

Long-term progression of inbreeding depression in a Mediterranean ornithophilous shrub

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Abstract

The ornithophilous species *Anagyris foetida* L. is a Mediterranean shrub with highly fragmented populations and a mixed mating system. In a previous study, we analyzed the first 3 years of the life cycle of two progenies (selfed and outcrossed) grown from seed obtained by hand pollination and planted in an experimental garden in 2005. In that study, we found that inbreeding depression (ID) was manifested both reproductively and vegetatively throughout the life cycle, with male reproductive function being the most affected trait. In the present study, our main aim was to check the progression of the two progenies 12 years after transplantation. For this we analyzed their survival and their vegetative and reproductive traits. According to our results, levels of ID were similar to those obtained in the previous study, with some factors decreased and thus varying with the age of the studied individuals. Vegetative parameters were found to have a greater influence than reproductive ones ($\delta = 0.56$ vs. $\delta = 0.36$) on overall ID. As indicated by the global ID ($\delta = 0.72$) the populations have a mating system that is intermediate between outcrossing and a mixed system. Furthermore, the lower male reproductive capacity of the selfed individuals has been maintained over time. Our study also demonstrates the importance of studying the ID value throughout the life cycle of plants.

KEYWORDS

fitness, inbreeding depression, nectar production, phenology, plant survival

1 | INTRODUCTION

The mating system, which is a key determinant of plant population genetic structure, varies from complete selfing to complete outcrossing, with mixed mating systems also occurring relatively frequently (Duminil et al., 2009; Goodwillie et al., 2005; Goodwillie & Knight, 2006; Winn

et al., 2011). One factor modulating the mating system is inbreeding depression (ID), which is defined as fitness reduction due to self-fertilization (selfing) or fertilization between similar or closely related genotypes (inbreeding) (Byers & Waller, 1999; Charlesworth & Charlesworth, 1987; Lloyd, 1980). ID varies among taxa, populations, and individuals, with its level frequently dependent on life history

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(Abdusalam & Li, 2019; Nebot et al., 2020; Webber et al., 2012) and the responsible genetic mechanism. ID may be expressed at different stages of the life cycle and can negatively affect different components of plant fitness, such as seed development (Ågren & Schemske, 1993; Severns, 2003), seed size (Hull-Sanders et al., 2005; Vaughton & Ramsey, 2006), germination (Ishida, 2006; Nebot et al., 2020), seedling size and growth (Ishida, 2006; Karron, 1989; Naito et al., 2005), and reproductive capacity (Busch, 2005; Carr & Dudash, 1997; Ellmer & Andersson, 2004; Ramsey et al., 2003).

Plants that reproduce predominantly by outcrossing are generally expected to exhibit higher ID than selfers during seed production, germination, and pre-reproductive growth stages (Husband & Schemske, 1996). Winn et al. (2011) have reported a lower ID in selfers versus outcrossers, thus supporting the “purging of genetic load” hypothesis (Charlesworth & Charlesworth, 1987; Lande & Schemske, 1985). Some initial theoretical studies (Lande & Schemske, 1985) demonstrated the existence of a bimodal distribution of outcrossing rates, thereby implying that natural selection leads to the evolution of outcrossing and selfing as two discrete strategies (Bartoš et al., 2020). In mainly selfing populations, deleterious recessive alleles could thus be purged by natural selection, whereas a decrease in fitness due to ID would occur in outcrossing populations (Lande & Schemske, 1985). Only two extreme mating systems were therefore assumed to be evolutionarily stable: inbreeding (selfing) with low rates of ID (values of <0.5) and outcrossing with high rates (values of >0.5). A significant proportion of plant species has mixed mating systems (Bartoš et al., 2020; Goodwillie et al., 2005; Goodwillie & Knight, 2006; Griffin et al., 2019), however, and several theoretical models have identified factors that would explain the stability of this phenomenon (Goodwillie et al., 2005; Sachdeva, 2019; Winn et al., 2011). In some cases, taxa with mixed mating systems have been found to have an ID similar to outcrossers at each life cycle stage (Griffin et al., 2019; reviewed in Winn et al., 2011).

Several authors have recently begun to differentiate between primary and secondary mating systems. The first-named system encompasses the initial stages of future progeny (fruit set, seed set, and seed germination), while the second also considers the late stages (survival and reproductive capacity). Populations of taxa with significant rates of selfing seeds and young seedlings therefore include relatively few or no mature selfed individuals, thus implying a high ID between both life stages (Duminil et al., 2016; Griffin et al., 2019; Hardy et al., 2019; Rodrigues et al., 2019). This situation leads to populations that have been considered to possess a mixed mating system because selfing is a major contributor to

seed formation, but in reality, this selfing does not play an important role in maintenance of these populations, which actually have outcrossing mating systems (Griffin et al., 2019).

ID is also an important parameter in endangered species conservation, both for wild populations and for those maintained ex situ. In wild populations, ID is of great importance when they have recently suffered a drastic size reduction or have undergone fragmentation (Byers & Waller, 1999; Hedrick & Kalinowski, 2000; Keller & Waller, 2002). The survival of these populations is threatened by an increase in ID associated with the expression of deleterious recessive or partially recessive alleles. The increased homozygosity responsible for this situation is a consequence of the elevated rate of inbreeding that frequently occurs in small populations because of reduced gene flow and limited pollinator activity (Byers & Waller, 1999; Hedrick & Kalinowski, 2000; Keller & Waller, 2002; Richards et al., 1999; Severns, 2003; Tambarussi et al., 2016). In populations maintained ex situ, which originate from a few individuals, increased selfing due to the founder effect is typically observed; ID levels are therefore initially high and then, over the medium term, become drastically lower because of the purging of deleterious alleles. ID is not only determined by genetic factors, but is also influenced by environment (Hedrick & Kalinowski, 2000; Jiménez et al., 1994; Suárez & Gianoli, 2005). Populations subjected to less stressful conditions should therefore have lower ID values (Armbruster & Reed, 2005), as has been seen in populations growing in greenhouse or controlled environments relative to wild populations (Dudash, 1990; Nebot et al., 2020; Villegas et al., 2020), but some studies have uncovered no relationship between stress intensity and ID (Angeloni et al., 2011; Sandner & Matthies, 2016).

