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RESEARCH ARTICLE

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Pollen competition in hybridizing *Cakile* species: How does a latecomer win the race?

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Abstract

Premise: Hybridization between cross-compatible species depends on the extent of competition between alternative mates. Even if stigmatic compatibility allows for hybridization, hybridization requires the heterospecific pollen to be competitive. Here, we determined whether conspecific pollen has an advantage in the race to fertilize ovules and the potential handicap to be overcome by heterospecific pollen in invasive *Cakile* species.

Methods: We used fluorescence microscopy to measure pollen tube growth after conspecific and heterospecific hand-pollination treatments. We then determined siring success in the progeny relative to the timing of heterospecific pollen arrival on the stigma using CAPS markers.

Results: In the absence of pollen competition, pollination time and pollen recipient species had a significant effect on the ratio of pollen tube growth. In long-styled *C. maritima* (outcrosser), pollen tubes grew similarly in both directions. In short-styled *C. edentula* (selfer), conspecific and heterospecific pollen tubes grew differently. *Cakile edentula* pollen produced more pollen tubes, revealing the potential for a mating asymmetry whereby *C. edentula* pollen had an advantage relative to *C. maritima*. In the presence of pollen competition, siring success was equivalent when pollen deposition was synchronous. However, a moderate 1-h advantage in the timing of conspecific pollination resulted in almost complete assortative mating, while an equivalent delay in conspecific pollination resulted in substantial hybrid formation.

Conclusions: Hybridization can aid the establishment of invasive species through the transfer of adaptive alleles from cross-compatible species, but also lead to extinction through demographic or genetic swamping. Time of pollen arrival on the stigma substantially affected hybridization rate, pointing to the importance of pollination timing in driving introgression and genetic swamping.

KEYWORDS

Brassicaceae, *Cakile edentula*, *Cakile maritima*, hybridization, invasion, pollination timing, post-pollination, reproductive barrier, species-specific markers

Interspecific hybridization is frequent between invasive or invasive and native taxa (Ellstrand and Schierenbeck, 2000; Mallet, 2005; Rius and Darling, 2014). It can contribute to invasion success through multiple mechanisms including demographic rescue (Mesgaran et al., 2016), hybrid vigor, or adaptive introgression (Whitney et al., 2010; Bock et al., 2018). Hybridization between invaders can also bear detrimental consequences for the co-occurring species due to potential demographic or genetic swamping (Todesco et al., 2016). We thus need a comprehensive understanding of the mechanisms that facilitate or limit hybridization during invasion to predict the evolutionary consequence of hybridization for the dynamics of co-occurring species (Yakimowski and Rieseberg, 2014).

The difference in the competitive ability of heterospecific pollen grains compared to conspecific pollen grains is a major driver of hybridization success. Siring success in flowering plants requires pollen production, pollen transfer, compatibility of pollen and stigma, and competition between pollen grains (Barrett and Eckert, 1990; Minnaar et al., 2019). Pollen germination and pollen tube growth can induce considerable variation in post-pollination processes, potentially influencing siring success (Rigney et al., 1993; Christopher et al., 2020) and even imposing reproductive barriers. These barriers may stem from several genetic and physiological mechanisms (Rieseberg and Willis, 2007; Harder et al., 2016) that impede reproduction (Rieseberg and Willis, 2007; Arceo-Gómez and Ashman, 2016; Ostevik et al., 2016) and alter the composition and genetic diversity of the progeny (Rieseberg and Willis, 2007). To determine the specific influence of post-pollination to the prezygotic component of reproductive success, it is critical to evaluate pollen–stigma compatibility and pollen tube competition between co-occurring species (Barrett and Eckert, 1990; Barrett et al., 2008; Streher et al., 2018).

The timing of pollen arrival on the stigma has been shown to influence the outcome of competition for fertilization (Karron et al., 2006; Burkhardt et al., 2009; Bruckman and Campbell, 2016b; Sorin et al., 2016). If pollen grains from different cross-compatible species are concurrently deposited on a stigma, the probability of siring success is likely to be correlated with the proportion of pollen types and the relative growth rate of pollen tubes (Rigney et al., 1993). However, when there is a time lag between the arrival of different pollen grains, the siring outcome is expected to change. If the interval between the time of pollen arrival is long, even a slow-growing pollen tube can still fertilize the ovule (Figueroa-Castro and Holtsford, 2009). In contrast, if the interval between the time of pollen arrival is short, a fast-growing pollen tube deposited later on the stigma may outcompete a slower-growing tube from a pollen grain that was deposited earlier (Christopher et al., 2020). Early arrival on the stigma is expected to confer a substantial advantage in siring (Snow et al., 2000), but it is not clear whether other pollen features might compensate for the lag. Hence, with respect to pistil length or pollen tube growth rate, this lag can provide a window of opportunity for pollen tube competition in some species (e.g., 48 h; Figueroa-Castro and Holtsford, 2009), while for others the time frame for competing pollen tubes to reach the ovules is much shorter (e.g., 1 h; Suárez-Mariño et al., 2019).

Differences in mating systems can also impose selection pressures that alter fertilization (Smith-Huerta, 1996; Mitchell et al., 2009; Mazer et al., 2018; Pickup et al., 2019) and hybridization outcomes (Karron et al., 2012; Willis and Donohue, 2017; Li et al., 2019) with potential consequences on the ability of species to colonize (Pannell, 2015; Ostevik et al., 2016). Several reproductive outcomes have been observed when a self-fertilizing and an outcrossing species hybridize (Brys et al., 2014; Li et al., 2019; Pickup et al., 2019; Li et al., 2021), but the specific effects of pollen deposition time and pollen tube growth on the hybridization of species with different mating systems have not been widely explored. It has been shown that with high selfing, pollen competition is irrelevant because pollen from different individuals does not interact (Brys et al., 2014; Pickup et al., 2019). By contrast, with outcrossing, sexual selection theory predicts the evolution of more competitive pollen due to the enhanced number of mates competing for access to the same ovules (Mazer et al., 2018; Peters and Weis, 2018). However, the outcome of pollen competition is influenced by the timing of pollen arrival in addition to pollen tube growth rate. Species with prior selfing relative to the timing of outcrossing may experience limited opportunities for outcrossing, which can thereby prevent hybridization. However, when a self-compatible (SC) and a self-incompatible (SI) species hybridize, early arrival of SI pollen on an SC stigma (before SC pollen is released) might provide a window of opportunity for the SI pollen to fertilize the SC species (Li et al., 2019). Fertilization success of the SI species could be further enhanced by faster pollen growth. Accordingly, these hypotheses can be powerfully investigated by testing the effect of pollen tube growth on siring success in reciprocal crosses between a self-incompatible and self-compatible species. The results would shed light on pollen growth and compatibility as one of the drivers of siring and hybridization success.

The *Cakile* species complex provides an illuminating model to analyze the siring pattern of cross-compatible, co-flowering species that are actively hybridizing yet have contrasting mating systems. Two sea rocket species (*Cakile* Mill., Brassicaceae) have both invaded Australia's southern and eastern coastlines (Rodman, 1986). These two species are morphologically different (Appendix S1) and have distinct flower characteristics with respect to flower size, anther size, and pistil length (Rodman, 1974). *Cakile edentula* is an annual self-compatible species introduced from eastern North America, while *C. maritima* is a self-incompatible species originating from Europe and North Africa and introduced to Australia decades after *C. edentula*. Although both species are invasive to Australia, *C. maritima* has been more successful in most regions where the two species co-occur and has frequently replaced *C. edentula* (Barbour and Rodman, 1970; Rodman, 1986; Boyd and Barbour, 1993; Cousens et al., 2013). In regions where *C. edentula* and *C. maritima* coexist, heterospecific pollen transfer is frequent and the chance of hybridization is substantial (Mesgaran et al., 2016). Experimental greenhouse studies have shown that *C. maritima* and *C. edentula* are highly crossable when using either species as the pollen donor (Rodman, 1974; Li et al., 2019, 2021); hybrids in both directions of crosses have also been observed in natural *Cakile* populations in Australia (Rodman, 1974; Ohadi et al., 2016; Rosinger et al., 2021). Previous studies on the hybridization outcomes of *Cakile* species revealed that pollen viability was equal in *C. edentula* and *C. maritima* (Li et al., 2019). Pollen germination rates for these reciprocal crosses were also similar, with pollen germinating quickly in both directions (Li et al., 2019, 2020). However, some degree of unidirectional incompatibility was observed between these self-compatible and self-incompatible species, as a consequence of lower pollen germination rate and tube

growth in the self-incompatible stigma, regardless of the pollen donor species (Li et al., 2020).

