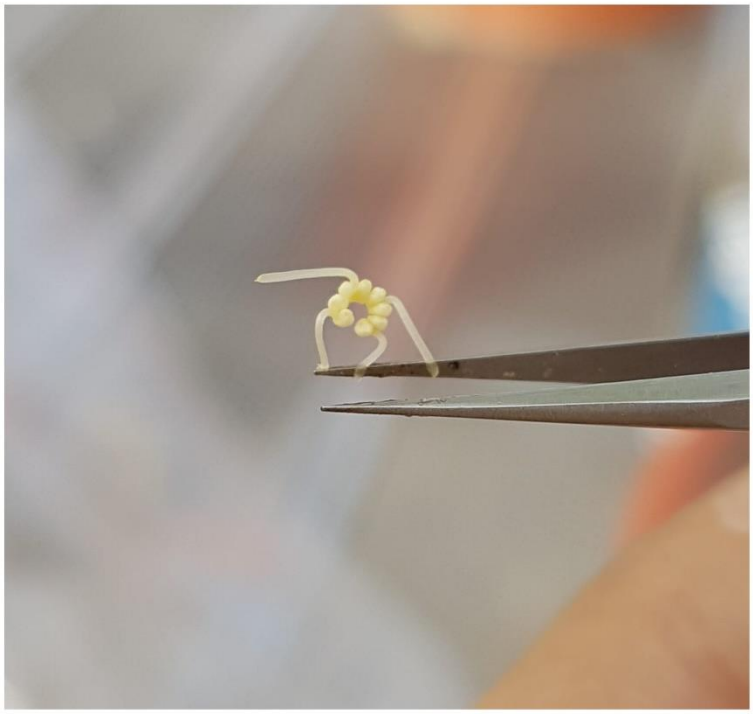
# Breeding strategies for resistance to sunflower broomrape: New sources of resistance and markers for resistance and avirulence genes



**TESIS DOCTORAL**

**Álvaro Calderón González**

TITULO: *Breeding strategies for resistance to sunflower broomrape: New sources of resistance and markers for resistance and avirulence genes*

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*“Que igual ser feliz no es ganar siempre, es no rendirse nunca.”*
*Defreds*

*Dedicado a mi familia.*

UNIVERSIDAD D CÓRDOBA

**Programa de doctorado**

Ingeniería Agraria, Alimentaria, Forestal y de Desarrollo Rural Sostenible

**Título de la tesis**

*Estrategias de mejora para resistencia a jopo de girasol: Nuevas fuentes de resistencia y marcadores para genes de resistencia y avirulencia*

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*Breeding strategies for resistance to sunflower broomrape: New sources of resistance and markers for resistance and avirulence genes*

**Directores**

Leonardo Velasco Varo

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**Fecha de depósito tesis en el Idep**: 6 de Abril de 2021

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSTITUTO DE AGRICULTURA SOSTENIBLE IAS

**TÍTULO DE LA TESIS: Breeding strategies for resistance to sunflower broomrape: New sources of resistance and markers for resistance and avirulence genes**

**DOCTORANDO/A: Álvaro Calderón González**

## 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 se ha realizado de forma satisfactoria por parte del doctorando, y se considera por parte de los directores que está preparada para su presentación y defensa. La tesis consta de tres capítulos basados en estudios sobre la planta parásita jopo del girasol (*Orobanche cumana* Wallr.) y la resistencia genética a la misma en el girasol.

Uno de los capítulos ha consistido en la elaboración de un mapa genético de jopo del girasol, el primero desarrollado en esta especie, como base para los estudios genéticos a desarrollar en esta especie. El correspondiente artículo científico se ha publicado en *Frontiers in Plant Science* (https://doi.org/10.3389/fpls.2019.00797).

Otro capítulo de la tesis ha consistido en un estudio genético sobre virulencia entre poblaciones de jopo de girasol. Estos estudios se han completado tanto a nivel fenotípico como genotípico. Se ha encontrado un gen de avirulencia en el parásito, lo que constituye un resultado espectacular, pues no hay antecedentes en la literatura. El correspondiente artículo científico se encuentra ya finalizado, a falta de enviar a una revista científica.

Por último, otro capítulo incluye un estudio de mapeo por asociación con tres poblaciones de jopo evaluadas en dos ambientes frente a en un panel de líneas de girasol, con el fin de localizar posibles fuentes de resistencia y genes de resistencia. El correspondiente artículo científico se encuentra ya finalizado, a falta de enviar a una revista científica.

En definitiva, se trata en nuestra opinión de un trabajo original de investigación que ha dado lugar a resultados de alto valor científico, y creemos que cumple todos los requisitos para su presentación y defensa como trabajo de tesis doctoral.

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

Córdoba, 5 de Abril de 2021

Firma del/de los director/es

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**TÍTULO DE LA TESIS:** Estrategias de mejora para resistencia a jopo de girasol: Nuevas fuentes de resistencia y marcadores para genes de resistencia y avirulencia

**DOCTORANDO/A:** Álvaro Calderón González

## INFORME RAZONADO DEL TUTOR

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

Juan Gil Ligero, Catedrático del Dpto. de Genética de la Univ. de Córdoba, informa que el trabajo de tesis ha tenido una evolución y desarrollo satisfactorio y ratifica el informe favorable del director.

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

Córdoba, 22_ de __marzo___ de __2021__

Firma del responsable de línea de investigación

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Fdo.: _Juan Gil ligero

## GENERAL INDEX

# AGRADECIMIENTOS/ACKNOWLEDGEMENTS

¡Por fin! Ha llegado el día tan esperado. Después de un duro camino que comenzó en mayo de 2016, tan lejos de mi casa, mi familia y mi entorno, en un sitio nuevo y tan diferente, esta etapa se ha cerrado.

Durante estos pocos años que ha durado mi doctorado, he pasado muchos altibajos, he conocido nuevos sitios, nueva gente y he vivido muchas aventuras que no podría haber experimentado de otra manera.

Quisiera empezar agradeciendo a las entidades que han hecho posible cumplir y culminar este trabajo. Al Ministerio de Educación por el contrato de Formación de Personal Investigador, a los proyectos AGL2017-87693-R y AGL2017-87693-R, Instituto de Agricultura Sostenible y al Laboratorio de Interacciones Planta-Microorganismo (LIPM-INRA).

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de haber coincidido poco tiempo conmigo, me ayudó mucho en todo momento y aún le tengo gran aprecio.

Como el día a día del investigador predoctoral está lleno de cosas buenas y malas, nunca faltan compañeros que te acompañan y, muchos de ellos pasan por lo mismo que tú. Mi trabajo y mi vida en Córdoba hubiera sido peor si no llega a ser por gente como las futuras doctoras Nuria Montes Osuna y Ludovica Rolando, ¡empezamos a la vez y vamos a acabar a la vez! Fueron las dos primeras personas que conocí al llegar al IAS y que a día de hoy se han convertido en amigas para toda la vida. La italo-andaluza Ludo, se ha pasado casi más tiempo en Andalucía en los últimos 5 años que en Italia, gracias a ella pude superar mi primer verano en Córdoba, turismo, tapeo, fiesta... 5 años después es mamá, casi doctora y una persona que, aún en la distancia, está, espero volver a verla pronto y me queda pendiente ¡visitar Roma! Nuria, qué voy a decir de Nuria, cualquier cosa que diga quedará pequeña en comparación a lo que ha significado esta persona para mí. Simplemente, no sé qué hubiera sido de mi sin ella en Córdoba. Más que una amiga, es ese tipo de persona que es familia y que sabe de sobra todo. Como yo soy difícil de aguantar, pero quien lo consigue, al final, me coge cariño, hay más gente que ha tenido la "suerte" de haberme acompañado estos años. La doctora Rosa Mérida García es de las personas que más mérito tiene en haber conseguido aguantarme hasta el día de hoy. Una gran persona, que siempre está para lo bueno y lo malo, con la que he compartido miles de desayunos e historias dentro y fuera del trabajo y junto al doctor Carlos Camino, ¡vaya dos!, la que me descubrió el Goiko y la que no hay día que no me metiera caña, pero, aunque tardamos en conocernos, a día de hoy una gran amiga. Su hermana la doctora Aida Mérida García, no se queda atrás, tan simpática como inteligente (gana todos los premios que hay así qué es extraordinaria como profesional y como persona), que también me ha tenido que aguantar, pero siempre con un buen consejo y una amplia sonrisa. Otra gran persona con mucha paciencia ha sido Valle, un gran apoyo para mí en los momentos buenos y malos, qué nos liaba a todos para tomar algo en el Cholondro y que fue muy importante en mi llegada a Córdoba. Echará de menos mis susurros en la ventana de cada mañana. El doctor Juan Manuel Arjona López, ¡un tío 10! con el que se puede hablar de todo y gran aficionado al ciclismo. Con el que tuve unas mini-vacaciones exprés en Portugal que salieron de lujo y que tenemos que repetir sí o sí. Buena experiencia en el Algarve también junto a los doctores Leticia Ayllón Egea y Manuel López López, con los que hay más historias fuera que dentro del IAS. Me ha venido muy bien la buena piña que se ha montado en el instituto gracias a la incorporación de Martina, que lo revolucionó todo para bien. Gran persona que ha podido "comprenderme" un poco, que me ha apoyado en

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sobreviviendo día a día al doctorado y a esta experiencia. Hay más nombres, solo he recalcado aquellas personas con las que más tiempo he pasado estos últimos años, pero, todo aquel que ha pasado por mi vida y ha sumado, sabe que le agradezco el tiempo compartido conmigo y, espero haber dejado un trocito de mi en todos ellos.

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Gracias a todas y cada una de las personas que han pasado por mi vida durante estos últimos 5 años para sumar, esta tesis no habría sido posible sin vosotr@s.

## SUMMARY

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant that causes significat yield losses to sunflower crops. While there are many studies associated with genetic resistance to broomrape in sunflower, the molecular tools that are available for research on *O. cumana* are very scarce. Genetic resistance to *O. cumana* in sunflower has been found in most cases following a gene-for-gene interaction, with dominant resistance genes in the host and dominant avirulence genes in the parasite. Due to this interaction, it is importat to know more about what the parasite is like and how it interacts, since a knowledge of both parts of the interaction is important for the development of long-term genetic resistance strategies, taking into account the rapid evolution in the virulence of the parasitic plant. The objectives of this Thesis focused on providing new data and molecular tools on the virulence/avirulence of *O. cumana* that are the basis for new approaches in the fight against the sunflower broomrape. In this work, SSR (simple sequence repeat) and SNP (single nucleotide polymorphism) molecular markers have been used to develop the first *O. cumana* genetic linkage map, successfully mapping a gene responsible for color, being the first step in the identification and mapping of other genes of interest. An avirulence gene, $Avr_{Hybrid2}$, has been positioned on the genetic map, thanks to the study of the inheritance of the trait using two *O. cumana* population currently found in Spain. Although resistance to the parasite has been found mainly qualitative, controlled by dominant genes, continuous racial evolution has given rise to the need for new sources of quantitative resistance. For this reason, a genome-wide association study (GWAS) in sunflower against different *O. cumana* races has revealed a series of markers associated with resistance and several candidate genes have been identified in these regions. They should be studied in more detail to discover new genes that lead to better and longer lasting sunflower broomrape resistance.

## RESUMEN

El jopo de girasol (*Orobanche cumana* Wallr.) es una planta holoparásita que causa importantes pérdidas de rendimiento en el cultivo de girasol. Si bien existen muchos estudios asociados con la resistencia genética en el huésped, las herramientas moleculares que se conocen para *O. cumana* son muy escasas. La resistencia genética a *O. cumana* se ha encontrado que sigue, en la mayoría de los casos, un modelo gen a gen, con genes dominantes de resistencia en el huésped y genes dominantes de avirulencia en el parásito. Debido a esta interacción, es importante conocer más sobre cómo es el parásito y cómo interactúa, ya que el conocimiento de ambas partes de la interacción es importante para el desarrollo de estrategias de resistencia genética a largo plazo, teniendo en cuenta la rápida evolución en la virulencia de la planta parásita. Los objetivos de esta Tesis se han centrado en aportar nuevos datos, así como herramientas moleculares relacionadas con la virulencia/avirulencia de *O. cumana*, que sirvan como base para el desarrollo de nuevos enfoques en la lucha contra esta planta parásita. En este trabajo se han utilizado marcadores moleculares de tipo SSR (secuencia de repetición simple) y SNP (polimorfismo de nucleótido simple) para desarrollar el primer mapa de ligamiento genético en *O. cumana*, mapeando además con éxito, un gen responsable del color, siendo el primer paso en la identificación y mapeo de otros genes de interés. Un gen de avirulencia, $Avr_{Hybrid2}$, ha sido mapeado en el mapa genético, gracias al estudio de la herencia llevado a cabo entre dos poblaciones de *O. cumana* presentes actualmente en España. Aunque la resistencia al parásito se ha encontrado principalmente cualitativa, controlada por genes dominantes, la evolución racial continua ha dado lugar a la necesidad de utilizar nuevas fuentes de resistencia de carácter cuantitativo. Por este motivo, un estudio de asociación de genoma completo (GWAS) en girasol evaluado frente a diferentes razas de *O. cumana*, ha revelado una serie de marcadores asociados a resistencia y, se han identificado varios genes candidatos en estas regiones que conviene estudiar con más detenimiento para descubrir nuevos genes. que logren conducir a una mejor y más duradera resistencia a jopo de girasol.

# CHAPTER I

# General Introduction and Objectives

## SUNFLOWER

### *Biology and history of its spreading*

Sunflower (*Helianthus annuus* L.) is one of the major oilseed crops in the world whose origin is North America. The cultivated sunflower is a species of the Asteraceae family grown commercially worldwide contributing to human health with nutritional benefits and offering important chemical applications (Guo et al., 2017; Adeleke & Babalola, 2020). The domesticated sunflower was introduced in Europe by Spanish explorers in the early 1500s (Putt, 1977, 1978), where its initial use was ornamental due to its attractive and flashy shape. Its cultivation spread through the whole continent to Russia. Currently, sunflower is present in every continent, except in Antarctica. It began to have an edible use as a snack and the first known patent for the use of sunflower oil dates from 1716, in England (for industrial use). The popularity of sunflower and thus its cultivation as well, increased in Russia thanks to the Orthodox Church, which prohibited the consumption of most oil foods but did not include sunflower in the list. Its rapid expansion in Russia led to the development of several local cultivars. Sunflower breeding work during the 19th century focused on early maturation, oil content and pest resistance. By the beginning of the 20th century, it became one of the major crops in Russia. Russian breeder Pustovoit achieved exceptional results, having managed to increase considerably the seed oil content (Pustovoit, 1964). After its expansion in Russia, sunflower was reintroduced to North America in the late 19th century (Semelczi-Kovacs, 1975; Heiser, 1976). The 20th century was characterized by rapid development in sunflower breeding. Major breeding advances were the discovery of cytoplasmic male sterility and the identification of fertility-restoration genes (Leclercq, 1969; Enns et al., 1970; Kinman, 1970), which enabled the development of commercial hybrids.

### *Economic importance and threats*

At present, sunflower is one of the most important oilseed crops in the world as a source of vegetable oil with a cultivated area of 27 million ha and a production annual that far exceeds 51 million metric tons, mainly concentrated in Russia, Ukraine, and the European Union (FAOSTAT, 2018). The international oilseed production is dominated mainly by soybean, followed by rapeseed and sunflower oil (Statista, 2020) (Figure 1).

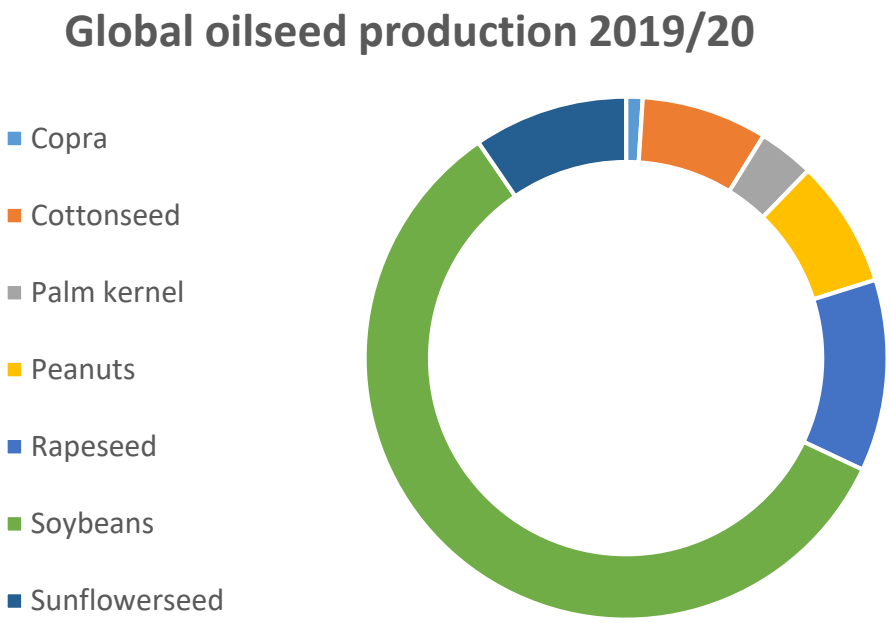


**Figure 1.** Worldwide oilseed production in 2019/2020, by type (in million metric tons). Statista 2020

Sunflower is considered as a high-quality vegetable oil source for edible and industrial uses (Pal et al., 2015). The wide agronomic adaptability of sunflower, which allows it to grow in different soil conditions and climatic regions, has expanded its cultivation throughout the world, making it a crop of great economic value in the global market (Forleo et al., 2018). The European Union is the third world producer of sunflower, behind Ukraine and Russia. Spain occupies the 5th place in the European Union behind Romania, Bulgaria, Hungary, and France. While the main oilseed crop in Europe is rapeseed followed by sunflower, the situation in Spain is the opposite. Sunflower is the first oilseed crop in Spain with production around 950 thousand metric tons, mainly cultivated in Castilla y León, Andalusia, and Castilla La Mancha (MAPA, 2020). The global sunflower production for 2020-2021 has been projected up to 56 million metric tons at the beginning of 2020 and even with the current world situation, FAO continues to estimate that its production will increase, reaching near-record values due to the increase in planted areas such as South America, China, Ukraine, and Russia, whereas in the European Union, its acreage will be slightly reduced in countries such as Romania and Spain (FAO, 2020).

Despite the progress that has been made with this oilseed crop, there are factors that affect the normal development of sunflower. Considering that sunflowers could be

cultivated worldwide, one of the challengeable issues is getting the plant to grow correctly to give good productivity without abiotic or biotic problems. Among the major limitations in the growth and production of sunflower is a parasitic plant called sunflower broomrape (*Orobanche cumana* Wallr.), which may cause devastating yield losses in sunflower crops in its areas where the parasite is present.

## SUNFLOWER BROOMRAPE

### *Parasitic plants*

Parasitic angiosperms obtain part or the totality of the necessary nutrients for their development from a host plant. Through a special organ known as haustorium, they manage to connect with the host plant to extract water and nutrients (Joel, 2013). There are two types of parasitic plants according to their mode of nutrition: those that need the host but have photosynthetic capacity (hemiparasitic plants) and others that are obligate parasites since they are unable to conduct photosynthesis, so they inevitably need the host for nutrition (holoparasitic plants). Among the parasitic angiosperms, there are species that parasitize crop plants and can be devastating for the agricultural systems. Parasitic plants of commercial importance include six genera belonging to three families: the genus *Cuscuta* within the Convolvulaceae family, genera *Orobanche*, *Phelipanch*e and *Striga* belonging to the Orobanchaceae family, and genera *Arceuthobium* and *Viscum*, both of the Viscaceae family (Schneeweiss, 2013). The genus *Arceuthobium* causes significant losses in forest species in North America and the genus *Striga* mainly affects maize, millets and sorghum in central Africa and Asia. In the Mediterranean area, the genera *Orobanche*, *Cuscuta* and *Viscum* are the most important, especially the first two ones (Parker & Riches, 1993).

### *Orobanche cumana Wallr.*

*Orobanche cumana* Wallr. (sunflower broomrape) is a holoparasitic weed which is one of the most important biotic restrictions for sunflower cultivation in the Old World, being not present in North and South America (Cantamutto et al., 2014). In the past, *Orobanche cumana* was considered as a subspecies of *Orobanche cernua*. Subsequent studies made it clear that that they were two separate species (Pujadas-Salvà & Velasco, 2000). *O. cumana* is a diploid species with 2n=38 chromosomes, with a genome size of 1.42 Gb obtained by densitometry (Weiss-Schneeweiss et al., 2006) or 1.40 Gb resulting from the assembly of DNA sequences (Pouilly et al., 2018), and with a first draft of the genome sequence (Gouzy et al., 2017).

### *Biology*

Like other holoparasitic plants devoid of chlorophyll, *O. cumana* haustorium penetrates the sunflower root system to obtain water and nutrients (Joel et al., 2015),

altering all sunflower growth parameters with a subsequent reduction of yield up to 100% in sensitive cultivars (Alcántara et al., 2006). Sunflower broomrape produces a large number of tiny seeds which are easily disseminated by water, wind, insects or other agents. In addition to the problem that each flower is capable of releasing thousands of seeds, the seeds remain viable in dormancy state for decades in the soil (Prider et al., 2013). *O. cumana* is characterized by flowering stems between 40 and 80 cm high without leaves but with several down-curved flowers vary in color from white to violet and arranged in racemes. The stem has a pale brown color due to the lack of chlorophyll. The seed has a teardrop shape with an elongated head and is densely pitted (Dor et al., 2019). The seeds only germinate in the presence of a nearby host. For germination, a starting conditioning period is required followed by chemical stimulation by the root exudates of the host (Louarn et al., 2012). It has been demonstrated that *O. cumana* seeds require at least four days at 21$^{o}$C as a conditioning period, to have a good response to the chemical stimulants (Lechat et al., 2015). Most chemical germination stimulants belong to a class of carotenoid-derived compounds called strigolactones (Yoneyama et al., 2013). Strigolactones are very usual root exudates of host and non-host plants. However, sunflower roots also produce a root exudate (dehydrocostus lactone) that specifically stimulates a response in *O. cumana* (Joel et al., 2011; Raupp & Spring, 2013). It has been reported that germination in *O. cumana* is also caused by exudates of cereals, legumes, rapeseed, and cotton (Fernández-Aparicio et al., 2009, 2011; Zhang et al., 2013; Ye et al., 2017) but without subsequent parasitism. After germination, the parasitic attachment is produced for a structure known as appresorium or pre-haustorium. Once the appresorium penetrates into the host root tissue, a connection with the host vessel system is established. A specialized organ named haustorium is the canal through which the parasitic plant uptakes the necessary water and nutrients from the host (Hibberd & Jeschke, 2001). When the haustorium is functional, the parasite acts by expressing genes involved in the metabolism of sucrose and generates a great absorption strength by taking mainly, auxin and sucrose form the host (Bar-Nun et al., 2008; Abbes et al., 2009). After a successful union and if the host mechanisms do not stop the parasitism, *O. cumana* will begin growing generating a structure known as nodule from which one or several stems are developed. They will emerge from the soil and shortly afterwards its flowers will produce thousands of small seeds that will start the cycle again (Figure 2).

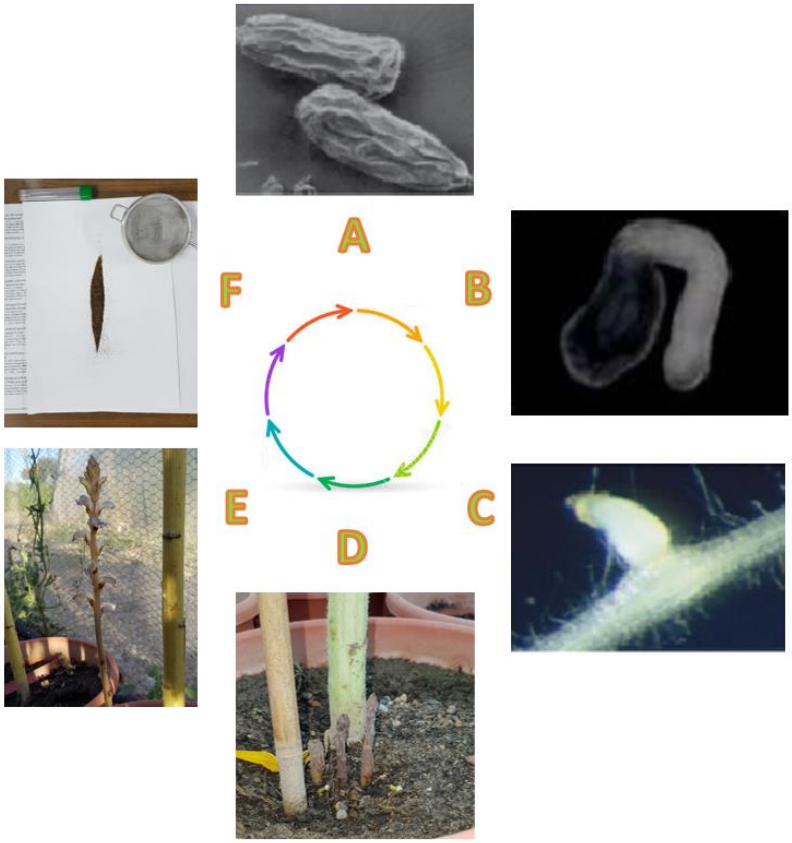


**Figure 2.** *Orobanche cumana* lyfe cycle. A) Seed germination; B) Appresorium development; C) Haustorium development; D) Emergence; E) Flowering; F) Seed production

### *Geographical spreading and racial evolution*

Sunflower broomrape is found in the wild as a parasite of wild Asteraceae species, mainly *Artemisia* spp., from central Asia to southeastern Europe (Pujadas-Salvà and Velasco, 2000). However, in crop plants it is only found parasitizing sunflower (Parker, 2013). First reports of broomrape parasitism in this crop date back to the late 19th in the former Soviet Union (USSR) and currently it has become a serious threat for this crop in several countries of the Old World, including Spain, countries around the Black and Caspian Seas, and China (Fernández-Martínez et al., 2015). In addition, over the past few years it has been found even in countries free of this parasite until now, such as France (Jouffret & Lecomte, 2010; Jestin, 2012; Jestin et al., 2014), Tunisia (Amri et al., 2012), Morocco (Nabloussi et al., 2018), and Portugal (González-Cantón et al., 2019).

*O. cumana* populations are commonly classified into physiological races based on the resistance reaction of different sunflower genotypes that are capable of avoiding parasitization (Molinero-Ruíz et al., 2006). The first sunflower variety resistant to *O.*

*cumana* (Race A) was at the beginning of the 20th century in western regions of the former USSR, developed by Plachek in 1918 (Skoric et al., 2010). However, the capacity of the parasite to overcome sunflower resistance led to the appearance of a new race (Race B) in other sunflower cropping regions of the country (currently independent countries of Moldova and Ukraine) (Duca, 2014; Miladinovic et al., 2014). It was followed by the new race C observed in Moldova in the 1960s (Alonso, 1998) as well as a different race C observed in Bulgaria in the 1970s (Skoric, 2012). Using different sunflower broomrape populations observed in Romania, Vranceanu et al., 1980, made the first comprehensive race classification of this parasitic weed, cataloging the *O. cumana* races from race A to E, in an ascending virulence range that would be given by a set of sunflower differential lines developed by the same researchers, which carried, respectively, five dominant resistance genes named *Or1*, *Or2*, *Or3*, *Or4* and *Or5*. Until the middle 1990s, races D and E were the predominant and they were controlled by the resistance gene *Or5*. However, populations overcoming *Or5* (race F) were detected in 1995 in Spain, and shortly after in Romania and several other countries (Molinero-Ruíz et al., 2006). Currently, *O. cumana* race F is the most commonly found but newest virulent races known as G and H have also been recently described (Kaya, 2014). Therefore, during the last 30 years more virulent broomrape races have been appearing, probably as a consequence of crop intensification, host selective pressure and a rapid evolution in the complexity of the interaction (Kaya et al., 2014).

Despite eight *O. cumana* races have been reported to date, named with letter from A to H, it exists confusion in terminology used for this race identification, since the same letter is used for diverse parasite populations in different countries without the appropriate racial studies using the same set of differential lines (Molinero-Ruiz et al., 2015; Cvejic et al., 2020). Currently, there have been reported cases of races F, G and H in Bulgaria (Shindrova, 2006; Batchvarova, 2014; Encheva, 2018), the presence of all races (A-H) in China and race G in Mongolia (Ma & Jan, 2014; Shi et al., 2015; Zhang et al., 2018), race F in Bulgaria (Zoltan, 2001; Hargitay, 2014; Molinero-Ruíz et al., 2014), races C and G in Kazakhstan (Antonova, 2014), less virulent races (up to E) in the central region of Moldova and, more virulent races (G and H) found in northern and southern regions of the country (Gisca et al., 2013; Duca, 2014; Duca et al., 2017, 2019), race G from 2005 in Romania and races more virulent observed in later research (Pacureanu et al., 2009; Risnoveanu et al., 2016), race E in Serbia with scattered points of race F (Miladinovic et al., 2012; Dedic et al., 2018), races F and G in Spain (Martín-Sanz et al., 2016; Malek et al., 2017), and races F, G and H in Ukraine (Hablak & Abdullaera, 2013; Duca et al., 2019). In summary, nowadays, there is a

general predominance of races F and G, with some reports of more virulent races (Table 1). The most virulence races are commonly found in areas close to the Black Sea.

The continuous appearance of new broomrape races that overcome the resistance of sunflower jeopardizes sunflower production in areas with very virulent races. Additionally, despite sunflower broomrape has a high specificity for sunflower, a recent study has reported *O. cumana* infestations in other crops such as tomato and tobacco at a local level (Dor et al., 2019).

**Table 1.** Worldwide race spreading of Sunflower Broomrape until 2020. UK* = unknown the race of the parasite

| World race evolution in Sunflower Broomrape | | | |
|---|---|---|---|
| **Country** | **Former Races** | **Current Races** | **References** |
| **Bulgaria** | A, B, C, D, E | E, F, G, H | Shindrova, 2006; Skoric, 2012; Batchvarova, 2014; Encheva, 2018 |
| **China** | A | A, B, C, D, E, F, G | Ma & Jan, 2014; Shi et al., 2015; Zhang et al., 2018 |
| **France** | | NK* | Jouffret & Lecomte, 2010; Jestin, 2012; Jestin et al., 2014 |
| **Hungary** | A, B, C, D | E, F | Zoltán, 2001; Hargitay, 2014; Molinero-Ruíz et al., 2014 |
| **Kazakhstan** | UK | C, G | Antonova, 2014 |
| **Moldova** | B, C | E, F, G, H | Alonso,1998; Gisca et al., 2013; Duca, 2014; Duca et al., 2017, 2019 |
| **Mongolia** | | G | Ma & Jan, 2014; Shi et al., 2015 |
| **Morocco** | | G | Nabloussi et al., 2018 |
| **Portugal** | | G | González-Cantón et al., 2019 |
| **Romania** | A, B, C, D, E | F, G | Vranceanu et al., 1980; Pacureanu-Joita et al., 2004, 2008, 2009; Risnoveanu et al., 2016 |
| **Rusia** | A, B, C, D | D, E, F, G, H | Kaya et al., 2009; Shindrova & Penchev, 2012; Antonova et al., 2013; Skoric, 2012; Antonova, 2014 |
| **Serbia** | B, E | E, F | Mihaljcevic, 1996; Masirevic & Medic-Pap, 2009; Masirevic et al., 2012; Miladinovic et al., 2014; Dedic et al., 2018 |
| **Spain** | B, C, D, E | E, F, $G_{GV}$ | González-Torres et al., 1982; Melero-Vara et al., 1989; Saavedra Del Río et al., 1994; Alonso et al., 1996; Molinero-Ruiz et al., 2008, 2014; Fernández-Escobar et al., 2008; Martín-Sanz et al., 2016; Malek et al., 2017 |
| **Tunisia** | | E, G | Jebri et al., 2017 |
| **Turkey** | D, E | F, G | Bulbul et al., 1991; Kaya et al., 2004, 2012; Molinero-Ruíz et al., 2014 |
| **Ukraine** | A, B, C, D | E, F, G, H | Alonso, 1998; Burlov & Burlov, 2010; Hablak & Abdullaera, 2013; Pototskyi, 2014; Miladinovic et al., 2014; Maklik et al., 2018 |

### *Molecular and genetic diversity studies*

Comparative studies between different *O. cumana* populations from different regions serve to unify genetic criteria and facilitate the knowledge of the virulence evolution of this parasite. The initial genetic variability studies in this parasitic weed were based on morphological characters, which did not allow differentiation of populations (Román, 2013). Isozymes were the first molecular markers used for diversity studies in *O. cumana* populations. These are protein markers that catalyze the same reaction but which, depending on structural modifications, move differently in an electrophoresis reaction. They were used for the study of populations of broomrape infesting sunflower in the south of Spain (Castejon-Muñoz et al., 1991). However, differences caused by environmental conditions, plant developmental stage or the type of tissue used did not allow a reproducible practical use. DNA-based molecular markers can overcome some of the disadvantages of the protein-based markers and their use in genetic diversity studies is widely known in different plant species. The first study of genetic diversity among *O. cumana* populations from different countries using DNA-based markers was carried out with RAPD (Random Amplified Polymorphic DNA). These markers were widely used to distinguish biotypes in several weed species and crops since they were simple, rapid, inexpensive, did not require prior knowledge of the genome of the plant and need minimal amounts of DNA, although they had limitations such as allele dominance and poor reproducibility (Harris,1999). Gagne et al. (1998) identified, using RAPDs, low intrapopulation and large interpopulation genetic variation in *O. cumana* populations from different countries, concluding the existence of two main genetic pools, one comprising the populations from Eastern Europe and another one including the populations from southern Spain. According to these authors, little gene exchange within populations was attributed to the fact that *O. cumana* was a self-pollinated species. However, a rate of cross pollination exists in this species as it was found in a study done with an unpigmented mutant lacking anthocyanin pigmentation (Rodriguez-Ojeda et al., 2013). Molinero-Ruiz et al. (2014) used also RAPD markers to show the genetic differences between the populations of Southern and Central Spain. Both groups were identified as distant pools with low intra- and inter-population genetic variation within each pool, probably due to different founder effect events, one in Cuenca province in central Spain and other in the Guadalquivir Valley in southern Spain. Although most of these studies reported low intra-population genetic variability, greater genetic diversity was observed among populations geographically distant (Ciuca et al., 2004; Atanasova et al., 2005). A comparative study between RAPD and a different group of markers, AFLP

(Amplified Fragment Length Polymorphism) concluded that both types or markers provided very similar results but that AFLP had the main advantage that they presented a higher degree of resolution when discriminating closely related genetic materials (Gagne et al., 2000).

Another type of DNA-based markers used later in *O. cumana* were single sequence repeats (SSRs), also known as microsatellite markers, and their closely related inter-simple sequence repeats (ISSR). SSRs have been used in several genetic diversity studies due to their high polymorphism level, reproducibility and codominant inheritance. Benharrat et al., 2002 used ISSR markers for taxonomic discrimination between *O. cernua* and *O. cumana*. A set of *O. cumana* SSRs was developed by Pineda-Martos et al. (2014). Using these SSR, Pineda-Martos et al. (2013) studied several populations from central and southern Spain, concluding also the existence of two Spanish gene pools, one in Cuenca and another one in the Guadalquivir Valley. The authors also identified a few populations in which individuals from both gene pools co-existed, and even documented some cases of hybridization between them, which resulted in increased intra-population polymorphism. Additionally, the set of microsatellites described in Pineda-Martos et al. (2014) has been used in different articles after its publication, in molecular characterization and comparative racial studies (Gutchel et al., 2014; Martín-Sanz et al., 2016; Duca et al., 2017; Malek et al., 2017; Jebri et al., 2017; Yonet et al., 2018; Ziadi et al., 2018; Duca et al., 2020), in the construction of the first genetic linkage map of *O. cumana* together with single nucleotide polymorphism (SNP) markers (this PhD thesis; Calderón-González et al., 2019), in the discovery of a new population of sunflower broomrape in Israel that has not only parasitized on sunflower, but also tomato and tobacco (Dor et al., 2019), and also for genetic diversity studies in the closely-related species *O. cernua* (Belay et al., 2020).

### *Control strategies*

*O. cumana* produces an extraordinary number of long-lived seeds with easy dispersal and a genetic adaptability to host resistance that makes it a challenge to find ways to avoid their infestations (Rubiales et al., 2009). Different methods of control used to fight this parasitic plant are described below.

- Agronomic control

The use of trap crops in rotation has been successful in reducing the seedbank. Trap crops refer to other non-host plant species that are capable of stimulating the

germination of sunflower broomrape seeds (suicide germination) but, as they are not natural hosts, they do not suffer from parasitism. Different crops have been tested to stimulate the germination of sunflower broomrape seeds, such as corn, sorghum, cotton, rice, eggplant, cauliflower, wheat, pepper and sugar beet plants (Rodriguez-Ojeda et al., 2001; Lang et al., 2017; Kitis et al., 2019; Hayat et al., 2020). A study conducted by Fernández-Aparicio et al. (2009) identified the following crops as potential trap crops for sunflower broomrape, listed here in decreasing order of germination induction: cotton, oat, wheat, sorghum, pearl millet, chickpea, fava bean, rapeseed and triticale. High germination percentages have been also reported for soybean cultivars (Zhang et al., 2013). Several lines of maize have produced germination percentages close to 40% and, subsequently, less broomrape infestations and increased sunflower biomass when used in the rotation (Ma et al., 2013; Ye et al., 2020). Rye root exudates also have the potential to induce germination of sunflower broomrape seeds (Cimmino et al., 2015). Similarly, compounds exudated by foxtail millet roots also stimulated germination of sunflower broomrape seeds (Ye et al., 2016).

- Chemical control

Initial studies indicated that herbicides of the imidazolinones group turned out to be quite effective in reducing sunflower broomrape infection regardless of the race composition in the soil. The application of imazapyr showed good results in pre and post-emergence treatments of the parasite (García-Torres et al., 1994, 1995), also very similar to those reported with imazapic (Aly et al., 2001; Eizenberg et al., 2009; Pincovici et al., 2018) and sulfonylurea (Salas et al., 2012). Thanks to the identification of imidazolinone-resistant sunflower mutants, it was possible to develop herbicide-resistant hybrids in which the treatment kills the broomrape but does not have a negative on the crop, provided that adequate doses and treatment stages are used (Tan et al., 2005). Breeding sunflower for resistance to herbicides is nowadays an important breeding objective (Eizenberg et al., 2013). Another chemical treatment that shows an activation of the defense mechanism in the host plant suggested by the lower number of emerged broomrape shoots and higher sunflower biomass is the treatment of sunflower seeds with salicyclic acid (Yang et al., 2016). Another compound tested for sunflower protection against broomrape is 5-aminolevolinic acid. This compound has no herbicide effect, but contributes to reduce the damage produced by the parasite (Li et al., 2019). Studies carried out with strigolactones (Lumbroso et al., 2016), dehydrococus lactones (Joel et al., 2011), sesquiterpene lactones (Raupp & Spring, 2013), non-sesquiterpene lactones (Ueno et al., 2014) and other biometabolites (Masi et al., 2019) have shown successful results as germination

stimulants that may contribute to reducing the number of broomrape seeds in the soil.

- Biological methods

The use of living organisms that are natural enemies of broomrape is an advantage since they are usually parasite-specific and do not contribute to environmental pollution. The fly *Phytomyza orobanchia* Kaltenbach was tested with good results and is a host-specific insect of *Orobanche* spp (Klein & Kroschel, 2002). They lay eggs inside the broomrape flowers, thus reducing their reproductive capacity. The validity of this approach for application at a commercial scale has not been demonstrated yet (Klein & Kroschel, 2002). A bioherbicide based on *Fusarium oxysporum* f.sp. *orthoceras* has achieved a significant reduction in the total number of sunflower broomrape after a simple soil application (Cohen et al., 2002; Shabana et al., 2003). Louarn et al. (2012) observed that the inoculation with arbuscular mycorrhizal fungi produces a negative impact on the germination of sunflower broomrape seeds, with exudates from the fungus providing also a protective effect to sunflower. Moreover, a culture filtrate of *Streptomyces enissocaesilis* had beneficial effects on the rhizosphere of infested sunflowers, reducing significantly the germination of *O. cumana* seeds (Chen et al., 2016).

- Genetic resistance

Genetic resistance is considered the most sustainable method for broomrape control. However, the continued use of vertical resistance mechanisms has resulted in a rapid evolution of broomrape populations towards more virulent genotypes. New resistance sources, carrying preferably different resistance mechanisms, are required for developing durable genetic resistance to broomrape in sunflower (Fernández-Martínez et al., 2015). This will be covered in more detail in the next section.

## *O. CUMANA* - SUNFLOWER INTERACTION

### *Sunflower resistance*

Breeding for broomrape resistance in sunflower is a successful and sustainable approach to control *O. cumana* (Pérez-Vich et al., 2013). The implementation of resistance is a continuous work, which includes a constant search for resistance genes and the development of sunflower genotypes resistant to new pathogenic races. Genetic variability is the base of any breeding program. The genetic resources existing in the wild sunflower species (*Helianthus*) are currently the most important reservoir of resistance genes against *O. cumana* (Seiler et al., 2017, 2019). Open pollinated cultivars, mainly those developed in the former Soviet Union in the initial breeding programs have also resulted in an important source of resistance genes against broomrape (Gorbachenko et al., 2011). The development of synthetic populations from resistant sunflower hybrids and inbred lines developed by diverse research centers in different countries has also proved to be an effective means to enhance sunflower resistance against the more virulent races of *O. cumana* (Cvejic et al., 2020). The success of the selection depends on the introgression of resistance genes to *O. cumana* from the resistant sources. In the case of wild species, this requires interspecific hybridization, which is complex process that requires recovery of favorable agronomic traits together with the resistance genes (Pérez-Vich et al., 2013).

In most crop species, resistance to *Orobanche* spp. is polygenic and non-race-specific. Contrarily, sunflower resistance to *O. cumana* is commonly controlled by qualitative or vertical genetic mechanisms, based in a gene-by-gene interaction (Pérez-Vich et al., 2013). Sunflower genetic resistance to broomrape conferred by dominant alleles at a single gene was first reported by Pogorletskiy & Geshele (1976). Vranceanu et al. (1980) confirmed the existence of vertical resistance to all broomrape races present at that time in Romania, named as race A to race E. The authors identified major resistance genes to the five races, which were named *Or1* to *Or5*, respectively. Additionally, five sunflower differential lines were established for race identification. The problem with this type of resistance is that the selection pressure exerted by the use of commercial sunflower hybrids carrying major resistance genes facilitates the appearance of new races of the parasite (McDonald & Linde, 2002). However, other resistance mechanisms not controlled by single major genes do also exist in sunflower. With the appearance of race F, a number of sources of resistance with different modes of inheritance were reported. They included resistance controlled by a single dominant gene (Pacureanu-Joita et al., 1998; Pérez-Vich et al., 2004), two partially dominant genes (Velasco et al., 2007; Akhtouch et al., 2016), two recessive

genes (Rodriguez-Ojeda et al., 2001; Akhtouch et al., 2002; Fernández-Martínez et al., 2004) or multiple quantitative trait loci (QTLs) (Pérez-Vich et al., 2004; Louarn et al., 2016; Imerovsky et al., 2019). For the new biotypes showing virulence higher than F (named as G or even H), resistance has been found to be controlled by single dominant genes (Velasco et al., 2012) and single recessive genes (Cvejic et al., 2020).

Genetic sources of horizontal or quantitative genetic resistance are important to provide durable resistance in combination with sources having vertical resistance (Pérez-Vich et al., 2013). Early studies by Pérez-Vich et al. (2004) identified a quantitative component of the resistance to broomrape determined by QTLs with small to moderate effect and associated with the number of broomrape shoots per plant, which did not determine total absence of broomrape as it is the case of the phenotype observed for major dominant *Or* genes, but a reduction in their number. Later studies revealed their role in controlling different broomrape developmental stages involved in minor and accumulative resistance mechanisms (Louarn et al., 2016), and also the importance of (i) "defeated resistance genes" corresponding to major resistance genes specific for a broomrape race which provide also moderate levels of resistance to a different-more virulent races (Imerovski et al., 2019) and (ii) resistance QTL present in susceptible cultivars (Pérez-Vich et al., 2004; Akhtouch et al., 2016). The combination of resistance genes with major (qualitative or vertical) and minor (quantitative or horizontal) mechanisms is a promising alternative for long-term strategies to ensure a durable sunflower protection against *O. cumana* (Pérez-Vich et al., 2013), as it has been demonstrated in other pathosystems involving viruses, fungi and nematodes (Brun et al., 2010; Fournet et al., 2013; Palloix et al., 2009).

Marker-assisted selection (MAS) for resistance breeding is a more efficient method for introgressing resistance genes into selected cultivars, compared to classical phenotype-based selection. It has been applied in sunflower mainly for major resistance genes. In recent years, the development of molecular markers for the localization and identification of region carrying resistance genes and their used in MAS have been successfully used for downy mildew, sunflower rust, broomrape and for characters such as seed oil quality (Dimitrijevic & Horn, 2018). In addition, the modern molecular tools for the analysis of the whole genome such as genome-wide association studies (GWAS), also called association mapping (AM), could lead to the discovery of novel resistance genes at a wider scale with a lower cost and time than classical breeding approaches. The use of GWAS for the assessment of marker-trait associations (MTAs) between phenotypes and genotypes has turned into a potential strategy in plant breeding programs and it has been demonstrated in multiple

species. The application of this new tool could help breeders to select indirectly genes of interest associated to markers. This technology has some limitations as well, since it requires high density of markers, efficient phenotypic information, good population structure, family relatedness, error correction and adequate computational resources, among other aspects. The development of better statistical models in recent years has contributed to improve the reliability of these studies (Gupta et al., 2019). In sunflower, the availability of high density SNP and the publication of the whole sunflower genome sequence (Badouin et al., 2017) allow to have available large amounts of genetic information for GWAS. However, association mapping studies in sunflower have only been carried out in relation to flowering time (Mandel et al., 2013; Cadic et al., 2013; Bonnafous et al., 2018), branching (Mandel et al., 2013; Nambeesan et al., 2015), abiotic stresses (Mangin et al., 2017), floral traits (Dowell et al., 2019) and fertility restoration (Goryunov et al., 2019; Talukder et al., 2019; Sajer et al., 2020). Thus far , only two studies associated with resistance to biotic stresses, focused on the disease caused by the Ascomycete *Sclerotinia sclerotiorum* (Lib.) de Baryresis (Fusari et al., 2012; Talukder et al., 2014). There are currently no GWAS studies related to the resistance to *O. cumana*, despite the great importance of this parasitic weed for sunflower production. In other crops, there are few GWAS studies related to parasitic weeds, such as the study in *Arabidopsis thaliana* that combined several abiotic and biotic stresses, including the response to the parasitic plant *Phelipanche ramosa* (Thoen et al., 2017) and, a very recent article in which SNPs significantly associated with defense mechanism of sorghum against the parasitic weed *Striga* sp. have been discovered (Kavuluko et al., 2020).