Although numerous studies have focused on ID, few have investigated ID in woody species during most or all of the plant life cycle, and most of the latter (e.g., Griffin et al., 2019) have centered on species of forestry interest. This paucity of studies is probably due to the difficulty of conducting long-term analyses (Husband & Schemske, 1996). Many studies relying on molecular markers to determine the paternity of individuals, however, have estimated levels of ID in wild tree populations (e.g., Aguiar et al., 2020; Duminil et al., 2016; Gonzaga et al., 2017; Griffin et al., 2019; Hardy et al., 2019; Lind et al., 2019; Pupin et al., 2019; Sabatti et al., 2020; Tambarussi, Boshier, et al., 2017; Tambarussi, Sebbenn, et al., 2017) and other perennial species (e.g., Duffy et al., 2020; Pinc et al., 2020; Wang, 2019).

In the present study, we analyzed the long-term progression and intensity of ID in *Anagyris foetida*, a leguminous Mediterranean shrub with a mixed mating system (Valtueña et al., 2008b) and the first native European

plant species in which ornithophily has been experimentally demonstrated (Ortega-Olivencia et al., 2005). We studied the survival and vegetative and reproductive traits of a 12-year-old population of two progenies derived respectively by manual self- and cross-pollination. This population has been growing in an experimental garden under environmental conditions similar to those of the source populations, which includes the main pollinators of *A. foetida*—the passerine birds *Sylvia melanocephala* and *Phylloscopus collybita*. In a study of this cultivated population during its first 3 years (Valtueña et al., 2014), we obtained the following ID values: vegetative ID ($\delta = 0.32$), reproductive ID (potential, $\delta = 0.33$; actual, $\delta = 0.54$), and global ID (potential, $\delta = 0.69$; actual, $\delta = 0.54$).

Given the above background, our main aim was to examine the progression of the 12-year-old cultivated population of this shrub species in the experimental garden. In particular, we tested the following hypotheses: (1) survival of selfed individuals are lower than that of outcrossed ones because of the lowered competitive

ability of the former, (2) because of higher competition, individual survival rates are lower in the center than at the periphery of the population, (3) the level of rewards (nectar and pollen) are lower in selfed individuals compared with outcrossed ones, and (4) ID values are higher than those previously observed during the 2008–2009 flowering period, thereby favoring outcrossing.

2 | MATERIAL AND METHODS

2.1 | Species and population studied

Anagyris foetida, a leguminous shrub of Mediterranean distribution, is deciduous in summer and flowers in autumn to winter. The flowers are papilionaceous, pendulous, odorless, and greenish-yellow when young and yellow when old (Figure 1a). These flowers, which are grouped into small racemes located on old branches or trunks, produce copious quantities of very dilute, hexose-dominant nectar (Ortega-Olivencia et al., 2005; Valtueña

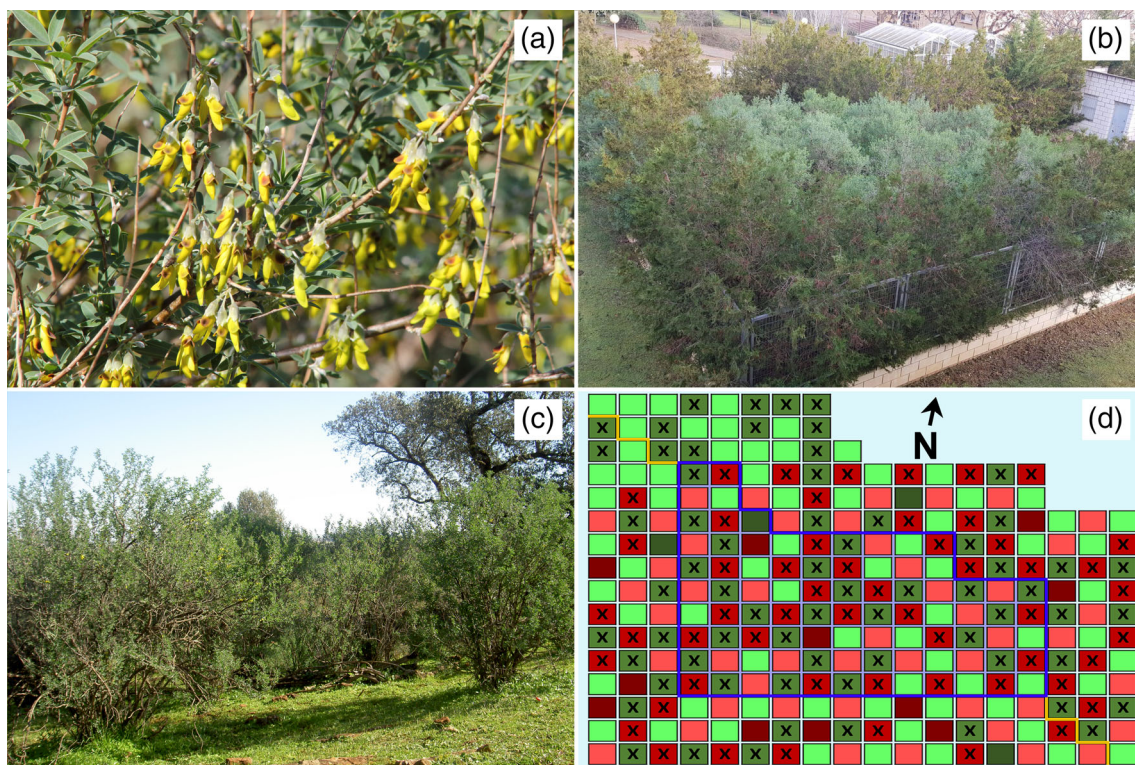


FIGURE 1 Details of the studied and wild populations. (a) Flowering branches of *Anagyris foetida*. (b) Image of the experimental garden showing *A. foetida* individuals, which have a bluish-green cast, in front of a *Platycladus orientalis* hedge. (c) Image of a wild population. (d) Positions of studied cohort individuals in the experimental garden. Outcrossed and selfed individuals are depicted with green and red squares, respectively. The status of each individual is symbolized as follows: dark-colored square, dead in the third year of life; medium-colored square with an X, died between the third year of life and the year of this study; light-colored square, still alive after 12 years. The arrow points north. To examine the effect of within-garden location on mortality, individuals were classified as central or peripheral. In the diagram, central individuals are surrounded by a blue polygon, and orange lines delimit peripheral individuals according to their proximity to northern and eastern versus southern and western boundaries

