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Journal

Annals of Botany, 125(4)

ISSN

0305-7364

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Publication Date

2020-03-29

DOI

10.1093/aob/mcz198

Peer reviewed

Inheritance of breeding system in *Cakile* (Brassicaceae) following hybridization: implications for plant invasions

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Received: 10 October 2019 Returned for revision: 10 November 2019 Editorial decision: 2 December 2019 Accepted: 4 December 2019
Published electronically 5 December 2019

- **Background and Aims** Hybridization is commonly assumed to aid invasions through adaptive introgression. In contrast, a recent theoretical model predicted that there can be non-adaptive demographic advantages from hybridization and that the population consequences will depend on the breeding systems of the species and the extent to which subsequent generations are able to interbreed and reproduce. We examined cross-fertilization success and inheritance of breeding systems of two species in order to better assess the plausibility of the theoretical predictions.
- **Methods** Reciprocal artificial crosses were made to produce F₁, F₂ and backcrosses between *Cakile maritima* (self-incompatible, SI) and *Cakile edentula* (self-compatible, SC) (Brassicaceae). Flowers were emasculated prior to anther dehiscence and pollen was introduced from donor plants to the recipient's stigma. Breeding system, pollen viability, pollen germination, pollen tube growth and reproductive output were then determined. The results were used to replace the assumptions made in the original population model and new simulations were made.
- **Key Results** The success rate with the SI species as the pollen recipient was lower than when it was the pollen donor, in quantitative agreement with the 'SI × SC rule' of unilateral incompatibility. Similar outcomes were found in subsequent generations where fertile hybrids were produced but lower success rates were observed in crosses of SI pollen donors with SC pollen recipients. Much lower proportions of SC hybrids were produced than expected from a single Mendelian allele. When incorporated into a population model, these results predicted an even faster rate of replacement of the SC species by the SI species than previously reported.
- **Conclusions** Our study of these two species provides even clearer support for the feasibility of the non-adaptive hybridization hypothesis, whereby the colonization of an SI species can be assisted by transient hybridization with a congener. It also provides novel insight into reproductive biology beyond the F₁ generation.

Key words: Breeding system, *Cakile edentula*, *Cakile maritima*, inheritance, invasion, population simulation model, reproductive isolation, self-(in)compatibility.

INTRODUCTION

Where reproductively compatible species occur in close proximity, such as when one invades the range of the other, there is the possibility of hybrids being produced. This can lead to various genetic outcomes with potentially important consequences for adaptation and evolution (Baack *et al.*, 2015). For example, hybrid speciation can occur when partial reproductive isolation between hybrids and parental species is observed (Ungerer *et al.*, 1998; Rieseberg, 2006). On the other hand, introgression may occur when hybrids do not show reproductive isolation (Ellstrand and Schierenbeck, 2000), potentially increasing the genetic diversity of parental species. Hybridization may result in the transfer of adaptive alleles, e.g. transgenes and other crop alleles spreading to wild or weedy populations of related taxa (Snow *et al.*, 2010).

It has recently been suggested that hybrids could have a transient but crucial role in the population dynamics of an invading species without necessarily having any long-term fitness

consequences for either species (Hall, 2016; Mesgaran *et al.*, 2016). Following long-distance dispersal, a self-incompatible (SI) colonizer may experience an Allee effect because of limited mate availability. However, if hybridization can occur with a resident species (or another invader), preferential backcrossing in further generations to the phenotypes most similar to the incoming species in future generations could reconstitute the species' genome (Mesgaran *et al.*, 2016). Thus, the incoming SI species can establish, persist and proliferate without any conspecific mates initially being available. Sensitivity analyses of simulation models have shown that the breeding systems of the species and their hybrids and the preferences of pollinators are two of the most important factors for this phenomenon to occur (Mesgaran *et al.*, 2016; Bouhours *et al.*, 2017), since these together drive the direction of backcrossing.

Although these simulation models for hybrid populations drew on published and as yet unpublished data for values of parameters, they also had to make crucial assumptions relating to factors such as density- and frequency-dependence

of pollinator behaviour, recombination at meiosis, the inheritance of breeding systems and the resulting fitness of F_1 and later generations (Bouhours et al., 2017; Mesgaran et al., 2016). Empirical data are thus essential for the further evaluation of the model predictions, including the importance of genotypic frequencies and the dynamics of mixed population persistence.

Hybridization studies rarely examine traits of offspring beyond the first (F_1) generation. Self-incompatibility in homoploid hybrids has usually been found to be dominant over self-compatibility (SC) and thus expressed in all F_1 offspring (Zeng and Cheng, 2014), although there are exceptions (Kakeda et al., 2000). The F_1 generation is only the first of a series of steps leading to the long-term population outcome of a hybridization event, and breeding systems of later hybrid generations cannot be generalized from a single generation or a single study. Breeding system transitions between generations, generally ascribed to a modification of the *S* locus, which controls incompatibility (Nasrallah et al., 2007), have significant evolutionary and ecological consequences (Pinheiro et al., 2015). For example, the shift from obligate outcrossing to selfing is usually accompanied by a consistent suite of changes in morphological traits such as pollen size and floral display (Tedder et al., 2015). Self-incompatibility has also been assumed to be responsible for the rejection of alien pollen following interspecific fertilization (Kitashiba and Nasrallah, 2014), where only a certain direction of hybrids can be generated, typically with the SI species being the pollen donor and the SC species being the receiver (Hiscock and Dickinson, 1993), i.e. ‘unilateral incompatibility’ or ‘the SI × SC rule’ (Harrison and Darby, 1955). As a result, in crosses between plants with differing breeding systems, SI parents are expected to ‘overpower’ SC parents (the weak inbreeder/strong outbreeder (WISO) hypothesis; see details in Brandvain and Haig, 2005). Therefore, such transitions between SC and SI will eventually affect reproductive isolation and gene flow between species and may contribute to species diversity and persistence (Pinheiro et al., 2015; Willis and Donohue, 2017).

The original simulation model for hybridizing populations was tested with parameters based on *Cakile edentula* (SC) and *Cakile maritima* (sporophytic SI) (Mesgaran et al., 2016). These two Brassicaceae species have the same number of chromosomes ($2n = 18$), evolved on different sides of the Atlantic (*C. maritima* is native to the coasts of Europe, the Middle East and North Africa while *C. edentula* is native to the east coast of North America) and have invaded either separately or consecutively along coastlines in many parts of the world, including western North America, Australia, New Zealand, Japan, western Asia, New Caledonia and eastern South America (Cousens et al., 2013). *Cakile edentula* invaded eastern Australia and spread widely before the arrival of *C. maritima*; subsequently, *C. edentula* has disappeared from much of its former range (this same pattern of invasion and replacement also occurred in California and New Zealand). All locations currently occupied by *C. maritima* in eastern Australia could thus have been occupied by *C. edentula* at some time in the past. *Cakile edentula* is now found only in a small and contracting area in Tasmania and in northern New South Wales/southern Queensland (Cousens et al., 2013). Bidirectional hybridization between the two species has been

shown to have occurred in Australia (Ohadi et al., 2015; Li et al., 2019) and in western North America (H. Rosinger, Monash University, Australia, pers. comm.). Chloroplasts of *C. edentula* have also been detected in populations consisting entirely of *C. maritima* phenotypes, along with evidence for nuclear introgression. Despite the presence of SI and the consequent possibility of an Allee effect upon first arrival on a beach, *C. maritima* has completely replaced *C. edentula* in most of southern and eastern Australia (Rodman, 1986), California (Barbour, 1972) and the North Island of New Zealand (Cousens and Cousens, 2011).