### *Avirulence genes in O. cumana*

The increasing accessibility to modern genetic and genomic tools allows not only advances in the knowledge of important crops such as sunflower but also the use of the same technologies in other plants, perhaps less attractive, as it is the case of parasitic plants. The discovery of resistance genes to *O. cumana* in sunflower is widely known (Molinero-Ruiz et al., 2015) but it is also known that gene-to-gene model is characterized by the recognition, for the host plant, of a protein encoded by an 'avirulence gene' complementary to the resistance gene, which would trigger the immune defense response in sunflower. Studies of avirulence genes have been mainly based on bacteria, fungi, viruses and nematodes (Rouxel & Balesdent, 2010). The only report of an avirulence gene in sunflower broomrape was described by

Rodriguez-Ojeda et al. (2013), who identified a gene named *Avr*$_{Or5}$ whose mutation allowed the parasite to surpass the resistance provided by *Or5* resistance gene in sunflower, resulting a more virulent *O. cumana* race F. However, the nature of this genes is still unknown, in part due to the lack of specific tools to conduct genetic research in this parasitic weed. In recent years, new tools such as the SSRs developed by Pineda-Martos et al., (2014), widely used in subsequent studies, or the first draft of the genome of this plant (Gouzy et al., 2017) are becoming available. Further studies such as genetic maps or mapping avirulence genes will provide additional tools and information to help control this parasitic weed. Both aspects are research objectives in this PhD Thesis.

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## OBJECTIVES

- Construction of a sunflower broomrape genetic map to provide a novel molecular tool for future genetic studies in this parasitic plant.
- Study of the inheritance of virulence/avirulence in the cross of two populations of sunflower broomrape of the Guadalquivir Valley in Southern Spain, classified as races $F_{GV}$ and $G_{GV}$, respectively, and identification of molecular markers associated with the trait.
- Identification of molecular markers associated with resistance to sunflower broomrape in sunflower through genome-wide association study (GWAS) in a set of sunflower accessions evaluated with three contrasting populations of broomrape.

# CHAPTER II

# An SSR-SNP linkage map of the parasitic weed *Orobanche cumana* Wallr. including a gene for plant pigmentation

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## ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant that causes major yield losses to sunflower crops in the Old World. Efforts to understand how this parasitic weed recognizes and interacts with sunflowers are important for developing long-term genetic resistance strategies. However, such studies are hampered by the lack of genetic tools for *O. cumana*. The objectives of this research were to construct a genetic linkage map of this species using SSR and SNP markers, and mapping the *Pg* locus that is involved in plant pigmentation. The genetic map was developed from the progenies of a cross between the *O. cumana* inbred lines EK-12 and EK-A1, which originated from populations belonging to two distant and geographically separated gene pools identified in Spain. The inbred lines also differed in plant pigmentation, with EKA1 lacking anthocyanin pigmentation (*pgpg* genotype). A genetic map comprising 26 SSR and 701 SNP markers was constructed, which displayed 19 linkage groups (LGs), corresponding to the 19 chromosome pairs of *O. cumana*. The total length of the map was 1795.7 cM, with an average distance between two adjacent positions of 2.5 cM and a maximum map distance of 41.9 cM. The *Pg* locus mapped to LG19 between the SNP markers OS02468 and OS01653 at 7.5 and 3.4 cM, respectively. This study constitutes the first linkage map and trait mapping study in *Orobanche* spp., laying a key foundation for further genome characterization and providing a basis for mapping additional traits such as those having a key role in parasitism.

Keywords: linkage map, plant pigmentation, segregating populations, sunflower, sunflower broomrape

## INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant found in the wild from south-eastern Europe to central Asia parasitizing a few species of the Asteraceae, mainly Artemisia spp. As a parasitic weed, it parasitizes on sunflower and represents one of the most serious production constraints for this crop in many sunflower-producing countries, particularly in Central and Eastern Europe, Spain, Turkey, Israel, Iran, Kazakhstan, and China (Fernández- Martínez et al., 2015). Moreover, the parasite has spread in recent years to new countries where it had not been reported before such as France (Jestin et al., 2014), Tunisia (Amri et al., 2012), and Morocco (Nabloussi et al., 2018), and in countries where the parasite had been traditionally observed in specific areas, it continues spreading to new regions, such as the North of Spain (Fernández-Escobar et al., 2009; Malek et al., 2017).

The genetic interaction between broomrape and sunflower is in most cases governed by the gene-for-gene model for plant-pathogen interactions, in which resistance reactions are governed by the interaction of host genes for resistance and the corresponding pathogen genes for avirulence (Rodríguez-Ojeda et al., 2013b). This kind of interaction has led to the development of resistant sunflower cultivars based on vertical resistance mechanisms, and determines the occurrence of physiological races of broomrape that are controlled by these resistant genes in sunflower (Fernández-Martínez et al., 2015). Vranceanu et al. (1980) described five broomrape races in the early 80s named as A to E and developed a set of sunflower differential lines to identify them, each carrying a single dominant gene (*Or1* through *Or5*, respectively) conferring resistance to the corresponding race. New races overcoming *Or5* resistance appeared from the middle 1990s onward in several countries such as Spain, Romania, Turkey, Bulgaria, Ukraine, and Russia (Fernández-Martínez et al., 2015). Initially, all of them were named as race F though the relationship between the different F races has not been studied. Nowadays, populations overcoming resistance sources to race F, named as races G and H, have been identified in most of these countries (Kaya et al., 2009; Pacureanu-Joita et al., 2009; Shindrova and Penchev, 2012; Antonova et al., 2013; Martín-Sanz et al., 2016). As mentioned for race F, no comparative studies have been conducted between races G and H reported in different countries. For races F and G, monogenic and dominant resistance in sunflower has also been reported (Pacureanu-Joita et al., 2004; Velasco et al., 2012).

As shown by broomrape race evolution, sunflower vertical resistance mechanisms are readily overcome by the parasite. For the development of long-term breeding strategies, it is essential to understand the genetic bases of the host-parasite

interaction. However, this is currently hampered by the limited availability of genetic tools in *O. cumana*, since most of the research has been carried out on the crop host, the sunflower. The few genetic studies in *O. cumana* have mainly focused on population structure and genetic diversity analyses (Castejón- Muñoz et al., 1991; Gagne et al., 1998; Pineda-Martos et al., 2013, 2014; Guchetl et al., 2014; Molinero-Ruiz et al., 2014; Martín-Sanz et al., 2016). These studies concluded that the populations parasitizing sunflowers were characterized by low intra-population diversity and, in general, low differentiation between populations. Additionally, different mechanisms were postulated for explaining race evolution, such as single-gene mutations within local populations (Pineda-Martos et al., 2013), or genetic recombination of avirulence genes (Martín-Sanz et al., 2016). Classical genetic analyses in *O. cumana* were started just a few years ago. This in part might be due to the fact that working on genetics of holoparasitic plants signifies additional difficulties to obtain the plant material and segregating generations required for such studies, in such a way that all the plants-segregating generations have to be obtained by artificial inoculation on the corresponding host, which is a labor intensive and timeconsuming procedure. Initially, Rodríguez-Ojeda et al. (2010) carried out basic studies for determining the feasibility of the use of inbreeding and hybridisation techniques for carrying out these genetic studies in *O. cumana*. Later, these authors isolated a line named EK-A1 from a natural mutant lacking anthocyanin pigmentation identified in a population of O. cumana from central Spain, and determined the inheritance of the trait evaluating the phenotype of segregating populations from crosses with plants of a normally pigmented line (Rodríguez-Ojeda et al., 2011). The authors concluded that the trait was controlled by partially dominant alleles at a single locus, which was named *Pg*. Therefore, this trait showing a monogenic inheritance turned out to be an excellent candidate for conducting trait mapping studies in *O. cumana* genome. Additionally, the segregating population generated by Rodríguez-Ojeda et al. (2011) probed to be highly polymorphic, since it was constructed from parental lines belonging to the two genetically distant *O. cumana* gene pools that co-exist in Spain (the Gualdalquivir Valley and the Cuenca gene pools, in southern and central Spain, respectively, Pineda-Martos et al., 2013).

Genetic linkage maps are essential for studying the genome structure and organization. They are also valuable resources in parasitic weeds for locating genes that control traits of interest, such as avirulence or host specificity and ultimately might permit the positional cloning of these genes. In *Orobanche* spp., there have been no attempts to develop genetic linkage maps, and no trait mapping studies have been carried out to date. Genetic linkage maps in other parasitic weeds are also extremely

limited. To our knowledge, the only study detailing a linkage map construction has been reported for *Striga hermonthica* (Del.) Benth., a parasitic weed of cereals, using amplified fragment length polymorphisms (AFLPs) (Pescott, 2013).

Since *O. cumana* genetic tools are essential to provide a better knowledge of the sunflower-*O. cumana* parasitic system for the development of knowledge-based control strategies, and these tools are so far extremely limited for this species and in general for *Orobanche* spp., the objectives of this research were to (i) develop a genetic linkage map in O. cumana using SSR and SNP markers, and (ii) map as a Mendelian trait the *Pg* locus involved in plant pigmentation, which was previously studied at the phenotypic level by Rodríguez-Ojeda et al. (2011).

## MATERIALS AND METHODS

### *Plant Material, Phenotyping and Mapping Population*

The mapping population consisted of 91 F2 plants and their corresponding F2V3 families derived from the cross between the *O. cumana* lines EK-A1 and EK-12, previously reported by Rodríguez-Ojeda et al. (2011). The EK-A1 plants lack pigmentation, having a yellow stem and a white corolla, whereas the EK-12 plants have normal pigmentation showing a bluish-violet stem and bluish to pale-violet corolla (Figure 1). This trait is controlled by partially dominant alleles at a single locus, referred to as *Pg*. This was demonstrated by Rodríguez- Ojeda et al. (2011) through the independent evaluation of sixteen F2 populations (F2 plant generation) segregating for the plant pigmentation trait and the progenies of 120 F2 plants (F3 plant generation) evaluated in different years. All plants and generations were grown on the sunflower susceptible cultivar DMM as reported by Rodríguez-Ojeda et al. (2011). The growth conditions and phenotypic characterization for plant pigmentation of F2 and F3 plants were previously described by Rodríguez-Ojeda et al. (2011). For the molecular study, a F2 population consisting in 91 F2 plants for which (i) F2V3 families were available and (ii) there was sufficient F3 tissue, was used. Stem and corolla colors were visually evaluated at each 91 F2 plants and their corresponding F2V3 families (F3 plants), considering the phenotypes of the parental lines (unpigmented-yellow stem in EK-A1 plants and a fully pigmented-bluish-violet stem in EK-12 plants, Figure 1), and of the F1-heterozygous plants (greenish-intermediate pigmented, Figure 1; Rodríguez-Ojeda et al., 2011). Plant stems were scored as unpigmented (yellow) and pigmented (bluish violet-fully pigmented and greenish-intermediate pigmented). Pigmented plants (bluish violet and greenish)

were included in the same class because of the large number of broomrape plants in some pots which made it difficult to distinguish greenish from bluish-violet plants. A Chi-square test was used to evaluate the proposed segregation ratio for the population used in the molecular study.

In order to evaluate the potential of the SNPs reported in this study for their use in genotyping other published *O. cumana* segregating populations and for diversity analyses, three further *O. cumana* populations used as parental lines in *O. cumana* crosses described in Rodríguez-Ojeda et al. (2013b) were used. These three populations belonged to the Guadalquivir Valley gene pool and were (i) OC-94 (race E, collected in Sevilla, Spain in 1994), (ii) EK-23 (race F, collected in Córdoba, Spain in 1995), and (iii) SP (race F, collected in Sevilla, Spain in 2004) (Rodríguez-Ojeda et al., 2013b).

### *Tissue Collection and DNA Extraction*

Broomrape shoots were first collected from individual F2 plants (single broomrape shoots), and individual plants from the parental lines EK-A1 and EK-12, and the other three O. cumana populations used for a polymorphism and diversity analysis (OC-94, EK-23, and SP). In order not to affect the F3 seed production, the apical bud of each F2 broomrape shoot was removed after most of the flowers had been formed. Because of the small amount of tissue collected in F2, tissue was also collected from F2V3 families (about 20 young F3 shoots per family) in order to recreate their corresponding F2 genotype. This is an accurate approach useful for species lacking enough tissue in individual F2 plants to yield enough DNA for marker analysis (Kochert, 1994). In this case, 20 young F3 shoots from each F2V3 family were collected before flowering, and bulked by mixing an equal tissue amount of the F3 shoots from each F2V3 family. In all cases, tissue was immediately frozen at -80 $^{o}$C, lyophilized, and ground in a laboratory ball mill. DNA was then extracted following the procedure reported by Pineda-Martos et al. (2013).

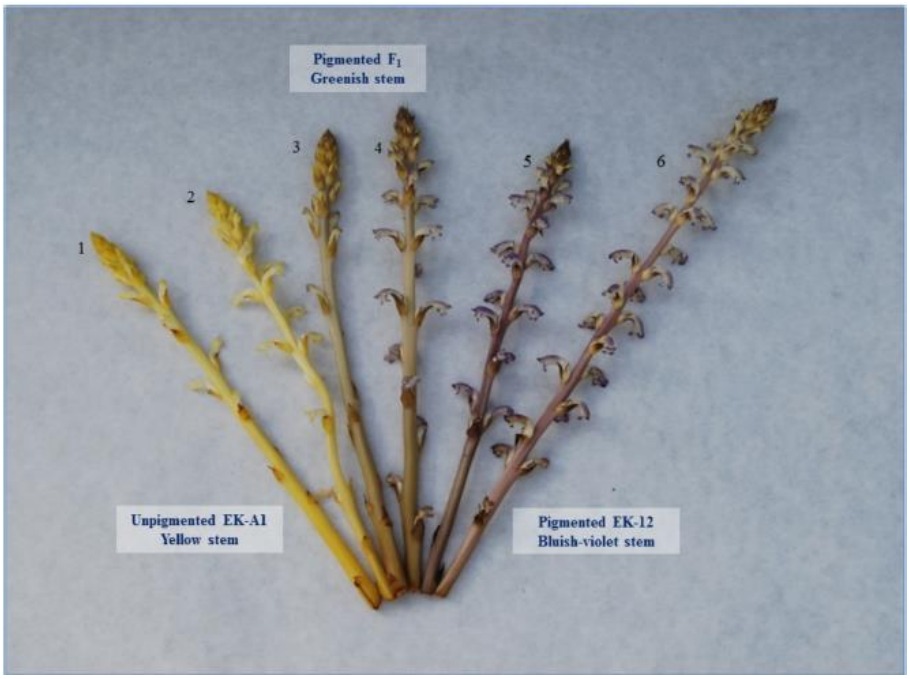



**Figure 1**. Unpigmented *O. cumana* EK-A1 plants (1, 2), partially pigmented heterozygotes (3, 4), and fully pigmented EK-12 plants (5, 6) showing yellow, greenish, and bluish-violet stems, respectively.

### *Genotyping with SSR Markers*

The complete set of 298 *O. cumana* SSR markers developed by Pineda-Martos et al. (2014) was tested for polymorphism in the parental lines EK-12 and EK-A1, and six individuals from the mapping population. PCR amplification was carried out in 30 mL reaction mixtures, consisting of 50 ng of template DNA, 0.03 U/mL of Taq DNA polymerase (FirePol Taq polymerase, Solis BioDyne, Tartu, Estonia), 1 x PCR buffer, 2.5 mM MgCl2, 200 mM dNTPs (Solis BioDyne, Tartu, Estonia), and 0.3 mM of primers. A touchdown PCR program was used on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, United States), which consisted of an initial denaturation step of 94 °C for 2 min, followed by one cycle of 94 °C for 30 s, final annealing temperature ($T_A$) + 10 °C for 30 s, and 72 °C for 30 s, 9 cycles in which the annealing temperature was decreased by 1 °C, and 32 cycles at 94 °C for 30 s, $T_A$ for 30 s, and 72 °C for 30 s, with a final extension of 10 min at 72 °C. Amplified products were separated on 3% MetaPhor (BMA, Rockland, ME, United States) agarose gels in 1 x TBE buffer with SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Huntingdon, United Kingdom) 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 for the DNA fragments. The resultant gel images

were scored manually with the aid of Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, United States). The amplification profile for each microsatellite was scored visually and independently. A set of 28 polymorphic SSR markers showing clearly codominant and scorable fragments was selected and genotyped in the mapping population (91 individuals), following the same approach described above.

### *Genotyping with SNP Markers*

A set of 1536 *O. cumana* SNP markers developed by Coque et al. (2016) through exome capture was used to genotype the parental lines EK-12 and EK-A1 and the mapping population (ninetyone individuals) using competitive allele-specific PCR assays based on KASP™ technology (LGC genomics, Teddington, United Kingdom) on the Limagrain genotyping platform. For this SNP discovery study, a set of 12 broomrape populations representing different level of virulence or aggressiveness and different countries was submitted to transcriptome sequencing. This approach led to the discovery of approximately 368000 bi-allelic SNP among which 1536 were selected for genotyping a Biogemma broomrape collection (around 500 populations). A genetic diversity analysis was conducted and an optimized subset of 198 SNP capturing the maximum of the genetic diversity analysis was selected. Details of the 198 *O. cumana* SNP markers are reported in Supplementary Table S1. The remainder of the SNP marker information is proprietary. For genetic mapping purpose, monomorphic markers were excluded for the linkage map construction.

In order to evaluate the potential of the SNPs reported in this study for their use in genotyping other *O. cumana* segregating populations, the number of markers polymorphic between parental genotypes of published mapping populations were determined as follows. The 198 *O. cumana* SNP marker subset from Supplementary Table S1 was genotyped in 10 individuals from each of the parental genotypes EK-A1, EK-12, EK-23, OC- 94, and SP, used to develop the segregating populations EK-12 x EK-23, and OC-94 x SP, segregating for avirulence/virulence (Rodríguez-Ojeda et al., 2013b), and EK-A1 x EK-12, segregating for plant pigmentation (Rodríguez-Ojeda et al., 2011, this study). The individuals were genotyped for these SNP using the KASP™ genotyping assay service provided by LGC Genomics (Herts, United Kingdom). A total of 38 markers and 7 individuals that failed genotyping or had >10% missing data, respectively, were excluded from the analysis. Therefore, polymorphism and genetic diversity analysis was carried out with 160 SNPs markers and the following parameters were calculated: percentage of polymorphic loci (P)

and Shannon's diversity index (I) within each population and percentage of polymorphic loci (P) between populations. In addition, pairwise genetic distances between populations were calculated as the genetic distance coefficient GST using 1000 random permutations to assess significance. The matrix of GST pairwise distances was used as input for a principal coordinates analysis (PCoA). All analyses were carried out using GenAlEx version 6.5 (Peakall and Smouse, 2012).

### *Genetic Linkage Map Construction and Genetic Mapping of the Pigmentation Gene*

Only co-dominant markers were used for the linkage map construction. The segregation of alleles at the SSR or SNP marker loci was checked against the expected ratios for codominant (1:2:1) markers using a chi-square test. The genetic linkage map was constructed with MAPMAKER/EXP (version 3.0b) (Lincoln et al., 1993) using genotyping data from polymorphic and codominant markers from the 298 SSR and 1536 SNP marker sets. Map distances in centiMorgan (cM) were converted from recombination fractions using the Kosambi mapping function. Two-point analysis was used to identify linkage groups (LGs) with an LOD score of 6.0 and a maximum distance of 40 cM, except for LGs 13 and 15, in which specific markers were grouped with a LOD value of 3.5. Three-point and multipoint analyses were used to determine the order and interval distances between the markers in each LG. Loci whose position were ambiguous (i.e., those placed automatically at a lessstrict LOD of 2.0) were noted. Markers that had the most skewed segregation ratios ($P < 0.0001$) were excluded from the map. Linkage maps were drawn using MapChart 2.1 software (Voorrips, 2002). The linkage groups were randomly numbered as there are no previously reported *O. cumana* genetic maps. Simple correlation coefficients (r) between the total number of markers per linkage group and the total linkage group length were calculated. The significance of the correlation coefficients was calculated by the standard testing procedures for $r = 0$ null hypothesis (Snedecor and Cochran, 1989).

As the "plant pigmentation" trait is controlled by a single gene (*Pg*, Rodríguez-Ojeda et al., 2011), it was mapped as a Mendelian locus. Accordingly, the genotypes for the *Pg* gene were inferred from the pigmentation phenotypes in F2 plants and their corresponding F2V3 families. F2 plants were classified as homozygous dominant for the pigmentation gene if they showed a stem similar to EK-12, and showed uniformly pigmented plants in their respective F3 progeny, heterozygous if their F3 segregated for stem color, and homozygous recessive if they were similar to EK-A1 and showed uniformly unpigmented plants in their respective F3 progeny. Linkage analysis for

the pigmentation gene was run with MAPMAKER/EXP (version 3.0b) using segregation data for SNP and SSR marker loci and for the *Pg* locus. *Pg* mapping was carried out as indicated for SSR and SNP markers, excepting that a LOD threshold of 10 and a maximum distance of 30 cM were used as linkage criteria. Finally, potential candidate genes for the *Pg* locus were identified using BLAST searches for plant pigment biosynthesis genes at the flavonoid/anthocyanin and carotenoid biosynthesis pathways (MetaCyc, version 20.0; Caspi et al., 2018) against a 622 contigs first draft of the *O. cumana* sequence genome (Gouzy et al., 2017).

## RESULTS

### *Phenotypic Evaluation of the Mapping Population*

Phenotypic evaluation of the mapping population revealed 15 unpigmented F2 plants showing homogeneously unpigmented F3 progenies (a total of 675 unpigmented F3 plants for all the 15 F2V3 families) and 76 pigmented F2 plants. From these, 30 presented homogeneously bluish-violet pigmented F3 progenies (a total of 1146 bluish-violet F3 plants), and 46 showed segregating F3 progenies with both pigmented and unpigmented plants (a total of 1305 pigmented and 452 yellow-unpigmented F3 plants). The number of plants observed in each phenotypic class [15 (unpigmented): 46 (segregating): 30 (fully pigmented)] did not differ significantly from a 1:2:1 genetic proportion expected for one-gene segregation ($2 = 4.96, P = 0.08).

### *Genetic Linkage Map Construction*

From the 1536 O. *cumana* SNPs, 1285 were successfully genotyped. Among these, 722 (56.2%) were polymorphic between the parents EK-A1 and EK-12 and segregated accordingly in the mapping population. From the 298 *O. cumana* SSRs, 168 showed high quality amplification, and 33 (19.6%) were polymorphic (28 co-dominant and 5 dominant). After excluding markers with extremely distorted segregation ($P < 0.0001$) and dominant markers, 737 markers were used for the linkage analysis. The final genetic linkage map was constructed using 26 SSR and 701 SNP polymorphic and codominant markers. Two additional markers that remained unlinked and 8 markers that remained "unmapped" (grouped in LGs of two or three markers) were not included. The 727 SSR and SNP loci were arranged in 19 linkage groups, which correspond to the 19 chromosome pairs of the *O. cumana* genome (Schneeweiss et al., 2004; Piednoël et al., 2012; Figure 2 and Supplementary Table S2). The total map length was 1795.7 cM. The average distance between two adjacent positions across the whole map was 2.5 cM, but there were 12 regions on 11 LGs with intervals greater

than 20 cM, with the largest interval (41.9 cM) being observed in LG13 (Figure 2 and Supplementary Table S2).

The number of markers on different LGs ranged from 13 on LG11 to 80 on LG19. The length of different LGs ranged from 40.5 cM on LG16 to 137.6 cM on LG6 (Figure 2 and Supplementary Table S2). The LG length was correlated with the number of markers per linkage group ($r = 0.66$, $p = 0.002$, $n = 19$). LG2 was the most skewed linkage group from the linear fitting, probably due to an excess of markers (a total of 28) clustering in two groups of 0 cM (Figure 2). Excluding this LG resulted in a higher correlation ($r = 0.74$, $p = 0.0004$, $n = 18$). A highly significant ($P < 0.001$) distorted segregation was observed in LG1, LG4, the bottom of LG9, and LG16 (Supplementary Table S2).

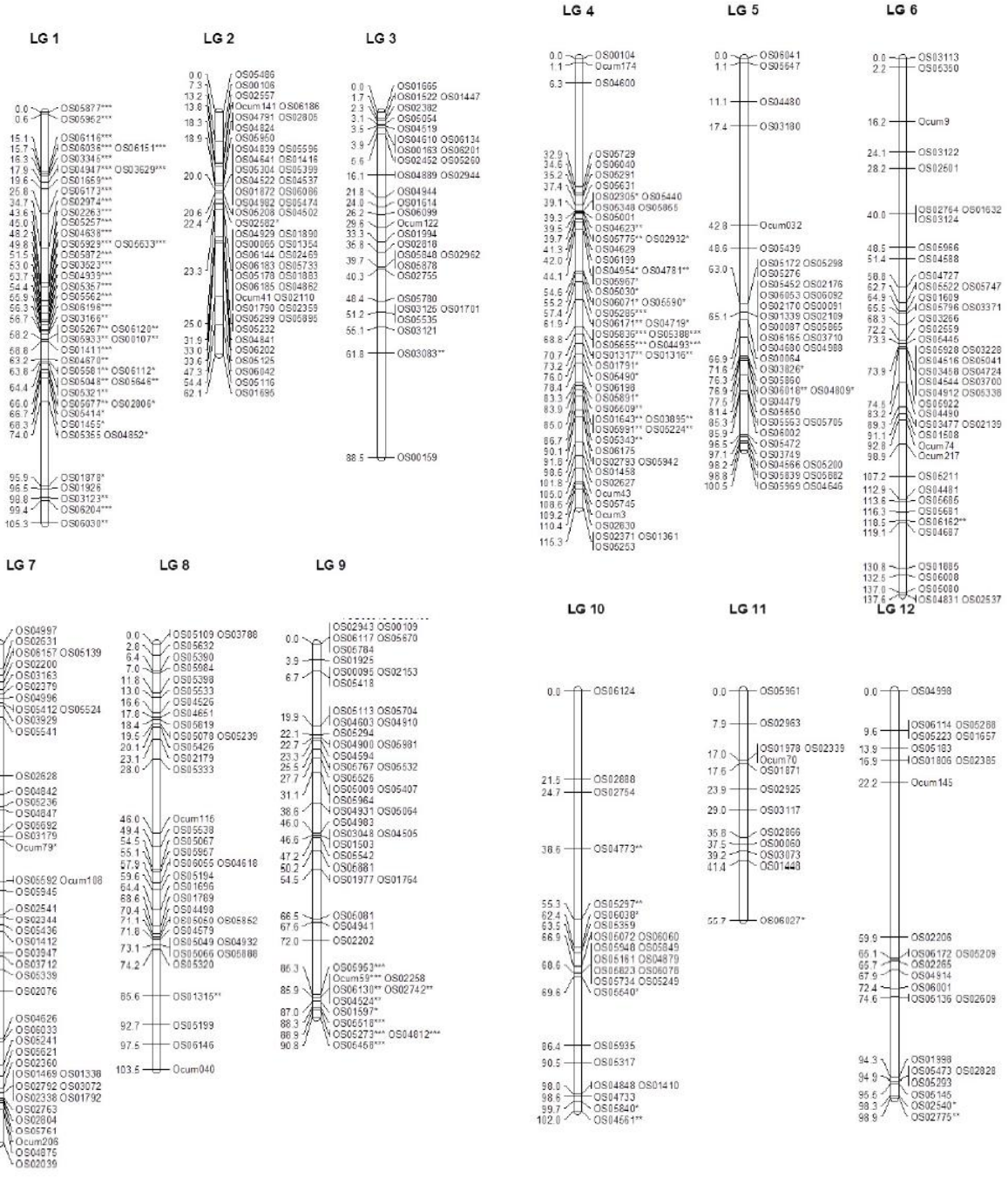
### *Genetic Mapping of the Pg Pigmentation Gene*

Using the genotypic classification of F2 plants (homozygous unpigmented: segregating: homozygous pigmented) based on the F3 evaluation, the locus *Pg* associated with plant pigmentation mapped as a Mendelian trait was located 28.2 cM downstream from the upper end of LG19, between the SNP markers OS02468 and OS01653, which were 7.5 cM distal and 3.4 cM proximal, respectively, of the *Pg* locus (Figure 2). Based on the physical position of context sequences of markers flanking *Pg* on the *O. cumana* draft genome (OS01653 and OS02571 at positions 4.17 and 4.07 Mbp, respectively, in contig OcIN23s039, and OS02468 and OS04605 at positions 2.51 and 4.41 Mbp, respectively, in contig OcIN23s036), candidate genes for plant pigment biosynthesis at the flavonoid/anthocyanin and carotenoid biosynthesis pathways were searched locating at these contigs. Two contiguous genes for flavonoid biosynthesis and carotenoid biosynthesis were found in contig OcIN23s036. Both genes were coding for carotenoid/flavonoid glucoside glucosyltransferases. The genes were a flavonoid glucoside glucosyltransferase (at position 3.61 Mbp) and a crocetin glucoside glucosyltransferase (at position 3.60 Mbp) whose physical position was between that of OS02468 and OS04605 SNP markers in contig OcIN23s036.

### *Diversity and Polymorphism SNP Analysis*

Evaluation of the potential of SNPs (using the 198 SNP subset from Supplementary Table S1) for genotyping existing segregating populations in *O. cumana* revealed an extremely low number of polymorphic markers between parental genotypes coming from populations of the Guadalquivir Valley gene pool (OC-94, SP, EK-12, and EK-

23), in contrast to a much higher number of polymorphic markers found between EK-A1 from the Cuenca gene pool and the remaining genotypes from the Guadalquivir Valley gene pool, including EK-12 (Table 1). This was coupled with a very low intra-population variation and interpopulation diversity for populations from the same gene pool, as shown by diversity parameters (Table 1). Principal Coordinate Analysis revealed a clear separation between parental genotypes from the Guadalquivir Valley gene pool (OC-94, SP, EK-12, and EK-23), and the Cuenca gene pool (EK-A1) (Figure 3), separated along Coordinate 1 that explained 94.14% of the total variation.


LG 1
LG 2
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LG 9
LG 10
LG 11
LG 12

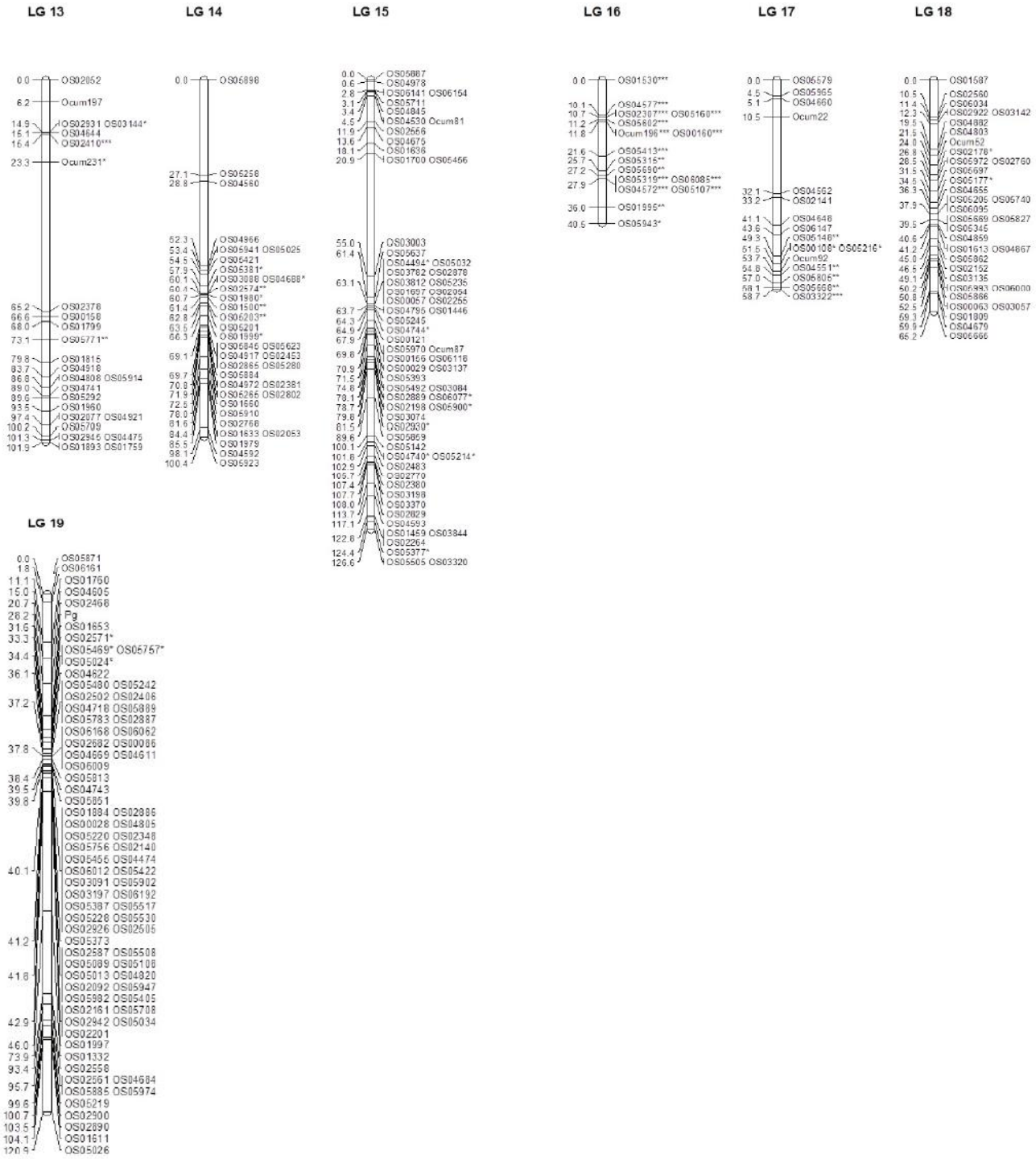


**Figure 2.** SSR and SNP *O. cumana* genetic linkage map containing the pigmentation locus *Pg*. The map is based on segregation of 727 co-dominant SNP and SSR markers, and *Pg*, in 91 individuals. Linkage groups (LG) follow a randomly selected numeration. Genetic distances are given in centiMorgans (Kosambi) on the left of each LG. The position of the *Pg* gene associated with plant pigmentation in O. cumana, mapped as a Mendelian trait, is shown at LG19. Prefix Ocum is for SSR marker loci and prefix OS for SNP marker loci. SNP and SSR marker loci labeled as *, **, or *** showed distorted segregation at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

**Table 1**. Percentage of polymorphic SNP loci between the parental genotypes EK-A1 (Cuenca gene pool), EK-12 (Guadalquivir valley gene pool), EK-23 (Guadalquivir valley gene pool), OC-94 (Guadalquivir valley gene pool), and SP (Guadalquivir valley gene pool) from *O. cumana* segregating populations.

| | EK-A1 | EK-12 | EK-23 | OC-94 | SP |
|---|---|---|---|---|---|
| EK-A1 | 0.00%; 0.000 | 56.88% | 56.88% | 56.88% | 56.88% |
| EK-12 | | 0.63%; 0.004 | 0.63% | 1.88% | 0.63% |
| EK-23 | | | 0.63%; 0.004 | 1.88% | 0.63% |
| OC-94 | | | | 1.88%; 0.008 | 1.88% |
| SP | | | | | 0.63%; 0.004 |

*In the diagonal axis intra-population genetic diversity parameters are indicated as follows: Percentage of polymorphic loci and Shannon's diversity index.*

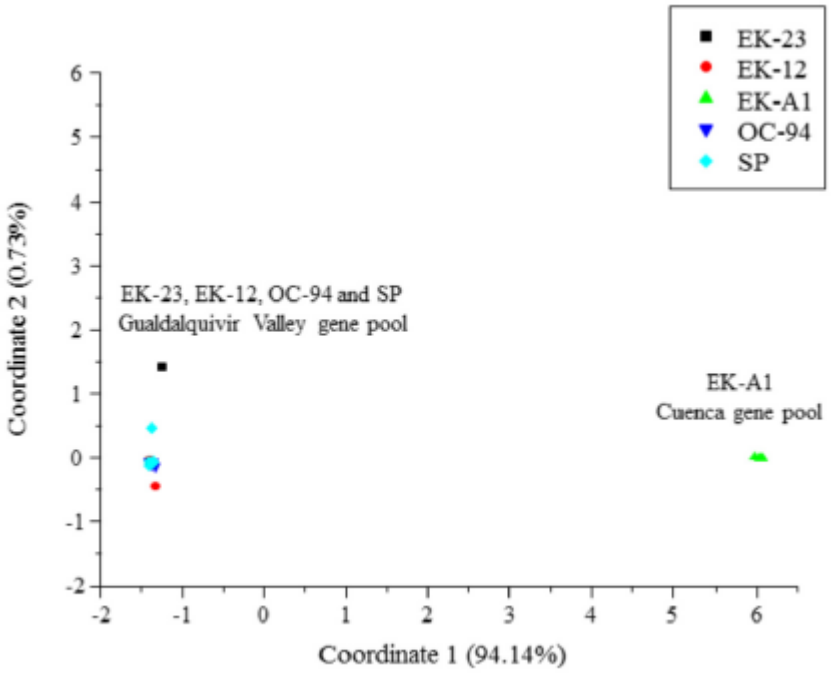


**Figure 3.** Principal coordinates analysis of the five *O. cumana* parental genotypes EK-A1, EK-12, EK-23, OC-94, and SP.

## DISCUSSION

Genetic studies in *O. cumana* have been very scarce till date. Rodríguez-Ojeda et al. (2011) studied the inheritance of the unpigmented plant trait in line EK-A1. The trait was found to be controlled by partially dominant alleles at a single locus, named *Pg*. Another genetic study at the phenotypic level was conducted by Rodríguez-Ojeda et al. (2013b), who evaluated the segregation of virulence in progenies from crosses between race E and race F lines developed from populations collected in southern Spain. Differences in virulence between both types of populations were found to be controlled by a single gene, suggesting that the evolution from race E to race F in that area was produced by a point mutation. Genetic studies in *O. cumana*, conducted initially at the phenotypic level, can be now be expanded at the molecular level thanks to genetic and genomic advances in this species, such as the development of SSR markers (Pineda-Martos et al., 2014), SNP markers (reported for the first time in this study), or a first draft of the genome sequence (Gouzy et al., 2017). The present study, in which SNP markers have been reported, a genetic linkage map using SSR and SNPs has been constructed and the *Pg* gene (characterized previously at the phenotypic level by Rodríguez-Ojeda et al., 2011) mapped, is in the same line as these previous studies, providing new genetic tools for understanding genetic mechanisms in this parasitic weed, and ultimately contributing to the development of durable genetic resistance in sunflower.

The development of a genetic linkage map is an important pre-requisite for the identification of genes or QTL underlying important traits. The *O. cumana* map developed in the present research is the first linkage map reported for any *Orobanche*/*Phelipanche* species and represents a significant advance in the study of genome structure and organization, and for mapping genes of importance on the *O. cumana* genome. This *O. cumana* genetic map contains 19 LGs, coincident with the basic number of chromosomes in this species (Schneeweiss et al., 2004; Piednoël et al., 2012), a relatively high marker density (a total of 727 SNP and SSR marker loci) and a resolution of 2.5 cM. From 737 polymorphic markers used for linkage analysis, only 10 remained "unmapped," with 98.6% of the markers falling into one larger LG (with a minimum number of markers of 13, and coverage >40 cM). Therefore, the map developed appears to cover the vast majority of the *O. cumana* genome. The genetic linkage map information from this study combined with the availability of an *O. cumana* genome sequence draft and the contig and genetic linkage position of SNP and SSR markers, will be of utility to anchor contigs into scaffolds and ultimately to obtain the chromosome sequences, contributing in this way to the development of new genetic resources for this species.

The total *O. cumana* map length was 1795.7 cM. Considering the size of its genome to be 1.42 Gb or 1.40 Gb resulting from either densitometry (Weiss-Schneeweiss et al., 2006) or assembly of DNA sequences (Pouilly et al., 2018), respectively, the *O. cumana* genome-wide recombination rate was estimated as 1.27 cM/Mb. This value is slightly lower than the average value of 1.85 cM/Mb previously reported by Stapley et al. (2017) for plants (averaged from 189 species), and also lower than the average value of 2.6 cM/Mb (ranging from 1.6 to 3.9 cM/Mb) detailed by these authors for five plant species of the Lamiales order, to which the Orobanchaceae family belongs. Interestingly, Stapley et al. (2017) compared the genome-wide recombination rate of parasitic or pathogenic species with free-living species and found that parasitic or pathogenic species had a higher recombination rate compared to their free-living counterparts in animals, but they found no differences in fungi. Unfortunately, no data were reported for parasitic plants as parasitic/pathogenic plant species were not included in their dataset.

The distribution of markers between the 19 linkage groups was fairly uniform with, in general, the largest groups containing the most markers. However, there were markers clustered observed in some regions of the map, especially on LG2, LG5, and LG19. Marker clustering has also been reported in genetic maps in other plant species, and it has been associated with centromeric regions due to the suppression of recombination in the heterochromatic regions surrounding these regions (Haanstra et al., 1999), or to the lack of recombination around genes with evolutionary significance (Jessup et al., 2002; Hao et al., 2004). In addition, despite the short average distance between adjacent markers on the map (2.5 cM), there were 12 gaps larger than 20 cM. These gaps have also been reported in several genetic linkage maps in plant species and may be due to the lack of marker polymorphism and a shortage of marker detection in these regions (Berry et al., 1995) or due to recombination hot spots (Mézard, 2006).

Around 20.5% (149 out of 728) of the mapped markers in the map had significantly distorted segregation ratios. They were mainly clustered on LG1 and LG16 and on specific regions in LG4 and LG9, suggesting that these may be of biological significance. Distorted segregation in specific genomic regions is an inevitable feature observed in many of the marker-based linkage maps in plants. It has been attributed to a range of causes, including deleterious recessive alleles (Berry et al., 1995), self-incompatibility alleles (Barzen et al., 1995), structural rearrangements (Quillet et al., 1995), or differences in DNA content (Jenczewski et al., 1997). It is worth noting that the allele differences that contribute to the relative success of parasitism could also lead to segregation distortion (Thomas et al., 2012). Interestingly, all markers showing segregation distortion clustering at LG1, LG9, and LG16 favored the allele from the pigmented

parent EK-12. Although the pigmentation gene *Pg* did not map in any of those regions, and the lack of pigmentation in *O. cumana* plants has not been associated to parasitism (González- Torres et al., 1982; Rodríguez-Ojeda et al., 2011), this might suggest a fitness benefit for progenies that inherited specific EK-12 alleles.

Overall SNP polymorphism in the EK-A1 and EK-12 cross was relatively high (more than 50% polymorphic loci). This cross involved parents from two distant gene pools identified in Spain, with the EK-A1 population belonging to the Cuenca gene pool in Central Spain and the EK-12 population being from the Guadalquivir Valley gene pool in Southern Spain (Rodríguez-Ojeda et al., 2011; Pineda-Martos et al., 2013). In addition to these two divergent gene pools in Spain, Pineda-Martos et al. (2013) also described an extremely low inter and intra- population diversity within each of these two gene pools using SSR markers, which was attributed to a founder effect. This lack of polymorphism within the Guadalquivir Valley gene pool has also been confirmed in this study using SNP markers. Therefore, using parental lines from already described *O. cumana* distant gene pools to avoid factors decreasing diversity such as founder effects is desirable for genetic mapping and construction of highly saturated genetic maps in *O. cumana*, since the possibility of detecting polymorphism among parents is increased, resulting in a higher number of segregating loci.

*Orobanche* spp. are often highly variable in regard to their size, coloration, and pubescence (Rumsey and Jury, 1991). Unpigmented plants (likely lacking anthocyanin pigmentation) have been observed in the populations of several *Orobanche* spp. (Kreutz, 1995; Rumsey, 2007), including *O. cumana* (González-Torres et al., 1982). Results previously reported by Rodríguez-Ojeda et al. (2011) showed that the absence of pigmentation in *O. cumana* was the result of a single-gene mutation, probably involved in anthocyanin biosynthesis. Later, Rodríguez-Ojeda et al. (2013a) proved the unpigmented trait in *O. cumana* to be very useful for studies on its biology, allowing the determination of cross-fertilization rate in this species. In this study, the pigmentation locus *Pg* has been mapped to LG19 in the SSR-SNP *O. cumana* genetic linkage map, between the SNP markers OS01653 and OS02468. This constitutes the first trait mapping study in *Orobanche* spp. Even though the development of molecular markers for the pigmentation trait may not have drawn interest because the trait is easily distinguishable visually, the location of a gene with a known phenotypic effect in the *O. cumana* genetic map may be an important reference for future mapping and molecular marker studies in this species. In addition, it might contribute to the identification of causal genes and mutations for stem and flower-color variations in *O. cumana*. In fact, candidate gene analysis revealed two contiguous genes for carotenoid/flavonoid glucoside glucosyltransferases mapping close to the *Pg* locus. Glycosylation is often the

final step in the biosynthesis of plant secondary metabolites, which enhances their water solubility and chemical stability and alters their biological activity (Yang et al., 2018). The attachment of additional sugar to flavonoid glycosides has also been related to modifications in physiological properties such as color. For instance, anthocyanin color is influenced by glycosylation pattern (Zhang et al., 2014), and its possible role on *Orobanche* plant pigmentation might be further investigated.

## CONCLUSION

In conclusion, this work represents the first genetic linkage map and trait mapping study for *O. cumana*, and for any *Orobanche*/*Phelipanche* spp., a species and a genus for which there are very limited genetic/genomic information published. Results from this study will contribute to understand the genetic basis of the sunflower-*O cumana* interaction, which is required for the development of new knowledge-based strategies for broomrape management. In this sense, the reported SNPs and the saturated genetic map constitute valuable genetic resources for different downstream applications such as new SNP-based genetic diversity and population structure analyses, further genome characterization and sequence assembly of the *O. cumana* genome, and the identification of genes/QTL underlying relevant traits. The mapping of the *Pg* locus determining plant pigmentation provides one example, and mapping genes/QTL associated to parasitism and virulence using segregating populations generated from parental lines differing for these traits is under way. Locating and eventually cloning genes responsible for these traits will bear direct implications for practical agriculture, since they will represent new targets for rational design of control strategies to this devastating parasitic weed.

