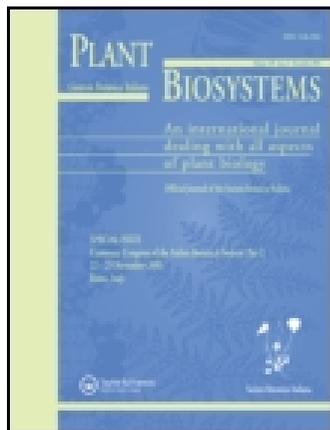


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ORIGINAL ARTICLE

Investigating reproductive incompatibility barriers in a Mediterranean rockrose (*Cistus ladanifer*)

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Abstract

Cistus ladanifer L. (Cistaceae) is a shrubby species, primarily found as a dominant species of the Mediterranean evergreen scrub. Intervarietal (var. *ladanifer* and var. *maculatus*) cross compatibility and self-incompatibility (SI) were studied in six Iberian populations of *C. ladanifer*. In addition, temporal variation in SI across 2 years and variability in the strength of SI throughout the flowering season were analysed in a subset of two populations. The two coloured floral morphs of *C. ladanifer* were compatible, and pollination had statistically similar ability to fruit set when crossing individuals of the two varieties (440 flowers). Results from hand self-pollination indicate negative self-compatibility, regardless of individuals being sampled from different populations, varieties and years (160 flowers in 2003 and 1620 flowers in 2006). In addition, SI strength did not vary along the flowering season. All these results lead us to argue that, in contrast to the results of its sister species (*Cistus salvifolius*), the highly polymorphic *C. ladanifer* possesses an extremely stable SI system but a weak reproductive barrier between varieties.

Keywords: *Breeding systems, Cistaceae, flowering phenology, multipopulation approach, spatial and temporal variation*

Plant self-incompatibility (SI) is the inability of individual plants to produce seeds when they are self-pollinated. It is thought that SI mechanisms have evolved to constrain the selfing that hermaphrodite flowers may enhance (because of the close proximity of anthers and stigmas) and to avoid the deleterious effects of inbreeding (Charlesworth 2006). However, selfing may also be advantageous (i.e. reproductive assurance when pollinators are scarce or colonization of new areas from a unique individual) and the evolutionary transition to self-compatibility has been described as one of the most common mechanism in flowering plants (Stebbins 1974; Barrett 2002). The breakdown of SI to self-compatibility can be rapid and complete (Foxe et al. 2009) or gradual and incomplete (Levin 1996). Species exhibiting quantitative variation among plants in the strength of SI are called partial or pseudo-self-compatible (Levin 1996; Good-Avila et al. 2008). Environmental conditions (such as temperature, light and humidity) (Lewis 1942; Horisaki & Niiikura 2008), developmental factors (such as the time of the flowering season when

a flower is produced and flower ageing) (Stephenson et al. 2000; Travers et al. 2004) or mutations affecting SI genes (Good-Avila & Stephenson 2002) could cause an increase in self-compatibility in populations with functional SI.

Cistaceae are one of the at least 68 angiosperm families that, despite the advantages that self-pollination offers, includes species exhibiting some kind of SI (de Nettancourt 2001; Silva & Goring 2001). Herrera (1992) considered that selfing occurred almost exclusively in annual Cistaceae species. However, as studies on Cistaceae reproductive system increase, a more complex breeding system evolution of Cistaceae genera was observed (i.e. Güemes & Boscaiu 2001; Aragón & Escudero 2008; Carrió et al. 2008). According to Herrera (1992), strict allogamy seems to be predominant within *Cistus* species (Blasco & Mateu 1995; Boscaiu & Güemes 2001; Talavera et al. 2001 and references therein). However, selfing has been reported in Canarian *Cistus* endemics (Gard 1910) and two continental species of *Cistus* are partially self-

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incompatible (Bosch 1992; Blasco & Mateu 1995). Interestingly, Bosch (1992) tested that *C. albidus* and *C. salviifolius* become self-fertile under certain (and still unknown) circumstances.