In this study, we addressed answer the following questions for hybrids of *C. edentula* and *C. maritima*: (1) Which breeding system predominates in the F_1 , F_2 and backcross generations? (2) Does hybridization result in differences in reproductive isolation and gene flow, thus potentially affecting the performance of hybrids and their population dynamics? (3) How do these results affect the predictions of the model by Mesgaran et al. (2016)? Artificial F_1 , F_2 and backcross generations were produced and observations were made on the inheritance of breeding system and associated reproductive traits (pollen viability, pollen germination and pollen tube growth, and reproductive output).

MATERIALS AND METHODS

Seed collection

Two Tasmanian populations of *Cakile edentula* were sampled, from Raspins Beach (EPop_{RB}), within the current sympatric region where both species are common, and from Sloping Main (EPop_{SM}), where *C. edentula* dominates and just a few *Cakile maritima* and hybrids have been found. *Cakile maritima* seeds were collected from three populations in Tasmania: Ulverstone (MPop_{UL}) ~300 km north-west of the current sympatric zone, i.e. ‘behind’ the invasion front; Marion Bay (MPop_{MB}), a morphologically homogeneous *C. maritima*-only population within the sympatric zone; and Raspins Beach (MPop_{RB}), a mixed population within the current sympatric zone. Multiple populations were included to ensure that a wide range of *S* alleles (self-sterility genes) would be sampled and to minimize the possibility of inbreeding depression in the later generations. In each population, individual collections of 100–200 seeds were made from at least ten plants, air-dried and then stored in separate paper bags at room temperature.

Cakile edentula populations on a beach ahead of the invading *C. maritima* are pure representatives of the species post-colonization; they may differ from the home-range source population as a result of drift, mutation or adaptation. They have been found (Ohadi, 2015) to display the high level of SC typical throughout the native range of the species and in other invaded ranges (Rodman, 1974; Donohue, 1998; Willis et al., 2014). Populations of *C. maritima* from behind its invasion limit are the likely source of propagules in regions containing *C. edentula*; these have been found to be primarily obligately SI (Thrall et al., 2000; Ohadi, 2015; A. Gendall, La Trobe University, Australia, pers. comm.), again similar to studies in other regions (Rodman, 1974; Willis et al., 2014). However, a low frequency of SC plants has been recorded at some sites

(Thrall *et al.*, 2000). The range of morphological variation in Australian *C. maritima* also encompasses the variation within the native range (Cousens *et al.*, 2013) but prior introgression from *C. edentula* cannot be discounted. If a species is actively moving through the domain of another species, hybridizing and backcrossing as it goes (the mechanism of the Mesgaran *et al.*, 2016 model), we expect the presence of introgressed *C. edentula* alleles in most *C. maritima* populations; and since we are concerned with the authentic dynamics occurring at an actively expanding range margin and not at the (highly idiosyncratic) very first point of colonization of the second species, it is immaterial that the '*C. maritima*' phenotypes we collected may not represent populations of the pure species. They represent the actual entities coming into contact and interacting with *C. edentula*.

Experimental crossing design

Ten families (i.e. mother plants grown from individual collections of each population) of each parental species were randomly selected for the crossing experiment: five from EPop_{SM} (E1–E5) and five from EPop_{RB} (E6–E10) for *C. edentula*, and three from MPop_{RB} (M1–M3), three from MPop_{UL} (M4–M6) and four from MPop_{MB} (M7–M10) for *C. maritima*. Specifically, 20 seeds from each individual collection of the parental species were put into Petri dishes containing one layer of filter paper (Whatman No.1) saturated with distilled water. Once imbibed, the seeds were carefully scratched using fine pins to break the seed coat on one side to ensure germination (Gormally and Donovan, 2011) and then the dishes were transferred into a glasshouse. As they germinated, seedlings were distributed evenly into three plastic pots with potting mix (20 % pine bark and 80 % sand) such that every pot contained the same range of germination times. After 2 weeks, the seedlings were thinned to only one individual in each pot to avoid competition.

All individuals were assessed beforehand to confirm that the breeding systems were expressed as expected, i.e. SI in *C. maritima* and SC in *C. edentula* (the methods are described below). Artificial crossing was conducted on ~50–100 buds per individual plant per cross direction, depending on bud availability. Buds were emasculated by physical removal of stamens while petals and sepals were left intact (details can be found in

Li *et al.*, 2019) and then enclosed in 20 cm × 10 cm pollinator-proof bags to prevent accidental pollination. When the emasculated bud was open, a fresh flower (day 1) from the designated pollen donor was used as a source of pollen. One anther was removed and carefully brushed across the targeted stigma to spread pollen evenly. The pollinator-proof bags were kept over the racemes thereafter, to ensure the retention of dehiscent fruits. Fruits were harvested when ripe and stored in paper bags at room temperature for later experimentation or subsequent crossing.

We produced F₁ hybrids by artificial crossing between the two species from different populations and families. Seeds from each field-collected family were sown in late spring 2015 and seedlings were used in reciprocal crosses to produce F₁ seeds in a polythene tunnel at ambient temperatures. Plants started flowering by the end of summer in 2015. At this time, outcrossed seeds within the parental species were also generated for further testing the breeding system within each species. There were 30 families of the F₁ hybrid class produced in total. Codes used for the plants descended from a given crossing direction are given in Table 1.

The *Cakile* species used in this study are effectively annual plants and rarely survive into a second year (Rodman, 1986; Cousens *et al.*, 2013); consequently, siblings of the original parental species plants, i.e. seeds from the same collection, had to be used in producing backcrosses. The F₁ plants and plants of the parental species populations were grown in a glasshouse during the winter of 2015. All these F₁s were derived from EM crosses only (i.e. *C. edentula* maternal plant with *C. maritima* pollen donor), due to insufficient availability of ME fruits (see Results section). All crossing was again between individuals derived from different populations to avoid inbreeding depression. The temperature was controlled at 25 °C during the day and 15 °C at night; lights were provided to produce an effective daylength of 16 h.

Finally, plants of the parental species, F₁s, F₂s and backcrosses were grown together in one experiment at the field station of the Burnley Campus, during late spring of 2016. Both hand-outcrossed parent species (MM and EE) and plants from the original field-collected *C. maritima* and *C. edentula* were included. Each family of hybrids and of the parental groups was represented by three replicate seedlings, giving a total of 381 plants.

TABLE 1. Crossing directions and lineage of corresponding progeny

| Generation | ♀ | ♂ | Plant type (code) | Number of families | Number of crossed buds |
|------------------------|------------------------|------------------------|------------------------|--------------------|------------------------|
| Parental species | <i>C. maritima</i> (M) | <i>C. maritima</i> (M) | MM | 4 | 429 |
| | <i>C. edentula</i> (E) | <i>C. edentula</i> (E) | EE | 4 | 400 |
| F ₁ | <i>C. edentula</i> (E) | <i>C. maritima</i> (M) | EM | 15 | 952 |
| | <i>C. maritima</i> (M) | <i>C. edentula</i> (E) | ME | 15 | 936 |
| F ₂ | F ₁ (H) | F ₁ (H) | HH | 13 | 600 |
| | BC | F ₁ (H) | <i>C. maritima</i> (M) | HM | 17 |
| <i>C. maritima</i> (M) | | F ₁ (H) | MH | 17 | 850 |
| F ₁ (H) | | <i>C. edentula</i> (E) | HE | 15 | 750 |
| <i>C. edentula</i> (E) | | F ₁ (H) | EH | 15 | 750 |

'Parental species' represents plants grown from the original collected seeds and the interspecifically outbred seeds of both parental species.

For simplicity, when denoting the crossing directions and corresponding progeny, initials of species are used (E for *C. edentula*, M for *C. maritima* and H for F₁ hybrids), with the female placed before the male. For example, a cross made by introducing pollen from *C. maritima* to the stigma of *C. edentula* is denoted as 'EM'.

