Association between inbreeding depression and floral traits in a generalist-pollinated plant

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Abstract

Individual variation in the magnitude of inbreeding depression (ID) in plants and its association with phenotypic traits may have important consequences for mating system evolution. This association has been investigated only scarcely, and always considering traits functionally related to autogamy. Here, we explore the association between individual variation in ID and plant traits associated with pollinator attractiveness (related to plant size, corolla size and corolla shape) in two populations of *Erysimum mediohispanicum* (Brassicaceae). ID was calculated along the entire life cycle of the plants. In addition, we also explored the relationship between phenotypic traits and the individual levels of heterozygosity. We found significant associations between ID and corolla diameter and stalk height, being taller plants with larger corollas those undergoing a lower intensity of ID. Furthermore, we found a negative relationship between corolla diameter and heterozygosity, suggesting that plants with large flowers have purged their genetic load. Finally, we found a significant effect of corolla diameter on the intrapopulation genetic structure. All these findings suggest that plants with large flowers have secularly suffered frequent inbreeding in the study populations. Because corolla diameter is a trait frequently selected by pollinators in *E. mediohispanicum*, we believe that the observed relationship between this trait and ID could be mediated by pollinators, probably throughout an increasing in biparental inbreeding, geitonogamy or autogamy.

Introduction

The evolution of self-fertilization has been the focus of a long-standing debate (Darwin, 1876; Fisher, 1941; Baker, 1955; Kimura, 1959; Nagylaki, 1976; Lloyd, 1979; Takebayashi & Morrell, 2001). Several genetic and ecological reasons may explain the evolution of selfing in plants. Selfing may evolve because it has an automatic advantage over outcrossing (OC) (automatic selection) with respect to transmission. Whereas out-

crossers pass on only half set of genes to their offspring, selfers transmit their entire set (Fisher, 1941; Nagylaki, 1976; Busch & Delph, 2012). Selfing may also evolve because it allows plants to maintain their reproductive output when mates or pollinators are scarce, a phenomenon called reproductive assurance (Darwin, 1876; Baker, 1955; Lloyd, 1979). Finally, self-pollination may evolve because it is more efficient energetically (Darwin, 1877; Waller, 1979; Aarsen, 2008). Despite these advantages, Goodwillie *et al.* (2005) and Barrett & Eckert (1990) found that only 14% and 25% of 345 and 129 studied plants, respectively, were predominantly selfers (OC rates lower than 20%). Inbreeding depression (ID), the reduced survival and fertility of the offspring of genetically related individuals, was soon

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recognized as a major obstacle to the evolution of self-fertilization (Darwin, 1876; Charlesworth & Charlesworth, 1987). ID occurs as a result of an increase in homozygosity in loci with partially recessive detrimental alleles (partial dominance hypothesis) or in loci with a heterozygous advantage (overdominance hypothesis) (Charlesworth & Charlesworth, 1987; Roff, 2002; Charlesworth & Willis, 2009).

When selfing is frequent and ID is intense due to deleterious alleles, plant populations can be expected to purge their genetic load and thereby counteract ID (Husband & Schemske, 1996; Byers & Waller, 1999; Crnokrak & Barrett, 2002). Decreasing levels of ID via efficient purging of the genetic load would favour the transition from OC to selfing in a given population within a relatively low number of generations (Charlesworth & Charlesworth, 1987; Charlesworth et al., 1990). That is easier in organisms in which recessive deleterious mutations compound the genetic load, which use to be expressed in early life-history stages (Husband & Schemske, 1996). Recent evidence suggests that genetic purging may occur over a short time period in many plants (Crnokrak & Barrett, 2002; Roels & Kelly, 2011). Selfing is associated with small flower size (Elle & Carney, 2003) and some floral traits in self-compatible plants, such as decreased spatial and temporal separation between anthers and stigmas, herkogamy and dichogamy, respectively (Brunet & Eckert, 1998; Takebayashi & Delph, 2000; Elle & Hare, 2002; Takebayashi et al., 2006; Fishman & Willis, 2008; Kalisz et al., 2012). Plant traits may also influence selfing rate with facilitated selfing, because, by their effect on the pollinator attraction, they could increase the amount of self-pollen deposited on stigmas (facilitated self-pollination) or modifying the amount of outcross pollen deposited on stigmas if the amount of self-pollen deposited on stigmas is unchanged (Lloyd & Schoen, 1992). Floral display size or plant size, for example, may increase the selfing rate through geitonogamy (Karron et al., 2004; Williams, 2007; Duminil et al., 2009). Attractive plant traits may also correlate with inbreeding in general when it occurs as a consequence of biparental inbreeding (Ritland, 1984; Goodwillie et al., 2010). Biparental inbreeding, the mating between relatives, is influenced in plants by biotic or abiotic factors that affect the seed and pollen dispersal, which result in the rise of subpopulation structures (Uyenoyama, 1986; Griffin & Eckert, 2003). Flowering phenology, for example, may increase the probability of reproduction between relatives by increasing population structuring and assortative mating (Weis, 2005; Elzinga et al., 2007). Furthermore, species with limited dispersal and spatially structured populations may have reduced genetic neighbourhoods, implying an increase in mating between relatives (e.g. Carrillo-Angeles et al., 2011). Those relatives would be more similar phenotypically and different between individuals of surrounding neighbourhoods, attracting pollinator types differing in behaviour or morphology.

