

Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible *Hypochaeris salzmanniana* (Asteraceae)

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- **Background and aims** The transition from outcrossing to selfing is a repeated pattern in angiosperm diversification and according to general theory this transition should occur quickly and mixed reproductive systems should be infrequent. However, a large proportion of flowering plants have mixed reproductive systems, even showing inbreeding depression. Recently, several theoretical studies have shown that mixed mating systems can be stable, but empirical studies supporting these assumptions are still scarce.
- **Methods** *Hypochaeris salzmanniana*, an annual species with populations differing in their self-incompatibility expression, was used as a study case to assess the stability of its mixed reproductive system. Here a descriptive study of the pollination environment was combined with measurements of the stability of the self-incompatibility system, outcrossing rate, reproductive assurance and inbreeding depression in four populations for two consecutive years.
- **Key Results** The reproductive system of populations exhibited a geographical pattern: the proportion of plants decreased from west to east. Pollinator environment also varied geographically, being less favourable from west to east. The self-incompatibility expression of some populations changed markedly in only one year. After selfing, progeny was mainly self-compatible, while after outcrossing both self-incompatible and self-compatible plants were produced. In general, both reproductive assurance and high inbreeding depression were found in all populations and years. The lowest values of inbreeding depression were found in 2014 in the easternmost populations, which experienced a marked increase in self-compatibility in 2015.
- **Conclusions** The mixed reproductive system of *H. salzmanniana* seems to be an evolutionarily stable strategy, with selfing conferring reproductive assurance when pollinator attendance is low, but strongly limited by inbreeding depression. The fact that the highest frequencies of self-compatible plants appeared in the environments most unfavourable to pollination suggests that these plants are selected in these sites, although high rates of inbreeding depression should impede the complete loss of self-incompatibility. In *H. salzmanniana*, year-to-year changes in the frequency of self-incompatible individuals are directly derived from the balance between reproductive assurance and inbreeding depression.

Key words: Breeding system evolution, inbreeding depression, mixed mating system, pollinator environment, self-incompatibility transmission.

INTRODUCTION

The transition from outcrossing to selfing is a repeated and predominant pattern in angiosperm diversification (Stebbins, 1974; Takebayashi and Morell, 2001; Iqic *et al.*, 2008; Goldberg *et al.*, 2010). Inbreeding depression, the reduction of fitness of selfed progeny relative to that of outcrossed progeny, is the main factor regulating the evolution of breeding systems and maintenance of outcrossing and/or selfing in the species (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). General theoretical models predicted that reproductive systems should evolve towards two extreme reproductively stable situations: either selfing or outcrossing (Jarne and Charlesworth, 1993; Holsinger, 1996). Outcrossing should evolve when the fitness of the selfed progeny is less than half that of the

outcrossed progeny (Jarne and Charlesworth, 1993; Holsinger, 1996); above this threshold value, selfing should evolve due to the numerical transmission advantage (3:2) of a selfing allele over an outcrossing allele (Fisher, 1941; Barrett, 2010; Busch and Delph, 2012). Theory also predicts that the transition from outcrossing to selfing should occur quickly, within hundreds of generations, since plants will quickly purge genetic load and lose inbreeding depression when they start selfing (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). At this point, mixed reproductive systems should be in rapid evolutionary transition towards selfing and thus be infrequent (Winn *et al.*, 2011). However, despite this theoretical framework, at least one-third of the flowering plants studied show mixed reproductive systems (Goodwillie *et al.*, 2005). In fact, Winn *et al.* (2011) shows that average inbreeding depression

of species with mixed reproductive systems is high, indicating that allele purging does not always occur and strongly supporting the idea that many mixed mating systems are evolutionarily stable. Other recent theoretical studies also predict a number of evolutionarily stable mixed reproductive systems (Vallejo-Marín and Uyenoyama, 2004; Porcher and Lande, 2013).

Genetic self-incompatibility constitutes a broadly distributed barrier to selfing in angiosperms (Igic et al., 2008). When self-incompatibility is broken, an increase in selfing rates may evolve, unless the advantages of selfing are counterbalanced by reduced fitness of selfed progeny due to inbreeding depression (Charlesworth and Charlesworth, 1987; Byers and Waller, 1999). However, self-fertility mutations can be beneficial in populations with few compatible mates and/or severe pollen limitation and may result in stable mixed reproductive systems even if there are high levels of inbreeding depression (Busch and Schoen, 2008; Good-Avila et al., 2008). In any case, in species with delayed autonomous selfing, which occurs after opportunities for outcrossing at the end of floral anthesis, any selfing rate could be beneficial, as it incurs no pollen or seed discount (Lloyd, 1992; Schoen and Lloyd, 1992).

Theoretical consideration of the evolutionary transition from self-incompatibility to self-compatibility indicates that it may be caused by a single factor: selection for reproductive assurance (Lloyd, 1992; Lloyd and Schoen, 1992; Herlihy and Eckert, 2002; Barrett, 2008; Busch and Delph, 2012). Population traits such as density and size affect pollinator attraction, and reproductive success commonly increases with population size (Groom, 1998; Hendrix and Kyhl, 2000; Hackney and McGraw, 2001; Moeller, 2004) or co-specific density (Fausto et al., 2001; Talavera et al., 2001; Knight, 2003; Moeller, 2004). Mate limitation in small populations increases the probability of population extinction (Cheptou, 2004; Berjano et al., 2013); under these conditions, selection should favour new mutations that disable or modify the self-incompatibility reaction, favouring selfing (Hiscock, 2000a; Busch and Schoen, 2008), and thus an increase in self-compatible plants can be expected. This could be particularly important for self-incompatible annual weeds whose opportunities to set seeds are reduced to one flowering season, and if reproduction fails population survival will be at risk. Therefore, reproductive assurance through selfing is an important adaptation in annual weeds, contributing to their success (Shivanna, 2015).