Although population-level variation in mating systems has been reported in some plant genera (i.e. Busch 2005; Foxe et al. 2010), description of SI for a large number of species has been historically based on a single population per species because of the difficulties in handling a high number of flowers and the high number of angiosperm species to be studied. The same is true for *Cistus ladanifer* from which individuals from a population previously studied were completely self-incompatible (Talavera et al. 1993). However, the fact that *C. ladanifer* is the species of *Cistus* with the highest level of morphological differentiation (three subspecies, variable ovary divisions and two petal colouration patterns) (Demoly & Montserrat 1993; Narbona et al. 2010) suggests potential diversity of breeding system in populations across its wide distribution range. In addition, the sister species (*C. salviifolius*) of *C. ladanifer* (Guzmán et al. 2009) is self-compatible for certain individuals (Bosch 1992). This led us to hypothesize that SI could have been a labile trait in the evolution of this pair species (Guzmán et al. 2009). Therefore, testing multiple populations to examine the potential self-compatibility under different temporal and spatial conditions will give us a better perspective of the breeding system of *C. ladanifer*.

Pollen compatibility is susceptible to be lost as phylogenetic divergence is more profound. Only 16–34% of plant families and 6–16% of genera have at least one reported intergeneric or interspecific hybrid (Rieseberg 1997). Contrarily, the absence of barriers to gene flow for intervarietal crosses is predictable since the varieties are usually based on small morphological differences (Bharathi et al. 2012). Although in some cases, barriers are not completely lost producing some degree of reproductive isolation between co-occurring varieties (Hannan & Prucher 1996). *Cistus ladanifer* × *C. salviifolius* rendered low levels of fertility (Gard 1910; Demoly 1996), which suggests the occurrence of genetic barriers related to taxonomic differentiation. However, Talavera et al. (1993) found compatibility between individuals of the same variety within a population of *C. ladanifer*. The question remains as to when genetic barriers are built up in the course of differentiation of *C. ladanifer*. The only taxonomic character recognized within *C. ladanifer* is the presence of a dark coloured blotch just above a yellowish stain on the petals (var. *maculatus* Dunal) versus the white petals with only the yellowish stain of the type variety (var. *ladanifer*) (Rouy & Foucaud 1895). The varietal divergent phenotypes suggest a genetic control for the dark coloured blotch; there-

fore, the shortage of individuals with intermediate morphs could indicate intervarietal incompatibility. *Cistus ladanifer* offers the opportunity to test when incompatibility appears as a result of morphological divergence. Specifically, the objectives of the present study on *C. ladanifer* were to investigate (1) the existence of SI variation among populations of the same variety; (2) whether expression of SI is related to flowering season; (3) SI variation across 2 years and (4) the intervarietal pollination compatibility.

Materials and methods

Species characteristics and study area

Cistus ladanifer L. (gum rockrose) is an evergreen shrub native to the western Mediterranean region (Southern France, Iberian Peninsula and Northern Africa), where it is found as a dominant species in evergreen shrub (Demoly & Montserrat 1993; Ribeiro et al. 2012). The gum rockrose flowers during spring (March to June) produce a high number (>500) of ephemeral (1–3 days) flowers. Solitary, terminal and large (5–8 cm) flowers of *C. ladanifer* are visited by a wide diversity of generalist insect pollinators (mainly flies, bees and beetles) (Talavera et al. 1993). Successful pollination is promoted by a wide-open floral architecture, the presence of numerous stamens (>100) located around a large and sessile stigma and the presence of a nectar disc under the ovary that produces a low amount of nectar (Herrera 1985; Bosch 1992). *Cistus ladanifer* varieties have exactly the same vegetative characters, being only distinguished by the individual-dependent petal colour polymorphism (Guzmán et al. 2011). Both varieties can occur in monomorphic populations or mixed in the same localities. The size and shade of the dark coloured blotch can be highly variable among individuals; however, individuals with intermediate characters are scarce.