Uyenoyama & Waller (1991a–c) have explored the association between individual ID and modifiers as a consequence of selfing evolution. They found that when ID is caused by deleterious recessive mutations (dominance hypothesis), individuals with traits promoting selfing should exhibit lower levels of ID than individuals with traits that promote OC (Uyenoyama & Waller, 1991a). This is because individuals with selfing-associated traits belong to lineages with a longer history of selfing, which have exposed their deleterious recessive alleles to genetic purging by selection more frequently than more heterozygotic lineages. Consequently, individuals bearing selfing-associated traits would have a lower frequency of deleterious recessive alleles than other individuals in the population, reinforcing the association between trait value and selfing tolerance (Uyenoyama & Waller, 1991a, c). In keeping with the theoretical expectations of Uyenoyama & Waller (1991a), some studies have found that the traits influencing the selfing rate, such as herkogamy, may be associated with a decrease in ID (Takebayashi & Delph, 2000; Stone & Motten, 2002). However, more studies are necessary to address the generality of the association between individual ID and phenotypic traits.

Plant–pollinator systems range from specialist to generalist. Specialized pollination systems have long been recognized as the result of important co-evolutionary processes that promote the effective transfer of outcross pollen by a few pollinators (Stebbins, 1970; Fenster et al., 2004). In contrast, generalist plants are visited by a wide range of flower visitors that differ dramatically in their pollinator effectiveness, foraging behaviour and preference patterns (Gómez & Zamora, 1999; Wilcock & Neiland, 2002; Perfectti et al., 2009). Consequently, the floral traits in generalist plants may undergo contrasting selective pressures both inter- and even intrapopulationally (Gómez et al., 2009a, 2011). In this scenario, plants from the same population but with a different phenotype may be visited by different subsets of floral visitors, with varying preference patterns and pollination efficiencies (Gómez et al., 2011). This intrapopulation variation in pollination environments may produce concomitant intrapopulation variations in selfing rates and, even, genetic structure. Generalist systems are, therefore, appropriate scenarios for exploring any putative association between ID and floral traits that favour both spontaneous autogamy and pollinator-mediated selfing (Uyenoyama & Waller, 1991a). Here, we investigate the association between pollinator-attracting floral traits and ID in a generalist plant, *Erysimum mediohispanicum* (Brassicaceae). Its flowers are visited by over 500 insect species belonging to six orders (Gómez et al., 2014), with varying attributes as pollinators as regards the floral traits they prefer and the magnitude and direction of the selection they exert (Gómez et al.,

2006, 2008a,b, 2009a). Specifically, in this study, we quantify the association between pollinator-attracting floral traits and the individual levels of both heterozygosity and ID.

Materials and methods

Study system

Erysimum mediohispanicum is a monocarpic hermaphroditic herb endemic to the Iberian Peninsula. As a facultative biennial, the plants spend 2–3 years as a rosette in a vegetative stage. After that period, they display anything from a few to several hundreds of flowers located on 1–8 stalks (Gómez, 2003), which can display from few to tens of flowers in anthesis simultaneously. Its flowers produce 20–30 ovules each and they are slightly protandric (Nieto-Feliner, 1993), presenting in the area included in the current study reverse herkogamy (-0.65 ± 0.10, $N = 180$, M. Abdelaziz, A. J. Muñoz-Pajares, F. Perfectti, J. M. Gómez , unpublished). *Erysimum mediohispanicum* is visited by a highly diverse assemblage of insects (Gómez *et al.*, 2007) with contrasting pollination effectiveness and preference patterns (Gómez *et al.*, 2008a, 2009a, b). These pollinators exert significant selection on many floral traits (Gómez *et al.*, 2008a, b).

Experimental design

In September 2009, 60 juvenile plants of *E. mediohispanicum* from two populations (Em25 and Em17; 30 plants per population) in the Sierra Nevada (Spain), located at 2064 and 2182 m a.s.l., respectively, were transplanted to individual pots (11 × 11 × 15 cm) and moved to a common garden at the University of Granada (700 m a.s.l. approx.). At the beginning of May 2010, a total of 30 plants had survived and before they started blooming they were moved to a greenhouse to isolate them from pollinators. We carried out three treatments on each of these plants: (i) OC, in which flowers were emasculated and hand-pollinated with pollen from different individuals from the same population; (ii) hand self-pollination (HS), in which flowers were emasculated before opening and hand-pollinated with their own pollen; and (iii) autonomous selfing (AS), in which flowers were not manipulated and left to self-pollinate spontaneously. This latter treatment was a procedural control for our manipulations. For treatments 1 and 2, we supplied sufficient pollen to ensure fertilizing all ovules per flowers. In addition, because plants were growing in a greenhouse, seed production was not resource-limited. In total, 717 flowers were used in the experiment, with a mean of 22 ± 14 experimental flowers per plant (3 ± 2 OC flowers, 10 ± 6 HS flowers and 10 ± 8 AS flowers). Once the blooming period was over, we recorded the number of flowers per plant and treatment setting ripe fruits or aborted without producing fruits. The total number of ovules, unfertilized ovules, aborted seeds and ripe seeds produced per ripe fruit were recorded in the laboratory using magnifying glasses. Fifteen seeds per plant and treatment were taken at random and sown haphazardly in a greenhouse. Their germination was recorded twice a week during the first month, and seedling survival was recorded every month during the next 10 months.