In light of these slight differences, determining how heterospecific pollen competitive ability differs between these two species with divergent mating systems is critical. Here, we hypothesized that heterospecific siring success will depend on the relative performance of synchronously deposited pollen grains from different species and that the rate of success will be positively correlated with the relative timing of heterospecific pollen deposition in sequential pollinations. Determining the paternity of the seeds produced using species-specific molecular markers offers a means to understand how post-pollination and post-zygotic processes influence the genetic composition of the offspring (Karron et al., 2012). The aims of this study were thus to quantify (1) the variation in pollen tube growth in conspecific versus heterospecific pollination treatments, (2) how the timing of pollen deposition influences siring success, and (3) how the likelihood of hybrid seed formation is affected by post-pollination processes.

MATERIALS AND METHODS

Plant material

Plants were grown from seeds collected from a natural population of *Cakile edentula* from Lighthouse Jetty beach, Tasmania, Australia (43°27′02.4″S, 147°08′52.0″E) and of *Cakile maritima* from Marion Bay, Tasmania, Australia (42°49′12.7″S, 147°52′09.2″E). Seeds were germinated on 1% w/v agar in 9-cm sterile Petri dishes, sealed with parafilm and placed in a growth chamber with day/night temperatures of 16/25°C and 12 h light/12 h dark. After a week, the seedlings were transferred to 5-L pots filled with pre-mixed soil (4:1 fine sand to pine bark and slow release NPK fertilizer [16:4:10+TE; Macracote Coloniser Plus, NSW, Australia]) and kept in a glasshouse at The University of Melbourne, Burnley Campus. To control aphids, Eco-Oil (Multicrop, Australia) insecticide at a rate of 10 mL/L was sprayed before flowering when necessary. We then selected seven *C. maritima* and seven *C. edentula* plants. Three plants of each species were randomly selected as pollen donors, and the remaining four plants were assigned as pollen recipients. For avoiding environmental variation due to different pollination conditions, as all pollen recipient plants flowered, open flowers from each plant were removed, then plants were covered by a netted tent to prevent insect visitation before the pollination treatments.

Pollen grain and style measurement

At the time of anther dehiscence, 10 anthers from the three *C. edentula* pollen donor plants and 10 anthers from the three *C. maritima* pollen donor plants were randomly selected and transferred into separate Eppendorf tubes, then 100 μL 70% ethanol was added to each tube and pipette-mixed gently to homogenize the pollen grains. Then, 10 μL of each sample was transferred to a microscope slide, left for 5 min to air dry and observed with an optical microscope (Leica M250A). Images of the sample on each slide were captured using a Leica DMC2900 HD camera for further analysis. Ten pollen grains on each slide were randomly selected, and the major axis diameter of the pollen grains was measured using ImageJ (https://imagej.nih.gov/ij/). Consequently, 300 pollen grains for each species (3 donor plants × 10 anthers from each plant × 10 pollen grains from each anther) were sampled and analyzed to measure the diameter of the pollen grains. Differences in the diameters of the pollen grains between *C. edentula* and *C. maritima* were analyzed using a linear mixed model. Pollen grain diameter was fitted as the response variable, plant species as the fixed effect, and anthers nested in individual plant were treated as the random effect.

To measure style length, we randomly selected five flowers that had bloomed from each of the four *C. edentula* pollen recipient plants and five flowers from each of the four *C. maritima* recipient plants. We then removed the petals and anthers, and placed the pistils on a white background. The pistils were then photographed using a Nikon D7500 camera, and style length was measured for 20 flowers per species, using ImageJ (https://imagej.nih.gov/ij/). Differences in pistil length between *C. edentula* and *C. maritima* was analyzed using a linear mixed model. Pistil length was fitted as the response variable, plant species as the fixed effect, and individuals within each species were treated as the random effect.

The effects in these linear mixed models were tested through ANOVA using type III analysis of variance with Satterthwaite's method and the anova function in the R package lmerTest (Kuznetsova et al., 2017) in R v4.0.2 (R Core Team, 2008).

Pollination techniques

A randomized complete block design with four replications was used for the experiments. Because of the different levels of self-incompatibility of each individual, each plant was considered as a block (pollen recipient). To avoid self-pollination, we emasculated every bud of the pollen recipient plants 24 h before anther dehiscence. The petals were left intact, and after flowers opened, emasculated flowers on the racemes of each plant were hand-pollinated with an equal amount of pollen using pollen from the donor plants. To assure efficient pollination after flower emasculation, we increased the glasshouse humidity by turning off the ventilation and minimizing the air flow in the experimental environment. To prepare the pollen source, we selected three plants as the source of *C. edentula* pollen, and three plants as the source of *C. maritima* pollen. To minimize the possible effects of self-incompatibility, we used mixed pollen loads from the three pollen donor plants from each species. To apply pollination treatments, the pollen grains were scooped from the donor plants and finely mixed on a microscope slide. Then, with a microspatula, an equal number of pollen grains (mean ± SD = 342 ± 23.6, see

methods below) was deposited on each stigma surface on the emasculated flowers. To ensure consistent quantities of pollen grains applied on the stigmas, we controlled the amount of pollen deposited on the stigma by using a microspatula with a calibrated 0.2-mm blade width. To estimate the number of pollen grains that were scooped with the microspatula, 10 anthers from each species were randomly selected, and pollen from selected anthers was scooped separately. Then the microspatula was washed with 70% ethanol on a microscope slide to ensure that all pollen grains were washed from the spatula. The pollen grains on each microscope slide were counted using an optical microscope (Leica DM2500). For the hand-pollinations, a mobile phone was adjusted at a fixed position above the flowers, so that the Android phone application Magnifying Glass version 1.2.2 (Pony Mobile, Hong Kong) magnified the small *Cakile* flowers and stigmas by × 10 to ensure pollen grains were properly deposited on the stigma.

Before measuring pollen tube growth, to ensure successful fertilization of the pollen donors in both species, we set up positive control treatments involving a separate application of conspecific and heterospecific pollen on stigmas of *C. edentula* and *C. maritima* and examined them for fertilization after 48 h. Three random flowers on each pollen recipient plant (four recipient plants per species) received conspecific pollen and three random flowers received heterospecific pollen. Hence, we had 24 control positive samples for each species to confirm successful fertilization in different crossing directions.

Experiment 1: Pollen tube growth measurement in the absence of competition

Pollination treatments included *C. edentula* and *C. maritima* pollen recipients that received conspecific or heterospecific pollen grains for a duration of 1, 2, or 4 h (from when pollen was deposited on the stigma until the pistil was removed to measure pollen tube growth). Two pollen recipient species, two pollen donor species, three pollination durations ($2 \times 2 \times 3 = 12$ pollination treatments), and four pollen recipient replicates resulted in 48 pollinations. Each of the pollination combinations was randomly assigned to one raceme on each recipient plant. All pollinations were performed from 6 May to 22 May 2020.

