *et al.*, 2003) or ISSRs (Benharrat *et al.*, 2002), as well as those based on ecological, morphological and biochemical data (Pujadas-Salvà & Velasco, 2000), which clearly support the separation between *O. cumana* and *O. cernua* and the treatment of both taxa as different species. It is also interesting to note that genetic diversity observed in *O. cumana* was considerably lower than in *O. cernua*, despite the reduced and 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.

We found that SSR markers have high resolving power in *O. cumana*, allowing the clustering of populations according to their different geographical origins and hosts. Because this resolving power, the SSRs described in this research should prove useful for several downstream applications. SSRs can be used to improve our understanding of the genetic diversity in *O. cumana*. As an example, a highly polymorphic subset of 15 of these SSR markers has been used successfully to determine genetic relationships within and among *O. cumana* populations from Spain (Pineda-Martos *et al.*, 2013). Further studies on *O. cumana* diversity in its current distribution area are required for understanding global genetic variability, dispersal routes and evolutionary characteristics of *O. cumana* parasitism. In conclusion, an informative and functional set of SSR markers of good quality was developed. This represents a major advance in the development of molecular tools for research in *O. cumana* and related species, such as *O. cernua*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 List of *Orobanche cumana* populations and number of individuals within each population used for SSR development.

Table S2 Full length sequence of 4200 non-redundant unique and consensus *Orobanche cumana* reads containing a SSR motif. SSR motifs are highlighted with lower case letters.

Table S3 Characteristics of 298 SSR markers developed in *O. cumana*, and validation parameters of a set of 217 SSR markers, in which the quality of the amplification in a screening panel of four *O. cumana* populations, the informativeness in a characterisation panel of 18 *O. cumana* populations and the quality of the amplification in four *O. cernua* populations were evaluated.

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Genetic diversity of *Orobanche cumana* populations from Spain assessed using SSR markers

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Summary

Orobanche cumana (sunflower broomrape) is found in Spain as an allochthonous species parasitising exclusively sunflower. For many years, it was distributed in the Guadalquivir Valley and Cuenca province, but in recent years, it has spread to new areas. The objective of this research was to study genetic diversity of *O. cumana* populations from Spain using robust co-dominant molecular markers. Cluster analysis on a set of 50 populations using 15 microsatellite markers revealed the existence of two distant gene pools, one in Cuenca province and another one in the Guadalquivir Valley. Within each gene pool, both inter- and intrapopulation variability were extremely low. This population structure probably reflects a founder effect, with the two genetically distant gene pools deriving from separate introduction events. Different races occurred within the same gene pool, suggesting that current races might have evolved through mutation from a common genetic background. Most of the populations from new areas were identical to the populations from the Guadalquivir Valley. Only a few populations showed larger intrapopulation variation. In these cases, our results suggested the co-existence of both gene pools within the same population, as well as the occurrence of genetic recombination between them. Genetic recombination between distant gene pools is an important mechanism for creating new variation, which might also have an effect on race evolution. These results will contribute to the establishment of improved crop breeding and management strategies for *O. cumana* control.

Keywords: genetic recombination, interpopulation variation, intrapopulation variation, sunflower broomrape.

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Introduction

Orobanche cumana Wallr. (sunflower broomrape) is a holoparasitic plant that parasitises 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 (Škorić *et al.*, 2010), and more recently in France (Jouffret & Lecomte, 2010) and Tunisia (Amri *et al.*, 2012). In most of these areas, *O. cumana* causes severe yield losses in sunflower crops (Fernández-Martínez *et al.*, 2012). In Spain, *O. cumana* is an allochthonous species exclusively found on cultivated sunflower, in contrast to the closely related species *Orobanche cernua* L., which is an autochthonous species that only parasitises wild

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Compositae hosts, mainly *Artemisi*a spp. (Pujadas-Salvà & Velasco, 2000). Infestations of *O. cumana* in Spain were first observed in 1958 in Toledo province on confectionary sunflower (Díaz-Celayeta, 1974). The presence of *O. cumana* in oilseed sunflower fields was detected from the beginning of the 1990s in wide areas of Cuenca province in Castilla-La Mancha region (central Spain) and the Guadalquivir Valley in Andalucía (southern Spain), infecting all commercial hybrids available at that moment (Alonso, 1998). Nowadays, both cultivation areas are seriously affected by *O. cumana*. Additionally, the parasite has spread to other areas of Andalucía, Castilla-La Mancha and Castilla y León (northern Spain) (Fernández-Escobar *et al.*, 2009).

The *O. cumana*-sunflower parasitic system generally follows the gene-for-gene model (Fernández-Martínez *et al.*, 2012), in which resistance reactions are governed by the interaction of host genes for resistance (*Or* genes) and the corresponding pathogen genes for avirulence (*avr* genes) (Flor, 1971). As a vertical resistance controlled by major genes, the virulence of a given *O. cumana* population is commonly measured by evaluating susceptibility or resistance reactions on a set of sunflower differential lines, in which the observed traits are incidence and degree of attack, that is, number of emerged shoots per sunflower plant (Vrânceanu *et al.*, 1980; Fernández-Martínez *et al.*, 2012). Following this approach, Vrânceanu *et al.* (1980) identified five races of *O. cumana* in Romania, designated as A to E, with a set of five sunflower differential lines carrying the dominant resistance genes *Or1* to *Or5*, respectively, that provide accumulative resistance to the races. Initial racial studies in Spain using the same differential lines concluded that the races with parasitism profiles similar to Romanian races A, C, D and E were present (Alonso, 1998). *Orobanche cumana* populations overcoming all the known resistance genes to races A to E have been identified, both in the Guadalquivir Valley (Alonso *et al.*, 1996), as well as in Cuenca (Molinero-Ruiz *et al.*, 2008). Populations from Cuenca and the Guadalquivir Valley have been reported to differ in their aggressiveness on some differential lines (Molinero-Ruiz *et al.*, 2008).

Molecular studies on genetic diversity within and among populations of *O. cumana* are scarce. Castejón-Muñoz *et al.* (1991a) studied intrapopulation diversity in five *O. cumana* populations from southern Spain using isoenzymes. They found low genetic diversity in four populations, but greater genetic diversity in the fifth population. Gagne *et al.* (1998) used RAPD markers to study genetic variability in three populations from Bulgaria, one population from Turkey, one population from Romania and three populations from Spain (Guadalquivir Valley). They identified low intrapopulation and large interpopulation genetic variation. They also found that the Spanish populations were closer to the Romanian population, than to the populations from Bulgaria and Turkey (Gagne *et al.*, 1998).

Studies on genetic variability of *O. cumana* populations from Spain have been based on restricted numbers of populations and marker types. A larger-scale evaluation would contribute to a better understanding of genetic structure and dynamics of *O. cumana* populations. Accordingly, the objective of this research was to study genetic diversity in a large set of *O. cumana* populations from Spain, using robust, neutrally evolving and co-dominant SSR markers.

Materials and methods

Orobanche cumana populations

Seeds of fifty *O. cumana* populations were collected from 1988 to 2008 in sunflower fields of Spain, both in the two traditional areas of *O. cumana* occurrence (Cuenca province in Central Spain and Guadalquivir Valley in southern Spain; 39 populations), as well as in areas where infestations have only recently been observed (11 populations) (Fig. 1 and Table 1). From 50 to 300 mature, *O. cumana* plants were collected and bulked at each population. Seeds were stored in glass jars at 4°C in the dark. A previous study showed that *O. cumana* seed stocks maintained their viability and

Fig. 1 Map of Iberian Peninsula showing provinces in which *Orobanche cumana* populations were collected, both in traditional infestation areas (grey) as well as areas of recent broomrape infestations (white). AB = Albacete, BA = Badajoz, CA = Cádiz, CO = Córdoba, CR = Ciudad Real, CU = Cuenca, HU = Huelva, SE = Sevilla, VA = Valladolid.

Table 1 Origin of the Spanish *Orobanche cumana* populations used in this study

Population	Collecting site (Region, Province, City)	Collection year	Race
AB01	Castilla-La Mancha, Albacete, Albacete	1999	E
AB02	Castilla-La Mancha, Albacete, Albacete	2006	E
BA01	Extremadura, Badajoz, Azuaga	2006	F
BA02	Extremadura, Badajoz, Azuaga	2003	F
CA01	Andalucía, Cádiz, Tarifa	1998	E
CA02	Andalucía, Cádiz, Rota	2002	F
CA03	Andalucía, Cádiz, Villamartín	2007	F
CA04	Andalucía, Cádiz, Arcos de la Frontera	2008	F
CO01	Andalucía, Córdoba, La Carlota	1992	E
CO02	Andalucía, Córdoba, Aldea Quintana	1995	E
CO03	Andalucía, Córdoba, La Carlota	1995	F
CO04	Andalucía, Córdoba, La Carlota	1996	E
CO05	Andalucía, Córdoba, El Carpio	1998	ND
CO06	Andalucía, Córdoba, La Carlota	2001	F
CO07	Andalucía, Córdoba, Córdoba	2004	<E
CR01	Castilla-La Mancha, Ciudad Real, Ciudad Real	1998	E
CU01	Castilla-La Mancha, Cuenca, Villarejo de Fuentes	1988	E
CU02	Castilla-La Mancha, Cuenca, La Almarcha	1995	E
CU03	Castilla-La Mancha, Cuenca, Carrascosa del Campo	1995	E
CU04	Castilla-La Mancha, Cuenca, Palomares del Campo	1996	F
CU05	Castilla-La Mancha, Cuenca, La Almarcha	1996	E
CU06	Castilla-La Mancha, Cuenca, Montalbo	1996	F
CU07	Castilla-La Mancha, Cuenca, Carrascosa del Campo	1996	E
CU08	Castilla-La Mancha, Cuenca, Carrascosa del Campo	1996	F
CU09	Castilla-La Mancha, Cuenca, Montalbo	1996	F
CU10	Castilla-La Mancha, Cuenca, Carrascosa del Campo	1996	E
CU11	Castilla-La Mancha, Cuenca, Montalbo	1998	F
CU12	Castilla-La Mancha, Cuenca, Palomares del Campo	2008	F
CU13	Castilla-La Mancha, Cuenca, Segóbriga	2000	E
CU14	Castilla-La Mancha, Cuenca, Villaescusa de Haro	1994	E
HU01	Andalucía, Huelva, Manzanilla	2003	F
SE01	Andalucía, Sevilla, El Coronil	1989	<E
SE02	Andalucía, Sevilla, El Coronil	1991	<E
SE03	Andalucía, Sevilla, Écija	1994	E
SE04	Andalucía, Sevilla, Écija	1997	F
SE05	Andalucía, Sevilla, Marchena	1998	F
SE06	Andalucía, Sevilla, Écija	1999	F
SE07	Andalucía, Sevilla, Los Palacios	1999	E
SE08	Andalucía, Sevilla, Alcalá de Guadaira	2001	F
SE09	Andalucía, Sevilla, Écija	2006	F
SE10	Andalucía, Sevilla, Écija	2007	F
SE11	Andalucía, Sevilla, Los Morales	2008	F
SE12	Andalucía, Sevilla	2000	F
SE13	Andalucía, Sevilla, Écija	1994	E
SE14	Andalucía, Sevilla, Osuna	1988	ND
SE15	Andalucía, Sevilla, Écija	1990	<E
SE16	Andalucía, Sevilla, Écija	1994	E
SE17	Andalucía, Sevilla, Écija	1994	E
SE18	Andalucía, Sevilla, Écija	2004	F
VA01	Castilla-León, Valladolid, Valladolid	2008	F

ND: not determined.

infectivity after 17 years stored in the dark under laboratory conditions (Molinero-Ruiz *et al*., 2008). *O. cumana* populations were putatively assigned to a given race on the basis of the resistance characteristics of the sunflower host on which they were collected and confirmed in tests of artificial inoculation with sunflower differential lines (Vrânceanu *et al*., 1980). Artificial inoculation tests were carried out as described below. Populations were classified as race E, race F or race below E (<E). In the latter case, the specific race was not determined because of the lack of appropriate differential lines.

Population multiplication and tissue collection

Orobanche cumana populations were multiplied in 2009 on plants of the sunflower line B117, derived from a confectionary landrace population collected by L. Velasco in Valdepeñas (Jaén, Andalucía). B117 was used because it is very susceptible to all *O. cumana* populations tested thus far in our research programme, and accordingly, it is not expected to distort the structure of the populations.

Small pots 7 × 7 × 8 cm were filled with a mixture of sand and peat (1:1 by vol). The soil mixture was carefully mixed with 50 mg of *O. cumana* seeds to obtain a homogeneously infested substrate. Sunflower seeds of line B117 were germinated on moistened filter paper in Petri dishes, and 2-day-old seedlings were planted in the pots. Three pots per *O. cumana* population were used. The plants were maintained in a growth chamber for 21 days at 25°C/20°C (day/night) with a 16-h photoperiod for incubation. After this time, the plants were transplanted to pots containing 3 L of an uninfested sand-silt-peat (2:1:1 by vol) soil mixture and maintained under open-air conditions. The plants were grown in the spring–summer season, from mid-March to mid-August. They were watered as needed and were not fertilised. Fresh tissue samples from 20 individual *O. cumana* plants of each population were collected before the beginning of flowering, except for a few populations in which a lower number of parasites emerged. Freshly harvested tissue was stored at −80°C.

DNA extraction, amplification and electrophoresis

The plant tissue was lyophilised and ground to a fine powder in a laboratory mill. DNA was isolated from individual *O. cumana* plants using a modified version of the protocol described in Pérez-Vich *et al.* (2004). For the study of interpopulation diversity, equal amounts of DNA of *O. cumana* plants from each population were pooled and used as a template for PCR amplification. For the study of intrapopulation diversity, DNA from 12 individual plants was used as a template for PCR amplification, with the exception of population CU03 in which 11 individual plants were used. DNA samples within each population were randomly chosen.