Quantification of phenotypic traits

A single flower in anthesis per plant was measured on 29 of the 30 experimental plants used in this study (one was excluded because we could not quantify its floral traits during blooming peak, and floral phenotype greatly vary at the end of the flowering period, Nieto-Feliner, 1993). A. J. Muñoz-Pajares, M. Abdelaziz, F. Perfectti, J. M. Gómez (in prep.) have analysed the intra-individual variation in floral traits, by quantifying corolla shape and size in 16 plants from two populations, using 3–16 flowers per plant. By means of a discriminant analysis, it was found that intraspecific variation in these floral traits is negligible. Thus, the following phenotypic traits were quantified per plant: (i) stalk height: the height of the tallest stalk from the ground to the top of the stalk at the end of flowering period; (ii) number of flowers: total number of flowers produced by the plant; (iii) corolla diameter: the distance between the edge of two opposite petals (± 0.1 mm error); (iv) corolla tube width: the diameter of the corolla tube aperture as the distance between the bases of two opposite petals; (v) corolla tube length: the distance between the corolla tube aperture and the base of the sepals; and (vi) corolla shape: determined by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variations in the location, orientation and scale of the specimens (Zelditch *et al.*, 2004). We took a digital photograph of one flower per plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects. We defined 32 coplanar landmarks located along the outline of the flowers and the aperture of the corolla tube, the number of landmarks being chosen to provide comprehensive coverage of the flower shape (see Gómez *et al.*, 2006 and Abdelaziz *et al.*, 2011 for a detailed description of the landmark locations and software used). The two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the generalized Procrustes analysis (GPA) superimposition method (Zelditch *et al.*, 2004). After GPA, the relative warps (RWs, principal components of the covariance matrix of the partial warp scores) were

computed (Zelditch *et al.*, 2004). This procedure generates $2p - 4$ orthogonal RWs (p = number of landmarks). Each RW explains a given variation in shape between specimens. Thus, RWs summarize shape differences between specimens, and their scores can be used as a data matrix to perform standard statistical analyses (Zelditch *et al.*, 2004). The first four shape components (RWs) were associated with similar changes in corolla shape in both experimental and natural plants (Fig. 1), explaining altogether more than 78% of its variance. In all subsequent analyses, we used the first four RWs, because each explained more than 5% of variation in shape.

Estimation of fitness components

The following pre- and post-dispersal components of plant reproductive output were quantified for each treatment and plant: (i) fruit set, the proportion of flowers setting fruit; (ii) seed production, number of seeds produced per fertilized ovule in a given fruit; (iii) seedling emergence, calculated as the proportion of seeds germinating and emerging as seedlings; and (iv) seedling survival, calculated as the proportion of seedlings surviving until the end of the experiment. Afterwards, we calculated the cumulative predispersal absolute fitness (W_{pre}) as fruit set × seed production and the cumulative total absolute fitness (W_{tot}), as fruit set × seed production × seedling emergence × seedling survival. Finally, the total number of flowers (F_1 flower production) was quantified on 85 plants resulting from OC crosses, and on 31 plants and 44 plants coming from HS and AS crosses, respectively.

Estimation of inbreeding depression

We calculated ID (δ) per plant at each fitness component as: $1 - w_S/w_X$, when $w_S < w_X$; and $w_X/w_S - 1$, when $ws > wx$, where w_S and w_X are the fitness associated with HS and OC crosses, respectively (Ågren & Schemske, 1993). Then, we calculated the average δ values in the population of experimental plants for each fitness component. Only for the total number of flowers produced by offspring (F_1 flower production), we averaged the value per treatment (HS and OC) and then we calculated the δ as aforementioned. The statistical significance for every ID values was calculated by bootstrapping, using the packages stats and boot in R (R Development Core Team, 2011).

Estimation of heterozygosity and inbreeding coefficient (F_{IS})

The association between phenotype and heterozygosity was determined during 2007 in 60 additional individu-
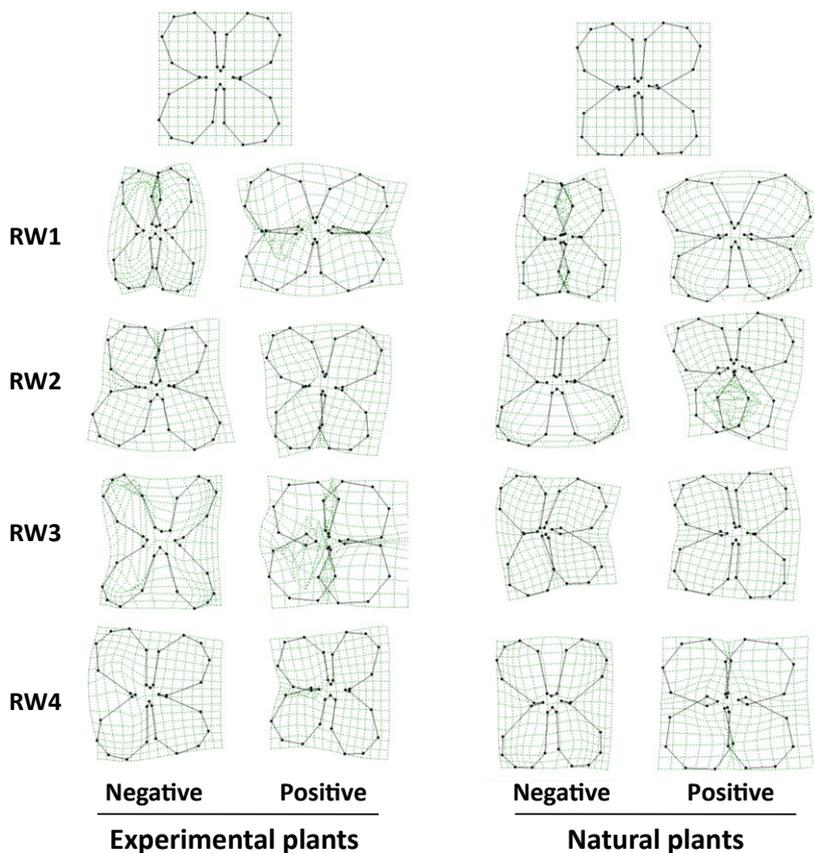
Fig. 1 Summary of the geometric morphometric analyses for the experimental ($N = 29$) and natural plants ($N = 60$) used in this study. Consensus morphology (uppermost panels) and relative warps explaining more than 5% of the overall variation in shape for both groups of plants are shown.