et al., 2007). The main pollinators are several passeriform species (Ortega-Olivencia et al., 2005). *Anagyris foetida* is a self-compatible species with a mixed breeding system in which cross-pollination has no clear advantage over self-pollination (Valtueña et al., 2008b). The reproductive success of this species is very low as a result of strong resource limitation (Valtueña et al., 2012) and because fruit initiation is limited by pollen availability (Valtueña et al., 2010).

The individual plants analyzed in this study belong to a cultivated population located in the Botanic Experimental Garden (Figure 1b) of the Badajoz University campus of the University of Extremadura, Spain. The garden is situated approximately 2 km from the city of Badajoz. The individuals originated from seeds obtained by hand pollination (both selfed and outcrossed) of two wild populations (Figure 1c) located approximately 20 km from the experimental garden and 5 km apart from each another. The experimental population therefore consisted of a mixture of two different progenies (selfed and outcrossed). More information on the wild and experimental populations, including seed derivation, can be found in Valtueña et al. (2014). Briefly, the experimental population was studied from seed germination in December 2005 (147 seeds from each progeny) until the end of the second flowering year (2008–2009 season). Individuals were not differentiated on the basis of their ancestral population because the two wild populations were not significantly different (Valtueña et al., 2014). In a sampling carried out in June 2009, 101 selfed and 137 outcrossed living individuals, corresponding to respective survival rates of 68.7% and 93.2% relative to the total number of initial seeds, were counted in the experimental population (Figure 2).

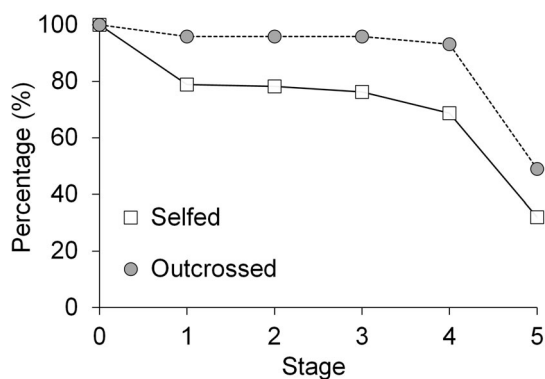


FIGURE 2 Survival trajectory of outcrossed and selfed progenies. Percentages of seeds and individuals are indicated relative to the number of planted seeds as follows: 0, planted seeds; 1, germinated seeds; 2, emerged seedlings; 3, seedlings transplanted into the experimental garden; 4, live plants at the third year of life (2009); 5, live plants sampled in this study (2019)

2.2 | Parameters and methods

2.2.1 | Survival rate and reproductive individuals

After 12 years of cultivation in the experimental garden, plant survival was quantified by counting the number of resprouting individuals during the 2018–2019 flowering season. To assess the effect of location on individual survival, two groups of individuals were differentiated: those at the center and those near the edge (Figure 1d). The latter, peripheral group was also delimited according to each individual's proximity to the four edges of the garden (north and east vs. south and west), as individuals along the southern and western peripheries were adjacent to a hedge of *Platycladus orientalis* surrounding the garden.

To quantify the number of reproductive individuals in a general way (i.e., without taking the location or closest edge into account), we counted the number of plants producing at least one inflorescence during the flowering season.

2.2.2 | Vegetative parameters

In January 2019, the following vegetative parameters were recorded: number of trunks per individual, thickness of each trunk at 10 cm from the ground, and the maximum height of each trunk.

2.2.3 | Flowering parameters

Flower and inflorescence production

At the end of the flowering period (early March 2019), we determined the number of inflorescences and the number of flowers/inflorescence on each individual plant. For individuals with fewer than 100 inflorescences, we counted these two parameters directly; for the remaining individuals, the values were estimated by extrapolating from counts of one to two marked, main branches per individual.

Floral rewards: Nectar

Nectar production (amount of sugar and volume) from six flowers per individual (two flowers/inflorescence and three inflorescences/individual) was measured on the first day of anthesis (opening of the corolla standard) according to Valtueña et al. (2014).

Investment in non-sexual whorls: Calyx and corolla weights

The flowers selected for measurement of nectar production were also used to estimate resource allocation by both progenies (selfed and outcrossed) to the sterile

whorls. For this purpose, the flowers were taken to the laboratory, dissected, and dried for 48 h in an oven at 80°C. The dry weights of the calyx and corolla were then determined on a precision balance (± 0.1 mg).

Investment in sexual whorls: Ovule and pollen production

The number of ovules and pollen grains in six flower buds (two buds/inflorescence and three inflorescences/individual) in the first/second inflorescence whorl of each individual were counted according to Valtueña et al. (2014).

2.2.4 | Fruiting parameters

Fruit set, seed set, and seed weight

The number of fruits produced by each individual was determined during the fruiting season following the same methodology used to calculate flower and inflorescence production, thus allowing the calculation of fruit set. We randomly collected 20 fruits per individual—or all of the fruits if fewer than 20 were present—and counted and weighed the fertile seeds according to Valtueña et al. (2008b).

2.2.5 | Comparison of the characteristics of surviving versus non-surviving individuals

To determine whether individuals that had died after our previous 2009 sampling were vegetatively or reproductively inferior to those that survived until 2019, we compared the height and flower production of the two sets of individuals using the data recorded in 2009.