## AUTHOR CONTRIBUTIONS

LV and BP-V conceived the study and planned and supervised the research. SM supervised a part of the genotyping. MC conducted the SNP development and genotyping. ÁC-G and NP conducted the SSR genotyping and collaborated in the SNP genotyping. BP-V, XG, and ÁC-G conducted the statistical analyses and map construction. ÁC-G, LV, and BP-V wrote the draft of the manuscript. All authors critically read the manuscript and revised its final version.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00797/full#supplementary-material

**Table S1.** Details of 198 *O. cumana* SNP markers, including their context sequence and map positions.

| index | code_OS | Alias | context_sequence |
|---|---|---|---|
| 1 | OS02153 | SNcum-1 | GTTTCCTGATGCTATTTTCACTCTTTTTGCTACCGCCCTGACTATCCATCTCATTTTCATCATGTCCTCTGATCTGACAGGCATCC TCATGGGAAACGTC[C/T]TTCTTCTCACCCTTTTGGAGCCTCACAGACTGTTTCTCAGTCATCCCTTCAGGATGAACCTTCTTGT GAAACATCTTTATAGCCTTGGAGAGCTTTTTCT |
| 2 | OS03091 | SNcum-2 | TGTGGGATTCGTTTCATCAGAGAATTCATGGTCCGACACGGTGAACTCCGGATTCATTTCATCAATATGAGGAGAGGTGAGTT GA[A/C]GTGACTTCCTATCCGAGATGGCCCAGACGAAAGATTCAATCATTTCTGTCGATCTCTCCAGATATTTAKCCAGCAGA CGGCTGAC |
| 3 | OS01875 | SNcum-3 | AGAAATAAAGCTGGTAAGTGTRGAGGCCATGAAGAAATAAAGCTGGGATCCTGAGGAAGGAAAARATAAGCGTGAGAGAG ATTGCGAATGCAGTGTGGAT[G/T]ATGAGATTGTTTGTAGGGAWAATTAGTGACAYTGGCGAGCSCATTATSATGAGATTTTAA TGCGCASARGCTGTTYGCTGGATTGTCTCRGCGACGRTGT |
| 4 | OS02579 | SNcum-4 | GAATACGTCTCTCTCTCTCCATATATACAGCCTTTCTTGCGTTTCTGTTTTCTTTCTCCCCTTTCTGCCAGTTTCTACAAGTCCGG ATTCTGGAGAGAAA[G/C]AGAGCCGACACCGAGCAAAAGTATTGCTAAAACTATATCCACAACCACAAGCTAGAAAGAATT TCCCACTCGGTTGAGCCGATTCGATTAATAATCTTTA |
| 5 | OS02202 | SNcum-5 | AGTTACGAGACGTGATGTCAGAGGGACTGGTTTTATGTGCTTCGAGTAAAGACCATACAGTTGTCGAGCCTCTACTTGCTCCA GAGGGTGCTAAAGTTGG[C/G]GAATGTGTTACTTTTTCYGGACATGACGGCAAGCCAGAAGAGRTTTTGAACCCYAAAAAGA AGCAGTTGGATAAAWTATATCCGCATCTTTCGACTGATG |
| 6 | OS06168 | SNcum-6 | TAATTTCTTGTTCCATAGTTCCAATGGTGAATATACACACACATCATGGCCAAGTTTGGAGCAATTATAA[G/T]CGAACAAGG AATATATGTAAAACTAAACTTAGTAAGCGTATGTCATTGCTAAGTATCGAGCAATTATAATCGATAACTGGGCACATATCAG CTAATATCG |
| 7 | OS02198 | SNcum-7 | CATCTACGATTCTTGAGCTGCTCAAGACAGCTGCACCTGAAGACCTGGTTGCTCCTTCTGGTTCAAGAGACCTATTATCTCCGC AGGGGGGTGAGGATGA[A/C]GTAGTAATATGCAATCTAGAATCATCATACACATCATGATGAGTCCCTTCATCAGCATTCTCG CTTGCCCAGGACTGACCCTTTCGAATTGTCACAGACC |
| 8 | OS06165 | SNcum-8 | ATGGCAGTGAATTCGTATTAAGCACAGAAAACCTGGCTCCAGATTCGACTTCAAGGACGAAAACTTATACCTCATTCAATCG TCGCCAAGACTGTTTACC[C/G]GGACTTTTAAACAACCCAATTGGCCCTAAGCAGGATGATATTCTTATAGCTAAACTTGGATC TGGACAGGAAATCGAGCTGGAAGCACATGCTGTGAAAG |

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| 9 | OS04740 | SNcum-9 | AGTAATTGAGTTTTTTTTWACTCGGATCTCAATTCCTTGAAGACTAATSCCAAACTTCTCTCTCCCRCTTTTCACTTCCCTCAAG CTCATCTTCAGTTCC[G/C]TGCYGTCACTACCTTTCTTGTTGAAATAGTCGCCCAACTCAACTTCATACCACCCATCGGCTCTCC GTACCGGATATTGGGGACCAGTAAATTCAGGATA |
| 10 | OS00106 | SNcum-10 | CGGGAACCCCATTTTCTGACTCCATTCCTCCTAGGGGAAAAAAAAAGATACAAGTCAAGCAAACCATGACAAACGGATCCCA CAACATGGAAGTATAATCA[C/G]AATCAGTGTATTACATAATCATAACTGGGGTATCACATAGTTATAGTGAAAAAATTTCGC CTAGTGTATCATAATTGATGTATCACATAATCACATTSAA |
| 11 | OS01411 | SNcum-11 | AACACAAACCCAGCCGGAGGATTCGGCAGASGCAGCGCCGAGTACGGCGACTGAGCTGATTCTGACGGTGACTCCATCACC TCCACTTCACCACCACACT[C/T]CTCGATCTCAAAGTCATCTCCGACCAACTTCTTCCTATTACTCTTACTACTCGGTTTCCTTTT CTTCTTCGTCGTCGTTGTCGGCGGCGCAGCGCCGCCC |
| 12 | OS03072 | SNcum-12 | TCTAAACTGATACATTAAATAATGGGATTAGGCTCATCCATTGTGTATATCTGCAATTCTCTTTAAGTCCTTGTTCTTGTGTGGC GTGTACTCTATAAAT[A/C]AAGTGGAGTTATCTTAGTATAAGCATATAAATTACAACAGAGAGAAAGGGGTCGAGAGAAGTT AAAGAAAATTGGGAAGTCTATAAGTTTGACTTGGCAT |
| 13 | OS00157 | SNcum-13 | CTCCACCAGAGAATCCTGTGCGGCTATTAGACGGGCAACAGTTTTCTTACTCGACTCTTGAAGTTCGCCAGCAATTAATATTT CATCCAATATGTAGTAA[A/G]CCTTGTGGAAATTAAAGATCAAATCCAGTTCACAAACACTTCCGAAGTAGCGGTCTAGTATC TCAACGAAGTGGTGGATAATTTCAAGTATCTCTAATTC |
| 14 | OS00159 | SNcum-14 | TTCCGTACCCTTGCCCTCTTATACCAAAGCACAAAAATTCTCCGGCGACATATTCGATCGTAACTACCATTCCCGACAACATC CTTCGTCCTCMATATCC[A/G]GCGKTCTTCTATTTTTGTTTCGGGCTAAAGAGCTTTAAGGGMTCTTTTTATCTGACCTCTCGGA ACGACACCCATTTATTCACCCAC |
| 15 | OS05160 | SNcum-15 | TGTCTTCCCCTTCGTCCTACCTTTTTGTGCTTGGAAGATTTGGACCCTACGGCTGGCCTTCGGATGTTTCCTTCACTATTCTTGTT CATTTCTGTCCTAT[C/T]GTTATTATGCTTCTTGGCTCGAGGTTTGTTCGATTTACCCTTTTGAAAATCAGGATGTCCGGCTCTTA CAAAAGATTTCTCACTATTCACTTTCTCGTCC |
| 16 | OS03124 | SNcum-16 | GCGGYACTCTGGCCGCCTCGTATGCTTCCTTTATTTCTATCCAACTTGAATGCTTTTTCGCTGCACAAAGGCAGTTCCAAAGAA CTCATCCTACT[T/A]CTCTTTTCATATATTTCCCTGGCTGCWTTTCCAACGRCCACCTATCTTCCTCCGTTCGATTTTCCTGAGAA AAAAAAAATGTTATCACCTCTAGTCCCTT |
| 17 | OS05836 | SNcum-17 | AGATTCRGACAAAGCTTTTTGAACAGACATCCGAGAATGAAGCGAAATCATCATATATATTAATTTTCTCTTGTTTGTGATAA CTATTACTAAGATGTAT[A/G]TATACGTGAATTTGAAAGTCAAAAAAAGAATCATCATCTCTACCACCCGCACACATAATAAA AATTGCGTTCTCATTAGCAGTATGGAATTTATCATAAT |

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| 18 | OS01799 | SNcum-18 | TCTTGTCTGTCTCACTGTACAACCCTAATTCCAATTCCCTGGGTCTGCCTCCTATCGCTACTGCGCAGATCAATTAAGGTTTGTT CTACGCCGGTCAACT[C/T]AAATCAAAACCCTAATTCGGTCCAGCTCTAATAAATCAAGAGACATTTGAAGAAAAAGCGCCG ACGAGAGTAATGGCGATGGCGGCGCCGGAGCAGCTAA |
| 19 | OS01883 | SNcum-19 | TGTGAACCTACACTTGAAATGGCAATCAGGCATTGTAAATAAAAAGCATACGTAGTCCCGCAATTACATATAAACAAGAAC ATACACAAAACTTCAGCTT[C/T]TATGGTACTGCCTCTTCAAGCAGCTGGTTGCTTCACTCCAGAATGTGGCTCCATTCCTACC AGATCCAGTTCTTCATCAGAATCGATAACATCTTCAAAA |
| 20 | OS03166 | SNcum-20 | ACCKTTACATAATTTTAATTTAAAACATCCAGCGGGAGATATTTCCCAGATAAGGAGAGCATCAACCCCAGAATAAACAGGA CAAATAAAATATTAAATA[G/A]ATAAAATAAGGTCAAATAGAAGAATGGTTACAGAAGGGGTAATAGAAATAAGGATTGTT TGTTTATACGTCAATGTTTTGTCATCATGATGAGTGAAATT |
| 21 | OS03749 | SNcum-21 | CGTTGGTGGATAAGGCGACGGGCGGGAGGTGGCTGATGAGTGATTTGAACGGAGGGAGGTTGGAGAATTTTTATTACATGCT TGGGGTTCTCGGCGTGCT[A/G]AATTTCGGTTTGTTTCTTGTTTTCGCCACGAGGCATGAGTATAAGGTGCAGAACTATATTGTT GCTGAAAATCATGGAGAAGGAGAGCTTAAGGAAGAAC |
| 22 | OS02557 | SNcum-22 | TCCAAGGACATCCCACTCAAATCCACTATTTCCACCTTGTTTTGATCATCTTCATCTTCTTCTATCCCGGTATTACTCCTCCGGT TATCCATCATATGTA[T/C]GTGCCTTTTATTCCTCGTATTGTTCGTCCTCACTTGTTGTTGCTGTTGCTGCTGCTCATACATCATCC TCACATAAATTAATATATCTAACTATATATAA |
| 23 | OS02963 | SNcum-23 | CGTTTTTCTCACTTCCATCAACACTTTTCTTCATGAAAATTCAGCATCWTAAGGCATATGGTATGCAAATTTTACGCGTTTCTT GTMACTTACACCAACT[T/C]GTATCTTAGGTCAAAAGAAACGTGATGCAGCAAAATTCAGCCTCATAAGGCATGTTTTATGCC TATTTTATGCGTTTTTTCAACTGCATCAACACTTATC |
| 24 | OS01999 | SNcum-24 | TTAGAGTCCTATGAGACGGCCCAGATGACCTGTGCATATGATGCCTTGATCTATATGACTTTTGAGTATCATCGGATGCCATA CTCGAATTGTTCCAMTG[T/C]CCATGTTCAAGTTCCGGGTTGTCTTCTTCTTCGTTCCCTGTAGGAGCTTCAATTACGCTTAAAG CATTCCTTTGTAGCTTCACCTCAATCCCATTTGTGA |
| 25 | OS04505 | SNcum-25 | TGTGATGGGATCACCGGAGCCTTTACCTTTTATTTGTATCTTCGCACCGGTTTCCTTTTCCATTTTCTTCTGKGTGATTCCCTTTG GTCCGAGAATGAGA[C/T]CGATGAAATTATACGTAGGATTCTCTTTATCCGGAATGAACAGTTTCTTCACAAGTTTCTGCTTAG GGGGTACATGCGGGTCCAAATTAGCTGCGACTAG |
| 26 | OS05631 | SNcum-26 | AATAATGCAGTCCAATTTAGAATTATATACTCTGACCTACCTATCTGAAACTCGATTTAATGATTGATTTGACTAATGATTCCG TACTAAACCTGTACAG[T/G]AATAAAGGAAGTAATTTTTGTCCACAGTTATACATTTTAAATACTTAGGAAATATATAATTCGT GTGATTTTTCATCGCAATTTTTGTTGGCGAACTCAG |

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| 27 | OS02053 | SNcum-27 | GTTGGATTTCCCGATCCCCTCACATACCTTTTAGACATCAGTAATTGTTKGATTTCCACATCCCCTCACATACCTTTTATACATA CCTTTTATATATCAG[T/C]AATTGTTGGATTTCCACATCCCCTCACATACCTTTAACAATTACTGAATAGCTATAAATAGGGGG ATAGGTGTGTGAGGGGATGGGATGCACATCATTAG |
| 28 | OS05647 | SNcum-28 | ATCGGAAACACTGGTAACAAAAAGTTACAACATTAACCAAATATGACTCTTCAAGTGKGATGGATGGAGTCTACAAGTTTCT ACAAACACTCCCGACAAC[C/T]GAGAACTTTTCGAGACTTTCGTTACATRAAGAAAGTATGTCCAAAAGTGCTTAATAGCAAA GTTTCTATCACCACCGCGATAAAAAGAAGGCAATGGCTT |
| 29 | OS06124 | SNcum-29 | CTTCTACTAGGTGGATATCAAACAGGTCAGAAGGCATCATTTACCTCCTTGCAATTGCCGTTTGTGGTTTTGCCGCTGGTGTCC TCTTTCGTTTCGGCTC[T/G]TCGTATTTTTTCCTGATTGTACCTTCCGTTCTTGCCATCTTTTCTGCTGCGGCTCTATATTTGCGAC AAATTTATATTGATCCACCGGGTTTTTCCTGCC |
| 30 | OS04862 | SNcum-30 | AACAAAGTTGATCCCAAGCAACTAAAGCTCTTTTGGAAGATTGAGAAGTTCCTGTCCACAAAAAAATTCTACATGCAGTTTG AATTATCTGAAGAACTCT[T/A]TTTGGCAATTGAAATATTTAGCACCAATACATTTGACCTCCAAAAATCTCTGACTTAATGAG CTGAATTCGTCCAGCATAAGTCAGCAATCTTGAACTCC |
| 31 | OS01884 | SNcum-31 | AAACTAMTAGGCAGAGAAGGTTGTAATTCTGAACAATGTGTTTTCTTAAMTTCATCTTGGGATCTGATGATCTCCGAGGTTTC ACCTCGACTAAGAGAAT[T/C]AACTGAAGCACTGGAAATCCCRTGGCGAGATGATTGCGAATCACAAAAAGAATGGAAAGA TTCATCATCATCSGATGAATGAGCTTCCTCAGGAAAAAAA |
| 32 | OS04537 | SNcum-32 | CATATACTATGGCGGAGACACCACGACAGTAACGTTCCCACATAGAACGAAATCTACGTTGCCCACCCAAGTCCCAAAGCTT TATTGTCACATTTCCTTT[A/C]GTGATTTTACGCATATTAAACCCCACGGTTGGAATCATATCCTCGCTATGTCCTCCTGTAGCA ACAACATTAACAAGTGATGTTTTTCCTGCATTTTGAA |
| 33 | OS03123 | SNcum-33 | GCCATCAACTATGACCACAACTTAAGGAACATATTGGGCATTTAAGTACATACCACACCTATATAGAGAGAATCTCTTATTTT GGTTTACTTACTATTTG[G/T]TTACTCTGCTATGCGTTGGTGCAATATTATTGTAGGTCACCTGGTTCATTGTGCGTTTCATAGAT GGTACAGAGGATCATGAAGGGCATATATTCTATCT |
| 34 | OS01886 | SNcum-34 | GCGAAGACTTATAATCCCAGCGCTGTTGAGAACTCTTGGTATGAATGGTGGGAGAAGTCTAATTTCTTTGAGGCAGACCCGA AAAGCTCCAAACCGTCTT[C/T]TGTAATTGTATTTCCTCCGCCTAATGTCACTGGGGGCCTTCATATCGGACATGCCCTTACTGC TGCCATCCAGGACACAAAAATCCGGTGGAGGCGGATG |
| 35 | OS04917 | SNcum-35 | GATAGCAGCCTCTGTTAGAATATTGGCAAAAAAAACATTCCATATGACAAAGTATATAACTTTGATGCATGTACTTCTTTTCC GGCCACTACGAGATATG[G/A]AGCCCTCCAATGTAGAGAATACCATCATTAAGGGAGCGACAATGTATAGAAACAATGAAT ACAACACTAGGTAGATATCCACTGATCAGCTGTTCCAAAA |

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| 36 | OS01905 | SNcum-36 | TGACTCGAACTCGRYAAGCTCRACTYGAYTYGGTTYGATAAGAAAACGAGCTCGAGYYCGAACAAAAGATTTTGTCCGCTCA GCTTATCTAGCTTGGCTC[G/C]GCTCGGCTCCATAAGAAAATGTTCAAACAAACTCGAGCTCGATAGTTCAGAATAAGGCTCG ACTCGGTTCTTTGACACCACTAATAGTATAATCATCACA |
| 37 | OS04572 | SNcum-37 | GAGTCACGTTGGTGAAATCAGTTCTTACTGCAATCCCGATATATCAGTTGTCCTTCTCCATTATATCATTTTGTTGCAATTTCTT GTGGGGGGAAGGGGC[G/A]AGAGTGATAGAAGGATGGCGTGGATTTGATGGGAAGATTTATGTCTCTGTCTTCGGGAAGGGG GATTAGGTTTTAAAGATTTAGGAGCTTTCAATAAAGC |
| 38 | OS02254 | SNcum-38 | TAAAACTGTTCCGCTTGAAACTTTCTGCTCGCTCTGACATAACTTTATTCCTCATATGGTGGAGCTTCTCATTASTCACAAATTT TATGGTTARAATGGC[C/A]TAGTGACTTAATCAACCTTCCATTKTTTTACTTTTTCTTTTGTAGAATRTCCTGTCATTATAKSAGR AACCCTCTAATAGYGCTTTTARAWAGCTTTGYR |
| 39 | OS02128 | SNcum-39 | TTTTGCCAATTTTGAAGGAGTGGTCACCCCCTTYGATCACATGCAATGCAGTATTGGYTTTCATTTTTTYCCTAACAGCTTCCAA CTGTTCTAGCGGGCA[G/C]AGCCCATCTTTGCTACCCTGCACAAATATTACAGGGACCGTGAGCTGCAGTAGAGTCTCATCCC GCACTGCACCATTCACGCCCTTTAGAGGGTATCCCA |
| 40 | OS05654 | SNcum-40 | CWCCCCATGAGCTKGTTCCGGTATTTCCAAGTCGWTGTGGATCGTAGACTGCTAGCTACATGCATTGCTGCATGGGATTATTA TT[T/C]GGTTCGGGAGTCTGGAAATCGTTTGCCATATCCCAGAACATGTTCTGCAGCTCCTCCATGCTGTAATCCTTTTGCTCAA CATGCTGTAATCCTAATTATG |
| 41 | OS02134 | SNcum-41 | CCCCTATGCCTTGTGCTCTGTGGGATATTTTTGCAGCAGCATTTAAGCAAAGAATTATTCCCACCACCTGAACTATTGAGGAC ACGGCAAAATCACCCCC[G/A]TTGATGACAGTAATCATCCCGTTATACCCAGTTGTCTGAAACAGTGTCATAAACTGGCTTAT AGTGACTATCACAAACTGTAGAAGGAGATATATTCTAA |
| 42 | OS02161 | SNcum-42 | GGATCAATCTCATCCGTCCGTGRCTTATATTTAAAGTCACCTTTTGTAACCCTAGCCTCATTTCCTAACCTTCTGTTTCTCTCTC ATCTCTCTCTCTCTC[G/T]CCCGCCGCCATGCTGATATCTTCTCCTCCCCGTTTCGRACGGCAGWTCCGTTCCGATTTGGAAGGR AATCGAAACCCGTATCAATCTAATGATAYGWTAG |
| 43 | OS04921 | SNcum-43 | GTGTTCCTGATGAGATGACTGTAGAAATAGATGGTTCTTCATCCCAAGTCCAAACCGCTCAGCAGTTGATACAGAATTCGCTT GCTGATGCTGCAAGCAG[T/C]GGACAAAATCCTGCTGGAGGGCCCGCAAACCAAGGTTATAATCCTTACCCATCTCATGGGCC TGGCTATTCTTCTATGCCGTCTACAGGTGGTTATGCCC |
| 44 | OS02200 | SNcum-44 | GGAAAGATGCATGTGATATACGTCATGTCACCTCATTTGTGGTAAAAGCCTATATATTACAGTGTTTATAACCAGAATATATA CACAAAATATCTTCCGC[A/T]CAGCTATGTAAATTATGCCTGCCATATAACTGAACGTACCCCCAGAACAAAGTTTCCAAGGG CACAACACATGCTTATACGACAAGAAACAAAAATAAGA |

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| 45 | OS06130 | SNcum-45 | TGCCCCTCTCTATATTTATTCAGGTACAAAAAGAACAAACATGCAGCTATATGAAACGCTACAATGACATCTGTACTGGTATA CAAAGATAAGTGTCCAC[G/A]TTCTCATCAATGTTATCGGCCCTTCGTAACTGTTCTACACTAGAATTGACACGAATACAATA AATGCATGGAGGAGTAAAGACCCGGAGTTGGAAAGCGA |
| 46 | OS04988 | SNcum-46 | AAGCAACTAATGATAACGATCAGTGAGTACTAGGCATGTTTGAGAAGGTCCAGAGTTTGTCGTCCAGCATTGCTCAGTTGTG GGTGTCTGAAAGTCAGTC[A/G]TGTCGTCTTCCTGTTGCGGCGGTTGTCGAGTTACTCTTACCTTTTTTATAATTTGTGTCAAGTT GCTGTATTAATTGCATCATCCATCACCAAAGATTTT |
| 47 | OS02414 | SNcum-47 | AGCAGGAGTATCTATAGAACTCGTCACTGGATTGCTATACCATGAACCGATTTTATCATTATCTTCATCTTCAGCCTTCTTGTC CTCATTGTTCGTCTTC[A/G]CCTCTTCCTCTTCATCAGACTCGGAATAAGGATTGACGAATACATCTATACTAATAATCTTGATC TCTTCCAGAGGCCGGTCATGATCATCATCTACGGG |
| 48 | OS05982 | SNcum-48 | AGTCTTGAAGAAAGAATCTCTATAACAACTTTAAAGATAAAGTTACGCAGCACAATCAGTCTGAATGTGTCCACTTTATCTGC TCCAGCAGTTTTAGGAA[G/A]TACAAAACTCGAGTTAATACCTACCGGAAAGTTACTCGAATCGAAGAAAACCTGGACTGCC AACACTACATTCGATGCGATCACATCTCAGGTTKTGCTA |
| 49 | OS04831 | SNcum-49 | CTGCCATTCAATTGGGTAACGATGTACGACTACATAACCCTGATTGTGAGACCGGAAKCTTCCCAAAATTTTCTTGTAGGAAT ACGAACCGGAGTTCGAT[G/T]AGATAACGTCAAAATTGGTTTGGGATGAAGTGGACCGGCTCAGTTATGCACTGGGTTCGGAG GTTGCTACGTGAGGAGGAACTAAGGAATTAAATATGTT |
| 50 | OS02559 | SNcum-50 | ATCAAACTTCCTCTTTCTCCAGTCTCCGATCCTATCTCCGGGCACTCTCCGACACGCCGAACCGCCTCCTCCGTCGGGCTTGTT CCGCATCAACATCCTC[C/T]GACGAAATGAGTCAGGCCCGACAACGATCGGGCTCGAGCATGAAGCGAACCCTCCGMTGGTA CGATCTCGTCGGTTTCGGGCTCGGGGGGATGGTCGGTG |
| 51 | OS05307 | SNcum-51 | ATGCTGAAGACGAGTTCGTATTTGATTGTGATTGATCTGTTCGCATTTGTACCTTCAGCATTTTCTTCAGTTTTTATGGATATTCC ATCAGGATAATTTC[C/T]GATATTGGTTTCTGTCTGGTCTTTGCTGAAAGAAATCTCAGCAGCTTCAAACTCTGTATGTTGAAAC CCTKCTCGTTGATTGCTCCAAGTTCTCCAGGAT |
| 52 | OS05579 | SNcum-52 | TGCGGCTGCCGCTGCTGGTCGTGGTCTGAGCTMGTATTGTGTCTCAGAATGACGTCGGCGCCTGCGGTGGRCAAGGATCGGC GGAACCGCCGGGAGAGGT[A/G]CCGAAGGAGAAAGTAAACTGAGGCGGAGACGATAATGGCAGAGGCKATGATGAYAATC GCTACGATAATGGACGGCGAAGAGGCGGAGGTATTCTCAGAT |
| 53 | OS04598 | SNcum-53 | GCCTCRRRCGAGTCAACTCGTAAACGAGTYAACTGTCCGCCTCRAAGATCAAGACTYGTATGTTTCTATKYATGTGTCTAAYTC GTAAGCCTCGACYCGC[A/C]ACCYRTMCGCCTCGCCTCGACTCGCGAGTYAACTCGTACRAGTCAAGYGTTYTTTAAAACACT GGTTCATGCACTCTCCATATGAAGAACACTTCAAGCA |

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| 54 | OS02676 | SNcum-54 | CCATAGAAAAAGRAGCATCTAATAACTCACCTTGCTTYCTAGACACAGTTTTGAAATTGTCTGAAACAATAACAGGCCGAAA CCCATGCACCTTTGCAAA[T/C]CGAGACTTAAAATTTTAGGAATTMACCTTTAACTGCAGCTGGATCTATCTCCCACACTCCTT CTTTCATAATTCCTTTTATCGCCGATTGCCTTCTCTTT |
| 55 | OS02762 | SNcum-55 | TCTCCAACCAACTCAAGAGGYTGYACAAATTGGGTGCTCGTAARTTTGTCTTAATGGCGATATATCCTAATGGTTGCAGCCCA ATGGCTACGGCTAGGGT[C/A]CCGACAAGTAATGGTTGTGTAGAAAGTCTAAACAGAGCAGCTCAGATGTTCAATGTCCAGTT GAAGAACTTGGTTGATAATATCCGGCCTMAAATGCCGG |
| 56 | OS05761 | SNcum-56 | TGMTRCTCGTKATGATGATCGGTTGATACTCAATGATATCAGTTGTTGACAAAGATTAAACATAAGTKGGTTGTTCATAATAA TGAAATGTACATCTAAG[T/C]TGTGGAGGGAGAGGGGAAGAGTTAATTACCTTTGTAGATAGTTGTGGTCTTTGTGGAGGTCT ATCTTCTGGTGTAGTTTGAGCCTCTATATATAGAGCCA |
| 57 | OS02815 | SNcum-57 | TGTSAAGTACRAAGAAGCACAGGGTTATGTRCTCCARACMAGRTTCMATGCMGTAATAGAARTGAGTATATGTTTTGGGATA ATTTTCATCCTACCGAGA[C/T]TGYCAATMRAGYTAYGGCCTCRAGATCATACAAYGCGAYGCTGCMGKCCGATGCCTATCCA GTTGATATTC |
| 58 | OS03427 | SNcum-58 | TGAAGGATGATTGCCTCCAAGTTCAYTGCTGTGTYGGKGTTGTTRGGTCTCACACAGAGGGACCTAAGATCTACTCAATATCA TTGCCCCCATCAGACAT[C/T]GGTCAGCATTTCGGGCAGCTGCTGGAAAGTGGAAAAGGGACTGATGTAACCTTTGAAGTTGA TGGAGACACTTTTTCTGCTCACAAGTTAGTTCTTGCTG |
| 60 | OS03003 | SNcum-60 | CTTCCCACGCAAATCCCGCCCATCATGTCGATGTCCATRTCAGCCTCCTMACTGCCGTCTGAGGGCCGCACCGACAACACAA GTCCTTCGTTGTTCAACC[T/C]AACACCACTATCTTGTTTTAGACGATGAAATACTCCCCACAGGAAGTTCTTTCCCTGCCAAC ATTGACGATTCACAGGCAAAAGTTTRGAGGTAAAAACC |
| 61 | OS03017 | SNcum-61 | TTAAGTTCTTCAGTTCACCAAGCTCAGGTGGCAAAACTGATAGCTTGTTGTTATCAAGGTATAGTTTTTCGAGAAGAGGAAGA CGTTATCTCAACTGGCA[A/C]TGCGGTTAATCCGAGACCGCAGAGGCTAACAACAGCAACATTCCGCCAATGATCTGCGCAG CCAGAACCCACGGACCCTCCATCTGCAACCCCATTGKCA |
| 62 | OS03325 | SNcum-62 | CTTCGGATTTCAGATCGTTCAACGTGACAACGAATTCTTGATTCCTTTCAAAATCTTGAACTCTAAAAGTGATATTCGCGCCGA CTTGTTCCTCTCTTTT[T/A]GGAATGCAAGCTACTGCCCCCAGGACCTCGACTTGAAATTTCCTAAATACGGAATGAGTATACA GCCCAGCCAGATGCTTCTCAAATGGAGAAGGGGACT |
| 63 | OS04677 | SNcum-63 | TCTTAACACAGTGTTYYAAGACAAGTTCAAGTTAGTTTGACATACCAGAATATTARCACCAACACTAGTGCCAACTGCAAAT AGAGGAGCTTCAGGGTAT[T/C]GGCKATGAATRAGGCCAATTACTGATCGTACATCATCCGYCCTTTMAGCAGTGTAGAAGCA ATCAGAYTGTTTGYTYGCAGRATAGAGTTAAACAARTCA |

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| 64 | OS03117 | SNcum-64 | TCATCCCAGTTCCTGGGTACATTACCTATAAACAGCTTATGTTTTGCTTGAGACGAAGAACACTTCAGCTTTTTCCCCTTCAAT TCAGTACTATTAAGCT[C/G]CTTAATAGCCTTGGAAGCCAATTCCTTGGTCCTGAAAGTTACAAAAGCATAGCCCTTGTTTTCA TTTGAATCTTTTCCCTTCATAATTCTGACCTCTGTA |
| 65 | OS05604 | SNcum-65 | CACATAATACAATGCGAGTTAAGCACGAGACAGAAAGTGAGGATGAGAGAACAAGAATTGATGCAAGGAATTACCATTAC GGCCCCTTTGCCCCTGCAAA[C/T]GTGCCCAAGGTCGGAGGATCTCATGACGAAAAAGGAAAACGGGTTGTTGATTGGGAAA GCCTATCTTCTGCTTATCGGCCTCAGTATCATAACCAAGMTC |
| 66 | OS03180 | SNcum-66 | AAAAAAATTTGTCACTGGGTATGCTCCAACTTGAGTTCCTTTGATGTCTTTCTCAAGTCCTATKTTTCTT[T/C]SGATKTTCATTTT ACTYCCGCTGAATTTATCTACCCTCGCACTAGATTTCACAGTAGAATTAATTTATGACCCKTCCATGGCAGTTGATGTTTAATG AAA |
| 67 | OS04783 | SNcum-67 | TGGATCTTCAAATACCCACCTGAACGACCCTATTGCCTTCCTTAATCGCGCGTCATTGGCCAGTGGCTTTCTTTCACAAACAGA CAGATAAAGAGG[A/G]GGGGAGGGGGCTTCTGATCGCAGCAACACGCAAGTGCGGCTCTTGTCACAGATTCACAATTTTACA GTTACTCGCTACACCACCTTCCAGCCACTAATCG |
| 68 | OS03893 | SNcum-68 | CGGCCGGGTAAATAGCCATTTGGGTTTTTCCAAACGGTCCGCCCGGTCACTCTCATGTCCTTGAGTTCCGGATCGCAGGTTAT AAAGAATAAATTGTACA[G/T]CCCTGTCCTTTTTATGTCGACTTCTTTATTGTTTTTCATATGGGTGGATAGCATGTTTCCTCTGA AATAAACATTTATAACAACCGGCCAGTTTTTATCG |
| 69 | OS03331 | SNcum-69 | GCAGTACCTGTCTTTTCATTTGACTTCTCAATAATCTCCTTCCGGTCTGCATTGCTTCGCAAATCCATGCCTGACGAGCAAAAG TCTTTTGTGCCCTCTC[T/C]AGATGGCATATTATCAGAAGAATGAGATTGTGCACGTTTCTCATCATAACACCGGTCCAAAACA GCCTGTTTCTTGCCATCACATTCAGCATCACTGTAA |
| 70 | OS04485 | SNcum-70 | GGAACATCTTGGGAAAGTGAGCTTAGATGGGTCAGCTAATCATTCTGTTCAAATTGTTGCTTCAGTAAACTGTGAKAATGTTG GTGTA[G/A]TCAGTGTTGATGATGGCTCTCAGAGTGTGAAAAYGGCTGTTGCTAGTTGTGATAGTATTGGTGGTGGTAAAAATG TGGAAGGAGTTGGTGGAAATGAGAA |
| 71 | OS04551 | SNcum-71 | AGGGCATCGAAATTGGTGAAATTGAATTGTGGAATCCCTTCTTCACAGGCTATAAACATATCTGCTACAACTATGCCTACAAT ACCATTCCCGACTTCTG[T/G]TGCTCCAGGAAGACAAGCTTCCGGAGCCGAMGAAACTCTAGACAGTGAGAAACAAGCTACT CAGATACAACTTCCGACTGATGTCGACTCAACCTTGCTT |
| 72 | OS03606 | SNcum-72 | CGAACGCCTATTTAAGTTACTGAGGGGAAACCACCAGAGTGTGTGGTTATGGCAGTGTCGTGCCCCCATAGTGGCCCCCTCG ATCATACAAAAGTTTCAT[C/A]TCTATATTTCGCGCCATCTGCATGGCCATCTCGTGATCCTGTGGATCCCATCCTGGACTTGCT CAGGCCATTCACAAAAATATCCCAAGTAGTGCTCCTC |

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| 73 | OS05331 | SNcum-73 | ATCTTTTTTTGAGGAATCGGATATGTTTCTACAATTGGTATCAAGAAGACAGTTCAGCTGTAATAATATTCCATGGACCGAATTTGATATGATATGGCTT[T/C]GCTGACGAGGTAACTCTGAGGCAATATTGAAGATGACAGCTGGCAGCTTCTCATTGGCTACCAGACCTGTTAGAGCTCCGGATGCGAGTACACGAATCCG |
| 74 | OS03352 | SNcum-74 | AGGATAGTTGCAGTTTAACTGCTGTCTTTCGGAACATCCATCCCCACTGTATCCATGAAAGCATCGGCATTGACCCAAATCACGATTACAAATTCCTTGG[A/C]CACTACAGTCATTTTTGCAGCTCTTGCCTCCAATTTTCTCAACAATTTCAACATATGTGAAGTTCGAGTCACAGACAGATAGCCACCGGCCAATCTCAGC |
| 75 | OS03371 | SNcum-75 | TGGTGATGATGATGCCGATGGAACCCGGCGCTCGACTCGCTCGCCATGTGTTTGTGCAAATGATTCACCCAATCATGAGAAAAAAACTACGGCGACGCTA[A/C]TTGACGGTTTTCAGATATACATCTGCTGCAGTAAAGGTTTCCTTTTCTGCAGCGTCTCGCATTACTGAGTCACTGCAATCTCGGTCGAATGATCTCAAGT |
| 76 | OS04771 | SNcum-76 | AAAGCCCGACTGGCTCGTCTCTGCACGAAGCCTCGACGTCAGAAAGCTCGGACTCGACGCTGTGCGGGTTCGACAATATCGGCCTCGACGTTGTTCCACT[G/A]AAATCGATCTGAAAATTCGTAAGCGTAAACTCCTTTTCACTATCGGGTATATTTTTCGCTGGACTATTCTCGACCGGATTTCCTAGCTGACCATTACTGT |
| 77 | OS04724 | SNcum-77 | ATGGATAATTTCAAGAAAATTGTATTTTATGATGACAAACCAAGATATACTGAACAATATTTTTTTTTCCTATAGTGAAYGTG[T/C]GAGTTACATCAAGATATCAGAAAAAAAAAGAGTTGGAATCCGAGGTCGAAACAAAACACAACTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGC |
| 78 | OS04904 | SNcum-78 | GAAAGAACAATTTTGGCCAACACAAAAAGGGTAACTTCATCTTTAGGMTATACTCCAGAAACTATGTTGAGTTCGTATCTTCGGCCACCAWATGGTCCGT[C/A]ACATACACAACGAAACTACAATGCAAACTTTTTTTTTTTTTTTAGTTCACTTACCATTAGTGTTTCCATGCTTGTGAAA |
| 79 | OS05130 | SNcum-79 | CTCTCTCATACTCTTCTCCATCAGAATCAGTTTCTTCTTCTTCATTATCGTCCACAAGTTTCTTGATTTTCAGATATGATGACATCATCATGTGTTGCAT[G/T]TTGTTGGGATCCATTTTTTTTTTCCTCCTCTGTTAGTGGGTTTTCAGGAATTTAGAGAAGGGTAAAGGCAAGG |
| 80 | OS03527 | SNcum-80 | TCTAGGGTTCTTAATTGAGCTAGTTGATTTTTTTTTCTGTCKTTTTTTTATTGCGTTGAATTGCGCTTTTCGTTTTAGCGTATTAATGTTGAGGAAATKC[C/T]TGTAGTTTCATGATATCAAGRAYTTGGATTTCGGGGGGTTTGAAATAGACATATGATGTCGAATTTGAATCGATTG |
| 81 | OS04568 | SNcum-81 | CCTCCAAAAGGACATGAGTGCCATAAATGTTGTTCTTGGTGAACTCAAAACTGTTTCCGAAAGAATTATCCACATGGGTTTGGGCGGCAAAGTGCATGAT[T/A]GTGTCGATGTTTTCGGTAATTAAAAGGTAGTTCACAAGGTCCGCGCTACCAATGTCGCCCTTAACGAACTTGAAGTTGGGAGAAGGGTTTGAAGGAAGAA |

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| 82 | OS03930 | SNcum-82 | TTGGAACTTGGAAGTACCTTMAACCATGAGTATGGCGAATTTGATTCACCAGTTGATGCYCGACTTGTAGTCCGTCTTGATGA GTTGTTTTATTTTTCAC[T/C]TTTGGTAAACTATTGCCGAGTAAGATCATATGTGAAATTTACAGTTAACCTTGCGTACATGTGA TATATGAAGYAATTCAMGTGTGCAGGAGATAGTAAY |
| 83 | OS05084 | SNcum-83 | TCCGAGTTATGTGCCAAATAACGTTTCAAATCCACAAAGCACCTCCGCTCCAAGGACATGCTAGCACAAGCATGAGAACTAG AATCAGGCAACAACTTTT[C/T]AAGGACTTGATACACCAATATCTGTGTAACTCCACGTAAAGTGTGATGATGGGAGGTCAAT AATGGAACCATAGGAGGAAGTAGTTCATCCAGGTGTTCA |
| 84 | OS03692 | SNcum-84 | ATCAGTAGTAGCAGCCAAARTAAMTTCAGAGATAATGTCCMACRAAGACTGGARAAAGGAAGCATTGCAGCCATCAGGGA ACTTAAATAAAAACAGCCGG[T/C]CTGACTTCCTCCGAGGGAGTCTCACCAAGATGGGGTCGTCAGAAAAGAGGGGAGTGTA ATGGTAGAYAACAGATTGTGGAATAAGGACAKAATCAGAAAC |
| 85 | OS04912 | SNcum-85 | ACTCCTTCCAAGTTCAAACACTCCGTTCAAGTTCAACACTTCGTTCAATAATGGTGTAGTGTAGAGATACCATGTAGCTTCAA GTTCAAGTTTACAAGCT[C/T]AATAGGGGTTCAATTAGGGCTTCAAGTTCAAGTTCAAGTTCAAGTTCAATTAGTGTTCGATTA GGGTTTCGATTAGGGCTTCGGTGTTTCGATTAGTTCT |
| 86 | OS05609 | SNcum-86 | GTACCCTAAAAATACAGATCTTGTGCTGAAACATTTTGCTTCAAAAAAATTTTTGACGTTTATTCTGTTAGATAGGG[T/G]GTCT ATTYATCAGAGTTCGCCTAGGGCCAACAAAATGTCAGGACCGGCCCTGGATATTGGGCCTATTCATGTTCTCACTGATTCTCT CTTGGCGATTCAT |
| 87 | OS05042 | SNcum-87 | TAATTTTTTCACACTTAGATCTTTCATTTCCTCTCCAGAAACCTCACCATTCAGAAAATCTAAACCACATTCTTCTTTCACTTTT ACACCCTCCATCCGC[T/G]CTTGCTTTACAATATTCAGCCCATAAGGAGATTTCCCCTCACTGATCCCATTAACCCCGTCCTCG AGATTATTCGATGAACTTACCCGTAGACTGGATAT |
| 88 | OS05980 | SNcum-88 | ACTACTACTAGCTTGCATTGTGTTTGGATCGAGAGTTTAGGGAAAAGGAAGTGAAATCCATAGTTCAAATCCATTAATTGATT CCAAATCCTTTTTCAAA[T/A]TTTTCATTTATTGTATATTTAACATTTGTCCTAAAACCYGCGCATTTGAGATCCAATTTTTTATT ATGTTA |
| 89 | OS05885 | SNcum-89 | TCTTCAAAATTCACGATGTTGGCCCGAATCATGCCATCTATCTTGCCATGCTCATCTGGAGAAACACCACTTTCTCTTTCATTTT CTTGTTCAGCTATTC[C/T]GTTGGCAGTCACAGGTACCATCGCCCCCCCATCAAAAGTAAGCAAATCTTTTAATTCCTTATATA CCCCTGACGACACTATTACATTACTACTACTAGTA |
| 90 | OS05038 | SNcum-90 | AAGTATAAATCTAGTTAAACRCAAGAATCTCATTTGATGATAACATCGGCAATAACGGTTGGGAGAGACATCCAAATTCGAA ACATGTCTCAACTTGTGG[T/G]GACAAGTTTCAATCCTATTTTGTTCACGGGTAGAGTCATCCTAAGTTGAGTTTATTTGAAGAT GGAAATTTTACCTTATCCGATGGGAAGGTCGCCCACA |

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| 91 | OS03788 | SNcum-91 | TAATCTGATGTCTCTCTGAAAAGCCTTTCTACTCCAATATGTGAGGACTGATCACAATTTGGAATCATCCTTTTCAGAATTCCC AGCTTCCTTTCGACCG[A/G]ATCCCGGGCCCCAACGCAACGATCGGAACGCGCCCTTGATCTCTTCATGATCTCTACTGCACTG CTGCACCTTCTTTTTTTTCTTCAGGAAATTATGAGC |
| 92 | OS05106 | SNcum-92 | RGTGRTAAKTGWYCAATYCATGAAMCAATTGRTCATGGGTTCAACTGCTGCCGAGGGTCCAAAAAATGGAAATAGCCTCTCT ACCCAAGGGCAAGGCTGC[T/G]TACTATCTTACCTTCCTCGGACCCTGCAAATGCGGGATATACTGAGTACGTTCTGTTTCGTT CYGTTTCCGTGTTCCGTTTCCGTGTACTACCAATCTTC |
| 93 | OS03799 | SNcum-93 | CTGATGTTCATGTGATGGATAATGTGATGACCKAAAAGATACGATATTCATATTATCCGTCATATCAGCAAGATATAATGAAA AATTAATTCAAAATTGC[G/A]AGAGAATAGTGGTTCAACTATCAGCATAAACACGATGCTCCTCTCTCTAATCATCCTTGCATC ACATGATCTTTAGGTTATATAGATAAAGTTCACTATC |
| 94 | OS05831 | SNcum-94 | CTGAGGAATGGCGCCTTAGCAGAGCCCATTCCGAAGCTTGGCCCTGCAAAAGATTGGTGCACATGGCCATCTCGCCACATGT AACATCAGCACCCAAAAT[T/C]TTGCACACCTGGCGAAAAGGTAGATTTCCTACTGTCGTGAGGGGAGCAAGATAGAGCTTTT CACGGAAGTCAATGAGTTTCCTTTCCCTTGGGTGTAACT |
| 95 | OS05974 | SNcum-95 | TGACCCTCAAGAAGTATGAGTTCGATGTCCAAAACAAGGTCTCCGTCGATTGGCTTCTCGGGTTAGGCCCGAGCCTCGGCATT GTGCGCGTCCGGGGTGT[T/C]GGGATCATCCACACCGAGCTCKTTTCGGGAATCCCGGCTATTTTCTCTCACTTTGTCACCAAT CTTCCCGCCTTTCACCAAGTCCTTGTTTTCCTTTGCG |
| 96 | OS03818 | SNcum-96 | CAGTAAGATATGCTTTTTTCTGAGTAATTCGACAAGCGGCCAAAGCGGAAATCCATTATTTGAGCTTTCTAAGACTGAGGAGA CAGAGTTGAAGTTGCTC[A/G]GACCTTCTACTGTTGACTCGATCGGCGTCAGCTGGTGGAAGTGCTTGRCGAGAGCAGATATG GCATTAAGAGTTGCAGCACCAGAACCGATGCGCTGACC |
| 97 | OS03824 | SNcum-97 | AACCCGAGCACAATCCCTCCTCTCTCCTCACACTAGACGAAAAAATTCGGCAGCTTTCCAACAACGCCTCCGCCGTTCCTA GGCTCGGCATTCCATCG[A/T]ACGAGTGGTGGTCGGAGTCTCTCCACGGTATCGCGTCCAATGGCCCCGGCGTTTCCTTCGACG GCGCCATCAAATCAGCCACAGGCTTCCCCCAARTCAT |
| 98 | OS04641 | SNcum-98 | ACTAGGGAATTTAGAAGCACATGGTAAGCAATCGTGTCCAAGTCAACATTCTGAAACCGCATTCTACCAAACAGTTGGAGTG CAGTCTCACATTTACCAG[C/A]CACAGCATAACCAATGACCAAAATATTGTAATACATTACTTTATGAACATACCTCTGCTTCA TATAGTTTTGTAAG |
| 99 | OS03878 | SNcum-99 | CTTAAATAAATAGTTTAGTGTAACTTCTGGCTAATGAAAATCAACTGATAAGGAACATTCCAAATTGTACCGCTTTAGCTGAT AACAGAAAGTTGGTTGC[A/G]ACCCTGATCACGCAATTGACCAGAAAGTAATGAAAAAAAGATTATGGGCTCCCTGGCTTAG TGAGTTGAGTCGGTTCCCGGTAGCTATGCGAAATGTCAA |