Sporophytically controlled self-incompatibility systems are widely represented in Asteraceae (Hiscock, 2000b; de Nettancourt, 2001; Ferrer and Good-Avila, 2007). Asteraceae species often show broad variation in the self-incompatibility response. Some species have both strictly self-incompatible and self-compatible populations, while others have partially self-compatible populations in which a mixture of self-incompatible and self-compatible individuals co-occur; even partially self-compatible plants appear in some populations. Thus, in Asteraceae, mixed reproductive systems occur at different levels: species, populations and individuals (Ferrer and Good-Avila, 2007). Although the general models assume that partially self-incompatible populations or individuals represent transient states between full self-incompatibility and full self-compatibility (Levin, 1996), the idea that partial self-incompatibility is a stable condition is gaining strong support in theoretical works (Vallejo-Marín and Uyenoyama, 2004; Goodwillie et al., 2005;

Shivanna, 2015). Given that partially self-incompatible systems confer reproductive assurance, an increase in this mixed system is predicted in the coming decades to cope with pollinator impoverishment due to habitat destruction (Shivanna, 2015). Yet while theoretical works predict a number of factors that could explain evolutionary stability of partial self-incompatibility systems, empirical studies to assess the importance of these factors in natural populations are scarce.

Hypochaeris salzmanniana (Asteraceae) is an endangered annual weed inhabiting sands in coastal ecosystems and adjacent woodlands in northern Morocco (Africa) and southern Spain (Europe) (Ortiz et al., 2004). *Hypochaeris salzmanniana* originated in Morocco and spread northward to Spain (Ortiz et al., 2006) probably during the Pleistocene (Ortiz et al., 2007). The species displays a sporophytic self-incompatibility system, with only a few known populations. Moroccan populations are strictly self-incompatible, but Spanish populations show a mixture of self-incompatible and self-compatible individuals. It has been suggested that a bottleneck during expansion caused loss of genetic variability and the breakdown of self-incompatibility in some populations (Ortiz et al., 2007). All Spanish populations occur in the coastal sands of Cadiz Province, and their level of self-compatibility is higher when nearer to Point Marroquí (Tarifa; Ortiz et al., 2006), the point where the Strait of Gibraltar is narrowest. This area is characterized by recurrent extremely strong winds that increase on approaching the narrowest part of the Strait of Gibraltar. In general, the activity of bees, the main pollinators of *H. salzmanniana*, ceases with winds stronger than 20 km h⁻¹, and only some bee species remain active up to 30 km h⁻¹ (Vicens and Bosch, 2000). Stronger winds are markedly common around the Strait of Gibraltar, and pollinator attendance is most likely to be limited. The flower heads of *H. salzmanniana* show nyctinastic movement; thus, selfing is possible at the end of the day (delayed selfing) in self-compatible individuals. This kind of autonomous self-pollination, which occurs after all opportunities for outcross pollen receipt have passed (delayed selfing), incurs no pollen or seed discounting costs (Lloyd, 1992). Under these circumstances, any self-compatibility mutation would spread, because a self-compatible plant can increase fitness by selfing if not outcrossed. If we assume that transition from outcrossing to selfing should occur quickly, and that Spanish populations of *H. salzmanniana* originated during the Pleistocene, its mixed reproductive system should be stable. These populations constitute an excellent opportunity to assess the importance of reproductive assurance in an uncertain pollination environment and of inbreeding depression as selective forces driving the evolution of a stable mixed reproductive system. Here we combine a descriptive study of the pollination environment with experimental measurements of the stability of the self-incompatibility system, outcrossing rate, reproductive assurance and inbreeding depression in four populations of *H. salzmanniana* during two consecutive years.

MATERIALS AND METHODS

Species and populations studied

Hypochaeris salzmanniana is an annual, endangered species that grows mainly on coastal sands. In field conditions each

plant produces from one to ten heads with 33–86 flowers, all of which are hermaphroditic. Flower heads show nyctinastic movements, and generally they open only for 2–4 h each day for a period of 3–4 d.

We studied four populations in southern Spain (Cadiz province) occurring along a cline from west to east: Conil, Barbate, Zahara and Tarifa. All of them occur on the first sand dune of the coast, with the exception of Barbate, where plants grow on a fixed dune under a forest of *Pinus pinea*. Three of these populations were previously sampled in 2006, when their percentages of self-compatible plants were 50 % at Conil, 40 % at Barbate and 100 % at Zahara (Ortiz et al., 2006).

Pollination environment

In the studied populations, traits related to pollinator attraction were recorded. Plant density was checked in 2014 and 2015 by means of 50-m transects initiated at a point near the middle of each population and oriented to each of the four cardinal points. The total number of plants was counted in two 1-m² plots located every 2 m at each side of the transect. In total, 200 plots per population were checked and population density was calculated as the average number of plants per square metre. Since floral display is highly relevant to pollinator attraction, the number of flower heads per plant in each plot was also recorded.

Wind records at three meteorological stations, one near the Conil population, a second in Barbate and a third near Zahara and Tarifa, were obtained from AEMET (Spanish Meteorology Agency) for each flowering season studied. Although winds stronger than 20 km h⁻¹ preclude the activity of most bees, some of them can fly with winds of up to 30 km h⁻¹. We have considered 30 km h⁻¹ as the limit over which pollinators cannot fly, thus taking a conservative estimate of the days unfavourable for pollinators. For each population and year, we calculated the number of days during the flowering period with winds stronger than 30 km h⁻¹ and the length of the periods (consecutive days) with these stronger winds as indicators of pollinator environment.

