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# THE CONTRIBUTION OF MATING SYSTEM VARIATION TO REPRODUCTIVE ISOLATION IN TWO CLOSELY RELATED *CENTAURIUM* SPECIES (GENTIANACEAE) WITH A GENERALIZED FLOWER MORPHOLOGY

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**In closely related plant species that display strong similarities in phenology and pollinator communities, differences in breeding system and associated shifts in floral traits may have important effects on the magnitude and direction of heterospecific pollen flow and hybridization. Here, we quantified the strength of several pre- and postzygotic barriers acting between the facultatively outcrossing *Centaurium erythraea* and the predominantly selfing *C. littorale* via a suite of experiments, and estimated the frequency of hybridization in the field using molecular markers. The reproductive barriers primarily responsible for preventing hybridization were essentially prezygotic and these acted asymmetrically. Due to differences in floral display, pollen production, and pollen transfer rates, heterospecific pollen flow occurred predominantly from *C. erythraea* to *C. littorale*. In *C. littorale*, on the other hand, close anther–stigma positioning and resulting higher capacity for autonomous selfing functioned as an efficient barrier to counterbalance the higher risk for hybrid mating. In both species the action of all reproductive barriers resulted in a small opportunity for hybrid establishment, which was confirmed by the occurrence of only ~1% putative hybrids in the field. Our findings confirm that differences in breeding system affect heterospecific pollen transfer patterns and that autonomous selfing may efficiently prevent hybridization.**

**KEY WORDS:** **Flowering phenology, herkogamy, hybridization, pollen competition, pollinator fidelity, reproductive asynchrony, self-pollination.**

The maintenance of species integrity between sexually compatible sympatric species largely depends on the strength of several reproductive barriers that together determine the reproductive isolation acting between them (Mayr 1992; Schluter 2001; Coyne and Orr 2004). In flowering plants, reproductive isolation is the result of the multiple action of both prezygotic and postzygotic isolating mechanisms, but prezygotic barriers generally make a greater contribution than postzygotic ones (Lowry et al. 2008; Widmer 2009). Prezygotic barriers include spatial (Ramsey et al. 2003; Kay 2006), temporal (Martin and Willis 2007; Botes et al. 2009), and pollinator isolation (Schemske and Bradshaw 1999; Pelmyr 2003), but can also be brought about by mechanical properties of flowers affecting pollen deposition/capture (Linder and Midgley 1996; Kay 2006) or competitive gametic interactions (Emms et al. 1996; Fishman et al. 2008).

Differences in mating system between co-occurring plant species can be expected to shape the above-mentioned prezygotic barriers and therefore have important effects on the direction

of heterospecific pollen flow and the extent of hybridization. Lowe and Abbott (2004), for instance, reported that in the recent evolution of *Senecio eboracensis* from *S. vulgaris,* predominant self-fertilization in *S. eboracensis* contributed to strong reproductive isolation and ecological differentiation. Martin and Willis (2007) also showed that in *Mimulus nasutus* almost exclusive autogamy offered nearly complete isolation from its close relative *M. guttatus* when they were growing in sympatry. Coyne and Orr (2004), on the other hand, argued that exclusive autogamy differs profoundly from other isolating barriers, and is in fact not an isolating barrier, because gene flow between individuals of different taxa is as much impeded as gene flow between individuals of the same taxon. In many cases, however, plants are neither exclusive selfers nor obligate outcrossers, but show a breeding system that represents an intermediate form between both extremes (so-called mixed-mating species) (Goodwillie et al. 2005).

Self-compatible species that are capable of selfing autonomously often show important changes in floral morphology, such as reductions in floral display, pollen and/or nectar production, and corolla size (Ornduff 1969). These morphological differences can, in turn, be expected to have an impact on interspecific pollen transfer patterns and thus the extent and direction of hybridization. Apart from the autonomous selfing mechanism itself (Fishman and Wyatt 1999; Smith and Rausher 2006), the reduced attractiveness of selfing species may lower the risk of being visited and pollinated with heterospecific pollen (Ferguson et al. 1999). Lower pollen production and a smaller floral display can, on the other hand, reduce a species' contribution to the potential total pollen pool, which in turn increases the risk of being pollinated with heterospecific pollen when visited by a pollinator foraging on both plant species (Ducarme and Wesselingh 2013). Besides prepollination interactions, postpollination interactions can also be expected to influence the risk of hybrid formation in the field, because pollen of selfing species often shows a lower germination and pollen tube growth compared to that of outcrossing relatives (Montgomery et al. 2010; Runquist 2012).