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| 100 | OS05601 | SNcum-100 | TTTCTAACAACTTTATGATCGATAAACACCGAATAAACCGASGCCTGTTCCGGCAGGTGMCACTCRTACTGATGCTCGAAGCT CCCCCTCCAGAAGTAGA[C/T]GGGTATGGATCCCGCCAGCATGCAGTCGAATGCGGATCTTCGGGTATACCCGTCACCTTTGG GCTGCAAGCAGAAATCCGAATCCAGAAACGACTCCAGT |
| 101 | OS04769 | SNcum-101 | GAGGAGATTGTTAACGAGAGCAGACAGCCCGAATCGAGTGAGATTGGCAGGTATGGAGATAGAAGCTTGCGGTATTTTGTAT GGGGRTTTTAGCTTCGTC[G/A]CGAAGCGGACTTGCACTTGCCTCGATGACACGTCATCCGCCCCTCCGCCTTCGTCAACGTCC ATTATCGTACTGCGTGTGGGGTGG |
| 102 | OS03929 | SNcum-102 | AATTATCCCTATTTTTTGAGGTCGGAAAACCTGCCTGAGTAGATCCTCGAATATGATAGGAAGGCTTTTCCCTCTCAGATGGCT TCTTTGCTGCATTTTC[C/G]CTCTCAGACAAATCAATCACTAGACGCATCAATGTCWTCCACCACAAGTATCGATCTATTCTCC GTAGAAATTAGCAGATTCCTCAAATCCGAATTGCGT |
| 103 | OS04664 | SNcum-103 | ATCAAATTTTGAAGGAAGTTGGTGATAGCTGAGGAAAAACTCTGGTTTATTGATGAAATGAAGATCAAGATAAACCCCAATC TGGCGGAGATGATACTGC[A/G]ATCGTTGCCCACGAGAGCCTCCTGATACACTCACCACTGCAGCAGATTTATCAGCAAAAA CATTTGGAGGCCTTGATGCCCAGTCAATTGCATTCTTCAG |
| 104 | OS04526 | SNcum-104 | CAGCTCGAGCTTTTCTTGATTCATATCTGATGTGTTTGTCATACCTTCTCGTTTTCTTTTTCTCTTTGTAGCGTGAAATTGCATAC TCTCGTTCGTGGCT[A/G]TTCAATTCACGTGATCCAGCTATAGGAAGCACTTGAGAGGTCTTGAAATCAAAAGGACCATACGA CTGAACTCCTTGGAAACCATTTGCAGTACTCGTAT |
| 105 | OS06018 | SNcum-105 | ACATAACAGATTCCAAAGTTTTATGCCACGACCATCTCTCATGAGACTCCCAYTAATAAAACCCGTTTGGACGAAATTTAGAT AACAAAAAGTTAGGTCC[G/A]TACAGGGAATTTACTTGCTGGCACTTAGTTCAGATTTTCGATCTTGAAGGTTCAGCAACAGC TCATTAGAGTTCAAGTACACCTTGTTGGTTGAAGCAGC |
| 106 | OS04486 | SNcum-106 | ACTTCGCCTCGTCTCTCTTACTCTGCYGCTGCATTTTTAACTTGAAATGCCYACAAAGAGGGACCATGCAGTATTCAGGGTCGC TGCACATGCGGGAATG[C/T]AGTTCATGCCACATGCGTTTACAATGGACACAGCCAGGGAACTCTGGCCTTACAGCCATAGA AGTGACGGACCAGAGTCTCGAGCCCTTTGCAGGCCGGG |
| 107 | OS04498 | SNcum-107 | AGCTCAAGCGGATCAAAGAAAGACAAAAGCTCGCAATACAGCAAAAAAAGAACGCTAAGTCTACCACGGACCAGGCCCGG AGGAAGAAAATGGCCCGAGC[C/G]CAAGACGGCATTTTGAAATACATGTTGAAGCTCATGGAAGTCTGCAAGGCTCGTGGAT TCGTGTACGGCATTATTCCCGAGAAGGGAAAGCCCGTCAGCG |
| 108 | OS05098 | SNcum-108 | TCCACCACACAATTTCCATTCKCACAATCTMCAMCACACAATCCCTTTAAAAARTCAACAATACACACAAAAATTGATTTGC GTGCCCACAACTGTGAAA[A/T]CTAATCTGGGACAGAGGGAGTATGCAACATATATGTACACCACAGGCTTTTATATACCGTT AGCCTTAGTAAACGAAAGGGATTATTGCGTAACGGTTTC |

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| 109 | OS04550 | SNcum-109 | TAGGTTTTCCAAAATGTACCGTTCGAGTAATGTCAGTTGTTCCATCTAGATACTGTGCTCCTGAATCAAATAGGTACATACTAT TCGGATCAAGTTCAGC[A/G]CATGTTTCTGCCTCAGGACTATAATGTATAACCGCACCATTTGGACCAACAGATGAGATAGTC GGGAAACTCAACCCCGTAAAATGCTGTTAATAAAACA |
| 110 | OS04555 | SNcum-110 | GCTTTTAAGTTTTGCAACAGTCGCATCATGTGATGAGGCTGAACATGGTTTGGAAGCCCCTAACTATCCTTGGCCCCACAAAG GCATTCTAAGTTCATAC[A/G]ACCATGCTTCGATTCGTCGTGGTCACCAGGTTTATCAGCAGGTCTGTGCATCCTGCCATTCTAT GTCCTTAGTTTCATATCGTGACTTGGTAGGTGTGGC |
| 111 | OS04704 | SNcum-111 | CAGACTCCCAAGTTTCGAAGATACAGATATCAGATACGAGAAGAAGAAGAAACTGTGCCGCGCGTGGAAATCTATGTATCG AGTGAGAAAAGAGATACTA[T/C]TAAGAAAGAAGGGTTTCGCCGACAGGGTTTTAATAAGGAGATCGATGTCGGAGATCGA GAATCCGAGGAGAAAATTGGAATTTCGGAGATGCAGAGGTAA |
| 112 | OS04570 | SNcum-112 | CGAGAACGAGTTTAAWTTCTCAAYCCTCAATCCGAAATTCAACTCCCGCTGAATTWCGATTTTAAGTTTCTGCSCGACGGATA TACATACACATCATTTT[C/T]CATGGAGTGCATGAATCTGATATCCTTAAGGTCGACATCCTTTGCGGCGAAAGCAACGCAGG GATTCTCTTTTCAACRCTCSCYCAATCCAATACCCCAA |
| 113 | OS04585 | SNcum-113 | GCGTAAGACATGGATGACAGTTTCAGGATGACTGCCATAGCTAGTGCAAAGGTTGGAYGTTGTGGGCATTCTCTGGAATCAA GGTGTATTCGTGACCAAG[A/C]GTTCCTGCACACCAAGCCAAAGAAAAATTTTTAGTTTATTCGGCAATTTAGACTTCCAAATT TATGTMCAACGGGTTGAATTGGCAYTGGAGCCATATCA |
| 114 | OS04587 | SNcum-114 | TGTACATCTGTGCCCTGGAAAAACTGTTCAGTCTCTTCATCATGGGTAGCCATCAAACCATCTTTCTTCAACAAACCCACGGA GGTTCGGTCGTCCCAAA[C/T]GAGCATAAGAACCCGAACACCTTCACTTGCTTTCTTCTTAAGGAGCTCACCTAGGGTTATGTC ACCTCCGGGCTTTTGTCTCCTGGAGTCCCTTATCAAA |
| 115 | OS04603 | SNcum-115 | CCAGATCCGGATCTCGCTTATCAGTATTATTACCAGATGGTGAGGTCGAAGAAAAGCAGACTTTCTGGCCGAAAAAAGACTC AGCGCAGAGGGCATCGAA[T/C]GGAGTGAAAATCGACATCGACATGGCCAGCTGAAATTAGATTATTCTTTTCCTGCGGAAG RTTTAAGAGAGAGGGAGAATTGAGAAAACTTTCAATGTTT |
| 116 | OS05532 | SNcum-116 | TTCTCGTTGGCTTTTCCGTCTTCACTTCTCATTTTCTTCAATTCTTCGATCTGTTTTCGTGAGCTTTCGAGCTTCTGAAGAAGGTC GGATTTTCCGGTTT[C/T]GGCCTTCTCTTTTTGAATTCTCCAAATAAGTAAGCCCATCAGATGGKCACTTCCTTTGAKCATCATG TTTCTTATTTCTAACCATTGGTCCTCAGATACT |
| 117 | OS06055 | SNcum-117 | CCGCAAGCCCTCACACCAGCCATGAAATGCTGTCGACAATCTTCACCAATGCGTTCTATGAATTTGGTGAAATGAGCAAAAG AATTGACAGTAAAGATAA[A/C]TGGCCGATATTCAACCTTGTCCCCGTCTTGGGTGCTGGAAGGTCTGCGACATCCATGGACG ATAATATATTCACATGTGAGCTATTTGCCAGGAAATCCA |

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| 118 | OS05886 | SNcum-118 | TGAAGAATTGGGACAAGAGTATAAACTCGGTCACAATAGTAGCTGGGGAGTGTATTCCCATAAACGGGATGAAGTCAACCT<br>GAAGCCAACTCTCCGCTGC[C/T]GAGCCGGCTAGTGCACCAGCTACGAGCCCTCCAATCATTATCACAGTTGCTTTCCCTAACT<br>TGACGTTTTTCTTGGTCATGAAGTATAAGGATGCCCCAA |
| 119 | OS05257 | SNcum-119 | CTTATCGAGGGTATTGKGACTTTGTTTGGAGTCCTCCAGGAAGTACTGGGACTACAGGAATATTTGAAGTGATTCGAGGAACA<br>AATTTTAAAGTTGTAGT[G/A]GAAACGGATGAACAGGTAGAGGTGTCGTTCTCAAGGACATGGAATATTTCCCTAGAGGGCG<br>AGTTTGGACCATTAAACATAGACAAAAGATTCGTGATGC |
| 120 | OS05399 | SNcum-120 | CGTGTGRTCATGCATTCTATCACCTGATCCCAGAGTCCATTGCTCACTTCTGCATCATCCGGCAACCGCATAATGATCTCAGA<br>AACAATCTGACAAGCCC[T/G]AGACCTGGCGACCTTGTCAGCCGCAATTGCTGGCACAAGAAGAAACTTAAGGAAATTTTCG<br>AGGAATTCCTTGCCGCCGTTGCCCTTTGGGTCGTACGAG |
| 121 | OS04663 | SNcum-121 | TCTTGGATCTCGACTTATTGACTCTCTTTCTCTTTGGCAACTCTGTTGTCAATGCATAAATCTTGACAGCAAATAGTCCATCAG<br>GAAATCCCTTTTGATC[T/C]TGACCACAACAAAATACTATGACTACATTAAACAGGTTAGTTTCCCAGACCAAAGCCACACCT<br>CAAATCTCCAGGAAATATGAGCAATCATCAGTATTCT |
| 122 | OS04808 | SNcum-122 | GTAACAAATACAGTTTGCTGGAGGAACGGAGTTAGTCGTGTAGTTTAATTTAGTAGAGGAGTGCTTAAGAGTAGCAAAGGTA<br>GCAGTTGATTTGGTAAAA[G/T]AAGGATGTATAGTATYAAAAATAGTAGTCAATARCGCCGCTTTAGYGCRCTMTAGCRTTTA<br>GYRTAGCRAAGCGGAGCMCMATCGCTAAACGYYRTTAAG |
| 123 | OS05121 | SNcum-123 | TCCGCTCATCATCTTGTCGAATACAAAGAAAAACACAAGAGTGAAGAGGTTGCATATAGACTTGGAGTCAATACGAAGACTT<br>GCACATGGTATAAACTGC[C/A]GCAAAAGCAGTGGGAAGATGGATAAGAGTTGGCTGCTCAGAGCCCACACCAGTGCCATTC<br>TGCCAGACCTTACAGCTCCTAATAAGTTGCTAAGGTTAAC |
| 124 | OS04687 | SNcum-124 | AGTGGTACAGCTGAAGGTGGGATAATGGCTGCTGGGACTGAGAAAGAACGTGCGTTATCAGGGGAAGATGCAGTTTGTTGTA<br>TTTGCTTGGCGAAGTACG[C/T]GAACAACGACGAGCTACGTGAGTTGCCTTGTTCTCATTTCTTTCACAAAGAATGTGTCGATA<br>AATGGCTCAAAATCAACGCATCGTGTCCGCTCTGCAAA |
| 125 | OS04688 | SNcum-125 | TATACAACGAGGTGTTAGTCCTCCCACCGTGTGCTATGCTGATACCATCCACAACCCTATAATCCTCGACCACAGACTCCATG<br>CTCGTTTCGTAGAATAC[A/G]CTGTCGTCCCCCACAGCCATCGACTTCATTCTTATCAACTTTGTGTCTTCGAATTGGATGAGAA<br>GACCCGTTCTCTGGCTAAAGTATCCCCATATCGTGT |
| 126 | OS04706 | SNcum-126 | TCCTACAAGAAKAGCATGTGGGACAGCCAATGACSYCTTCGGCAAGTCATAGTCCAAACCCTTGAGTAGTGGACCAATGAAC<br>TCRAAACTGTTGACAGGA[C/A]TACCATCAGATATGAAGTAGGGTTGACCAGCAGCAACTGGCTGTCCTACTCTTCCAGGAAT<br>ATCATCCAAAAGGCCCATGCTAGCTAACAGCAGGGCCAG |

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| 127 | OS05911 | SNcum-127 | AATAAATAGGGCAACGCAGAAAAGGAGAGAGACAGATTCGAGGAGTGCGTTAGAGGGGGTTAGGGTTAAATATAGAGGGG TGCTTTTTTCCTACGACGAA[G/T]ATCGAGTGATGGTGCGATCTTAGACGTTGGTTGGCCTTGGGGCGAAGGTGTGAAGCATCT AGAATTTTCTAC |
| 128 | OS05571 | SNcum-128 | ATAAAATAGATACCAACATAAAGGTTCATGCTTGTTTGATGTGATTGCTGTTAGAGAATTAATTTGGTTGAGTAAGTATTTATT AATAATTGTTTTTGTA[T/C]GATTTGGTCGATTAGTTAATAATCATCATCCTCTGCACATTAATCCTAATTAGGTCGTGGTCAGT GACATGAAGAATATAGTGTTAATTAATGGTCAAAA |
| 129 | OS04725 | SNcum-129 | TCGTATTTGAGTTCAATGCAGTCATACGGGTTCGAGGAGACGACAAATCCATAATCTAGCAGAAAAAGATCGTTATTCAAAC AGCCGTAATTAAGTTCCA[C/A]AGGATCATCCTGTTTAATATGTGCTCCAGCAATGACCTTCACAAGCATGTTACCATTGTTTG CTTCTTTTTCTTGTACAATTTCAGCRTTTGGCGTGAAG |
| 130 | OS04728 | SNcum-130 | ACAGCTGTAATGGCGGGAAGGCCACCTCCTCGAATTCCAAAATACTTTGTTAGAAAAATGGCGCTTCTGCATATAGCAAAGG ATACAGCAAGGGCAGCCG[C/T]GGTCTGCAGTACAGATATCTTGCCACCGGAATTCGGTTCTTCATCAAGACCGGCACCGGCT GCTGATGATGACGACTCGGCAGGTATTTTAGACGCCAAT |
| 131 | OS05128 | SNcum-131 | TCAGACCCTTCATCAGAAAAAATAGAAACAAGGTGGCTGATTGATGGTGTAGGGATGTTGTTATTTGTTTGAGAATGAATTGA TCCATTTTAATCGCTTA[G/A]ATACTTCCATCCTTGTGATGTAAAGGACTTTGAAGAGAACATGCCAAACCCTGAATTCGCACT GCTCTCAAGTGATGTCTGCAAATTTGATGCTAACATA |
| 132 | OS05218 | SNcum-132 | TTAGGTTAGGATTGATAAGGACCTGTTTGGTGACGGGGCATAATGTTGATGCATTAGTACTGTTCTTGTTATTGGGGGAGGAA ATCAGCAGCCACTTCTC[C/G]ATGCTTTCCCTGTCGTAGGTTATCCCCGTCGAGACGGTGACGGGATCCTTCATTAGCTGCATG GAGATGGGGCAGAGGAAATAACACGGGATCTCGACTT |
| 133 | OS04739 | SNcum-133 | AAACCAAGACAGACAAAAATGGCTACTTCTTCTTCATGCCACAGAAACTGACTACGGCGGCGTTTCACAAGTGCAAGGTTTT CCTAGTTTCTTCTCCGGT[C/G]GCGGCATGCAACGTCCCCACCAACCTCCACGGCGGAGTCGCCGGCGCTTCACTGATCCCCA CTGCAAAGTCGCCGGTTAAGGCGCCGTTCCAGCTTTTCA |
| 134 | OS06060 | SNcum-134 | CTCCCACCCACAATCGGCATTTCTCTCACGACATCCATCACATGGTTCCGCCCAAGCATGCTTATGCTGCTCCC[A/G]TTGTAC TCCCCTTCAGTGAATGAAAACGTCACAACCATTAGCAACGCCAGGTCATTTTGTGCGGAAAATGCATATGTTCCTTGTGCCCG TCCGATGATCT |
| 135 | OS05820 | SNcum-135 | AACACATGATTTTCTTCTAATCATTCAACAATTTTGACGACTGTCATATTCCCTAACAGAACCTATACCCACACAGAAAAAGG GCGAACCAATAACCCCC[G/A]GAAAATACAAAGGTACCACCAAGAACACACAATCACTTTGATCCAGCAAATTTACTCATCA TCTTCGCTATGATTGGAGCCACCTTTGGGTTACCTTGAT |

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| 136 | OS05856 | SNcum-136 | TCAAATAAACCAGATACGAACTAAATAAGAAGGAACAGCTCAAGAGATTTTACATTGACATAATTCCCATACATTCCTGGGC TGCGAGGACATTCAATAC[T/C]AGAATTGGCATCTGCATCAGGGCAAGGGGTGCATTTTCTAAAAAGTGGCAATCCAAATGA AATAACGGAAGTTATCAAGGACACAATACACACTTCAATA |
| 137 | OS04773 | SNcum-137 | CAAGCATACTCCAAGTGATTATACGATACCACCAATCCATCGCCCAGATTTTGGCCCCCTTTCTTCATAAACTAGTCGCCCCC CGTCATCACTGGAATCG[C/T]CGCCATGGCCGATGGCGCTGATTTTGGAGCTTTGGGCCCACCAAGAAAAGCCATGTCTTTTAT GGGATGAGATTAGAGAACTAGTCTTGGCTGGAAATTA |
| 138 | OS06036 | SNcum-138 | CAAAGTCATCTGAGCCAGGAGTATCAAAACCTTTATCCCAAGCAAGTTCAAGCCACCTTTGAAGTGATCGGATATTAGGTAC GAATCCACAACCACCATA[T/C]AGAACGCTTTTCACTTCTTCATGTTGGCTAAGCAAATAAGAGCTAAGCATTTGGATATTAC GCCAACCACATCCCCAACCAACATCCCAAGATTCAATGC |
| 139 | OS05652 | SNcum-139 | CTTTCAGTACCATCAATTTTTCCAGGGGAGTTAACCATATGGAACTGAAATTCCTCGTCATCAAGACCCTCCATAAGCTCCCA AGTGTTGATAACCGAGT[C/T]TGGGGATAGAGGGTCCTCAGAGCTACCAAAATCGCGAACCTTAATCATCTGGGCCGTCGGAT TCGCAAAATCCCGGCCGTTGAGACTGGGTTTTGTGGCT |
| 140 | OS05734 | SNcum-140 | GCCTGAGTTTGTTAACATCGGATTGCTGARCTGGCTTCAGGAAGAATTCTTCAGCCCCTTGTTCCAAGCAACTGTCGATTCTCG ACGGAATATTTTCCGA[T/C]GACATTATCACAACTGGGATGTCCTTCAACATCTCCGATTCCTTTATTTTTCTCAGAAGATCGAA ACCGGTTAGTGCAGGCATAGAGTAATCTGTGATGA |
| 141 | OS05060 | SNcum-141 | GACGACACGCTTTTCTTCATCAGAACCCTCACTGTCGGTAGTGCGAATATATTTGTTGGAAGGCACTTCCGTAGTGTTCTCCAC TGGTGCATTTTCCCCC[C/T]CACTGCCATAGTCGTCACTCTCTTCATCCTCGGTGTCACTGTCACCACGCTGAAAAAATTTCGAC ATCGTCTCTAAAACAACGACAGTAAACTTCTCAGT |
| 142 | OS05125 | SNcum-142 | TTAAACAAGTCGTCCCAACAGGTGAAGCATTTTCACACAAAATGAGCAYGAGAAGTGCTGTTCGCAAAGACAATAAATTTGT CAAATACATGAAAGCCCC[A/C]ATCAGAGTCCTGGCCCGAGCCCGTGATTTCTACGTGCAGAGCCTCACGTCCTGCGCCGGCG GCGCAGCCTATTACGGCAACAACATGGGCTGCTCATATC |
| 143 | OS05231 | SNcum-143 | GTAGCCACGCCACTTTCCTCATTAATCGACGGCGACACGAAACCGTTCGCGGCAATGAATGTAGCCCTGTTGGTCGGAAGCT CAGTCGTACGGTTGTCTC[G/T]GGAGCGACCGCGGCGAAGTGGTTGGATGTTGCGAGTGGAGGTGGTCGGCGAGTCGGAGGC CATGAGCTCCACCTTGCTGAGGGAGACCACCACGTTGTTT |
| 144 | OS05316 | SNcum-144 | AAGGTGGGGGTGGCCCACTCAAGTTCGACTGCCCCGTTAACTTAATAAAAGTCCGCTTGAGCCTACGCAGCTCCGGCAGACC AACGGGCCTCGAGATCTC[A/C]ACATTGGGCAAGTTCGGCCCGAGTGAAACACCATTGAGCGGGCCTCCCATGGCCCGTGAC TGTGCTTCTACAATCATATTCACGACCTCTAGAGCCGCTC |

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| 145 | OS05049 | SNcum-145 | ATGATTGGCATTCACGTATATACGAGAATATATTATGATTCTTCATTTGTTTGCCGGTAATTTTGGATGGATTTTGTAATTTGTT AAAAAGATCACTTCA[G/A]TGATCCGATTATAAAAATGAAAATTCAGTTCAATGATGCAATTGAAATTTGAGACCAAGTACA GTTACAGTGACAAAAGATATATTTAACCCTAAGATAG |
| 146 | OS05690 | SNcum-146 | ARAACCCGCCCTATCATAYGTGTCCTTATCCCTACTTCTATACCCCGAACCATCAGATTTTGCATGAGWGTGTGTGACCACAT CRTTCACATAATCTCTA[T/G]ATCGGTGCTCTTTMTCGGACCTTGAGTAATCCGAGTTAATGTTGTATCGTGAGTCCCGGCTAG AACGATAGGATGACTTAGAATAACCTCGATCATAGTC |
| 147 | OS05406 | SNcum-147 | AAGGTTGTAATAGAMCATTCCATGTTGATTGTATGGGAGGAAGGKAGCAAGATACTCCTTGGAACTTTGATTGTGAGGTATG CTTATGTGAGAAACAACT[C/T]GTGGTCTTGAAAAAATATTGCGAGTCCCAGAATAGGGATGACCAGAAACAAAATCGCAGT CATTCAGGAAAGTCTTCTCAAGCTACAGGCAGAGTCACAA |
| 148 | OS05265 | SNcum-148 | CTACCTAATCGGATAAAAACTCAACCGCGCTCCATTTTGGAGCAAATAATCCTCTTTGTTTTCGTTTAGCTTACTTCTTGTTGAT GGGTTCCGTAGAATG[A/G]ATAAACTCCATATGCAGCGTACATGTTTGGGTTGTGAGGGGGTGGCATATGATATCCATAGCCA TCAAAGAAAGGCCCACCATAGTATGCTCCAGTCCAT |
| 149 | OS04990 | SNcum-149 | GTCCGTTCCTATTATGAGAGGGAAGTGATTTTTTTAGGGCCGTGAATCTGGCGAACAGATCGTCGTCCGCCGCCGCGCCGTGG ACTTCACTGGCGGAATT[G/A]GTATCCATTTTCTCAGGAACCTTTTTCAGAGGATCGTCGATTTGCGATGGCTTCTGGGGTCGTT TTAGGGCTTCGAAGCGGCGGTTGAGATCTGGATCGA |
| 150 | OS05343 | SNcum-150 | GTATTCAGTATTGTGTAAATGACATCGAAAAGGTAGCTACACTTGCAGAGGACGAGGACCCAAATTTTGGACCCACTGACGT AACAAAAAACTGCCACGA[C/A]GATTCGACCATTCCAAAAACGTGTATGACAATGTCTTTAAGCAGTGACGATAAAAAATTA CCGGATGAACTAAAATGTTATGCGGATAAAAATGGGCGAA |
| 151 | OS05001 | SNcum-151 | ATGATCAGGTTCAAATCGAGTTTGATGTGCCTCAGAATGACGGACTTTACAAATGTAAGTTGGAAACTCTTCACAGCCATCAC ACACGAAGCGTAAAAGA[T/C]GTAAATGAATTCTCCACTTTAGGGAACTCATTCACAATCAGAAAAGTGAAAAGAGGGTGTG ATAAAGGGAAGGGCATCCCACAGTCAATTCGAGCATTTT |
| 152 | OS05048 | SNcum-152 | GCCGGAGTCTCCCCCATTTGCAAGAAACTAGGTATCATTCTCCGTTCCTTAACCACAGGAAACTGTGGCAGCGATTGAGCAA ATGGTACCTCGTCAGCCG[T/C]CTCTACATAGTCTCTGATCTCTCTAATAGCCCCTGAACTCACTAAACAGTCAGCCAGAACCT CTGAAGCACCAGTAAACCCTTGCGCCATGCCCAAGTCT |
| 153 | OS05957 | SNcum-153 | TGATGATGGTCAATATGGTGTTAGAGCAATCTAKCTTTGTGAATAAGAATATCAATATTATTCCATTCTTGAGCCAAATCTTTC ATTGGGGTATAGAAAT[T/G]TGTTAAATTCCATTGGCAGGGGTATTAGTTGACGATGACATGCATTGTGAAAAGTCCTAAAAT TGGGACTCATGTGTTGTCTGT |

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| 154 | OS06082 | SNcum-154 | TACGCCTCTTCGTTGAAGGTATCTAACGACAACCCAATGATGGAGATAAGTTTCGTGAAGTGGGATCGGGCTCCCTTTTTCGT CAATCACTTCGGCGTCA[A/G]AGCTCTTTATAGCAATATGACCTTTTGGGAAGTCGATGTTGTAGTAGTATTTGTTGGAGACTG ATCCGGGTTCCAATTCGAATTTAGGTGAGAGGAAGAC |
| 155 | OS05968 | SNcum-155 | GCCAAGCTCGAAGAACCGAAACCGCTCTTTCGGGTAAACCTCTCTTAGGTCTCCACACATCATTATTTTCCATAATTCCGAGT TGCTGCTGCTGCTGTTG[T/C]TGCAATGCGCGTTGCTGTCGGAGATGATGATCGATGAATTTTAATCTCGAGAAKKCCATTTTCC CTCCAACTGCATTCTCTTCTTCTCCTAGGCTTTTGC |
| 156 | OS06085 | SNcum-156 | TGCACAATGGCTACTTAAAGATCCCTMCTTGAGTATCCTCACATCYGGACCTWATGGACTCATAKTTTCTTCATTTRTACCCTT TTATTTCGATATCCCC[A/G]TYTCAACGCRGTTTCGTGTGTTTAGCCTCAACTTCTCGGACAAGACTTTTATWTAYTTAGCTGGA GTTCAGCTGTTCTTGTCGTCCTGGAAAAGATCYAT |
| 157 | OS05697 | SNcum-157 | CATAGCGACCGGAACGGTCAAATATTGCACAATACACAGCATTCCTATGTCCTCTTAACTTCTTCACGTTTTGCATCTTCGGCA CCATAGCTGATGGTTT[G/A]GCAATTGCATAACATGCAACACGAATAGACGGAGCACGGTGGTGTTTTGCAAAGCCCCCTCCA ATTTCTCTCAAGCTAAGCCCTCGAACCTGATCAGCTT |
| 158 | OS05788 | SNcum-158 | TCACGCCAAGTGACTCCGCCACCCTGTCCAACCCACCATAAAGCCCCTGACAGAATTTCATCAAATGTTTTACATCATAAACC CGGTTTCCAAAAATGAC[C/T]TTCATCAAATTCAGAAACTCGGAAAGAGTAGATAACCAAAATCGTACCCACTGTGGAAGGT GATGTAAGTAATATTCTCGTTGCACAACAGGCCAGAAGA |
| 159 | OS05153 | SNcum-159 | AGAAATGGCAGCTCTATTGAAGTTGCTCCTCTGCATTGCCGGAATACTTTGCTRTACGAAAACCGCTCGCGCGAAWATCSMC GGCSTTTATACCGGCGGC[G/A]AMTGGSAARCCGCYCACGCCACCTTCTACGGCGGCRRYGAYGCCTCYGGMACCATGGGCGG TGCKTGTGGSTACGGAAAYCTGTACAGCCAGGGCTACGG |
| 160 | OS05151 | SNcum-160 | ACTCAAATCAGACCCAGGTTTATWCAACCCGTTTCAACCCGTACTCAGACCCATCACCGCTCCAGTTTTCGCCTGCCATCACC AATCAGTGGAGGACAAT[A/G]GCCGATGTTTTTTTGTTTCGCTGCAATCCGGCCGCCTRAATTCTRTGTATACTCRTGTGTGTGT TTATASGGGCGTTTTCTGTGTGATTTAGAGGGAGAG |
| 161 | OS05165 | SNcum-161 | TTATGTTGGCAAGCCGACATTAAAATTACACAATATTGTCTTGCAAATTAATAGATTGGCGGCCTTACTTGCACTTGGAAAAG AAAGACGCCTATGGTTG[A/G]CCCAAGTGGTCGAGCCTAAGTCATAATTTGTGGTGGTGGGGCCCACGTTCACACAGTGCCGA CTAGTCAATTAGTCGCTCAGTTAGACCTCTATCGACTC |
| 162 | OS05421 | SNcum-162 | AAAAAACAGCGTCAATTCAGTGATTTTCTTAAAGCTATTCCAAGGTTGAACAATGATGGATTATACTAMTGAAAAACTCTTA ACAGAAAAGTTCAMTGTG[G/A]GTATTCTACAAATAAAAAGAATGGGGCATAAATAGTATAAATTTGCAACARAACGGCAT ATTCCAAGATRTCCAAGATTAGRGGAAAAGAAGTAATACTC |

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| 163 | OS05681 | SNcum-163 | TATGAATTGTGGATACAGGATACGCAACACAAAAAATCCTTCAAACATTTTGAAAATGAGAAACATCTTTCCGACAAGAGG<br>AGCCGATAACAGTCAAAAC[A/G]ACACTGGCACATATAACATCAAAAGTTTAAAACGATAAACTCGATGGGCTTAAAAAAA<br>AATTCCTATCGTCCCATCAATAACACGAAAAAAAAAAAAGAA |
| 164 | OS05805 | SNcum-164 | ACGCAGCCATTAACACGTTAAAACTATAGATATTCGGCATACATCCAAGTCTCGCAAATTCTCCCCATAATTCCTCGACCTTA<br>TTTGTCAAACCTTCTCT[G/A]TAAAAACCGACCATAACCACATTCAATGTATTCGCATTCGGGAAAATTCCCTTACCGCATTTC<br>CCATTTCTAGCCACATTCTCGACATCACMACTAAAGA |
| 165 | OS05920 | SNcum-165 | TTGAGCACCAAAACTTGAACTCGGGAGGAGTTCAGCCTGTGGGAACGAACCATTCGACGGGACATCGAGCAGCAGATCCTG<br>TTCATGGCTTTCATCACCA[C/T]AATCTGAACAAAAGGGGCTTTCCAGACTGAAGAAAGACGAGCAACCCTTATCTATTATTA<br>CTTCATCCTCATTGTAGCTTGGCCACATCGTTATCCTTTT |
| 166 | OS05646 | SNcum-166 | CACATCGGCGGTACCACCACCAAAGCTAATTCTTATGACCTTATAGTCGTCGGTTTCGGGATCGTATCCGAGACCATTGTCGG<br>ATAAATAGGTATCAGGG[A/C]TGGTGGGCGGGGGAAGTAATTTAAGTTGTTTGGTTGTTAAGTTGACAATAACAGCCTTGACA<br>TCCTCAACATCAACATCACAATCAAAACTATTAAAGTA |
| 167 | OS05394 | SNcum-167 | CTAGATGGACCGGCTTCTACAAGATCAGATTTGAGGCCGTTTTCAGTGTGGGTATTTGTGGAGGTGGCTGAAGAACTCGTGCC<br>TGCGTAGGGATCGACAG[C/G]TGGCAGTCTTTCGTGGGAGGCGGATTGAAGGGAGGCGTTAATAGCCATGGCTAGCTGTTCGT<br>CTTCGGAAGATGGAGGTGCAGTTGCTGGGGCAGTAGAG |
| 168 | OS05416 | SNcum-168 | TTACTAATGTTGCTCGGTGCCGTTACCAGCGCCAACTCCCACCCTGAGGTGTTTCCGGTTCCGTCTAACGCATTATTAGTTGGT<br>AGTGGTGCATCATTGT[C/T]ACGTTGAACAATTGCAAKGGCCAATGCCTTGCTTTTCTCTAACTCTCCGGCTCTCTGGTTTACTT<br>CATTCAATCCGAGTAGATCAACGGGCTCCCGAGAG |
| 169 | OS05428 | SNcum-169 | CTCATTTGTTTCCTTTAATTTATCAATCGSGTCAAAGGACCAACGATACTCAAYYGGAATYCAYAACTCGATTATTGCTCCAAA<br>CACCAACTCCGRACTC[A/G]GAYTCATTCCGTGGGTCTAATTACACCAATCYRCTCGTAGACCAATTATCAAAACCTCCATTAA<br>GCCTAYATMTTCYCCRTTCGCAATTCC |
| 170 | OS05458 | SNcum-170 | CATCATCACGTAACCCCATCTCCATCTTTCACCAAATCAAAATTTAAAACCCCAACTAGTCYGAATCTGACCCAAATCGCACA<br>AACTGAGAACCCAAATT[C/T]CCGTTCAAACGAGACCCATCGCGGCTAACCTTGTACCCAACAGTATCGGACCTTACATATTT<br>CACTCCACCGGAGGGCGAGTAGCCAACCCKCCGCYGAG |
| 171 | OS05483 | SNcum-171 | ACTCCATCTTTTGAGAGGCTCTTGTACCGAGTTATTTCGTGTTATGTGGATAAAACTTCATATGAGCTCATCTTACCATTCAGA<br>AACGACCGGACATGGC[A/G]GTATTAAATAGAGGACTGGAGAAGTACTTGCGGCGCCTTCATGATTTAGCGGCCAAAACAGT<br>GGAGCCGATGGCTTCCATTTGAGGCGGTTTACGACAGA |

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| 172 | OS05524 | SNcum-172 | GCAGTGCGTTGTAAATCAGCCCTACTTCACTGGTGATTCCAGCAGGGTACAACAATAGAAACGTGCTGTACCTCAGCCAAAG AAGCCACGATGGCGCGAA[C/T]CCGAATGTCTCCTTGACGCCGAAGAACGAGTAGCGGATTATCTCGGTGACTGACCAGCTA ATGACGAGGGATACAACGAAGAAATGGTCCCGGATCTCGG |
| 173 | OS05525 | SNcum-173 | GTTCATTTCAATTAAACCCCTACTCAATACTCTAAGACAAGGAAAGCACGGMCGCCCCCTTGCTATTCGMGYTCCCGAGATT TATT[T/G]GGCATTTGAAGTAAAACCCATAACGAGCCGAGCRTACGCARATCAGAAGATGAYGAAGGCATCATCAGAACCTC CGATGAARTCCAACGATSGCGATAGA |
| 174 | OS05590 | SNcum-174 | AGAAAGTCCAAAGGTTTCGATTTGAAATATCCGATTGGGGGTTTGTTAACTGGAAGTTGAGCGCCGGAGGGAGATGAGGGA GTTTCAAAACCCTAGCCGA[A/G]AGGAGGGTAAGAACTGAAAAAGGGAAGAGTGAGCGCCAAAAGATGAAACTTCTATTYT TGTTTTGGCGGCGTCGAGGAAGT |
| 175 | OS05790 | SNcum-175 | GTGGRCAACGTGGGAATAACCTTCAAGAAAATCCTGCAGGGAAATGATTACTTGAGTGAGAGAGTCAGCCTGTTCTTGGTGG AGGGAAATAGGAGGGCGA[G/A]GATGATGCTGGAGTTGATCTATAATTTGCATTACGGAAACTAGAGATCCATAGGCAGCCA TTTCCGACTTTTTTTTCTGGGTTTTAGAACAGAGCACCAG |
| 176 | OS05626 | SNcum-176 | GAGGCCGKAGATGGTGGCGCAGACGGAGGAGGTRGTGGCGCRGACGCARACGGAGRAGRTGGTGGCGCRRACGCCGGRGR AGGTGGTGTCAGCCAGTTGT[C/G]ACCAATGACAAAGTGAACTACGATCCCTTCCTCTGCCTCCAAAGCTCGTCGTTTCTCTCC TTGTGTGTTAAACTCCACAACCATCAAGTATTTCCTCCTT |
| 177 | OS05748 | SNcum-177 | GGCGGCCGTCCCCGACATTTAGCTGCATCTCTTTGCAAGCAAACCACATGGCAACAAGTGTATGAGTCCATGCAATGCCGGC GACGGAGGCAACGACGCA[A/C]ATGCTATGGMGGTCAAATTTGCATCGGAAAAAAATTTGGATTATTCCGAAAATTGATGAA CCCAGTCCAGAAATTCCAGCAAGCAAAGCAAACAATCCTG |
| 178 | OS05712 | SNcum-178 | GGGAGTCYATGCATATGGMATGCGATTTGTGATTTATTGTTTTGATGTGTATTTTGATTTTTTTTATGTCTGGTTTWAAATAAGC GATGAGATATTTGTA[C/T]GAGCCCACGGAAGCCGCAAAAGATACTGTTGATATGTCTTTTGAAAGAGGACCAGCTTTAGATC GTGTATCACTTAAATTAGAAGACTAGAGAAACATCC |
| 179 | OS05807 | SNcum-179 | CTATAACCTAAACTACACTCTATCAGTAAAACTCAAAATAAGACAAAACTCGATTAGACGAGCACGTCAAAATCTCATCCTG RCCGTCAATACGGCTGGA[A/G]ATGAATATATTACAAGACAAAACTCGGTTAGACRAGCACGTCAAAATCATCAAAATCATC TGTTTCCTCACCTTGCGAAACGTAGTCACAAACTTCATCC |
| 180 | OS05854 | SNcum-180 | ACTTTTCTAAGCTGGTCGTGAGAGAGAGGATCAAAAACTACAATTTCATCCAGCCGGTTCAGCAACTCGGGCTTGAAATGCT TTCTTACCTCTTGCATCA[A/C]CATCTCACGGGCATTCTCCATCGTACACTTCCCCACCAGTCCCCTTAAGAGGTACTCGGCCC CGAGATTGGACGTCATGATAATAACAGTATTCGTGAAG |

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| 181 | OS05996 | SNcum-181 | CTTTTTGGTCACATAATTACTCTGGGTGATTTTTTCTTTTTTGCACTTTTGGTCCTCCACCGAGCTCAAYATAGTTG[G/A]GATTA AGGTGAAGTTGATGATGATGCACCTTTGGTTCCCGTGGAGTTTTTGTTTTGCTTTTAGCTAATTAAA |
| 182 | OS05916 | SNcum-182 | ATCCAATCCGGTTTTGTTCCTTTCTCTCTCATCTCGTTGAACAGATCATGTGGCATTTGCACAAACAGTTTCCTACCATCTTCCA AAACTCCACACTTGC[A/C]GTACATGCTAATCAATGAGGTGCCTACAGTCGGGTCGAAATATATAGGTAATTTGTACATAAGC TGATGAAATTGTTTCCCAAGTTTCAACATGGATAAA |
| 183 | OS05922 | SNcum-183 | ACGTCAAAGATTTCATGTAATTGATCCGGCAGGTGCAGGTGGCTCGCCCTTCTCAGGACCAGGATCTGCAGAACATTAGGAA GCACAACAAACATGTGAA[A/G]CAGAATTCAAGGTGAGCAGGTGAAAAGTATAATCGATTTGTCTAATAGCAGCTCCTGCTG TTAATATTTCTCAAAGAGATCAATTCATGATCGGGCTTGA |
| 184 | OS05924 | SNcum-184 | AAAAAGACAGGTGCCTCGGAGTTTAACTCTTCCACTCTCATCTTCCAAAACTATATAATCGTCCGAGTGAATAAAATTATGCG GTTTGACAAGTGGGCTT[C/G]CTGTCCTCTCCTTTGAGTACTCATCAAGTACTGAAGGTTTGAGCTTCATGTGCTTATAAAGGGT TCCAACAATGATGCATTCCTTTCCTTTTTCTAGTCC |
| 185 | OS05925 | SNcum-185 | CATCTGMATTTACACCTCCATCTATTCCTTCCTCCGACCCAATTCCTTCTCCGCCTCCCGACCACCACTGCCAGAGGCGCCAG GCTACTGCYGTCCATTC[A/G]CCGAGTTTCGTGACCAACTCCATTATGCTGATCAACGCTCCAGACACTGGCTCTCACAATTGA TCGACGAGATTTGATTGGAGAGCAAGTGAAT |
| 186 | OS05928 | SNcum-186 | GCCTGGATCGTCGTCAACCTCTCCGCCAAGGAAGGAATGCCAACAATGGATTTGTCTATTCTAGAAAGCAAGCGGTTATTTTC GAAAAGGTTCTCCTCTT[C/T]CGCCCGCGCCTCCTCGTAGGTCTCATCGCCGATGCGGTTTCTGACGCAGATGTAGCCGAGGCC GATGTTGACGTCGTCGGCGACGATCTTCTCGAGCAGT |
| 187 | OS05929 | SNcum-187 | TGGGTGTTGCATCAACCCGAAACATAAATTCTGTCATCACTTTATCTAATGACAGATTTCCAGACCTGGGAATCCTAGGGACC CCAAAGCGAGGCCATCT[G/A]GAAAAGGTTCGCAACTTATGAGGGGGGAGATAATGTAAATTCCAGAATTTGATTAACCATG CTAAATCGTGATGACCAAGACTCACAATTGTTGAATCAT |
| 188 | OS05933 | SNcum-188 | GGCCTGGATGTCCTGGATCTGGGTTGGAGACAACTGGTTGAGCCCAATAGGTGTGTGGTGCTGCTGACCCAGACCATATATAT AGTCATGAGCAGAGGCC[C/T]GATACATACTGGGTTGCTCAGATTGAGCGGATGAATAGTAGGAATTGTATGGAAAGGAACT GGAGGTGGAAGGAGAGAAGGTGGAATTAGAATAATATTG |
| 189 | OS05935 | SNcum-189 | AGCCATCATGTGCAAAACAGACTGTCCCTTCAAATAGTATTCATGGAGCCATTTCACTTAAAGTTTTTATTTCTGATTCCGCAG AACCCAAAGCAGTGTA[G/A]TGTAAGCCAGTTTATTCTAGGTAGGTAAATGAATTAATCCTAAGCMCCCTTCAGTTTTAGACC TGCAGACAGCATCCTAAGAGATAAGAGACTTCCTATA |

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| 190 | OS05937 | SNcum-190 | TCGGACTCRGGATCCCGACGTTGAAATCGAGATGGGATAGGGGCAATGTGGAAGATATCGAATTTAATCCTCCGATTAATCC<br>GTTTGGTCCGACTCCGAG[C/T]TCCAAGCTCGAGTTGGGCATCGGCGGGCAAAGGGACGAAATGGGCCGGCCCAAAAACTTG<br>CCGGCTAAAGAGCAAACCCGGTCGAGTTCGTCTTTTAGCC |
| 191 | OS05959 | SNcum-191 | CGTGAGTTGGACGGGAACAAAAGAAAAAGACGGATAAGTCCGATGTCACAAAAGTCTGAGCGGATATCCAAAACAAAACG<br>AAAAATAGAAAAAAATAAAA[C/A]GATAGAGGGTCTTACAAGGTCAAGAAAAGGTCTTACAAGGTCAAGAAAAGGTCTTAC<br>AAGATCAAGAAAACCGCAAAACAAAGAAGGTAACAAAAAAATA |
| 192 | OS06007 | SNcum-192 | TCCCTTTCTTCAGATGTTTCCTTTCACGCCCTCTCTTTCTCCTTTGGTGCGTAATCAACTGCAGAGGAGACCTAATGGCTCCACG<br>CAATTTCCACATAAG[A/C]AAGAGGTAGTGTATACATAATATGTACAAGGAGTAGGAGTCCCTAGCCGCCAATATGTATCATT<br>GGTCTRTTTGGWGTCTTWGCGATGTCRAACATTCCA |
| 193 | OS06008 | SNcum-193 | TTGTTCTGGCCTCGATACACAATAGCACCTTTAGAAGGACCCATGAGCCGATTACGGACAAACAGAAATCCAGCCCATTGCC<br>CGTTCTGAATGACTTGAG[G/C]GAAAGGGGATTGCCAGACACTGCCTTCCCAACTGTGATGCCGATCGAATTGAAGGTTGCCT<br>CCGGTGGCATTGAAAATCATGCAGAGGGTCGAGGTCCCA |
| 194 | OS01997 | | TGAGAAGATAASGAAAAAATTGCTTSGTTGGAACATAGGTMTAYTATYACAARGAGGAAGATGGATTATTATCCARAGTGTA<br>CTATCCTCAATGCCMGTT[C/T]ACCTAYTGCAAATATKCTCTCMYCATGCTTCTGTTAGRCTCTATCATYTCYAATGTTTTCYGG<br>GRCAYYRYTGATAATTCTARAAAGATACATTGGTCTW |
| 195 | OS02468 | | CCGGTCTCTCCACCAGGCGCTGCCCATTCCGCTACTGGATCCACCTGCACCGCATCCATTGCCTGGGTCTCACTGTACCTGTCC<br>ATATATCACAAACGCA[C/T]TCACTTTCACGCCCTCTCTCTCTCTCTCTCTCTCTACAGATACAAAAAGTATGAACAAATATGT<br>AAAGATATTTACAGAGGTATCTTATGGGTGTCTAGA |
| 196 | OS04605 | | CTCGGGCCGAATTCTCCGACGGCTGGCTTTGATCAGGTTCGGGTCCGGATCCGGATTCGATGTCAGSAAATGGAGCGTAAAG<br>GGTTTCGTCGAGGTTGAA[G/A]TCCAGGAATTCGGACCAGTCGAAGGAGGCGGCGTCGGCGGAGGCCATTTCGGGGTCTCTG<br>GGTAGGTGGGCTTGAGCTGGTATTGGAGATGGTGGGTTCT |
| 197 | OS01760 | | ACCTTTCAATTGGTGAACATCAGCATCAATCTTTTTAAAGTCCACATTCTGCTGATCCAAAGCTATAGTGAGCTCATTGAGAA<br>TTCTTTCAGAATCATCA[A/G]AGAAAATAGACACAACTTCAGACACAAAATCAGGATTGCTCTCATCTTGAAGCTGTTGCAAT<br>TGACCAAACTGCCCATCCAAGAATCCCTCACGAATTAA |
| 198 | OS06161 | | CCACAATCAAGAAACAGTTAAGAACAGAAAGACGTGTATACATTTCAACCGACCTCTCAACAAAGAACCGAAACAATTTTC<br>CCATACCGACTAATGAAGC[A/G]GCAAACAAGTTTGAAAACCCGAGTTTCTTCTGCACGAATTGACTGATACCTCGATATATA<br>CTGAAATATCAACTGCAAGTCCAACCTCTGATATATCAAC |