To ascertain floral visitation, 15-min censuses by both direct observation and video recording were performed on sunny days with gentle wind (<30 km h⁻¹) throughout the daily anthesis period and the peak of flowering (end of April to early May) in 2014 and 2015. Between 3 and 8 h of censuses per population and year were made, totalling 37 h of censuses. In each census, the number and identity of visitors were recorded, as well as the number of heads visited by each one and the number of open heads in the plot censused, in order to calculate a rate of pollinator visits.

Annual variation in self-incompatibility of populations

To determine the stability of the self-incompatibility system at the population level, 30–40 randomly distributed individuals were selected in 2014 and two flower heads on each plant were tagged and bagged before anthesis in each plant. One bagged head was cross-pollinated with a mixture of pollen from three plants of the population and kept bagged to avoid

fruit loss when ripened. The other bagged flower head was left to self-pollinate automatically; we had previously checked that in bagged heads all the stigmas had received self-pollen after their anthesis period. For each plant, the number of flowers, the number of fruits (fruit production) and the fruit-set (proportion of flowers that set fruits) of both self- and cross-pollinated heads were recorded. Only seeded fruits were considered in the estimation of fruit production and fruit-set. In 2015, the same procedure was carried out in the same areas to assess the stability of the self-incompatibility system between the two years.

The index of self-incompatibility (ISI) is usually calculated using the formula $ISI = \text{extent of fruit set in self-pollinated flowers} / \text{extent of fruit set in cross-pollinated flowers}$. Thus, a plant is considered fully self-compatible (FSC) when ISI is ≥ 1 , partially self-incompatible when ISI is ≥ 0.2 but < 1 , mostly self-incompatible when ISI is < 0.2 but > 0 and fully self-incompatible (SI) when ISI is 0 (Zapata and Arroyo, 1978). Since both partially self-incompatible and mostly self-incompatible plants represent mixed reproductive strategies, we will consider them together [partially self-compatible (PSC)]. Moreover, given that FSC plants were not represented in some populations (see the Results section), for most purposes we will consider all plants with any degree of self-compatibility together, i.e. PSC plants plus FSC ones; they will be referred to as SC plants. Anyway, consideration of SC as a unique category seems the most appropriate way to assess reproductive assurance since all these plants can produce some fruits without depending on pollination services.

To ascertain how self-incompatibility is transmitted across generations, in two populations, Barbate and Zahara, fruits from free-pollinated heads were collected from SI plants ($n = 22$ plants in Barbate and $n = 29$ in Zahara), and fruits from both free-pollinated and automatic-self-pollinated (bagged) heads were collected from SC plants ($n = 15$ plants in Barbate and $n = 14$ in Zahara). The fruits from SI plants were strictly outcrossing, automatic self-pollinated fruits were strictly selfing, and those from free-pollinated SC plants were derived from a mixture of outcrossing and selfing. The three types of fruits were sown in similar conditions in glasshouses, and when the resulting plants flowered their self-incompatibility system was assessed as explained above.

Outcrossing rate of populations and reproductive assurance

We estimated the percentage of progeny derived from selfing or outcrossing in SC plants for each population using SSR markers. In 2014 we selected one SI plant and three SC plants per population, and fresh leaves of each plant were dried in silica gel until DNA extraction. For each plant, heads were tagged at anthesis on windless days at peak of flowering. For progeny array analyses, all mature fruits from the tagged heads were collected and 20 of them were randomly selected for germination. In the laboratory, germination reached nearly 100 %, reducing the potential bias in outcrossing rate estimates due to inbreeding depression. Samples of leaves from each of the resulting seedlings were also dried in silica gel to obtain the outcrossing rates by using microsatellite markers. Between seven and 21 seedlings were studied for each SI plant and between ten and 21 seedlings for each SC plant. In each population, leaves from

the selected SI plant and their progeny were used as controls for outcrossing.

Twenty-five microsatellite loci previously characterized and available for *Hypochoeris* species (Mix et al., 2004; Ruas et al., 2009; Lúcio et al., 2011) were tested for amplification following the polymerase chain reaction (PCR) conditions provided with a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). Ten of them produced clear amplicons of the expected size on agarose gels [Hsalz5, Hsalz7, Hsalz12, Hsalz14, Hsalz25, Hsalz30 and Hsalz35 from Ruas et al. (2009); and HrGA-9, HrGA-10 and HrGA-12 from Mix et al. (2004)]. Forward primers were then labelled with fluorescent dyes (VIC, PET, NED and FAM from Applied Biosystems) and PCR products were sequenced on an Applied Biosystems 3730xl DNA Analyzer, using LIZ-500 as internal lane size standard. We selected three highly polymorphic SSR loci (*HrGA-12*, *Hsalz30* and *Hsalz35*) to determine the percentage of outcrossing. First we measured the resolution of outcrossing in seedlings of SI plants in which all progeny resulted from outcrossing. Secondly, we calculated percentages of progeny originating from outcrossing in seedlings of SC plants, by detecting newly acquired alleles that differed from the mother-donor genotype. We measured the multilocus outcrossing rate (t_m) using the program MLTR (Ritland, 2002), obtained using 1000 bootstraps, by resampling progenies among families and by including the known genotype of maternal donors.