To get a better understanding of the strength and nature of diverse isolating barriers, Martin and Willis (2007) recently developed a methodology that allows calculating the impact of several sequentially acting prezygotic barriers in diminishing the chance of hybrid formation. This method is primarily based on the assumption that the sequential action of each barrier alters the frequency of heterospecific (and conspecific) pollen in the pollen pool and thus determines the net gene flow resulting from these interspecific differences. In contrast to previous methods (e.g., Ramsey et al. 2003), this method accounts for differences in the frequency of both parental species, flower production, pollination patterns, differential pollen availability, and/or pollen competition, and therefore allows quantifying the chance of heterospecific pollen flow and investigating whether reproductive isolation evolved asymmetrically between taxa (Tiffin et al. 2001).

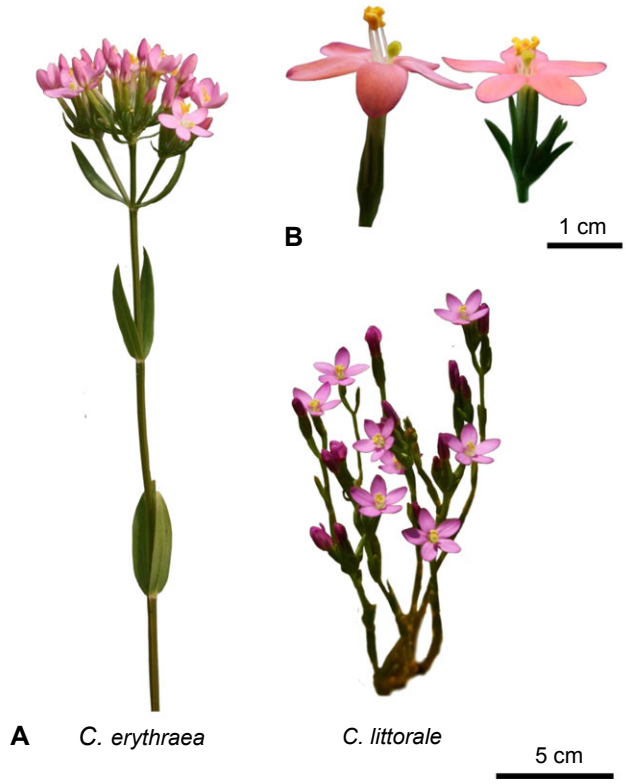


**Figure 1. Illustration of *Centaurium erythraea* and *C. littorale* (A) in situ and (B) detail of typical flower morphology under sympatry.**

In this study, we investigated the likelihood and frequency of hybrid mating in two closely related *Centaurium* species that display strong similarities in overall flower morphology (Fig. 1) and pollinator communities (*C. erythraea* Rafn and *C. littorale* [D. Turn.] Gilm.), but show substantial differences in floral traits related to their mating system. *Centaurium littorale* is characterized by close stigma–anther positioning (Fig. 1B) resulting in a higher capacity of autonomous selfing than in *C. erythraea* (autofertility index = 0.68 ± 0.09 and 0.55 ± 0.06, respectively) (Brys and Jacquemyn 2011). We also found that the timing of selfing differs significantly between the two species, with *C. littorale* showing early (competing) selfing and *C. erythraea* delayed selfing. In addition, *C. littorale* has a smaller floral display size and produces less pollen per flower than *C. erythraea*. These differences in mating system and floral traits suggest that asymmetries in heterospecific pollen flow may arise when both species co-flower. In particular, the lack of herkogamy and early timing of selfing in *C. littorale* may function as a protection mechanism against heterospecific pollen deposition and hybrid seed formation.

To test these hypotheses, a series of field and laboratory experiments was performed to estimate the strength of four

sequentially acting prezygotic barriers (flowering phenology, pollen production, pollination patterns, and pollen competition) acting between *C. erythraea* and *C. littorale*. In addition, the fitness of hybrid and parental offspring and gamete (in)viability were determined to assess potential fitness costs following hybrid formation and to calculate their role in functioning as potential postzygotic barriers. Finally, to test the efficiency of the above described reproductive barriers in preventing gene flow between both species in the field, the frequency of hybrids in a natural population was assessed using molecular markers.