als growing in the same populations in which our experimental plants were collected. For estimating heterozygosity, we collected fresh tissue that was stored in silica gel for their subsequent genotyping, using 10 microsatellite markers described for *E. mediohispanicum* (Muñoz-Pajares *et al.*, 2011). DNA was isolated using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). PCR was performed in 15 μL of reaction mixture containing 0.17 ng μL^{-1} of template genomic DNA, 1 × buffer (ref. M0273S; New England BioLabs, Ipswich, MA, USA), 0.16 mM each dNTP (Sigma-Aldrich), 0.33 μM each forward (fluorescently tagged; Applied Biosystems, Foster City, CA, USA) and reverse primer, and 0.02 U μL^{-1} *Taq* polymerase (ref. M0273S; New England Biolabs). PCR was conducted in a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany) with an initial 30 s of denaturation at 94 °C, 35 cycles at 94 °C for 15 s, annealing temperatures per single microsatellite marker described by Muñoz-Pajares *et al.* (2011) for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 3 min. PCR products were diluted 1 : 15 and analysed by MACROGEN analysers (Geumchun-gu, Seoul, Korea; http://www.macrogen.com) using 400HD ROX (Applied Biosystems) as standard. Alleles were called using Peak Scanner Software version 1.0 (Applied Biosystems), with which we counted the number of heterozygotic and homozygotic loci to calculate the proportion of heterozygotic loci for each individual.

The inbreeding coefficients (F_{IS}) were estimated by means of Bayesian inference using BayesAss v 3.0 (Wilson and Rannala, 2003), for each studied populations, and after obtaining similar positive values for both of them, we estimated the overall F_{IS}. Analysis lasted for 10 million MCMC iterations, sampling each 1000 generations, optimizing the mixing parameter for allele frequencies and for inbreeding coefficients. After that, we removed the first 10% of total iterations and we checked trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the independent Bayesian MCMC runs. The number of multilocus genotypes, the expected and observed heterozygosity, together with the mean number of alleles per locus and mean allelic richness were also calculated per population and with an inclusive analysis for both populations together using the package hierfstat v. 0.04-6 (Goudet, 2005) or using scripts developed by ourselves, both in R (R Development Core Team, 2011).

Data analysis

Between-treatment differences in reproductive output during each life-cycle stage and for the cumulative fitness estimates were tested with the nonparametric Kruskal–Wallis test, using the software JMP 7.0 (SAS, Cary, NC, USA). Generalized linear models (GLM), including populations and their interaction with plant phenotype as random factors, were used to explore the potential relationship between individual ID and the phenotypic traits on the experimental plants. Because no single interaction was significant (Appendix S1), we explored the relationship between ID and the phenotypic traits using Spearman's rank correlation pooling the two populations. We decided to use nonparametric correlations due to our small sample size, and in order to avoid any assumption about linearity in the relationship between variables. Spearman's correlations were also used to explore the correlations between phenotypic traits, and the correlations between the plant phenotypes and their heterozygosity, in each population separately and also pooling the two populations. These analyses were performed using the package stats in R (R Development Core Team, 2011). Finally, we quantified the relationship between intrapopulation genetic structure and corolla diameter using distance matrices by means of a permutational analysis of variance (PERMANOVA) implemented in the vegan package. Bruvo method (Bruvo *et al.*, 2004) was used to estimate the genetic distance matrix between genotyped plants using the polysat package in R.

Results

Reproductive output was significantly higher in outcrossed flowers than in selfed flowers for all fitness components except for *seedling survival* and F_1 *flower production*, where no differences were found (Table 1). However, no difference in reproductive output was found between autonomous and hand self-pollination for any of the life-cycle stages analysed in this study, but for seed production and W_{pre} (Table 1). This indicates that our experimental manipulations did not produce significant side effects and that fruits from autonomous selfing presented slightly, but not significantly, higher number of aborted seed that those from hand-pollinated selfing (AS = 0.47 ± 0.064, HS = 0.55 ± 0.66, $\chi_1^2 = 0.84$, $P = 0.36$). No significant differences in number of unfertilized ovules were found between autonomous selfing and any of the other two treatments (data not shown), being only hand self-pollination and OC the treatments differing in the number of unfertilized ovules (HS = 0.43 ± 0.05, OC = 0.27 ± 0.3, $\chi_1^2 = 5.31$, $P = 0.02$). ID was significant for every life-cycle stage except *seedling survival* and F_1 *flower production* (Table 1). The highest intensity of ID was found for fruit set (0.69), W_{pre} (0.83) and W_{tot} (0.89). Remarkably, there was evident variation in ID between maternal plants (Fig. 2). Thus, 16 of 24 families in W_{pre} and 10 of 14 families in W_{tot} (67% and 71%, respectively) showed ID values higher than 0.8, which means that most families exhibit high levels of ID (Fig. 2).

There was significant positive correlation between corolla diameter and corolla tube length and stalk height, and between stalk height and the number of

Table 1 Performance (mean ± SE) of plants under each of the three treatments carried out in the experiment, and mean of the individual inbreeding depression (δ) values calculated using outcrossing (OC) and hand self-pollination treatment per family (bold values are significant at 95% confidence interval).