Reproductive assurance is usually determined experimentally by comparing the seed production of free-pollinated intact flowers, which can be both cross- and self-fertilized, with those of free-pollinated emasculated flowers on the same plant, which depend on pollinators to be cross-fertilized. The index of reproductive assurance is calculated as [1–(seed set of emasculated flowers/seed set of intact flowers)] (Schoen and Lloyd, 1992; Eckert et al., 2010; Brys et al., 2011). In *H. salzmanniana*, as in many Asteraceae, emasculation of flower heads causes the excretion of latex, which impedes successful pollination; thus an index of reproductive assurance per plant as described above cannot be obtained. To determine the possibility of reproductive assurance in *H. salzmanniana*, we compared the fruit-set in free-pollinated SI individuals, which depend only on pollinators to be cross-fertilized, with that of SC individuals, which can be both cross- and self-fertilized by pollinators, and can be self-fertilized automatically in the absence of pollinators. Given that in Asteraceae each flower has only one ovule, the fruit-set value indicates reproductive success. Thus, we calculated an index of reproductive assurance (RA) for each population and year as [1–(open fruit set of SI plants/open fruit set of SC plants)]. This index shows the relative contribution of individuals capable of producing some seeds through selfing compared with those in which no seed is set by selfing, and it shows the advantage of self-fertilization when mating is limited, giving an idea of the magnitude of reproductive assurance. To this end, we selected 15–32 SI and SC individuals in each population and year; before anthesis, one flower head on each plant was tagged. We tried to mark bud heads at similar phenological stages (estimated by size), but it was not possible to determine whether head anthesis occurred synchronously in each population. Fruit heads were collected when ripe, their unfertilized flowers and developed fruits were counted, and open fruit production and open fruit-set per plant were calculated from these counts.

Annual variation in inbreeding depression of populations

Inbreeding depression at different life-history stages was studied under glasshouse and field conditions. Hand pollinations with self- and cross-pollen were made in the field in SC plants from the four studied populations and years. The two types of treatment were carried out for each plant. The sample size was $n = 10$ plants in Conil, $n = 24$ in Barbate, $n = 26$ in Zahara and $n = 38$ in Tarifa in 2014, and $n = 16$ in Conil, $n = 10$ in Barbate, $n = 44$ in Zahara and $n = 56$ in Tarifa in 2015. Fruit production and fruit-set were calculated for each plant. From each plant, a subset of selfed and outcrossed fruits were sown in Petri dishes and placed in a germination chamber; germinated seedlings were cultivated in a glass-house, and survival to reproductive age was recorded. Another subset of selfed and outcrossed fruits were sown in their natural populations in 2014 and 2015, using fruits from only three populations (Conil, Zahara and Tarifa, 120 of each type per population). Fruits were placed in individual cardboard pots using sand from each natural population as substrate. Each potted seed was treated as an independent experimental unit. Sowing was carried out in November, and plants were harvested at the end of May. Pots were visited every 15 d, and both seed germination and seedling survival were recorded. In 2015 the pots placed in Conil were destroyed and no data were recorded.

Partial inbreeding depression coefficients (δ_i) were calculated at each life-stage recorded (fruit set, δ_f ; germination, δ_g ; and seedling survival, δ_s) for each population and year. These coefficients were calculated using the expression proposed by Ågren and Schemske (1993):

$$\delta_i = (W_{io} - W_{is}) / W_{imax}$$

where W_{io} is fitness derived from outcrossing at a given life-stage, W_{is} is that derived from selfing and W_{imax} is maximum fitness (W_{io} or W_{is}) at this stage. When $W_{io} > W_{is}$, values of δ are positive, indicating inbreeding depression, while when $W_{is} > W_{io}$ values of δ are negative and outbreeding depression may occur. Moreover, in those populations and years with estimates of inbreeding depression throughout life, the cumulative inbreeding depression coefficients (δ_T) were also calculated. To this end, we estimated cumulative fitness (W_T) for each cross type as the number of final survivors from 100 ovules, i.e. $W_T = [100 \times (\text{mean fruit-set}/100) \times (\text{mean percentage of germination}/100) \times (\text{mean percentage of survival}/100)]$. As in this study cumulative fitness from outcrossing (W_{To}) was always higher than cumulative fitness from selfing (W_{Ts}), following Ågren and Schemske (1993), δ_T was calculated by using the expression $\delta_T = (W_{To} - W_{Ts}) / W_{To}$.

Data analyses

Differences in ISI, percentage of self-incompatibility of progeny, pollinator attendance, fruit production, fruit-set, seed germination and seedling survival were analysed using generalized linear models (GLMs). To discover whether there were differences in pollinator attendance (number of visits) or in the self-incompatibility index, we used population and year as fixed factors and took their interaction into consideration. The

TABLE 1. Traits of studied populations of *Hypochaeris salzmanniana* and values of reproductive assurance index in 2014 and 2015

Population traits	Year	Populations (from west to east)			
		Conil	Barbate	Zahara	Tarifa
Plant density (plants m ⁻²)	2014	0.07	1.62	1.02	2.53
	2015	0.11	3.3	2.19	4.98
Heads per plant	2014	8.64	4.5	4.46	2.03
	2015	8.89	4.96	4.21	2.11
Head density (heads m ⁻²)	2014	0.60	7.29	4.54	5.13
	2015	0.97	16.36	9.22	10.5
No. days available to pollination (wind <30 km h ⁻¹)	2014	50	33	21	21
	2015	47	33	22	22
Length of periods with strong winds (d)*	2014	1.57 (1–3)	3.11 (1–6)	3.08 (1–10)	3.08 (1–10)
	2015	1.56 (1–3)	2.33 (1–6)	3.55 (1–11)	3.55 (1–11)
Reproductive assurance index	2014	0.02	0.45	0.03	0.10
	2015	0.30	0.14	-0.02	0.16

*Mean length and range in parentheses

differences in self-incompatibility category of the progeny derived from selfing or outcrossing from both SC and SI plants were analysed with population, type of pollination (selfing or outcrossing) and category of incompatibility system of the mother plant (SC or SI) as principal factors. To analyse the possibility of reproductive assurance, fruit-set was compared using population, self-incompatibility system (SC plants versus SI plants) and year as fixed factors, and taking the three-way interaction into consideration. Similarly, in comparisons of fruit production, fruit-set, seed germination and seedling survival to explore the possibility of inbreeding depression, the factors of population, treatment (selfing or outcrossing) and year were considered fixed, and the three-way interaction was also considered. Data were analysed using GLMs with different link functions and error distributions, depending on the type of response variable modelled. The binomial distribution of errors and the logit link function were used to analyse germination, survival, fruit-set and percentage of self-incompatibility in populations and progeny. Poisson distributions with log link were used to analyse fruit production and pollinator attendance. All these analyses were carried out using the GLM module of SPSS (IBM SPSS Statistic 23, 2015, USA) with type III tests. When GLMs showed significant differences, the means of treatment were compared using *t*-tests based on standard errors calculated from the specific model.