After the pollen treatments of the stigmas, flowers were removed at the defined times (1-, 2- and 4-h pollination durations) to be stained using procedures that were based on those from previous studies (Dionne and Spicer, 1958; Martin, 1959; Alexander, 1987; Dashek, 2000). Floral tissues including petals, sepals, and filaments were carefully removed, and only the pistil was kept. The pistils were immediately transferred to 1.5 mL tubes, and 500 µL of the fixation solution was added. The fixation solution was a 3:1 mixture of 100% ethanol and glacial acetic acid. Pistils were completely bleached in this step to remove all pigments that might generate interfering fluorescence. Following tissue bleaching, a sodium buffer was used to soften the pistils (maceration). This buffer was made of a strong sodium hydroxide solution (8*N*). To make NaOH solution with normality 8 N, 32 g of NaOH pellets was added to 100 mL of distilled water and mixed gently. Pistils were then transferred to new 1.5 mL tubes, and 500 µL of NaOH solution was added to each tube and then incubated at 60°C for 30 min. After maceration, pistils were rinsed in distilled water and gently transferred to a microscope slide. Finally, pollen tubes were stained in a 0.1% w/v solution of aniline blue in 0.1 N K_3PO_4. One drop of the stain was placed on the pistil, which was covered with a coverslip and pressed very gently to spread the pistil into a thin layer. The aniline blue stain penetrates the macerated pistils very quickly in a few minutes. Pollen tubes were then viewed with a fluorescence microscope (Leica DM2500) using excitation filter BP 450–490 nm. Images of the sample on each slide were captured using a HD camera (Leica DMC2900), then blue-greenish fluorescing branches were counted and lengths measured using ImageJ (https://imagej.nih.gov/ij/). Pollen tube length was normalized over the distance from the style to the first ovule as the ratio of pollen tube length to distance between the stigma and first ovule.

Experiment 2: Paternity determination

Nine pollination treatments were applied to the two species: a single-donor pollen load of *C. maritima* pollen or *C. edentula* pollen as control treatments and seven combinations of a two-donor pollen load (50:50 mixture of *C. maritima* pollen and *C. edentula* pollen) with different time lags between the application of the two donor loads (Table 1). To apply the pollen loads, half of the lobe of the stigma received one species' pollen load and the other half of the stigma lobe received the second species' pollen load. After the pollinations, flowers were covered with pollination bags. All treatments were done from 31 October to 30 November 2018. Ripening capsules were collected after each pollination treatment individually. Then, 10 seeds (only from the upper segment of the fruit) from each treatment on each plant were collected to determine offspring paternity with genetic markers.

Developing species-specific markers

We developed species-specific cleaved amplified polymorphic sequence (CAPS) markers to assess paternity in pollen competition experiments between the two species *C. edentula* ($2n = 18$) and *C. maritima* ($2n = 18$). Single nucleotide polymorphisms (SNPs) that were potentially fixed between the species were selected based on the allelic frequencies of SNPs derived from the native ranges of *C. edentula* and *C. maritima* (where the species are allopatric) using genotype by sequencing data (Rosinger et al., 2021). From the 98 divergent SNPs that were identified between *C. edentula* and *C. maritima*, 16 candidate fragments with potential restriction enzymes that could cut one specific allele over another at the SNP site were

TABLE 1 Crossing directions and time of controlled hand-pollination treatments to determine paternity. For two-donor treatments, a mixture of (50:50 ratio) pollen from both species were applied at the same time or after a lag of 20 min, 40 min, and 60 min pollination time difference.

Pollen recipient	First pollen donor	Second pollen donor	Pollination lag (min)
C. maritima	*C. maritima*	–	–
	C. edentula	–	–
	C. maritima	*C. edentula*	0
			20
			40
			60
	C. edentula	*C. maritima*	20
			40
			60
C. edentula	*C. maritima*	–	–
	C. edentula	–	–
	C. maritima	*C. edentula*	0
			20
			40
			60
	C. edentula	*C. maritima*	20
			40
			60

found. Primers were designed to amplify the specific sequences using a draft genome assembly of *C. maritima* (GenBank: MK637688.1; https://genome.jgi.doe.gov/portal/CakmarStandDraft/CakmarStandDraft.info.html) and the program Primer3 (Untergasser et al., 2012). All the selected targets were amplified by PCR. Restriction enzymes differentiating the two SNP alleles were identified to develop using the program RestrictionMapper version 3 (http://www.restrictionmapper.org). Eight specific sequences that were consistently amplified for both parental species were chosen as the final PCR target for the rest of the experiment (see Appendix S2). From these, four species-specific fragments were retained, and restriction enzymes (see Appendix S2) were used to distinguish the species.

Genotyping parents and F_1 progeny

The presence of the *Cakile* parental alleles and the homozygosity of all parents at the marker loci were confirmed using the species-specific markers. Genomic DNA from all plants that had been used as pollen recipients and pollen donors was extracted using the DNeasy Plant Mini Kit (QiaGen, Hilden, Germany). Details on the DNA extraction and PCR protocol can be found in the supporting information (Appendix S2). The PCR to amplify the target DNA sequences and subsequent restriction digestion were done for all samples.

To distinguish paternity for F_1 progeny that were produced in the controlled pollination experiment, the same species-specific genetic markers were used. Ten seeds from each treatment were collected on each pollen recipient plant to determine offspring paternity. The collected seeds were sown in the glasshouse, then leaf samples from each seedling were collected, labeled, and kept in a −80°C freezer for later DNA extraction. From each pollination treatment, five of the 10 samples were randomly selected for DNA extraction using the DNeasy Plant Mini Kit (QiaGen, Hilden, Germany). Four *C. edentula* and *C. maritima* individuals that were assigned as pollen recipient plants, received one of nine different pollination treatments (Table 1), and five seedlings from each mother within each treatment were randomly selected for DNA extraction. Hence, 180 F_1 progeny from *C. edentula* mothers and 180 of the F_1 progeny from *C. maritima* pollen recipient plants were selected for DNA extraction and paternity identification. The extracted DNA from the progeny were then used for PCR amplification and restriction digestion to determine the paternity of each progeny with the CAPS markers. The same PCR target sequences, primers, restriction enzymes, and markers used in the parental species genotyping were used for the offspring genotyping experiment.

Statistical analyses

Number of pollen tubes and pollen tube length (Experiment 1)

A linear-mixed model (lmer function in the lme4 package; Bates et al., 2015) was used to test the effects of controlled pollination treatments (pollen recipient species × pollen donor species, hereafter referred to as cross) and duration after pollination (hereafter, time) and their interaction (cross × time) on the number of pollen tubes developed in the styles and on pollen tube length. The number of pollen tubes (48 observations) or pollen tube growth (322 observations) was treated as the response variable, while cross and time were treated as the fixed effects, and block (recipient plants) was modeled as a random effect. The block effect was nonsignificant (fit by REML) and discarded from the following analysis of variance.

The effects in these linear mixed models were tested through ANOVA (Tables 2 and 3) using type III ANOVA with Satterthwaite's method (anova function in lmerTest package; Kuznetsova et al., 2017). Time was modeled at discrete time points in the analyses. Modeling time as a continuous variable required using quadratic regression terms to accommodate the nonlinear response which was ruled out due to the difficulty of providing a quantitative, biological interpretation of the coefficients when both nonlinear and linear effects are jointly estimated. Additionally, the effect of pollen donor and pollen recipient species and the interaction between them on pollen

TABLE 2 Analysis of variance table from the linear mixed model fitted on controlled crosses between *C. edentula* and *C. maritima* during time on the number of pollen tubes growing in the style. In this model, pollen recipient, donor and pollination time were fitted as the fixed effects.

Effect	SS	MS	df	df residual	*F*	*P*
Pollen recipient	0.01	0.01	1	37	0.002	0.95
Pollen donor	0.09	0.09	1	37	0.02	0.87
Time	209.26	209.26	1	37	56.18	**6.26e-09**
Pollen recipient × pollen donor	3.01	3.01	1	37	0.80	0.37
Pollen recipient × time	5.18	5.18	1	37	1.39	0.24
Pollen donor × time	14.00	14.00	1	37	3.75	0.06
Pollen recipient × pollen donor × time	0.43	0.43	1	37	0.11	0.73

Notes: SS, sum of squares; MS, mean square.

TABLE 3 Analysis of variance shows the effect of pollination treatments on pollen tube growth. In this model, we analyzed the effects of controlled pollination treatments and time after pollination on pollen tube growth in *C. edentula* and *C. maritima*.