The study was carried out in 2003 and 2006 in six Iberian populations (Table I) where *C. ladanifer* is the dominant species. These populations represent the two taxonomic varieties and two distant areas (Madrid and Huelva provinces).

Intervarietal reciprocal crosses and interpopulation variation in SI

During the spring of 2003, reciprocal crosses between var. *maculatus* and var. *ladanifer* were performed in 10 individuals from four populations located in Sierra de Guadarrama (Madrid, Spain) and two populations located in Andalusia (Huelva, Spain) (Table I). Monotypic populations for petal colour polymorphisms were randomly chosen; however, in those populations (ESC, MAN, ATA

Table I. Sites (ordered by decreasing latitude), geographical parameters, climatic characteristics, infra-specific level and cross-pollination results of the studied populations of *C. ladanifer*.

Population code	Locality	Variety	Latitude	Longitude	Altitude (m a.s.l.)	PREC (mm)	T (°C)	Cross combination (♀ × ♂)	Fruit set (mean ± SE)
ATA	El Atazar, Madrid	<i>ladanifer</i>	40°55'N	3°28'W	1000	692	12.5	ATA × CAB	0.80 ± 0.07
CAB	La Cabrera, Madrid	<i>maculatus</i>	40°52'N	3°37'W	1000	692	12.5	CAB × ATA	0.82 ± 0.11
MAN	Manzanares el Real, Madrid	<i>ladanifer</i>	40°43'N	3°52'W	900	725	12.7	MAN × ESC	0.52 ± 0.10
ESC	El Escorial, Madrid	<i>maculatus</i>	40°34'N	4°07'W	900	637	13.4	ESC × MAN	0.67 ± 0.12
VAL	Valverde del Camino, Huelva	<i>maculatus</i>	37°34'N	6°45'W	250	782	19.2	VAL × HIN	0.37 ± 0.09
HIN	Hinojos, Huelva	<i>ladanifer</i>	37°17'N	6°22'W	80	618	17.1	HIN × VAL	0.55 ± 0.08

Notes: PREC, mean annual precipitation; T, mean annual temperature. Climatic data from the Worldwide Bioclimatic Classification System (<http://www.ucm.es/info/cif/data/indexc.htm>).

and CAB) where both varieties coexist, individuals from the predominant variety were selected. To avoid consanguinity problems and to ensure lack of gene flow via pollen, crossed populations were separated at least 20 km. Crossings are listed in Table I. Four to six flowers per individual were cross-pollinated and two flowers per individual were self-pollinated following the procedure previously used by Gard (1910) and Talavera et al. (1993). Prior to anthesis, flowers were bagged with polyester mesh bags (pore size ca. 2 mm × 2 mm) and tightened around the base of the peduncle. After 1–2 days, when flowers open, bags were removed and hand pollination was performed by applying fresh distant (intervarietal treatment) or self-pollen (self-fertility treatment) to stigmas. Flowers were bagged again with tea bags after pollination and allowed to senesce.

A test of seed germination was carried out in a subset of two populations (HIN and VAL) to explore variability in the percentage of germination between seeds from natural (same variety) and artificial crossings (different varieties). Both populations are monotypic and located about 40 km apart, thus natural pollination can only generate crosses among plants of the same variety. Four fruits (two from natural and two from artificial crossings) from 10 individuals were randomly selected. A total of 150 seeds (three replicates of 50 seeds each) were pretreated with dry heat (10 min at 100°C) (Corral et al. 1990; Pérez-García 1997) before sowing them in a Petri dish with two layers of filter paper. Distilled water was added to each Petri dish and replaced regularly. Seed incubation took place in a germination chamber (IBERCEX, Madrid, Spain) under a 16-h light (23°C)/8-h dark (13°C). After the start of incubation, germinated seeds measured as the proportion of seeds showing radicle emergence (Baskin & Baskin 1998) were scored and eliminated daily.