Pearson correlations were calculated between pollinator visits, and flower head density, fruit-set in free pollination and the reproductive assurance index, and also between the index of self-incompatibility and population parameters. The mean values of these parameters per population and year were used in these correlations.

RESULTS

Pollination environment

Plant density differed among populations, increasing from west to east in both 2014 and 2015 (Table 1). Plant size, measured as heads per plant, differed among populations (Wald $\chi^2=175.13, 3$ d.f., $P=0.000$) but not between years (Wald $\chi^2=0.114, 1$ d.f., $P=0.735$). Thus, in both years, the production of heads/plant decreased from west to east (Table 1).

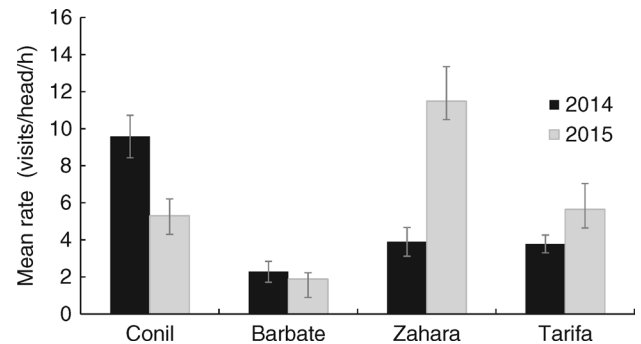


FIG. 1. Mean rate of visits to *H. salzmanniana* plants in the four populations and years studied. Means and standard errors are shown.

The flowering period was about 60 d in 2014 and 56 d in 2015. The number of days favourable to pollinator activity (mean winds <30 km h⁻¹) during the flowering period decreased markedly from west to east, halving in the easternmost populations, closest to the Strait of Gibraltar, compared with the westernmost (Table 1). Moreover, the mean length of periods of consecutive days unfavourable to pollinator activity increased from west to east (Table 1). On sunny windless days, pollinator attendance was generally high. Medium-sized bees were the most common visitors to *H. salzmanniana*. Syrphid and *Bombylius* flies were less frequent, representing <0.5 % of total visits. Pollinator attendance varied significantly among populations but not between years; population \times year interaction was also significant (Table 2). In 2014 the lowest number of visits was found in Barbate, followed by Zahara and Tarifa with fewer than four visits per head and hour (Fig. 1). In 2015 the number of visits increased in Zahara and Tarifa, decreased in Conil and was again lowest in Barbate. The mean number of visits was not correlated with head density ($P > 0.05$).

Annual variation in self-incompatibility of populations

In 2014 most plants in Conil, Barbate and Zahara were SI while in Tarifa PSC plants were the most frequent (Fig. 2). In 2015, in Conil and Barbate SI plants were again the most frequent, but in Zahara and Tarifa the proportion of SI plants

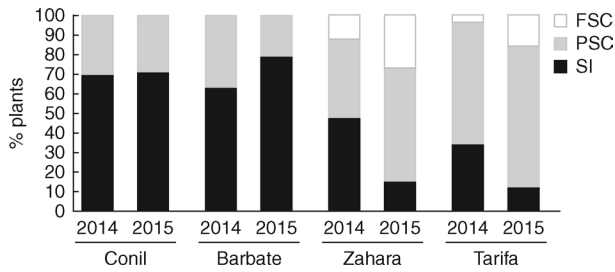


Fig. 2. Proportion of SI, PSC and FSC plants in the four studied populations of *H. salzmanniana* during 2014 and 2015.

decreased and PSC plants were the most frequent. In both years, FSC plants only appeared in Zahara and Tarifa. The mean ISI differed among populations and years (Table 2); the population \times year interaction was also significant, because two populations differed between years. Conil and Barbate populations showed similar ISI in both years ($P > 0.05$), while in Zahara and Tarifa ISI increased markedly in 2015 (Zahara: Wald $\chi^2 = 4.42$, 1 d.f., $P = 0.036$; Tarifa: Wald $\chi^2 = 8.12$, 1 d.f., $P = 0.003$). At population level, mean ISI was positive and significantly correlated only with plant density (Pearson's $r = 0.940$, $n = 8$, $P = 0.001$).

The self-incompatibility category of a progeny depended mainly on the type of pollination from which it came (Wald $\chi^2 = 4.97$, 1 d.f., $p = 0.026$), but not on the incompatibility system of the mother plant (Wald $\chi^2 = 0.17$, 1 d.f., $P = 0.677$) or on population (Wald $\chi^2 = 0.0$, 1 d.f., $P = 0.988$). As a whole, free-pollination in both SI and SC plants produced a mixture of SI and SC plants (67 ± 5.4 % SC). In contrast, the progeny derived from selfing in SC plants was mainly SC (96 ± 3.6 %; Fig. 3).

Outcrossing rate of populations and reproductive assurance

Multilocus outcrossing rate of SC plants calculated from all families in 2014 was 50.2 ± 2.4 % and ranged from 0 to 100 % among all families. Outcrossing rates varied significantly among populations, ranging from a minimum of 13.7 ± 6.3 % in Barbate to 97.7 ± 1.1 % in the Conil population. The remaining two populations exhibited moderate levels of outcrossing: 39.5 ± 4.6 % in Zahara and 62.7 ± 9.7 % in Tarifa. The outcrossing rates found for the control plants (SI plants) ranged from 83.1 ± 12.1 to 100 %. The small number of genetic loci used could be responsible for the failure to detect 100 % outcross progeny in all the control SI plants.