Life-cycle stage	Autonomous selfing	Hand self-pollination	OC	χ^2 (AS-HS)	P	χ^2 (HS-OC)	P	δ	95% Confidence interval†
Fruit set	0.24 ± 0.05	0.27 ± 0.04	0.80 ± 0.05	0.35	0.551	29.19	0.000	**0.69**	0.57 to 0.77
Seed production	0.50 ± 0.06	0.29 ± 0.06	0.71 ± 0.04	5.59	0.018	19.55	0.000	**0.58**	0.45 to 0.75
Seedling emergence	0.28 ± 0.07	0.28 ± 0.07	0.61 ± 0.06	0.00	0.990	10.35	0.001	**0.49**	0.34 to 0.79
Seedling survival	0.89 ± 0.06	0.87 ± 0.08	0.93 ± 0.03	0.34	0.558	0.02	0.888	0.13	−0.07 to 0.20
W_{pre}	0.22 ± 0.03	0.10 ± 0.02	0.58 ± 0.04	7.71	0.005	31.94	0.000	**0.83**	0.77 to 0.89
W_{tot}	0.06 ± 0.02	0.05 ± 0.01	0.39 ± 0.05	0.50	0.478	22.99	0.000	**0.89**	0.80 to 0.93
F_1 flower production	97.64 ± 6.21	102.64 ± 9.38	104.92 ± 5.52	0.14	0.706	0.08	0.767	0.02*	−0.15 to 0.20

*δ was calculated for the F1 flower production using the average values for each treatment.
†The 95% confidence interval was obtained by bootstrapping.
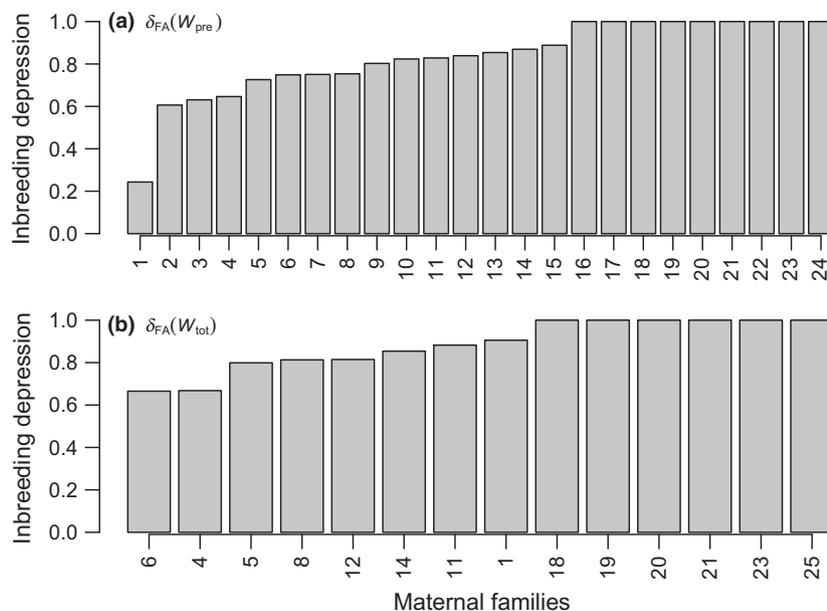
Fig. 2 Differences among *Erysimum mediohispanicum* families in inbreeding depression (ID), calculated as: (a) predispersal absolute fitness (W_{pre}) and (b) cumulative total absolute fitness (W_{tot}). Families are arranged in order of increasing ID.

flowers, in experimental plants (Table 2). No other traits were correlated in the experimental plants (Table 2). However, only two correlations, stalk height vs. number of flowers and corolla diameter vs. corolla tube length, were significant in natural plants (Table 2). Corolla diameter and corolla tube length were the only two phenotypic traits presenting a significant positive correlation considering the complete set of natural plants ($r_s = 0.747$, $P < 0.0001$; Table 2) and when considering both populations separately ($r_s = 0.543$, $P < 0.0028$ in Em17; and $r_s = 0.728$, $P < 0.0001$ in Em25).

We found significant correlation between the intensity of individual ID and some phenotypic traits. Plants with larger corollas suffered lower ID during most of their progeny's life-cycle stages (Table 3 and Fig. 3). Plants with taller flowering stalks and with more flowers also expressed lower ID in at least three and one of the life-cycle stages, respectively (Table 3 and Fig. S1).

Individual heterozygosity was significantly and negatively correlated with corolla diameter and corolla tube length ($r_s = -0.536$, $P < 0.0001$ and $r_s = -0.532$, $P < 0.0001$, respectively) when analysis was carried out using the whole set of natural plants. However, when analysis was performed per population separately, only corolla diameter presented a significant negative correlation with heterozygosity in both populations ($r_s = -0.419$, $P < 0.027$ in Em17 and $r_s = -0.5636$, $P < 0.002$ in Em25). No other phenotypic traits presented a significant correlation with the individual level of heterozygosity.

The genetic diversity was similar in the studied populations (Table 4). In this sense, the observed heterozygosities were lower than expected under panmixia in both populations and when pooling the populations ($H_O = 0.59$ and $H_E = 0.68$, respectively). Inbreeding coefficients showed significant positive values for both

Table 2 Phenotypic trait means and standard errors (SE) associated with the *Erysimum mediohispanicum* plants included in the study: *experimental plants* ($N = 29$) refers to plant flowering at greenhouse conditions; *natural plants* ($N = 60$) refers to plants flowering in the field.