2.2.6 | Relative fitness, cumulative relative fitness, and ID

For each parameter, relative fitness (F_S , corresponding to the fitness relationship of selfed and outcrossed progenies) was calculated as the ratio of the value of that parameter in the selfed progeny to that in the outcrossed one (Husband & Schemske, 1996). Relative cumulative fitness (F_r) was then calculated as the product of all F_S values and used to calculate the ID value ($\delta = 1 - F_r$).

Positive and negative values of δ indicate the existence of ID and outbreeding depression, respectively, with the intensity in both cases proportional to the value of δ .

As detailed in Valtueña et al. (2014), ID was calculated at both vegetative and reproductive levels, with reproductive ID also differentiated into potential versus actual. At the vegetative level, we took into account the following parameters: individual survival, number and diameter of trunks, and individual height. To calculate potential reproductive ID, two sets of parameters were considered: (a) the percentage of flowering individuals, the number of flowers/individual, numbers of ovules and pollen grains/flower, and the percentage of viable pollen and (b) the previous parameters plus floral parameters whose influence on *A. foetida* reproductive success was not directly known but which were expected to be positively correlated, namely, nectar production and weight of the non-sexual whorls. For calculation of ID at the actual reproductive level, the previous parameters plus fruit set (F), number of seeds/fruit (S), and seed weight (SW) were considered. Flowering peak, which has an undetermined effect on reproductive success, was not used in the calculation of ID.

Finally, global ID was calculated based on all the parameters used to obtain vegetative and actual reproductive ID values.

2.3 | Statistical analysis

All statistical analyses were performed using the IBM Statistics SPSS 20 package. Pearson chi-square tests with 2-factor $R \times C$ contingency tables were used to assess the effect of location (central vs. peripheral) and closest edge on individual survival, for both the entire population and each progeny, and to compare survival and flowering rates of the two progenies. To compare vegetative and reproductive variables between progenies, we carried out one-way ANOVAs, with a Brown–Forsythe correction applied when the variances of the data were not homogeneous. The normality and homoscedasticity of these variables were verified using a Kolmogorov–Smirnov test with a Lilliefors correction and a Levene test, respectively (Sokal & Rohlf, 2012).

To check for relationships among various vegetative and reproductive parameters, we performed Pearson correlation analyses for the entire population and each of

TABLE 1 Vegetative parameters studied in outcrossed and selfed individuals of *Anagyris foetida* (mean \pm SD)

Parameter	Crossed	Selfed	Statistic
Number of trunks	2.41 \pm 1.19	1.91 \pm 1.01	$F = 5.13, p = 0.026$
Height (cm)	284.7 \pm 66.1	281.2 \pm 61.6	$F = 0.79, p = 0.780$
Diameter (cm)	2.48 \pm 0.87	2.20 \pm 0.71	$F = 3.13, p = 0.080$

Note: Significant differences between progenies are indicated in bold.

the two progenies. In the case of reproductive parameters, the following products were considered: $F \times S$, $S \times SW$, and $F \times S \times SW$. In all cases, the p -values obtained were adjusted using the false discovery rate.

3 | RESULTS

3.1 | Survival rate and reproductive individuals

In the autumn of 2018, 72 outcrossed and 47 selfed individuals had resprouted, which corresponds to survival rates of 52.6% and 45.5%, respectively, relative to the previous sampling (June 2009) and to 51.1% and 40.9%, respectively, relative to the number of emerged seedlings; the difference in survival rates obtained using the two approaches was not

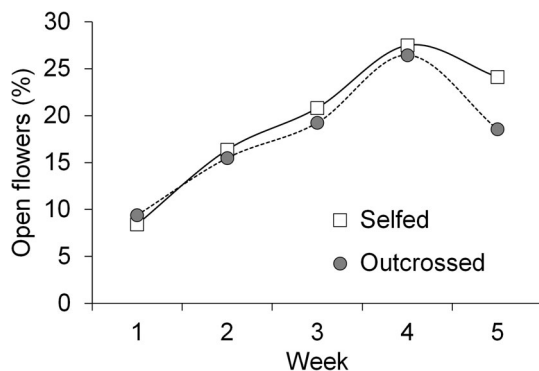


FIGURE 3 Percentage of flowers in anthesis in the two progenies during the weeks of the study

significant between the two progenies ($\chi^2 = 0.843$, $p = 0.359$; $\chi^2 = 2.646$, $p = 0.104$, respectively). Although peripheral individuals had a slightly higher survival rate than central individuals, this difference was not significant: location had no significant effect on survival, with respect to neither the entire population ($\chi^2 = 2.835$, $p = 0.092$) nor each progeny (outcrossed: $\chi^2 = 3.711$, $p = 0.054$; selfed: $\chi^2 = 0.061$, $p = 0.805$). Similarly, the closest edge position of peripheral individuals did not affect survival in any of the cases (entire population: $\chi^2 = 0.613$, $p = 0.433$; outcrossed: $\chi^2 = 0.345$, $p = 0.557$; selfed: $\chi^2 = 0.691$, $p = 0.406$).

A total of 59 outcrossed and 34 selfed individuals (81.9% and 72.3% of living plants, respectively) produced at least one inflorescence, but no significant difference was found between the two progenies ($\chi^2 = 1.536$, $p = 0.215$).

3.2 | Vegetative parameters

Although values of the three studied vegetative parameters were higher in outcrossed individuals than in selfed ones, the number of trunks was the only parameter significantly different between progenies (Table 1).