Intervarietal pollination fruit set was compared with generalized linear models using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). We used a logit link function and binomial error structure, in which the factor “population” was nested within “variety” (Crowley 2007). The same analysis was carried out to compare the percentage of germination between seeds from natural and artificial crossings; factor “individual” was nested within “population” and “fruit” was nested within “individual” and “population”.

Self-pollinations along the flowering season and between years

In the spring of 2006, an SI test was carried out in a subset of two populations (ESC and CAB) to explore whether SI is maintained along its flowering period and between non-consecutive years. Prior to flowering, we randomly selected 32 (CAB) and 37 (ESC)

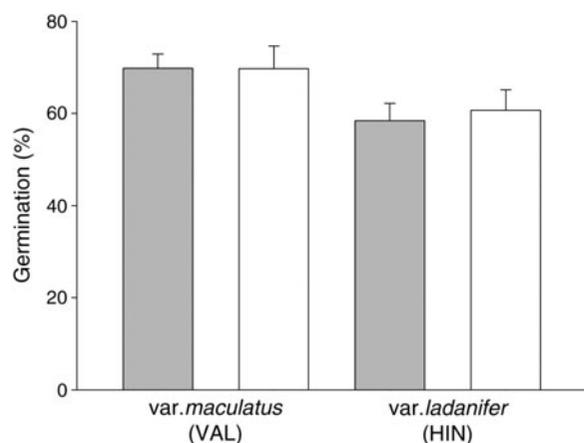


Figure 1. Germination percentages (mean \pm SD) of seeds from natural (same variety, grey bars) and artificial (between varieties, white bars) crossings in *C. ladanifer* var. *maculatus* (VAL) and var. *ladanifer* (HIN) populations.

individuals. In 2006, the selected individuals from the two populations of Madrid flowered in May. We self-pollinated all the newly open flowers of these plants during every day of the flowering period (5th May to 6th June). Hand pollinations were performed following the procedure described earlier. Over 1600 (CAB: 742 and ESC: 878) self-pollinations were made. Six (ESC) and seven (CAB) marked individuals were used in the tests of 2003 and 2006 to check SI variation between years.

Results

Intervarietal reciprocal crosses

The two floral morphs of *C. ladanifer* were clearly compatible. The six crosses performed in 2003 between six Iberian populations of var. *ladanifer* (3) and var. *maculatus* (3) were found to be highly fertile (Table I). Mean fruit set of populations of var. *ladanifer* ranged from 0.55 to 0.80 and var. *maculatus* ranged from 0.37 to 0.82 (Table I). The difference among populations was statistically significant (Wald $\chi^2_{4,54} = 31.26$, $p < 0.001$). Considering all populations together, pollen from both varieties was equally successful in setting fruit on individuals of a different variety (mean fruit set of var. *ladanifer* = 0.63 ± 0.09 and var. *maculatus* = 0.62 ± 0.13 ; Wald $\chi^2_{1,54} = 0.22$, $p = 0.64$).

Seed germination reached high values in both HIN (var. *ladanifer*) and VAL (var. *maculatus*) populations irrespective of the crosses performed (Figure 1). Percentage of germination of seeds from artificial crossings and from natural crossing (same variety) was not significantly different (Wald $\chi^2_{1,179} = 0.67$, $p = 0.41$).

Interpopulation variation in SI and temporal variation in the strength of SI

None of the six populations set any fruit from self-pollinated flowers in 2003. In addition, none of the six (ESC) and seven (CAB) marked individuals set any fruit from self-pollinated flowers in 2006. Moreover, individuals from ESC and CAB populations were self-incompatibly independent of the stage of floral phenology. Only three of the 742 (CAB) and one of the 878 (ESC) self-pollinated flowers set fruit in 2006. However, these four fruits were morphologically abnormal and a very low number of seeds were developed.