Open fruit-set differed in relation to the self-incompatibility system of the plants among populations, but not between years (Table 2). The SI plants always showed lower fruit set than SC plants (0.87 ± 0.006 versus 0.93 ± 0.005 %), although there were marked differences between populations. Each year, the lowest RA index was found in the population with the highest pollinator attendance (Conil in 2014 and Zahara in 2015; Table 1 and Fig. 1). In 2014 the highest RA index was found in Barbate, the population with fewest pollinators; but in 2015 the highest RA index was found in Conil, where pollinator attendance showed an intermediate value (Table 1 and Fig. 1). Open fruit-set of populations was not correlated with mean number of pollinators visits ($P > 0.05$).

TABLE 2. Summary of GLM results of different effects: population (Conil, Barbate, Zahara and Tarifa), year (2014, 2015), self-incompatibility system (SIS) and treatment (selfing, outcrossing) and their interactions on different traits measured in *Hypochaeris salzmanniana*

Dependent variable	Effects	Wald χ^2	d.f.	<i>P</i>
ISI	Population (P)	78.55	3	0.000
	Year (Y)	6.4	1	0.011
	P \times Y	15.74	3	0.001
Pollinator attendance	Population	193.79	3	0.000
	Year	5.373	1	0.173
	P \times Y	59.264	3	0.000
Open fruit-set (RA index)	Population	266.91	3	0.000
	SIS (SC/SI)	51.588	1	0.000
	Year	0.028	1	0.866
Fruit production (ID index)	P \times SIS \times Y	532.623	10	0.000
	Population	479.13	3	0.000
	Treatment	1120.42	1	0.000
Fruit-set (ID index)	Year	7.078	1	0.008
	P \times T \times Y	378.834	10	0.000
	Population	250.74	3	0.000
Germination (ID index)	Treatment	634.428	1	0.000
	Year	10.889	1	0.001
	P \times T \times Y	180.297	10	0.000
Survival (ID index)	Population	79.098	2	0.000
	Treatment	9.090	1	0.003
	Year	0.723	1	0.395
	P \times T \times Y	36.633	4	0.000
	Population	14.276	2	0.000
	Treatment	2.408	1	0.121
	Year	14.263	1	0.000
	P \times T \times Y	6.868	4	0.231

ID, inbreeding depression; RA, reproductive assurance.

Annual variation in inbreeding depression of populations

In SC plants a mixture of ripe fruits and abortions were found in fruit heads. Fruit production and fruit-set were significantly different among populations, between years and between treatments (Table 2). Fruit production after selfing (18.1 ± 0.5 , $n = 117$) was markedly lower than after outcrossing (57.2 ± 0.9 , $n = 112$). Similarly, fruit-set was 53 ± 1.4 versus 90 ± 0.5 % after selfing and outcrossing, respectively. These trends were found in both years and in all populations, but interactions were significant as there were variations in the magnitude of differences among populations, being lower in Zahara and Tarifa. The lowest inbreeding depression indexes at the fruit-set stage were found in Zahara and Tarifa in both years (Table 3).

In glasshouses, neither germination nor survival differed between treatments or populations; germination was nearly 100 % and all seedlings reached reproductive age. In contrast, germination was generally low in natural populations, ranging from only 14 % in Zahara to 52 % in Conil. Differences were not found between years, but differences between both treatments and populations were significant (Table 2); germination was higher in outcrossed seeds than in selfed seeds (24 ± 2 versus 16 ± 1.7 %). The lowest inbreeding depression at this stage was found in Tarifa in both years (Table 3). Seedling survival was also low and differed among populations (Table 2), ranging from 24 % in Tarifa to 43 % in Zahara. The survival of the seedlings was 27 ± 5.5 % for selfed ones and 40 ± 5.5 %

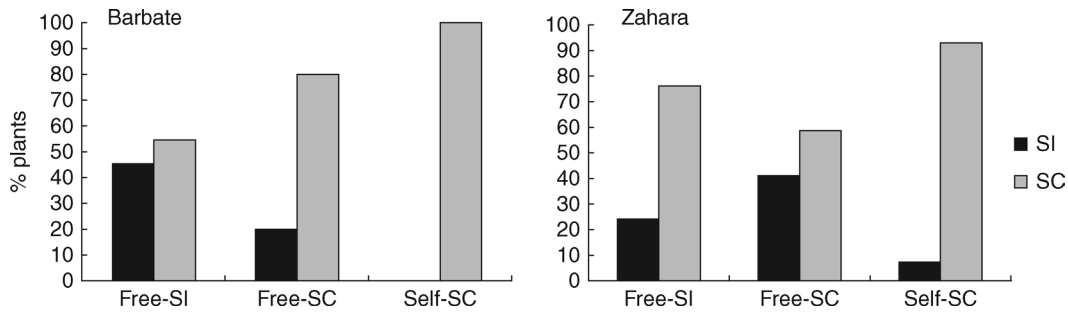


FIG. 3. Compatibility system of F_1 progenies derived from selfing and free pollination of SC plants and from free pollination of SI plants from two wild populations of *H. salzmanniana*.