3.3 | Reproductive parameters

3.3.1 | Phenology and flowering peak

During the first week of the study (mid-January 2019), approximately 10% of all flowers produced during the entire phenological period by individuals of both

Parameter	Outcrossed	Selfed	Statistic
Flowering peak (week of study)	3.83 ± 0.85	3.99 ± 0.97	$F = 0.64$, $p = 0.426$
Number of inflorescences	115.4 ± 154.1	90.0 ± 171.8	$F = 0.54$, $p = 0.465$
Number of flowers	971.5 ± 1262.0	719.3 ± 1249.4	$F = 0.87$, $p = 0.354$
Flowers/inflorescence	8.81 ± 2.00	8.27 ± 1.86	$F = 1.69$, $p = 0.196$
Nectar volume (μl)	8.07 ± 3.79	6.91 ± 4.54	$F = 1.70$, $p = 0.195$
Nectar sugar amount (mg)	0.771 ± 0.360	0.692 ± 0.470	$F = 0.80$, $p = 0.373$
Calyx weight (mg)	13.19 ± 2.42	12.57 ± 2.32	$F = 0.23$, $p = 0.234$
Corolla weight (mg)	17.86 ± 2.58	16.55 ± 2.98	$F = 4.82$, $p = 0.031$
Ovules/ovary	8.53 ± 0.60	8.49 ± 0.60	$F = 0.09$, $p = 0.763$
Pollen grains/flower (×1000)	244.0 ± 53.4	239.5 ± 72.3	$F = 0.11$, $p = 0.737$
Aborted pollen grains (%)	3.45 ± 1.78	10.13 ± 6.03	$F = 63.20$, $p = 0.000$
Fruit set (%)	9.77 ± 6.29	12.4 ± 9.24	$F = 2.56$, $p = 0.113$
Seeds/fruit	3.34 ± 0.88	3.17 ± 1.01	$F = 0.69$, $p = 0.410$
Seed weight	394.0 ± 52.1	356.6 ± 49.8	$F = 10.96$, $p = 0.001$

Note: Significant differences between progenies are indicated in bold.

TABLE 2 Reproductive parameters studied in outcrossed and selfed individuals of *Anagyris foetida* (mean ± SD)

TABLE 3 Correlations among vegetative, flowering, and reproductive traits of *Anagris foetida* individuals, with selfed and outcrossed progenies considered together

	Number of trunks	Height	Trunk diameter
Number of flowers ($n = 95$)	$r = \mathbf{0.288}$ $p = \mathbf{0.020}$	$r = \mathbf{0.248}$ $p = \mathbf{0.048}$	$r = \mathbf{0.378}$ $p = \mathbf{0.000}$
Number of fruits ($n = 95$)	$r = \mathbf{0.367}$ $p = \mathbf{0.000}$	$r = \mathbf{0.347}$ $p = \mathbf{0.008}$	$r = \mathbf{0.518}$ $p = \mathbf{0.000}$
Fruit set (F) ($n = 95$)	$r = -0.105$ $p = 0.310$	$r = 0.154$ $p = 0.136$	$r = 0.196$ $p = 0.057$
Seeds/fruit (S) ($n = 88$)	$r = -0.059$ $p = 0.310$	$r = 0.238$ $p = 0.051$	$r = 0.187$ $p = 0.081$
Seed weight (SW) ($n = 88$)	$r = 0.098$ $p = 0.363$	$r = 0.092$ $p = 0.393$	$r = 0.104$ $p = 0.334$
F \times S ($n = 88$)	$r = -0.090$ $p = 0.403$	$r = 0.095$ $p = 0.337$	$r = 0.159$ $p = 0.140$
S \times SW ($n = 88$)	$r = -0.015$ $p = 0.893$	$r = \mathbf{0.253}$ $p = \mathbf{0.048}$	$r = 0.188$ $p = 0.079$
F \times S \times SW ($n = 88$)	$r = -0.088$ $p = 0.415$	$r = 0.108$ $p = 0.317$	$r = 0.176$ $p = 0.101$

Note: Significant correlations are indicated in bold.

progenies were in bloom. This percentage continuously increased until week 4, when more than 25% of the total number were in anthesis, but had decreased by the final sampling (week 5) (Figure 3). Although the peak of flowering, during week 4, occurred slightly earlier in the outcrossed progeny than in selfed individuals, this difference was not significant (Table 2).

3.3.2 | Flowering parameters

Only two studied flowering parameters were significantly different between progenies. In particular, corolla weight was significantly higher in the outcrossed progeny, whereas the percentage of aborted pollen was significantly higher in the selfed progeny (Table 2).

3.3.3 | Fruiting parameters

The fruit set percentage and number of seeds per fruit were slightly higher in selfed and outcrossed progenies, respectively, but these differences were not significant. Finally, seeds of the outcrossed progeny were significantly heavier than those of selfed individuals (Table 2).

3.4 | Correlations among vegetative and reproductive traits

At the population level, trunk number, height, and diameter were significantly positively correlated with the number of flowers and fruits produced (Table 3). The height of the

individuals was also significantly positively correlated with investment in seeds (S \times SW) (Table 3). The number of flowers and fruits was significantly positively correlated with trunk height and diameter in the outcrossed progeny and with the number of trunks in the selfed progeny (Table 4).

3.5 | Comparative characteristics of surviving and non-surviving individuals

In the outcrossed progeny, neither height nor flower production in 2009 was significantly different between individuals alive in 2019 and those that had died (height: 155.2 and 155.7 cm, respectively, $F = 0.008$, $p = 0.929$; flowers: 370.7 and 392.9, respectively, $F = 0.129$, $p = 0.720$). In the selfed progeny, in contrast, surviving individuals in 2019 exhibited higher values of both parameters in 2009 (height: 150.4 cm; flowers: 295.8) than individuals in 2009 that subsequently died (height: 137.8 cm; flowers: 162.8) (height: $F = 4.859$, $p = 0.030$; flowers: $F = 4.236$, $p = 0.042$).

3.6 | Relative fitness, cumulative relative fitness, and ID

Relative fitness values for all studied parameters and their corresponding ID values are shown in Table 5. The only parameters showing significant differences between progenies were the number of trunks, corolla weight, the percentage of viable pollen, and seed mean weight; the ID values (δ) for these parameters were 0.21, 0.07, 0.07, and 0.09, respectively.