Effect	SS	MS	df	df residual	*F*	*P*
Pollen recipient	0.14	0.14	1	55	7.14	**0.009**
Pollen donor	0.13	0.13	1	310	6.46	**0.011**
Time	7.39	7.39	1	310	365.70	**<2.2e-16**
Pollen recipient × pollen donor	0.15	0.15	1	310	7.72	**0.005**
Pollen recipient × time	0.01	0.01	1	310	0.64	0.424
Pollen donor × time	0.17	0.17	1	310	8.71	**0.003**
Pollen recipient × pollen donor × time	0.12	0.12	1	310	6.35	**0.012**

Notes: SS, sum of squares; MS, mean square.

tube growth were analyzed for each time separately to test how the effect of recipient species changed in each time. *F*-tests were done separately for the three groups (1 h, 2 h, and 4 h times; Appendix S3, Tables S5–S7), comparing a model where the effect is included against a null model where the effect was not included. Differences between each cross or each time were compared using Tukey's honest significant difference (HSD) test. Shapiro-Wilks normality test was used to check the normality of residuals as implemented in R (v4.0.2) (R Core Team, 2008).

Paternity determination (Experiment 2)

The results from the progeny genotyping experiment were analyzed using a logistic regression model. The same model was used for each pollen recipient species. In these two models, the proportion of fertilization by a pollen donor species was treated as the response variable, assuming a binomial distribution (success or failure of the conspecific pollen). Time lag between conspecific vs. heterospecific pollen deposition was treated as the independent variable. Block effect (individual) was nonsignificant and not further included in the final regression model. Additionally, we used a chi-squared test to determine whether the observed proportion of conspecific vs heterospecific progeny was different from the following null expectations: (1) all offspring were sired by conspecific pollen; (2) all offspring were sired by either species in equal proportions.

RESULTS

Species differences in pollen and style traits

Pollen grains of both species had an oval shape and similar diameter (mean ± SD: 84.40 ± 3.9 µm for *C. edentula*, 84.20 ± 3.4 µm for *C. maritima*; ANOVA, $F_{1,569}$ = 0.0.8, P = 0.36; Appendix S1). However, pistils of *Cakile maritima* had significantly longer styles than those of *C. edentula* (mean ± SD: 5.9 ± 0.1 and 4 ± 0.1 mm, respectively; ANOVA, $F_{1,35}$ = 1980, P < 2e-16). Fluorescent images of the negative control pollination treatments (no pollination, emasculated flowers) displayed similar location and structure of vascular elements in the style and structure of the ovule before fertilization (Figure 1A,B,D,E). Thus, we could differentiate vascular bundles from growing pollen tubes in the image analysis. Vascular bundles (Figure 1C) had a spiral structure, while pollen tubes had brightly fluorescing callose plugs (Dashek, 2000). Positive control treatments, which involved a separate application of conspecific and heterospecific pollen on both *C. edentula* and *C. maritima*

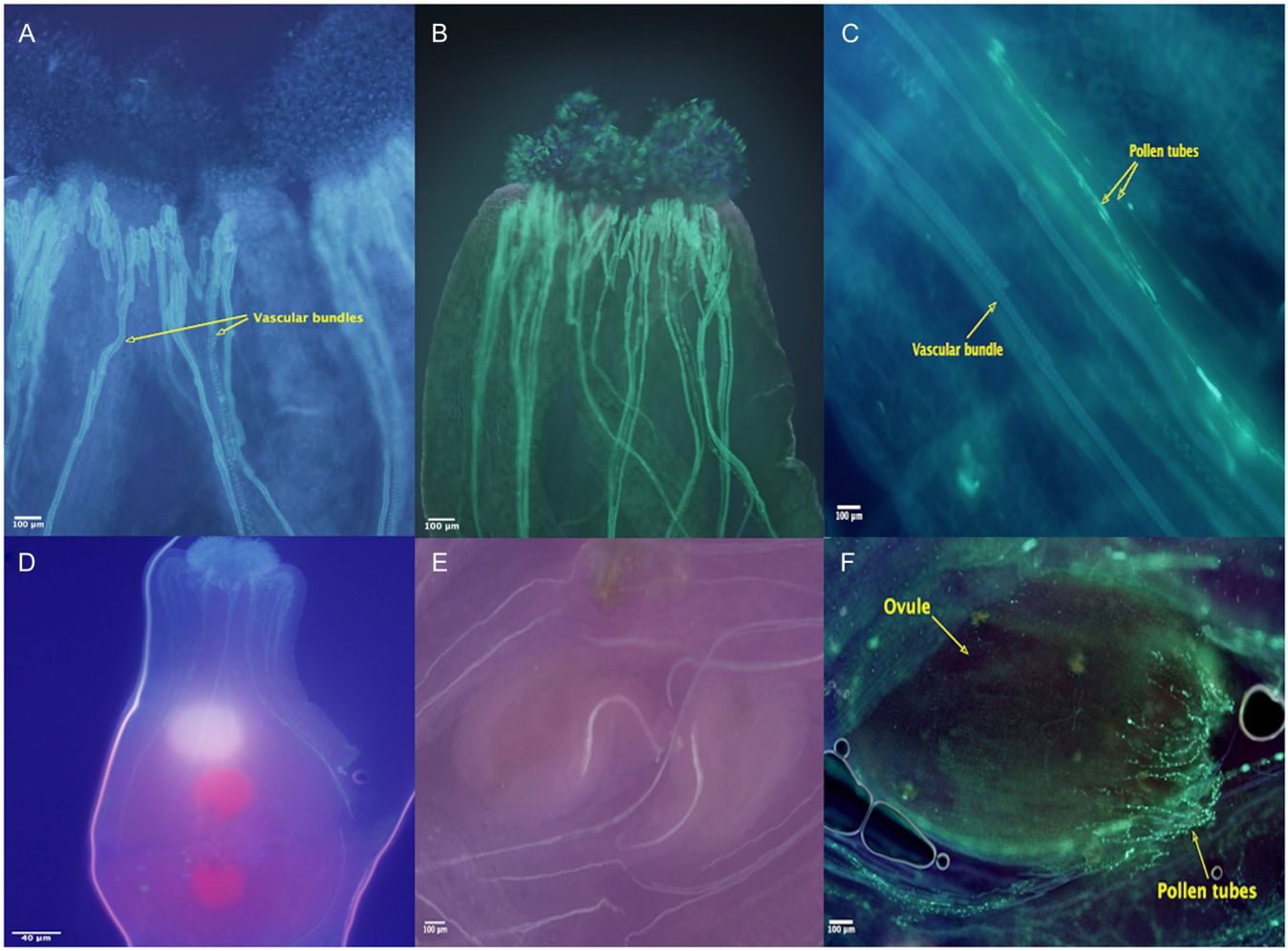

FIGURE 1 Fluorescent micrographs of *Cakile edentula* unpollinated stigma (A), *C. maritima* unpollinated stigma (B) and pistil (D), structure of vascular bundle vs. pollen tube (C), *C. maritima* ovule before fertilization (E), and fertilized ovule after pollen tube penetration (F).

stigma, confirmed successful fertilization of the pollen donors in both species after 48 h (Figure 1F).

The effect of conspecific versus heterospecific pollen on pollen tube growth

Each duration after pollination ("time": 1 h, 2 h and 4 h) had a consistent effect on the number of pollen tubes developing in the style across all treatments (ANOVA, $F_{1,37} = 56.18$, $P = 6.262e\text{-}09$; Figure 2 and Table 2), regardless of the pollination treatment (pollen recipient × pollen donor; ANOVA, $F_{1,37} = 0.8$, $P = 0.37$). However, testing the specific effect of pollen donor species showed that *C. edentula* pollen produced more pollen tubes irrespective of the maternal plant (ANOVA, $F_{1,46} = 6.506$, $P = 0.01$), revealing the potential for a mating asymmetry between the two species whereby *C. edentula* pollen had an advantage relative to *C. maritima*.

In contrast, pollen tube growth differed across pollination treatments (Table 3). Growth was consistent across replicates, validating the experimental approach (low residual variance and no significant difference between replication blocks). The pollen recipient species (ANOVA, $F_{1,55} = 7.14$, $P = 0.009$; Table 3), the pollen donor species ($F_{1,310} = 6.46$, $P = 0.01$; Table 3), and time ($F_{1,310} = 365.70$, $P < 2.2e\text{-}16$) all had a significant effect on pollen tube growth (Table 3). Furthermore, the pollen donor × time interaction and the pollen donor × pollen recipient × time interaction had a significant effect (Table 3), highlighting differences in pollen tube growth rate across the different treatments.