TABLE 3. Estimates of inbreeding depression in *H. salzmanniana*. W_s and W_o are fitness measures at each stage or total for the life cycle (cumulative), expressed as percentages, after selfing and outcrossing, respectively; δ_i is the inbreeding depression coefficient at each stage or the cumulative value

	Fruit-set				Germination			Survival			Cumulative		
	Conil	Barbate	Zahara	Tarifa	Conil	Zahara	Tarifa	Conil	Zahara	Tarifa	Conil	Zahara	Tarifa
2014													
W_s	44 ± 4.5	40 ± 2.4	64 ± 1.6	61 ± 2.2	41 ± 4.9	9 ± 2.6	27 ± 2.6	37 ± 6.1	17 ± 10	24 ± 7.9	6.67	0.98	3.95
W_o	91 ± 1.0	76 ± 2.1	87 ± 1.0	99 ± 0.3	63 ± 4.8	10 ± 2.7	24 ± 3.9	37 ± 7.5	18 ± 11	13 ± 5.8	21.21	1.57	2.71
δ_i	0.52	0.47	0.26	0.38	0.34	0.10	-0.11	0.0	0.05	-0.45	0.68	0.37	0.38
2015													
W_s	27 ± 6.6	48 ± 3.9	73 ± 1.4	66 ± 1.3	–	13 ± 3.0	8 ± 2.4	–	60 ± 12	22 ± 14	–	5.70	1.16
W_o	81 ± 1.8	77 ± 2.4	94 ± 0.6	85 ± 0.9	–	27 ± 4.1	13 ± 3.1	–	82 ± 6.7	44 ± 12	–	20.8	4.86
δ_i	0.67	0.38	0.22	0.22	–	0.55	0.46	–	0.36	0.5	–	0.72	0.76

for outcrossed ones, although differences were not significant (Table 2), probably due to the low number of survivals. Inbreeding depression at this stage ranged from -0.45 in Tarifa in 2014, to 0.5 for this same population in 2015. Cumulative inbreeding depression varied among populations and between years; it was <0.5 only in Zahara and Tarifa in 2014 (Table 3).

DISCUSSION

In both years studied, the populations nearest to Point Marroquí in Tarifa (the narrowest part of the Strait of Gibraltar) showed a lower proportion of SI plants, as previously reported (Ortiz *et al.*, 2006). In addition to this west-to-east pattern, populations of *H. salzmanniana* varied in self-incompatibility expression between years. In two populations, Zahara and Tarifa, the proportion of SI plants decreased markedly in 2015. Three of the populations studied were also sampled by Ortiz *et al.* (2006), and the percentages of self-incompatibility were also different from those shown here. In one of these populations, Zahara, all plants were SC in 2006, and thus in only a few years a population of an annual species changed from 100 % SC plants to almost 50 % SI plants. This drastic, rapid variation highlights the need for caution when studying self-incompatibility in annual species if only one year is sampled.

Changes in self-incompatibility of populations of *H. salzmanniana* could be directly associated with pollination due to the transmission pattern found in this study when progeny plants were grown in glasshouses. After selfing, practically all

the progeny were SC, while after free pollination the progeny showed both SI and SC breeding systems. This implies that if outcross or compatible pollen is limited in the wild, SC plants will produce a great proportion of progeny for the next year as they can self-pollinate automatically; consequently, the proportion of compatible plants would increase in only one generation. However, the high level of inbreeding depression found at germination and survival stages in the wild would limit this increase. Pollinator attendance was generally high; even in Barbate, where the fewest pollinators were observed, there was a mean of two visits per head and hour on days with gentle winds. Results from microsatellite markers were consistent with those from pollinator attendance, as the lowest outcrossing rate in 2014 was found in Barbate, with the highest in Conil, where pollinators were most abundant. However, outcrossing rates were obtained from heads exposed to pollinators during days with low wind speed. The number of auspicious days for pollinator activity during the flowering period of *H. salzmanniana* decreased markedly from west to east, pollinator activity being possible on only a third of flowering days in easternmost populations. Thus, during the whole flowering period, pollinator attendance is much more limited in Zahara and Tarifa. Moreover, in the easternmost populations, up to ten or 11 consecutive days unfavourable to pollinator activity were recorded in both years; given that a head lasts 3–4 d, its chance of successful pollination depends on coincidence or not with one of these periods. Thus, pollinator environment is not only worse as we go east, but also much more unpredictable. Given the low number of heads per plant found in the easternmost populations,

high variation in pollination success would be expected between plants. In addition, among all populations of *H. salzmanniana* studied by Ortiz *et al.* (2007), those near Tarifa were the most genetically impoverished; these populations are expected to have a lower number of incompatibility alleles, thus being more prone to compatible pollen limitation. Taking into account both pollination environment and self-incompatibility transmission, it is tempting to speculate that the increase in self-compatibility in Zahara and Tarifa in 2015 could be a result of low pollinator attendance in 2014. In contrast to this, the maintenance of high proportions of SI plants in the western populations could reflect higher levels of outcrossing permitted by longer periods of favourable conditions for pollinator activity.

Reports on temporal variation in mating patterns are scarce (Yin *et al.*, 2016) and, to the best of our knowledge, year-to-year variation in self-incompatibility at the population level has not been previously reported in connection with the mating pattern of the preceding year, as seems to occur in *H. salzmanniana*. The genetic basis of self-incompatibility in *H. salzmanniana* remains unknown, but its self-incompatibility system is probably controlled by a multi-allelic S locus, as in other Asteraceae species (Hiscock, 2000a, b). It has been proposed that the presence of PSC plants could be due to low expression levels of S-allele products, or to the action of modifiers that limit the efficacy of the self-incompatibility reaction (Busch and Schoen, 2008). In fact, unlinked modifier loci that modulate the self-incompatibility reaction have been reported in other Asteraceae, such as *Senecio squalidus* and *Chrysanthemum* spp. (Ronald and Ascher, 1975; Hiscock, 2000b; Brennan *et al.*, 2011). Further study is needed to explain and characterize the genetic or physiological mechanism underlying the aforementioned year-to-year variation in *H. salzmanniana*.