Pooled DNA samples from four populations were screened with a set of 217 SSR primers developed for *O. cumana* (Genoscreen, Lille, France). From these, 157 SSR were selected, because they produced intense and consistent amplification products. This set of SSR primers was screened for polymorphism in 10 populations, selected from different geographical origins. Monomorphic markers were excluded from the analysis, leaving a total of 15 high-quality polymorphic SSR primers (Table 2) that were used for genotyping the complete set of populations both in the study of interpopulation and intrapopulation diversity. The PCR reaction mixture (30 μL) contained 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP (dNTP Set and Mix, Solis BioDyne, Tartu, Estonia), 0.3 μM of primer, 0.05 U μL^{-1} of *Taq* DNA polymerase (FIREPol® DNA Polymerase, Solis BioDyne, Tartu, Estonia) and 50 ng of DNA template. DNA amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) as described for SSRs by Pérez-Vich *et al.* (2004). Amplification products were resolved by electrophoresis on 3% Metaphor agarose (BMA, Rockland, ME, USA) gels in 1 × TBE buffer at 100 V constant, with SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Huntingdon, UK)

Table 2 Simple sequence repeats markers used for evaluating genetic diversity in 50 *Orobanche cumana* populations from Spain

Marker	5′ → 3′ Forward	5′ → 3′ Reverse	Size	Motif	Alleles
Ocum-52	CATGTCTAAGCTTTTGGCTCG	CAAGACTTGGAACAAGCAAATC	108	$(AG)_{10}$	2
Ocum-59	TCTTGATTTGTATATGTCTGATGCAAT	ATGCTACAATAGAAATACACAACGAAC	90	$(TC)_{11}$	2
Ocum-70	AAGCTGTAAACAATGCCTGAA	CCTCCTCCAGTACCACTAGGC	96	$(TG)_{11}$	3
Ocum-74	CCTAAAATTGAAACCTTAAGGAAA	ACTTTCCGTGAGACGGAGTC	99	$(GA)_{12}$	2
Ocum-75	TGTGGATAGAGTATAAGCTACCAGTTC	TTCCCGTAGCTTGGAGAATG	110	$(CA)_{12}$	2
Ocum-81	TTACAAGGTGAAACCACCCA	CAGCTACTGTCCGCAAGAAA	90	$(CA)_{13}$	2
Ocum-87	TTCTCGACAGCTTTGGGTAAA	ATGCCAACTTCGAGTGATCC	134	$(TTC)_{13}$	3
Ocum-108	TCGTTAATAAGTGGTTCACGAAAA	TGACTAAAAATAAAATGTACGGGTG	143	$(GTAT)_6$	2
Ocum-122	GGAATACATCATTAAAGTAGTTGTCCG	GAAGGAGTCATTAAACTCCGTGA	241	$(AGTGTG)_6$	2
Ocum-141	CAGCAACTGTTTCTTCCATAGAG	TCCAAGAAGAGGAAAAGAAGTGA	191	$(CTT)_6$	2
Ocum-160	TGAGGGTTTGTAAAGTGGGC	CGTACCTTATCCCTCCGTCA	136	$(AG)_7$	2
Ocum-174	CAACCAACAAACAAGTAGTGACG	TCTTGCGGCAAAACCATT	190	$(AAG)_7$	2
Ocum-196	GTATGTGCGCCCGTCTTG	GGGGATGACTGTGTTTCGAT	192	$(GT)_7$	3
Ocum-197	AGAGACGGCATCATCAATCA	GTGATCGTGCAGGCACCTA	95	$(GA)_7$	2
Ocum-206	CCGATTGCTGTTTATGTTGTATT	TGTAGGAGATGCCCAGTTCA	119	$(TG)_8$	2

incorporated in the gels and visualised under UV light. A 100-bp DNA ladder (Solis BioDyne, Tartu, Estonia) was used as a standard molecular weight marker to get an approximate size of DNA fragments. Bands were scored manually with the aid of Quantity One® 1-D Analysis Software (Bio-Rad Laboratories Inc, Hercules, CA, USA).

Statistical analysis

Amplified fragments were scored for the presence (1) or absence (0) of homologous bands. Data were compiled into a binary data matrix. Dice similarity index (Dice, 1945) was calculated as: $S_{ij} = 2a/(2a + b + c)$, where a is the number of bands common to population i and population j, b is the total number of bands present only in population i and c the total number of bands present only in population j. A cluster analysis based on the similarity matrix was performed using the UPGMA (unweighted pair group method with arithmetic mean) method of NTSYSpc ver. 2.21o (Exeter Software, Setauket, NY, USA) (Rohlf, 2010). The cophenetic correlation coefficient was calculated, and Mantel's test (Mantel, 1967) was performed to check the goodness of fit of cluster analysis to the matrix on which it was based. The randomisation procedure included 1000 permutations.

Eight populations were selected for the study of intrapopulation variation. Descriptive population genetic statistics (total and effective number of alleles, observed heterozygosity and expected heterozygosity) were calculated using GeneticStudio 0.7 (Richmond, VI, USA) (Dyer, 2009). Cluster analysis was conducted as described previously. The corrected average pairwise distance between individuals within each population was calculated using Arlequin ver. 3.5.1.3 (Excoffier & Lischer, 2010) as a measure of intrapopulation diversity. Analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was conducted on the distance matrix to separate total variance into variance attributable to differences between individuals within a population and variance attributable to differences between populations.

Results

Analysis of interpopulation diversity

Polymorphisms among 50 populations of *O. cumana* collected in different areas of Spain were detected using 15 SSR primers that amplified between 2 and 3 alleles, with a total number of 33 detected alleles (Table 2). All the primer pairs produced PCR products with the expected sizes. Cluster analysis resulted in a dendrogram with a high cophenetic value ($r = 0.96$, $P < 0.01$) that separated the populations into two main clusters (Fig. 2). One of the clusters contained most of the populations from the traditional distribution area of Cuenca (13 of 14), together with one population from a new distribution area (AB01), with no polymorphism being detected between these populations. Two populations were at a larger genetic distance, one of them from the traditional distribution area of the Guadalquivir Valley (SE02) and another one from a new area (CA01). A second cluster contained most of the populations from the provinces of Córdoba and Sevilla in the traditional area of the Guadalquivir Valley (23 of 25), together with eight populations from new areas (BA01, BA02, CA02, CA03, CA04, CR01, HU01, VA01), with no polymorphism within this group. There were three populations at a larger distance, one population from the Guadalquivir Valley (SE07), one population from Cuenca (CU03) and another population from a new area (AB02). The two main clusters were very distant genetically, showing a similarity index below 0.1 (Fig. 2). Clustering was not influenced by the level of virulence of the populations, as populations from different races (Table 1) were clustered together.

Analysis of intrapopulation diversity

The five populations showing larger genetic distance within the two main clusters, that is, SE02 and CA01 from the Cuenca cluster and AB02, CU03 and SE07 from the Guadalquivir Valley cluster, were selected for studying intrapopulation diversity. However, CA01 was discarded, because the number of individuals was low. Additionally, two populations from the uniform group of the Cuenca cluster (CU05, CU08) and two populations from the uniform group of the Guadalquivir Valley cluster (CO02, SE10) were included, to have a reference for the two main gene pools. A total of 95 individuals from the eight populations were scored for each of the 15 SSR markers. Descriptive diversity statistics for populations from the two main gene pools (CU05, CU08, CO02 and SE10) showed the expected values for monomorphic loci, that is, one single allele and values of zero for heterozygosity estimations. Populations showing higher genetic diversity (SE02, AB02, CU03 and SE07) showed significant variation for number of alleles per locus, number of alleles with frequencies p_i >5%, effective number of alleles, observed heterozygosity and expected heterozygosity (Table 3). The average number of alleles per locus ranged from 1.40 in population SE02 to 2.13 in population AB02. The effective number of alleles ranged from 1.20 in population SE02 to 1.97 in population AB02. A total of 31 heterozygous individuals

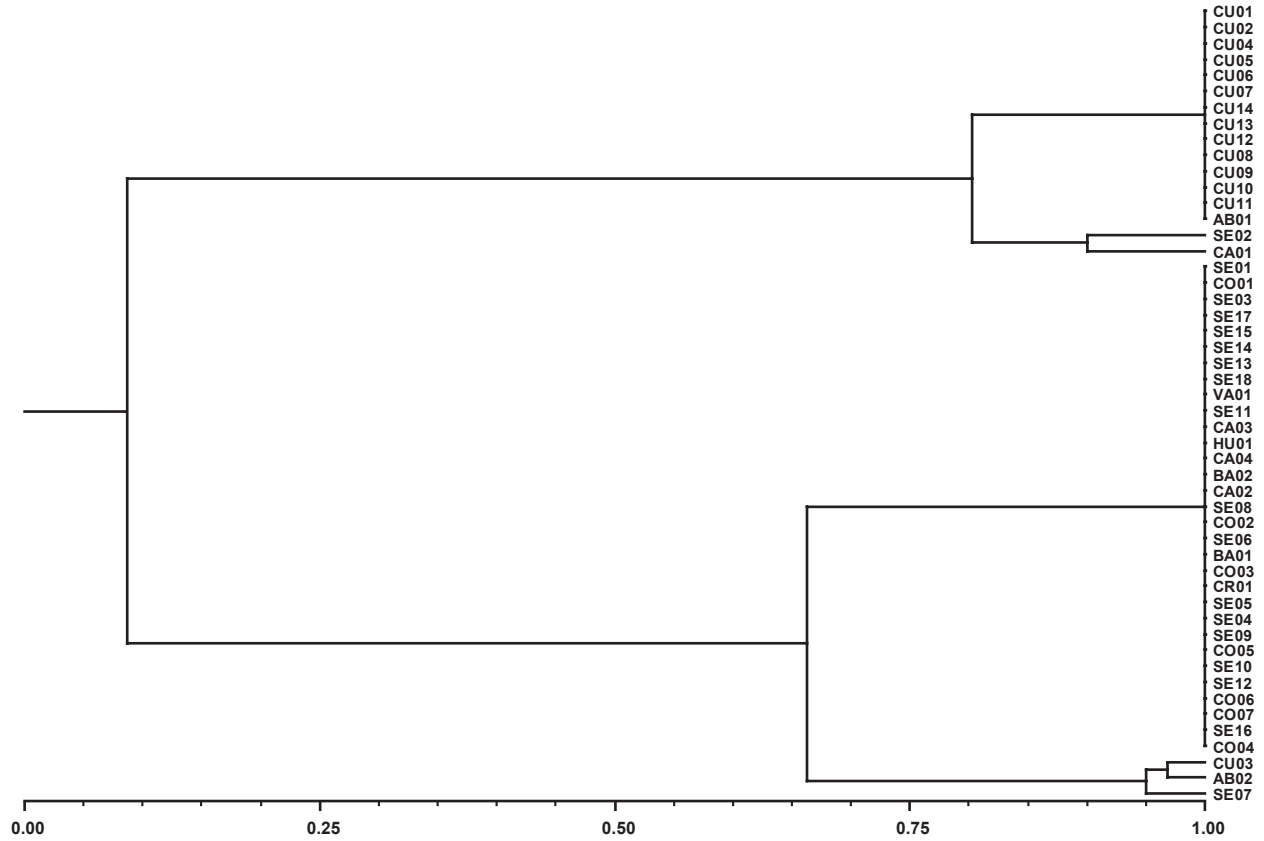

Fig. 2 Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Dice similarity matrix between 50 populations of *Orobanche cumana* (sunflower broomrape) populations collected in Spain using 15 SSR markers. Two-letter prefixes refer to the province in which the population was collected (see Table 1).

Table 3 Mean descriptive population genetic statistics for each of the four populations genetically distant to the main genetic pools (SE02, SE07, CU03, AB02) of *O. cumana* followed by standard deviation values

Population	A	$A_{0.95}$	Ae	Ho	He
AB02	$2.13^{a}\pm0.35$	$2.07^{a}\pm0.26$	$1.97^{a}\pm0.11$	$0.02^{ab}\pm0.04$	$0.49^{a}\pm0.02$
CU03	$2.00^{a}\pm0.00$	$2.00^{a}\pm0.00$	$1.65^{b}\pm0.05$	$0.00^{a}\pm0.00$	$0.39^{b}\pm0.02$
SE02	$1.40^{b}\pm0.51$	$1.33^{b}\pm0.49$	$1.20^{c}\pm0.32$	$0.06^{b}\pm0.09$	$0.12^{c}\pm0.18$
SE07	$1.93^{a}\pm0.46$	$1.93^{a}\pm0.46$	$1.61^{b}\pm0.30$	$0.09^{c}\pm0.10$	$0.35^{b}\pm0.16$

A: number of alleles at a locus; $A_{0.95}$: number of alleles with frequencies $p_i > 5\%$; Ae: effective number of alleles; Ho: observed heterozygosity; He: expected heterozygosity.
Within columns, means that have no superscript in common are significantly different from each other at 0.05 level of probability (Duncan's multiple range test).

were identified at 12 SSR loci. Population SE07 showed the highest heterozygosity value (0.09), whereas population CU03 showed no heterozygous individuals. Values for the mean expected heterozygosity ranged from 0.12 in population SE02 to 0.49 in population AB02. Allelic variation of the individuals of the eight populations at two SSR loci is shown in Fig. 3.