Determination of self-incompatibility

Self-incompatibility prevents incompatible pollen, including self-pollen, from germinating and pollen tubes from growing. Therefore, the germination and tube growth of self-pollen on the stigma surface in a plant should ideally be used to determine whether the SI has been inherited in progeny (Nasrallah et al., 2007). However, due to the large number of crosses in this study, seed set following artificial selfing was used to indicate whether individual plants were SC. Breeding system was determined on the same plants that were used to generate the next generation of crosses. A raceme was randomly chosen on each plant and covered with a bag to exclude pollinators. Instead of emasculating and pollinating buds, newly open flowers (day 1) of the bagged racemes were simply squeezed to make sure the stigma surface had been fully covered by self-pollen (previously used in *Cakile maritima* by A. G. Young, CSIRO, Australia, pers. comm. to R. D. Cousens). This was repeated on all flowers of the bagged racemes until no further flowers were available. On each plant, at least 50 flowers were tested in this way and, after plants senesced, the number of successfully set seeds was counted. Since even in SI species occasional spontaneous fruit set and seed production can occur ('matromorphy'; Eenink, 1975), plants with only one spontaneous fruit (i.e. <2 % fruit set) were regarded as effectively SI.

The proportion of SC versus SI plants was calculated and compared with that expected in each generation according to simple Mendelian inheritance, assuming a single recessive SC gene. Generally, the shift from SI to SC is ascribed to a modification of the *S* locus, which controls incompatibility (Nasrallah et al., 2007), and the SC gene is normally recessive to the SI gene in homoploid hybrids (Zeng and Cheng, 2014). In this case, all F_1 s should be SI; furthermore, if random crosses of F_1 s were made, SI would be expected to segregate as a simple dominant trait with a typical F_2 segregation ratio of 3:1 SI:SC. We acknowledge, however, that such calculations based on Mendelian inheritance do not allow for post-zygotic reproductive barriers that might occur upon interspecific hybridization.

Reproductive isolation

Hybridization could affect pollen viability in progeny, rates of pollen germination and pollen tube growth and thus the reproductive outcome of crosses after hybridization. This may or may not be a consequence of the confused self-(in)compatibility system, but nonetheless it is an important component of the fitness of hybrid plants. Therefore, quantitative measures of pollen viability, pollen performance on the recipient style (i.e. pollen germination and pollen tube growth) and corresponding fruit and seed set were used to explore whether there was any possible reproductive isolation in subsequent generations following hybridization.

Pollen viability. The viability of pollen was assessed using fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison, 1970). Fresh anthers were collected from open flowers in the morning on day 1 of flowering and carefully brushed onto the centre of a clean glass slide. The released pollen grains were then stained with one drop of FDA solution and observed under an optical microscope (Leica M250A) in

blue light (wavelength 495 nm) (Li, 2011). The number of viable pollen grains, as well as the total, was counted to determine the percentage pollen viability. All families within plant types were included, and each family had three replicates (plants). In each replicate, three samples were collected for pollen viability and their average percentage viability was used.

Pollen germination and tube growth. Pollen tube growth was measured using aniline blue fluorochrome on the same plants as those used for artificial crosses. Buds were emasculated before they opened and pollen from the appropriate pollen donor was then applied to their pistils. Samples were harvested at intervals of 2, 4 and 6 h after pollination. Three pollinated buds were collected at each interval (i.e. nine buds from a single plant). Samples were stored at 4 °C in 1.5-mL tubes containing 150 μ L of a fixing solution (Lu, 2011). As required, the preserved samples were removed and stained with aniline blue fluorochrome, which caused the callose in the pollen tube to emit blue fluorescence under UV light (Lu, 2011). The intention was to collect samples from outcrossed progeny of parental species and all generations; however, *C. edentula* and some F_1 hybrids ceased flowering too soon so that there were insufficient buds available. The experimental design for pollen tube measurement was therefore incomplete and unbalanced. Pollen germination and pollen tube growth were observed by fluorescence microscopy (Leica DM 2500). Pollen tube length was assessed using ImageJ (<https://imagej.nih.gov/ij/>).

Fruit and seed set. Fruit and seed set were recorded after artificial pollination in each generation. Fully developed fruits were counted about 2 weeks after pollination and harvested after the plants matured. Fruit set proportion (R_f) for a given cross was calculated as $R_f = \frac{\text{total number of fruits set}}{\text{total number of pollinated buds}}$. As *Cakile* fruits have two segments, the numbers of fully developed proximal (N_p) and distal segments (N_d) were also counted. The fruit wall in both segments was removed and fully developed seeds were counted; aborted or abnormal seeds (obvious to the naked eye) were omitted. The total number of fully developed seeds (N_s) was used to calculate the seed set proportion as $R_s = \frac{N_s}{N_p + N_d}$.

Model simulations

To explore potential changes in population dynamics of the *Cakile* system predicted by the original model, we re-ran the simulation model of Mesgaran et al. (2016) with updated selfing rate values based on the experimental results from this study. The model is based on the life cycle of an annual plant with no persistent seedbank. At time t the population consists of N seeds, $N(t)$, that may germinate and survive to maturity as a function of both density-dependent and density-independent factors, represented by a Beverton–Holt type model: $M(N) = \frac{L}{1+bN}$, where M is the number of adult plants, L is the proportion of seeds surviving to maturity independent of population density, while b represents the density-dependent survivorship. Each surviving plant may produce a maximum of θ ovules in the absence of competition, and fecundity declines with population density (i.e. number of adult plants M) following a rectangular hyperbola function: $F(M) = \theta \left[1 - \frac{dM}{1+dM} \right]$, where

F is per capita ovule number and d indicates the rate of decline in ovule production per unit of density. A fraction s of ovules may become fertilized with self pollen, while the remaining $(1 - s)$ fraction is available for outcrossing. The probability of successful outcrossing (pollination), P , was assumed to be positively related to the population density (i.e. there is an Allee effect) following a Holling type III functional response:

$P(M) = \frac{(\rho M)^\gamma}{1 + (\rho M)^\gamma}$, where γ is a shape parameter and the reciprocal of ρ gives the population density at which 50 % of plants are likely to be pollinated by pollinators. The difference equation for a single species' population dynamics is therefore given by:

$$N(t+1) = M(N(t)) \theta F(M(N(t))) [s + (1-s)P(M(N(t)))].$$

The above model was then expanded to simulate the population dynamics of multiple species (genotypes):

$$N_{x(t+1)} = \sum_y M_y \theta_y F_y(M) \left[s_y H(x : y, y) + (1 - s_y) \sum_z C(y, z) V_y P(M) H(x : y, z) \right]$$

where subscripts x and y denote the three plant types i.e. *C. edentula*, *C. maritima* and hybrid. The difference between genotypes in degree of attractiveness towards pollinators was included by adding parameter V_y , which describes the relative pollinator preference for genotype y . The compatibility of genotype y with genotype z is given by $C(y, z)$ which was experimentally quantified in this study. The identity (and frequency) of offspring, x , resulting from a cross between genotypes y and z is determined by $H(x : y, z)$. When $y = C. edentula$ and $z = C. maritima$, all the resulting offspring will be hybrids, i.e. $x =$ hybrid, but for a cross between any parental species and a hybrid (i.e. backcross), half the offspring will be hybrid and the other half will possess the identity of the parent as predicted by a Mendelian model of inheritance. All simulations were coded and run using MATLAB (Version R2019a); codes and original parameter values can be found at <https://www.pnas.org/content/suppl/2016/08/31/1605626113.DCSupplemental>.