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| 199 | OS05871 | CCCGATATAGCGTGCACCGGCTGATCTTCGAACTCCGGGCGGCGACTTGCTGGAACTCAGTGCAGAAAATTAGCCACAAATTTTAGCTGTACTGTCCAGC[T/A]ATCGATTGCCCGCACATATTTTGCGCTAGCTTCGAAGTTCTTTTAACACAGTGGTTGCAGCCTCCGTTAATGGCAGACAGTGAGCCGTCTTCGGACTGTC |

**Table S2.** Map positions of 727 SNP and SSR marker loci and the pigmentation locus *Pg* in the *O. cumana* genetic linkage map. Prefix Ocum is for SSR marker loci and prefix OS for SNP marker loci. Marker loci labelled as *, **, or *** showed distorted segregation at $P < 0.05$, $P < 0.01$, or $P < 0.001$, respectively. Loci whose position were ambiguous (i.e., those placed automatically at a less-strict LOD of 2.0) were labelled in red. Markers highlighted in bold and underlined are SSR or those SNP detailed in Supplementary Table S1.

| LG | order | Marker | cM (intervals) | cM (absolute) | Distorted |
|---|---|---|---|---|---|
| 1 | 1 | OS05877 | 0.6 | 0 | *** |
| 1 | 2 | OS05952 | 14.5 | 0.6 | *** |
| 1 | 3 | OS06116 | 0.6 | 15.1 | *** |
| 1 | 4 | OS06036 | 0 | 15.7 | *** |
| 1 | 5 | OS06151 | 0.6 | 15.7 | *** |
| 1 | 6 | OS03345 | 1.6 | 16.3 | *** |
| 1 | 7 | OS04947 | 0 | 17.9 | *** |
| 1 | 8 | OS03629 | 1.7 | 17.9 | *** |
| 1 | 9 | OS01659 | 6.2 | 19.6 | *** |
| 1 | 10 | OS06173 | 8.9 | 25.8 | *** |
| 1 | 11 | OS02974 | 8.9 | 34.7 | *** |
| 1 | 12 | OS02263 | 1.4 | 43.6 | *** |
| 1 | 13 | **OS05257** | 3.2 | 45 | *** |
| 1 | 14 | OS04638 | 1.6 | 48.2 | *** |
| 1 | 15 | **OS05929** | 0 | 49.8 | *** |
| 1 | 16 | OS05633 | 1.7 | 49.8 | *** |
| 1 | 17 | OS05872 | 1.5 | 51.5 | *** |
| 1 | 18 | OS03523 | 0.7 | 53 | *** |
| 1 | 19 | OS04939 | 0.7 | 53.7 | *** |
| 1 | 20 | OS05357 | 1.5 | 54.4 | *** |
| 1 | 21 | OS05562 | 0.4 | 55.9 | *** |
| 1 | 22 | OS06196 | 0.4 | 56.3 | *** |
| 1 | 23 | **OS03166** | 1.5 | 56.7 | ** |
| 1 | 24 | OS05267 | 0 | 58.2 | ** |
| 1 | 25 | OS06120 | 0 | 58.2 | ** |
| 1 | 26 | **OS05933** | 0 | 58.2 | ** |
| 1 | 27 | OS00107 | 0.6 | 58.2 | ** |
| 1 | 28 | **OS01411** | 4.4 | 58.8 | *** |
| 1 | 29 | OS04670 | 0.6 | 63.2 | ** |
| 1 | 30 | OS05581 | 0 | 63.8 | ** |
| 1 | 31 | OS06112 | 0.6 | 63.8 | * |
| 1 | 32 | **OS05048** | 0 | 64.4 | ** |
| 1 | 33 | **OS05646** | 0 | 64.4 | ** |
| 1 | 34 | OS05321 | 1.6 | 64.4 | ** |
| 1 | 35 | OS05677 | 0 | 66 | ** |
| 1 | 36 | OS02806 | 0.7 | 66 | * |
| 1 | 37 | OS05414 | 1.6 | 66.7 | * |

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|---|---|---|---|---|---|
| 1 | 38 | OS01455 | 5.7 | 68.3 | * |
| 1 | 39 | OS05355 | 0 | 74 | |
| 1 | 40 | OS04852 | 21.9 | 74 | * |
| 1 | 41 | OS01878 | 0.6 | 95.9 | * |
| 1 | 42 | OS01926 | 2.3 | 96.5 | |
| 1 | 43 | **OS03123** | 0.6 | 98.8 | ** |
| 1 | 44 | OS06204 | 5.9 | 99.4 | *** |
| 1 | 45 | OS06030 ------ | | 105.3 | ** |
| 2 | 1 | OS05486 | 7.3 | 0 | |
| 2 | 2 | **OS00106** | 5.9 | 7.3 | |
| 2 | 3 | **OS02557** | 0.6 | 13.2 | |
| 2 | 4 | **Ocum141** | 0 | 13.8 | |
| 2 | 5 | OS06186 | 4.5 | 13.8 | |
| 2 | 6 | OS04791 | 0 | 18.3 | |
| 2 | 7 | OS02805 | 0 | 18.3 | |
| 2 | 8 | OS04824 | 0.6 | 18.3 | |
| 2 | 9 | OS05950 | 1.1 | 18.9 | |
| 2 | 10 | OS04839 | 0 | 20 | |
| 2 | 11 | OS05596 | 0 | 20 | |
| 2 | 12 | **OS04641** | 0 | 20 | |
| 2 | 13 | OS01416 | 0 | 20 | |
| 2 | 14 | OS05304 | 0 | 20 | |
| 2 | 15 | **OS05399** | 0 | 20 | |
| 2 | 16 | OS04522 | 0 | 20 | |
| 2 | 17 | **OS04537** | 0 | 20 | |
| 2 | 18 | OS01872 | 0 | 20 | |
| 2 | 19 | OS06086 | 0 | 20 | |
| 2 | 20 | OS04982 | 0 | 20 | |
| 2 | 21 | OS05474 | 0.6 | 20 | |
| 2 | 22 | OS05208 | 0 | 20.6 | |
| 2 | 23 | OS04502 | 1.8 | 20.6 | |
| 2 | 24 | OS02582 | 0.9 | 22.4 | * |
| 2 | 25 | OS04929 | 0 | 23.3 | |
| 2 | 26 | OS01890 | 0 | 23.3 | |
| 2 | 27 | OS00065 | 0 | 23.3 | |
| 2 | 28 | OS01354 | 0 | 23.3 | |
| 2 | 29 | OS06144 | 0 | 23.3 | |
| 2 | 30 | OS02469 | 0 | 23.3 | |
| 2 | 31 | OS06183 | 0 | 23.3 | |
| 2 | 32 | OS05733 | 0 | 23.3 | |
| 2 | 33 | OS05178 | 0 | 23.3 | |
| 2 | 34 | **OS001883** | 0 | 23.3 | |
| 2 | 35 | OS06185 | 0 | 23.3 | |
| 2 | 36 | **OS04862** | 0 | 23.3 | |
| 2 | 37 | **Ocum41** | 0 | 23.3 | |
| 2 | 38 | OS02110 | 0 | 23.3 | |
| 2 | 39 | OS01790 | 0 | 23.3 | |

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|---|---|---|---|---|---|
| 2 | 40 | OS02359 | | 1.7 | 23.3 | |
| 2 | 41 | OS05299 | | 0 | 25 | |
| 2 | 42 | OS05895 | | 0 | 25 | |
| 2 | 43 | OS05232 | | 6.9 | 25 | |
| 2 | 44 | OS04841 | | 1.1 | 31.9 | |
| 2 | 45 | OS06202 | | 0.6 | 33 | |
| 2 | 46 | **OS05125** | | 13.7 | 33.6 | |
| 2 | 47 | OS06042 | | 7.1 | 47.3 | |
| 2 | 48 | OS05116 | | 7.7 | 54.4 | |
| 2 | 49 | OS01695 | ---------- | | 62.1 | |
| 3 | 1 | OS01665 | | 1.7 | 0 | |
| 3 | 2 | OS01522 | | 0 | 1.7 | |
| 3 | 3 | OS01447 | | 0.6 | 1.7 | |
| 3 | 4 | OS02382 | | 0.8 | 2.3 | |
| 3 | 5 | OS05054 | | 0.4 | 3.1 | |
| 3 | 6 | OS04519 | | 0.4 | 3.5 | |
| 3 | 7 | OS04610 | | 0 | 3.9 | |
| 3 | 8 | OS06134 | | 0 | 3.9 | |
| 3 | 9 | OS00163 | | 0 | 3.9 | |
| 3 | 10 | OS06201 | | 1.7 | 3.9 | |
| 3 | 11 | OS02452 | | 0 | 5.6 | |
| 3 | 12 | OS05260 | | 10.5 | 5.6 | |
| 3 | 13 | OS04889 | | 0 | 16.1 | |
| 3 | 14 | OS02944 | | 5.7 | 16.1 | |
| 3 | 15 | OS04944 | | 2.2 | 21.8 | |
| 3 | 16 | OS01614 | | 2.2 | 24 | |
| 3 | 17 | OS06099 | | 3.4 | 26.2 | |
| 3 | 18 | **Ocum122** | | 3.7 | 29.6 | |
| 3 | 19 | OS01994 | | 2.5 | 33.3 | |
| 3 | 20 | OS02818 | | 3.9 | 35.8 | |
| 3 | 21 | OS05848 | | 0 | 39.7 | |
| 3 | 22 | OS02962 | | 0 | 39.7 | |
| 3 | 23 | OS05878 | | 0.6 | 39.7 | |
| 3 | 24 | OS02755 | | 8.1 | 40.3 | |
| 3 | 25 | OS05780 | | 2.8 | 48.4 | |
| 3 | 26 | OS03125 | | 0 | 51.2 | |
| 3 | 27 | OS01701 | | 0 | 51.2 | |
| 3 | 28 | OS05535 | | 3.9 | 51.2 | |
| 3 | 29 | OS03121 | | 6.7 | 55.1 | |
| 3 | 30 | OS03083 | | 26.7 | 61.8 | ** |
| 3 | 31 | **OS00159** | ---------- | | 88.5 | |
| 4 | 1 | OS00104 | | 1.1 | 0 | |
| 4 | 2 | **Ocum174** | | 5.2 | 1.1 | |
| 4 | 3 | OS04600 | | 26.6 | 6.3 | |
| 4 | 4 | OS05729 | | 1.7 | 32.9 | |
| 4 | 5 | OS06040 | | 0.6 | 34.6 | |
| 4 | 6 | OS05291 | | 2.2 | 35.2 | |

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| 4 | 7 | **OS05631** | 1.7 | 37.4 | |
| 4 | 8 | OS02305 | 0 | 39.1 | * |
| 4 | 9 | OS05440 | 0 | 39.1 | |
| 4 | 10 | OS05348 | 0 | 39.1 | |
| 4 | 11 | OS05855 | 0.2 | 39.1 | |
| 4 | 12 | OS05001 | 0.2 | 39.3 | |
| 4 | 13 | OS04623 | 0.2 | 39.5 | ** |
| 4 | 14 | OS05775 | 0 | 39.7 | ** |
| 4 | 15 | OS02932 | 1.6 | 39.7 | * |
| 4 | 16 | OS04629 | 0.7 | 41.3 | |
| 4 | 17 | OS06199 | 2.1 | 42 | |
| 4 | 18 | OS04954 | 0 | 44.1 | * |
| 4 | 19 | OS04781 | 0 | 44.1 | ** |
| 4 | 20 | OS05967 | 10.5 | 44.1 | * |
| 4 | 21 | OS05030 | 0.6 | 54.6 | * |
| 4 | 22 | OS06071 | 0 | 55.2 | * |
| 4 | 23 | **OS05590** | 2.2 | 55.2 | * |
| 4 | 24 | OS05285 | 4.5 | 57.4 | *** |
| 4 | 25 | OS06171 | 0 | 61.9 | ** |
| 4 | 26 | OS04719 | 6.9 | 61.9 | * |
| 4 | 27 | **OS05836** | 0 | 68.8 | *** |
| 4 | 28 | OS05388 | 0 | 68.8 | *** |
| 4 | 29 | OS05655 | 0 | 68.8 | *** |
| 4 | 30 | OS04493 | 1.9 | 68.8 | *** |
| 4 | 31 | OS01317 | 0 | 70.7 | ** |
| 4 | 32 | OS01316 | 2.5 | 70.7 | ** |
| 4 | 33 | OS01791 | 2.8 | 73.2 | * |
| 4 | 34 | OS05490 | 2.4 | 76 | * |
| 4 | 35 | OS06198 | 4.9 | 78.4 | |
| 4 | 36 | OS05891 | 0.6 | 83.3 | * |
| 4 | 37 | OS05509 | 1.1 | 83.9 | ** |
| 4 | 38 | OS01643 | 0 | 85 | ** |
| 4 | 39 | OS03895 | 0 | 85 | ** |
| 4 | 40 | OS05991 | 0 | 85 | ** |
| 4 | 41 | OS05224 | 1.7 | 85 | ** |
| 4 | 42 | **OS05343** | 3.4 | 86.7 | ** |
| 4 | 43 | OS06175 | 1.7 | 90.1 | |
| 4 | 44 | OS02793 | 0 | 91.8 | |
| 4 | 45 | OS05942 | 6.8 | 91.8 | |
| 4 | 46 | OS01458 | 3.2 | 98.6 | |
| 4 | 47 | OS02627 | 3.2 | 101.8 | |
| 4 | 48 | **Ocum43** | 3.6 | 105 | |
| 4 | 49 | OS05745 | 0.6 | 108.6 | |
| 4 | 50 | **Ocum3** | 1.2 | 109.2 | |
| 4 | 51 | OS02830 | 4.9 | 110.4 | |
| 4 | 52 | OS02371 | 0 | 115.3 | |
| 4 | 53 | OS01361 | 0 | 115.3 | |

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| 4 | 54 | OS05253 | 0 | 115.3 | |
| 5 | 1 | OS06041 | 1.1 | 0 | |
| 5 | 2 | **OS05647** | 10 | 1.1 | |
| 5 | 3 | OS04480 | 6.3 | 11.1 | |
| 5 | 4 | **OS03180** | 25.4 | 17.4 | |
| 5 | 5 | **Ocum32** | 5.8 | 42.8 | |
| 5 | 6 | OS05439 | 14.4 | 48.6 | |
| 5 | 7 | OS05172 | 0 | 63 | |
| 5 | 8 | OS05298 | 0 | 63 | |
| 5 | 9 | OS05276 | 2.1 | 63 | |
| 5 | 10 | OS05452 | 0 | 65.1 | |
| 5 | 11 | OS02176 | 0 | 65.1 | |
| 5 | 12 | OS06053 | 0 | 65.1 | |
| 5 | 13 | OS06092 | 0 | 65.1 | |
| 5 | 14 | OS02170 | 0 | 65.1 | |
| 5 | 15 | OS00091 | 0 | 65.1 | |
| 5 | 16 | OS01339 | 0 | 65.1 | |
| 5 | 17 | OS02109 | 0 | 65.1 | |
| 5 | 18 | OS00087 | 0 | 65.1 | |
| 5 | 19 | OS05865 | 0 | 65.1 | |
| 5 | 20 | OS06165 | 0 | 65.1 | |
| 5 | 21 | OS03710 | 0 | 65.1 | |
| 5 | 22 | OS04680 | 0 | 65.1 | |
| 5 | 23 | **OS04988** | 1.8 | 65.1 | |
| 5 | 24 | OS00064 | 4.7 | 66.9 | |
| 5 | 25 | OS03826 | 4.7 | 71.6 | * |
| 5 | 26 | OS05860 | 0.6 | 76.3 | |
| 5 | 27 | **OS06018** | 0 | 76.9 | ** |
| 5 | 28 | OS04809 | 0.6 | 76.9 | * |
| 5 | 29 | OS04479 | 3.9 | 77.5 | |
| 5 | 30 | OS05650 | 3.9 | 81.4 | |
| 5 | 31 | OS05563 | 0 | 85.3 | |
| 5 | 32 | OS05705 | 0.6 | 85.3 | |
| 5 | 33 | OS06002 | 10.6 | 85.9 | |
| 5 | 34 | OS05472 | 0.6 | 96.5 | |
| 5 | 35 | **OS03749** | 1.1 | 97.1 | |
| 5 | 36 | OS04566 | 0 | 98.2 | |
| 5 | 37 | OS05200 | 0.6 | 98.2 | |
| 5 | 38 | OS05839 | 0 | 98.8 | |
| 5 | 39 | OS05882 | 1.7 | 98.8 | |
| 5 | 40 | OS05969 | 0 | 100.5 | |
| 5 | 41 | OS04646 | ---------- | 100.5 | |
| 6 | 1 | OS03113 | 2.2 | 0 | |
| 6 | 2 | OS05350 | 14 | 2.2 | |
| 6 | 3 | **Ocum9** | 7.9 | 16.2 | |
| 6 | 4 | OS03122 | 4.1 | 24.1 | |
| 6 | 5 | OS02501 | 11.8 | 28.2 | |

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| 6 | 6 | OS02764 | 0 | 40 | |
| 6 | 7 | OS01632 | 0 | 40 | |
| 6 | 8 | **OS03124** | 8.5 | 40 | |
| 6 | 9 | OS05966 | 2.9 | 48.5 | |
| 6 | 10 | OS04588 | 7.4 | 51.4 | |
| 6 | 11 | OS04727 | 3.9 | 58.8 | |
| 6 | 12 | OS05522 | 0 | 62.7 | |
| 6 | 13 | OS05747 | 2.2 | 62.7 | |
| 6 | 14 | OS01609 | 0.6 | 64.9 | |
| 6 | 15 | OS05796 | 0 | 65.5 | |
| 6 | 16 | **OS03371** | 2.8 | 65.5 | |
| 6 | 17 | OS03266 | 3.9 | 68.3 | |
| 6 | 18 | **OS02559** | 1.1 | 72.2 | |
| 6 | 19 | OS05445 | 0.6 | 73.3 | |
| 6 | 20 | OS05928 | 0 | 73.9 | |
| 6 | 21 | OS03228 | 0 | 73.9 | |
| 6 | 22 | OS04516 | 0 | 73.9 | |
| 6 | 23 | OS05041 | 0 | 73.9 | |
| 6 | 24 | OS03458 | 0 | 73.9 | |
| 6 | 25 | **OS04724** | 0 | 73.9 | |
| 6 | 26 | OS04544 | 0 | 73.9 | |
| 6 | 27 | OS03700 | 0 | 73.9 | |
| 6 | 28 | **OS04912** | 0 | 73.9 | |
| 6 | 29 | OS05338 | 0.6 | 73.9 | |
| 6 | 30 | **OS05922** | 8.7 | 74.5 | |
| 6 | 31 | OS04490 | 6.1 | 83.2 | |
| 6 | 32 | OS03477 | 0 | 89.3 | |
| 6 | 33 | OS02139 | 1.8 | 89.3 | |
| 6 | 34 | OS01508 | 1.7 | 91.1 | |
| 6 | 35 | **Ocum74** | 6.1 | 92.8 | |
| 6 | 36 | **Ocum217** | 8.3 | 98.9 | |
| 6 | 37 | OS05211 | 5.7 | 107.2 | |
| 6 | 38 | OS04481 | 0.7 | 112.9 | |
| 6 | 39 | OS05685 | 2.7 | 113.6 | |
| 6 | 40 | **OS05681** | 2.2 | 116.3 | |
| 6 | 41 | OS06162 | 0.6 | 118.5 | ** |
| 6 | 42 | **OS04687** | 11.7 | 119.1 | |
| 6 | 43 | OS01885 | 1.7 | 130.8 | |
| 6 | 44 | OS06008 | 4.5 | 132.5 | |
| 6 | 45 | OS05080 | 0.6 | 137 | |
| 6 | 46 | **OS04831** | 0 | 137.6 | |
| 6 | 47 | OS02537 | ---------- | 137.6 | |
| 7 | 1 | OS04997 | 5.9 | 0 | |
| 7 | 2 | OS02631 | 1.7 | 5.9 | |
| 7 | 3 | OS06157 | 0 | 7.6 | |
| 7 | 4 | OS05139 | 1.7 | 7.6 | |
| 7 | 5 | **OS02200** | 1.7 | 9.3 | |

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| 7 | 6 | OS03163 | 1.1 | 11 | |
| 7 | 7 | OS02379 | 1.1 | 12.1 | |
| 7 | 8 | OS04996 | 1.1 | 13.2 | |
| 7 | 9 | OS05412 | 0 | 14.3 | |
| 7 | 10 | **OS05524** | 0.6 | 14.3 | |
| 7 | 11 | **OS03929** | 2.9 | 14.9 | |
| 7 | 12 | OS05541 | 14.2 | 17.8 | |
| 7 | 13 | OS02628 | 4.6 | 32 | |
| 7 | 14 | OS04842 | 3 | 36.6 | |
| 7 | 15 | OS05236 | 0.6 | 39.6 | |
| 7 | 16 | OS04847 | 5.5 | 40.2 | |
| 7 | 17 | OS05692 | 1.2 | 45.7 | |
| 7 | 18 | OS03179 | 0.8 | 46.9 | |
| 7 | 19 | **Ocum79** | 9.8 | 47.7 | * |
| 7 | 20 | OS05592 | 0 | 57.5 | |
| 7 | 21 | **Ocum108c** | 2.2 | 57.5 | |
| 7 | 22 | OS05945 | 5.7 | 59.7 | |
| 7 | 23 | OS02541 | 0.6 | 65.4 | |
| 7 | 24 | OS02344 | 2.2 | 66 | |
| 7 | 25 | OS05436 | 1.7 | 68.2 | |
| 7 | 26 | OS01412 | 5.1 | 69.9 | |
| 7 | 27 | OS03947 | 0.8 | 75 | |
| 7 | 28 | OS03712 | 3.3 | 75.8 | |
| 7 | 29 | OS05339 | 5.3 | 79.1 | |
| 7 | 30 | OS02076 | 11.7 | 84.4 | |
| 7 | 31 | OS04626 | 0.6 | 96.1 | |
| 7 | 32 | OS06033 | 5.7 | 96.7 | |
| 7 | 33 | OS05241 | 2.8 | 102.4 | |
| 7 | 34 | OS05621 | 2.8 | 105.2 | |
| 7 | 35 | OS02360 | 1 | 108 | |
| 7 | 36 | OS01469 | 0 | 109 | |
| 7 | 37 | OS01338 | 0 | 109 | |
| 7 | 38 | OS02792 | 0 | 109 | |
| 7 | 39 | **OS03072** | 0 | 109 | |
| 7 | 40 | OS02338 | 0 | 109 | |
| 7 | 41 | OS01792 | 1.1 | 109 | |
| 7 | 42 | OS02763 | 0.3 | 110.1 | |
| 7 | 43 | OS02804 | 0.3 | 110.4 | |
| 7 | 44 | **OS05761** | 0.6 | 110.7 | |
| 7 | 45 | **Ocum206** | 1.7 | 111.3 | |
| 7 | 46 | OS04875 | 8.3 | 113 | |
| 7 | 47 | OS02039 | ---------- | 121.3 | |
| 8 | 1 | OS05109 | 0 | 0 | |
| 8 | 2 | **OS03788** | 2.8 | 0 | |
| 8 | 3 | OS05632 | 3.6 | 2.8 | |
| 8 | 4 | OS05390 | 0.6 | 6.4 | |
| 8 | 5 | OS05984 | 4.8 | 7 | |

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| 8 | 6 | OS05398 | 1.2 | 11.8 | |
| 8 | 7 | OS05533 | 3.6 | 13 | |
| 8 | 8 | **OS04526** | 1.2 | 16.6 | |
| 8 | 9 | OS04651 | 0.6 | 17.8 | |
| 8 | 10 | OS05819 | 1.1 | 18.4 | |
| 8 | 11 | OS05078 | 0 | 19.5 | |
| 8 | 12 | OS05239 | 0.6 | 19.5 | |
| 8 | 13 | OS05426 | 3 | 20.1 | |
| 8 | 14 | OS02179 | 4.9 | 23.1 | |
| 8 | 15 | OS05333 | 18 | 28 | |
| 8 | 16 | **Ocum115** | 3.4 | 46 | |
| 8 | 17 | OS05538 | 5.1 | 49.4 | |
| 8 | 18 | OS05067 | 0.6 | 54.5 | |
| 8 | 19 | **OS05957** | 2.8 | 55.1 | |
| 8 | 20 | **OS06055** | 0 | 57.9 | |
| 8 | 21 | OS04618 | 1.7 | 57.9 | |
| 8 | 22 | OS05194 | 4.8 | 59.6 | |
| 8 | 23 | OS01696 | 4.2 | 64.4 | |
| 8 | 24 | OS01789 | 1.8 | 68.6 | |
| 8 | 25 | **OS04498** | 0.7 | 70.4 | |
| 8 | 26 | OS05050 | 0 | 71.1 | |
| 8 | 27 | OS05852 | 0.7 | 71.1 | |
| 8 | 28 | OS04579 | 1.3 | 71.8 | |
| 8 | 29 | **OS05049** | 0 | 73.1 | |
| 8 | 30 | OS04932 | 0 | 73.1 | |
| 8 | 31 | OS05066 | 0 | 73.1 | |
| 8 | 32 | OS05888 | 1.1 | 73.1 | |
| 8 | 33 | OS05320 | 11.4 | 74.2 | |
| 8 | 34 | OS01315 | 7.1 | 85.6 | ** |
| 8 | 35 | OS05199 | 4.8 | 92.7 | |
| 8 | 36 | OS06146 | 6 | 97.5 | |
| 8 | 37 | **Ocum040** | ---------- | 103.5 | |
| 9 | 1 | OS02943 | 0 | 0 | |
| 9 | 2 | OS00109 | 0 | 0 | |
| 9 | 3 | OS06117 | 0 | 0 | |
| 9 | 4 | OS05670 | 0 | 0 | |
| 9 | 5 | OS05784 | 3.9 | 0 | |
| 9 | 6 | OS01925 | 2.8 | 3.9 | |
| 9 | 7 | OS00095 | 0 | 6.7 | |
| 9 | 8 | **OS02153** | 0 | 6.7 | |
| 9 | 9 | OS05418 | 13.2 | 6.7 | |
| 9 | 10 | OS05113 | 0 | 19.9 | |
| 9 | 11 | OS05704 | 0 | 19.9 | |
| 9 | 12 | **OS04603** | 0 | 19.9 | |
| 9 | 13 | OS04910 | 2.2 | 19.9 | |
| 9 | 14 | OS05294 | 0.6 | 22.1 | |
| 9 | 15 | OS04900 | 0 | 22.7 | |

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| 9 | 16 | OS05981 | 0.6 | 22.7 | |
| 9 | 17 | OS04594 | 2.2 | 23.3 | |
| 9 | 18 | OS05767 | 0 | 25.5 | |
| 9 | 19 | **OS05532** | 2.2 | 25.5 | |
| 9 | 20 | OS05526 | 3.4 | 27.7 | |
| 9 | 21 | OS05009 | 0 | 31.1 | |
| 9 | 22 | OS05407 | 0 | 31.1 | |
| 9 | 23 | OS05964 | 7.5 | 31.1 | |
| 9 | 24 | OS04931 | 0 | 38.6 | |
| 9 | 25 | OS05064 | 7.4 | 38.6 | |
| 9 | 26 | OS04983 | 0.6 | 46 | |
| 9 | 27 | OS03048 | 0 | 46.6 | |
| 9 | 28 | **OS04505** | 0 | 46.6 | |
| 9 | 29 | OS01503 | 0.6 | 46.6 | |
| 9 | 30 | OS05542 | 3 | 47.2 | |
| 9 | 31 | OS05881 | 4.3 | 50.2 | |
| 9 | 32 | OS01977 | 0 | 54.5 | |
| 9 | 33 | OS01764 | 12 | 54.5 | |
| 9 | 34 | OS05081 | 1.1 | 66.5 | |
| 9 | 35 | OS04941 | 4.4 | 67.6 | |
| 9 | 36 | **OS02202** | 13.3 | 72 | |
| 9 | 37 | OS05953 | 0.6 | 85.3 | *** |
| 9 | 38 | **Ocum59** | 0 | 85.9 | *** |
| 9 | 39 | OS02258 | 0 | 85.9 | |
| 9 | 40 | **OS06130** | 0 | 85.9 | ** |
| 9 | 41 | OS02742 | 0 | 85.9 | ** |
| 9 | 42 | OS04524 | 1.1 | 85.9 | ** |
| 9 | 43 | OS01597 | 1.3 | 87 | * |
| 9 | 44 | OS05518 | 0.6 | 88.3 | *** |
| 9 | 45 | OS05273 | 0 | 88.9 | *** |
| 9 | 46 | OS04812 | 1.9 | 88.9 | *** |
| 9 | 47 | **OS05458** | ---------- | 90.8 | *** |
| 10 | 1 | **OS06124** | 21.5 | 0 | |
| 10 | 2 | OS02888 | 3.2 | 21.5 | |
| 10 | 3 | OS02754 | 13.9 | 24.7 | |
| 10 | 4 | **OS04773** | 16.7 | 38.6 | ** |
| 10 | 5 | OS05297 | 7.1 | 55.3 | ** |
| 10 | 6 | OS06038 | 1.1 | 62.4 | * |
| 10 | 7 | OS05359 | 3.4 | 63.5 | |
| 10 | 8 | OS05072 | 0 | 66.9 | |
| 10 | 9 | **OS06060** | 1.7 | 66.9 | |
| 10 | 10 | OS05948 | 0 | 68.6 | |
| 10 | 11 | OS05849 | 0 | 68.6 | |
| 10 | 12 | OS05161 | 0 | 68.6 | |
| 10 | 13 | OS04879 | 0 | 68.6 | |
| 10 | 14 | OS05823 | 0 | 68.6 | |
| 10 | 15 | OS06078 | 0 | 68.6 | |

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| 10 | 16 | **OS05734** | | 0 | 68.6 | |
| 10 | 17 | OS05249 | | 1 | 68.6 | |
| 10 | 18 | OS05540 | | 16.8 | 69.6 | * |
| 10 | 19 | OS05935 | | 4.1 | 86.4 | |
| 10 | 20 | OS05317 | | 7.5 | 90.5 | |
| 10 | 21 | OS04848 | | 0 | 98 | |
| 10 | 22 | OS01410 | | 0.6 | 98 | |
| 10 | 23 | OS04733 | | 1.1 | 98.6 | |
| 10 | 24 | OS05840 | | 2.3 | 99.7 | * |
| 10 | 25 | OS04561 | ---------- | | 102 | ** |
| 11 | 1 | OS05961 | | 7.9 | 0 | |
| 11 | 2 | **OS02963** | | 9.1 | 7.9 | |
| 11 | 3 | OS01978 | | 0 | 17 | |
| 11 | 4 | OS02339 | | 0 | 17 | |
| 11 | 5 | **Ocum70** | | 0.6 | 17 | |
| 11 | 6 | OS01871 | | 6.3 | 17.6 | |
| 11 | 7 | OS02925 | | 5.1 | 23.9 | |
| 11 | 8 | OS03117 | | 6.8 | 29 | |
| 11 | 9 | OS02866 | | 1.7 | 35.8 | |
| 11 | 10 | OS00060 | | 1.7 | 37.5 | |
| 11 | 11 | OS03073 | | 2.2 | 39.2 | |
| 11 | 12 | OS01448 | | 14.3 | 41.4 | |
| 11 | 13 | OS06027 | ---------- | | 55.7 | * |
| 12 | 1 | OS04998 | | 9.6 | 0 | |
| 12 | 2 | OS06114 | | 0 | 9.6 | |
| 12 | 3 | OS05288 | | 0 | 9.6 | |
| 12 | 4 | OS05223 | | 0 | 9.6 | |
| 12 | 5 | OS01657 | | 4.3 | 9.6 | |
| 12 | 6 | OS05183 | | 3 | 13.9 | |
| 12 | 7 | OS01806 | | 0 | 16.9 | |
| 12 | 8 | OS02385 | | 5.3 | 16.9 | |
| 12 | 9 | **Ocum145** | | 37.7 | 22.2 | |
| 12 | 10 | OS02206 | | 5.2 | 59.9 | |
| 12 | 11 | OS06172 | | 0 | 65.1 | |
| 12 | 12 | OS05209 | | 0.6 | 65.1 | |
| 12 | 13 | OS02265 | | 2.2 | 65.7 | |
| 12 | 14 | OS04914 | | 4.5 | 67.9 | |
| 12 | 15 | OS06001 | | 2.2 | 72.4 | |
| 12 | 16 | OS05136 | | 0 | 74.6 | |
| 12 | 17 | OS02609 | | 19.7 | 74.6 | |
| 12 | 18 | OS01998 | | 0.6 | 94.3 | |
| 12 | 19 | OS05473 | | 0 | 94.9 | |
| 12 | 20 | OS02828 | | 0 | 94.9 | |
| 12 | 21 | OS05293 | | 0.6 | 94.9 | |
| 12 | 22 | OS05145 | | 2.8 | 95.5 | |
| 12 | 23 | OS02540 | | 0.6 | 98.3 | * |
| 12 | 24 | OS02775 | ------ | | 98.9 | ** |

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| 13 | 1 | OS02052 | 6.2 | 0 | |
| 13 | 2 | **Ocum197** | 8.7 | 6.2 | |
| 13 | 3 | OS02931 | 0 | 14.9 | |
| 13 | 4 | OS03144 | 0.2 | 14.9 | * |
| 13 | 5 | OS04644 | 0.3 | 15.1 | |
| 13 | 6 | OS02410 | 7.9 | 15.4 | *** |
| 13 | 7 | **Ocum231** | 41.9 | 23.3 | * |
| 13 | 8 | OS02378 | 1.4 | 65.2 | |
| 13 | 9 | OS00158 | 1.4 | 66.6 | |
| 13 | 10 | **OS01799** | 5.1 | 68 | |
| 13 | 11 | OS05771 | 6.7 | 73.1 | ** |
| 13 | 12 | OS01815 | 3.9 | 79.8 | |
| 13 | 13 | OS04918 | 3.1 | 83.7 | |
| 13 | 14 | **OS04808** | 0 | 86.8 | |
| 13 | 15 | OS05914 | 2.2 | 86.8 | |
| 13 | 16 | OS04741 | 0.6 | 89 | |
| 13 | 17 | OS05292 | 3.9 | 89.6 | |
| 13 | 18 | OS01960 | 3.9 | 93.5 | |
| 13 | 19 | OS02077 | 0 | 97.4 | |
| 13 | 20 | **OS04921** | 2.8 | 97.4 | |
| 13 | 21 | OS05709 | 1.1 | 100.2 | |
| 13 | 22 | OS02945 | 0 | 101.3 | |
| 13 | 23 | OS04475 | 0.6 | 101.3 | |
| 13 | 24 | OS01893 | 0 | 101.9 | |
| 13 | 25 | OS01759 | ---------- | 101.9 | |
| 14 | 1 | OS05898 | 27.1 | 0 | |
| 14 | 2 | OS05258 | 1.7 | 27.1 | |
| 14 | 3 | OS04560 | 23.5 | 28.8 | |
| 14 | 4 | OS04966 | 1.1 | 52.3 | |
| 14 | 5 | OS05941 | 0 | 53.4 | |
| 14 | 6 | OS05025 | 1.1 | 53.4 | |
| 14 | 7 | OS05421 | 3.4 | 54.5 | |
| 14 | 8 | OS05381 | 2.2 | 57.9 | * |
| 14 | 9 | OS03088 | 0 | 60.1 | |
| 14 | 10 | **OS04688** | 0.3 | 60.1 | * |
| 14 | 11 | OS02574 | 0.3 | 60.4 | ** |
| 14 | 12 | OS01980 | 0.7 | 60.7 | * |
| 14 | 13 | OS01500 | 1.4 | 61.4 | ** |
| 14 | 14 | OS05203 | 0.7 | 62.8 | ** |
| 14 | 15 | OS05201 | 2.8 | 63.5 | |
| 14 | 16 | OS01999 | 2.8 | 66.3 | * |
| 14 | 17 | OS05845 | 0 | 69.1 | |
| 14 | 18 | OS05623 | 0 | 69.1 | |
| 14 | 19 | **OS04917** | 0 | 69.1 | |
| 14 | 20 | OS02453 | 0 | 69.1 | |
| 14 | 21 | OS02865 | 0 | 69.1 | |
| 14 | 22 | OS05280 | 0.6 | 69.1 | |

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|---|---|---|---|---|---|
| 14 | 23 | OS05884 | 1.1 | 69.7 | |
| 14 | 24 | OS04972 | 0 | 70.8 | |
| 14 | 25 | OS02381 | 1.1 | 70.8 | |
| 14 | 26 | **OS05265** | 0 | 71.9 | |
| 14 | 27 | OS02802 | 0.6 | 71.9 | |
| 14 | 28 | OS01660 | 5.5 | 72.5 | |
| 14 | 29 | OS05910 | 3.6 | 78 | |
| 14 | 30 | OS02768 | 2.8 | 81.6 | |
| 14 | 31 | OS01633 | 0 | 84.4 | |
| 14 | 32 | **OS02053** | 1.1 | 84.4 | |
| 14 | 33 | OS01979 | 12.6 | 85.5 | |
| 14 | 34 | OS04592 | 2.3 | 98.1 | |
| 14 | 35 | OS05923 | ---------- | 100.4 | |
| 15 | 1 | OS05887 | 0.6 | 0 | |
| 15 | 2 | OS04978 | 2.2 | 0.6 | |
| 15 | 3 | OS06141 | 0 | 2.8 | |
| 15 | 4 | OS06154 | 0.3 | 2.8 | |
| 15 | 5 | OS05711 | 0.3 | 3.1 | |
| 15 | 6 | OS04845 | 1.1 | 3.4 | |
| 15 | 7 | OS04530 | 0 | 4.5 | |
| 15 | 8 | **Ocum81** | 7.4 | 4.5 | |
| 15 | 9 | OS02556 | 1.7 | 11.9 | |
| 15 | 10 | OS04675 | 4.5 | 13.6 | |
| 15 | 11 | OS01636 | 2.8 | 18.1 | |
| 15 | 12 | OS01700 | 0 | 20.9 | |
| 15 | 13 | OS05456 | 34.1 | 20.9 | |
| 15 | 14 | OS03003 | 6.4 | 55 | |
| 15 | 15 | OS05637 | 1.7 | 61.4 | |
| 15 | 16 | OS04494 | 0 | 63.1 | * |
| 15 | 17 | OS05032 | 0 | 63.1 | |
| 15 | 18 | OS03782 | 0 | 63.1 | |
| 15 | 19 | OS02878 | 0 | 63.1 | |
| 15 | 20 | OS03812 | 0 | 63.1 | |
| 15 | 21 | OS05235 | 0 | 63.1 | |
| 15 | 22 | OS01697 | 0 | 63.1 | |
| 15 | 23 | OS02054 | 0 | 63.1 | |
| 15 | 24 | OS00057 | 0 | 63.1 | |
| 15 | 25 | OS02255 | 0.6 | 63.1 | |
| 15 | 26 | OS04795 | 0 | 63.7 | |
| 15 | 27 | OS01446 | 0.6 | 63.7 | |
| 15 | 28 | OS05245 | 0.6 | 64.3 | |
| 15 | 29 | OS04744 | 3 | 64.9 | * |
| 15 | 30 | OS00121 | 1.9 | 67.9 | |
| 15 | 31 | OS05970 | 0 | 69.8 | |
| 15 | 32 | **Ocum87** | 0 | 69.8 | |
| 15 | 33 | OS00156 | 0 | 69.8 | |
| 15 | 34 | OS06118 | 1.1 | 69.8 | |

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|---|---|---|---|---|---|
| 15 | 35 | OS00029 | 0 | 70.9 | |
| 15 | 36 | OS03137 | 0.6 | 70.9 | |
| 15 | 37 | OS05393 | 3.3 | 71.5 | |
| 15 | 38 | OS05492 | 0 | 74.8 | |
| 15 | 39 | OS03084 | 3.3 | 74.8 | |
| 15 | 40 | OS02889 | 0 | 78.1 | |
| 15 | 41 | OS06077 | 0.6 | 78.1 | * |
| 15 | 42 | **OS02198** | 0 | 78.7 | |
| 15 | 43 | OS05900 | 1.1 | 78.7 | * |
| 15 | 44 | OS03074 | 1.7 | 79.8 | |
| 15 | 45 | OS02930 | 8.1 | 81.5 | * |
| 15 | 46 | OS05859 | 10.5 | 89.6 | |
| 15 | 47 | OS05142 | 1.7 | 100.1 | |
| 15 | 48 | **OS04740** | 0 | 101.8 | * |
| 15 | 49 | OS05214 | 1.1 | 101.8 | * |
| 15 | 50 | OS02483 | 2.8 | 102.9 | |
| 15 | 51 | OS02770 | 1.7 | 105.7 | |
| 15 | 52 | OS02380 | 0.3 | 107.4 | |
| 15 | 53 | OS03198 | 0.3 | 107.7 | |
| 15 | 54 | OS03370 | 5.7 | 108 | |
| 15 | 55 | OS02829 | 3.4 | 113.7 | |
| 15 | 56 | OS04593 | 5.7 | 117.1 | |
| 15 | 57 | OS01459 | 0 | 122.8 | |
| 15 | 58 | OS03844 | 0 | 122.8 | |
| 15 | 59 | OS02264 | 1.6 | 122.8 | |
| 15 | 60 | OS05377 | 2.2 | 124.4 | * |
| 15 | 61 | OS05505 | 0 | 126.6 | |
| 15 | 62 | OS03320 | ---------- | 126.6 | |
| 16 | 1 | OS01530 | 10.1 | 0 | *** |
| 16 | 2 | OS04577 | 0.6 | 10.1 | *** |
| 16 | 3 | OS02307 | 0 | 10.7 | *** |
| 16 | 4 | **OS05160** | 0.5 | 10.7 | *** |
| 16 | 5 | OS05602 | 0.6 | 11.2 | *** |
| 16 | 6 | **Ocum196** | 0 | 11.8 | *** |
| 16 | 7 | OS00160 | 9.8 | 11.8 | *** |
| 16 | 8 | OS05413 | 4.1 | 21.6 | *** |
| 16 | 9 | OS05315 | 1.5 | 25.7 | ** |
| 16 | 10 | **OS05690** | 0.7 | 27.2 | ** |
| 16 | 11 | OS05319 | 0 | 27.9 | *** |
| 16 | 12 | **OS06085** | 0 | 27.9 | *** |
| 16 | 13 | **OS04572** | 0 | 27.9 | *** |
| 16 | 14 | OS05107 | 8.1 | 27.9 | *** |
| 16 | 15 | OS01995 | 4.5 | 36 | ** |
| 16 | 16 | OS05943 | ---------- | 40.5 | * |
| 17 | 1 | **OS05579** | 4.5 | 0 | |
| 17 | 2 | OS05965 | 0.6 | 4.5 | |
| 17 | 3 | OS04660 | 5.4 | 5.1 | |

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| 17 | 4 | **Ocum22** | | 21.6 | 10.5 | |
| 17 | 5 | OS04562 | | 1.1 | 32.1 | |
| 17 | 6 | OS02141 | | 7.9 | 33.2 | |
| 17 | 7 | OS04648 | | 2.5 | 41.1 | |
| 17 | 8 | OS06147 | | 5.7 | 43.6 | |
| 17 | 9 | OS05148 | | 2.2 | 49.3 | ** |
| 17 | 10 | OS00108 | | 0 | 51.5 | * |
| 17 | 11 | OS05216 | | 2.2 | 51.5 | * |
| 17 | 12 | **Ocum92** | | 1.1 | 53.7 | |
| 17 | 13 | **OS04551** | | 2.2 | 54.8 | ** |
| 17 | 14 | **OS05805** | | 1.1 | 57 | ** |
| 17 | 15 | OS05668 | | 0.6 | 58.1 | ** |
| 17 | 16 | OS03322 | ---------- | | 58.7 | *** |
| 18 | 1 | OS01587 | | 10.5 | 0 | |
| 18 | 2 | OS02560 | | 0.9 | 10.5 | |
| 18 | 3 | OS06034 | | 0.9 | 11.4 | |
| 18 | 4 | OS02922 | | 0 | 12.3 | |
| 18 | 5 | OS03142 | | 7.2 | 12.3 | |
| 18 | 6 | OS04882 | | 2 | 19.5 | |
| 18 | 7 | OS04803 | | 2.5 | 21.5 | |
| 18 | 8 | **Ocum52** | | 2.8 | 24 | |
| 18 | 9 | OS02178 | | 1.7 | 26.8 | * |
| 18 | 10 | OS05972 | | 0 | 28.5 | |
| 18 | 11 | OS02760 | | 3 | 28.5 | |
| 18 | 12 | OS05697 | | 3 | 31.5 | |
| 18 | 13 | OS05177 | | 1.8 | 34.5 | * |
| 18 | 14 | OS04655 | | 1.6 | 36.3 | |
| 18 | 15 | OS05205 | | 0 | 37.9 | |
| 18 | 16 | OS05740 | | 0 | 37.9 | |
| 18 | 17 | OS06095 | | 1.6 | 37.9 | |
| 18 | 18 | OS05669 | | 0 | 39.5 | |
| 18 | 19 | OS05827 | | 0 | 39.5 | |
| 18 | 20 | OS05345 | | 1.1 | 39.5 | |
| 18 | 21 | OS04859 | | 0.6 | 40.6 | |
| 18 | 22 | OS01613 | | 0 | 41.2 | |
| 18 | 23 | OS04867 | | 3.8 | 41.2 | |
| 18 | 24 | OS05862 | | 1.5 | 45 | |
| 18 | 25 | OS02152 | | 2.6 | 46.5 | |
| 18 | 26 | OS03135 | | 1.1 | 49.1 | |
| 18 | 27 | OS05993 | | 0 | 50.2 | |
| 18 | 28 | OS06000 | | 0.6 | 50.2 | |
| 18 | 29 | OS05866 | | 1.7 | 50.8 | |
| 18 | 30 | OS00063 | | 0 | 52.5 | |
| 18 | 31 | OS03057 | | 6.8 | 52.5 | |
| 18 | 32 | OS01809 | | 0.6 | 59.3 | |
| 18 | 33 | OS04679 | | 5.3 | 59.9 | |
| 18 | 34 | OS05665 | ---------- | | 65.2 | |

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|---|---|---|---|---|---|
| 19 | 1 | **OS05871** | 1.8 | 0 | |
| 19 | 2 | **OS06161** | 9.3 | 1.8 | |
| 19 | 3 | **OS01760** | 3.9 | 11.1 | |
| 19 | 4 | **OS04605** | 5.7 | 15 | |
| 19 | 5 | **OS02468** | 7.5 | 20.7 | |
| 19 | 6 | $P_g$ | 3.4 | 28.2 | * |
| 19 | 7 | OS01653 | 1.7 | 31.6 | |
| 19 | 8 | OS02571 | 1.1 | 33.3 | * |
| 19 | 9 | OS05469 | 0 | 34.4 | * |
| 19 | 10 | OS05757 | 0 | 34.4 | * |
| 19 | 11 | OS05024 | 1.7 | 34.4 | * |
| 19 | 12 | OS04622 | 1.1 | 36.1 | |
| 19 | 13 | OS05480 | 0 | 37.2 | |
| 19 | 14 | OS05242 | 0 | 37.2 | |
| 19 | 15 | OS02502 | 0 | 37.2 | |
| 19 | 16 | OS02406 | 0 | 37.2 | |
| 19 | 17 | OS04718 | 0 | 37.2 | |
| 19 | 18 | OS05889 | 0 | 37.2 | |
| 19 | 19 | OS05783 | 0 | 37.2 | |
| 19 | 20 | OS02887 | 0.6 | 37.2 | |
| 19 | 21 | **OS06168** | 0 | 37.8 | |
| 19 | 22 | OS06062 | 0 | 37.8 | |
| 19 | 23 | OS02682 | 0 | 37.8 | |
| 19 | 24 | OS00086 | 0 | 37.8 | |
| 19 | 25 | OS04669 | 0 | 37.8 | |
| 19 | 26 | OS04611 | 0 | 37.8 | |
| 19 | 27 | OS06009 | 0.6 | 37.8 | |
| 19 | 28 | OS05813 | 1.1 | 38.4 | |
| 19 | 29 | OS04743 | 0.3 | 39.5 | |
| 19 | 30 | OS05851 | 0.3 | 39.8 | |
| 19 | 31 | **OS01884** | 0 | 40.1 | |
| 19 | 32 | OS02886 | 0 | 40.1 | |
| 19 | 33 | OS00028 | 0 | 40.1 | |
| 19 | 34 | OS04805 | 0 | 40.1 | |
| 19 | 35 | OS05220 | 0 | 40.1 | |
| 19 | 36 | OS02348 | 0 | 40.1 | |
| 19 | 37 | OS05756 | 0 | 40.1 | |
| 19 | 38 | OS02140 | 0 | 40.1 | |
| 19 | 39 | OS05455 | 0 | 40.1 | |
| 19 | 40 | OS04474 | 0 | 40.1 | |
| 19 | 41 | OS06012 | 0 | 40.1 | |
| 19 | 42 | OS05422 | 0 | 40.1 | |
| 19 | 43 | **OS03091** | 0 | 40.1 | |
| 19 | 44 | OS05902 | 0 | 40.1 | |
| 19 | 45 | OS03197 | 0 | 40.1 | |
| 19 | 46 | OS06192 | 0 | 40.1 | |
| 19 | 47 | OS05387 | 0 | 40.1 | |

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|---|---|---|---|---|---|---|
| 19 | 48 | OS05517 | | 0 | 40.1 | |
| 19 | 49 | OS05228 | | 0 | 40.1 | |
| 19 | 50 | OS05530 | | 0 | 40.1 | |
| 19 | 51 | OS02926 | | 0 | 40.1 | |
| 19 | 52 | OS02505 | | 1.1 | 40.1 | |
| 19 | 53 | OS05373 | | 0.6 | 41.2 | |
| 19 | 54 | OS02587 | | 0 | 41.8 | |
| 19 | 55 | OS05508 | | 0 | 41.8 | |
| 19 | 56 | OS05089 | | 0 | 41.8 | |
| 19 | 57 | OS05108 | | 0 | 41.8 | |
| 19 | 58 | OS05013 | | 0 | 41.8 | |
| 19 | 59 | OS04820 | | 0 | 41.8 | |
| 19 | 60 | OS02092 | | 0 | 41.8 | |
| 19 | 61 | OS05947 | | 0 | 41.8 | |
| 19 | 62 | **OS05982** | | 0 | 41.8 | |
| 19 | 63 | OS05405 | | 1.1 | 41.8 | |
| 19 | 64 | **OS02161** | | 0 | 42.9 | |
| 19 | 65 | OS05708 | | 0 | 42.9 | |
| 19 | 66 | OS02942 | | 0 | 42.9 | |
| 19 | 67 | OS05034 | | 0 | 42.9 | |
| 19 | 68 | OS02201 | | 3.1 | 42.9 | |
| 19 | 69 | **OS01997** | | 27.9 | 46 | |
| 19 | 70 | OS01332 | | 19.5 | 73.9 | ** |
| 19 | 71 | OS02558 | | 2.3 | 93.4 | ** |
| 19 | 72 | OS02561 | | 0 | 95.7 | |
| 19 | 73 | OS04684 | | 0 | 95.7 | |
| 19 | 74 | **OS05885** | | 0 | 95.7 | |
| 19 | 75 | **OS05974** | | 3.9 | 95.7 | |
| 19 | 76 | OS05219 | | 1.1 | 99.6 | |
| 19 | 77 | OS02900 | | 2.8 | 100.7 | |
| 19 | 78 | OS02890 | | 0.6 | 103.5 | |
| 19 | 79 | OS01611 | | 16.8 | 104.1 | |
| 19 | 80 | OS05026 | ---------- | | 120.9 | |

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# CHAPTER III

# Genetic analysis of increased virulence in a sunflower broomrape population from southern Spain

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## ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant species that jeopardizes sunflower production in most producing areas of Europe and Asia. Recently, populations with increased virulence, classified as race $G_{GV}$, have been identified in the Guadalquivir Valley in southern Spain. These populations overcome resistance genes present in hybrids resistant to the race $F_{GV}$, which is the predominant race in the region. The objective of this study was to study the inheritance and mapping position of avirulence genes segregating in the cross between individuals of both races. Plants of EK23 (race $F_{GV}$) were crossed with plants of population IN201 (race $G_{GV}$), and their $F_1$, $F_2$, and $F_3$ generations were obtained. A commercial hybrid, named here as Hybrid 2, was used as a differential between both races. The $F_1$ population was virulent on Hybrid 2, suggesting that virulence was dominant over avirulence. The F2 genotype was inferred by the evaluation of the corresponding $F_{2:3}$ families. From 144 $F_{2:3}$ families evaluated, 29 of them were avirulent on Hybrid 2, whereas the other 115 families were virulent, with an average number of broomrape shoots from 0.17 to 16.33. The ratio of avirulent to virulent families was not significantly different to 1:3 ($\chi 2$=1.81; P=0.18), indicating monogenic control of the avirulence/virulence reaction. The $Avr_{Hybrid2}$ locus was mapped 14.9 cM upstream from the upper end of LG 2 of the *O. cumana* genetic map. This study confirmed the existence of a gene-for-gene interaction in the sunflower-*O. cumana* parasitic system and mapped for the first time an avirulene gene in this species. The results will contribute to a better understanding of the interaction and to the development of more durable resistance to *O. cumana* in sunflower.