One hour after pollination, conspecific pollination (*C. edentula* [E] pollen on *C. edentula* style, EE) resulted in faster pollen tube growth compared to heterospecific pollination treatments (*C. maritima* pollen on *C. edentula* style, EM; Figures 3, 4A,4B). *Cakile edentula* pollen tubes in *C. edentula* styles (EE) had the fastest growth compared to those in the other pollination treatments (Figure 3). In contrast, pollen tubes from *C. maritima* [M] pollen on *C. maritima* styles, MM, and from *C. edentula* pollen on *C. maritima* styles, ME, did not differ in growth (Figures 3, 4C,4D).

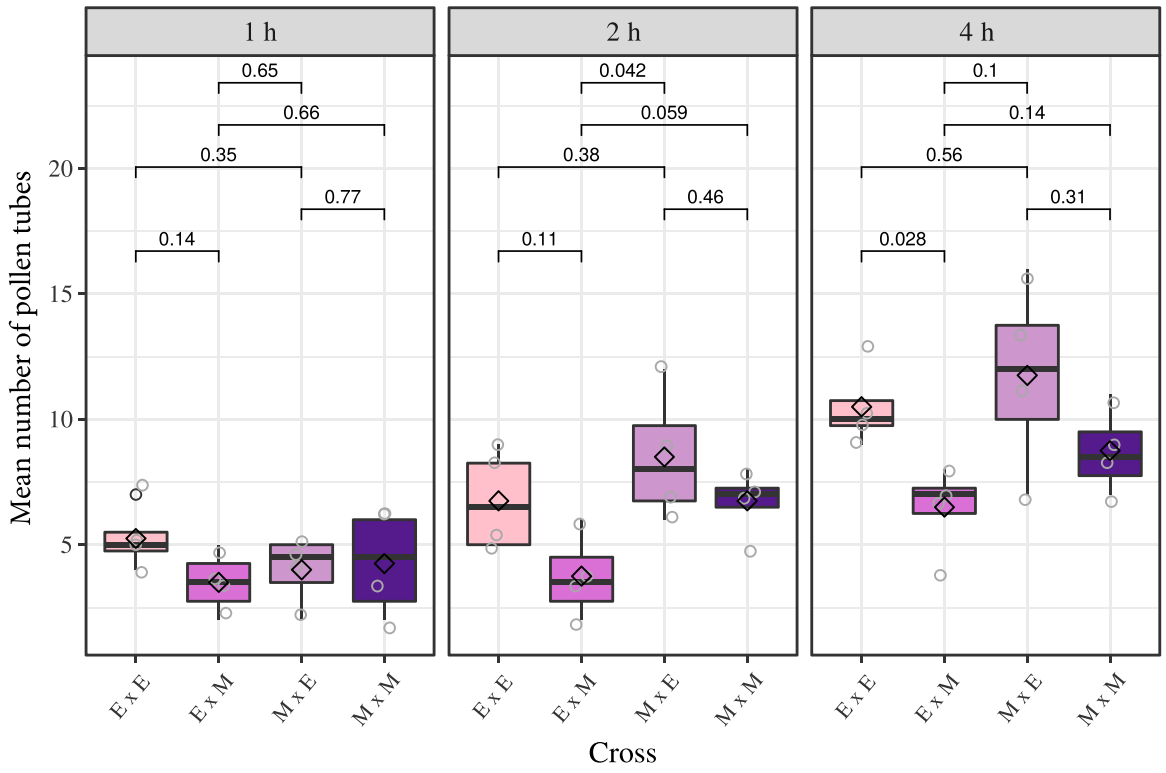

FIGURE 2 Mean number of pollen tubes in the pistil after different pollination treatments 1, 2, or 4 h after the controlled pollination of *Cakile edentula* (E) and *C. maritima* (M). The box and whisker indicate interquartile range: the first quartile (Q1/25th percentile shows the lowest 25% of the observations) and third quartile (Q3/75th percentile shows the upper 25% of the observations). The midline inside the box indicates the median. In crossing directions, the first species is the pollen recipient, and the second species is the pollen donor.

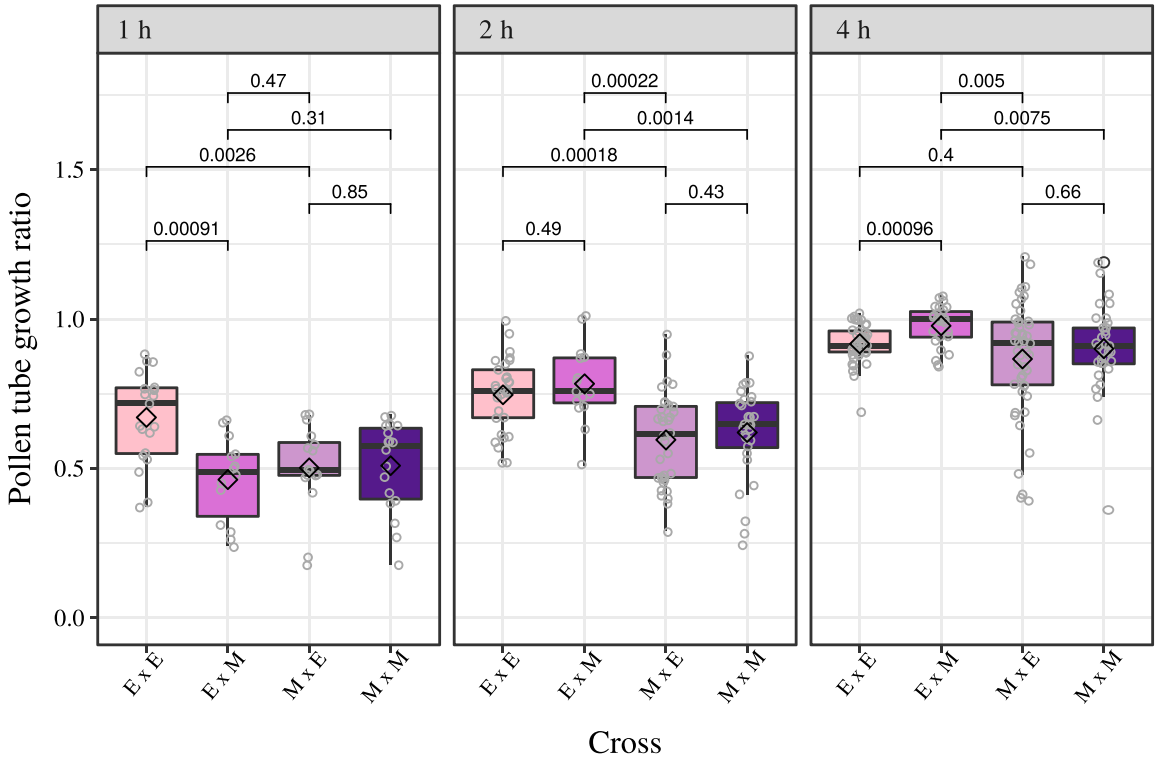

FIGURE 3 The ratio of pollen tube growth to the distance between stigma and ovule after different pollination treatments 1, 2, or 4 h after controlled pollination. The diamond inside the box indicates the mean and the midline shows the median. The box and whisker show interquartile range: the first quartile (Q1/25th percentile shows the lowest 25% of the observations) and third quartile (Q3/75th percentile shows the upper 25% of the observations). The numbers above the boxplots show *P*-values from pairwise comparison using Tukey's HSD test.