Two main changes were implemented in the model relating to the selfing rates of *C. edentula* and hybrids. Based on literature review and some preliminary data, these rates had been assumed to be 80 and 20 %, respectively, in the original model (Mesgaran et al., 2016). As will be seen, our new study showed that under field conditions the selfing rate of *C. edentula* is much lower, i.e. 48 % (though under glasshouse conditions it can be as high as 81 %), and that of hybrids, averaged over F_1 , F_2 and backcrosses, does not exceed 2 %. We also ran the

simulations with different combinations of the selfing rates in both hybrids and *C. edentula* to further explore the interactive effects of breeding system on the population dynamics of *C. maritima*. In all simulations, *C. maritima* was treated as SI, with 1 % spontaneous fruit set, and all other parameters of the model were kept unchanged.

Data analysis

In this study, SAS 9.4 (SAS Institute, Cary, NC) was used to perform data analysis unless otherwise stated. To analyse the breeding system data, we used a binary variable to score the breeding system of a plant, using 1 to indicate an SC system and 0 an SI system. The occurrence of SC (i.e. probability of event being 1) was analysed with a generalized linear mixed model (PROC GLIMMIX) using plant type as the fixed factor and family as a random effect. A binomial distribution with logit link function were used. Comparisons were made between plant types. Pairwise examined in SI frequency in the hybrid classes as well as between expected (based on a Mendelian inheritance model) and observed SI proportions in hybrid classes were also conducted with Fisher's exact test.

Pollen viability was analysed using a generalized linear model (PROC GLM) since the data were approximately normally distributed. The proportion of viable pollen was the response variable while plant type was the fixed factor. The family effect was discarded due to its insignificant impact on the response variable. The difference between means of each plant type was examined using Tukey's *post hoc* test.

Pollen tube growth was expressed as an ordinal variable with four levels – zero, low, medium and high – since many pollen tubes were obscured and unable to be counted or measured accurately. A generalized linear mixed model (PROC GLIMMIX) was used to analyse the data in which pollen tube growth was the response variable while plant type was the fixed factor, with family as a nested random factor within plant type. Multinomial distribution and cumulative logit were the default distribution and link function. Odds ratios were compared between each plant type. The number of germinated pollen grains and the total number of adhered pollen grains were also recorded in the first generation of crosses to assess any difference between the two crossing directions; the event/trial syntax was used for analysis.

Fruit set and seed set were analysed using a generalized linear model (PROC GLM), with plant type used as a fixed effect; the data fitted a normal distribution reasonably well. Family was treated as a replicate for seed set since there was no further replicate within family itself. Differences between plant types were assessed using pairwise comparisons based on Tukey's *post hoc* test.

TABLE 2. Proportion of seeds set as a result of artificial or autogamous selfing in *C. edentula*

| Site | Treatment | Number of plants | Number of tested buds | Selfing proportion | s.e. |
|---------------|---------------------|------------------|-----------------------|--------------------|------|
| Glasshouse | Artificially selfed | 10 | 462 | 0.82 ^a | 0.01 |
| Glasshouse | Natural selfing | 10 | 535 | 0.81 ^a | 0.02 |
| Field station | Natural selfing | 12 | 1200 | 0.48 ^b | 0.04 |

Significance groupings (lower-case letters) are based on Tukey's *post hoc* test. Means with the same letter are not significantly different ($P > 0.05$).

TABLE 3. Breeding system in the parental species and their progeny

| Generation | Plant type | Total | SI plants | SC plants | SC proportion | | R_{as} (s.e.) |
|------------------|--------------------|-------|-----------|-----------|---------------|----------|-----------------|
| | | | | | Observed | Expected | |
| Parental species | <i>C. maritima</i> | 27 | 27 | 0 | 0 | 0 | – |
| | MM | 12 | 12 | 0 | 0 | 0 | – |
| | <i>C. edentula</i> | 33 | 0 | 33 | 1.00 | 1 | 0.82 (0.01) |
| F_1 | EE | 6 | 0 | 6 | 1.00 | 1 | 0.50 (0.18) |
| | EM | 42 | 42 | 0 | 0 | 0 | – |
| F_2 | ME | 45 | 43 | 2 | 0.04 | 0 | 0.13 (0.03) |
| | HH | 36 | 33 | 3 | 0.06 | 0.25 | 0.45 (0.15) |
| BC | EH | 33 | 30 | 3 | 0.09 | 0.50 | 0.22 (0.04) |
| | HE | 45 | 36 | 9 | 0.20 | 0.50 | 0.27 (0.15) |
| | HM | 51 | 46 | 5 | 0.10 | 0 | 0.10 (0.04) |
| | MH | 51 | 48 | 3 | 0.06 | 0 | 0.10 (0.03) |
| | | | | | | | |

R_{as} represents the seed set proportion in SC plants (if any) through artificial selfing. ‘–’ indicates a predominance of SI and thus R_{as} is not available. Plants with fewer than two fruits were regarded as SI.

Explanation of plant types and their codes can be found in Table 1.

Expected proportion of SC plants is based on the assumption of a single recessive SC allele.

C. maritima and *C. edentula* were field-collected plant types while MM and EE were the intraspecific outcrossed ones.

RESULTS

Determination of self-incompatibility

All plants of *C. edentula* were SC (Table 2), while *C. maritima* was mostly SI (Table 3), with a few spontaneously set fruits on a single plant. In *C. edentula*, the seed set rates of autogamously selfing and artificially selfed plants did not differ significantly ($P > 0.05$) but were much higher in the glasshouse than under natural selfing in the field ($P < 0.05$) (Table 2).

In hybrid classes, F_1 , F_2 and backcrosses were mostly SI (Table 3). Only a small proportion of hybrids were SC and the SI:SC ratios observed did not conform to the Mendelian segregation ratios expected for a single-locus biallelic trait (Table 3). All F_1 plants were expected to be SI but two plants with *C. edentula* as the maternal parent (i.e. EM) were SC. In all cross types where segregation was expected, there were significantly lower proportions of SC individuals than expected ($P < 0.01$). Only three out of 36 F_2 s were SC, significantly deviating from the expected proportion of 0.25 ($P < 0.05$). The number of SC plants in the backcrosses to *C. edentula* tended to increase relative to the F_1 , especially in HE, but were still significantly lower than the expected frequency of 0.5 ($P < 0.01$). Some backcrosses to *C. maritima* were also SC when none were expected under the assumption of a single dominant gene.

Reproductive isolation

The parental species showed little difference in pollen viability from their hybrids (Fig. 1). There were no significant differences between subsequent generations. It should be noted that there were two families in ME and F_2 found to be completely male-sterile, i.e. no pollen grains fluoresced after FDA staining (see staining results in Supplementary Data Fig. S1).

Pollen germinated rapidly in both reciprocal crossings and there was no significant difference in the number of germinated pollen grains between crossing directions ($P > 0.05$). Three out

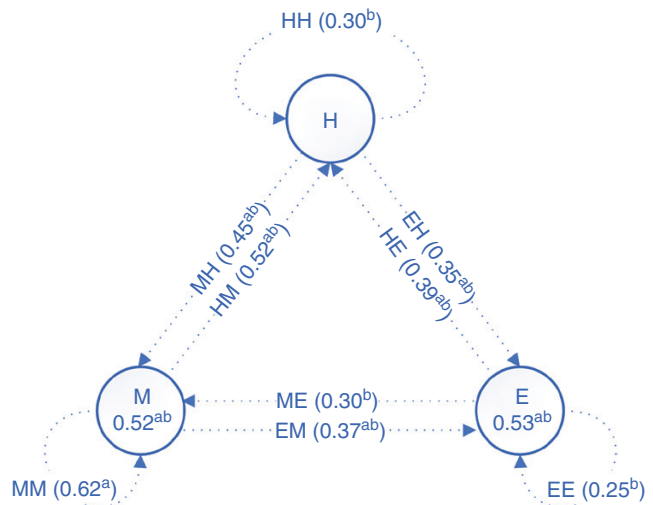


FIG. 1. Pollen viability in the progeny of various reciprocal crosses between *C. edentula*, *C. maritima* and their hybrids. Dotted arrows indicate the crossing direction used to generate each plant type: for example, MH plants (one of the possible backcrosses) were produced by applying H pollen to M stigmas while HH plants (i.e. F_2 s) were produced by crossing H plants with other H plants. The values within circles give the pollen viability of plants grown from field-collected seeds. Significance groupings (lower-case letters) are based on Tukey's *post hoc* test (pairwise comparisons with a significance level of $P < 0.05$). Means with the same letter are not significantly different.

of ten plants in *C. maritima*, however, had no germinated pollen of *C. edentula* on their stigmas, although some pollen grains were successfully attached (Fig. 2A). Such unsuccessful germination was not observed with pollen from *C. maritima* on the stigmas of *C. edentula*.