Keywords: Avirulence genes – genetic mapping – gene-for-gene interaction – *Orobanche cumana* – parasitic plants

## INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant species with a very narrow range of hosts. In the wild, it has been observed on a few species of the Compositae, mainly on *Artemisia* spp., whereas in cultivated fields this species only parasitizes on sunflower (Velasco et al., 2016). Probably linked to this reduced range of hosts, the species is also characterized by the presence of physiological races, i.e. populations with defined virulence patterns (Molinero-Ruiz et al., 2015). Both aspects clearly differentiate *O. cumana* from other related *Orobanche* spp. and *Phelipanche* spp. parasitizing on agricultural crops, which are characterized by broader ranges of crop hosts and absence of well-defined physiological races (Pérez-Vich et al., 2013).

Another important difference between *O. cumana* and other related species of agricultural importance is the genetic control of the avirulence/resistance interaction. Thus, whereas in most parasitic systems such an interaction is under polygenic control (horizontal resistance), the avirulence/resistance interaction between *O. cumana* and sunflower generally follows a gene-for-gene relationship, i.e. vertical resistance under monogenic control (Velasco et al., 2016). This was first reported by Vranceanu et al. (1980) for the sunflower resistance to the races A to E found in Romania. Later studies confirmed the existence of monogenic inheritance to newly evolved races in several geographical areas, although more complex inheritance patterns have been also described in several sunflower resistant lines (Cvejić et al., 2020).

The existence of a gene-for-gene interaction in the parasitic system involving *O. cumana* and sunflower has been not only evaluated from the perspective of the resistance in the host, but also through changes of virulence observed in the parasite. Antonova and Ter Borg (1996) found that the virulence of *O. cumana* race D against genotypes carrying the *Or3* resistance gene providing resistance to race C populations was caused by the absence of extracellular excretion of peroxidases from the apex of broomrape radicles, which eluded the formation of lignin layers barriers in sunflower roots. Rodríguez-Ojeda et al. (2013) studied the inheritance of avirulence/virulence in the $F_1$ and $F_3$ plant generations from the cross between *O. cumana* plants of races E and F, using the differential line P-1380, resistant to race E and susceptible to race F. They concluded that race-E avirulence was dominant and controlled by a single gene, as expected for the gene-for-gene interaction (Flor, 1951).

In the Guadalquivir Valley area, one of the main sunflower cultivation areas in Spain, race F became predominant in the middle 1990's and has continued without apparent changes of virulence till 2014, when spots of populations attacking race-F resistant hybrids were detected (Martín-Sanz et al., 2016). That study evaluated the genetic composition of the populations and concluded as preliminary hypothesis that the newly detected virulence was probably caused by recombination between individuals of the two gene pools of *O. cumana* identified in Spain, distributed mainly in the Guadalquivir Valley and Cuenca province, respectively (Pineda-Martos et al., 2013).

The objective of the present study was to conduct a comparative genetic analysis between two populations of the Guadalquivir Valley (GV) area, one of them of race $F_{GV}$ and the other one incorporating the new virulence and classified accordingly as race $G_{GV}$ based on the nomenclature proposed by Martín-Sanz et al. (2016).

## MATERIAL AND METHODS

### *Plant materials*

*Orobanche cumana* population EK23 is a race $F_{GV}$ population representative of the classical and uniform gene pool of the Guadalquivir Valley (Pineda-Martos et al., 2013). In that study, the population was identified with code CO03. It was collected in Córdoba in 1995 (Rodríguez-Ojeda et al., 2013). IN201 is a sunflower broomrape population that overcomes resistance provided by race-$F_{GV}$ resistant hybrids cultivated in the Guadalquivir Valley area. It was collected in Ronda (Málaga, Spain) in 2012.

Six sunflower commercial hybrids resistant to race $F_{GV}$ were used for the characterization of the virulence of broomrape population IN201. All the hybrids used had been identified as race $F_{GV}$ resistant by the Spanish Plant Variety Registration Office and were active in the Spanish market at the time of the study. The names of the hybrids and the producing seed companies are not provided to not interfere with the commercial interests of the companies, as we did not include hybrids from all companies present in the Spanish market. A hybrid resistant to race E but susceptible to race F and the inbred line P96 were also included in the evaluation. P96 is resistant to race $F_{GV}$ (Fernández-Martínez et al., 2004) and has been reported to be also resistant to race $G_{GV}$ but susceptible to other populations of race G from Eastern Europe (Martín-Sanz et al., 2016). One

of the race-$F_{GV}$ hybrids used in this evaluation, named as Hybrid Race F-2 in Table 1 and abbreviated as Hybrid 2 in the manuscript, was used as a differential between broomrape populations EK23 and IN201 in the genetic study. B117 is a confectionery population very susceptible to all *O. cumana* populations evaluated so far (Martín-Sanz et al., 2016). It was used as a susceptible control and also for multiplication of the $F_1$ and $F_2$ populations.

**Table 1.** Number of emerged shoots per sunflower plant of *Orobanche cumana* population IN201 against a set of commercial hybrids resistant to race $F_{GV}$ cultivated in Spain in 2017, a race-E resistant hybrid used as susceptible control, and inbred line P96, resistant to races $F_{GV}$ and $G_{GV}$. Data are given as mean ± standard error.

| Hybrid/Line | N. emerged shoots |
|---|---|
| Hybrid Race F-1 | 2.90 ± 0.67 |
| Hybrid Race F-2 | 0.20 ± 0.20 |
| Hybrid Race F-3 | 0.00 ± 0.00 |
| Hybrid Race F-4 | 1.56 ± 0.75 |
| Hybrid Race F-5 | 0.70 ± 0.42 |
| Hybrid Race F-6 | 1.10 ± 0.50 |
| P96 | 0.00 ± 0.00 |
| Hybrid Race E | 58.70 ± 7.02 |

### *Inheritance study*

Plants of EK23 were emasculated and pollinated with pollen from plants of IN201, following the procedures described by Rodríguez-Ojeda et al. (2010). $F_1$ seeds were inoculated on both B117 and Hybrid 2 as described below. The genetic study could not be based on evaluation of the $F_2$ generation on the differential Hybrid 2, as avirulent genotypes would remain undetected. Accordingly, $F_2$ plants from the crosses EK23 x IN201 were grown on plants of the susceptible population B117 to produce $F_3$ seed, and the inheritance study was based on the evaluation of $F_{2:3}$ families, i.e. $F_3$ seeds derived from individual self-fertilised $F_2$ plants. To ensure self-pollination, $F_1$ and $F_2$ plants were isolated with

microperforated bags as described by Rodríguez-Ojeda et al. (2010) and harvested individually.

$F_3$ seeds from 144 $F_2$ plants were evaluated for virulence/avirulence on six plants of Hybrid 2. Additionally, $F_3$ seeds were also inoculated on three plants of the susceptible population B117 to test the viability of the seeds. Case that no infection was observed on both B117 and Hybrid 2, the $F_3$ family was discarded because of no viability of the seeds. Emerged broomrape shoots were counted on each sunflower plant at sunflower maturity. Virulence of the $F_1$ generation on Hybrid 2 was evaluated at the same environment as the $F_3$ generation. Inheritance of avirulence / virulence of *O. cumana* populations was assessed by the chi-square goodness of fit between expected and observed phenotypic ratios for avirulence / virulence of $F_2$ genotypes determined through the evaluation of their corresponding $F_3$ progenies on Hybrid 2, as described above.

### *Tissue collection and DNA extraction*

Apical tissue from the parental populations EK23 and IN201 and the $F_3$ plants of each $F_2$ genotype growing on B117 was cut shortly before flowering and bulked. The tissue was maintained in a cooler bag with ice packs during the time of tissue collection and immediately after frozen at -80 ºC. The tissue was then lyophilized and ground in a laboratory ball mill. DNA was extracted following an adapted version of the protocol developed by Rogers and Bendich (1985).

### *Genetic mapping*

A set of 192 *O. cumana* SNP markers reported and mapped by Calderón-González et al. (2019) was used to genotype the parental lines EK23 and IN201 and the mapping population (144 genotypes) using competitive allele-specific PCR assays based on KASP™ technology at LGC genomics (Teddington, Middlesex, UK). For genetic mapping purpose, monomorphic markers were excluded. The segregation of alleles at the SNP marker loci was checked against the expected ratios for codominant (1:2:1) markers using a chi-square test. The genetic linkage map was constructed with MAPMAKER/EXP (version 3.0b) (Lincoln et al., 1993) using genotyping data from polymorphic markers from the 192 SNP marker set. Map distances in centiMorgan (cM) were converted from recombination fractions using the Kosambi mapping function. Two-point analysis was used to identify

linkage groups (LGs) with an LOD score of 10.0 and a maximum distance of 30 cM. Three-point and multi-point analyses were used to determine the order and interval distances between the markers in each LG. Linkage maps were drawn using MapChart 2.1 software (Voorrips, 2002).

The avirulence trait was mapped as a Mendelian locus, since its inheritance was determined by one single gene (named as $Avr_{Hybrid2}$), as it is detailed in the Results section. Accordingly, the genotypes for the $Avr_{Hybrid2}$ gene in each $F_2$ plant were inferred from the avirulence/virulence phenotypes in their corresponding $F_{2:3}$ families. $F_2$ plants were classified into $Avr_{Hybrid2}Avr_{Hybrid2}$ (homozygous for the avirulent allele, with no emerged broomrape on the differential Hybrid 2 in their corresponding $F_{2:3}$ families) versus $Avr_{Hybrid2}avr_{Hybrid2}$ + $avr_{Hybrid2}avr_{Hybrid2}$ ($F_{2:3}$ families showing emerged broomrape on the differential Hybrid 2) genotypes. $Avr_{Hybrid2}$ mapping was carried out as indicated for SNP markers, excepting that a LOD threshold of 14 was used as linkage criteria. Additionally, the significance of each marker's association with the phenotypic trait was determined by one-way analysis of variance (ANOVA) using the statistical package SPSS Statistics v 27.0 (SPSS for Windows; SPSS Inc., Chicago, IL, USA), with marker genotypes being classes.

### *Diversity analysis*

A genetic diversity analysis was conducted on the parents EK43 and IN201, and the $F_1$ and $F_2$ generations. Additionally, based on the hypothesis of Martin-Sanz et al. (2016) that the new race $G_{GV}$ might be the result of genetic recombination between individuals of races $F_{GV}$ and $F_{CU}$, the latter from Cuenca province in central Spain, the parents and the $F_2$ population from the cross between the broomrape populations EK12 ($F_{GV}$) and EKA1 ($F_{CU}$), previously published by Calderón-González et al. (2019), were also included in the study. The study was conducted using 99 SNP markers from the 192 *O. cumana* set, which were polymorphic among all the seven parental or $F_2$ populations used in the study. SNP markers were genotyped as described above for EK43, IN201, and their $F_1$ and $F_2$ generations, and for EK12 and EKA1 and their $F_2$ as described by Calderón-González et al. (2019), who used the same SNP marker set.

The intra-population percentage of polymorphic loci, Shannon's information index (I), observed heterozygosity (Ho), Nei's expected heterozygosity (He), and Nei's unbiased expected heterozygosity (uHe) were computed for each

population. A principal coordinate analysis (PCoA) was conducted using the abovementioned seven populations and the 99 polymorphic markers. An additional PCoA was conducted using only the four populations of the genetic study (EK23, IN201 and their $F_1$ and $F_2$ populations) and 77 SNP markers that were polymorphic in this set. GenAlEx 6.501 (Peakall and Smouse, 2012) was used for these analyses.

### *Inoculation and plant cultivation*

In all experiments, soil inoculation and plant cultivation followed the procedures described by Rodríguez-Ojeda et al. (2010). In short, small pots 7 x7 x 7 cm were filled with a mixture of sand and peat and 50 mg of broomrape seeds. The mixture was shaken in a plastic bag to distribute broomrape seeds uniformly and put back in the pot. Sunflower seeds were germinated in moistened filter paper at 25 ºC in the dark for 48 h. After this time, germinated seeds were planted in the pots. The pots were maintained in a growth chamber at 25ºC / 20 ºC (day/night) with 16 h photoperiod for eight weeks, then transplanted into 5 L pots containing a soil mixture of sand, silt and peat in a proportion 2:1:1. The pots were maintained either in a greenhouse (parents for the crosses and $F_2$ generation), growth chamber at the abovementioned conditions ($F_1$ generation to produce $F_2$ seeds) or open-air conditions in the spring-summer period ($F_1$ and $F_3$ generation).

## RESULTS

### *Virulence of IN201*

Population IN201 was virulent against five of the six commercial hybrids with resistance to race $F_{GV}$ used in the study (Table 1). It was also virulent against the race-E resistant hybrid and avirulent against P96. The degree of attack, i.e. the number of emerged broomrape shoots per sunflower plant was low in all susceptible hybrids with race-F resistance, from 0.63 ± 0.20 (mean ± standard error) to 2.24 ± 0.75. On the contrary, the degree of attack was very high in the race-E resistant hybrid (58.70 ± 7.02; Table 1).

### *Inheritance study*

The race-$F_{GV}$ parent EK23 was avirulent on Hybrid 2 and virulent on the susceptible control B117 (23.33 ± 1.58 shoots per plant given as mean ± standard error). The race-$G_{GV}$ parent IN201 was virulent on both B117 (22.50 ±0.99) and Hybrid 2 (4.33 ± 0.76). $F_1$ plants from the cross between EK23 and IN201 were also virulent on both B117 (8.50 ± 1.52) and Hybrid 2 (6.25 ± 1.07).

All the 144 $F_{2:3}$ families ($F_3$ plants from each individual $F_2$ plant) were virulent on B117, with an average number of shoots per plant from 1.00 to 60.00. On the contrary, 29 $F_{2:3}$ families were avirulent on Hybrid 2, whereas the other 115 families were virulent, with an average number of broomrape shoots from 0.17 to 16.33. The ratio of avirulent to virulent families was not significantly different to 1:3 ($\chi^2$=1.81; P=0.18). Considering those families virulent on Hybrid 2, there was a positive correlation between the number of shoots per plant on B117 and Hybrid 2 (r=0.63; P<0.01), suggesting that part of the differences in the shoot number between families were caused by differences in germination ability and/or seed vigor.

### *Diversity analysis*

The parent population EK23 showed values of zero for all the parameters evaluated (Table 2). The other parent population, IN201, showed a 77.78% of polymorphic loci, a Shannon's information index (I) of 0.30, and values of 0.07, 0.19, and 0.19 for Ho, He and uHe, respectively. The $F_1$ population showed 16.16% of polymorphic loci, and values of 0.11 for I, and 0.16, 0.08, and 0.11 for Ho, He, and uHe, respectively. In the $F_2$, the percentage of polymorphic loci was similar to the $F_1$, 17.17%, and the indexes I, Ho, He, and uHe were 0.12, 0.08, 0.08, and 0.09, respectively. In the parents and $F_2$ population used as a control, the parents EK12 and EKA1 showed values of zero for all parameters, whereas the $F_2$ population showed a high percentage of polymorphic loci (89.90%) and much higher values of the diversity parameters than the $F_2$ between EK43 and IN201 (Table 2).

**Table 2.** Percentage of polymorphic loci (PL), Shannon's information index (I), observed heterozygosity (Ho), Nei's expected heterozygosity (He), and Nei's unbiased expected heterozygosity (uHe) in the parents EK23 (race $F_{GV}$) and IN201 (race $G_{GV}$), their $F_1$ and $F_2$ populations, and the control populations EK12 (race $F_{GV}$), EKA1 (race $F_{CU}$) and the $F_2$ population from their cross.

| | PL (%) | I | Ho | He | uHe |
|---|---|---|---|---|---|
| EK23 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| IN201 | 77.78 | 0.30 | 0.07 | 0.19 | 0.19 |
| $F_1$(EK23 x IN201) | 16.16 | 0.11 | 0.16 | 0.08 | 0.11 |
| $F_2$(EK23 x IN201) | 17.17 | 0.12 | 0.08 | 0.08 | 0.09 |
| EK12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| EKA1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| $F_2$(EK12 x EKA1) | 89.90 | 0.60 | 0.41 | 0.43 | 0.43 |

The three first axes in the principal coordinate analysis conducted using the populations derived from the crosses EK23 x IN201 and EK12 x EKA1 explained 35.05%, 10.58%, and 6.19% of the total variation. Fig. 1 shows the biplot of the two first axes. The two race-$F_{GV}$ populations EK23 and EK12 were grouped together in the same area as the $F_2$ population from EK23 and IN201. The latter population showed large dispersion. The $F_1$ population between EK23 and IN201 was located in the middle of the $F_2$ population, very close to EK23 and far from the $F_{CU}$ population EKA1. The $F_2$ population from EK23 and IN201 showed three apparent groups, although it was not possible to assign all individuals unequivocally to the groups in this plot.

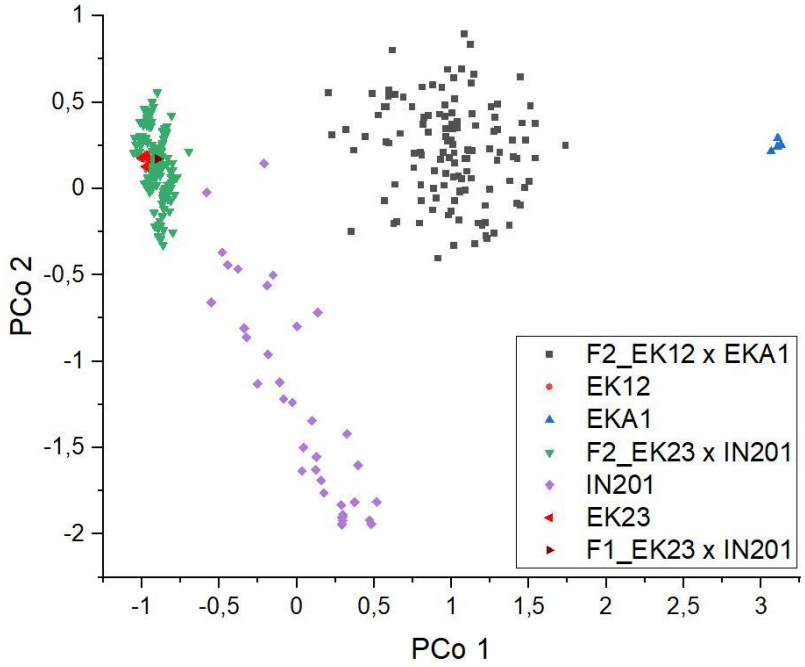



**Figure. 1.** Principal coordinate analysis of the *Orobanche cumana* parents EK23 ($F_{GV}$) and IN201 ($G_{GV}$), their $F_1$ and $F_2$ populations, and the reference populations EK12 ($F_{GV}$) and EKA1 ($F_{CU}$) and their $F_2$ population, based on their genotype at 99 polymorphic *O. cumana* SNP markers.

PCoA was repeated focusing only on the four populations of the genetic study EK23 x IN201. In this case, only 77 SNP markers that were polymorphic in the set of the four populations (EK23, IN201, $F_1$, and $F_2$) were retained (Fig. 2). As in the previous analysis, the $F_1$ population was located in the middle of the $F_2$, but more distant to the EK23 parent. The three groups of genotypes in the $F_2$ were more clearly observed in this analysis. Although the classification of all genotypes was not unequivocal, they were tentatively grouped in three groups formed by 42 (lower group), 68 (middle), and 34 genotypes (upper), which fitted a 1:2:1 distribution ratio ($\chi^2$=1.33; P=0.51).

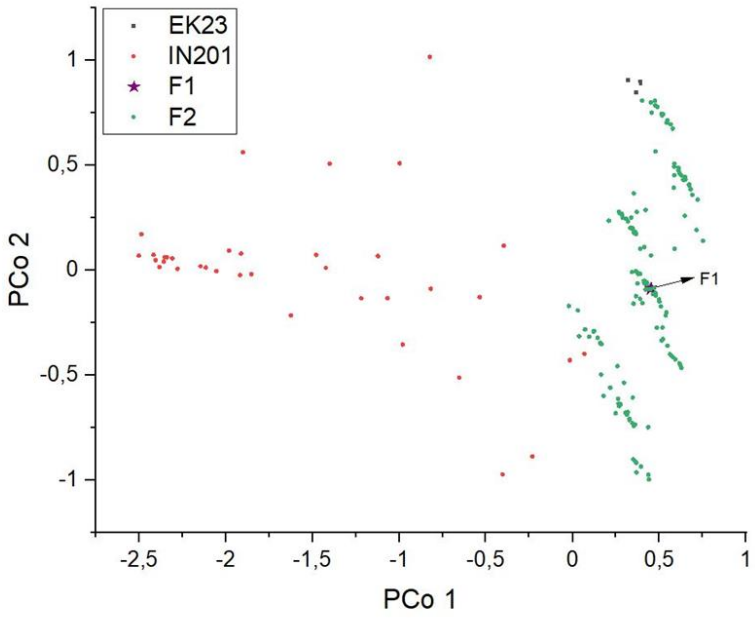


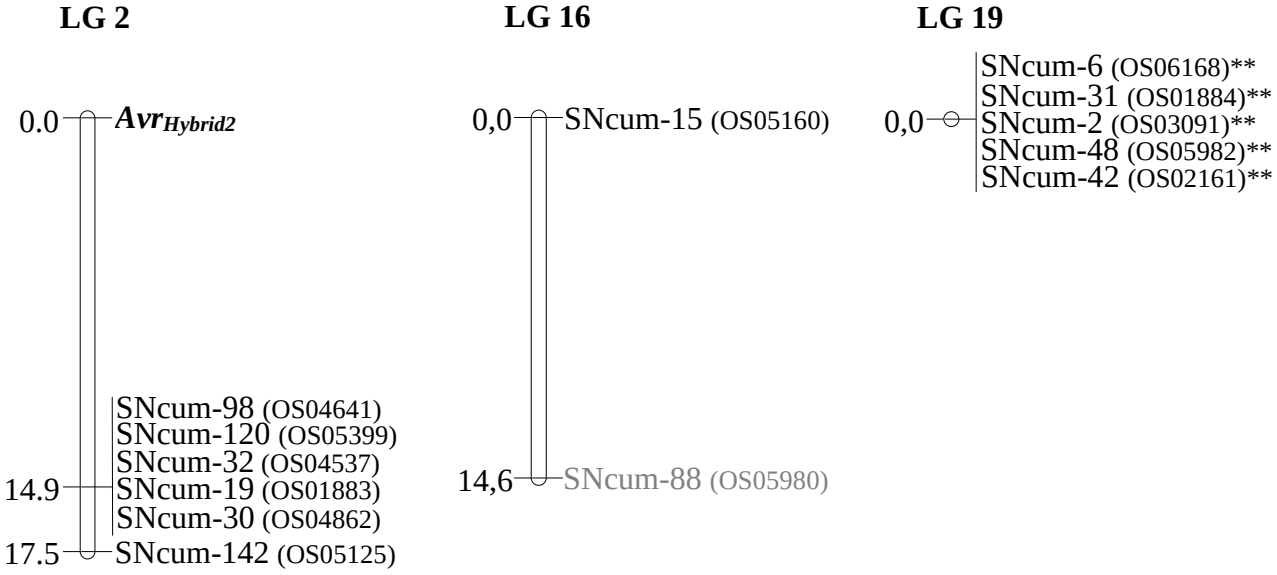

**Figure. 2.** Principal coordinate analysis of the *Orobanche cumana* parents EK23 ($F_{GV}$) and IN201 ($G_{GV}$), and their $F_1$ and $F_2$ populations based on their genotype at 77 polymorphic SNP markers.

To test whether the grouping might be related to the virulence of the genotypes, we calculated the correlation coefficients between the score for PCo1 and PCo2 and the number of broomrape shoots per plant on Hybrid 2 for each $F_{2:3}$ families. The results showed a significant negative correlation ($P<0.01$) of the number of broomrape shoots with both PCo1 ($r=-0.38$) and PCo2 ($r=-0.42$).

### *Genetic mapping the Avr$_{Hybrid2}$ gene*

From the 192 *O. cumana* SNPs, only 18 were polymorphic and segregated in the EK23 x IN201 mapping population (144 individuals). Seventeen of the SNP markers were codominant. The 18 SNP loci were arranged into 3 linkage groups (LGs) which had 6, 5 and 2 markers, respectively. The remaining five SNP loci were unlinked. Twelve out of the 13 markers arranged in LGs were previously mapped by Calderón-González et al. (2019), which made it possible to assign them to LG 2 (6 SNPs), LG 19 (5 SNPs), and LG 16 (2 SNPs, one of them unmapped previously) (Fig. 3). All marker loci from LG 19 showed distorted segregation at $P<0.01$, and mapped clustered at 0 cM. Also, the majority of markers on LG 2 mapped clustered, although distorted segregation was not

detected for any marker in this LG (Fig. 3). Using the genotypic classification of $F_2$ plants based on their $F_{2:3}$ families evaluation, the locus *Avr*$_{Hybrid2}$ was mapped 14.9 cM upstream from the upper end of LG 2 (Fig. 3). ANOVA analyses with genotypes at the SNP marker loci used as classes indicated clear significant differences ($P < 0.001$) between the marker class means for the total number of broomrapes in the differential Hybrid 2 at all LG 2 markers, while no significant differences were found for this trait for the remaining mapped markers (Table 3) or unlinked markers. Also, no significant differences between the marker class means were found at any marker for the total number of broomrapes in the susceptible control line B117 (Table 3).




Unlinked SNP loci: SNcum-4 (OS02579), SNcum-36 (OS01905), SNcum-52 (OS05579), SNcum-63 (OS04677), and SNcum-104 (OS04526).

**Figure. 3.** Genetic mapping of the avirulence gene *Avr*$_{Hybrid2}$ from the cross between *Orobanche cumana* populations EK23 and IN201. Linkage groups (LG) follow the numeration of Calderón-González et al. (2019). Genetic distances are given in centiMorgans (Kosambi) on the left of each LG. The position of the *Avr*$_{Hybrid2}$ gene, mapped as a Mendelian trait, is shown at LG 2. Prefix OS and its equivalent alias SNcum- are for the SNP marker loci, and follow the nomenclature of Calderón-González et al. (2019). SNP marker loci labelled as ** showed distorted segregation at $P<0.01$. Unlinked SNP loci are also included. SNP loci also polymorphic between the parental lines EK12 ($F_{GV}$) and EKA1 ($F_{CU}$) and mapped in the EK12 x EKA1 mapping population described by Calderón-González et al. (2019) are highlighted in black, and those not mapped by Calderón-González et al. (2019) are indicated in grey.

**Table 3.** Association between SNP mapped marker loci (for markers mapping clustered at 0 cM, only one representative marker per cluster is included) and the total number of broomrapes (per plant) emerged in either the differential Hybrid 2 or the susceptible control line B117 determined by variance analysis in the IN-23 x IN201 population. Marker closest to the *Avr*$_{Hybrid2}$ gene is highlighted in bold.

| LG | Marker | Sunflower host | No. individuals within each marker class | | | Mean ± SD for the number of broomrapes per plant within each marker class[a] | | | ANOVA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | A | H | B | A (EK23) | H | B (IN201) | *F* | *P* |
| 2 | **SNcum-120** | **Hybrid 2** | **36** | **59** | **42** | **0.17±0.4** | **3.8±3.1** | **4.7±4.1** | **24.47** | **<0.001** |
| | | **B117** | | | | **15.8±8.8** | **20.3±13.4** | **18.8±14.1** | **1.45** | **0.24** |
| 2 | SNcum-142 | Hybrid 2 | 40 | 58 | 41 | 0.7±1.7 | 3.4±2.9 | 5.1±4.3 | 20.46 | <0.001 |
| | | B117 | | | | 15.7±8.8 | 19.0±14.0 | 19.3±14.1 | 1.00 | 0.37 |
| 16 | SNcu-15 | Hybrid 2 | 37 | 72 | 30 | 2.8±3.6 | 3.4±3.7 | 2.7±2.9 | 0.71 | 0.49 |
| | | B117 | | | | 16.9±12.2 | 19.1±13.5 | 18.3±11.7 | 0.37 | 0.69 |
| 16 | SNcu-88 | Hybrid 2 | 36 | 77 | 27 | 2.9±3.5 | 3.5±3.7 | 2.3±2.6 | 1.33 | 0.27 |
| | | B117 | | | | 15.3±10.1 | 20.5±13.9 | 16.1±11.3 | 2.64 | 0.08 |
| 19 | SNcum-42 | Hybrid 2 | 51 | 65 | 24 | 2.7±2.9 | 3.4±3.8 | 3.1±3.9 | 0.65 | 0.52 |
| | | B117 | | | | 17.2±13.0 | 19.2±13.0 | 17.0±12.4 | 0.43 | 0.65 |

[a]Mean number of broomrapes per plant± standard deviation (SD) are presented in different genotypic classes: A, homozygous with respect to the avirulent allele derived from EK23; B, homozygous with respect to the virulent allele derived from IN201; H, heterozygous.

Allelic diversity at SNP markers segregating in the mapping population (Fig. 3) was investigated in detail at the parental population level (EK23 and IN201), and compared to alleles found in the parental populations EK12 (race $F_{GV}$ from the non-variable Guadalquivir Valley gene pool) and EKA1 (race $F_{Cu}$ from the non-variable Cuenca gene pool) from Calderón-González et al. (2019), and the $F_2$ populations EK23 x IN201 and IN12 x EKA1 (Table 4). As expected, the parental populations EK23, EK12 and EKA1 showed no allelic diversity. At all SNP marker loci, the allele present in EK12 was identical to that of EK23, and EKA1 had the alternative allele (excepting the two markers SNcum-63 and SNcum-88, which were monomorphic). At the 16 polymorphic loci, the parental population IN201 showed a mixture between the allele present in the Guadalquivir Valley gene pool (EK23 and EK12) and that present at the Cuenca gene pool (EKA1), with a number of heterozygous individuals also observed (Table 4).

**Table 4.** Percentage (%) individuals carrying each SNP allele at all SNP loci segregating in the mapping population EK23 x IN201 in the parental populations EK23 and IN201, and EK12 and EKA1, and, respectively, in their corresponding segregating $F_2$ EK23 x IN201 (this study) and EK12 x EKA1 (Calderón-González et al., 2019). SNP markers are ordered according to their mapping position (UL= Unlinked).

| LG | Allele present at SNP | | % of individuals carrying the SNP allele at each parental population or segregating $F_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | EK23 (n=19) | EK12 (n=10) | EKA1 (n=10) | IN201 (n=40) | $F_2$ (EK23xIN201) (n=144) | $F_2$ (EK12xEKA1) (n=125) |
| 2 | SNcum-98 | A:A | 100,0 | 100,0 | 0,0 | 2,5 | 25,4 | 27,7 |
| | | A:C | 0,0 | 0,0 | 0,0 | 15,0 | 43,3 | 42,0 |
| | | C:C | 0,0 | 0,0 | 100,0 | 82,5 | 31,3 | 30,3 |
| 2 | SNcum-120 | G:G | 100,0 | 100,0 | 0,0 | 2,5 | 26,3 | 28,7 |
| | | G:T | 0,0 | 0,0 | 0,0 | 15,0 | 43,1 | 40,0 |
| | | T:T | 0,0 | 0,0 | 100,0 | 82,5 | 30,7 | 31,3 |
| 2 | SNcum-32 | C:C | 100,0 | 100,0 | 0,0 | 3,1 | 27,8 | 27,0 |
| | | C:A | 0,0 | 0,0 | 0,0 | 12,5 | 40,6 | 43,4 |
| | | A:A | 0,0 | 0,0 | 100,0 | 84,4 | 31,6 | 29,5 |
| 2 | SNcum-19 | T:T | 100,0 | 100,0 | 0,0 | 2,5 | 25,4 | 30,2 |
| | | T:C | 0,0 | 0,0 | 0,0 | 15,0 | 44,2 | 39,7 |
| | | C:C | 0,0 | 0,0 | 100,0 | 82,5 | 30,4 | 30,2 |
| 2 | SNcum-30 | A:A | 100,0 | 100,0 | 0,0 | 2,5 | 24,6 | 27,9 |
| | | A:T | 0,0 | 0,0 | 0,0 | 15,0 | 44,8 | 43,4 |
| | | T:T | 0,0 | 0,0 | 100,0 | 82,5 | 30,6 | 28,7 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2 | SNcum-142 | C:C | 100,0 | 100,0 | 0,0 | 5,0 | 28,8 | 26,3 |
| | | C:A | 0,0 | 0,0 | 0,0 | 22,5 | 41,7 | 44,7 |
| | | A:A | 0,0 | 0,0 | 100,0 | 72,5 | 29,5 | 28,9 |
| 16 | SNcum-15 | T:T | 100,0 | 100,0 | 0,0 | 82,5 | 26,6 | 40,8 |
| | | T:C | 0,0 | 0,0 | 0,0 | 5,0 | 51,8 | 47,5 |
| | | C:C | 0,0 | 0,0 | 100,0 | 12,5 | 21,6 | 11,7 |
| 16 | SNcum-88 | A:A | 100,0 | 100,0 | 100,0 | 82,5 | 25,7 | 100,0 |
| | | A:T | 0,0 | 0,0 | 0,0 | 10,0 | 55,0 | 0,0 |
| | | T:T | 0,0 | 0,0 | 0,0 | 7,5 | 19,3 | 0,0 |
| 19 | SNcum-6 | G:G | 100,0 | 100,0 | 0,0 | 85,0 | 37,8 | 25,8 |
| | | T:G | 0,0 | 0,0 | 0,0 | 5,0 | 45,2 | 56,5 |
| | | T:T | 0,0 | 0,0 | 100,0 | 10,0 | 17,0 | 17,7 |
| 19 | SNcum-31 | T:T | 100,0 | 100,0 | 0,0 | 87,2 | 37,5 | 24,4 |
| | | C:T | 0,0 | 0,0 | 0,0 | 5,1 | 44,9 | 57,7 |
| | | C:C | 0,0 | 0,0 | 100,0 | 7,7 | 17,6 | 17,9 |
| 19 | SNcum-2 | A:A | 100,0 | 100,0 | 0,0 | 85,0 | 35,8 | 23,8 |
| | | C:A | 0,0 | 0,0 | 0,0 | 5,0 | 47,0 | 58,2 |
| | | C:C | 0,0 | 0,0 | 100,0 | 10,0 | 17,2 | 18,0 |
| 19 | SNcum-48 | G:G | 100,0 | 100,0 | 0,0 | 85,0 | 36,7 | 25,8 |
| | | A:G | 0,0 | 0,0 | 0,0 | 5,0 | 46,8 | 55,8 |
| | | A:A | 0,0 | 0,0 | 100,0 | 10,0 | 16,5 | 18,3 |
| 19 | SNcum-42 | G:G | 100,0 | 100,0 | 0,0 | 85,0 | 36,4 | 25,0 |
| | | T:G | 0,0 | 0,0 | 0,0 | 5,0 | 46,4 | 56,7 |
| | | T:T | 0,0 | 0,0 | 100,0 | 10,0 | 17,1 | 18,3 |
| UL | SNcum-4 | C:C | 100,0 | 100,0 | 0,0 | 92,5 | 26,6 | 25,4 |
| | | C:G | 0,0 | 0,0 | 0,0 | 5,0 | 47,6 | 45,6 |
| | | G:G | 0,0 | 0,0 | 100,0 | 2,5 | 25,9 | 28,9 |
| UL | SNcum-36 | G:G | 100,0 | 100,0 | 0,0 | 32,5 | 79,9 | 76,8 |
| | | ? | 0,0 | 0,0 | 100,0 | 67,5 | 20,1 | 23,2 |
| UL | SNcum-52 | A:A | 100,0 | 100,0 | 0,0 | 92,1 | 29,4 | 23,0 |
| | | G:A | 0,0 | 0,0 | 0,0 | 7,9 | 41,3 | 55,7 |
| | | G:G | 0,0 | 0,0 | 100,0 | 0,0 | 29,4 | 21,3 |
| UL | SNcum-63 | C:C | 100,0 | 100,0 | 100,0 | 70,0 | 25,3 | 100,0 |
| | | C:T | 0,0 | 0,0 | 0,0 | 12,5 | 44,0 | 0,0 |
| | | T:T | 0,0 | 0,0 | 0,0 | 17,5 | 30,8 | 0,0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| UL | SNcum-104 | G:G | 100,0 | 100,0 | 0,0 | 77,5 | 28,1 | 23,5 |
| | | G:A | 0,0 | 0,0 | 0,0 | 2,5 | 45,9 | 50,4 |
| | | A:A | 0,0 | 0,0 | 100,0 | 20,0 | 25,9 | 26,1 |

The predominant allele in IN201 at all SNP markers from LG2 was that present in the Cuenca gene pool (EKA1), with more than 70% of the analysed individuals carrying it, while in the other SNP markers the predominant allele was of the Guadalquivir Valley gene pool. The segregating populations EK23 x IN201 and EK12 x EKA1 showed the expected proportions according to an $F_2$ segregation, excepting markers SNcum-63 and SNcum-88 which did not segregate in EK12 x EKA1.

## DISCUSSION

The results of the present study revealed that the difference in virulence between populations EK23, of race $F_{GV}$ and IN201, of the new race $G_{GV}$ is caused by one single gene, which has been named as *Avr*$_{Hybrid2}$. The gene-for-gene interaction, in which alleles of a single locus in the pathogen condition virulence or avirulence in the differential host cultivar carrying a single resistance gene, was initially demonstrated in the parasitic system involving *O. cumana* and sunflower by genetic studies of Rodríguez-Ojeda et al. (2013). These authors showed, in crosses between broomrape individuals from race E (population EK12) and race $F_{GV}$ (population EK23) populations from the Guadalquivir Valley gene pool, that these two races were allelic, with the more virulent allele (race $F_{GV}$) was able to overcome the resistance conferred by the dominant gene *Or5*. The avirulent gene was named *Avr*$_{Or5}$. In this study, we moved a step forward. Crosses between the same $F_{GV}$ population used by Rodríguez-Ojeda et al. (2013), and the new race $G_{GV}$ also from the Guadalquivir Valley have indicated that the $F_{GV}$ to $G_{GV}$ change of virulence is also determined by one single gene (*Avr*$_{Hybrid2}$), and confirmed the gene-for-gene relationships. Single genes associated to avirulence phenotypes have been described in different gene-for-gene interactions involving pathogens other than parasitic plants such as bacteria, fungi or nematodes (Rouxel and Balesdent, 2010).

The *Avr*$_{Hibrid2}$ has been mapped close to the upper end of LG 2 of the *O. cumana* genetic map. This is the first report on mapping an avirulence gene within parasitic plants, and opens up new opportunities to gain insight into the genetic mechanisms underlying the evolution of virulence in O. cumana populations. A*vr*$_{Hybrid2}$ has been mapped 14.9 cM upstream a cluster of five markers including SNcum-98. According to the chromosome level assembly of the *O. cumana* genome (Muños et al., unpublished), physical position of this marker is at 23.5 Mbp on LG2. According to the LG 2 map of Calderón-González et al. (2019), the mapping distances from this study and the physical position of markers in close proximity to A*vr*$_{Hybrid2}$, this gene might be close to a telomeric region of LG 2. Mapping *Avr* genes close to chromosomal telomeric regions has been described previously in gene-for-gene interactions for example between rice and the fungus *Magnaporthe grisea* (Orbach et al., 2000), or between potato and the oomycete *Phytophthora infestans* (van der Lee et al., 2001). Since telomeric regions in eukaryotes are flexible and unstable regions in the genome, avirulence genes in these regions may facilitate the adaptation of the pathogen to resistance mechanisms in the host. In fact, the study of Orbach et al. (2000) demonstrated that the pathogen could escape the disease resistance gene-mediated recognition by diverse mechanisms, including point mutations, insertion mutations, and deletions of Avr gene sequences, and that this potential source of *Avr* gene hypervariability was related to its location near a telomere. On the other hand, this study also showed clustering of markers on LG 2 associated to *Avr*$_{Hybrid2}$, but also on LG 19. Marker clustering was also observed on these two linkage groups in the map developed by Calderón-González et al. (2019). This phenomenon has also been reported in genetic maps in other plant species, and it has been associated with centromeric regions due to the suppression of recombination around these regions (Haanstra et al., 1999), to the lack of recombination around genes with evolutionary significance (Jessup et al., 2002; Hao et al., 2004) or to DNA sequence divergence (Lukacsovich and Waldman, 1999).