AMOVA analysis revealed no polymorphism within populations CO02, SE10, CU05 and CU08, with all the genetic variation corresponding to interpopulation diversity (Table 4). Conversely, AMOVA conducted on populations SE02, SE07, AB02 and CU03 revealed that most of the variance (68.2%) was attributable to differences between individuals within each population, whereas the remaining 31.8% of the variance corresponded with differences between populations (Table 4), even though populations derived from separated geographical areas. The corrected average pairwise distance between individuals within these populations was

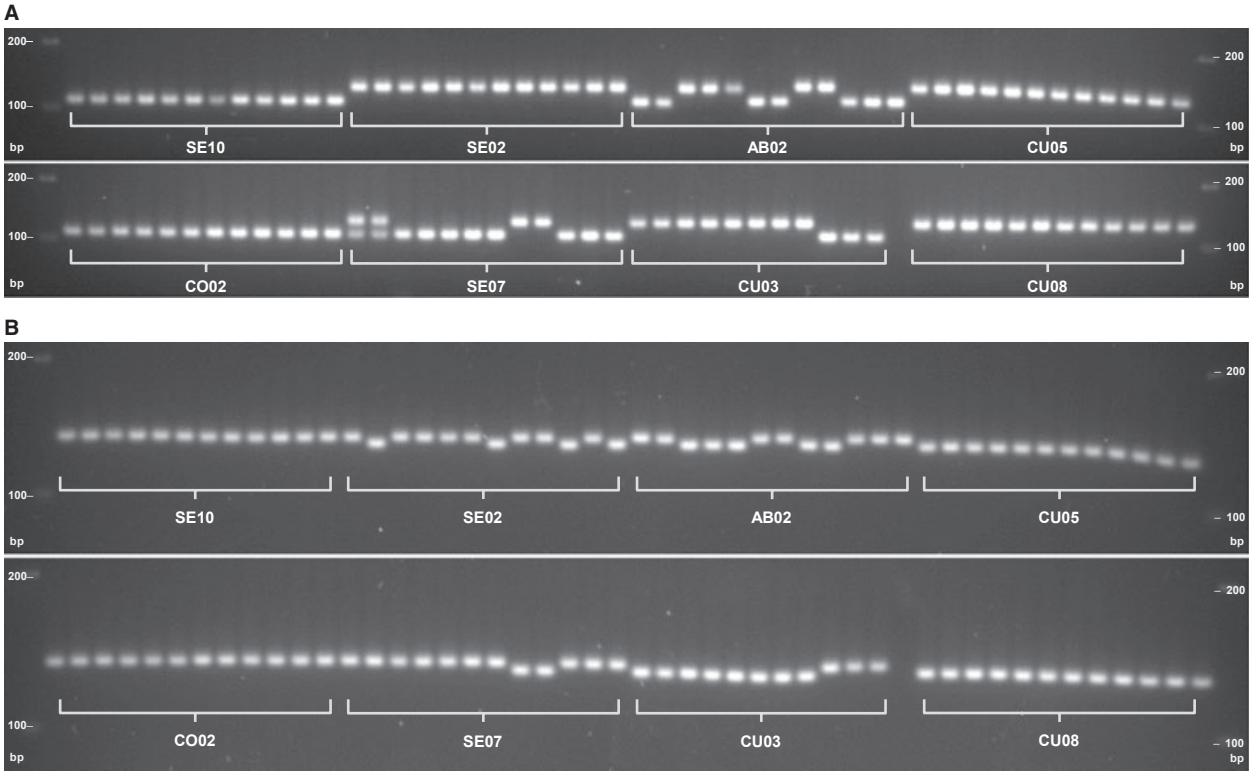

Fig. 3 Allelic variation at the Ocum-52 (A) and Ocum-160 (B) SSR loci in 12 single plants of eight *Orobanche cumana* populations, with the exception of CU03 for which 11 individual plants were included.

Table 4 Analysis of molecular variance in two groups of populations of *O. cumana* from Spain: Group I, including four populations representative of the two main genetic pools identified in Guadalquivir Valley (CO02 and SE10) and Cuenca (populations CU05 and CU08) respectively; Group II, including four populations genetically distant to the main genetic pools (SE02, SE07, CU03, AB02)

Source of variation	Sum of squares	Variance components	%Variation	P-value
Group I				
Among populations	356.00	9.89	100.00	<0.01
Within populations	0.00	0.00	0.00	<0.01
Group II				
Among populations	102.52	2.46	31.80	<0.01
Within populations	226.92	5.28	68.20	<0.01

3.6 ± 2.0 for SE02, 10.7 ± 5.2 for SE07, 16.3 ± 7.8 for AB02 and 12.2 ± 6.0 for CU03.

Cluster analysis on the individual plants from the eight populations also resulted in the separation of two distant clusters (Fig. 4), one of them containing all the individuals of the homogeneous populations of Cuenca (CU05, CU08) and another one containing all the individuals of the homogeneous populations of the Guadalquivir Valley (CO02, SE10). The former cluster also included all the individuals from population SE02, which were genetically distant from the individuals of the homogeneous populations (Fig. 4). Individuals of populations AB02, CU03 and SE07 were distributed among both clusters. Thus, population AB02 included five individuals in the Cuenca cluster, four of them genetically distant to the main group, and seven individuals in the Guadalquivir Valley cluster, all of them genetically similar to the main group. Population CU03 included eight individuals in the Cuenca cluster and three individuals in the Guadalquivir Valley cluster, all of them integrated in the respective main groups in which no polymorphism was identified. Finally, four individuals from population SE07 were grouped in the Cuenca cluster, at a certain genetic distance from the main group (similarity index <0.7; Fig. 4), and eight individuals were grouped in the Guadalquivir Valley cluster, six of them showing no genetic distance to the main group and the other two showing some genetic distance to the main group, with a similarity index below 0.9 (Fig. 4).

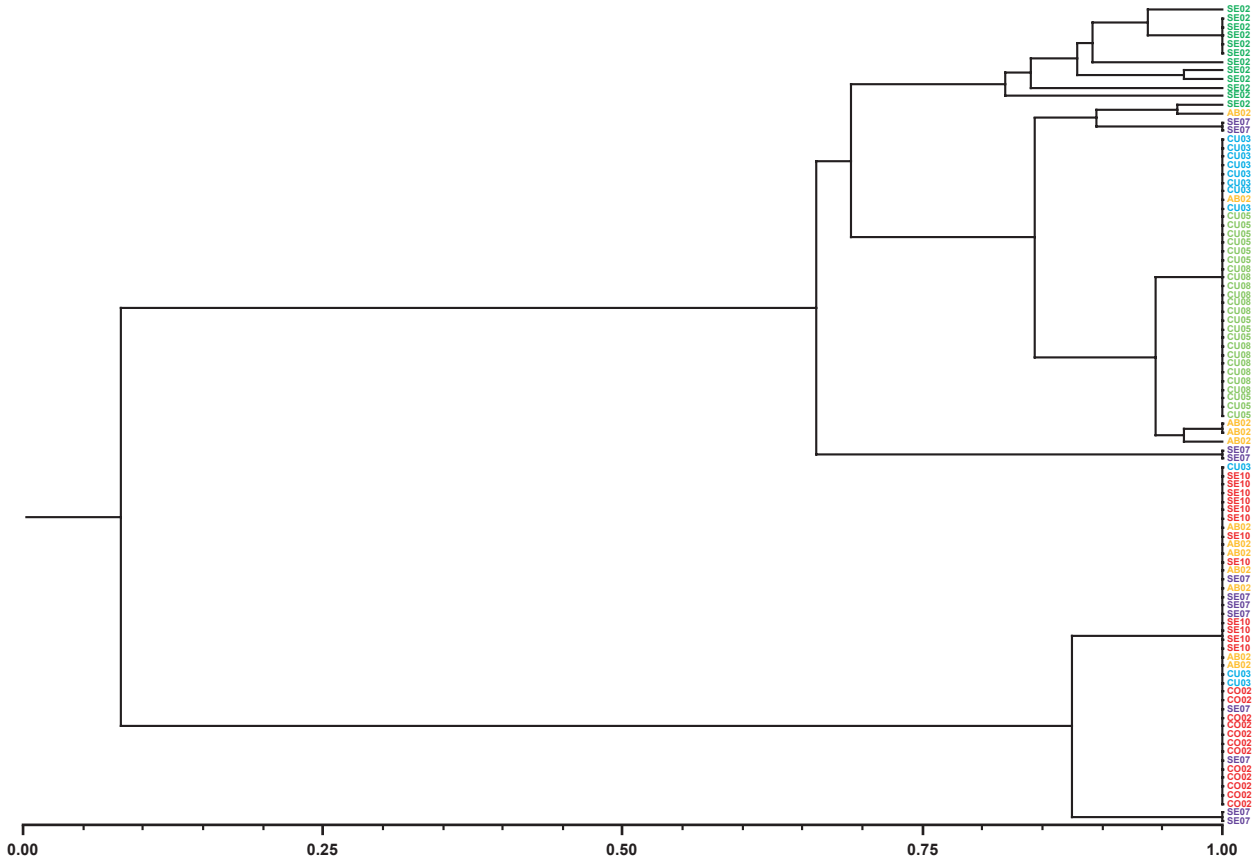

Fig. 4 Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Dice similarity matrix between individual plants of eight *Orobanche cumana* (sunflower broomrape) populations collected in Spain using 15 SSR markers. Two-letter prefixes refer to the province in which the population was collected (see Table 1).

Discussion

Orobanche cumana has a natural area of distribution from Central Asia to South-eastern Europe, where it is found parasitising wild species of the Compositae, mainly *Artemisia* spp. (Beck-Mannagetta, 1930). However, shortly after the expansion of sunflower cultivation in Russia at the end of the 19th century, *O. cumana* plants started attacking this crop (Vrânceanu *et al.*, 1986). In the Iberian Peninsula, *O. cumana* is not found in the wild, but exclusively within sunflower fields (Pujadas-Salvà & Velasco, 2000). Therefore, Spanish populations of *O. cumana* originated from seed introductions from other areas. The great genetic separation between populations of Cuenca and the Guadalquivir Valley suggests that they may derive from seed introductions from different areas. The parasite was observed in Cuenca in 1974 and only 5 years later in southern Spain (González-Torres *et al.*, 1982). The occurrence of extremely low inter- and intrapopulation genetic diversity in most of the populations from Cuenca and the Guadalquivir Valley could be attributed to the founder effect (Mayr, 1954), that is, the two main groups of populations most likely originated from a very small number of individuals introduced in the area from larger populations, together with population isolation. Our results suggest that the original populations from which the current gene pools of Cuenca and the Guadalquivir Valley evolved might be genetically distant. A similar situation of very low intra- and interpopulation diversity has been described for *Orobanche minor* populations in the US, as well as for *Striga gesnerioides* (Willd.) Vatke populations parasitising either cowpea (*Vigna unguiculata* (L.) Walp.) in West Africa or *Indigofera hirsuta* L. in Florida. The low genetic diversity was explained on the basis of a common origin of the populations from a limited number of founder plants (Westwood & Fagg, 2004; Botanga & Timko, 2005; Dube & Belzile, 2010). In the case of the *O. minor* study, the authors also found two clearly different groups of populations, which suggested that they developed from two separate introduction events, as is probably the case for the Cuenca and the Guadalquivir Valley gene pools of *O. cumana* reported in the present study.

In addition to populations from traditional areas of Cuenca and the Guadalquivir Valley, most of the populations from new areas belonged to one of the two gene pools, in most cases to the Guadalquivir Valley gene pool. *Orobanche cumana* populations in Spain exclusively occur in sunflower fields, so that it is conceivable that they spread to new areas due to agricultural practices. Thus, the production of a high number of long-lived tiny seeds (Molinero-Ruiz *et al*., 2008) that can be disseminated by vehicles, machinery and sunflower achenes has been reported as a major factor in the spread of *Orobanche* spp. populations (Castejón-Muñoz *et al*., 1991b; Parker, 1991; Rubiales *et al*., 2009).

A previous study on genetic diversity in eight *O*. *cumana* populations from Spain, Bulgaria, Romania and Turkey reported low intrapopulation variation and therefore little gene exchange between different geographical regions (Gagne *et al*., 1998). Similarly, Castejón-Muñoz *et al*. (1991a) identified low intrapopulation diversity in four of five *O*. *cumana* populations from southern Spain using isoenzymes, but greater genetic diversity in the fifth population. Their results are in line with those obtained in the present research, in which most of the populations fell into two major groups characterised by low intra- and interpopulation diversity, but with a few populations showing great intrapopulation genetic diversity. In these, the presence of individuals genetically similar to those of the two main genetic pools (Cuenca and Guadalquivir Valley) indicated the existence of seed interchange between them, which was probably followed by genetic recombination between plants of both gene pools. Allelic variation at SSR markers provided clear evidence of the existence of genetic recombination between gene pools (e.g. Ocum-52 marker in population SE07; Fig 4), with outcrossing revealed by observed values of heterozygosity from 0.02 to 0.09 in populations SE07, SE02 and AB02. This suggests the possibility of genetic recombination after plants from the two main genetic pools came into contact, giving rise to new genetic variability. *Orobanche cumana* has been considered as a self-pollinated species, on the basis of the occurrence of low intrapopulation genetic diversity (Gagne *et al*., 1998) and flower morphology (Satovic *et al*., 2009). However, a recent study using a gene that determines absence of anthocyanin pigmentation estimated the occurrence of a relatively high rate of cross fertilisation in *O*. *cumana* plants, from 14.8% to 40.0% (Rodríguez-Ojeda *et al*., 2013). The occurrence of genetic recombination between distant populations, documented in the present research, may be an important driving force of race evolution in *O*. *cumana,* with reassortment of avirulence genes conferring specificity against resistance genes, as proposed also by Joel *et al*. (2007).

Clustering of populations was not associated with virulence groups. Populations from traditional areas of Cuenca and the Guadalquivir valley that showed extremely low intra- and interpopulation genotypic diversity within each cluster included several virulence groups. This indicated that virulent populations do not share a common origin and strongly suggests that they might be the result of independent mutational events. Studies on *S*. *gesnerioides* populations collected on cowpea also showed lack of correspondence between genetic diversity and virulence groups in populations showing low genetic variability between and within populations (Dube & Belzile, 2010). Similarly, Semblat *et al*. (2000) found that population structure was not related to race formation in their study of genetic diversity of populations of root-knot nematodes. They concluded that most of the DNA polymorphism was independent of the virulence, as might be the case in the *O*. *cumana*-sunflower gene-for-gene interaction.