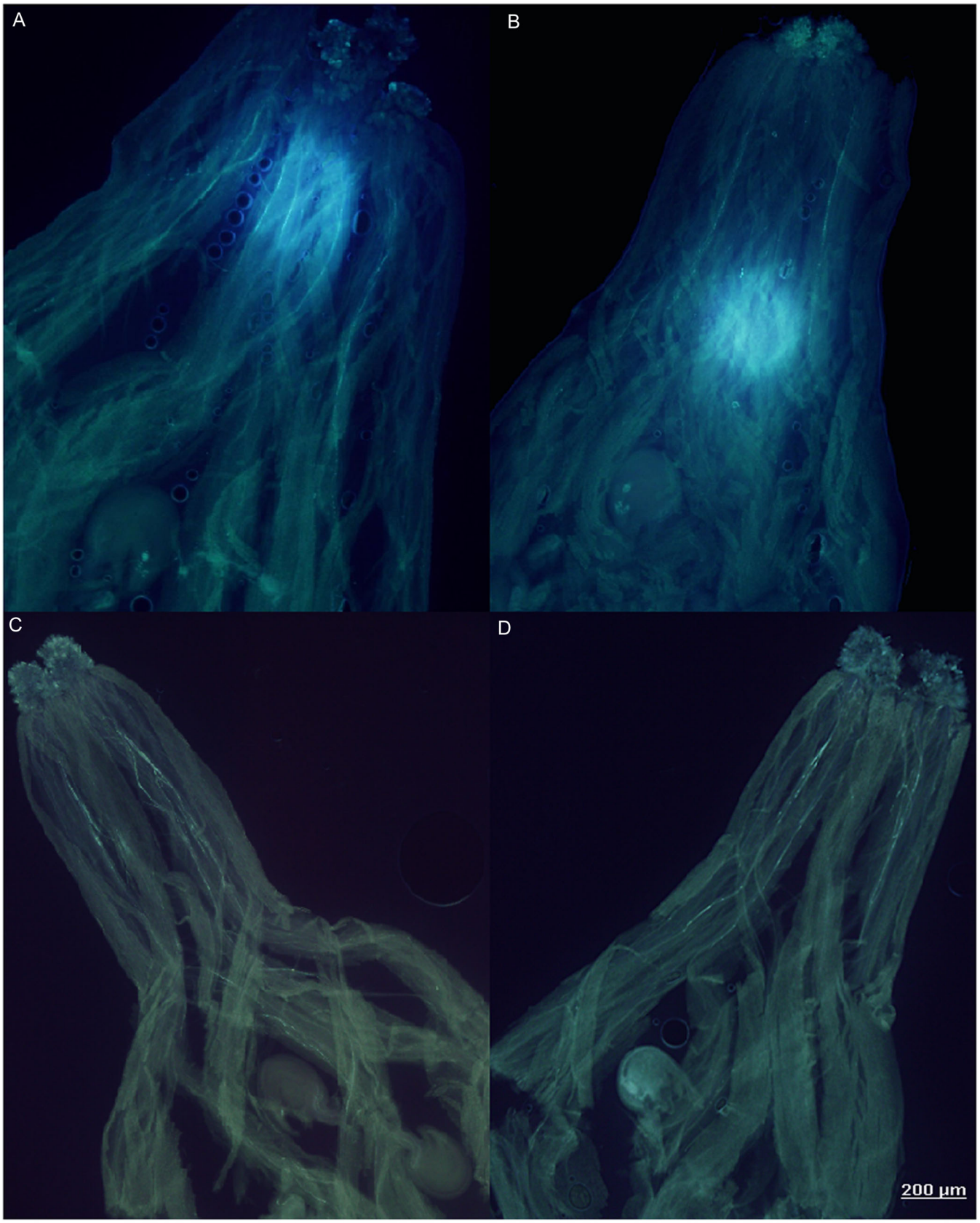

FIGURE 4 *Cakile edentula* pistil 1 h after pollination with *C. edentula* pollen (A), 1 h after pollination with *C. maritima* pollen (B), *C. maritima* pistil 1 h after pollination with *C. maritima* pollen (C), and 1 h after pollination with *C. edentula* pollen (D).

Two hours after pollination, the advantage of conspecific pollination in *C. edentula* style identified in the first hour was gone, and no difference in pollen tube growth between pollen types was evident (Tukey HSD, $P = 0.49$; Figure 3). Conversely, pollen recipient species had a significant effect on pollen tube growth in all pollination treatments after 2 h (ANOVA, $F_{1,103} = 28.70$, $P = 5.16e\text{-}07$; Appendix S3, Table S6) regardless of the pollen donor species (ANOVA, $F_{1,103} = 1.04$, $P = 0.31$; Appendix S3, Table S6). Moreover, the interaction between pollen donor and recipient was not significant (ANOVA, $F_{1,103} = 0.04$, $P = 0.83$; Appendix S3, Table S6), suggesting that both E and M pollen reached the ovule quicker in *C. edentula* styles than in those of *C. maritima*.

Four hours after pollination, pollen recipient species had a significant effect on pollen tube growth (ANOVA, $F_{1,142} = 6.77$, $P = 0.01$; Appendix S3. Table S7), while pollen donor species no longer had a significant effect (ANOVA, $F_{1,142} = 3.88$, $P = 0.05$; Appendix S3, Table S7). However, the difference in pollen tube growth across the cross type was less pronounced as most pollen tubes had reached the ovule. The average pollen tube length in EM pollination treatment was significantly greater than that of EE pollination ($P = 0.0009$; Figure 3). In contrast, there was no significant difference between E pollen and M pollen growth in *C. maritima* mothers after 4 h. However, heterospecific pollination treatment in *C. maritima* (ME) displayed a high variance in the extension of pollen tubes with values greater than one, indicating that some of the *C. edentula* pollen tubes reached the first ovule and continued growing toward the second ovule.

Genotyping of the offspring of *C. edentula* as pollen recipient

The two CAPS markers used for genotyping unambiguously distinguished the parental species and returned consistent results across all parents and offspring (markers 1 and 2; Appendix S2, Table S4). All pollen donor and recipient parents were homozygous for the expected species-specific allele. A total of 180 progenies produced from *C. edentula* mothers were genotyped for the two markers. Forty individuals with single-donor pollination (i.e., control treatments: either *C. edentula* pollen or *C. maritima* pollen) and 140 individuals with double-donor (both *C. edentula* and *C. maritima* pollen) pollination treatment were genotyped, resulting in 111 (61.66%) EE genotypes and 69 (38.3%) EM genotypes. The analysis of double-donor pollination treatments (140 individuals) showed that the two markers yielded identical results; therefore, we report a single analysis for both markers.

In double-donor pollination treatments, the proportion of conspecific vs. heterospecific progenies differed significantly from the null expectations that either *C. edentula* pollen would fertilize the ovules in all treatments (assortative mating, 1:0 ratio, $\chi^2 = 57$, df = 1, $P = 4.37e\text{-}14$), or that both species had an equal chance of siring success (equilibrium, 1:1 ratio, $\chi^2 = 5.84$, df = 1, $P = 0.01$). As an alternative, siring success was shown to be influenced by the timing of pollen deposition (logistic regression, time of deposition z-score = 6.08, $P = 1.16e\text{-}09$). The logistic model estimated a probability of siring success of 100% for *C. edentula* and 66.5% for *C. maritima* pollen when deposited earlier than the second species. These results suggested that conspecific pollination was more likely overall (Figure 5). Interestingly, when *C. maritima* pollen was applied 20 min or 40 min earlier than *C. edentula* pollen, the conspecific pollen (E pollen) could compete with the heterospecific pollen, resulting in almost 50% conspecific siring. However, when *C. maritima* pollen was applied 1 h before *C. edentula*, M pollen sired 95% of the seeds. When *C. maritima* pollen was applied earlier, a greater proportion of hybrid offspring was formed (Figure 5). When pollen grains of both species were deposited at the same time on stigmas, there was no difference between conspecific versus heterospecific fertilization rate (55% and 45%, respectively; logistic regression, z-score = 0.44, $P = 0.65$).

Genotyping of the offspring of *C. maritima* as pollen recipient

The genotyping of 180 progenies produced from *C. maritima* mothers resulted in 113 (63%) MM genotypes and 67 (37%) ME genotypes (markers 3 and 4; Appendix S2, Table S4). In double-donor pollination treatments, the observed proportion of conspecific vs heterospecific among 140 progenies again differed significantly from either the 1:1 or 1:0 expectations ($\chi^2 = 136.27$, df = 1, $P < 2.2e\text{-}16$ or $\chi^2 = 7.10$, $P = 0.007$, respectively). The timing of pollen deposition thus influenced the paternity outcome in *C. maritima* progeny (logistic regression, z-score = –5.936, $P = 2.92e\text{-}09$). When *C. maritima* pollen was applied first, conspecific siring occurred at a very high rate (95%). When *C. edentula* pollen was deposited first on *C. maritima* stigma, the time lag significantly affected fertilization (60% siring success for E pollen when it was deposited earlier). In the concurrent pollination treatment, the siring success of *C. maritima* pollen was higher than that of *C. edentula* (60% and 40%, respectively; Figure 5), but the difference was not statistically significant (logistic regression, z-score = 0.38, $P = 0.70$).