Mapping *Avr*$_{Hybrid2}$, together with the advent of new genomic tools developed for *O. cumana* (Gouzy et al., 2017; Calderón-González et al., 2019) will contribute to understand the molecular basis of the *O. cumana*-sunflower gene-for-gene interaction through *Avr*$_{Hybrid2}$ map-based cloning or candidate gene strategies, which were not possible until now. Since the nature of avirulence genes in parasitic plants remains poorly understood, the term "avirulence gene" is used in this study in a broad sense (Lee et al., 2009) in such a way that it indicates a gene that encodes any determinant of the specificity of the interaction with the

host, regardless of its function or role in parasitism. Recent studies in gene-for-gene interactions between crops and parasitic plants evidence that parasitic plant-host interactions might be governed by similar rules as in other plant pathogen-host interactions (Delavault, 2020), and therefore the gene *Avr*$_{Hybrid2}$ might be related to pathogen-associated molecular patterns (PAMPs), pathogen effectors, or any other gene underlying variation in the activity of these molecules. In this way, it has been demonstrated that root parasitic plants can produce effector proteins that suppress host plant immunity. This is the case for the interaction between *Striga gesnerioides* race SG4z parasitizing cowpea (*Vigna unguiculata*) cultivar B301 (Su et al., 2019), and also for PAMPs that are recognized by host receptors leading to defense responses in resistant hosts, such as the PAMP GRP (glycine-rich protein) from *Cuscuta reflexa*, which acts as a binding ligand for the tomato (*Solanum lycopersicum*) receptor protein *CuRe1* (a leucine-rich repeat receptor-like protein) triggering plant defense responses (Hegenauer et al., 2020).

Although *Avr*$_{Hybrid2}$ position on the *O. cumana* map must be refined, it might reveal putative candidate genes. These included a cell-wall degrading putative glycoside hydrolase (OcIN23r2Chr02g0039901) at physical position of 22.5 Mbp close to SNcum-98. Glycoside hydrolases have been described as a virulence factor in other plant pathogens (Mathews et al., 2019; Shinya et al., 2021), and have been reported to be highly expressed in compatible interactions between *O. cumana* race G and sunflower (Yang et al., 2020).

*Orobanche cumana* populations are notorious for the appearance of new races that are able to overcome monogenic dominant resistance in sunflower (Molinero-Ruiz et al., 2015). The existence of a new race-G population of sunflower broomrape that overcame the resistance provided by all the race-F resistant hybrids cultivated in the Guadalquivir Valley area of Southern Spain was reported for the first time by Martín-Sanz et al. (2016). The authors found that the increased virulence was associated with larger intra-population diversity, and hypothesized that the new virulence might be caused by the genetic recombination of avirulence genes of the two gene pools in Spain, in the Guadalquivir Valley and Cuenca Province (Central Spain). Genetic recombination between individuals of both gene pools had been first observed by Pineda-Martos et al. (2013), although with no virulence implications. The results of the present study strongly support the hypothesis of Martin-Sanz (2016) that the new race G$_{GV}$ is the result of mixture and hybridization of the two Spanish broomrape gene pools, that of the Gudalquivir Valley and that from

Cuenca Province, with a likely introgession of virulent alleles from the Cuenca gene pool into the Guadalquivir Valley one. This was clearly seen by the fact that the vast majority (88.9%) of the SNP loci that were heterozygous in the $F_1$ and $F_2$ populations between EK23 and IN201, both of the Guadalquivir Valley gene pool, were also polymorphic between EK23 and EKA1 (Cuenca gene pool) (Fig. 3). The prevalence of the Cuenca allele (which is the virulent allele, alternative to that present in the avirulent parent EK23), in the IN201 parental population exclusively at those SNP loci of LG 2 associated to the $Avr_{Hybrid2}$ gene, also points to the introgression of genes from the Cuenca gene pool into the Guadalquivir Valley gene pool (Table 4). Genetic exchange within species and recombination has already been described as a mechanism that can give rise to novel virulent phenotypes, and different examples can be found in fungi, oomycetes and nematodes (Sacristán et al. 2021), such as increased virulence in downy mildew (*Plasmopara halstedii*) races infecting sunflower in France due to admixture and hybridization between the main introduced races in that country (Ahmed et al. 2012).

The fact that the virulent allele was that predominant in the parental population IN201 (race $G_{GV}$) only at the SNP markers associated to $Avr_{Hybrid2}$, which are able to overcome sunflower immunity provided by Hybrid 2, suggest that the resistance in host is the main driver for the evolution of virulence. This was likely to be facilitated by the widely cultivation of these type of race $F_{GV}$ resistant hybrids in the Guadalquivir valley area from Suthern Spain for many years (Martín-Sanz et al., 2016). Gene-for gene resistance is important in sunflower due to its relative ease to use and bred true (Fernández-Martínez et al., 2015). However, as it has been shown, its efficiency widely relies on the *Avr* gene structure of *O. cumana* populations, since the strong selection pressure created on the *Avr* gene by repeated cropping of resistant genotypes determines that any evolution of this gene that implies that its product is not recognized by the resistance genes, considering $Avr_{Hybrid2}$ as an effector, would become rapidly predominant in the pathogen population (Hu et al., 2020). Different breeding strategies aimed at reducing this strong selection pressure have been proposed in order to attain a more durable resistance in sunflower; these include pyramiding major genes controlling different mechanisms of resistance, alternation in time or in "multihybrids" of different major resistance genes, and also the back-up of these major genes through quantitative ressitance mechanisms (Vear, 2004; Molinero-Ruiz et al., 2015).

In conclusion, the present study demonstrated for the first time that classic genetic studies based of the evaluation of avirulence/virulence of $F_1$ and $F_3$ generations combined with genetic mapping of the corresponding *Avr* gene can provide useful information to define precisely races (in this study, specifically race $G_{GV}$) of the parasite on the basis of the presence of specific alleles at markers closely linked to the *Avr* gene. Although the molecular systems responsible for plant parasitism have been extensively studied in the last decade (Hu et al, 2020; Mutuku et al., 2020), very few studies regarding avirulence genes in gene-for-gene systems have been conducted in parasitic plants. This study provides for the first time a map position for an avirulence gene within parasitic plants, new evidence that the sunflower-*O. cumana* parasitic system conforms to the gene-for-gene model, and shows the *Avr* gene implication in determining the structure in broomrape populations subjected to selection pressure posed by a vertical resistance gene. This is an important step towards the precise identification of the $AVR_{Hybrid2}$ gene through map-based or candidate gene strategies. Finally, studying *Avr* genes in the parasitic weed and their interaction with resistance genes in the host would provide more detailed information on how sunflower resistance could be affected, which can be exploited to enhance the durability of resistance.

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# CHAPTER IV

# Association mapping for broomrape resistance in sunflower

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## ABSTRACT

Breeding for sunflower resistance to the parasitic plant sunflower broomrape (*Orobanche cumana* Wallr.) requires the identification of novel resistance genes. In this study, we used genome wide association study (GWAS) as a powerful tool to identify QTLs by examining the marker-trait associations across a germplasm set composed of 104 sunflower accessions. They were genotyped using 23,473 SNP markers selected from a sunflower Affymetrix AXIOM Genome-Wide array with an initial set of 586,985 SNPs. The accessions were evaluated for resistance to three populations of *O. cumana* with varying levels of virulence in two environments. The analysis of the genetic structure of the germplasm set revealed the presence of two main groups. Application of optimized treatments based on the general linear model (GLM) and the mixed linear model (MLM) identified allowed the detection of 14 SNP markers significantly associated with broomrape resistance. The highest number of marker-trait associations were identified on LG 3, clustered in two different genomic regions of this LG. Other associations were identified on LG5, 10, 13, and 16. Candidate genes for the main genomic regions associated with broomrape resistance were studied and discussed. The results of this study have confirmed the role of some QTL on resistance to sunflower broomrape and have revealed new ones that may play an important role in the development of durable resistance to this parasitic weed in sunflower.

Keywords: Broomrape resistance – Genome-Wide Association Mapping – GWAS – Orobanche cumana – Parasitic plants

## INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is one of the main biotic stresses affecting sunflower at a world scale. This holoparasitic plant parasitizes sunflower roots causing devastating effects if resistant cultivars and/or herbicide treatments are not used (Cvejić et al., 2020). The parasite has been traditionally present in many sunflower producing areas of Europe and Asia (Fernández-Martínez et al., 2015) and more recently has started to be detected in African countries such as Tunisia (Amri et al., 2012) and Morocco (Nabloussi et al., 2018).

Broomrape control strategies in sunflower have largely focused on the use of resistant cultivars, since their development was possible due to the existence of good sources of resistance, especially in wild *Helianthus* species, simple-trait inheritance in most cases, and efficient and controlled broomrape screening methods (Fernández-Martínez et al., 2015; Cvejić et al., 2020). Genetic resistance to broomrape was introduced into sunflower in the early breeding programs in the former USSR in the first years of the 20th century (Velasco et al., 2016). However, the introduction of new resistance sources was followed by the appearance of new *O. cumana* pathogenic races that overcame resistance (Fernández-Martínez et al., 2015). Thus far, eight broomrape races designated with letters from A to H have been reported, based on their virulence on sunflower differential lines (Cvejić et al., 2020). However, the current distinction between broomrape races in the main infested areas remains unclear, as there is little information of the correspondence of races with the same name reported in different countries (Cvejić et al., 2020). Races D and E were predominant till the middle 1990s, and they were satisfactory controlled by the resistance gene *Or5*, widely used in commercial hybrids. Populations overcoming *Or5* resistance were detected in 1995 in Spain (Alonso et al., 1996), and shortly after in Romania, Turkey and several other countries (Skoric et al., 2010). Currently, it seems clear that races more virulent than E (races F, G and H) are predominant in most sunflower producing areas of the Old World (Cvejić et al., 2020).

Genetic resistance to broomrape in sunflower has been found in most cases to be controlled by vertical resistance mechanisms that follow a gene-for-gene interaction, in which a dominant gene for host resistance interacts with a dominant avirulence gene in the parasite (Rodríguez-Ojeda et al., 2013). The genetic control of broomrape resistance by a single dominant gene was first reported by Pogorletsky and Geshele (1976). Shortly after, Vranceanu et al., 1980, identified five differential lines that had accumulative resistance to broomrape races A to E controlled by five dominant resistant genes *Or1* to *Or5*, respectively. Several other studies confirmed monogenic dominant resistance to race E (Sukno et al., 1999; Lu et al., 2000; Pérez-Vich et al., 2004). One dominant gene has also been reported

controlling races overcoming *Or5* resistance, such as *Or6* conferring resistance to race F from Romania (Pacureanu-Joita et al., 2004), *Or7* controlling race F from Spain, and also other more virulent races (Duriez et al., 2019) such as $Or_{Deb2}$ for race G from Turkey ($G_{TK}$) (Velasco et al., 2012), $Or_{SII}$ determining resistance to races F and G (Martín-Sanz et al., 2020), and $Or_{Pra1}$ for race $G_{TK}$ (Sayago et al., 2018). A number of these major dominant genes have been located on the sunflower genetic map. *Or5* has been mapped to a telomeric region of linkage group (LG) 3 (Lu et al., 2000; Tang et al., 2003; Pérez-Vich et al., 2004). Later, Imerovski et al. (2013, 2016), also found simple sequence repeat (SSR) markers of LG3 strongly associated with resistance genes other than *Or5* such as *Or2*, *Or3*, and *Or6*. Recently, Duriez et al. (2019) have mapped *Or7* to LG7, and Martín-Sanz et al. (2020) and Gao et al. (2018) have located $Or_{SII}$ and $Or_{Deb2}$, respectively, to the upper half of LG4.

In addition to studies on qualitative resistance, molecular studies have revealed a more complex control of broomrape resistance in sunflower. Pérez-Vich et al. (2004) described the existence of a quantitative component in *O. cumana* resistance, in addition to the qualitative component determined by major genes. This is conferred by quantitative trait loci (QTLs) that contribute with small-to-moderate effects to decreasing the number of emerged broomrapes (Pérez-Vich et al., 2004; Akhtouch et al., 2016; Imerovski et al., 2019). It has been demonstrated that resistance QTL may act at different broomrape developmental stages, providing accumulative resistance mechanisms (Louarn et al., 2016). Within this quantitative component, the role of (i) "defeated resistance genes" corresponding to major resistance genes specific for a broomrape race which provide only moderate levels of resistance to a different-more virulent race (Imerovski et al., 2019) and (ii) resistance QTL present in susceptible cultivars (Pérez-Vich et al., 2004; Akhtouch et al., 2016), has also been demonstrated. The combination of major resistance genes with quantitative resistance factors is seen as a promising alternative to ensure a durable sunflower protection against *O. cumana* (Pérez-Vich et al., 2013).

Genome wide association study (GWAS) is a powerful tool to identify QTLs by examining the marker-trait associations across a set of diverse germplasms. Compared to traditional genetic linkage analysis based on bi-parent populations, GWAS increases mapping resolution, reduces research time, and includes a greater number of alleles (Zhu et al., 2008). The availability of high density SNP genotyping data, linkage maps and the full genome sequence (Badouin et al., 2017), together with sufficient LD decay (Kolkman et al., 2007), have made it feasible to carry out large scale GWAS in sunflower. Association mapping studies in this crop have focused on flowering time (Cadic et al., 2013; Mandel et al., 2013; Bonnafous et al., 2018), branching pattern (Mandel et al., 2013; Nambeesan et al., 2015), fertility restoration (Goryunov et al., 2019; Talukder et al., 2019) and floral traits (Dowell et al., 2019). However, very few studies have been conducted on disease resistance, all of them

on fungal diseases (Fusari et al., 2012; Talukder et al., 2014). No association mapping research has been published so far on *O. cumana* resistance and are very limited for resistance to parasitic plants, and centred on the interaction between *Striga hermonthica* and cereal crops (Adewale et al., 2020; Kavuluko et al, 2020).

In this study, we have used GWAS on a population of 104 diverse sunflower accessions with varying levels of resistance to *O. cumana*. The accessions were genotyped using a suflower Affymetrix AXIOM Genome-Wide array and evaluated for resistance to three populations of *O. cumana* with varying levels of virulence in two environments. The main objective of the study was to detect loci associated with resistance to this parasitic weed and to identify resistance candidate genes.

## MATERIALS AND METHODS

### *Sunflower germplasm*

The sunflower germplasm set included 104 accessions (Table S1) selected from the germplasm collections of the USDA-ARS (38), INRA (46), and IAS-CSIC (20). Around one third of the accessions (34) were selected because we had previous indication that they possessed non-dominant resistance against broomrape, particularly to race F, but also in some cases to populations of race G. In general, the resistance of these lines was incomplete, i.e. they showed reduced infection but not immunity like the germplasm with dominant, vertical resistance. They were in most cases unpublished material, but some of the accessions have been reported previously, e.g. L86, K96, P96 and R96 (Fernández-Martínez et al., 2004), AM1, AM2 and AM3 (Pérez-Vich et al., 2006), and LR1 (Louarn et al., 2016).

### *Sunflower broomrape populations*

Resistance of the sunflower accessions was evaluated with three contrasting *O. cumana* populations from different origin and degree of virulence. SP is a population belonging to race $F_{GV}$ of the Guadalquivir Valley collected in Écija, Andalusia region, Spain. Bourret is a population of race $E_{FR}$ collected in Bourret (Tarn et Garonne), Occitanie region, France. GT is a population belonging to race $G_{TK}$ collected in Çeşmekolu, Thrace region, Turkey. Broomrape nomenclature follows Martín-Sanz et al. (2016).

### *Phenotypic evaluation*

Sunflower accessions were evaluated for resistance to broomrape populations Bourret and GT in pots in 2016 and 2017 in Córdoba, Spain. For population SP, evaluation was conducted

in pots in 2017 and in the field in 2018 in the same location. SP population, which belongs to the race $F_{GV}$, widely distributed in the area of the experiments, was the only population that could be evaluated under field conditions without risk of introduction on foreign populations in the area. In all cases, included the experiment in the field, all plants were inoculated with broomrape seeds as detailed below.

In all experiments, sunflower seeds were germinated in moistened filter paper at 25 ºC in the dark for 48 and sown in small pots 7 x7 x 7 cm filled with a mixture of sand and peat and 50 mg of broomrape seeds. The soil mixture contained broomrape seeds had been strongly shaken in a plastic bag to distribute broomrape seeds uniformly. The pots were maintained in a growth chamber at 25ºC / 20 ºC (day/night) with 16 h photoperiod for six weeks, then transplanted into 5L pots containing a soil mixture of sand, silt and peat in a proportion 2:1:1 or to the field in the case of the field experiment in 2018. The pots were maintained under open-air conditions in the spring-summer period and watered as required. In the field, plants were watered with drip irrigation. Sowing dates were 9 to 11 March in 2016, 6 to 8 March in 2017, and 26 to 28 February in 2018. In pot experiments, seven pots per accession were used. In the field, the experiment included three replicates of eight plants each. The number of emerged broomrape shoots (NEBS) was counted at each individual sunflower plant at the end of sunflower flowering.

Analysis of variance (ANOVA) was conducted on the number of emerged broomrape shoots using the accessions, the broomrape populations, and the environments (nested to the broomrape populations) as fixed factors. Mean squares values were used as an estimate of the relative weight of the factors on the number of emerged shoots. Pearson's correlation coefficients were also computed between environments for a given broomrape population and between the two-year average NEBS value of the accessions for each broomrape population. Analyses were conducted using SPSS statistical package version 27.

### *Tissue collection, DNA extraction and plant genotyping*

Genomic DNA for the 104 accessions was extracted from leaves tissue using the Kit DNeasy Plant Mini Kit (Qiagen©). The DNA concentration was adjusted to 10ng/µl in water. The genotyping experiments were performed by the Gentyane platform (Plateforme Gentyane, UMR INRA/UBP 1095 Génétique Diversité et Ecophysiologie des Céréales, Clermont-Ferrand, France) on a GeneTitan® (Affymetrix) according to the manufacturer's instructions. The AXIOM array was built using a set of 586985 SNPs. Genotypic data were obtained with the software Axiom Analysis Suite (http://www.affymetrix.com).

### *Genetic diversity and population structure analysis*

The genotyping data were imputed by genetic linkage group by means of BEAGLE (Browning and Browning, 2009). We filtered genotyping data by keeping a single SNP when redundant to others and we removed SNPs showing minor allele frequency (MAF < 5%). A final filtering was done with software TASSEL v5.2.59 (Bradbury et al., 2007) removing a total of twelve markers classified as unmapped which were discarded to create the definitive set of markers that were used for subsequent analyses. The kinship matrix (K-matrix) was calculated using the Centered-IBS method on this set of high quality filtered SNP markers. Finally, we kept a set of 23,743 SNPs for further analysis after removing redundant markers.

For the genetic diversity analysis, we used 6,264 SNP bi-allelic markers, i.e. one out of every fourth marker. Shannon's information index (I), observed heterozygosity (Ho), Nei's expected heterozygosity (He), and the fixation index (F) were computed. A principal coordinate analysis (PCoA) was also conducted. GenAlEx 6.501 (Peakall and Smouse, 2012) was used for these analyses.

The analysis of the genetic structure and kinship patterns of the population was computed using STRUCTURE ver. 2.3.4 (Pritchard et al., 2000). An admixture model following the Hardy-Weinberg equilibrium was used. The analysis was repeated ten times for each value of K (from 1 to 10) using a burn-in period of 100,000 Markov Chain Monte Carlo (MCMC) iterations and a run length of 100,000. The number of groups in the population was determined using Structure Harvester (Earl and von Holdt, 2012) with the Evanno correction (Evanno et al., 2005). The output of STRUCTURE analysis was subjected to the FullSearch algorithm of CLUMPP ver. 1.1.2b (Jakobsson and Rosenberg, 2007), and the output was used to produce bar graphs of the population structure using Origin Pro 9.1 software (OriginLab Corporation, Northampton, MA, USA).

### *Genome-wide association analysis and linkage disequilibrium*

A panel of 23,743 SNP markers with MAF > 5% was used for GWAS. A preliminary analysis evaluated the performance of the general linear model (GLM) and the mixed linear model (MLM) using either the Q-matrix or PCA covariates as cofactors. Additionally, the kinship (K) matrix was added to the MLM models as a way to avoid spurious associations linked to the genetic relatedness. For MLM models, we tested also several compression and variance component estimation options. The analyses were conducted using phenotypic data (average emerged broomrape shoots per sunflower plant) for each broomrape population and environment, and the average values for each broomrape population in the two environments. Quantile-quantile plots (QQ-plots) were constructed from the observed

versus expected -$\log_{10}$(p) values of each model. The significance of marker-trait associations (MTAs) was checked based on Bonferroni and false discovery rate (FDR) corrections at 5% and 20% (Benjamini and Hochberg, 1995). Furthermore, the range of linkage disequilibrium (LD) was computed using a sliding window of 50 kbp. Manhattan-plots were generated with the position and the p-value. The analyses were performed using TASSEL software v. 5.2.56. The matrix of p-value was used to estimate the FDR with the QVALUE package (Storey, 2002) in R.

### *Candidate gene analyses*

The significant marker-trait associations obtained were mapped on the HanXRQr2.0-SUNRISE reference sunflower genome sequence (https://www.heliagene.org/HanXRQr2.0-SUNRISE). After the physical positions were extracted, the genomic regions of the significant SNPs were examined to identify the annotated protein-coding genes located in or close to the significant SNPs. Exploration of the genomic regions for identification of candidate genes was carried out as follows: (i) if a cluster of significant marker-single trait associations was found, the SNP and the physical region spanned by the significant markers (+/- 250-Kb) was explored for high confidence genes with predicted biological function; and (ii) if only one single SNP marker constituted the significant marker-single trait association, genes which may function in plant disease and parasitic plant-resistance pathways among the genes containing or immediately adjacent to the SNPs (within a window of 250-Kb) were identified. Finally, if no candidate genes were found using these criteria, the closest candidate gene with known function in disease and parasitic plant-resistance pathways was also selected. The nature of most significant annotated candidate genes, and of all the genes coding for uncharacterized proteins, unknown function, or directly annotated but without description, was verified in the NCBI *Helianthus annuus* annotation release 101 (2020-09-02), and through BLAST searches using the sunflower sequences.

## RESULTS

### *Phenotypic evaluation of sunflower genotypes*

The analysis of variance showed a marked effect of the environment on the number of emerged broomrape shoots, accounting for 67.3% of the total estimated variance (Table 1). It was followed by the broomrape population, which accounted for 28.4% of the estimated variance, and the sunflower accession, which contributed with 3.3% to the total variance. These three main factors, as well as the interactions, were significant ($P<0.01$), although the interactions were of very low magnitude (Table 1).

**Table 1.** Analysis of variance for the number of emerged broomrape shoots in a set of 104 sunflower accessions evaluated for three broomrape populations in two environments for each population.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | % MS | F | P |
|---|---|---|---|---|---|---|
| Accession | 103 | 464458 | 4509 | 3.3 | 40.4 | <0.01 |
| Broomrape population | 2 | 76607 | 38303 | 28.4 | 343.5 | <0.01 |
| Environment (Br. Population) | 3 | 271712 | 90571 | 67.3 | 812.3 | <0.01 |
| Acession x Br. Population | 206 | 109738 | 533 | 0.4 | 4.8 | <0.01 |
| Accession x Environment | 308 | 190394 | 618 | 0.5 | 5.5 | <0.01 |
| Error | 3359 | 374543 | 112 | 0.1 | | |
| Total | 3982 | 3267402 | | | | |

The average number of broomrape shoots per sunflower plant ranged from 6.91 in the evaluation for broomrape population SP in 2018 to 36.3 for broomrape population GT in 2017 (Table 2). For the three populations, there was a variable number of sunflower accessions that showed a high degree of resistance. Considering the accessions that showed less than one broomrape shoot in the average of both evaluations, we observed 23 accessions for population SP, seven accessions for Bourret, and two accessions for GT. If we consider the six evaluations, two accessions showed less than one broomrape per plant (Table S1).

**Table 2.** Mean, standard deviation (SD), minimum and maximum values of emerged broomrape shoots in a set of 104 sunflower accessions evaluated for broomrape populations Bourret, SP and GT in two years.

| Population/Year | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|
| Bourret 2016 | 22.13 | 14.98 | 0.00 | 75.75 |
| Bourret 2017 | 22.89 | 17.37 | 0.00 | 75.86 |
| SP 2017 | 17.30 | 16.48 | 0.00 | 63.14 |
| SP 2018 | 6.91 | 6.54 | 0.00 | 25.04 |
| GT 2016 | 10.27 | 6.34 | 0.13 | 28.00 |
| GT 2017 | 36.32 | 19.29 | 0.00 | 79.29 |

Despite the large influence of the environment on the number of emerged broomrape shoots, correlation coefficients between the evaluations for the same population in different environments or even for the evaluation of different populations in different environments were in all cases positive and statistically significant. Correlation coefficients between the two evaluations for each population ranged from 0.59 for population GT to 0.82 for population SP. Considering the correlation coefficients between different populations in individual environments, they ranged from 0.50 (GT in 2016 vs. Bourret in 2016) to 0.77 (Bourret in 2017 vs. SP in 2017), whereas the correlation coefficients between populations considering the average value of the two evaluations ranged from 0.72 (GT vs. Bourret) to 0.78 (Bourret vs. SP) (Table 3).

**Table 3.** Correlation coefficients between emerged broomrape shoots in a set of 104 sunflower accessions evaluated with three broomrape populations in two environments each one. Correlation coefficients between the average values in the two environments for each population are also included.

| Evaluation | SP_2018 | Bourret_2016 | Bourret_2017 | GT_2016 | GT_2017 | Bourret_Average | GT_Average |
|---|---|---|---|---|---|---|---|
| SP_2017 | 0.82** | 0.66** | 0.77** | 0.59** | 0.76** | | |
| SP_2018 | | 0.55** | 0.63** | 0.55** | 0.65** | | |
| Bourret_2016 | | | 0.67** | 0.50** | 0.61** | | |
| Bourret_2017 | | | | 0.51** | 0.66** | | |
| GT_2016 | | | | | 0.59** | | |
| SP_Average | | | | | | 0.78** | 0.78** |
| Bourret_Average | | | | | | | 0.72** |

### *Genetic diversity, population structure and linkage disequilibrium analysis*

Single nucleotide polymorphism (SNP) markers were evenly distributed across the whole genome, from 629 SNPs in LG 7 to 2605 SNPs in LG 8 (Table 4). The number of SNPs per Mbp ranged from 5.1 in LG 15 to 17.1 in LG 8.

Genetic diversity analysis revealed that the means of the effective and observed allele numbers for the collection were 2.0 and 1.75, respectively. The expected heterozygosity (Nei's gene diversity) and Shannon's information index were 0.42 and 0.60, respectively. The observed heterozygosity and the fixation index were 0.04 and 0.92, respectively.

The average pairwise genetic distance computed with Genalex 6.5 was 10.475 and ranged from 126 for accessions CD and HA89 and 13.423 for accessions UD and PI578010. Principal Coordinate Analysis (PCoA) revealed that the three first axes explained a low proportion of the total variance, 8.1, 6.4, and 4.3%, respectively. Fig. 1 shows the bi-plot for PCo 1 and PCo 2. Some accessions were grouped very closely, for example accessions PO7-28, PO7-34, PO7-38, PO7-61 and PO7-63. They were developed in a recurrent selection program starting from a random mating population with selection for broomrape race F (unpublished). Their relatedness was unknown at the beginning of the research.

The analysis of the genetic structure of the germplasm set suggested the existence of two main groups, as indicated by a K=2 using the Delta K method (Fig. 2). LD was calculated using all the SNP markers and the LD decay was estimated about 0.25 $x10^6$ bp for all the chromosomes (Fig. 3), which is consistent with other studies in which it was observed that the linkage disequilibrium rapidly decays in sunflower (Liu and Burke, 2006; Kolkman et al., 2007; Fusari et al., 2008).

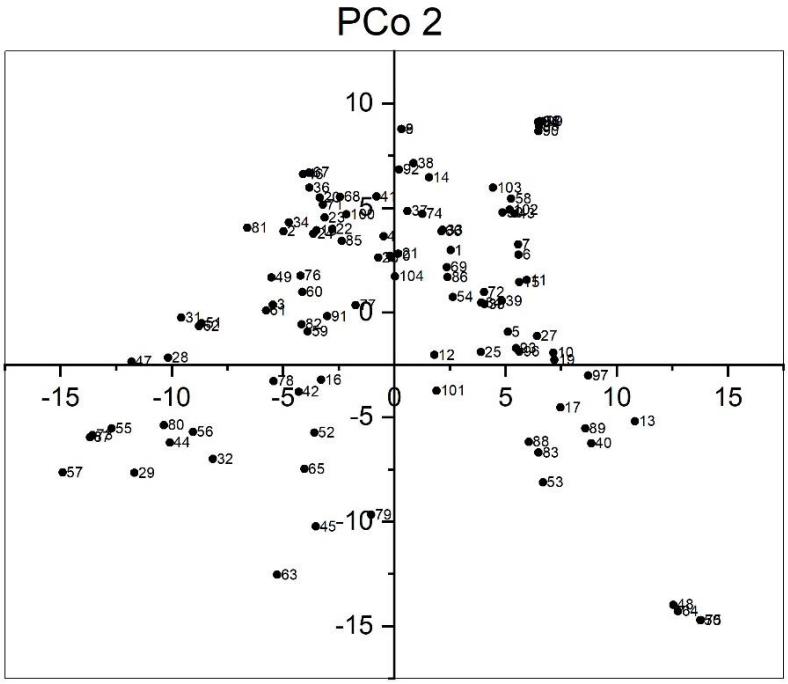


**Figure 1.** Principal coordinate analysis of 104 sunflower accessions genotypes with SNP markers.

**Table 4.** SNPs distribution across all the chromosomes with the position (bp) of the first and the last molecular marker per linkage group.

| Linkage group | Number of SNPs | Length (Mbp) | SNPs/Mbp | Physical position of the extreme markers in each chromosome (Mbp) |
|---|---|---|---|---|
| **1** | 2045 | 153.3 | 13.3 | 0 (0.01) - 2044 (153.3) |
| **2** | 1605 | 177.7 | 9.0 | 2045 (1.4) - 3649 (179.1) |
| **3** | 1360 | 167.8 | 8.1 | 3650 (0.6) - 5009 (168.5) |
| **4** | 1234 | 178.5 | 6.9 | 5010 (0.2) - 6243 (178.8) |
| **5** | 2069 | 218.6 | 9.5 | 6244 (0.4) - 8312 (219.1) |
| **6** | 703 | 102.5 | 6.9 | 8313 (1.0) - 9015 (103.5) |
| **7** | 629 | 103.8 | 6.1 | 9016 (0.05) - 9644 (103.8) |
| **8** | 2605 | 152.4 | 17.1 | 9645 (0.04) - 12249 (152.5) |
| **9** | 1206 | 207.8 | 5.8 | 12250 (1.3) - 13455 (209.1) |
| **10** | 2101 | 245.3 | 8.6 | 13456 (0.9) - 15556 (246.2) |
| **11** | 1055 | 167.9 | 6.3 | 15557 (0.01) - 16611 (167.) |
| **12** | 989 | 165.6 | 6.0 | 16612 (0.05) - 17600 (165.7) |
| **13** | 1360 | 195.7 | 6.9 | 17601 (1.1) - 18960 (196.8) |
| **14** | 1108 | 173.9 | 6.4 | 18961 (0.5) - 20068 (174.3) |
| **15** | 874 | 169.8 | 5.1 | 20069 (1.4) - 20942 (171.2) |
| **16** | 1976 | 187.8 | 10.5 | 20943 (0.7) - 22918 (188.5) |
| **17** | 2027 | 214.7 | 9.4 | 22919 (0.01) - 24945 (214.7) |

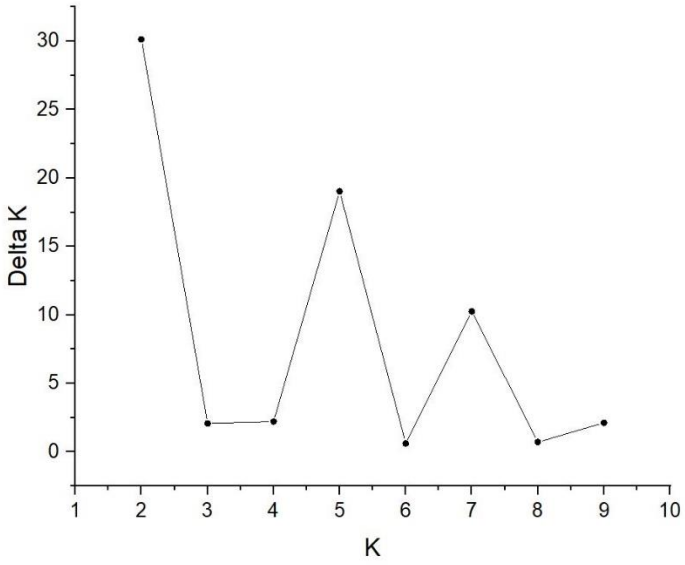


**Figure 2.** Delta K for values of K in admixture analysis of genetic structure in a germplasm set of 104 accessions of sunflower.

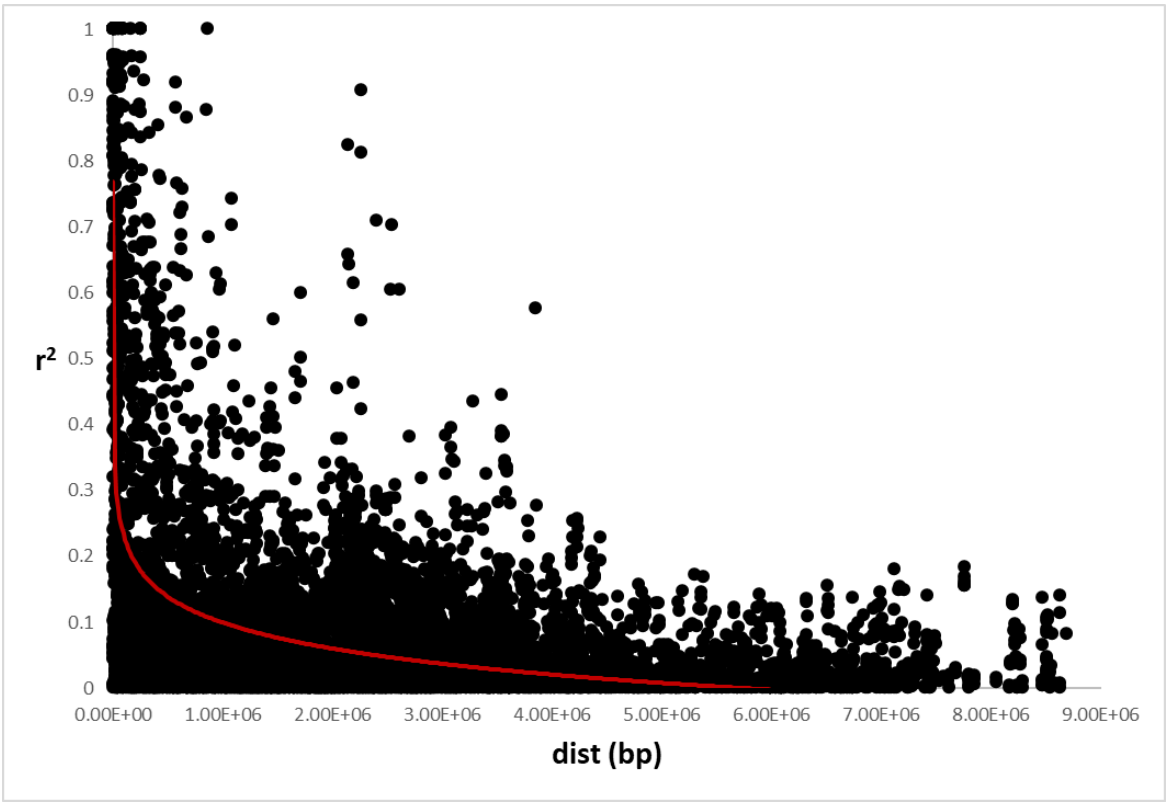


**Figure 3.** Linkage disequilibrium decay using SNP markers data set. Estimation of $r^2$ versus distance in base pair (bp) was represented. LD decay was established around $0.25e^6$ bp.

### *Marker trait association*

Genome-wide association was performed in a panel of 104 sunflower accessions using a set of 24,946 SNP markers. The best fitting models were chosen analysing the quantile-quantile plots (QQ-plots). The deviation of observed vs expected $-\log_{10}$ p-values was smaller for the mixed linear models (MLM) than for general linear models (GLM), with the best results within each group using GLM+PCA and MLM+K+PCA combinations with optimum level of compression and re-estimation after each marker (Fig. 4).

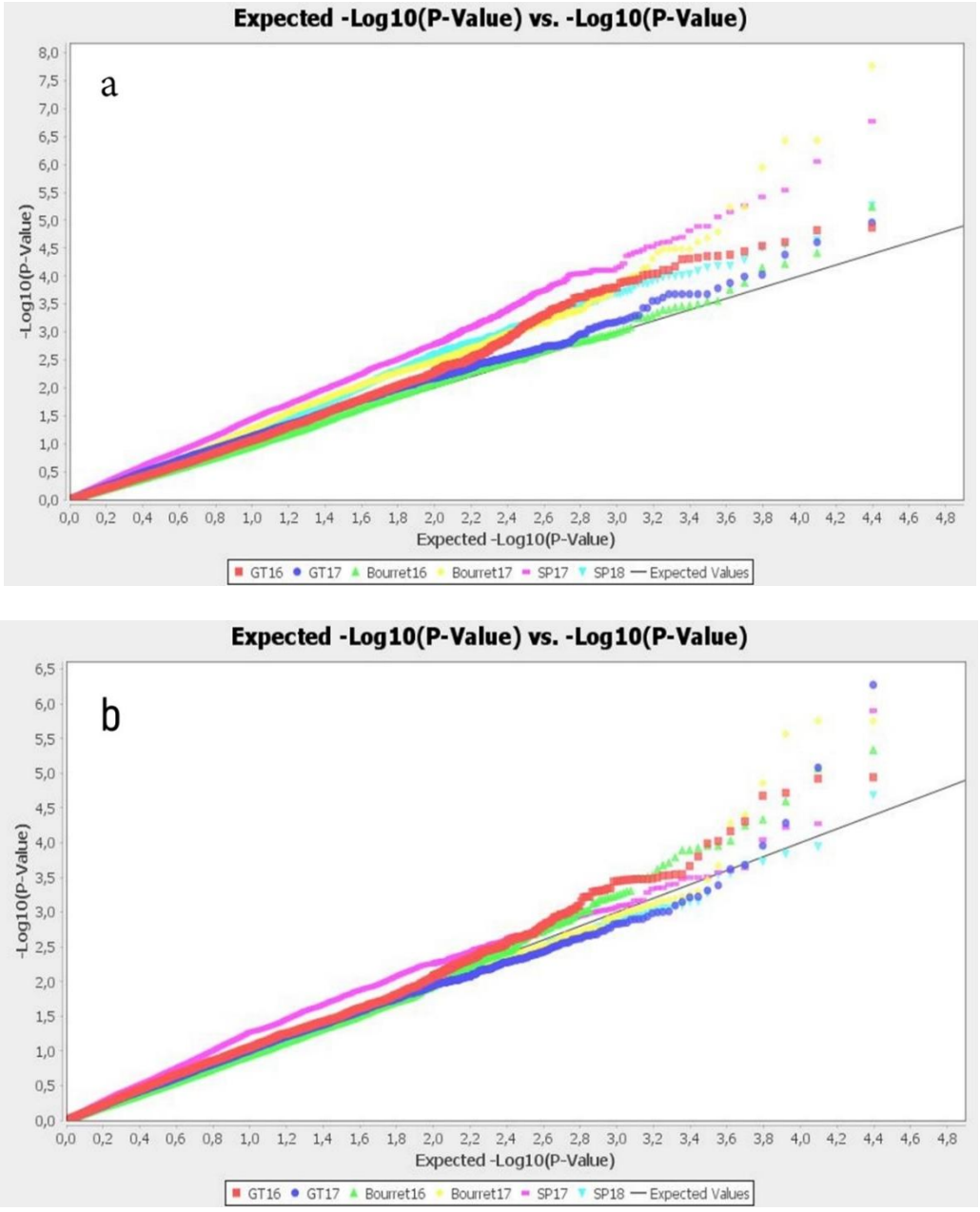


**Figure 4.** Quantile-quantile (Q-Q) plots of observed versus expected P values of the GWAS results using GLM+PCA (a) and MLM+K+PCA (b). The straight line represents concordance of observed and expected values.

Manhattan plot from GLM+PCA (Fig. 5) and MLM+K+PCA (Fig. 6) revealed a total of 14 SNP markers significantly associated with the number of emerged broomrapes. There were six of them in GLM+PCA and four of them in MLM+K+PCA with p < 2e-06 (5%) and 5 additional in GLM+PCA and 2 in MLM+K+PCA with p < 8.01e-06 (20%) (Table 5). Significant associations were identified on 6 different chromosomes from the HanXRQr2.0-SUNRISE reference sunflower genome assembly (https://www.heliagene.org/HanXRQr2.0-SUNRISE) (Table 5) and for most of the broomrape populations and environments, except for GT16, being cases in which there was presence of a marker for two different broomrape populations and/or environments such as: AX-105943713 for Bourret17 and SP17, and AX-105776042 for SP17 and SP18 (Table 5). The trait variation explained by each marker varied from 14 to 24% (Table 5). The most significant peaks detected above the 5% Bonferroni threshold and identified both with GLM+PCA and MLM+K+PCA were observed on LG3, which, in addition, showed by far the highest number of marker-trait associations. Two regions which contained clustered associations were observed on this LG. The first one was a 5.2 Mbp region spanned by the two markers AX-105943713 and AX-147199586 [coordinates 85486771-90700620 (HanXRQr2.0-SUNRISE)] and associated to both race E (Bourret17) and race F (SP17) of broomrape. The second one with markers AX-105705204, AX-105776042, AX-105655280, and AX-105768536 ranged from physical position 129889814 bp to 136591650 bp (6.7 Mbp) (HanXRQr2.0-SUNRISE) and it was associated only to broomrape race F (SP17 and SP18) (Table 5). Other significant single marker-trait associations were identified on LGs 5, 10 and 13 for race E populations (Bourret17 for LG5, and Bourret16 for LGs 10 and 13), LG 15 for race F population (SP17), and LG 16 for race $G_{TK}$ population (GT17) (Table 5).

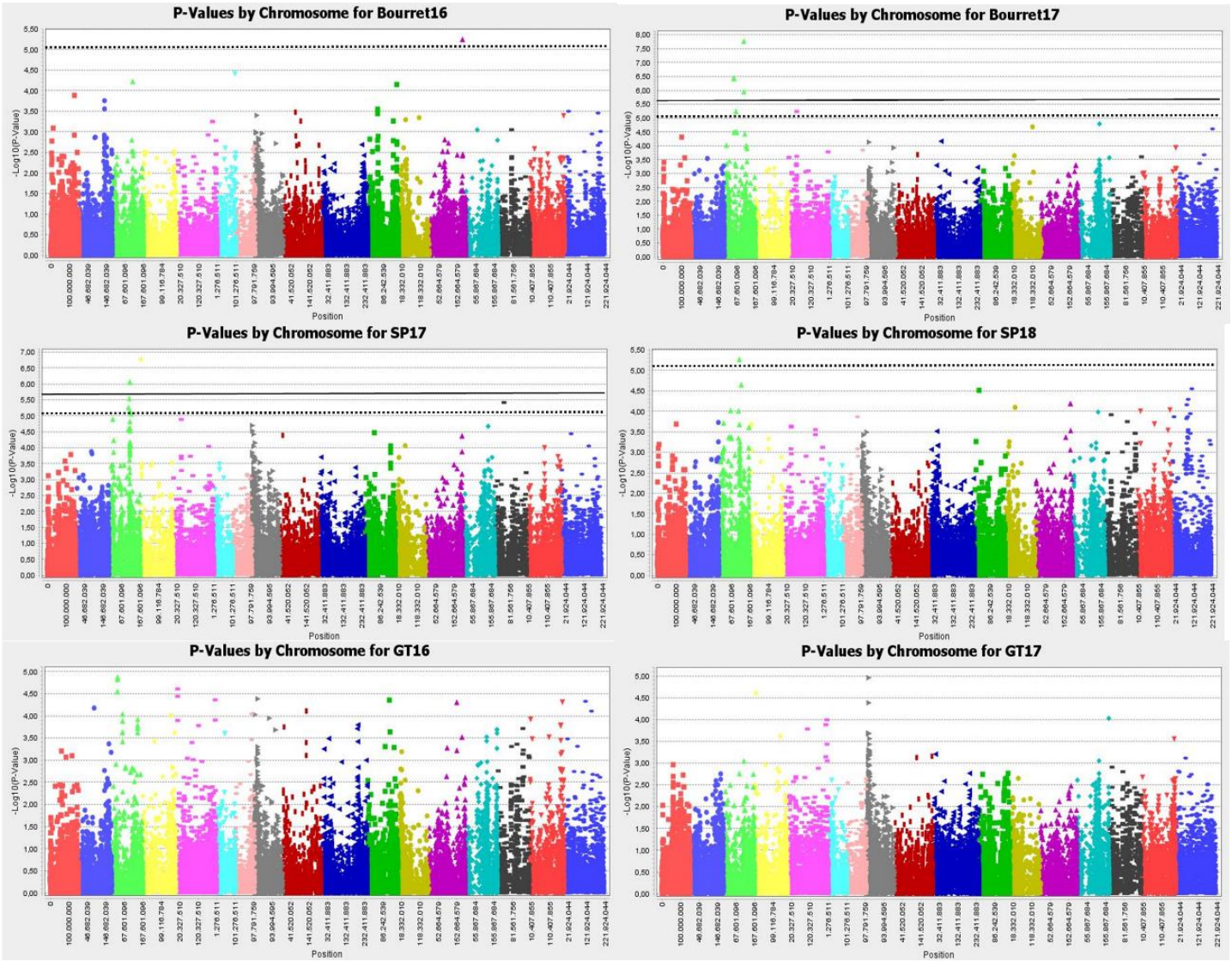


**Figure 5.** Manhattan-plots illustrating significant associations for resistance to three broomrape populations (SP, Bourret and GT) in a panel of 104 sunflower accessions evaluated in two environments each using GLM+PCA. The P values were adjusted using the Bonferroni threshold and false detection rate (FDR) correction (5% and 20%) to reduce false positive associations. The solid line corresponds to the 5% threshold and the dotted line to the 20% threshold. The vertical axis indicates –log10 of p-value and horizontal axis indicates chromosomes and physical positions of SNPs

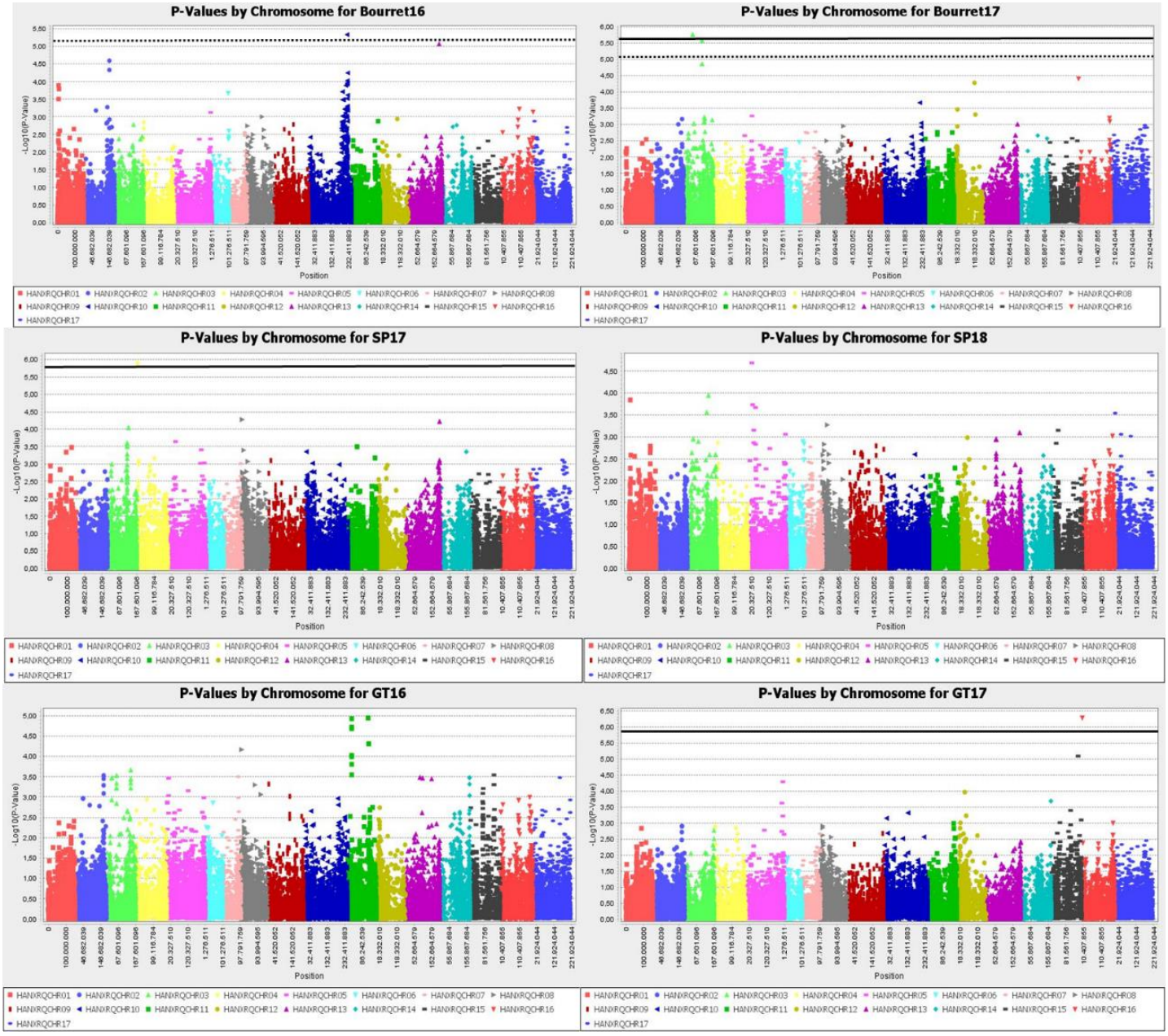


**Figure 6.** Manhattan-plots illustrating significant associations for resistance to three broomrape populations (SP, Bourret and GT) in a panel of 104 sunflower accessions evaluated in two environments each using MLM+K+PCA. The P values were adjusted using the Bonferroni threshold and false detection rate (FDR) correction (5% and 20%) to reduce false positive associations. The solid line corresponds to the 5% threshold and the dotted line to the 20% threshold. The vertical axis indicates –log10 of p-value and horizontal axis indicates chromosomes and physical positions of SNPs.