Experimental plants	Mean ± 1 SE	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3
Stalk height	29.71 ± 2.12								
No. flowers	87.69 ± 10.27	0.7034****							
Corolla diameter	11.50 ± 0.31	0.5098**	0.3154						
Corolla tube length	10.36 ± 0.25	0.1808	0.1762	0.4788*					
Corolla tube width	1.19 ± 0.11	−0.1154	−0.1380	0.0239	−0.3684				
RW1	0.01 ± 0.02	0.1987	0.0391	0.3191	0.1443	−0.1090			
RW2	0.00 ± 0.01	0.0822	0.0831	−0.2218	−0.1602	0.1635	0.0376		
RW3	0.00 ± 0.01	0.0840	0.0406	−0.2158	−0.1252	−0.2817	0.0882	0.0012	
RW4	0.00 ± 0.01	0.1884	0.0447	−0.1686	−0.0347	−0.0815	−0.0836	0.0281	−0.0114

Natural plants	Mean ± 1 SE	Stalk height	No. flowers	Corolla diameter	Corolla tube length	Corolla tube width	RW1	RW2	RW3
Stalk height	27.00 ± 1.48								
No. flowers	132.24 ± 12.57	0.3534**							
Corolla diameter	12.32 ± 0.25	0.1024	0.1489						
Corolla tube length	10.96 ± 0.24	0.0984	0.0567	0.7468****					
Corolla tube width	1.36 ± 0.10	−0.2365	−0.0209	0.1894	0.1249				
RW1	−0.01 ± 0.01	0.2313	0.2465	−0.2235	−0.1444	−0.0037			
RW2	−0.01 ± 0.01	−0.2520	0.0779	0.0375	−0.0508	0.2069	0.1043		
RW3	0.01 ± 0.01	−0.2350	−0.1876	−0.1422	0.0498	−0.1053	−0.0917	0.1443	
RW4	0.01 ± 0.01	0.0841	−0.1726	−0.2415	−0.2139	−0.0471	0.1126	−0.2644	−0.1092

Spearman's correlations were calculated between the phenotypic characters at each plant group (*experimental* and *natural*) values between them. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Table 3 Spearman's rank correlation between phenotypic traits and inbreeding depression ($N = 29$) calculated at each life-cycle stage and their cumulative fitness: predispersal absolute fitness (W_{pre}) and cumulative total absolute fitness (W_{tot}).

| Traits	δ_{HS} Fruit set	Seed production	W_{pre}	Seedling emergence	Seedling survival	W_{tot}
Stalk height	**−0.546****	−0.192	−0.370	**−0.609***	0.007	**−0.600***
No. flowers	**−0.500***	−0.138	−0.289	−0.393	−0.240	−0.242
Corolla diameter	**−0.427***	**−0.503***	**−0.562****	**−0.824****	0.362	**−0.850*****
Corolla tube width	−0.286	0.007	−0.100	−0.372	0.065	−0.441
Corolla tube length	0.122	0.196	0.057	−0.092	0.329	0.067
RW1	−0.093	0.000	0.031	−0.190	0.165	−0.170
RW2	0.034	0.405	0.064	0.383	0.225	0.323
RW3	−0.099	0.031	−0.061	0.425	0.164	0.411
RW4	0.193	0.145	0.226	0.102	−0.265	0.083

*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$. Significant values are shown in bold.

populations (Table 4). These results revealed an excess of homozygotes with respect to the Hardy–Weinberg equilibrium. Finally, the PERMANOVA found a low but significant effect of corolla diameter on the intrapopulation genetic structure ($R^2_{1,57} = 0.044$, $P < 0.002$).

Discussion

The magnitude of ID in *E. mediohispanicum* (up to 0.85) was very high, much higher than the values found in most OC and mixed-mating plants (Barrett & Eckert, 1990; Husband & Schemske, 1996; Goodwillie *et al.*, 2005; Winn *et al.*, 2011). This high ID was found even though we performed the experiment in controlled conditions, where the strength of ID is usually significantly smaller than in the field (Dudash, 1990; Willis, 1993; Montalvo, 1994; Koelewijn, 1998). In addition, studies evaluating the impact of ID tend to focus on a single life-history stage, or only a few. However, studies accounting for lifetime ID have shown that this tends to accumulate across life-history stages (Szulkin *et al.*, 2007; Grindeland, 2008; Grueber *et al.*, 2010). In our
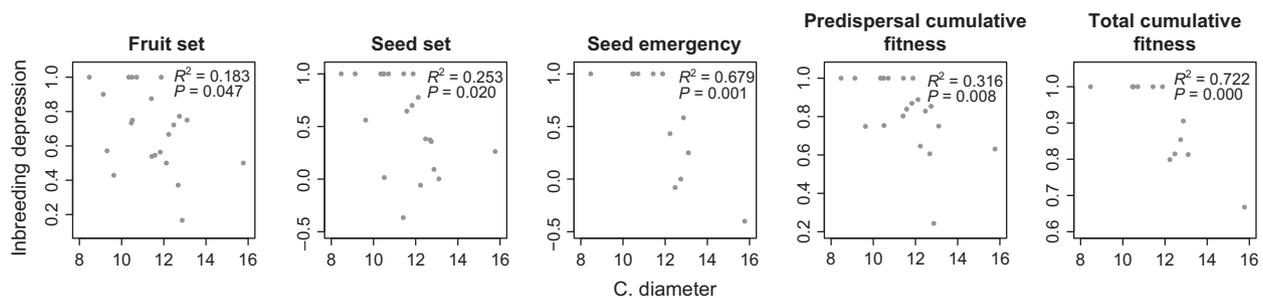
Fig. 3 Correlation between corolla diameter and inbreeding depression across different life-cycle stages. The regression coefficients (R^2) and their *P*-values are given.