Understanding variability within and between *O*. *cumana* populations is important for determining crop breeding strategies for *O*. *cumana* control. The fact that the majority of the populations from new areas belonged to one of the two main gene pools and that there are situations in which the two main genetic pools became into contact indicate the importance of avoiding *O*. *cumana* seed transportation to new areas, such as detection and elimination of contaminated sunflower seed stocks (Dongo *et al*., 2012). Results from this study also suggested that more aggressive *O*. *cumana* genotypes that arose and are fixed within homogeneous gene pools might be a consequence of mutation followed probably by selection, owing to the use of dominant resistance genes (*Or* genes) in sunflower. In this sense, the extensive use of sunflower oilseed cultivars carrying monogenic dominant resistance seems not to be the optimal strategy for long-term *O*. *cumana* control. Additionally, recombination has been shown as a mechanism generating new genetic variability in *O*. *cumana*, which might impact strategies for the breeding of resistant sunflower lines using combination of major *Or* genes. Incorporation of certain levels of quantitative resistance would contribute to attaining long-term resistance to *O*. *cumana*, as has already been suggested (Pérez-Vich *et al*., 2004; Joel *et al*., 2007; Molinero-Ruiz *et al*., 2008; Fernández-Martínez *et al*., 2012.).

In conclusion, this research revealed the existence of two distant gene pools of *O*. *cumana* in Spain, one in Cuenca province in central Spain and another one in the Guadalquivir Valley in southern Spain. The latter has spread to other areas to a larger extent than the

genetic pool from Cuenca, probably through contaminated sunflower seed. Both gene pools have been maintained under a high degree of isolation, which determines low intrapopulation genetic diversity in most of the populations evaluated. However, both gene pools have been found to co-exist in a few populations. Also, different degrees of genetic recombination between both gene pools were detected in some of these populations. Genetic recombination between distant gene pools may be an important mechanism of creating new variation, and consequently, it might be relevant for race evolution.

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Research Article

The Genetic Structure of Wild *Orobanche cumana* Wallr. (Orobanchaceae) Populations in Eastern Bulgaria Reflects Introgressions from Weedy Populations

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Orobanche cumana is a holoparasitic plant naturally distributed from central Asia to south-eastern Europe, where it parasitizes wild Asteraceae species. It is also an important parasitic weed of sunflower crops. The objective of this research was to investigate genetic diversity, population structure, and virulence on sunflower of *O. cumana* populations parasitizing wild plants in eastern Bulgaria. Fresh tissue of eight *O. cumana* populations and mature seeds of four of them were collected *in situ* on wild hosts. Genetic diversity and population structure were studied with SSR markers and compared to weedy populations. Two main gene pools were identified in Bulgarian populations, with most of the populations having intermediate characteristics. Cross-inoculation experiments revealed that *O. cumana* populations collected on wild species possessed similar ability to parasitize sunflower to those collected on sunflower. The results were explained on the basis of an effective genetic exchange between populations parasitizing sunflower crops and those parasitizing wild species. The occurrence of bidirectional gene flow may have an impact on wild populations, as new physiological races continuously emerge in weedy populations. Also, genetic variability of wild populations may favour the ability of weedy populations to overcome sunflower resistance mechanisms.

1. Background

Broomrapes (*Orobanche* spp. and *Phelipanche* spp.) are a group of around 170 holoparasitic plant species mainly distributed in the northern hemisphere. They do not have photosynthetic activity and entirely depend on a host plant for nutrition [1]. Even though most of the *Orobanche* spp. only parasitize wild plants, some of them have become noxious weeds on a variable range of cultivated hosts [2]. This is the case of *Orobanche cumana* Wallr. (sunflower broomrape), which is nowadays one of the most limiting factors for sunflower (*Helianthus annuus* L.) production in Europe and Asia [3].

Orobanche cumana is naturally distributed from central Asia to south-eastern Europe, where it parasitizes wild Asteraceae species, mainly *Artemisia* spp. [4]. Even though it has been considered by some authors as an intraspecific taxon of *Orobanche cernua* L. [5], its treatment as a separate species is nowadays widely accepted [2, 6, 7]. The Black Sea coast in eastern Bulgaria is one of the main natural distribution areas for *O. cumana*, where this species is mainly found parasitizing *Artemisia maritima* L. [8].

Though domesticated in eastern North America and widely used as a staple food in the pre-Columbian period [9], the transformation of sunflower into one of the major world oil crops started in Russia in the second half of the nineteenth

century [10]. Plants of *O. cumana* parasitizing sunflower were observed for the first time in Russia in the 1890s [11]. In Bulgaria, *O. cumana* parasitization on sunflower was first detected in 1935 [12]. Currently, *O. cumana* is present in the main sunflower-producing countries around the world, particularly in Central and Eastern Europe, Spain, Turkey, Israel, Russia, Ukraine, Iran, Kazakhstan, and China [2]. Moreover, the parasite has spread to new areas in recent years [13, 14]. Broomrape seed transport has been suggested as one of the main factors in the dispersion of the infestation [15]. Broomrape seeds are extremely small (dust-like seeds), and individual plants can produce an impressive number that remain viable in the soil for up to 20 years, which are easily dispersed by water, wind, animals, humans, machinery, or though attachment to sunflower seeds [15, 16].

Unlike most weedy *Orobanche* spp., which have a broad range of host crops, weedy *O. cumana* only parasitizes sunflower [2]. The high host specificity of *O. cumana* is probably associated with the mode of inheritance of genetic resistance in sunflower. Whereas in most host crops genetic resistance to *Orobanche* spp. is horizontal, that is, polygenic and nonrace specific, resistance to *O. cumana* in sunflower is primarily vertical, that is, monogenic, dominant, and race specific [16]. The development of sunflower resistant cultivars has been paralleled by the appearance of *O. cumana* populations that overcame sunflower genetic resistance, a recurrent process that has continued until today [11]. Several physiological races of *O. cumana* have been reported. Vrânceanu et al. [17] identified races A through E using five sunflower differential lines carrying the dominant resistance genes *Or1* through *Or5*, respectively. More virulent races named as F, G, and H were later detected in the main sunflower cultivation areas of the Old World [3]. In Bulgaria, races D and E were predominant till few years ago [18], but a more virulent race G has become increasingly important in recent years [19].

There are few studies on genetic interactions between wild and weedy forms of parasitic plant species. Knowledge about such interactions is important because wild vegetation may play a role as reservoir of genetic diversity for overcoming genetic resistance mechanisms in the host crops [20, 21]. But on the other hand, evolution of virulence in weedy populations may also have an impact on the distribution of the species in the wild [22]. Botanga et al. [23] used seeds of eight populations of the parasitic weed *Striga asiatica* (L.) Kuntz collected on wild hosts to conduct infestation experiments on susceptible maize and sorghum cultivars. None of the populations parasitized on sorghum, whereas five out of the eight populations failed to parasitize on maize. The authors concluded the occurrence of local adaptation of the parasite to a host species as well as a high degree of host specialization. Similarly, Botanga and Timko [24, 25] reported the stratification by host preference of *Striga gesnerioides* (Willd.) Vatke genotypes parasitizing cowpea [*Vigna unguiculata* (L.) Walp.] and the wild legume *Indigofera hirsuta* L. Conversely, Olivier et al. [26], using isozyme loci, showed little genetic differentiation based on host specificity among *Striga hermonthica* (Del.) Benth. populations parasitizing sorghum, pearl millet, maize, and wild grasses. Similarly, Vaz Patto et al. [20] found low genetic differentiation between populations of *Orobanche foetida* Poir. collected on a wild host and a population growing on cultivated vetch (*Vicia sativa* L.) using AFLP analyses.

Studies on genetic diversity within and between *O. cumana* populations are scarce and focused on weedy populations collected on sunflower. Gagne et al. [27] studied genetic diversity in eight populations from several countries using RAPD markers. They identified large interpopulation and low intrapopulation genetic variation, concluding the existence of two main gene pools, one comprising populations from Eastern Europe and another one including populations from Southern Spain. Pineda-Martos et al. [28] identified two main gene pools for *O. cumana* in Spain, comprising populations from the Guadalquivir Valley (Southern Spain) and Cuenca Province (Central Spain), respectively. Both groups were genetically distant, but both intra- and interpopulation genetic variation were in general extremely low within each gene pool due probably to a founder effect. However, a reduced number of populations exhibited larger genetic diversity, which was attributed to the presence of individuals from both gene pools and the occurrence of crosses between them. Even though *O. cumana* is considered to be primarily a self-pollinated species [29], the occurrence of a certain rate of cross pollination has been experimentally demonstrated [30].

There is no information on the population structure of *O. cumana* populations parasitizing wild species and their genetic relationship with weedy populations in areas where they coexist. There is also no information on their virulence on sunflower. The objective of this research was to investigate the genetic diversity, population structure, and ability to parasitize sunflower of *O. cumana* populations growing on wild plants in the Black Sea coast of Bulgaria, as well as their relationship with weedy populations parasitizing sunflower.

2. Materials and Methods

2.1. Orobanche cumana Populations. Two field expeditions were conducted in July 2006 and June 2012 along the Black Sea coast of Bulgaria, where the distribution of *O. cumana* in the wild has been largely documented [8, 31–33], to collect fresh tissue and mature seeds of *O. cumana* populations parasitizing wild Asteraceae species. Six populations were located in both expeditions, one of them in both years (Table 1, Figure 1). Samples from the latter population were managed separately in the study to evaluate potential changes between both collection dates. Voucher specimens of the populations are housed in the herbarium of the University of Córdoba, Spain (herbarium code COA). Duplicated specimens can also be found at the herbarium SOA (Agricultural University of Plovdiv, Bulgaria). Populations CUMBUL-1 (COA-45783 and COA-45784), CUMBUL-2 (COA-45789), CUMBUL-3 (COA-45790), CUMBUL-4 (COA-45785), CUMBUL-6 (COA-53262 and COA-54519), and CUMBUL-7 (COA-54510) were collected on *A. maritima* L. (Table 1). Figure 2 shows details of population CUMBUL-1. Population CUMBUL-5 was found parasitizing *Anthemis arvensis* L., *Chamaemelum nobile* (L.) All., and another species of the Asteraceae that could not be identified, though

Table 1: Host species, collecting site, characteristics, and number of individuals analyzed for the studied *Orobanche cumana* populations.

Population	Host species	Collecting site	Region	Latitude, Longitude, Altitude	Year	*n*
O. cumana populations collected on wild hosts						
CUMBUL-1	*Artemisia maritima*	Bulgaria, Burgas, Atanasovsko Lake	South-Eastern Bulgaria	42°33′02.7″N; 27°29′24″E; 14 m	2006	16
CUMBUL-2	*Artemisia maritima*	Bulgaria, Burgas, Pomorie-Aheloj	South-Eastern Bulgaria	42°37′02.8″N; 27°37′31.1″E; 17 m	2006	30
CUMBUL-3	*Artemisia maritima*	Bulgaria, Kranevo	North-Eastern Bulgaria	43°20′05.6″N; 28°3′41.9″E; 112 m	2006	6
CUMBUL-4	*Artemisia maritima*	Bulgaria, Balchik	North-Eastern Bulgaria	43°24′36.9″N; 28°9′23.5″E; 21 m	2006	29
CUMBUL-5_1	*Anthemis arvensis*	Bulgaria, Kavarna, Gorun-Tyulenovo	North-Eastern Bulgaria	43°29′12.6″N; 28°31′13.3″E; 44 m	2006	28
CUMBUL-5_2	*Chamaemelum nobile*	Bulgaria, Kavarna, Gorun-Tyulenovo	North-Eastern Bulgaria	43°29′12.6″N; 28°31′13.3″E; 44 m	2006	20
CUMBUL-6	*Artemisia maritima*	Bulgaria, Burgas, Poda Protected Area	South-Eastern Bulgaria	42°26′35.91″N; 27°27′58.64″E; 7 m	2012	23
CUMBUL-7	*Artemisia maritima*	Bulgaria, Burgas, Atanasovsko Lake	South-Eastern Bulgaria	42°33′05.88″N; 27°29′22.91″E; 8 m	2012	14
O. cumana populations collected on sunflower						
CUMBUL-8	*Helianthus annuus*	Bulgaria, Sadovo	Central Bulgaria	42°07′13.49″N; 24°54′53.40″E; 156 m	2012	20
CUMBUL-9	*Helianthus annuus*	Bulgaria, Plodiv	Central Bulgaria	42°03′35.43″N; 24°49′26.28″E; 189 m	2012	20
CUMBUL-10	*Helianthus annuus*	Bulgaria, Balgarevo	North-Eastern Bulgaria	43°24′58.14″N; 28°26′43.83″E; 81 m	2012	18
IASCum-2	*Helianthus annuus*	Spain, Sevilla, Écija	Southern Spain	37°34′24″N; 5°8′45″W; 181 m	2008	12
IASCum-3	*Helianthus annuus*	Spain, Sevilla, Osuna	Southern Spain	37°15′19″N; 5°3′49″W; 304 m	2008	12
IASCum-4	*Helianthus annuus*	Spain, Cuenca, Montalbo	Central Spain	39°51′03″N; 02°39′54″W; 838 m	2008	12

n, final studied sample size [including a number of plants excluded from the analysis because of lack of amplification, belonging to each of the populations CUMBUL-1 (four plants excluded), CUMBUL-4 (one plant), CUMBUL-5_1 (two plants), CUMBUL-5_2 (two plants), CUMBUL-6 (three plants), and CUMBUL-10 (two plants)].