**Table 5.** SNP markers associated with broomrape resistance in different environments (composed of three races evaluated over 2 years) according the GLM corrected with PCA and MLM corrected with kinship (K-matrix) and PCA. Unshaded data corresponds to the markers that exceed Bonferroni 5% threshold and shaded markers corresponds with Bonferroni 20%.

| | Trait | Marker | LG (HanXRQr2.0) | Position (bp) (HanXRQr2.0) | Initial mapped position (bp) in HanXRQr1.0 | p-value | marker_$R^2$ |
|---|---|---|---|---|---|---|---|
| GLM+PCA | | | | | | | |
| | Bourret17 | AX-105943713 | 3 | 85486771 | 105501538 | 1.13E-06 | 0.16272 |
| | SP17 | AX-105943713 | 3 | 85486771 | 105501538 | 5.48E-06 | 0.14168 |
| | Bourret17 | AX-105531030 | 3 | 85505366 | 105482945 | 1.72E-08 | 0.23265 |
| | Bourret17 | AX-105925988 | 3 | 86489659 | 51278297 | 3.73E-07 | 0.19847 |
| | Bourret17 | AX-105709192 | 3 | 87263090 | 51917942 | 3.73E-07 | 0.19847 |
| | Bourret17 | AX-147199586 | 3 | 90700620 | 62576510 | 5.79E-06 | 0.14342 |
| | SP17 | AX-105705204 | 3 | 129889814 | 4872459 | 1.70E-07 | 0.20398 |
| | SP17 | AX-105776042 | 3 | 133359785 | 110766840 | 2.89E-06 | 0.1716 |
| | SP18 | AX-105776042 | 3 | 133359785 | 110766840 | 5.43E-06 | 0.15702 |
| | SP17 | AX-105655280 | 3 | 135970618 | 113178523 | 8.84E-07 | 0.18535 |
| | SP17 | AX-105768536 | 3 | 136591650 | 113484242 | 7.08E-06 | 0.16095 |
| | Bourret17 | AX-105759358 | 5 | 24325063 | 44462658 | 5.82E-06 | 0.16604 |
| | Bourret16 | AX-105929368 | 13 | 157389651 | 178662062 | 5.70E-06 | 0.18655 |
| | SP17 | AX-105876346 | 15 | 38533277 | 49324811 | 3.79E-06 | 0.16838 |
| MLM+K+PCA | | | | | | | |
| | Bourret17 | AX-105531030 | 3 | 85505363 | 105482945 | 2.70E-06 | 0.21737 |
| | Bourret17 | AX-105925988 | 3 | 86489659 | 51278297 | 1.76E-06 | 0.22416 |
| | Bourret17 | AX-105709192 | 3 | 87263090 | 51917942 | 1.76E-06 | 0.22416 |
| | SP17 | AX-105705204 | 3 | 129889814 | 4872459 | 1.25E-06 | 0.23529 |
| | Bourret16 | AX-105891155 | 10 | 167173148 | 221096919 | 4.60E-06 | 0.20456 |
| | GT17 | AX-105925592 | 16 | 2751833 | 4266574 | 5.32E-07 | 0.24252 |

### *Candidate genes*

As mentioned above, significant associations were identified on linkage groups 3, 5, 10, 13, 15 and 16. The most relevant were found on two regions of chromosome 3. The first LG3 region was 5.2 Mbp long, delimited by markers AX-105943713 and AX-147199586. The candidate gene analysis was centred on those genes found on or around +/- 250 Kbp to the significant unique SNPs or on the interval delimited by tightly linked clustered SNPs (+/- 250 Kbp). Therefore, the five SNP markers in the 5.2 Mbp region were analysed as follows: (i) AX-105943713/AX-105531030, and AX-105925988/AX-105709192 as two clusters (and their +/- 250 Kbp window), and (ii) AX-147199586 as a single marker and its +/- 250 Kbp window. SNP markers AX-105943713 and AX-105531030 delimited a 18592 bp long area (coordinates from 85486771 to 85505363). Both AX-105943713 and AX-105531030 were found within a putative SWEET sugar transporter (HanXRQr2_Chr03g0103911 and HanXRQr2_Chr03g0103941, respectively) (Table 6 and Table S2). Two additional coding regions in this interval were found, corresponding to putative mitochondrial carrier domain protein (HanXRQr2_Chr03g0103921) and to a putative potassium channel, voltage-dependent, EAG/ELK/ERG (HanXRQChr03g0075331). Very close to this interval (+/- 250000 bp) two putative transcription factors of the C3H (HanXRQr2_Chr03g0103891) and the AS2-LOB (HanXRQr2_Chr03g0103951) families were identified (Table 6 and Table S2). The second LG3 area in the 5.2 Mbp interval showing a cluster of associations was 773431 bp long. It was flanked by SNP markers AX-105925988 and AX-105709192 (coordinates from 86489659 to 87263090), which were not found within a protein coding gene. The AX-105925988 and AX-105709192 (+/- 250000 bp) interval contained 17 protein coding regions (Table S2), three of them (HanXRQr2_Chr03g0104061, HanXRQr2_Chr03g0104071, and HanXRQr2_Chr03g0104081) corresponding to putative putative geraniol 8-hydroxylases (cytochrome P450 genes). Annotation of two of these cytochrome P450 coding regions was corrected in NCBI *Helianthus annuus* Annotation Release 101 and they were grouped as one single locus coding for a 7-ethoxycoumarin O-deethylase (LOC110929042), which was confirmed through Blast searches of its genomic and RNAs sequences. Also in this interval a putative non-specific serine/threonine protein kinase was identified (HanXRQr2_Chr03g0104051) (Table 6 and Table S2). Finally, within the 5.2 Mbp region, single marker trait association for race E (Bourret17) for marker AX-147199586 (position 90700620) was analysed for candidate genes. This SNP was found within a putative R-linalool synthase (HanXRQr2_Chr03g0104971). The AX-147199586 (+/- 250000 bp) area showed three protein coding genes, and among them a putative transcription factor interactor and regulator of the CCHC(Zn) family (HanXRQr2_Chr03g0104961) was identified. The above described candidate genes were those tightly linked to the significant SNPs; however, it is worth mention that exploration of the 5.2 Mbp region in the the AX-

105925988/ AX-105709192 to AX-147199586 interval outside the areas already described revealed an important proportion of protein kinase genes [out of 57 protein coding genes annotated in this region, 12 (21%) corresponded to protein kinases of the RLK-Pelle-LRR-I-1 f, RLK-Pelle-LRR-VIII-1, RLK-Pelle-LRR-XI-1, RLK-Pelle-CR4L, RLK-Pelle-SD-2b, RLK-Pelle-WAK, CMGC-GSK, and CMGC-CDK-CRK7-CDK9 families] (Table 6 and Table S2).

**Table 6.** Summary of the most relevant genes identified as closely associated to significant marker trait associations (detailed information in Table S2).

| Chr | Interval explored | Nº genes[a] | SNP markers (clustered or single-marker associations) | SNP position (bp) (HanXRQr2.0) | Trait | Most relevant genes in the SNP (shaded), in the interval spanned by clustered markers, within a 250 Kb window, or closely located but outside the 250 Kb window (in grey)[b] | Gene start position (bp) (HanXRQr2.0) | Description |
|---|---|---|---|---|---|---|---|---|
| 3 | AX-105943713 to AX-105531030 and their 250 Kb window | 11 | | | | HanXRQr2_Chr03g0103891 | 85268543 | Putative transcription factor C3H family |
| | | | AX-105943713 | 85486771 | Bourret17/SP17 | HanXRQr2_Chr03g0103911 | 85462527 | Putative SWEET sugar transporter |
| | | | | | | HanXRQr2_Chr03g0103921 | 85489385 | Putative mitochondrial carrier domain protein |
| | | | | | | HanXRQr2_Chr03g0103931 | 85489947 | Putative potassium channel, voltage-dependent, EAG/ELK/ERG |
| | | | AX-105531030 | 85505366 | Bourret17 | HanXRQr2_Chr03g0103941 | 85501458 | Putative SWEET sugar transporter [bidirectional sugar transporter SWEET17 (LOC110929598)] |
| 3 | AX-105925988 to AX-105709192 and their 250 Kb window | 17 | | | | HanXRQr2_Chr03g0103951 | 85631556 | Putative transcription factor AS2-LOB family |
| | | | | | | HanXRQr2_Chr03g0104051 | 86256736 | Putative non-specific serine/threonine protein kinase |
| | | | | | | HanXRQr2_Chr03g0104061 | 86283258 | Putative geraniol 8-hydroxylase |
| | | | | | | HanXRQr2_Chr03g0104071 | 86285554 | Putative geraniol 8-hydroxylase [7-ethoxycoumarin O-deethylase (LOC110929042)] |
| | | | | | | HanXRQr2_Chr03g0104081 | 86328955 | Putative geraniol 8-hydroxylase [7-ethoxycoumarin O-deethylase (LOC110929042)] |
| | | | AX-105925988 | 86489659 | Bourret17 | | | |
| | | | AX-105709192 | 87263090 | Bourret17 | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 | Bigger interval from AX-105925988/AX-105709192 to AX-147199586 outside their 250 Kb window | 57 | | | | | | 12 protein kinase genes (Four MDIS1-interacting receptor like kinase 2; one receptor-like protein kinase ANXUR1; one of the CMGC-CDK-CRK7-CDK9 family; one of the RLK-Pelle-SD-2b family; two of the RLK-Pelle-CR4L family, one of the RLK-Pelle-LRR-I-1 family, one of the RLK-Pelle-WAK family, and one of the CAMK-CDPK family) |
| 3 | AX-147199586 and its 250 Kb window | 3 | | | | HanXRQr2_Chr03g0104961 | 90567938 | Putative transcription factor interactor and regulator CCHC(Zn) family |
| | | | AX-147199586 | 90700620 | Bourret17 | HanXRQr2_Chr03g0104971 | 90699125 | Putative R-linalool synthase |

**Table 6 (continued).** Summary of the most relevant genes identified as closely associated to significant marker trait.

| Chr | Interval explored | Nº genesa | SNP markers (clustered or single-marker associations) | SNP position (bp) (HanXRQr2.0) | Trait | Most relevant genes in the SNP (shaded), in the interval spanned by clustered markers, within a 250 Kb window, or closely located but outside the 250 Kb window (in grey)[b] | Gene start position (bp) (HanXRQr2.0) | Description |
|---|---|---|---|---|---|---|---|---|
| 3 | AX-105705204 and its 250 Kb window | 22 | | | | HanXRQr2_Chr03g0116041 | 129875663 | Putative transcription factor interactor and regulator CCHC(Zn) family |
| | | | | | | HanXRQr2_Chr03g0116071 | 129888590 | Putative transcription factor TFIIIC, triple barrel domain-containing protein |
| | | | AX-105705204 | 129889814 | SP17 | | | |
| 3 | AX- AX-105776042 and its 500 Kb window | | | | | | | One Putative protein kinase RLK-Pelle-LRR-I-2 family (HanXRQr2_Chr03g0116831), and three putative transcription factors of the C2H2 family (HanXRQr2_Chr03g0116871), of the Hap3/NF-YB family (HanXRQr2_Chr03g0116881), and of the |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | AX-105776042 | 133359785 | SP17 | | | CCHC(Zn) family (HanXRQr2_Chr03g0116891) |
| 3 | AX-105655280 to AX-105768536 and its 250 Kb window | 35 | | | | HanXRQr2_Chr03g0117711 | 135874296 | Putative mitogen-activated protein kinase STE-STE11 family |
| | | | AX-105655280 | 135970618 | SP17 | HanXRQr2_Chr03g0117741 | 135961367 | Putative 1,4-alpha-glucan branching enzyme |
| | | | AX-105768536 | 136591650 | SP17 | | | |
| 5 | AX-105759358 and its 250 Kb window | 8 | AX-105759358 | 24325063 | Bourret17 | HanXRQr2_Chr05g0200451 | 24028576 | Putative transcription factor interactor and regulator CCHC(Zn) family |
| 10 | AX-105891155 and its 250 Kb window | 20 | AX-105891155 | 167173148 | Bourret16 | HanXRQr2_Chr10g0458681 | 167025903 | Putative transcription factor TIFY family [Protein TIFY 10c (LOC110886429)] |
| | | | | | | HanXRQr2_Chr10g0458741 | 167193456 | Putative transcription factor MYB-related family |
| | | | | | | HanXRQr2_Chr10g0458761 | 167211637 | Putative transcription factor MYB family [Transcription factor MYB3 (LOC110883374)] |

**Table 6 (continued).** Summary of the most relevant genes identified as closely associated to significant marker trait associations.

| Chr | Interval explored | Nº genesa | SNP markers (clustered or single-marker associations) | SNP position (bp) (HanXRQr2.0) | Trait | Most relevant genes in the SNP (shaded), in the interval spanned by clustered markers, within a 250 Kb window, or closely located but outside the 250 Kb window (in grey)[b] | Gene start position (bp) (HanXRQr2.0) | Description |
|---|---|---|---|---|---|---|---|---|
| 13 | AX-105929368 | 32 | | | | HanXRQr2_Chr13g0610991<br>HanXRQr2_Chr13g0611011<br>HanXRQr2_Chr13g0611021 | 157302732;<br>157322524,<br>157325775 | Three putative non-specific serine/threonine protein kinase genes |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | and its 250 Kb window | | | | | HanXRQr2_Chr13g0611031 | 157334169 | Putative disease resistance RPP13-like protein 1 (LOC110902132) |
| | | | | | | HanXRQr2_Chr13g0611041 | 157358197 | Putative protein kinase CK1-CK1 family |
| | | | AX-105929368 | 157389651 | Bourret16 | HanXRQr2_Chr13g0611081 | 157386420 | Putative splicing factor 3B subunit 5/RDS3 complex subunit 10 |
| | | | | | | HanXRQr2_Chr13g0611091 | 157419694 | Putative cytochrome P450 [Alkane hydroxylase MAH1 (LOC110899957)] |
| | | | | | | HanXRQr2_Chr13g0611131 | 157482837 | Putative cytochrome P450 [Alkane hydroxylase MAH1 (LOC110899958)] |
| 15 | AX-105876346 and its 250 Kb window | 23 | AX-105876346 | 38533277 | SP17 | HanXRQr2_Chr15g0687981 | 38520847 | Putative protein kinase RLK-Pelle-DLSV family |
| | | | | | | HanXRQr2_Chr15g0688011 | 38600086 | Putative transcription factor of the C2H2 family |
| 16 | AX-105925592 and its 250 Kb window | 23 | AX-105925592 | 2751833 | GT17 | HanXRQr2_Chr16g0724281 | 2553825 | Putative transcription factor bHLH family |
| | | | | | | HanXRQr2_Chr16g0724391 | 2748783 | Putative RNA recognition motif domain, mei2/Mei2-like RNA recognition [Protein MEI2-like 1 (LOC110914999)] |
| | | | | | | HanXRQr2_Chr16g0724411 | 2760903 | Putative transcription factor AP2-EREBP family [Ethylene-responsive transcription factor ERF114 (LOC110917506)] |
| | | | | | | HanXRQr2_Chr16g0724481, HanXRQr2_Chr16g0724491, HanXRQr2_Chr16g0724511, HanXRQr2_Chr16g0724531 | 2847073, 2902831, 2945156, 2982242 | Four putative chromatin regulators of the PHD family |

[a] Number of annotated genes in the specified interval.

[b] Shaded candidate genes corresponds to those identified outside the 250 Kb window, because (i) no protein coding regions were found within the window or (ii) the protein coding regions found in the interval were a priori not associated plant disease resistance mechanisms.

[b] In the AX-147199586 250 Kb window no protein coding regions were identified.

The second LG3 region showing a cluster of associations ranged from physical positions 129889814 bp to 136591650 bp (6.7 Mbp long). Due to the distance found between the four SNP markers in this interval, AX-105705204 and AX-105776042 were analysed a single markers and their +/- 250000 window, and AX-105655280, and AX-105768536 as a cluster and their +/- 250000 window. The AX-105705204 +/- 250000 window contained 22 protein coding genes (Table 6 Table S2). Among them and tightly linked to this SNP a putative transcription factor interactor and regulator of the CCHC (Zn) family (HanXRQr2_Chr03g0116041) and a putative transcription factor TFIIIC (HanXRQr2_Chr03g0116071) were found. The AX-105776042 +/- 250000 region had 9 annotated genes (Table S2), which were not clearly a priori associated to plant resistance to pathogens. However, when exploring a larger window of 500 Kb, three tightly linked transcription factors of the C2H2, Hap3/NF-YB, and CCHC(Zn) families were found 300 Kbp upstream this region, and other three of the CCHC(Zn) and C2H2 families were identified 500 Kbp downstream the above mentioned region (Table 6 Table S2). Finally, within the AX-105655280 to AX-105768536 interval (+/- 250000 bp) a putative mitogen-activated protein kinase of the STE-STE11 family was identified (Table 6 Table S2).

Exploration of the genomic region surrounding the unique markers (+/- 250000 bp) of the remaining chromosomes revealed close genes that included proteins that might be associated with disease resistance functions as a putative transcription factor interactor and regulator of the CCHC(Zn) family (HanXRQr2_Chr05g0200451) in chromosome 5; three putative transcription factors of the TIFY and MYB families (HanXRQr2_Chr10g0458681, HanXRQr2_Chr10g0458741, HanXRQr2_Chr10g0458761) in chromosome 10; five protein kinases, a putative virus X resistance protein-like, two putative transcription factor interactor and regulator of the CCHC(Zn) family and five putative cytochrome P450s (two of them renamed as alkane hydroxylases MAH1) in chromosome 13; a putative protein kinase of the RLK-Pelle-DLSV and a putative transcription factor of the C2H2 family in chromosome 15; and two putative transcription factors (of the bHLH and AP2-EREBP families) and four clustered putative chromatin regulators of the PHD family in chromosome 16 (Table 6 Table S2).

## DISCUSSION

Resistance to sunflower broomrape in commercial hybrids is mainly qualitative, controlled by dominant alleles at major genes. However, this type of resistance is easily surpassed by the parasite, leading to a continuous race evolution that makes it difficult the control of the parasite by means of genetic resistance (Fernández-Martínez et al., 2015). Alternative

sources of resistance, such as those under quantitative genetic control are required. To that end, genome-wide association study (GWAS) is an optimized approach to identify new genes associated with resistance to broomrape in sunflower.

Using Q matrix, K matrix and principal component analysis (PCA), which reduce the computation demand and solve the problems related to type I and type II error rates (Yu et al., 2006), a total of 14 single nucleotide polymorphisms (SNPs) exhibited a significant relation with resistance to sunflower broomrape, located on six sunflower chromosomes. On Chrom 3, two regions were distinguished, the upper one associated to both Bourret and SP populations, while the lowest one was only associated to SP. SNPs on Chrom 5, 10 and 13 were significant for population Bourret too, while those on Chrom 15 and 16 were for SP and GT populations, respectively. Although complete resistance has been found in the analysed material for all the broomrape populations analysed, the trait evaluated (the number of broomrapes per plant), showed mostly a continuous distribution in the accessions analysed. Considering the number of genomic regions identified and their minor effect, this study conformed the involvement of quantitative resistance mechanisms controlled by multiple minor QTLs with a small effect, distributed across the sunflower genome and affecting the number of broomrape shoots per plant, in genetic resistance to broomrape in sunflower, as described previously by Pérez-Vich et al. (2004), *O. cumana*. The complementary use of major genes with resistance mechanisms under quantitative genetic control has been proposed as an approach for developing more durable genetic resistant to sunflower broomrape (Pérez-Vich et al., 2013). A greater durability of such polygenic resistance compared to monogenic resistance has been demonstrated in other pathosystems involving viruses, fungi and nematodes (Brun et al., 2010; Fournet et al., 2013; Palloix et al., 2009). All these studies have observed a higher breakdown of the major resistance gene when it was introgressed into a susceptible genetic background compared to a partially resistant one, probably because of the protective effect of the partially resistant genetic background on the major gene.

Most of the significant markers found in this study were located in two different regions of LG 3. In this LG, genes conferring resistance to sunflower broomrape have been reported. Thus, Tang et al. (2003) and Pérez-Vich et al. (2004) identified on the upper half of LG3 the gene *Or5*, conferring major resistance to sunflower broomrape race E. Additionally, Imerovski et al. (2019) identified two regions in LG 3 associated with broomrape resistance: the region between 31.9 and 38.48 Mb (from HanXRQr1.0 assembly), named as QTL *or3.1* by the authors, which coincides with the same region where $Or_5$ was found, and the region between 97.13 and 100.85 Mb (from HanXRQr1.0 assembly), named QTL *or3.2*. The study of Imerovski et al. (2019) was based on bi-parent genetic populations and therefore analytical

and experimental procedures were completely different to those used in this study. In addition, these authors used only one broomrape population, race G from Serbia, also different to those evaluated in this research. The fact that in this study two different significant regions on LG3 were also identified, at close proximity of the *or3.1* and *or3.2* QTL intervals (comparing the SNP markers positions from this study using also the HanXRQr1.0 assembly, Table 5) indicates that these two QTL are likely to be the two significant regions detected on LG3 in this study, and that they are stable and expressed over a wide range of environments, analytical procedures and broomrape populations.

Following the same nomenclature of Imerovski et al. (2019), the *or3.1* region in which presumably *Or5* is located was 5.2 Mbp long in this study and spanned from 85.5 to 90.7 Mbp (HanXRQr2.0-SUNRISE)], delimited by markers AX-105943713 and AX-147199586. The *Or5* gene has been widely demonstrated shown to confer resistance to race E (Vranceanu et al., 1980; Sukno et al., 1999; Lu et al., 2000; Tang et al., 2003; Pérez-Vich et al., 2004), and also controls broomrape infection in the new race E from France (Muños and Velasco, unpublished data). It has been suggested that it also confers an incomplete resistance to races of virulence higher than E, acting as a "defeated gene" (Imerovski et al., 2019). In this study, markers in the *or3.1*–*Or5*-5.2 Mbp region were associated to both race $E_{FR}$ (Bourret17) and race $F_{GV}$ (SP17) of broomrape. Although the presence of the *Or5* in the germplasm analysed is not completely known, since, for the majority of the analysed lines, the pedigree and the germplasm to which the different accesions trace back was not public, its presence has been demonstrated in some of the lines used in this study, such as P96 (Pérez-Vich et al., 2004). Thus, the significant effect of *or3.1*–*Or5*-5.2 Mbp might be due to the presence of *Or5* in some of the accessions analysed, which would determine complete resistance for race $E_{FR}$ (Bourret population) and partial resistance for race $F_{GV}$ (SP population). Within the *or3.1*–*Or5*-5.2 Mbp region, three intervals containing 11 (Bourret17 and SP17), 17 (Bourret17) and 3 (Bourret17) candidate genes were identified at or tightly linked to the significant SNPs. Among these candidate genes, two that were respectively identified carrying the significant SNPs AX-105943713 and AX-105531030, were SWEET sugar transporter genes. SWEET (Sugars Will Eventually be Exported Transporters) transporters are mainly involved in the efflux of both mono- and di-saccharides from the site of synthesis to the sink organs, like grains, flowers or roots (Chen et al., 2010, 2012), and they play a critical role in important plant physiological processes such as pollen nutrition, nectar secretion, stress tolerance, phloem transport, and plant-microbe interactions (Jeena et al., 2019). It has been shown that pathogens use these genes as a means to extract sugars for their multiplication, and that SWEET genes are negative regulators of disease resistance (Devanna et al., 2021). During pathogen infection, the pathogen effector molecule TAL (transcription activator-like) precisely binds with a cis regulatory element of the SWEET

gene promoter and modulates its transcription for enhancing the efflux of sugars which are utilized by the pathogens. The loss of pathogen-induced transcriptional motivation alters the plant-pathogen reaction from susceptibility to resistance (Jeena et al., 2019; Devanna et al., 2021). In the context of a plant to plant parasitic relationship, sucrose transfer at the host-parasite interface, in addition to sucrose phloem unloading in the sink tissues of tubercle and shoot represent key processes in the parasite growth (Misra et al., 2019). Although there are not to date previous reports on the role of SWEET genes in resistance in parasitic plant systems, its importance in parasite dcvelopment and sunflower resistance might be considered for future studies. In addition to SWEET genes, the *or3.1*–*Or5*-5.2 Mbp region showed a putative non-specific serine/threonine protein kinase tightly linked to SNP AX-105925988, as well as a total of 12 protein kinase genes. So far, the only cloned gene (*Or7* on LG7) conferring resistance to *O. cumana* in sunflower has been identified as a receptor-like protein kinase gene (Duriez et al., 2019), so that these kinases genes also represent promising candidates for future investigations.

The *or3.2* region from Imerovski et al. (2019), associated to race G, was likely that delimited by AX-105705204 and AX-105768536 in this study, which ranged from physical positions 129.8.0 Mbp to 136.6 bp (6.7 Mbp) (HanXRQr2.0-SUNRISE) and it was exclusively associated to broomrape race $F_{GV}$ (SP17 and SP18) (Table 5). Using biparental populations, Akhtouch et al. (2016) also identified a QTL associated to recessive resistance to the same race $F_{GV}$ in line K-96 (used in this study) on LG 3, flanked by SSR markers ORS338 and ORS10. ORS338 blast searches against the HanXRQr2.0-SUNRISE assembly located this marker at 138.7 Mbp, very close to the *or3.2*- 6.7 Mbp interval from this study. Therefore, this region seems to be detected across environments, populations and it is presumably associated different broomrape races (at least $F_{GV}$ and G). Within the AX-105705204 and AX-105768536 interval, transcription factors of several families were tightly linked to the significant SNPs. Plant transcription factors play roles in diverse biological processes including defense responses to pathogens, in which they regulate genes related to pathogen-associated molecular pattern-triggered immunity, effector-triggered immunity, hormone signaling pathways and phytoalexin synthesis (Seo et al., 2015), and they appear to be hubs targeted by multiple pathogen effectors in diverse ways (Mukhtar et al., 2011). Yang et al. (2020) in their study of the transcriptional profile of the underground interaction between *O. cumana* and two contrasting sunflower genotypes (one susceptible and one resistant) showed that genes related to transcription factors were highly induced in the resistant cultivar after inoculation with a broomrape population of race G from China, while, in contrast, in the susceptible reaction more transcription factor genes were found down regulated than up-regulated.

For the Bourret population, chromosomes 5, 10, 13 also showed significant marker-trait associations. Pérez Vich et al. (2004), Akhtouch et al. (2016), Louarn et al. (2016) and Imerovski et al. (2019) using bi-parent populations, also found QTL in these chromosomes associated to races E, F or G. Although comparisons among the different studies is difficult since in most cases the sequences of the markers flanking the QTL were not published, among these QTL that on Chrom 13 was found to be highly stable across environments evaluated within the same study and associated to different races (E, $F_{GV}$, and G), and comparable in position to the QTL *or13.2* reported by Imerovski et al. (2019). Therefore, it might be a novel target for further investigate quantitative variations in broomrape resistance. Again, transcription factors and protein kinase genes were found tightly linked to the significant SNPs at these regions.

Finally, other two QTL at Chrom 15 and at 16 for the SP (race $F_{GV}$) and GT (race $G_{TK}$) populations, respectively, were detected. For race $G_{TK}$, this was the only significant marker-trait association found, which differed from results found for the other two races evaluated. The effect of a quantitative component determining partial resistance as a result of measuring the total number of emerged broomrapes per plant has been described for race G from Serbia (Imerovski et al., 2019). Although there are not previous reports for the population GT used in this study, the fact that only one marker-trait association was observed for this population was attributed in part to the expression of the race G resistance trait in the two years evaluated, especially in the GT16 assay which that showing the lowest correlation with the others. A putative ethylene responsive transcription factor ERF114 (AP2-ERF) was found tightly linked (at 9070 bp) to the significant SNP on Chrom 16. Interestingly, in the interaction between the parasitic weed *Striga hermonthica* and rice, the systemic acquired resistance (SAR) pathway is regulated by both jasmonic acid (JA) and salycilic acid (SA) in a cross talk mediated by WRKY45 (Mutuku et al., 2015) and regulated by ethylene responsive factor (AP2/ERF) transcription factor (Licausi et al., 2013), and, additionally, AP2/ERFs were found to be significantly associated with *S. hermonthica* resistance in maize in a GWAS study (Adewale et al., 2020).

The markers having significant associations with the *O. cumana* resistance trait could be useful for marker-assisted selection for improved and more durable sunflower resistance. Allelic variations at each significant SNP were associated 14 to 24% of the phenotypic variance, suggesting that they could be used to this end. Quantitative variation is more difficult to manage in breeding programs than major genes, but the resources developed in this study will contribute enhanced levels of resistance through gene and QTL pyramiding, especially for the two closely linked regions on Chrom 3.

In sunflower, several GWAS studies have been conducted in sunflower on traits such as basal and apical branching (Nambeesan et al., 2015), abiotic stresses (Mangin et al., 2017), flowering time (Bonnafous et al., 2018) or flower morphological traits (Dowell et al., 2019). However, there are no previous studies on the use of GWAS approach to analyse resistance to sunflower broomrape. The present study, using three broomrape populations with contrasting degree of virulence, revealed several genomic regions that are associated with broomrape resistance. Candidate genes putatively involved in broomrape resistance were identified in these regions. This information will serve as a basis for the identification and characterization of novel broomrape resistance genes of value for developing durable genetic resistance to this parasitic weed.

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## SUPPLEMENTARY MATERIAL

**Table S1.** Accession name, origin, and average number of broomrape shoots per plant for populations SP, Bourret, and GT in two years each.

| Accession | Source | GT_2016 | GT_2017 | Bourret_2016 | Bourret_2017 | SP_2017 | SP_2018 |
|---|---|---|---|---|---|---|---|
| BP | INRA | 5.71 | 37.86 | 49.88 | 20.43 | 38.71 | 11.27 |
| ADV | INRA | 11.50 | 36.43 | 23.50 | 15.86 | 40.14 | 13.27 |
| 97A7 | INRA | 22.57 | 54.86 | 41.00 | 12.57 | 27.00 | 15.71 |
| CT | INRA | 13.00 | 50.71 | 27.00 | 20.29 | 13.29 | 9.59 |
| OJQ | INRA | 24.75 | 64.00 | 75.75 | 58.29 | 41.43 | 13.38 |
| SAB | INRA | 16.00 | 21.57 | 25.00 | 17.67 | 3.29 | 1.48 |
| 92A6 | INRA | 9.33 | 29.43 | 50.00 | 13.14 | 19.29 | 9.73 |
| 2603RM | INRA | 28.00 | 60.43 | 35.50 | 34.86 | 35.14 | 13.23 |
| 2603 | INRA | 25.25 | 77.14 | 28.63 | 23.50 | 23.86 | 10.95 |
| OV | INRA | 17.63 | 54.57 | 50.50 | 18.29 | 9.43 | 1.08 |
| OLF | INRA | 11.86 | 11.00 | 14.00 | 10.14 | 0.86 | 0.04 |
| CAL | INRA | 2.50 | 20.33 | 13.13 | 7.86 | 0.00 | 0.05 |
| LR | INRA | 1.63 | 24.29 | 12.38 | 12.43 | 2.00 | 0.04 |
| FU | INRA | 7.57 | 28.67 | 6.63 | 4.50 | 0.29 | 0.39 |
| PI-578872 | USDA | 15.38 | 63.57 | 23.38 | 34.57 | 26.83 | 4.04 |
| PI-561921 | USDA | 6.86 | 52.29 | 30.60 | 35.29 | 45.29 | 10.76 |
| PI-597369 | USDA | 2.38 | 18.71 | 8.75 | 4.43 | 1.14 | 0.30 |
| PI-650353 | USDA | 11.25 | 79.29 | 21.88 | 51.29 | 50.71 | 15.43 |
| UD | INRA | 9.88 | 14.86 | 11.38 | 5.86 | 0.86 | 3.25 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 84P10C | INRA | 23.25 | 62.00 | 44.88 | 63.50 | 63.14 | 14.17 |
| BH | INRA | 7.25 | 17.71 | 17.25 | 23.00 | 20.71 | 5.63 |
| NEAGRA | INRA | 18.13 | 27.29 | 26.88 | 30.57 | 13.43 | 20.46 |
| CHILI | INRA | 14.13 | 47.43 | 39.38 | 75.86 | 46.14 | 13.67 |
| BAI2C | INRA | 18.00 | 56.50 | 35.50 | 33.40 | 31.00 | 13.46 |
| VAQ | INRA | 18.13 | 23.86 | 0.00 | 1.29 | 19.71 | 10.65 |
| WG | INRA | 11.50 | 32.67 | 45.00 | 31.67 | 19.29 | 11.91 |
| XWQ | INRA | 17.63 | 66.29 | 33.00 | 51.14 | 39.40 | 13.39 |
| PI-578010 | USDA | 10.13 | 33.20 | 31.88 | 35.20 | 21.60 | 10.96 |
| PI-578011 | USDA | 26.88 | 68.57 | 28.43 | 31.17 | 32.71 | 14.35 |
| PI-578873 | USDA | 19.38 | 33.71 | 24.50 | 27.57 | 28.57 | 6.05 |
| PAC2 | INRA | 14.50 | 65.86 | 59.25 | 58.67 | 60.57 | 20.67 |
| PI-578008 | USDA | 6.25 | 26.86 | 34.14 | 8.57 | 5.14 | 1.63 |
| PI-650541 | USDA | 10.38 | 30.71 | 23.38 | 29.71 | 14.57 | 5.56 |
| PI-599980 | USDA | 19.25 | 31.00 | 22.25 | 34.14 | 41.33 | 14.13 |
| PI-548997 | USDA | 12.63 | 61.29 | 24.50 | 35.00 | 14.71 | 10.36 |
| PI-509062 | USDA | 10.75 | 49.50 | 31.29 | 41.29 | 20.71 | 18.67 |
| SF | INRA | 11.88 | 30.00 | 26.38 | 19.14 | 26.50 | 8.09 |
| TJ | INRA | 10.75 | 51.14 | 31.63 | 17.43 | 26.86 | 15.38 |
| VZQ | INRA | 13.57 | 33.57 | 26.57 | 21.83 | 19.29 | 10.63 |
| SQ | INRA | 8.25 | 40.86 | 0.00 | 0.14 | 11.00 | 4.54 |
| SN | INRA | 9.63 | 79.00 | 31.40 | 19.00 | 29.29 | 18.63 |
| DPC6 | INRA | 4.29 | 7.14 | 23.63 | 2.00 | 4.57 | 6.32 |
| WJR1634 | INRA | 6.38 | 38.00 | 6.00 | 6.86 | 0.00 | 0.09 |
| PI-649793 | USDA | 10.13 | 40.14 | 13.50 | 34.71 | 16.57 | 4.00 |
| PI-578009 | USDA | 14.25 | 65.43 | 39.88 | 27.50 | 37.29 | 10.08 |
| PI-509061 | USDA | 8.63 | 54.00 | 42.75 | 45.43 | 27.29 | 10.59 |
| PPR8 | INRA | 18.63 | 50.43 | 28.25 | 50.14 | 28.57 | 10.65 |
| USSCL283 | INRA | 6.75 | 35.43 | 18.13 | 27.43 | 13.29 | 3.74 |
| PSM5 | INRA | 11.88 | 53.29 | 38.75 | 45.83 | 39.71 | 13.10 |
| CD | INRA | 8.25 | 13.33 | 14.40 | 3.00 | 0.29 | 1.33 |
| PST5 | INRA | 14.83 | 67.43 | 60.63 | 42.71 | 31.86 | 5.52 |
| SURES2 | INRA | 2.75 | 26.43 | 17.25 | 19.33 | 3.43 | 0.46 |
| R96 | IAS-CSIC | 3.00 | 19.83 | 13.88 | 14.33 | 2.00 | 0.88 |
| P96 | IAS-CSIC | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| RHA801 | USDA | 10.75 | 32.29 | 11.13 | 9.43 | 0.29 | 0.00 |
| PI-599775 | USDA | 6.38 | 18.00 | 22.50 | 16.29 | 7.57 | 0.54 |
| RHA274 | USDA | 6.63 | 47.29 | 33.13 | 14.50 | 13.86 | 2.32 |
| LC1093 | INRA | 17.50 | 43.71 | 0.25 | 0.71 | 22.86 | 6.43 |
| RHA266 | USDA | 11.50 | 56.80 | 32.13 | 34.29 | 35.71 | 13.64 |
| BAS3C | INRA | 10.00 | 66.43 | 17.25 | 45.14 | 41.43 | 23.25 |
| PSX9 | INRA | 9.63 | 52.43 | 9.00 | 33.50 | 17.29 | 3.82 |
| PAZ2 | INRA | 9.88 | 36.43 | 0.00 | 0.14 | 21.83 | 8.00 |
| PI-560145 | USDA | 6.50 | 42.00 | 29.63 | 18.00 | 20.43 | 4.19 |
| PI-561920 | USDA | 2.00 | 16.57 | 1.13 | 9.86 | 0.00 | 0.46 |

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|---|---|---|---|---|---|---|---|
| PI-543746 | USDA | 5.63 | 54.86 | 34.25 | 45.43 | 29.29 | 2.83 |
| PI-650582 | USDA | 11.57 | 15.17 | 5.00 | 22.33 | 4.57 | 1.29 |
| PI-509060 | USDA | 13.75 | 46.86 | 27.25 | 48.57 | 33.43 | 17.96 |
| PI-650794 | USDA | 12.57 | 45.00 | 20.88 | 40.33 | 25.57 | 11.78 |
| PI-549009 | USDA | 9.13 | 38.29 | 22.75 | 29.86 | 9.57 | 1.71 |
| PI-549006 | USDA | 19.29 | 36.00 | 24.88 | 46.00 | 27.00 | 6.00 |
| PI-509065 | USDA | 6.38 | 34.57 | 7.13 | 13.14 | 5.86 | 0.78 |
| PI-642777 | USDA | 8.13 | 46.14 | 13.38 | 33.43 | 13.71 | 1.26 |
| RHA396 | INRA | 8.88 | 45.71 | 31.75 | 52.57 | 25.14 | 13.50 |
| L86 | IAS-CSIC | 4.00 | 37.71 | 3.38 | 8.86 | 0.14 | 0.00 |
| HA89 | INRA | 8.88 | 17.14 | 12.29 | 14.20 | 0.57 | 2.09 |
| 83HR4 | INRA | 12.50 | 75.86 | 35.38 | 38.17 | 62.00 | 25.04 |
| LR1 | INRA | 2.88 | 23.86 | 19.50 | 23.57 | 4.33 | 0.41 |
| RHA439 | USDA | 2.63 | 7.33 | 19.88 | 3.43 | 3.57 | 1.63 |
| RHA464 | USDA | 0.25 | 2.14 | 2.38 | 0.00 | 0.00 | 0.13 |
| RSCOTT | INRA | 3.25 | 42.00 | 29.75 | 31.57 | 15.86 | 7.90 |
| PI-599764 | USDA | 24.50 | 43.14 | 29.00 | 52.86 | 36.29 | 16.86 |
| PI-655014 | USDA | 8.88 | 42.67 | 9.63 | 25.67 | 18.14 | 6.59 |
| PI-642771 | USDA | 15.00 | 31.43 | 26.88 | 34.14 | 13.43 | 8.32 |
| PI-642775 | USDA | 13.88 | 20.29 | 26.75 | 12.67 | 2.00 | 2.58 |
| PI-650842 | USDA | 12.75 | 46.00 | 38.63 | 35.14 | 51.71 | 13.80 |
| PI-650579 | USDA | 5.25 | 22.00 | 19.88 | 30.57 | 6.29 | 1.88 |
| PI-597373 | USDA | 6.63 | 16.43 | 23.50 | 41.29 | 15.00 | 14.25 |
| J2 | IAS-CSIC | 2.71 | 10.43 | 0.00 | 0.00 | 0.00 | 0.00 |
| J3 | IAS-CSIC | 0.13 | 12.00 | 1.13 | 1.00 | 0.00 | 0.00 |
| PO7-28 | IAS-CSIC | 8.17 | 11.50 | 11.75 | 6.14 | 0.14 | 0.25 |
| PO7-5 | IAS-CSIC | 15.63 | 40.71 | 11.25 | 9.29 | 1.14 | 0.75 |
| PO7-9 | IAS-CSIC | 8.25 | 29.00 | 18.25 | 11.00 | 3.43 | 5.38 |
| PO7-13 | IAS-CSIC | 0.63 | 4.43 | 6.25 | 5.83 | 0.00 | 0.04 |
| PO7-34 | IAS-CSIC | 4.50 | 14.57 | 3.83 | 2.29 | 0.00 | 0.50 |
| PO7-63 | IAS-CSIC | 5.00 | 13.00 | 6.14 | 3.17 | 0.00 | 0.00 |
| PO3-2 | IAS-CSIC | 0.17 | 1.29 | 2.60 | 3.00 | 0.00 | 0.00 |
| STR-1 | IAS-CSIC | 4.20 | 19.00 | 15.33 | 4.00 | 4.33 | 1.93 |
| PO7-61 | IAS-CSIC | 4.67 | 10.00 | 2.40 | 2.86 | 0.00 | 0.08 |

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|---|---|---|---|---|---|---|---|
| PO7-38 | IAS-CSIC | 3.00 | 10.57 | 9.00 | 3.57 | 0.00 | 0.41 |
| B117 | IAS-CSIC | 3.75 | 33.00 | 6.88 | 7.29 | 10.83 | 18.24 |
| AM3 | IAS-CSIC | 3.50 | 39.43 | 17.13 | 20.29 | 0.00 | 0.13 |
| K96 | IAS-CSIC | 9.63 | 20.57 | 0.00 | 0.00 | 0.00 | 0.00 |
| AM2 | IAS-CSIC | 6.57 | 33.14 | 26.13 | 17.71 | 1.57 | 0.43 |
| AM1 | IAS-CSIC | 4.50 | 32.57 | 19.25 | 7.29 | 2.86 | 5.48 |

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# CHAPTER V

# Conclusions

1. A genetic map for the parasitic plant sunflower broomrape (*Orobanche cumana* Wallr.) has been developed. This is the first genetic map developed for this species. Additionally, a gene controlling plant pigmentation (*Pg*) has been mapped. This genetic map will be of great value for future genetic studies on this parasitic plant.

2. The avirulenve/virulence trait in the cross between populations of race $F_{GV}$ and $G_{GV}$ is controlled by alles at a single gene. The $Avr_{Hybrid2}$ locus was mapped 14.9 cM upstream from the upper end of LG 2 of the *O. cumana* genetic map. This study confirmed the existence of a gene-for-gene interaction in the sunflower-*O. cumana* parasitic system and mapped for the first time an avirulene gene in this species. The results will contribute to a better understanding of the interaction and to the development of more durable resistance to *O. cumana* in sunflower.

3. Genome-wide association study (GWAS) using 23,473 SNP markers on a set of sunflower accessions, evaluated with three broomrape populations in two evironments, allowed the identification of 14 SNP markers significantly associated with broomrape resistance. Candidate genes have been identified in the genomic regions spanned by the marhers. The results of this study have confirmed the role of some QTL on resistance to sunflower broomrape and have revealed new ones that may play an important role in the development of durable resistance to this parasitic weed in sunflower.

# CHAPTER VI

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