DISCUSSION

In this study, we have highlighted two important features for understanding pollen competition: the time of pollen arrival on the stigma and pollen growth in the style. A key finding is that pollen tube growth in heterospecific versus conspecific pollination differed between the two *Cakile* species investigated. When *C. edentula* was the maternal plant, conspecific pollen germinated more readily and had a growth advantage during the first hour following pollination, which is consistent with the siring success assessed through molecular analysis. This growth advantage is likely to have contributed to the conspecific siring advantage of this species unless *C. maritima* pollen was given a moderate

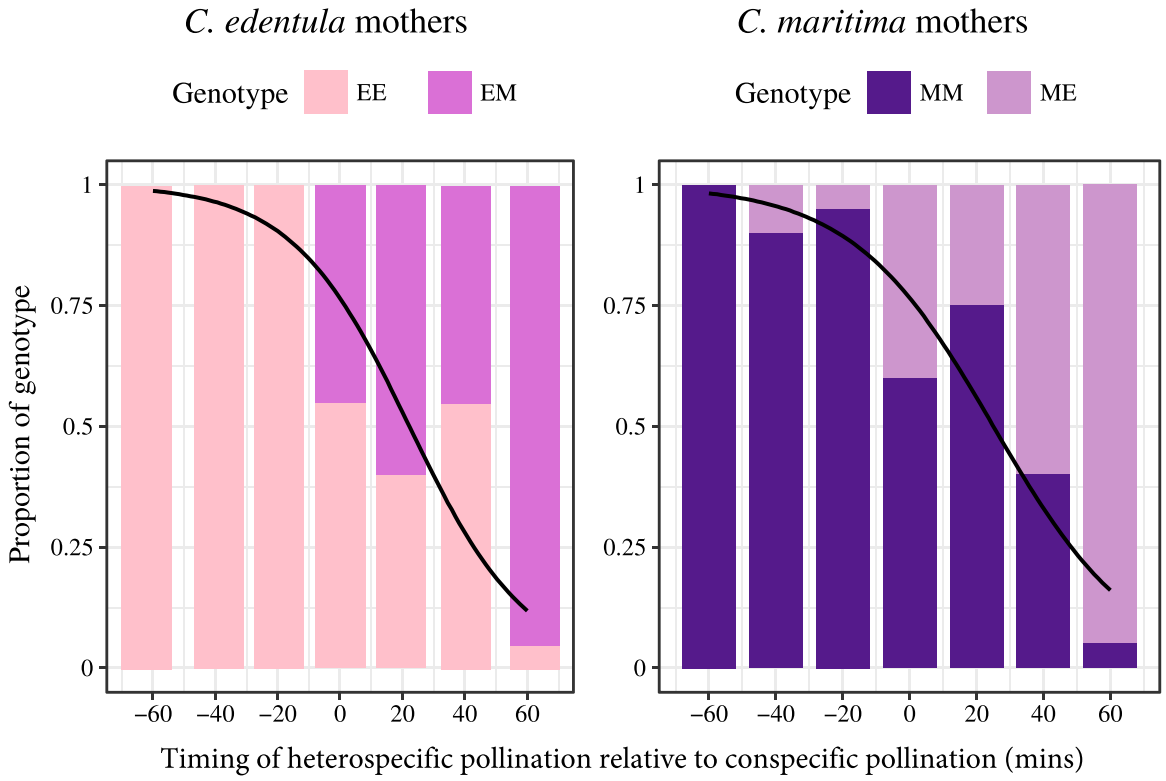

FIGURE 5 Fertilization probability relative to the time of pollen deposition in *Cakile edentula* (left) and *Cakile maritima* (right) mothers. The probability of fertilization for each pollination treatment was determined by analysis of paternity for 20 progenies per treatment. In the left graph, "EE" (light pink) indicates conspecific fertilization and "EM" (dark pink) indicates heterospecific fertilization in *C. edentula* mothers. In the right graph, "MM" (dark purple) indicates conspecific fertilization and "ME" (light purple) indicates heterospecific fertilization in *C. maritima* mothers. Negative values in time (*x*-axis) indicate that conspecific pollen load was deposited before heterospecific pollen load, whereas positive values indicate that conspecific pollen load was deposited after heterospecific pollen load. Time zero indicates pollen deposition at the same time.

head start. Interestingly, we did not see large differences in the number or growth of pollen tubes from conspecific and heterospecific pollen in *C. maritima* styles, although the siring advantage of conspecific pollen was still evident, suggesting that a post-zygotic mechanism(s) may contribute to the conspecific siring advantage.

Floral morphology and mating system create the opportunity for pollen competition

Morphological characteristics such as pollen size and style length have been shown to influence pollen competition (Williams and Rouse, 1990; Harder and Barrett, 2006; Lee et al., 2008; Figueroa-Castro and Holtsford, 2009; Harder et al., 2016; McCallum and Chang, 2016). For instance, pollen grains of a long-style species need to travel a longer distance from the stigma tip to the ovules, which may lead to the evolution of inherently faster growth of pollen tubes in long-style species (Figueroa-Castro and Holtsford, 2009). Hence, pollen of a long-style species may have a competitive advantage in a short-styled species. Similarly, larger pollen grains can produce tubes that grow faster in the style (Williams and Rouse, 1990). As a result, heterospecific pollination between species with distinct flower characteristics (e.g., style length and pollen size) may lead to asymmetric siring success (Williams and Rouse, 1990; Figueroa-Castro and Holtsford, 2009; Nista et al., 2015). We showed that pollen size and shape of the two *Cakile* species were the same, but the length of their styles differs, with *C. edentula* having shorter styles. This style-length difference likely explains the maternal effect found on the ratio of pollen tube growth, where 2 h after pollen deposition, both *C. edentula* and *C. maritima* pollen tubes travelled down a greater proportion of the *C. edentula* style than of the *C. maritima* style. In *C. edentula*, the pollen tubes need to travel a shorter distance to reach the ovule. After 2 h, some pollen tubes from both species reached the ovules in *C. edentula* styles, but no pollen tubes in *C. maritima* styles reached the ovules.

Although *C. maritima* pollen took longer to germinate on the *C. edentula* stigma and initial pollen tube growth appeared slower over the first hour (perhaps reflecting this delayed germination), this initial disadvantage disappeared after 2 h and was reversed after 4 h. These results suggest that *C. maritima* has enhanced pollen tube growth in the *C. edentula* style over time, compared to pollen tube growth after conspecific pollination. Due to the short length of *C. edentula* styles, there is only a short time when pollen tube growth can compensate for a delay in deposition, while the longer styles of *C. maritima* provide a greater distance to compensate for this delay.

The effect of floral morphology and pollen tube growth was in part consistent with the expectation based on the differences in the mating system between these two *Cakile* species. In a highly selfing species, a uniform and predictable amount of pollen is likely to be deposited on every stigma leading to lower pollen competition intensity between related pollen grains (Smith-Huerta, 1996). Self-fertilization was frequently shown to be associated with evolutionary changes in floral traits (Mazer et al., 2018). Smaller flowers and shorter styles are among the traits that have evolved in plant species with high selfing rates, and these can also be correlated with reduced pollen competitiveness (Duncan and Rausher, 2013; Mazer et al., 2018). In a self-incompatible species, larger flowers (i.e., with longer styles) attract more pollinators, and consequently, diverse pollen sources can be deposited in different proportions on each stigma. Thus, the number of competing pollen grains and the distance over which their pollen tubes will compete are also greater, leading to higher pollen competition intensity and selection against slower-growing pollen tubes (Travers and Shea, 2001; Mazer et al., 2018). Hence, rapid pollen germination and tube growth would be favorable traits under intense pollen competition between multiple donors in a self-incompatible species. In this study, we observed rapid growth of both heterospecific and conspecific pollen tube in *C. maritima* styles, which may occasionally contribute to successful hybridization between the two species.