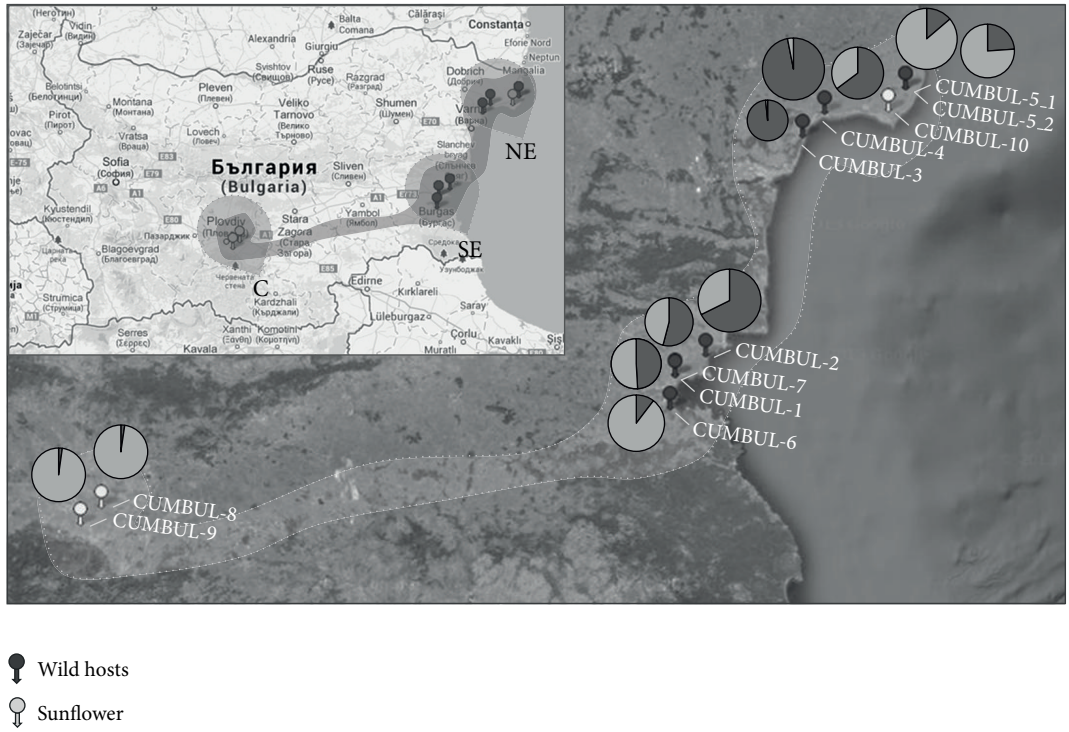

Figure 1: Geographical distribution of *Orobanche cumana* Bulgarian populations collected on wild and cultivated hosts (left side of the figure) and map of mean membership probabilities per population as obtained using Bayesian clustering analysis resulting from STRUCTURE at $K = 2$ (right side of the figure). Pie size is proportional to the size of each population.

Figure 2: Details of population CUMBUL-1 of *Orobanche cumana* parasitizing *Artemisia maritima* in Burgas, Bulgaria.

in the latter case only two plants were presen and they were not collected. Plants collected on *A. arvensis* (CUMBUL-5_1; COA-45791) and *C. nobile* (CUMBUL-5_2; COA-45792) were analyzed separately to evaluate potential differences associated with the host plant. The populations were located at a distance of less than 3 km from agricultural fields. Fresh tissue (young stalks) from 6 to 30 individual plants (Table 1), depending on population size, was collected *in situ* for each population and kept under drying conditions in ziplock bags with silica gel for subsequent freezing at −80°C. Fresh tissue of three *O. cumana* populations parasitizing sunflower crops in two different areas of Bulgaria (Table 1) was collected in the 2012 expedition. Additionally, fresh tissue was also collected *in situ* from three populations parasitizing sunflower in two different areas of Spain in which contrasting gene pools have been identified [28], to be used as a control (Table 1).

Mature seeds were collected in bulk from 5 to 30 mature plants of populations CUMBUL-1, CUMBUL-2, CUMBUL-4, and CUMBUL-5_1. No mature plants were available at the time of the collection expeditions for the other populations, including the *O. cumana* populations parasitizing sunflower in Bulgaria. Alternatively, seeds from three populations of *O. cumana* collected in sunflower fields in Bulgaria (OC-9, OC-11, and OC-13) were used for virulence studies. Populations OC-9 and OC-13 were kindly provided by Professor Rossitza Batchvarova, AgroBioInstitute, Sofia, Bulgaria. Population OC-11 was collected by one of the authors (K. Stoyanov). Spanish race F population OC-88 was also used as a control for virulence studies.

2.2. DNA Extraction and SSR Analysis. Frozen tissue was lyophilized and ground to a fine powder. DNA was extracted from individual *O. cumana* plants using a modified version of the protocol described in Pérez-Vich et al. [34]. Microsatellite analyses were carried out as described in Pineda-Martos et al. [28], using the same set of fifteen high-quality, polymorphic SSR primer pairs (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/150432). Amplification products were resolved by electrophoresis on 3% Metaphor agarose (BMA, Rockland, ME, USA gels in 1x TBE buffer at 100 V constant voltage, with SaveView Nucleic Acid Stain (NBS Biologicals Ltd., Huntingdon, UK) incorporated in the gels and visualized under UV light. A 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia) was used as a standard molecular weight marker to get an approximate size of DNA fragments. Bands were scored manually with the aid of Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) at least twice independently for each population.

2.3. Molecular Data Analysis

2.3.1. Genetic Diversity Analysis. For each SSR locus, the number of alleles (Na), observed and expected heterozygosity (Ho and He), and F_{ST} were calculated using GenAlEx ver. 6.5 [35]. Additionally, each locus was tested for departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium within each of the populations with Arlequin ver. 3.5.1.3 [36]. To characterize the genetic diversity of *O. cumana* populations collected on wild hosts and the control populations collected on sunflower, the percentage of polymorphic loci (P), the average observed number of alleles (Na), the number of different alleles with a frequency ≥ 5% (Na ≥ 5%), the number of effective alleles (Ne), the number of private alleles unique to a single population (Npa), the observed and expected heterozygosity (Ho and He), the Shannon's diversity index (I), and the fixation index (F_{is}) were calculated for all loci at each population. All calculations were carried out using GenAlEx ver. 6.5. F_{is} was used to estimate the selfing rate (S) from $S = 2F_{is}/(1 + F_{is})$ [37]. As additional measures of intrapopulation diversity, the mean number of pairwise differences between individuals within each population, estimated as the mean number of differences between all pairs of SSR haplotypes in each population, and the genotypic richness (R), defined as $(G-1)/(N-1)$, where G is the number of MLGs (the observed number of multilocus genotypes) and N is the number of samples per population, were determined using Arlequin ver. 3.5.1.3, and GenClone 2.0 [38], respectively.

2.3.2. Genetic Differentiation Analysis. To evaluate genetic differentiation between populations, initial frequency-based analysis was carried out by calculating pairwise genetic distances between populations using the genetic distance coefficient G_{ST} as implemented in GenAlEx ver. 6.5 using 1000 random permutations to assess significance. Pairwise distance matrices were also calculated using GenAlEx ver. 6.5 with other frequency-based estimators of population structure for codominant data such as Nei's G_{ST}, Nei's standardized G_{ST}, Hedrick's standardized G_{ST}, Hedrick's further standardized G_{ST} for small number of populations, and Jost's estimate of differentiation, following calculations detailed in [39]. The pairwise relationship between the genetic distance matrices was tested through a Mantel's test with 999 permutations. Since the different statistical measures were highly correlated ($r > 0.94$, $P = 0.001$ for all comparisons, excepting those including the Jost's estimate of differentiation in which $r > 0.90$, $P = 0.001$), only the results based on the genetic distance coefficient G_{ST} with the corrections of Nei and Chesser [40] and Nei [41] are presented. To assess genetic relationships among populations, the matrix of G_{ST} pairwise distances was used as input for a principal coordinates analysis (PCoA) using GenAlEx ver. 6.5. PCoA has the main advantage of not requiring strong assumptions about the underlying genetic model [42].

To identify genetically homogeneous groups (gene pools), Bayesian model-based clustering algorithms implemented in the software package STRUCTURE ver. 2.3.4 [43] were applied. Cluster grouping in STRUCTURE is based on iterative analysis using K number of groups previously defined by the user, with individuals in the sample being assigned probabilistically to one or several groups. The admixture model and the allele frequencies correlated model were used [44]. No prior information was used to define the clusters. For each value of K (from 1 to 14), 10 independent runs were made that were used to estimate the probability of the data $\Pr(X \mid K)$. For each run, 1,000,000 Monte Carlo Markov chain (MCMC) iterations were carried out after a burn-in period of 200,000 steps. To detect the number of genetically homogeneous groups (K) that best fits the data, the STRUCTURE HARVESTER website [45], which implements the Evanno method [46], was used. The 10 runs from the most probable number of K groups were averaged applying the FullSearch algorithm provided in the CLUMPP ver. 1.1.2b software [47] and the output was entered into Distruct ver. 1.1 for display [48]. To explore the genetic structure further, the STRUCTURE analyses were also carried out only with the 11 Bulgarian populations, as described above. We also used the program InStruct [49] for analyzing population structure, since this program is an extension of STRUCTURE that does not assume Hardy-Weinberg equilibrium and can incorporate selfing in the model. In addition, it can estimate the level of selfing in each population. Five independent chains were run for each K. Each chain was run for 1,000,000 iteration steps, with a burn-in of 500,000, and a thinning of 10. Graphical representations of population assignments from InStruct were produced from the program Distruct ver. 1.1 [48].

Finally, an analysis of molecular variance (AMOVA) [50] within populations, among populations, and among population groups (based on *a priori* grouping variables such as wild or cultivated host or based on the gene pools determined with clustering methods) was carried out to determine the distribution of variation at different hierarchical levels. The variance components were tested statistically by nonparametric randomization tests using 1000 permutations. Fixation indices (F-statistics) were also estimated by AMOVA. All calculations were carried out with Arlequin ver. 3.5.1.3.

2.4. Parasitization Ability and Virulence on Sunflower. Mature seeds were collected for wild *O. cumana* populations CUMBUL-1, CUMBUL-2, CUMBUL-4, and CUMBUL-5_1. However, the amount of available seed was very low, which restricted the number of sunflower genotypes for virulence studies as well as the number of plants per genotype. Accordingly, their parasitization ability and virulence on sunflower was evaluated in two separated experiments. The first experiment was aimed at determining whether the populations had the ability to parasitize sunflower genotypes with no genetic resistance to weedy *O. cumana* physiological races. Two confectionery sunflower landraces, B117 and B206, with no known resistance to any *O. cumana* race were used. Both landraces were collected by L. Velasco in isolated vegetable patches in Valdepeñas (Jaén Province, Spain) and Quintana de la Serena (Badajoz Province, Spain), respectively. *Orobanche cumana* population OC-88 [with known virulence (race F)] was used as a positive control. In a second experiment, the virulence of the populations was tested on a set of sunflower lines with varying levels of genetic resistance to *O. cumana* physiological races. Jdanovski 8281 (J8281) is a line incorporating resistance gene *Or2* that confers resistance to *O. cumana* race B [17]. AC03-1589 is a line incorporating resistance gene *Or3* that confers resistance to *O. cumana* race C, kindly provided by Dr. Maria Păcureanu, National Agricultural Research and Development Institute, Fundulea, Romania. S1358 is a line incorporating resistance gene *Or4* that confers resistance to *O. cumana* race D [17]. P-1380 is a line containing the resistance gene *Or5*, which determines resistance to *O. cumana* race E [17]. P96 is a line with recessive resistance to *O. cumana* race F [34]. B117 with no known resistance to any *O. cumana* race was used as positive control. Populations OC-9, OC-11, OC-13, and OC-88 were used as controls. Because the amount of seed of populations CUMBUL-1, CUMBUL-4, and CUMBUL-5_1 was not enough for evaluating them on all sunflower lines of the second experiment, it was decided not to test them on line S1358.

Seeds of *O. cumana* populations were used to inoculate small pots 7 × 7 × 8 cm filled with a mixture of sand and peat (1 : 1 by vol). Twenty-five mg of *O. cumana* seeds per pot was used. The soil mixture containing *O. cumana* seeds was carefully mixed to obtain a homogeneously infested substrate. Seeds of sunflower cultivars were germinated on moistened filter paper in Petri dishes and two-day-old seedlings were planted in the pots inoculated with *O. cumana* seeds. Eight pots (replications) per combination of sunflower cultivar and *O. cumana* population were used. The plants were maintained in a growth chamber for 21 days at 25°C/20°C (day/night) with a 16 h photoperiod for incubation. After this time, the plants were transplanted to pots containing 3 L of an uninfested sand-silt-peat (2 : 1 : 1 by vol) soil mixture and maintained under open air conditions. The plants were watered as needed and were not fertilized. The number of *O. cumana* shoots per sunflower plant was counted at sunflower maturity. Differences between mean numbers of *O. cumana* shoots per plant for each *O. cumana* population and sunflower cultivar were analyzed through one way ANOVA and Tukey's range test using IBM SPSS Statistics version 19.

3. Results

3.1. Genetic Diversity and Population Structure. All SSR markers were polymorphic (Table S1). The total number of alleles scored was 38, ranging from 2 to 4 for each SSR locus. Allelic diversity was generally low for all fifteen SSR loci when considering the whole set of 260 individual *O. cumana* plants (Table S1). All the loci exhibited an important heterozygote deficiency (Table S1). A significant deviation ($P < 0.05$) from Hardy-Weinberg equilibrium was found for almost all loci when all samples were considered. Linkage disequilibrium was significant ($P < 0.05$) in 238 out of 430 paired loci comparisons when considering all the samples. It has been established that linkage disequilibrium is predicted to approach zero for an ideal population, in the absence of forces such as genetic drift, population mixing, mutation, natural selection, or inbreeding [51]. High linkage disequilibrium observed suggested the existence of some genetic structure, apart from other factors determining the organization of genetic variation in the studied populations, as it will be further discussed below.