Table 4 Genetic diversity parameters and breeding characteristics for *Erysimum mediohispanicum* natural populations included in this study.

Population	N	N_G	n_a	H_O	H_S	R_S	F_{IS}
Em17	30	30 (30)	7 ± 1.12	0.54 ± 0.05	0.67 ± 0.07	6.58 ± 0.97	0.213 ± 0.045
Em25	30	30 (30)	7.8 ± 1.31	0.63 ± 0.04	0.70 ± 0.06	7.39 ± 1.30	0.363 ± 0.248
Total	60	60 (60)	7.4 ± 0.85	0.59 ± 0.03	0.68 ± 0.04	6.98 ± 0.79	–

For each population, parameters include: number of genotyped plants (N) with 10 nuclear microsatellite loci, number of multilocus genotypes (N_G), mean number of alleles per locus (n_a), mean observed heterozygosity (H_O), mean gene diversity (H_S), mean allelic richness (R_S) and inbreeding coefficient (F_{IS}). Mean and standard deviation (± SD) values for each population and total are also given.

case, we quantified ID during most of the life cycle of the progeny – a further explanation of why ID was so strong. All of these findings suggest that *E. mediohispanicum*, although self-compatible, greatly benefits from outcross pollen and behaves functionally as OC or mixed mating (Goodwillie, 2000). Strong ID would be expected to coexist with large OC rates because the high occurrence of mutation producing recessive deleterious alleles in these populations (Lande *et al.*, 1994). The similar fitness values between AS and HS suggest that pollen self-deposition occurs in *E. mediohispanicum*, reaching the maximum values allowed by its mating system to the self-reproduction. Our results also suggest that although the reproductive assurance is possible, this would be very inefficient, given the strong difference between the selfing and the OC treatments.

The main finding of our study is the significant correlation between the individual level of ID and some phenotypic traits. Takebayashi & Delph (2000) and Stone & Motten (2002) have previously reported association between phenotype and ID. The phenotypic trait associated with ID in those studies was the level of herkogamy, a trait directly affecting the rate of spontaneous autogamy. For this reason, this association between phenotype and ID is explained by invoking the occurrence of genetic purging, mostly in those genotypes exhibiting a lower magnitude of herkogamy and consequently a higher rate of spontaneous autogamy. This option is not possible under the overdominance hypothesis, when individuals with selfing-associated traits would show lower levels of fitness, due to their low level of heterozygosity as a consequence of promoting self-fertilization (Uyenoyama & Waller, 1991b). In our study, the trait associated with ID does not necessarily affect spontaneous autogamy. In contrast, the traits associated with *E. mediohispanicum* ID were corolla diameter and stalk height, two traits affecting the interaction with pollinators. However, these two traits were significantly correlated. Given that the only trait relating with individual heterozygosity was corolla diameter, we think that the observed relationship between ID and stalk height was spurious, produced by its correlation with corolla diameter.

We found that plants with larger corollas underwent a lower intensity of ID. This result might be caused by an effect of ID on phenotype, specifically on corolla diameter. As a reduction in fitness, ID can show effects on not only the survival and fertility of the offspring, but their phenotype, for example, reducing flower sizes. However, we dismiss this explanation because, after comparing the flower sizes of 75 and 147 plants resulting from self- and cross-fertilization, respectively, we found no significant differences ($\chi^2_1 = 1.5$, $P = 0.22$), indicating that the ID did not show a direct effect on flower size in *E. mediohispanicum*. Nonetheless, pollinators may affect the observed association between corolla diameter and ID through two nonexclusive ways: by favouring self-reproduction of individuals with larger corollas or by structuring the plant population based on corolla diameter and causing biparental inbreeding. Pollinators may augment the frequency of self-reproduction in plants with larger corolla by both autogamy and geitonogamy (Lloyd & Schoen, 1992; Barrett & Harder, 1996). In *E. mediohispanicum,* large corollas are

positively associated with floral reward (Gómez *et al.*, 2008b). Under these circumstances, pollinators could optimize their foraging behaviour by spending more time in plants with larger corollas, which are offering more reward (Pyke *et al.*, 1977). This optimal foraging behaviour may indirectly promote higher frequency of self-fertilization in plants with large corolla, favouring their genetic purging of deleterious recessive alleles. As an example, indeed, beetles were found selecting larger corollas on *E. mediohispanicum* (Gómez *et al.*, 2009a). In this sense, because the number of flowers was described as a trait with low heritability (Gómez *et al.*, 2009b) together with the absence of relationship we found between number of flowers per plant and ID, we suggest that autogamy would be more important than geitonogamy to explain the observed relationship between corolla diameter and ID (Barrett & Harder, 1996; Eckert, 2000).