	Number of trunks	Height	Trunk diameter
Outcrossed progeny			
Number of flowers ($n = 60$)	$r = 0.153$ $p = 0.243$	$r = 0.361$ $p = 0.005$	$r = 0.455$ $p = 0.000$
Number of fruits ($n = 60$)	$r = 0.280$ $p = 0.240$	$r = 0.396$ $p = 0.002$	$r = 0.518$ $p = 0.000$
Fruit set (F) ($n = 60$)	$r = -0.033$ $p = 0.803$	$r = 0.228$ $p = 0.079$	$r = 0.357$ $p = 0.005$
Seeds/fruit (S) ($n = 55$)	$r = -0.092$ $p = 0.506$	$r = 0.263$ $p = 0.052$	$r = 0.172$ $p = 0.210$
Seed weight (SW) ($n = 55$)	$r = 0.103$ $p = 0.456$	$r = 0.100$ $p = 0.465$	$r = 0.006$ $p = 0.965$
F \times S ($n = 55$)	$r = -0.051$ $p = 0.714$	$r = 0.139$ $p = 0.311$	$r = 0.290$ $p = 0.051$
S \times SW ($n = 55$)	$r = -0.030$ $p = 0.826$	$r = 0.308$ $p = 0.059$	$r = 0.163$ $p = 0.234$
F \times S \times SW ($n = 55$)	$r = -0.057$ $p = 0.678$	$r = 0.172$ $p = 0.209$	$r = 0.310$ $p = 0.042$
Selfed progeny			
Number of flowers ($n = 35$)	$r = 0.535$ $p = 0.004$	$r = 0.005$ $p = 0.977$	$r = 0.189$ $p = 0.278$
Number of fruits ($n = 35$)	$r = 0.558$ $p = 0.000$	$r = 0.239$ $p = 0.167$	$r = 0.259$ $p = 0.133$
Fruit set (F) ($n = 35$)	$r = -0.163$ $p = 0.349$	$r = 0.038$ $p = 0.828$	$r = 0.020$ $p = 0.911$
Seeds/fruit (S) ($n = 33$)	$r = -0.040$ $p = 0.827$	$r = 0.202$ $p = 0.261$	$r = 0.194$ $p = 0.280$
Seed weight (SW) ($n = 33$)	$r = -0.039$ $p = 0.829$	$r = 0.079$ $p = 0.661$	$r = 0.185$ $p = 0.303$
F \times S ($n = 33$)	$r = -0.121$ $p = 0.502$	$r = 0.053$ $p = 0.769$	$r = 0.044$ $p = 0.806$
S \times SW ($n = 33$)	$r = -0.078$ $p = 0.667$	$r = 0.160$ $p = 0.375$	$r = 0.168$ $p = 0.349$
F \times S \times SW ($n = 33$)	$r = -0.126$ $p = 0.486$	$r = 0.025$ $p = 0.890$	$r = 0.018$ $p = 0.923$

Note: Significant correlations are indicated in bold.

When all studied vegetative parameters were taken into account, the ID at the vegetative level was 0.39. When cohort germination and the rate of seedling survival relative to emerged seedlings in our previous study (Valtueña et al., 2014) were additionally considered, this value increased to 0.56.

The potential reproductive cumulative relative fitness without considering nectar production or perianth weight was 0.60, which was equivalent to $\delta = 0.40$; when these two parameters were considered, the value decreased to 0.41 ($\delta = 0.59$). The actual reproductive cumulative relative fitness was 0.64, with a corresponding δ of 0.36.

TABLE 4 Correlations among vegetative, flowering, and reproductive traits of *Anagyris foetida* individuals, with outcrossed and selfed progenies considered separately

Finally, the global ID of the studied cohort was high. On the one hand, the global ID value based on potential reproductive capacity was 0.82 when perianth weight and nectar production were taken into account and 0.73 otherwise. On the other hand, the global ID value based on actual reproductive capacity ($\delta = 0.72$) was similar to the potential value obtained without using those parameters.

4 | DISCUSSION

The first main finding of this study is that the effects of ID on the studied cohort in the experimental garden

TABLE 5 Statistical summary of studied parameters in outcrossed and selfed individuals of *Anagyris foetida*

Parameter	Outcrossed	Selfed	F _S	δ
Survival and vegetative parameters				
% Survival (respect to total seedlings)	51.1	40.9	0.80	0.20
% Survival (respect to June 2009)	52.6	46.5	0.89	0.11
Number of trunks	2.41	1.91	0.79	0.21
Height (cm)	284.7	281.2	0.99	0.01
Diameter (cm)	2.48	2.20	0.89	0.11
Flowering parameters				
Flowering/alive individuals (%)	83.3	74.5	0.89	0.11
Number of flowers	971.5	719.3	0.74	0.26
Number of inflorescences	115.4	90.0	0.78	0.22
Flowers/inflorescence	8.81	8.27	0.94	0.06
Flowering peak (week)	3.83	3.99	1.04	-0.04
Sterile whorls and nectar				
Calyx weight (mg)	13.19	12.55	0.95	0.05
Corolla weight (mg)	17.86	16.55	0.93	0.07
Nectar volume (μl)	8.07	6.91	0.86	0.14
Nectar sugar amount (mg)	0.771	0.692	0.90	0.10
Fertile whorls				
Ovules/Ovary	8.53	8.49	1.00	0.00
Pollen grains/flower (× 1000)	244.0	239.5	0.98	0.02
Viable pollen grains (%)	96.6	89.9	0.93	0.07
Fruiting parameters				
Fruit set (%)	9.6	11.9	1.24	-0.24
Seeds/fruit	3.34	3.17	0.95	0.05
Seed weight	394.0	356.6	0.91	0.09

Note: The value of each parameter in the two different progenies is provided along with its relative fitness (F_S) and inbreeding depression value (δ). Parameters with significant differences between progenies are indicated in bold.

did not vary substantially at 12 years compared with the third year of cultivation. Because some parameters no longer differed between the two progenies, however, the long-term effects of ID were somewhat less than those found during the first years of *A. foetida* cultivation. Second, our results demonstrate the importance of studying the ID value throughout the life cycle of individuals and also reveal that this value varies with the age and/or size of individuals in a population. Third, we verified that ID in this shrubby species is manifested mainly during early stages of the life cycle. Taking into consideration that selfed individuals with the worst vegetative and reproductive trait values have been eliminated and that only those individuals with values similar to those of outcrossed ones exist as mature shrubs in these populations, the level of ID should have no effect on population maintenance. In addition, the level of inbreeding in *A. foetida* is significant; this is mainly because individual plants

produce a large number of long-lived, self-compatible flowers simultaneously, which promotes pollinator behavior that favors geitonogamy. Although *A. foetida* is an adichogamous species, autogamy is not of major importance because a stigma-tripping mechanism acts as a barrier against self-pollination; hence, the level of spontaneous self-pollination is practically nil (Valtueña et al., 2008b).