Genetic diversity within each population, measured by the mean number of observed and effective alleles, the expected heterozygosity, and Shannon's diversity indexes, was in general low, and only one population (CUMBUL-4) contained a substantial number of private alleles (Table 2). As expected from previous studies, Spanish populations were characterized by extremely low level of intrapopulation genetic diversity due probably to a founder effect [28], with no polymorphic loci being detected in two out of the three populations (Table 2). In contrast, populations from Bulgaria exhibited higher diversity values, with the exception of population CUMBUL-3, which showed no polymorphic loci. However, it is important to note that this was the smallest population, in which only six individual plants could be collected. Amongst the other Bulgarian populations, the highest genetic diversity corresponded to the populations collected on wild hosts CUMBUL-2, CUMBUL-5_2, and CUMBUL-7, which showed He, I and pairwise difference (between individuals) values over 0.25, 0.4, and 3.5, respectively (Table 2). The lowest genetic diversity corresponded to populations CUMBUL-8 and CUMBUL-9, collected on sunflower, which showed He, I and pairwise difference (between individuals) values below 0.05, 0.1, and 0.5 respectively (Table 2). The other six populations, excluding CUMBUL-3, showed intermediate diversity values, ranging from 0.10 to 0.23 for He, from 0.18 to 0.35 for I, and from 1.8 to 3.1 for pairwise differences between individuals. The fixation index (F_{is}) and selfing rate (S) values were high for the populations studied (Table 2).

For measuring differentiation between populations, pairwise G_{ST} values were computed (Table S2). No significant or very low ($G_{ST} \leq 0.01$) differentiation was found for populations CUMBUL-8 and CUMBUL-9, collected on sunflower at close locations, CUMBUL-2 and CUMBUL-7, collected on *A. maritima* at near sites the same year, and CUMBUL-5_1 and CUMBUL-5_2, collected at the same location but on different wild hosts. Populations CUMBUL-1 and CUMBUL-7, which were collected at the same site but with a six-year difference, showed slightly higher G_{ST} values (0.107). The

Table 2: Genetic diversity parameters of *Orobanche cumana* populations from Bulgaria collected on wild hosts and on sunflower (prefix CUMBUL-) and from Spain collected on sunflower (prefix IASCum-).

Population	*P*	Na (±SE)	Na ≥ 5% (±SE)	Ne (±SE)	Npa (±SE)	Ho (±SE)	He (±SE)	*I* (±SE)	Pairwise differences	Genotypic richness *G*	Genotypic richness *R*	F_{is} (±SE)	*S*
O. cumana populations collected on wild hosts													
CUMBUL-1	66.7	1.733 (0.15)	1.667 (0.13)	1.391 (0.10)	0.000 (0.00)	0.004 (0.01)	0.229 (0.05)	0.349 (0.08)	3.111 (1.66)	7	0.40	0.984 (0.01)	0.992
CUMBUL-2	86.9	2.000 (0.14)	1.933 (0.15)	1.521 (0.10)	0.000 (0.00)	0.000 (0.00)	0.297 (0.05)	0.458 (0.07)	4.393 (2.20)	8	0.24	1.000 (0.00)	1.000
CUMBUL-3	0.0	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	1	0.00	—	—
CUMBUL-4	53.3	1.600 (0.16)	1.333 (0.13)	1.145 (0.05)	0.067 (0.07)	0.000 (0.00)	0.105 (0.03)	0.184 (0.06)	1.595 (0.96)	6	0.18	1.000 (0.00)	1.000
CUMBUL-5_1	80.0	2.000 (0.17)	1.867 (0.16)	1.250 (0.07)	0.000 (0.00)	0.032 (0.01)	0.171 (0.04)	0.306 (0.06)	2.449 (1.34)	11	0.37	0.776 (0.04)	0.874
CUMBUL-5_2	80.0	1.933 (0.15)	1.933 (0.15)	1.418 (0.10)	0.000 (0.00)	0.014 (0.01)	0.248 (0.05)	0.400 (0.07)	3.597 (1.86)	7	0.32	0.920 (0.04)	0.958
CUMBUL-6	73.3	1.800 (0.14)	1.667 (0.16)	1.294 (0.10)	0.000 (0.00)	0.003 (0.01)	0.181 (0.05)	0.300 (0.07)	2.629 (1.43)	10	0.41	0.968 (0.03)	0.984
CUMBUL-7	73.3	1.800 (0.14)	1.800 (0.14)	1.467 (0.12)	0.000 (0.00)	0.010 (0.01)	0.258 (0.05)	0.398 (0.08)	3.947 (2.04)	7	0.46	0.975 (0.01)	0.987
Mean	64.2	1.733 (0.06)	1.650 (0.12)	1.311 (0.03)	0.008 (0.01)	0.008 (0.002)	0.186 (0.02)	0.299 (0.03)	2.715 (0.05)	7.1	0.30	0.946	0.971
O. cumana populations collected on sunflower													
CUMBUL-8	40.0	1.400 (0.13)	1.133 (0.09)	1.039 (0.02)	0.000 (0.00)	0.021 (0.01)	0.034 (0.01)	0.071 (0.03)	0.446 (0.41)	3	0.11	0.194 (0.10)	0.325
CUMBUL-9	13.3	1.133 (0.09)	1.133 (0.09)	1.014 (0.01)	0.000 (0.00)	0.000 (0.00)	0.013 (0.01)	0.026 (0.02)	0.195 (0.25)	2	0.05	1.000 (0.00)	1.000
CUMBUL-10	46.7	1.467 (0.13)	1.467 (0.13)	1.175 (0.06)	0.000 (0.00)	0.015 (0.01)	0.123 (0.04)	0.201 (0.06)	1.825 (1.07)	8	0.41	0.915 (0.04)	0.956
Mean-Bulgaria	33.3	1.333 (0.10)	1.244 (0.11)	1.076 (0.05)	0.000 (0.00)	0.012 (0.01)	0.057 (0.03)	0.099 (0.05)	0.822 (0.51)	4.3	0.19	0.703	0.760
IASCum-2	0.0	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	1	0.00	—	—
IASCum-3	0.0	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	0.000 (0.00)	1	0.00	—	—
IASCum-4	6.7	1.067 (0.07)	1.067 (0.07)	1.026 (0.03)	0.000 (0.00)	0.000 (0.00)	0.019 (0.02)	0.030 (0.03)	0.290 (0.32)	2	0.09	1.000 (0.02)	1.000
Mean-Spain	2.2	1.022 (0.02)	1.022 (0.02)	1.009 (0.01)	0.000 (0.00)	0.000 (0.00)	0.006 (0.01)	0.010 (0.01)	0.097 (0.10)	0.7	0.03		

P: percentage of polymorphic loci, Na: average observed allele number, Na > 5%: number of different alleles with a frequency ≥ 5%, Ne: number of effective alleles, Npa: number of private alleles unique to a single population, Ho: observed heterozygosity, He: expected heterozygosity, *I*: Shannon's diversity index, Pairwise differences: mean number of pairwise differences between individuals within each population (±SD), *G*: number of distinct multilocus genotypes (MLGs), *R*: genotypic richness; F_{is}: fixation index, and *S*: selfing rate.

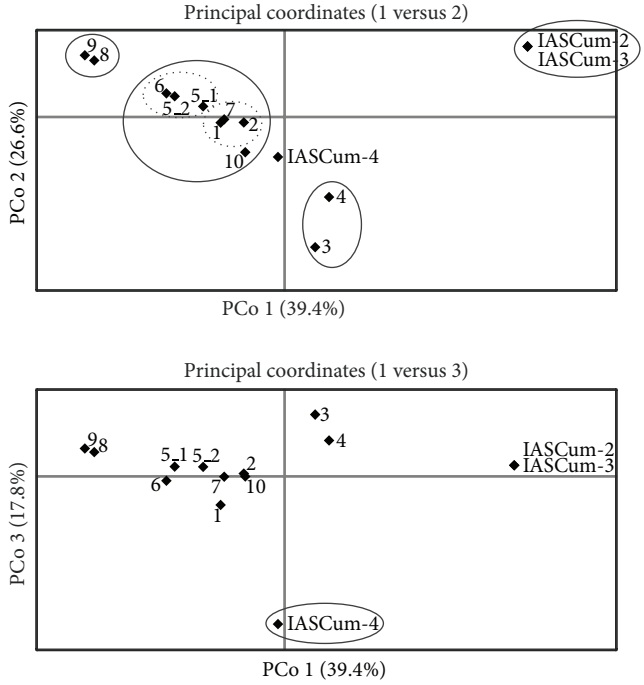

Figure 3: Principal coordinates analysis of pairwise genetic distances among 14 *Orobanche cumana* populations and subpopulations (260 individuals). Primary groups identified with either the 1st versus 2nd axis plot or with the 1st versus 3rd axis plot are highlighted with solid boxes. Populations from Spain (prefix IASCum) are named with their complete name, and populations from Bulgaria (prefix CUMBUL) are named with their number, without prefix.

highest differentiation values ($G_{ST} > 0.8$) were found between the following three groups of populations: (i) IASCum-2, IASCum-3, and IASCum-4 collected on sunflower in Spain, (ii) CUMBUL-8 and CUMBUL-9 collected on sunflower in Bulgaria, and (iii) CUMBUL-3 and CUMBUL-4 collected on wild *A. maritima* in Bulgaria (Table S2). In principal coordinate analyses, the first three axes explained 39.4%, 26.6%, and 17.8%, respectively of the variation, producing five differentiated groups of populations: (i) IASCum-2 and IASCum-3, (ii) IASCum-4, (iii) CUMBUL-8 and CUMBUL-9, (iv) CUMBUL-3 and CUMBUL-4, and (v) the remaining seven populations, six of them collected on wild hosts in Bulgaria and one of them collected on sunflower in Bulgaria (Figure 3).

Bayesian-based analysis of the structure of the whole set of populations including those from Spain and Bulgaria with STRUCTURE revealed a close relationship among populations whatever their geographical origin, with an optimal K value of 2 (Figures S1 and S2). Secondary peaks were observed at $K = 4$ and 7 (Figure S1), and the standard deviation of $\Pr(X \mid K)$ began to increase substantially at K values higher than these (Figure S1). Visualization of the cluster membership for $K = 2$ to $K = 7$ showed a general trend towards classification of populations IASCum-2, IASCum-3, IASCum-4, CUMBUL-3, CUMBUL-4, CUMBUL-8, and CUMBUL-9 within uniform pools, while the rest of the populations were included within mixed pools (Figure S2), assignments that recurred at all monitored levels of K (Figure S2).

Table 3: Proportion of membership of each Bulgarian *Orobanche cumana* population in inferred STRUCTURE groups for $K = 2$. Populations collected on wild hosts are highlighted in bold.

Population	Genetic group 1	Genetic group 2
CUMBUL-1	0.491	0.509
CUMBUL-2	0.677	0.323
CUMBUL-3	0.984	0.016
CUMBUL-4	0.969	0.031
CUMBUL-5_1	0.138	0.862
CUMBUL-5_2	0.239	0.76
CUMBUL-6	0.104	0.896
CUMBUL-7	0.541	0.459
CUMBUL-8	0.023	0.977
CUMBUL-9	0.022	0.978
CUMBUL-10	0.647	0.352

A more detailed analysis of population structure including only Bulgarian populations was carried out. STRUCTURE analyses indicated the existence of two ($K = 2$; Figure S3) major genetic groups, mainly represented by populations CUMBUL-3 and CUMBUL-4 on one hand (Gene Pool 1), and CUMBUL-8 and CUMBUL-9 on the other hand (Gene Pool 2) (Table 3; Figure 4(a)). The remaining seven populations were categorized in-between these two groups, although the average proportion of membership was shifted towards Gene Pool 1 for populations CUMBUL-2 and CUMBUL-10, whereas populations CUMBUL-5_1, CUMBUL-5_2, and CUMBUL-6 were clearly shifted towards Gene Pool 2 (Table 3; Figure 1). When the membership value of each individual for each population was analyzed in detail, it was shown that an important number of individuals from populations CUMBUL-5_1, CUMBUL-5_2, and CUMBUL-6 [19 individuals out of 28 (67.9%), 10 out of 20 (50%), and 15 out of 23 (65.2%), resp.] showed a high (>0.90) membership value for Gene Pool 2 (Figure 4(b)). Classifications of individuals at $K = 2$ by the algorithms of STRUCTURE and InStruct were very similar qualitatively (Figure 4(a)). Within-cluster selfing rates estimated from InStruct analyses were very high (on average, 0.947 for Gene Pool 1 and 0.951 for Gene Pool 2).