In all the samples in which pollen had successfully germinated, the pollen tubes grew rapidly into the style within the first 2 h (Fig. 2B–D). However, significant differences were detected in pollen tube growth between crosses (Table 4). Pollen, whether conspecific or interspecific, germinated and performed

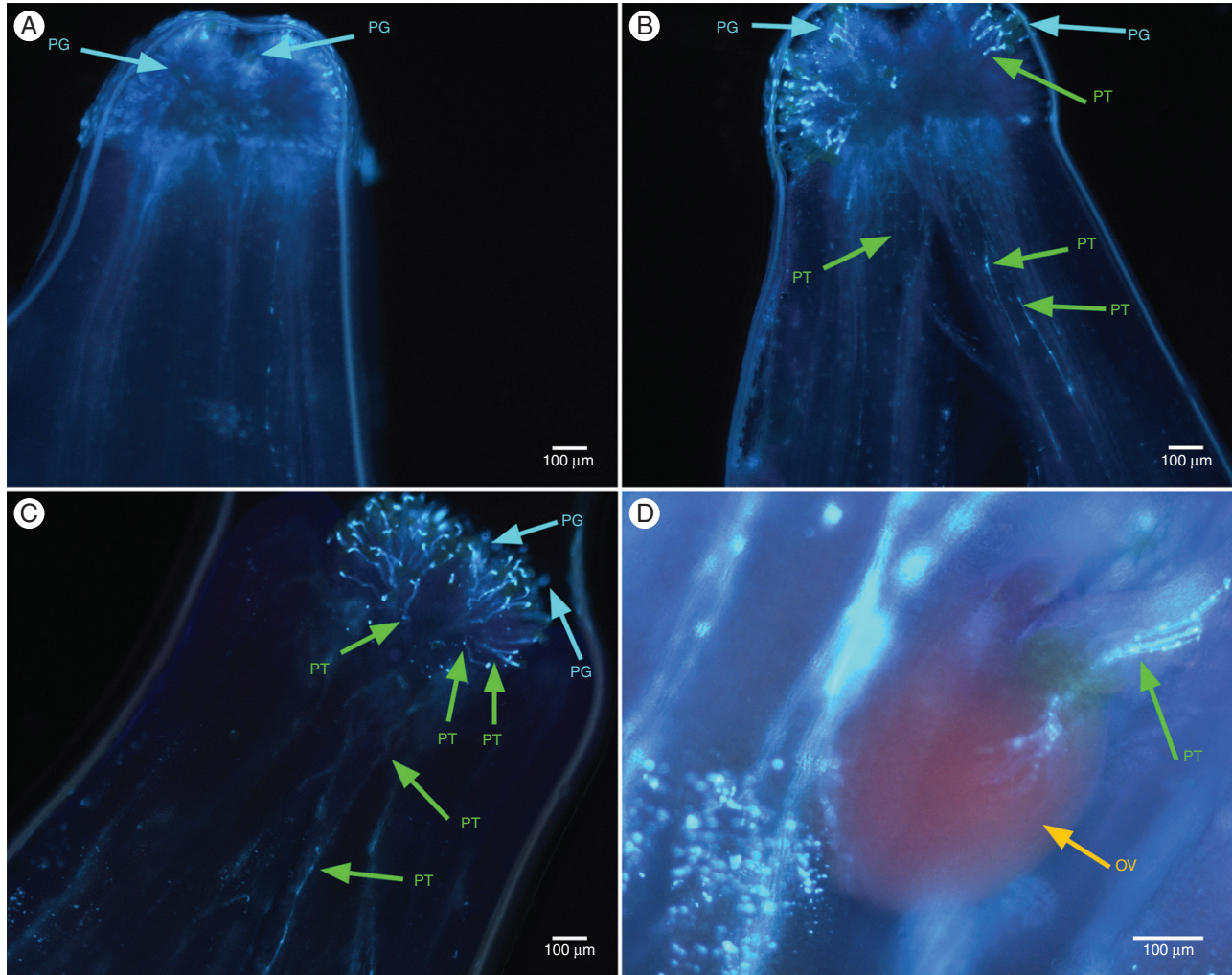


FIG. 2. Pollen germination and pollen tube growth under UV light 2 h after the pollen of *C. edentula* landed on the stigma of *C. maritima* (A and B) and vice versa (C and D). Blue arrows indicate pollen grains (PG), green arrows pollen tubes (PT) and the yellow arrow an ovule (OV).

much better on *C. edentula* stigmas than on *C. maritima* stigmas (Figs 2 and 3). Pollen from hybrids did not show a significant difference in performance between crossing directions (Table 4, Supplementary Data Fig. S2).

Fruit set was achieved in all pollination directions, but the proportion varied between and within generations (Fig. 4). Both crossing directions between *C. maritima* and *C. edentula* (i.e. EM and ME) set fruit. However, the proportion of fruits set was significantly higher when *C. edentula* was the maternal parent (Fig. 4A; $P < 0.001$). Similarly, fruit set was significantly different among the four directions in backcrosses, with the lowest proportion in the cross HE (0.14, $P < 0.01$). The seed set within generations did not show much variation. No significant difference was observed in seed set between the crosses EM and ME (Fig. 4B; $P > 0.05$). Likewise, no significant differences in seed set between backcrosses were detected. The only significant difference observed between directions was between EH and HE, with a much larger percentage of seeds set in EH ($P < 0.05$). The lowest seed set was for crossing F_1 individuals to produce the F_2 generation.

Model simulations

The model simulations indicated that the establishment of *C. maritima* and the replacement of *C. edentula* by *C. maritima* would be more rapid than previously predicted (Fig. 5). With the new parameters, *C. maritima* was predicted to account for >99 % of the mixed population after 11 generations, whereas the same event occurred after 16 generations in the original simulation.

Our sensitivity analysis showed that the system is non-linear and that the effect of hybrid selfing rate on invasion success (or failure) of *C. maritima* is not independent of the selfing rate of *C. edentula*. With a selfing rate of 80 % for *C. edentula*, *C. maritima* can only become the dominant species (i.e. relative frequency >0.99) when the selfing rate of hybrids falls within the range of 10–78 % (*C. maritima* will not be able to establish beyond these threshold values). In contrast, with the observed 48 % selfing rate under natural conditions in *C. edentula*, the lower the selfing rate in hybrids, the faster *C. maritima* can establish and dominate the mixed population (Fig. 6).