ID at the vegetative level in the studied cohort was higher after 12 years (δ = 0.56) than at the end of the third year (δ = 0.32) (Valtueña et al., 2014); this is because some considered parameters, such as trunk number and diameter, were not recorded in the first study. When those two parameters were eliminated from the calculation, leaving only germination, emergence, survival, and height of individuals to be considered, the value was reduced (δ = 0.36). This result implies that ID has a greater effect during early stages of the life cycle

(from germination to plant establishment), a phenomenon that has already been observed in some tree species, such as *Dipteryx alata* (Fabaceae; Tambarussi, Sebbenn, et al., 2017), *Ostrya rehderiana* (Betulaceae; Yang et al., 2018), and various conifers (Sorensen & Miles, 1982). The similar value of ID in late stages is probably due to the elimination of selfed individuals having the greatest deleterious load, which results in survivors with characteristics more similar to outcrossed individuals; this hypothesis is indirectly supported by the results of our comparison of heights in 2009 between surviving and non-surviving individuals in 2019. Furthermore, this reduction may also be due to the fact that heights of the two progenies (selfed and outcrossed) had become equal. From a vegetative point of view, in fact, most individuals would have already reached their maximum height, and available plant resources would be used to increase the number and/or diameter of trunks. At the end of the third year of life (Valtueña et al., 2014), neither of the progenies had yet acquired the typical bushy habit (e.g., production of numerous branches from the base of the plant) and still had only a main stem. An approach that takes into account trunk number and diameter in the calculation of ID (0.56 vs. 0.32 in the third year) therefore seems logical.

The survival of individuals was not affected by their positions in the experimental garden (i.e., central vs. peripheral). Compared with central individuals, peripheral plants experienced less intra-specific competition for light, water, and nutrients and also suffered from less humidity-related stress during the winter, the latter being a factor that very negatively affects individuals (Valtueña et al., 2010); in theory, greater survival should thus be observed in the less stressful (peripheral) areas (Armbruster & Reed, 2005). Our results are consistent with those of previous studies that detected no correlation between the level of stress and the ID values (Angeloni et al., 2011; Sandner & Matthies, 2016).

As far as reproductive parameters whose direct relationship with fitness is unknown, namely, nectar production and the weight of sterile whorls, only the weight of the corolla differed significantly between progenies, similar to results from the third year (Valtueña et al., 2014). The ID value of this parameter ($\delta = 0.07$) was very low, however, in agreement with other studies indicating that ID hardly affects the size of the flower or its components (Ellmer & Andersson, 2004; Thiele et al., 2010). In regard to nectar, the outcrossed progeny in the third year of sampling (Valtueña et al., 2014) produced significantly more sugar/flower, which was attributed to their larger floral size. We note that a correlation generally exists between floral size and nectar production (Kaczorowski et al., 2008; Pacini et al., 2003). The absence of significant

differences in the present study may be due to the fact that the plants with the lowest overall nectar production values were selfed individuals that did not survive, which would have reduced the difference in observed nectar production between progenies. To our knowledge, no other studies have quantitatively compared nectar production between selfed and outcrossed individuals. In terms of pollinator attraction, flowers of outcrossed individuals, which are larger and more nectariferous—although in the latter case not significantly—would surely be more frequently visited by pollinators compared with those of selfed individuals. If so, this situation would lead to a longer duration of visits and also a higher pollen discount due to geitonogamous selfing (Bartoš et al., 2020 and references therein). In any case, the ID values for these parameters were very low (between 0.05 and 0.14), thus confirming that these parameters are less affected by ID (Thiele et al., 2010).

Because nectar production and corolla size (corolla weight) can influence the degree of pollinator attraction and thus fruit and seed set, they may also contribute to reproductive fitness. When we considered these parameters in our calculation, potential reproductive fitness was reduced from 0.60 to 0.41. Taking into account that the actual relative reproductive fitness (0.64) was closer to that of potential reproductive fitness when nectar production and corolla size were not considered, the idea that these two parameters do not influence the reproductive capacity of individuals of this species seems logical. On the contrary, a tradeoff may exist between improved pollination due to greater attractiveness (increased fruit and seed initiation) and reduced final production of fruits and seeds due to greater competition for resources (Jacquemyn & Brys, 2020).

Except for some inter-individual variability, the two progenies had synchronous flowering peaks. As previously reported by Valtueña et al. (2008b), *A. foetida* is characterized by high floral synchrony, with the flowering of individuals in the wild source populations almost totally overlapping.

In the present study, flowering parameters (percentage of blooming individuals, total number of flowers and inflorescences/individual, and number of flowers/inflorescence) were all higher in the outcrossed progeny than in selfed plants, although not significantly. This situation contrasts with that observed in the third year of life, when the outcrossed progeny had significantly higher values of all parameters except for the number of flowers/inflorescence (Valtueña et al., 2014). As mentioned earlier, the lack of differences in flowering parameters between the two current progenies is probably due to the higher death rate of selfed individuals with the worst reproductive trait values, which would cause the survivors to have characteristics more similar to the

outcrossed individuals. This hypothesis is supported by the results of our comparison of flower production in 2009 between surviving and non-surviving individuals in 2019.