Different AMOVA analyses were carried out within the *O. cumana* populations collected in Bulgaria. First, AMOVA analyses were conducted on populations collected on wild hosts. When no population structure was considered, 53.6% of the genetic variance was attributable to differences among populations, while the remaining 46.4% was due to differences within populations (Table 4). When populations were structured according to clustering results, differences among groups accounted for 50.4% of the total variance, while differences among populations of each group only accounted for 17.6% (Table 4). When populations collected on sunflower were added to the model, no significant structuring according to the ecological status of the populations was detected (Table 4). Structured analysis based on clustering groups

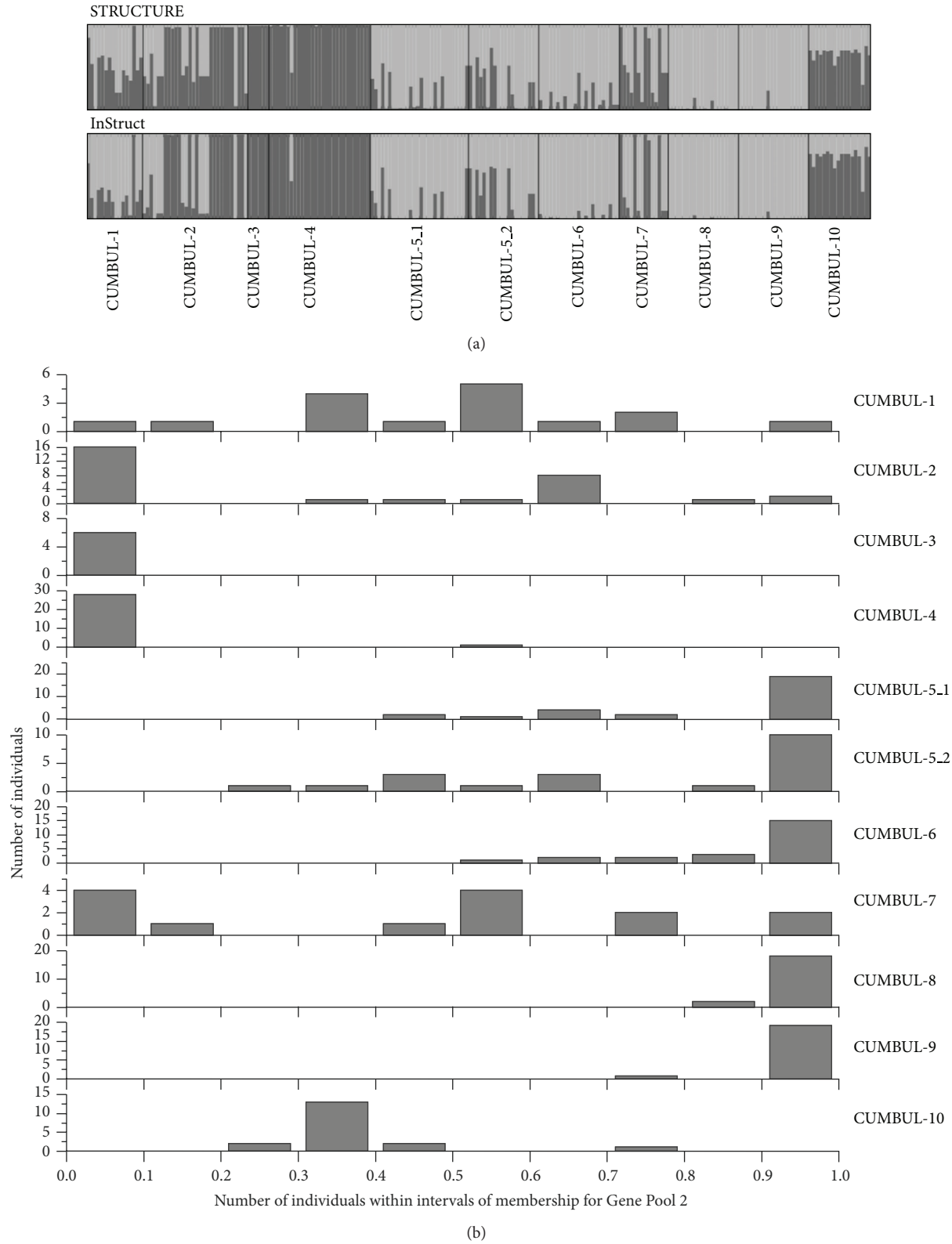

Figure 4: Results from STRUCTURE and InStruct analyses: (a) population structure obtained from STRUCTURE and InStruct analyses of eleven Bulgarian *Orobanche cumana* populations, with each individual being represented by a single vertical bar divided into two shades. Each shade represents one gene pool (K) and the length of the shaded segment shows the individual's estimated proportion of membership in that cluster and (b) number of *O. cumana* individuals from each Bulgarian population within intervals of membership for Gene Pool 2 in the STRUCTURE analyses.

Table 4: Analysis of molecular variance (AMOVA) of *Orobanche cumana* populations from Bulgaria.

Hierarchical structure and source of variation	AMOVA statistics				F-statistics[a]	P value
	df	Sum of squares	Variance components	% Variance		
Bulgarian populations collected on wild hosts (8 populations; 166 individuals)						
Not structured						
Among populations	7	491.05	1.69	53.64	$F_{ST} = 0.54$	<0.001
Within populations/group	324	473.19	1.46	46.36		
Structured based on gene pools[b]						
Among groups	1	294.36	2.29	50.37	$F_{CT} = 0.50$	0.032
Among populations/group	6	196.69	0.80	17.56	$F_{SC} = 0.35$	<0.001
Within populations/group	324	473.19	1.46	32.07	$F_{ST} = 0.68$	<0.001
Total of Bulgarian populations (wild and cultivated host) (11 populations; 224 individuals)						
Not structured						
Among populations	10	713.97	1.74	59.54	$F_{ST} = 0.60$	<0.001
Within populations/group	437	517.64	1.18	40.46		
Structured based on ecological status[c]						
Among groups	1	93.81	0.14	4.55	$F_{CT} = 0.05$	0.234
Among populations/group	9	620.16	1.68	56.05	$F_{SC} = 0.59$	<0.001
Within populations/group	437	517.64	1.18	39.40	$F_{ST} = 0.61$	<0.001
Structured based on gene pools[d]						
Among groups	2	423.05	1.51	42.01	$F_{CT} = 0.42$	0.002
Among populations/group	8	290.91	0.90	25.05	$F_{SC} = 0.43$	<0.001
Within populations/group	437	517.64	1.18	32.94	$F_{ST} = 0.67$	<0.001

[a] F-statistics represents differentiation among groups (F_{CT}), among populations within groups (F_{SC}), and among populations within the whole population (F_{ST}).
[b] The gene pools defined with clustering analyses comprised (i) populations CUMBUL-3 and -4 and (ii) populations CUMBUL-1, -2, -5_1, -5_2, -6, and -7.
[c] The structured groups based on the ecological status were (i) wild hosts (populations CUMBUL-1, -2, -3, -4, -5_1, -5_2, -6, and -7) and (ii) cultivated host (sunflower) (populations CUMBUL-8, -9, -10).
[d] The gene pools defined with clustering analyses were (i) populations CUMBUL-3, -4, (ii) populations CUMBUL-8 and -9, and (iii) populations CUMBUL-1, -2, -5_1, -5_2, -6, -7, and -10.

produced similar results to the analysis of populations collected on wild hosts alone; that is, variation among groups accounted for 42.0% of total variation, while variation among populations at each group accounted for 25.1% (Table 4).

3.2. Parasitization Ability and Virulence on Sunflower. A first experiment demonstrated that *O. cumana* populations CUMBUL-1, CUMBUL-2, CUMBUL-4, and CUMBUL-5_1, collected on wild hosts, had the ability to parasitize sunflower lines B117 and B206, with no resistance genes, though some differences between populations were observed (Table 5). On B117, populations CUMBUL-1 and CUMBUL-5_1 produced similar number of shoots per sunflower plant to the control population OC-88, while CUMBUL-2 produced around four times more shoots per plant and CUMBUL-4 produced about half of shoots per plant than the control. On B206, both CUMBUL-1 and CUMBUL-2 yielded more shoots per plant than the control, while CUMBUL-4 produced less shoots per plant than the control (Table 5). In a second experiment the virulence of the populations collected on wild hosts, together with Bulgarian populations collected on sunflower, was evaluated on sunflower lines with varying degrees of genetic resistance. On sunflower line J8281, resistant to *O. cumana* race B, the number of shoots per sunflower plant did not differ significantly between Bulgarian *O. cumana* populations collected on wild hosts and those collected on sunflower (Table 6). The results were similar on sunflower line AC03-1589, resistant to race C, except for a significantly higher number of shoots in population CUMBUL-5_1. Similarly, the only wild population evaluated on line S1358 resistant to

Table 5: Number of emerged *Orobanche cumana* shoots per sunflower plant (mean ± standard deviation) in the evaluation of *O. cumana* populations CUMBUL-1, CUMBUL-2, and CUMBUL-4, collected in Bulgaria on *Artemisia maritima*, CUMBUL-5_1, collected in Bulgaria on *Anthemis arvensis*, and control population OC-88, collected in Spain on cultivated sunflower, on two sunflower lines (B117 and B206) with no genetic resistance to *O. cumana*, conducted in pots in 2007[a].

	B117[b]	B206[a]
CUMBUL-1	14.5 ± 9.9^{b}	35.3 ± 13.1^{c}
CUMBUL-2	39.5 ± 7.7^{c}	36.3 ± 11.3^{c}
CUMBUL-4	5.2 ± 4.7^{a}	1.7 ± 1.0^{a}
CUMBUL-5_1	11.5 ± 8.9^{ab}	14.3 ± 4.5^{b}
OC-88	10.3 ± 6.0^{ab}	18.5 ± 7.2^{b}

[a] Eight pots per each combination of sunflower cultivar and *O. cumana* population.
[b] Means with different letters for each sunflower cultivar differ significantly ($P < 0.05$).

Table 6: Number of emerged *Orobanche cumana* shoots per sunflower plant (mean ± standard deviation) in the evaluation of *O. cumana* populations CUMBUL-1, CUMBUL-2, and CUMBUL-4, collected in Bulgaria on *Artemisia maritima*, CUMBUL-5_1, collected in Bulgaria on *Anthemis arvensis*, OC-9, OC-11, and OC-13, collected in Bulgaria on cultivated sunflower, and OC-88, collected in Spain on cultivated sunflower, on six sunflower lines with different levels of genetic resistance, conducted in pots in 2008[a]. The *O. cumana* race to which each sunflower line is expected to be resistant (if any) is given in parenthesis.

	B117[b]	J8281 (B)	AC03-1589 (C)	S1358 (D)	P-1380 (E)	P96 (F)
CUMBUL-1	17.7 ± 6.3^{bc}	2.6 ± 2.4^{ab}	1.1 ± 1.2^{a}	NE[c]	0^{a}	0
CUMBUL-2	20.6 ± 3.2^{c}	1.3 ± 1.7^{ab}	0.5 ± 1.1^{a}	2.0 ± 1.4^{a}	0^{a}	0
CUMBUL-4	10.9 ± 7.1^{ab}	0.1 ± 0.4^{a}	0.6 ± 0.7^{a}	NE	0^{a}	0
CUMBUL-5_1	12.6 ± 6.7^{ab}	2.0 ± 1.1^{ab}	5.1 ± 2.6^{b}	NE	0.4 ± 0.7^{a}	0
OC-9	13.8 ± 7.4^{abc}	3.0 ± 2.5^{ab}	0.5 ± 0.8^{a}	0.8 ± 1.0^{a}	0^{a}	0
OC-11	8.6 ± 5.3^{a}	2.5 ± 2.1^{ab}	0.2 ± 0.4^{a}	0.7 ± 0.8^{a}	0^{a}	0
OC-13	14.3 ± 7.9^{abc}	4.1 ± 1.9^{b}	1.1 ± 1.1^{a}	1.2 ± 0.8^{a}	0^{a}	0
OC-88	9.9 ± 6.9^{a}	16.6 ± 6.5^{c}	1.6 ± 1.3^{a}	1.2 ± 1.2^{a}	6.9 ± 4.3^{b}	0

[a]Eight pots per each combination of sunflower cultivar and *O. cumana* population.
[b]Means with different letters for each sunflower cultivar differ significantly ($P < 0.05$).
[c]NE = not evaluated.

race D (CUMBUL-2) did not differ from the Bulgarian populations collected on sunflower. When the populations were tested on sunflower line P-1380, resistant to race E, only population CUMBUL-5_1 produced a few number of shoots per plant, whereas neither the other populations collected on wild species nor the Bulgarian populations collected on sunflower did possess the ability to parasitize P-1380. None of the populations parasitized on race-F resistant line P96 (Table 6).

4. Discussion

The genetic structure of *O. cumana* populations analyzed in this study was not determined by the fact that the populations were collected on wild or cultivated hosts. This was an unexpected result, since, within a number of largely self-pollinated parasitic plant species, host specificity has been found as a mechanism of accelerating isolation and subsequently genetic divergence among populations, for example, in *Orobanche minor* Sm. [52–54], *Striga asiatica* [23], and *S. gesnerioides* [24, 25]. Conversely, Vaz Patto et al. [20] studied the genetic structure of five Moroccan *O. foetida* populations, four of them parasitizing wild plants (*Scorpiurus muricatus* L. and *Ornithopus sativus* Brot.) and another one parasitizing cultivated vetch. The authors found that the vetch-parasitizing population was closer to the three populations parasitizing *S. muricatus*, while the population collected on *O. sativus* was the most genetically divergent. This suggested that parasitization of wild or cultivated hosts was not among the main factors determining genetic differences between these populations. Since host specificity in *Orobanche* spp. is mainly determined by induction of seed germination by specific chemical stimulants exuded by the host root [55], host-induced selection is expected to have an impact on very small portions of the genome, probably even at a single locus by modifying the binding site of the stimulant receptor [56]. Such limited genetic modifications, despite having a huge phenotypic impact, might not be detected with overall genome scans such as the one carried out in this research, while the rest of the genome is predominantly shaped by other evolutionary sources, namely, recombination and migration [29].