Pollinators may also cause biparental inbreeding if they favour the rise of intrapopulation mating structure, where phenotypically and genetically similar individuals tend to mate with each other more frequently than with the rest of the population because they are visited by a similar subset of pollinators. *Erysimum mediohispanicum* flowers are visited in any population by many diverse insects varying in foraging behaviour, effectiveness and preference patterns (Gómez *et al.*, 2007, 2008a, 2009a). Consequently, phenotypically similar individuals are visited by the same subset of insects (Gómez *et al.*, 2011). In fact, corolla diameter is under significant selective pressures in different populations of this plant species (Gómez *et al.*, 2006, 2009a). For example, in a study including 720 plants from eight populations and over 1300 pollinator visits, corolla diameter significantly affected plant fitness in four of the populations (Gómez *et al.*, 2009a). In three of these four populations, small corollas were selected by large bees, small bees and bee flies. However, only beetles, very inefficient pollinators that tend to spend long time foraging in the same flower (Gross, 2005), significantly preferred large corollas (Gómez *et al.*, 2009a) probably because these bigger corollas offer more reward (Gómez *et al.*, 2008b). This reproductive structure probably means that genetically related individuals frequently mate with each other, causing pollinator-mediated biparental inbreeding in this plant species. As the probability of biparental inbreeding depends on the value of various phenotypic traits that influence the identity of the insects visiting the flowers, any genetic purging will be associated with the phenotypic traits mediating biparental inbreeding.

The negative correlation found between corolla diameter and heterozygosity level supports our argument. Thus, we found that plant families with corollas that promote self-fertilization were also more homozygous. This finding may indicate that these families have evolved after successive generations of self-fertilization

(Takebayashi & Delph, 2000). This reduction in heterozygosity would entail the purging of deleterious recessive alleles, producing reproductive lines that endure higher rates of selfing and thus present less ID. This may explain why, despite the high level of ID found in *E. mediohispanicum*, its inbreeding coefficient was fairly high (Table 4), closer to a mixed-mating species than to an OC one. If we consider that the pollinators of *E. mediohispanicum* produced this pattern, we can assert that a significant intrapopulation structure exists, as it is evidenced by the PERMANOVA.

In this sense, it is worth mentioning that different effects are expected to shape the evolution of mating systems and, consequently, the evolution of the phenotypic traits influencing them, depending on the prevalence of selfing or biparental inbreeding in the wild. The classical theoretical models (considering just selfing and OC) predict that populations will evolve from OC to self-fertilization or *vice versa*, attending to the equilibrium between the above-mentioned benefits and costs of producing offspring under these two types of reproductions (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). However, biparental inbreeding has the ability to reduce, in one hand, the advantage of alleles promoting self-fertilization by reducing the genetic costs of OC and, in the other, the fitness advantage of OC progeny by means of genetic purge of selfing progeny (Uyenoyama, 1986; Yahara, 1992). This combination generates a scenario in where mixed-mating systems arise when biparental inbreeding is the main mechanism driving self-reproduction (Griffin & Eckert, 2003). Therefore, in the former general scenario, the phenotypic traits favouring either selfing or OC will vary in frequency depending on the relative advantage of each reproductive strategy. In the latter scenario, in contrast, as selfing and OC coexist under a mixed-mating scenario, the phenotypic traits favouring each of the reproductive strategies will longer co-occur in the populations.

The presence of plants with different levels of ID and, consequently, different degrees of tolerance to selfing in the same population could help to explain the existence of mixed-mating systems (Chang & Rausher, 1999; Goodwillie *et al.*, 2005; Winn *et al.*, 2011). Under low or null ID, theoretical models predict the replacement of OC by selfing as the primary mating system (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987; Charlesworth *et al.*, 1990). In contrast, when ID is high, OC should be the most probable mating system. In this scenario, a mixed-mating population has been considered an evolutionary transition between OC and selfing (Winn *et al.*, 2011), but its high frequency (Barrett & Eckert, 1990; Goodwillie *et al.*, 2005) requires alternative theories to explain the stable levels of mixed-mating systems. Recently, Winn *et al.* (2011), after exploring the relationship between ID and mating system in 68 taxa, suggested that

mixed-mating systems could be explained by selective interference in many natural populations and called for more empirical studies focused on understanding mixed systems in plants. In the present work, we describe an association of a flower trait (corolla diameter) with the degree of tolerance to ID, the individual level of heterozygosity and intrapopulation genetic structure in *E. mediohispanicum*. In this species, hot and cold selection spots have been described on a geographic selective mosaic by contrasting significant selective pressures acting on them (Gómez *et al.*, 2009a, c), due to the differences in pollination effectiveness, foraging behaviour and preference patterns exhibited by its huge flower visitor assemblage (Gómez *et al.*, 2006, 2007, 2008a, b, 2009a). Indeed, in the study area, and associated with the high levels of intrapopulation phenotypic variability exhibited by *E. mediohispanicum* (Gómez *et al.*, 2006, 2009a), up to 44 different pollinator species belonging to six different orders were recorded in a single population (Gómez *et al.*, 2007, 2009a, 2010). This generalist pollination degree at intrapopulation level generates that different phenotypes will attract different types (or species) of pollinator. Under these circumstances, we suggest that these differences could play an important role in the differential inbreeding degrees and subsequent genetic purge experienced by the different families in a given population, producing significant intrapopulation genetic structure. In this respect, plants exhibiting phenotypic traits preferred by pollinators that increase selfing or biparental inbreeding would increase their fitness by genetic purge, thereby reducing their dependence on more effective, abundant or outcross-promoting pollinators.

In summary, in this study, we have shown a significant correlation between individual ID and the value of a floral trait related to pollination in *E. mediohispanicum*. We think that this outcome may be the result of different plant families experiencing contrasting selfing histories, thereby contributing to the long-term stability of mixed-mating systems. Nevertheless, further evidence is needed to demonstrate this hypothesis.

Conflict of interest

The author declares no conflict of interest.

Acknowledgments

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Supporting information

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

Appendix S1 Outcome of the GLMs testing the effect of population on plant reproductive outputs for each trait correlated significantly with ID.