In regard to female potential reproductive capacity, the number of ovules/flower did not differ between progenies, which was also true in the third year (Valtueña et al., 2014). This result suggests that ovary size (to maximize female reproductive capacity) is under such strong selection pressure that ID disappears. This hypothesis is supported by the low inter-individual and inter-population variation in this parameter (Valtueña et al., 2008b). As for male potential reproductive capacity, the percentage of aborted pollen grains was the only parameter that differed significantly among progenies; it was higher in the selfed progeny, just as in the third year of the cohort. The ID associated with this parameter was similar between samples (0.08 in the third year vs. 0.07 in this study). A decrease in pollen viability after selfing is a known phenomenon that has strong implications for population dynamics and the mating system (Carr & Dudash, 1997; Ellmer & Andersson, 2004; Willis, 1993). In regard to total pollen produced per flower, differences observed in the third year had disappeared after 12 years, when values of this parameter were similar to those of the outcrossed progeny in the third year of the study. One explanation for this result is that this parameter, similar to the number of ovules/ovary, is probably under strong genetic control; in addition, or alternatively, selfed individuals with less pollen production may have had lower survival rates.

Similar to the above results, two fruiting-related parameters—fruit set and number of seeds/fruit, which were significantly higher at 3 years of age in selfed and outcrossed individuals, respectively (Valtueña et al., 2014)—became identical between progenies over time. In contrast, seed weight was still significantly higher in the outcrossed progeny, although the ID at this level had slightly decreased by 9 years ($\delta = 0.09$ vs. $\delta = 0.14$). The balancing of resources between fruiting and seed parameters (number of seeds/fruit and seed weight) can explain these differences between progenies. The relative fitness value of the three parameters (fruit set, number of seeds, and seed weight) was 1.06, which indicates that the selfed individuals had greater reproductive success; this is probably due to the tradeoff between the level of pollination and fruit and seed set that we mentioned above when considering the investment in the perianth and nectar production. After 9 years, the relative fitness values of these parameters, both individually and jointly, were closer to 1 than values recorded after 3 years of growth, thus demonstrating their convergence in both progenies and a reduction in their differences over time. Most likely, this cultivated population of selfed and outcrossed individuals would be uniform, with the behavior

of the two types of individuals during pollination no longer clearly distinguishable. After 12 years of growth, these individuals indeed appear to form a dense population with barely separated individuals. Their canopies are often intertwined, with pollinators only needing very short flight distances to visit the two types of individuals indiscriminately. Selfed genotypes appear to have been selectively eliminated, however, as fewer surviving selfed individuals are present; consequently, most of these flights presumably occur between outcrossed individuals.

In regard to population-level relationships, we found that trunk height, trunk diameter, and the number of trunks per individual were positively correlated with individual reproductive capacity (i.e., more flowers and fruits) and that trunk height was also positively correlated with seed investment ($S \times SW$). When selfed and outcrossed individuals were considered separately, most of these correlations still held for the outcrossed progeny. These results demonstrate that reproductive competence is related to individual size (see Griffin et al., 2019). This conclusion mainly applies to outcrossed individuals, as selfed individuals have been eliminated in a greater proportion from the moment of germination, probably because of their lower competitive ability and higher load of deleterious alleles. Plants of *A. foetida* probably harbor large numbers of deleterious mutations, as somatic mutations can rapidly accumulate via the numerous mitotic cell divisions that take place in woody species between meiotic events (Griffin et al., 2019).

After 9 years of cultivation since the previous sampling, potential and actual ID values of the studied cohort ($\delta = 0.72$ and $\delta = 0.73$, respectively) were similar to those found in the third year ($\delta = 0.69$ and $\delta = 0.54$, respectively). Although some theoretical studies have indicated that only two mating systems are evolutionarily stable (selfing with ID <0.5 and outcrossing with ID >0.5 ; Lande & Schemske, 1985), the maintenance of selfing with high rates of ID has also recently been theoretically demonstrated (e.g., Sachdeva, 2019; Sakai, 2020). The values obtained in our study were similar to those estimated by Duffy et al. (2020) for *Aloe thraskii*, a perennial, succulent, shrubby plant with a mixed bird–bee pollination system. As in *A. thraskii*, the high number of flowers in anthesis at any given time per individual, as well as the behavior of pollinators, facilitates geitonogamy in *A. foetida*; because individuals are so close to each other, with intermixed canopies, inbreeding is increased. In addition, we note that a small population size is usually correlated with high values of ID (Nebot et al., 2020; but see Chai et al., 2019).

In this study, vegetative parameters had the highest ID value ($\delta = 0.56$). This ID mainly affected the first stages of the life cycle: germination and survival in the initial years. Reproductive parameters, with the passage of time, have

become equalized between progenies, which implies a decrease in ID relative to the third year of study (flowering, $\delta = 0.34$ vs. $\delta = 0.44$; potential and actual reproductive capacity, $\delta = 0.40$ vs. $\delta = 0.54$ and $\delta = 0.30$ vs. $\delta = 0.33$, respectively). The greater effect of ID during the early stages of development is consistent with observations in other perennial taxa possessing mixed mating systems (Ishida, 2006; Ismail et al., 2014; Naito et al., 2005; Nebot et al., 2020; Sorensen & Miles, 1982; Takeuchi et al., 2020; Tambarussi, Boshier, et al., 2017; Tambarussi, Sebbenn, et al., 2017; Yang et al., 2018).

Finally, the experimental population in this study has a reproductive output similar to that of natural ones. In natural populations of *A. foetida*, extensive herbivory by sheep (Valtueña et al., 2008a), along with other intrinsic factors, prevents seedlings from thriving despite good seed production. In the experimental population, sheep predation is not a problem. A large number of seeds are present in the soil, some of which germinate properly. The seedlings do not thrive, however, probably because of genetic issues and/or intra- and inter-specific competition. In both natural and experimental populations, we therefore conclude that seedling emergence, despite the presence of a major soil seed bank (Valtueña et al., 2008a), is the most critical stage for the long-term persistence of this species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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