In addition to the pre-zygotic barriers imposed by flower structure and pollen tube growth, post-zygotic mechanisms such as embryo abortion or seed formation can influence the reproductive outcome (Erbar, 2003; Willis and Donohue, 2017). Hence, assessing paternal contribution in the progeny is critical to examine the importance of any post-pollination barriers. In this regard, the molecular marker analysis provided unambiguous results: conspecific pollen sired significantly more seeds than heterospecific pollen in both *Cakile* species, regardless of the pollination time lag.

The timing of pollination can reduce assortative mating and increase rates of hybridization

In general, in both conspecific and heterospecific fertilization, the plants with the greatest siring success are expected to have the fastest pollen tube growth or their pollen arrives earlier on the stigma (Snow et al., 2000; Figueroa-Castro and Holtsford, 2009). Hence, evaluating the time of pollen arrival on the stigma is also an important component of siring competition. Clearly, earlier-arriving pollen has a substantial advantage over later-arriving pollen in sending pollen tubes through the style and thereby usurping ovules (Snow et al., 2000; Bruckman and Campbell, 2016a). We have shown that in *C. edentula*, siring success of conspecific pollen when deposited first was 100%, while siring success of heterospecific pollen when it was deposited first was only 66.5%. Likewise in *C. maritima*, siring success was 95% for the conspecific pollen when deposited first and 60% for the heterospecific pollen when deposited first. Offspring genotyping revealed that in both species, when a time lag was introduced between the heterospecific pollen (deposited first) and conspecific pollen (deposited second), the chance of conspecific pollination decreased dramatically. However, shorter time intervals between pollen depositions (20- and 40-min delay to synchronous deposition in our study) increased the competition between the early and late-arriving pollen, and some of the late-arriving conspecific pollen could sire the seeds. There is thus a narrow time interval when both conspecific and heterospecific pollen are equally likely to fertilize the ovule. When the interval between pollen depositions was longer (1 h in our study), the delayed pollen load was unable to access the ovules and pollen competition between the species was effectively eliminated. This critical below-1-h delay between pollen depositions is consistent with the time pollen tubes need to reach the ovule. Altogether, our findings suggest that the time to pollen germination and relative tube growth in the absence or presence of heterospecific pollen are likely to drive the intensity of pollen competition and substantially influence the formation of F_1 hybrids.

The effect of pollen arrival time on siring success has been investigated in other species (Epperson and Clegg, 1987; Snow et al., 2000; Karron, et al., 2006; Burkhardt et al., 2009). Similar to our finding, the timing of pollen deposition has been found to strongly determine the occurrence of assortative mating (Snow et al., 2000; Burkhardt et al., 2009). First-arriving pollen grains sired more seeds than later-arriving pollen grains in *Silene latifolia* (Burkhardt et al., 2009) and *Hibiscus moscheutos* (Snow et al., 2000). The variation in siring success between early- and late-arriving pollen is also often positively correlated with the interval between pollen deposition (Burkhardt et al., 2009). These studies in an intraspecific context suggest that if the time lag between sequential pollinator probes was short, pollen loads that were deposited in later probes may have an equal chance to fertilize the ovule (Epperson and Clegg, 1987; Karron et al., 2006). Our results extend these findings to an interspecific, hybridization context where further research is needed.

Several studies have examined how post-pollination processes influence the degree of assortative mating and the strength of reproductive barriers between species (e.g., Rahmé et al., 2009; Widmer et al., 2009; Ostevik et al., 2016). Similar to the results of our study, conspecific pollen performance is higher than heterospecific performance in *Ipomopsis arizonica* (Wolf et al., 2001), sympatric *Orchis* species (Luca et al., 2015), *Silene latifolia* (Rahmé et al., 2009; Montgomery et al., 2010), *Arabidopsis thaliana* accession Columbia (Swanson et al., 2016), and some species of the *Erica* genus (Coetzee et al., 2020). In particular, Rahmé and colleagues (2009) found that when mixing equal proportions of pollen from compatible *Silene latifolia* and *S. dioica* species, the differential success of conspecific vs.

heterospecific pollen revealed an asymmetric post-zygotic reproductive barrier toward hybrid formation. Our study did not show such asymmetry in siring success in the 50:50 mixed pollination assays. In some species such as *Campanulastrum americanum* and *Nicotiana longiflora*, heterospecific pollen had higher siring success in mixed 50:50 pollinations (Kruszewski and Galloway, 2006; Figueroa-Castro and Holtsford, 2009), indicating that any type of outcome could be expected. It would be interesting to assess how these kinds of asymmetry in siring are driven by pollen germination and growth rate.

Eco-evolutionary consequences

Our evaluation of pollen competition measured through pollen tube growth (focused on prezygotic aspects) and paternity assessment (capturing both pre- and post-zygotic aspects) provided valuable information on its effect on siring success and unidirectional gene flow among species and its consequence for formation of F_1 hybrids. Therefore, our findings on pollen competition and interactions between pollen grains can inform our understanding of hybridization in natural populations of these species. *Cakile maritima* is replacing *C. edentula* throughout much of the introduced range in hybrid zones in Australia and western North America, and many *C. maritima*-like individuals have some *C. edentula* ancestry, particularly in Australia (Ohadi et al., 2016). We have shown that there are weak post-pollination barriers to reproduction between these two species, but these barriers are strengthened when heterospecific pollen deposition occurs later than conspecific pollination. If self-fertilization in *C. edentula* is delayed (e.g., due to variation in floral development; see Li et al., 2019), pollinators preference for visiting *C. maritima* may be conducive to early heterospecific pollination of *C. edentula* and thus increasing the formation of F_1 hybrids. This may explain the relatively commonly observed mixed-hybrid composition of invasive populations. Heterospecific siring advantage driven by the timing of pollination could have aided the initial establishment of *C. maritima*, whereby Allee effects commonly experienced in small SI populations (e.g., Uesugi et al., 2020) could have been overcome via mating with the already established *C. edentula*. As stated by Todesco and colleagues (2016), assessments of mating patterns in natural mixed populations will allow us to determine whether demographic rescue or genetic swamping is contributing to the replacement of *C. edentula* by *C. maritima* in Australia, a hypothesis that is consistent with our data. Future studies of post-pollination processes in hybrids, and particularly the presence of preferential backcrossing direction vs. F_2 formation, will shed light on the mechanisms promoting or hampering asymmetrical introgression and species replacement.

CONCLUSIONS

Our pollen competition experiments allowed us to dissect some of the mechanisms shaping the reproductive barriers in these two *Cakile* species. The study highlights that even if pollen grains are, on average, better competitors on their own stigma, modest time differences in the arrival of pollen on a stigma could substantially interfere with the success of different pollen donors. Microscopic observations illustrate that despite some differences in the patterns of pollen tube germination and growth, the differences are not large and do not seem to have a net impact on siring success. In a situation where pollen grains of both *Cakile* species are concurrently deposited on one stigma, both pollen types stand a similar chance of fertilization. The plant-related characteristics involved in the post-pollination reproductive mechanisms and the differences in time of pollen arrival, which mainly depends on pollinator behavior and potentially other pre-pollination mechanisms, will drive the outcome of siring and hybridization success. However, the time of pollen arrival played a more important role in siring success. As a result, we postulate that the time of pollen arrival is critical, and given the narrow time window for pollen grains to usurp the ovules in *Cakile* species, later-arriving pollen grains are usually at a substantial disadvantage that cannot be overcome.

AUTHOR CONTRIBUTIONS

T.J. and A.F. conceived the ideas and designed the methodology. T.J. performed the experiments and collected the data. T.J. and A.F. analyzed the data with the help of H.R. T.J. wrote the first draft of the manuscript. A.F., K.H., and H.R. contributed to several revised versions of the manuscript.

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DATA AVAILABILITY STATEMENT

The data for this manuscript are available at the FigShare repository: https://melbourne.figshare.com/projects/Pollen_competition_in_hybridising_Cakile_species/135827.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Differences in floral morphology of *Cakile maritima* and *C. edentula*.

Appendix S2. Primer design, PCR protocols, and CAPS markers design.

Appendix S3. Analysis of variance tables to show the effect of pollen recipient and pollen donor species on pollen tube growth at each pollination time separately.

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