Head production per plant decreased from west to east; thus, populations with higher proportions of SI plants also produced higher numbers of flower heads per plant. In Asteraceae, the flower head is the functional unit for pollinators and a decrease in head production in the most compatible populations could be associated with the selfing syndrome. This syndrome causes reduction in flower size, floral display or flower reward of selfers in relation to outbreeders (Richards, 1986; Mena-Ali *et al.*, 2008; Goodwillie *et al.*, 2010; Sicard and Lenhart, 2011) as a consequence of reproductive independence from pollinator visits. This selfing syndrome has been previously suggested in *H. salzmanniana* (Ortiz *et al.*, 2006). However, an alternative explanation is that the lower production of flower heads on SC plants could be a consequence of reduced plant vigour due to the high inbreeding depression of this species.

Plants of *H. salzmanniana* with lower production of floral heads would attract a lower number of visits, given that a positive relationship between floral display size and frequency of pollinator visits has been repeatedly reported (Ohashi and Yahara, 1999). However, differences in flower head production among populations seem to be associated with differences in plant density, since the higher the density the lower the production of flower heads (and thus smaller plants). In the same way, the strong correlation between ISI and plant density within populations shows that density was markedly high in the most SC populations. Thus, the most compatible populations, Zahara and Tarifa, consist of smaller plants at higher densities. Although the low floral display of plants in these populations

could imply lower pollinator attraction, environmental factors such as plant density can modify pollinator behaviour, and a positive relationship between visitation rate and plant density is documented in some studies (Klinkhamer and de Jong, 1990; Grindeland *et al.*, 2005). However, we failed to find a correlation between head density and pollinator attendance; it is possible that other environmental factors, such as habitat suitability for pollinators or interspecific interactions, could be responsible for differences in pollinator attendance. Thus, Barbate, the only population growing under a pine forest, could be less suitable for pollinator activity than open scrublands, or perhaps competition with other species for pollinators is higher.

High levels of inbreeding depression in the fitness of selfed progeny relative to that of outcrossed progeny is the major selective force proposed as maintaining self-incompatibility (Barret, 1998). The four populations of *H. salzmanniana* showed high inbreeding depression rates at an early life stage (fruit-set), although important differences among populations were found. Inbreeding depression at this stage can be distinguished from sporophytic self-incompatibility, as in the latter there is no fertilization and fruit is not initiated. In the two years studied, easternmost populations showed the lowest values of inbreeding depression at an early life stage. Selfed and outcrossed fruits did not show differences in germination or survival in glasshouses, where inbreeding depression was zero. However, in field conditions there were important differences in germination and survival. This is a common pattern in many plant species in which inbreeding depression is usually greater when measured under field conditions than under glasshouse conditions (Cheptou *et al.*, 2000; Armbruster and Reed, 2006). The more favourable conditions of glasshouses, where plants have sufficient water and nutrients as well as low competition, diminish the expression of inbreeding depression, which is context-dependent (Cheptou and Donohue, 2010).

The impact of total inbreeding depression on the fitness of *H. salzmanniana* was generally high, in accordance with that of species that typically outcross (Husband and Schemske, 1996; Armbruster and Reed, 2006), but also with that of species with mixed mating systems (Winn *et al.*, 2011). If the breakdown of self-incompatibility in *H. salzmanniana* took place during the Pleistocene (Ortiz *et al.*, 2007), the relatively high levels of inbreeding depression found in our study are not compatible with a purge of deleterious alleles, nor with consistent selection towards selfing. However, the rates of cumulative inbreeding depression varied drastically among sites and from year to year in the two populations for which germination and survival data were available. In 2014, cumulative inbreeding depression in Zahara and Tarifa was <0.5 (0.37 and 0.38, respectively) but increased markedly in 2015 (0.72 and 0.76). The increase in self-compatibility in these populations in 2015 could be a result of the relatively lower inbreeding depression after selfing in 2014 (given the transmission pattern discussed above). However, the higher fitness of outbreeding plants would prevent the complete shift to selfing in these populations. These results indicate that environmental context is a vital factor for the impact of inbreeding depression, and differences between sites and years on the performance of selfing individuals relative to outcrossers would contribute to the maintenance of a mixed reproductive system in *H. salzmanniana*.

Another factor that could promote selfing is an increase in pollen limitation, under which reproductive assurance gains in importance. Outcrossing rates found in 2014 were in accordance with rates of pollinator visits to *H. salzmanniana*. This indicates that during days with low wind speed, pollinator activity is likely high and sufficient pollen for outcross fertilization is deposited on the flower heads; in fact, the reproductive assurance indexes were lower when pollinator attendance was high. In contrast, during inclement weather automatic self-pollination in SC plants would offer reproductive assurance, giving rise to selfed seeds. The fact that the highest frequencies of SC plants appeared in the easternmost populations suggests that these plants are selected in these unfavourable environments. However, the benefit of reproductive assurance cannot be determined without accounting for fitness costs associated with ovule and pollen discounting and inbreeding depression (Herlihy and Eckert, 2002). Because *H. salzmanniana* shows delayed selfing, pollen and seed discounting costs are probably negligible (e.g. Kalisz et al., 2004); thus, the survival of any progeny produced by delayed selfing would provide reproductive assurance (Kalisz et al., 2004), even under high inbreeding depression as recorded in *H. salzmanniana*.

In conclusion, the mixed reproductive system of *H. salzmanniana* seems to be an evolutionarily stable strategy. We have found spatial and temporal differences in self-incompatibility expression, inbreeding depression and reproductive assurance. Self-incompatible plants were less frequent in populations with consistently worse pollination environment, suggesting that reproductive assurance selects for selfing in these areas. However, the high levels of inbreeding depression limit the evolution of selfing and the complete breakdown of self-incompatibility. In addition, there seems to be a dynamic strategy, with year-to-year changes in frequency of SI individuals in populations of *H. salzmanniana* being the rule.

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