TABLE 4. Differences (estimated odds ratios) in the degree of pollen tube growth between plant types (i.e. pollen performance of different donors on different styles)

| Pollen tube growth | Odds ratio | 95 % CI |
|--|--------------------|----------------------------|
| Following interspecific outcrossing between parental species | | |
| M × E versus E × M | <0.001 | <0.001, 0.172 |
| Backcrossing compared with inter-parental outcrossing | | |
| M × H versus M × E | 0.085 | <0.001, 22.739 |
| M × H versus E × M | <0.001 | <0.001, 0.005 |
| Interparental outcrossing and backcrossing compared with intraspecific outcrossing within hybrids | | |
| M × E versus H × H | 1.183 | 0.003, 382.825 |
| E × M versus H × H | >999,999 | 12.021, >999,999 |
| M × H versus H × H | 0.155 | 0.001, 20.262 |
| Other pollen on <i>C. maritima</i> compared with intraspecific outcrossing within <i>C. maritima</i> | | |
| M × H versus M × M | <0.001 | <0.001, 0.041 |
| M × E versus M × M | <0.001 | <0.001, 0.802 |
| Other pollen on non- <i>C. maritima</i> stigma compared with intraspecific outcrossing within <i>C. maritima</i> | | |
| E × E versus M × M | >999,999 | <0.001, >999,999 |
| H × H versus M × M | <0.001 | <0.001, 0.488 |
| E × M versus M × M | 2.693 | 0.006, >999,999 |
| Other pollen on <i>C. edentula</i> compared with intraspecific outcrossing within <i>C. edentula</i> | | |
| E × M versus E × E | <0.001 | <0.001, >999,999 |
| Other pollen on non- <i>C. edentula</i> stigma compared with intraspecific outcrossing within <i>C. edentula</i> | | |
| M × M versus E × E | <0.001 | <0.001, >999,999 |
| M × H versus E × E | <0.001 | <0.001, >999,999 |
| M × E versus E × E | <0.001 | <0.001, >999,999 |
| H × H versus E × E | <0.001 | <0.001, >999,999 |

Pollen tube growth data following H × M, H × E and E × H were not available.

Estimated odds ratios are based on the higher value of pollen tube growth.

For example, pollen tube growth of F₁ in *C. maritima* style (M × H) had an estimated odds ratio of <0.001 compared with that of *C. edentula* on its own style, which means the former was more than 1000 times (= 1/0.001) likely to have lower pollen tube growth than the latter.

Confidence limits including the value 1 indicate the corresponding comparisons are not significant.

Significant comparisons are in bold. See Table 1 for abbreviations of plant types.

DISCUSSION

Although the experimental results presented here are, no doubt, to some extent peculiar to the two chosen species – and are of interest in their own right as case studies of the inheritance of breeding systems and reproductive isolation following hybridization – our motivation was the plausibility of a novel hypothetical mechanism for invasions by SI species. The predictions from the updated model were that the reconstitution of the SI species post-hybridization would be even more rapid than under previous parameter values. At least for this pair of species, then, the non-adaptive advantage of hybridization seems to hold up when tested with rigorously derived information on plant biology: the availability of a congeneric species

could aid colonization by a SC species. Whether the prediction will also be supported for other pairs of species remains to be determined.

Proportions of different breeding systems measured in the F₁ generation may be quite misleading if a single dominant allele is assumed when making predictions. We found that, for our species, F₁, F₂ and backcrosses were mostly, but not exclusively, SI while the proportions of SI and SC individuals in subsequent generations varied (Table 3). This suggests that Mesgaran *et al.* (2016) may have underestimated the potential hybridization-rescue effects between species since they assumed that 20 % of the total flowers in hybrids selfed. *Cakile edentula* had a lower selfing rate under natural conditions while hybrids were mostly SI and thus a greater proportion of flowers than assumed in the model would be available for outcrossing, providing the colonizing species (i.e. *C. maritima*) with more available mates. Therefore, *C. maritima* can establish and dominate the mixed population more rapidly than originally expected (Fig. 5).

The results of our study also indicate that the inheritance of breeding systems in interspecific hybrids may deviate from a Mendelian model. The co-existence of SI and SC in the F₁ and the unusual segregation ratios in the F₂ and backcrosses suggested that the dominance of SI over SC is more complicated than a simple biallelic dominance relationship (Table 3). It is possible that SI dominance over SC in *Cakile* species could be a trait controlled by alleles at multiple loci instead of a single-locus mutation (Ritland, 2002). Alternatively, it could be an anomaly due to the interspecific crossing that we used. Interspecific crossing, compared with intraspecific crossing methods commonly used to determine inheritance, is much more complicated as it involves both pre-zygotic and post-zygotic barriers (Roccaforte *et al.*, 2015; Christe *et al.*, 2016). Our design was also complicated by the choice of populations and families for crossing, in an attempt to avoid chance effects of the unknown number and identity of parental *S* alleles as well as inbreeding depression. Although artificial hybridization could be achieved in both directions, it is obvious that both the F₁ and the F₂ suffered from poor subsequent pollen performance (Table 4), indicating that there are barriers following hybridization between *Cakile* species. Such barriers then could distort the apparent segregation ratios. Mendelian ratios commonly apply in the case of alleles within the same species, where there are no barriers. In interspecific hybrids incompatibility between the parental genomes could interrupt normal segregation, leading to abnormal ratios (but see Nasrallah *et al.*, 2004). Having said this, the patterns observed across generations were not subtle and we are confident that they are strongly indicative of the sorts of values that need to be incorporated into population models.

Meanwhile, absolute unilateral incompatibility following SI × SC hybridization was not observed but significant differences between crossing directions was detected in both pollen performance and reproductive output. Quantitative differences in pollen tube growth between the parental species as well as in subsequent artificial crosses were detected, with poorer pollen performance on an SI style compared with an SC one, regardless of its pollen donor (Table 4, Figs 2 and 3), although there was no significant difference in pollen viability (Fig. 1). Moreover, fruits and viable seeds developed; however,