Nevertheless, an important observation in this study was that the genetic structure of wild *O. cumana* populations reflected introgressions from weedy populations parasitizing sunflower. This was shown not only by the analysis of population structure, but also by similar levels of virulence on sunflower of weedy and wild *O. cumana* populations. To the best of our knowledge, this is the first study on molecular diversity and virulence on sunflower of *O. cumana* populations parasitizing wild hosts. Previous studies focusing exclusively on weedy populations have shown the existence of several gene pools in this species, with low genetic diversity within each gene pool [27, 28, 57–59]. Gagne et al. [27] identified two gene pools, one of them comprising populations from eastern Europe (Romania, Bulgaria, and Turkey) and another one including populations from southern Spain. Studies on Spanish populations identified two well-separated gene pools, one of them in the south (Guadalquivir Valley) and another one in the central area (Cuenca Province) [28, 58, 59]. The study of Spanish populations [28] revealed that, although intrapopulation genetic diversity was in general extremely low, some populations showed larger diversity, which was hypothesized to be produced by genetic recombination between individuals from both gene pools. In the present research, two contrasting gene pools were identified in Bulgaria, one of them best represented by weedy populations from the central area (CUMBUL-8 and CUMBUL-9), and another one represented by wild populations from the eastern coast (CUMBUL-3 and CUMBUL-4), which showed in all cases low intrapopulation diversity. The fact that some wild populations had higher genetic diversity values and contained individuals that exhibited membership values very close to a weedy gene pool (>0.90) suggested the existence of genetic flow between both gene pools, which could be attributed to cross fertilization and/or seed movement. It is important to note that in the Black Sea coast of Bulgaria weedy and wild *O. cumana* populations coexist at short

distances. The existence of cross fertilization within this species has been demonstrated in controlled experiments at a local scale [30] as well as in the molecular evaluation of field-collected weedy populations, where heterozygous individuals for unique alleles of different gene pools have been identified [28]. In relation to gene flow through seed dispersal, *Orobanche* seeds are easily dispersed by water, wind, and animals. Individual broomrape plants produce an impressive number of seeds from 50,000 to 500,000 [1] that maintain their viability in the soil for up to 20 years [11]. These seeds are of near-microscopic size, from 250 to 380 μm long and from 150 to 240 μm wide, with a weight from 1.0 to 2.5 μg and are considered as "dust-seeds" [11, 15, 60, 61]. These factors are regarded as adaptations for being an obligate parasite, in order to be dispersed through vegetation so as to be as close as possible to the host plant and increasing the probability of finding an appropriate host [61]. Additionally, at a landscape scale, *Orobanche cumana* seed dispersion is highly influenced by human-derived agricultural and cultivation practices, as well as crop-seed trade and the use of contaminated sunflower seed stocks [15, 29], which might overpass spatial distances or barriers to gene flow common in natural ecosystems.

Wild and cultivated host plants represent different habitats for parasitic plants, especially when cultivated plants carry qualitative resistance genes, as is the case of the sunflower-*O. cumana* system [16]. The use of cultivars expressing vertical resistance mechanisms has contributed to a rapid development of *O. cumana* physiological races in most cultivation areas on the Old World, including Bulgaria [3, 19], which may explain why weedy populations of *O. cumana* generally show low genetic diversity, since new physiological races most likely evolve from single mutations events [62]. This is in general agreement with reports on plant pathogen-host interactions [22, 63, 64]. For parasitic plant-host interactions, higher intrapopulation variability was reported in a *Striga gesnerioides* population parasitizing the wild legume *Indigofera hirsuta* L. when compared to populations growing on cultivated cowpea [25]. Another study identified genetic diversity differences for a population of *S. hermonthica* grown on rice accessions of varying resistance to *Striga*, with the lowest diversity corresponding to a highly resistant rice accession [65]. This was not exactly the case of the present study, since we found genetic diversity values in wild populations similar or even lower than those reported in weedy *O. cumana* recombinant populations [28]. This could be explained on the basis of the existence of introgression from weedy populations into wild *O. cumana* populations. Studies in nonparasitic plant species, for example, in rice, have shown that introgression from cultivated species can considerably shape genetic diversity of wild populations [66, 67].

The study of *O. cumana* populations in their natural habitat provided new data about its breeding system. A clear heterozygote deficiency similar to that observed in populations parasitizing sunflower, deviation of genotypic frequencies at most loci from Hardy-Weinberg equilibrium, high inbreeding and selfing rate values, and the relatively low levels of genetic variation within populations coupled with substantial differences among populations, supported that wild populations of this species show a high degree of self-pollination, as reported previously for *O. cumana* populations parasitizing sunflower [27], and for other predominantly self-pollinating broomrape species such as *Phelipanche ramosa* (L.) Pomel [68].

Orobanche spp. differ for host specificity. Within this genus, *O. cumana* is one of species with the narrowest range of host plants. In the wild, it mainly parasitizes *Artemisia* spp. [4], whereas sunflower is the only crop in which *O. cumana* occurs as a parasitic weed [2]. *Orobanche cumana* belongs to the native flora of Bulgaria, where it parasitizes wild species of the Asteraceae, mainly *A. maritima* [8]. Conversely, the genus *Helianthus* is from North American origin [69]. The first report of *O. cumana* parasitization on sunflower dates back to the 1890s in Russia [11] and to 1935 in Bulgaria [12]. It is unknown whether *O. cumana* possesses natural ability to parasitize sunflower or this ability arose in particular genotypes following mutation [70]. The possibility that *O. cumana* possesses natural ability to parasitize sunflower cannot be discarded, since molecules of the same nature to those involved in *O. cumana* stimulation of germination by sunflower root exudates occur commonly in plant organs of Asteraceae species [55]. The results of the present research did not shed light on this aspect, since both the population structure analysis as well as the virulence study indicated that the wild populations used in the study contain introgressions from weedy populations. The existence of genetic flow between *O. cumana* populations parasitizing sunflower and those parasitizing wild species opens up an interesting field of research on how increasing virulence in weedy populations observed in recent years in Bulgaria [19] may influence the parasitization ability of *O. cumana* on wild species and on how genetic variability of wild populations may favour the ability of weedy populations to overcome sunflower resistance mechanisms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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07 Conclusions

> > >

From the Research Article **Pineda-Martos, R.; Velasco L.; Pérez-Vich, B. Identification, Characterisation and Discriminatory Power of Microsatellite Markers in the Parasitic Weed *Orobanche cumana*. *Weed Research* 2014, *54(2)*, 120–132**

I. A collection of 298 SSR markers was developed using next-generation sequencing (454 GS-FLX Titanium) of genomic *O. cumana* DNA enriched for microsatellite motifs.

II. Within SSR motifs, dinucleotides are the most abundant, with the frequency of SSRs decreasing while increasing motif length, and a preference of (A+T)-rich repeats in tri-nucleotide SSRs is observed.

III. An informative and functional set of 79 SSR primer pairs of good quality and high-level of polymorphism was identified, representing a major advance in the development of molecular tools for research in *O. cumana* and related species, such as *O. cernua*.

IV. The SSR markers showed important discriminatory power, allowing proper classification of *O. cumana* populations according to their different geographical origins and hosts.

V. The *O. cumana* SSR markers were highly transferable to the closely related species *O. cernua* and showed high species-specific alleles, resulting in a clear discrimination between populations of both species.

VI. Genetic diversity observed in *O. cumana* populations was considerably lower than in *O. cernua* populations, despite the reduced and geographically proximal population set concerning *O. cernua*.

From the Research Article **Pineda-Martos, R.; Velasco, L.; Fernández-Escobar, J.; Fernández-Martínez, J. M.; Pérez-Vich, B. Genetic Diversity of *Orobanche cumana* Populations from Spain Assessed Using SSR Markers. *Weed Research* 2013, *53(4)*, 279–289**

VII. Most of the populations fell into two major genetically distant gene pools in Spain, one in Cuenca province in Central Spain and another one in the Guadalquivir Valley in Southern Spain.

VIII. The great genetic separation between populations of Cuenca and the Guadalquivir Valley suggested that they may derive from separate seed introduction events from different areas.

IX. The occurrence of extremely low inter- and intra-population genetic variability in most of the populations from Cuenca and the Guadalquivir Valley could be attributed to the founder effect.

X. Clustering of populations was not associated with virulence groups. Different races occurred within the same homogeneous gene pool, suggesting that current more aggressive races might have evolved through independent mutational events – followed probably by selection – from a common genetic background.

XI. Most of the populations from new areas were identical to the populations of one of the two gene pools, in most cases from the Guadalquivir Valley gene pool.

XII. Only a few populations showed larger intrapopulation genetic variation, suggesting the existence of seed interchange and thus, co-existence of both gene pools within the same population, as well as the occurrence of different degrees of genetic recombination between them.

XIII. Genetic recombination between plants from the two main distant gene pools is an important mechanism for creating new variation, which might also have an effect on race evolution and new genetic variability.

From the Research Article Pineda-Martos, R.; Pujadas-Salvà, A. J.; Fernández-Martínez, J. M.; Stoyanov, K.; Velasco, L.; Pérez-Vich, B. The Genetic Structure of Wild *Orobanche cumana* Wallr. (Orobanchaceae) Populations in Eastern Bulgaria Reflects Introgressions from Weedy Populations. *The Scientific World Journal* 2014, *vol. 2014*, Article ID 150432, 15 pages. doi: 10.1155/2014/150432

XIV. The genetic structure of *O. cumana* populations was not determined by the fact that the populations were collected on wild or cultivated hosts.

XV. Two main contrasting gene pools were identified in Bulgarian populations, with most of the populations having intermediate characteristics.

XVI. Some wild populations had higher genetic diversity values and contained individuals with membership values very close to a weedy gene pool, suggested the existence of genetic flow between both gene pools that could be attributed to cross fertilization and/or seed movement.

XVII. Cross inoculation experiments also revealed that *O. cumana* populations collected on wild species possessed similar ability to parasitize sunflower to those collected on sunflower, even on cultivars carrying resistance genes.

XVIII. Wild populations of *O. cumana* show a high degree of self-pollination.

08 Contributions

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Other Scientific Contributions derived from the Doctoral Thesis are listed below

Kirilova, I.; Gevezova, M.; Dimitrova, A.; Kostov, K.; Batchvarova, R.; **Pineda-Martos, R.**; Pérez-Vich, B.; Maširević, S.; Škorić, D.; Medić-Pap, S.; Stoyanov, K.; Păcureanu, M.; Denev, I. Genetic Diversity of *Orobanche cumana* and *Orobanche cernua* Populations as Revealed by Variability of Internal Transcribed Spacers1/2 of Ribosomal Cistron and Ribulose-Bisphosphate Carboxylase Pseugene. In *Knowing the Parasite: Biology and Genetics of* Orobanche, *Proceedings of the Third International Symposium on Broomrape (*Orobanche *spp.) in Sunflower*, Córdoba, Spain, June 03–06, 2014; International Sunflower Association (ISA): Paris, France, 2014; pp 133–139.

Pineda-Martos, R. Diversidad Genética entre Poblaciones de *Orobanche cumana* Wallr. Españolas y Centroeuropeas. In *Proceedings of the First Congreso Científico de Investigadores en Formación*, Córdoba, Spain, October 15–16, 2009; University of Córdoba (UCO): Córdoba, Spain, 2009; pp 35–37.

Pineda-Martos, R.; Batchvarova, R.; Fernández-Martínez, J. M.; Velasco, L.; Pérez-Vich, B. Genetic Diversity of *Orobanche cumana* Populations from Spain and Eastern Europe. In *Proceedings of the Tenth World Congress of Parasitic Plants*, Kusadasi, Turkey, June 08–12, 2009; International Parasitic Plant Society (IPPS), 2009; pp 145.

Pineda-Martos, R.; Velasco, L.; Fernández-Martínez, J. M.; Pujadas-Salvà, A. J.; Pérez-Vich, B. Genetic Diversity of Wild *Orobanche cernua* L. Populations from Southeastern Spain. In *Proceedings of the Eleventh World Congress on Parasitic Plants*, Martina Franca, Italy, June 07–12, 2011; International Parasitic Plant Society (IPPS), 2011; pp 55.

Pineda-Martos, R.; Velasco, L.; Pujadas-Salvà, A. J.; Fernández-Martínez, J. M.; Pérez-Vich, B. Phylogenetic Relationships and Genetic Diversity among *Orobanche cumana* Wallr. and *O. cernua* L. (Orobanchaceae) Populations in the Iberian Peninsula. In *Knowing the Parasite: Biology and Genetics of* Orobanche, *Proceedings of the Third International Symposium on Broomrape (*Orobanche *spp.) in Sunflower*, Córdoba, Spain, June 03–06, 2014; International Sunflower Association (ISA): Paris, France, 2014; pp 127–132.

Rodríguez-Ojeda, M. I.; **Pineda-Martos, R.**; Alonso, L. C.; Fernández-Escobar, J.; Fernández-Martínez, J. M.; Pérez-Vich, B.; Velasco, L. A Dominant Avirulence Gene in *Orobanche cumana* Triggers *Or5* Resistance in Sunflower. *Weed Research* **2013**, *53(5)*, 322–327.

Rodríguez-Ojeda, M. I.; **Pineda-Martos, R.**; Alonso, L. C.; Fernández-Martínez, J. M.; Velasco, L.; Fernández-Escobar, J.; Pérez-Vich, B. Genetic Studies in Sunflower Broomrape. In *Knowing the Parasite: Biology and Genetics of* Orobanche, *Proceedings of the Third International Symposium on Broomrape (*Orobanche *spp.) in Sunflower*, Córdoba, Spain, June 03–06, 2014; International Sunflower Association (ISA): Paris, France, 2014; pp 116–120.

This Thesis aims to investigate the genetic diversity and population structure of the sunflower parasitic weed *Orobanche cumana* Wallr. through the use of microsatellite markers (SSRs). For this, it was necessary to develop such powerful approach and molecular tools, which were not available at the beginning of this research work. The genetic diversity of weedy *O. cumana* populations was studied in Spain, where this species is found as an allochthonous species parasitizing exclusively sunflower. Genetic diversity, population structure, and virulence on sunflower were investigated on *O. cumana* populations parasitizing wild plants from the Black Sea coast in Eastern Bulgaria, a country for which this species is autochthonous and where wild and weedy populations of *O. cumana* co-exist.

Sometimes the weeds turn into something beautiful

Cover Image 1. *From The Voynich Manuscript*
Cover Image 2. Population CUMBUL-8 of *O. cumana*. *From Pineda-Martos* et al. *2014b*

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