Discussion

The percentage of fruit set following hand self-pollination was approximately zero in all populations. The results presented in this comparative study (six populations) revealed that there is no variation in the expression of SI in each individual of *C. ladanifer* either at the population or varietal levels. These results are in agreement with those obtained for a single population of var. *ladanifer* and confirm the previously reported obligate xenogamy of the species due to a gametophytic mechanism of incompatibility (Talavera et al. 1993). Some other Cistaceae woody species (Herrera 1987; Brandt & Gottsberger 1988; Talavera et al. 1997) meet the “strictly allogamous nature of cistaceous shrubs”, hypothesized by Herrera (1992). In contrast, other species of white-flowered (*C. salvifolius*) and purple-flowered (*C. albidus*) lineages (Guzmán et al. 2009) appear to possess a more flexible SI system (Bosch 1992; Blasco & Mateu 1995). Other cases of selfing has been reported in some perennial shrubby species of genera *Helianthemum* (i.e. Aragón & Escudero 2008) and *Fumana* (i.e. Carrió & Güemes 2013). Variation in levels of the strength of SI within and among populations has been well documented for various species (Richards 1986). For example, mutations resulting in suppression in the expression of S-RNase alleles (Tsukamoto et al. 2003a) or S-alleles functionless (Tsukamoto et al. 2003b) seem to be responsible for the pronounced variation in self-fertility of populations of *Petunia axillaris*. In *Campanula rapunculoides*, a heritable (Good-Avila & Stephenson 2002) and positive correlation between self-fertility and floral age (Stephenson et al. 2000) has been documented. Different environmental or developmental conditions along the flowering season of *C. ladanifer* var. *maculatus* do not relax the maintenance of SI, and latest flowers exhibit an expression of SI as strong as first flowers. Reproductive assurance, i.e. seed production when pollinators are scarce, is considered one of the most important

mechanisms that trigger the evolution of self-compatibility from SI (Schoen et al. 1996; Busch et al. 2011). *Cistus ladanifer* are actively visited by a variety of generalist pollinators (Talavera et al. 1993) and usually their populations are monospecific and formed by thousands of individuals. Thus, reproductive assurance is expected to have little incidence in *C. ladanifer* and may help to explain the null spatio-temporal variability in SI found in our study. The SI response of *C. ladanifer* is an effective means of preventing self-fertilization and consequently avoiding negative consequences of inbreeding depression (Lloyd & Schoen 1992; Charlesworth 2006). As the gum rockrose has no other mechanism to elude selfing, as dichogamy or herkogamy, SI could represent substantial advantage for their fitness due to the forced outbreeding (Barrett 2002).

Results of the intervarietal hand pollination demonstrate that the two *C. ladanifer* varieties were highly compatible (producing viable seeds with successful germination), although rendered lower values of fruit set than those between individuals of the same variety (55% vs. 100%) (Talavera et al. 1993). However, this result should be cautiously considered, given that the individuals of the population were not the same and hand-pollination experiments were not identically performed. Differences in reproductive biology, including asymmetrical interfertility relationships, can influence levels of reproductive isolation (Lowry et al. 2008 and references therein). Overlapping flowering times in regions of sympatry and the apparent indifference of insect pollinators to petal colour variation (Guzmán et al. 2011) may permit any pollen flow from any variety of *C. ladanifer* to produce vigorous and fertile offspring (Guzmán & Vargas, unpublished data). Thus, reproductive isolation of any variety is not expected in mixed populations. In fact, no significant differences in fruit set and seed set between plants with blotched and unblotched flowers were found in mixed populations (Guzmán et al. 2011).

In summary, this study provides information on the lack of barriers to gene exchange for intervarietal *C. ladanifer* crosses. In addition, the results herein presented led us to argue that, in contrast to results of its sister species (*Cistus salvifolius*), the highly polymorphic *C. ladanifer* possess an extremely stable SI system, although further studies are needed to disclose the genetic basis of self- and interspecific incompatibility in the rapid evolving *Cistus* (Guzmán et al. 2009).

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