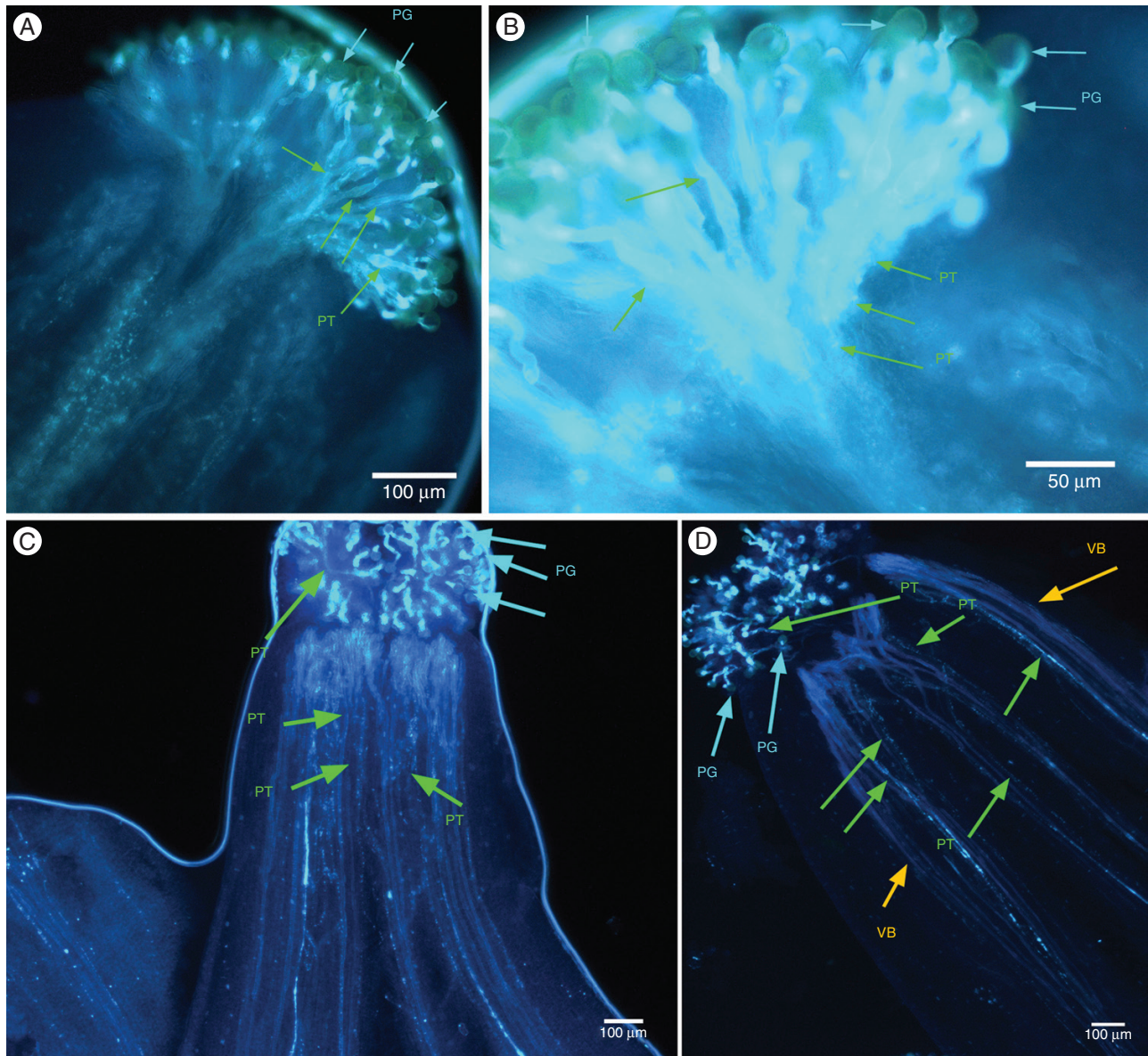


FIG. 3. Pollen germination and pollen tube growth under UV light 2 h after intraspecific pollen landed on its stigma in *C. edentula* (A and B) and in *C. maritima* (C and D). Blue arrows indicate pollen grains (PG), green arrows pollen tubes (PT) and yellow arrows vascular bundles (VB).

significantly greater numbers were found in certain crossing directions, especially when *C. edentula* was the maternal parent (Fig. 4). It should be noted that such bias was not observed when outcrosses occurred between different plants with the same breeding system (i.e. SI × SI and SC × SC). Such facts indicate that fertility is higher when SI species act as pollen donors to SC species, following the SI × SC rule (Hiscock and Dickinson, 1993; Pinheiro et al., 2015). These results suggest that although the SI system did not reject pollen that was expected to be incompatible, it had a significant impact on its pollen performance and thus on reproductive output following interspecific hybridization, i.e. subsequent backcrossing to certain parental species (e.g. ME versus EM and HE versus EH; Table 4, Fig. 4). Such results have also been reported previously in the genus *Cakile* by Willis and Donohue (2017), where the divergence of breeding systems induced significant early-stage reproductive isolation after hybridization.

Therefore, during the early stages of such hybridization events where there are only a few isolated SI plants, individuals from SC species can readily serve as the potential mates (Mesgaran et al., 2016). Selfing is most often regarded as a significant reproductive isolation barrier between species (Vekemans et al., 2014). However, this seems to be not the case for these two species despite *C. edentula* being a predominant selfer (Donohue, 1998). Morphological and genetic studies have suggested that bidirectional hybridization can be achieved readily between these species (Ohadi et al., 2015; Li et al., 2019) in both the glasshouse and the field. Moreover, the lower reproductive isolation when the SI species acts as pollen donor to the SC species observed in our study ensures that a considerable number of hybrids between these two species can be generated despite SI species being isolated (Fig. 5). This essentially provides the SI species with enough mates, overcoming demographic constraints and easing the

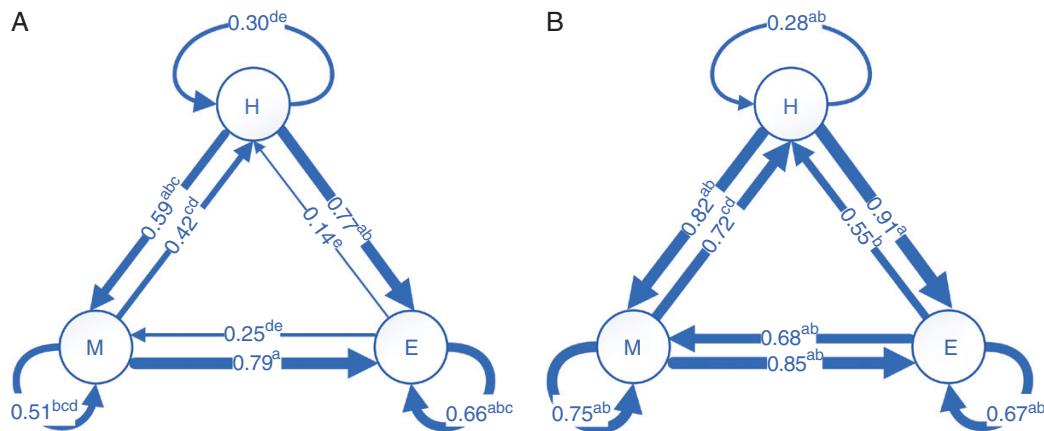


FIG. 4. Proportion of fruits set (R_f) (A) and of seeds set (R_s) (B) in all reciprocal outcrosses. Arrows indicate the direction of pollen transfer for each cross. Arrow thickness is proportional to relative success of fruit set/seed set in that cross. Significance groupings (lower-case letters) are based on Tukey's *post hoc* test. Means with the same letter are not significantly different ($P > 0.05$). Note that intraspecific outcrossing (curved arrows) instead of self-pollination was conducted to produce MM, EE and HH.

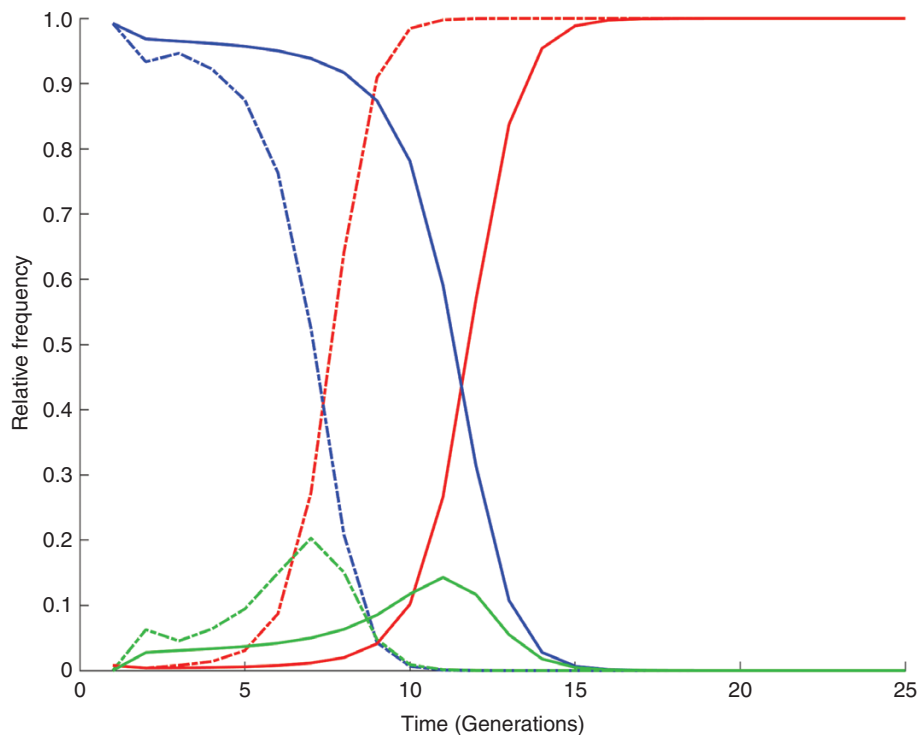


FIG. 5. Population dynamics of *Cakile* predicted by Mesgaran *et al.*'s model (2016). Solid lines represent simulation results in the original model while dashed lines indicate predictions with lower selfing rates estimated in this study (2 % versus 20 % in hybrids and 48 % versus 80 % in *C. edentula*). Selfing rate in hybrids was the averaged proportion of selfing flowers in a hybrid population, estimated from the selfing rate data in Table 3. The relative frequency of *C. maritima* reached 99 % in 16 generations (original simulation) and 11 generations (new simulation), respectively. Blue indicates *C. edentula*, red indicates *C. maritima* and green indicates hybrids.

Allee effects (Mesgaran *et al.*, 2016, 2017). However, with an increasing number of hybrids being produced, there is no guarantee that gene flow between species will eventually occur from *C. maritima* to *C. edentula*. This is because the direction of pollen flow (gene flow) after hybridization will also be regulated by the breeding system and pollinator behaviour (Sargent and Ackerly, 2008; Devaux *et al.*, 2014), as assumed in Mesgaran *et al.* (2016).

Thus, due to SI, the pollen flow as well as its subsequent gene flow in this study is most likely to occur between hybrids and their SI parent, agreeing with Mesgaran *et al.* (2016). Self-incompatible species generally rely on animals for pollination and thus have larger flowers, while selfing species, which are less reliant on pollinators, tend to reduce their allocation to attraction and thus have smaller and less showy flowers (Goodwillie *et al.*, 2010). In our study, significantly showier individuals were

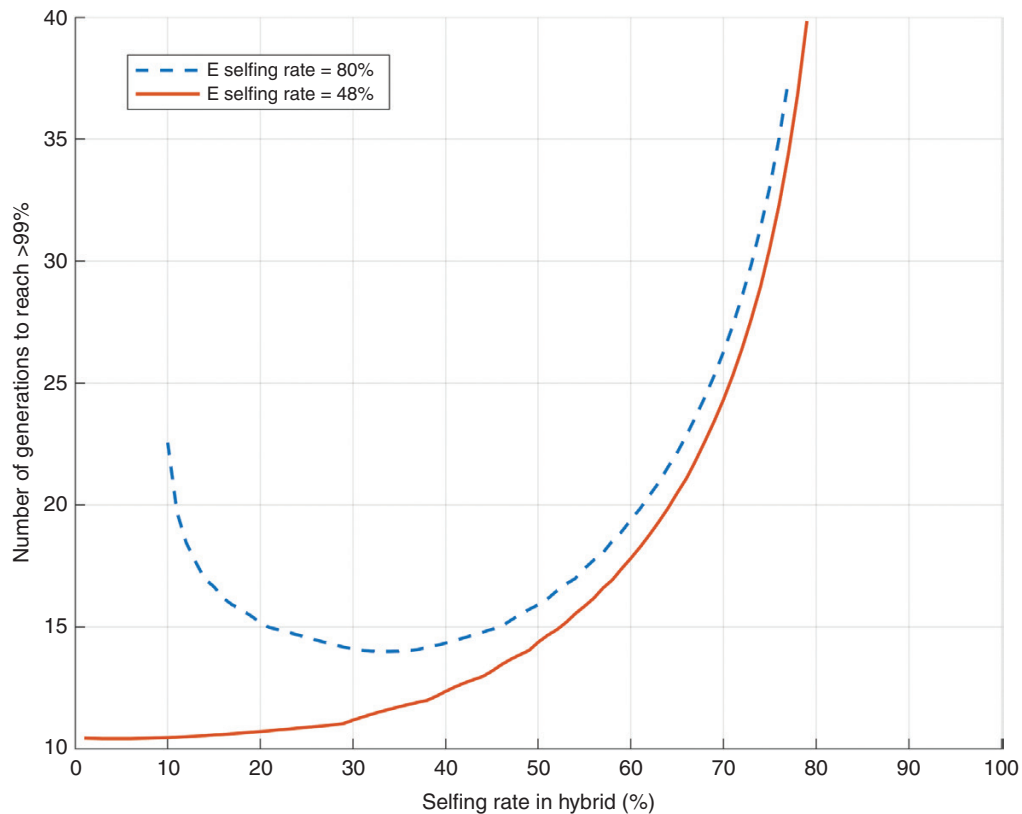


FIG. 6. Relationship between the selfing rate in hybrids and the time to dominance of *C. maritima* (i.e. relative frequency >0.99) at two fixed selfing rates of *C. edentula* (E; 48 % and 80 %), predicted using the simulation model developed by Mesgaran *et al.* (2016). Selfing rate in hybrids was the averaged proportion of selfing flowers in a hybrid population, estimated from the selfing rate data in Table 3.

observed in most hybrids (especially in the backcrosses to the SI parent) and in the SI parent compared with *C. edentula* plants (Supplementary Data Table S1). Therefore, SI hybrids and their SI parent should be more attractive to pollinators than the SC parental species (*C. maritima* has a much higher visitation rate than *C. edentula* in the field; see Supporting Information in Mesgaran *et al.*, 2016), indicating that the pollen flow is much more likely to occur between these SI individuals. Meanwhile, as suggested before, the SI in *Cakile* species, although not fully functional in the face of interspecific pollen, still showed significant negative impacts on its performance. Thus, even though there will be occasional pollinator visits and pollen transfer between the SC species and hybrids, such pollen flow would be largely reduced by the SI in hybrids (current research on the preferences of insects to *Cakile* hybrids is ongoing; T. Jalali, The University of Melbourne, Australia, pers. comm.). Consequently, most F_1 s cannot self and mainly rely on pollinators for pollination due to the presence of SI; they have a greater chance of outcrossing as well as backcrossing to their SI parental species.

In our study, the early generations of backcrosses between hybrids and the SI parent had high similarity in many traits to *C. maritima* and better performance compared with other hybrids, with showier floral display, greater flower production (Supplementary Data Table S1) and larger plants (Supplementary Data Table S2). Therefore, after repeated backcrossing, the breeding system in subsequent generations may shift over to completely SI with its corresponding phenotypic

characteristics. Such hybrids would be barely distinguishable from the original species since they have the same phenotypes and breeding systems. Support for this comes from the finding that phenotypes identified as *C. maritima* in the wild may contain genetic material from *C. edentula*, i.e. introgression (Ohadi *et al.*, 2015). This provides further support for Mesgaran *et al.*'s (2016) model, which concluded that preferential cross-pollination between hybrids and SI species (asymmetrical backcrossing) can facilitate the establishment of an SI invader.

In conclusion, while the non-adaptive mechanism of invasion following hybridization is supported in our study and it is tempting to generalize, caution is needed when making predictions in the absence of detailed information on plant breeding systems, their inheritance and reproductive isolation. More studies are needed in which the demographic implications of biological differences between species are explored over multiple generations. We also need studies of the genetic basis of SC in a greater range of species, rather than having to make assumptions that all species are similar to *Arabidopsis thaliana*. It is by no means certain that what happens within a single species is what will happen in hybridizing species, no matter if they are closely related.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following.

Figure S1: pollen viability test in the parental species and all hybrid generations using fluorescein diacetate. Figure S2: pollen germination and pollen tube growth under UV light 2 h after pollination in artificial backcrossing with *C. maritima* and in interspecific crossing within F_1 s. Table S1: means of petal area per flower (mm^2) and daily flower production in each plant type. Table S2: means of plant size (projected area) in all plant types at maturity.

FUNDING

C.L. was partially funded by the China Scholarship Council (grant 201306240013). M.B.M. was supported by Australian Research Council grant DP140100608.

ACKNOWLEDGEMENTS

We thank Phillip Salisbury, Ed Newbigin and Susan Hoebe for discussions of the ideas presented here.

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