# Materials and Methods

## STUDY SPECIES

*Centaurium erythraea* and *C. littorale* are monocarpic biennials that belong to the Gentianaceae and that are native to Europe. Both species are tetraploid ($2n = 40$) in the main part of their distribution range (central and north Europe) (Zeltner 1962, 1970; Ubsdell 1976). *Centaurium erythraea* generally prefers drier soil conditions than *C. littorale* (van Tooren et al. 1983; Schat et al. 1989). It is found in a wide range of habitats, varying from well-developed calcareous grasslands, over wood margins to coastal dunes and river banks (van Tooren et al. 1983; Schat et al. 1989). *Centaurium erythraea* also shows the largest distribution area of both species, extending from the south of Scandinavia over the United Kingdom toward North Africa, and up to southwest Asia in the east. *Centaurium littorale*, on the other hand, not only shows a higher tolerance to moist conditions, it also prefers slightly saline locations (Schat and Scholten 1985) and can be characterized as a flooding-tolerant species (Schat 1984). The distribution area of *C. littorale* is much more restricted to coastal regions, ranging from the south of Scandinavia, over the United Kingdom, the Netherlands, and Belgium, up to the North of France and some isolated locations in the Iberian Peninsula where it reaches its southern limits. At their overlapping ranges, such as the borders of dune slacks or river banks, both species often grow together.

The two species have a different growth form. *C. erythraea* is the tallest of both species (mean plant height: 22.7 ± 7.4 cm), and is characterized by a basal rosette from which usually one, but sometimes several erect stems arise (Fig. 1A). *Centaurium littorale* is much smaller (mean plant height: 13.2 ± 3.4 cm) and produces a basal rosette from which mostly several erect stems develop (see Fig. 1A). Both species produce similarly looking, showy pink flowers that are hermaphroditic and self-compatible (Brys and Jacquemyn 2011). In *C. erythraea*, flowers are produced in larger numbers that are grouped in compact cymes at the top of the plant. *Centaurium littorale*, on the other hand, develops its flowers more scattered around the plant, often into the internodes of its stems (Fig. 1A). Flowers do not produce any nectar, and both species largely share the same guild of pollinators (pollen-gathering hoverflies [Diptera, Syrphidae], bees [Hymenoptera, Apidae], and small flies [Empididae-Muscidae]) (Brys and Jacquemyn 2011, 2012). *Centaurium erythraea* is more frequently visited by foraging insects than *C. littorale* (pollinator failure index of 28.5 ± 4.9 and 56.9 ± 5.3 for *C. erythraea* and *C. littorale*, respectively) (Brys and Jacquemyn 2011). For both species, reproduction can only take place by means of seeds, which are very small (0.01 mg) and produced in large quantities (Brys and Jacquemyn 2011; Brys et al. 2011).

## ASSESSMENT OF PREZYGOTIC BARRIERS

### *Flowering phenology*

To investigate the flowering phenology of naturally occurring *C. erythraea* and *C. littorale* individuals, we established five 1 × 1 $m^2$ plots prior to the onset of flowering (beginning of June 2011) in a large sympatric population in a coastal dune area at the Western part of the Belgian coast close to Oostduinkerke. Plots were haphazardly situated within the population and monitored during the entire flowering period of both species. Within these plots, the total number of flowering plants and the total number of flowers that were open for each species were recorded every two days.

### *Rates of intra- and interspecific pollen transfer*

To assess pollen transfer patterns in the field, experimental arrays were setup within the same sympatric study population. In mid-July (2011), two experimental arrays were established more than 100 m isolated from each other. Per array, 36 potted transplants (18 *C. erythraea* and 18 *C. littorale* individuals) were placed in a 6 × 6 arrangement. In this arrangement, both species were positioned in a checkerboard fashion with a separation of 0.5 m among plants (Fig. S1a). All transplants were at the peak of flowering at the beginning of the experiment. Within each array, 12 "donor" plants were selected (six per species), on which dehiscing anthers were coated with colored powder with a pencil to track pollen movement (Fig. S1b). Two colors (orange and yellow) of fluorescent dye (Radiant Color Corp., Serie Radglo© R) were used as pollen analogues (one color per species). For each donor plant, all flowers that opened during the entire experiment were colored and recorded. Although previous investigations showed that marking anthers with dye did not change the behavior of pollinators and that no differences were observed between the two dye colors in terms of pollinator behavior (R. Brys, unpubl. data), the color-species combination was reversed between both experimental arrays to avoid any effect of dye color on pollen transition patterns. Before applying any dye on the flowers, plants were left untreated for several days in this arrangement. During a four-day period of dry sunny weather in July, plants were daily visited and marked, and the number of open flowers was recorded for each Q2

plant. On day 4, all flowers of the remaining and untreated "receptive plants that were open during the experiment were harvested and brought to the laboratory. In total, 1321 flowers were examined using a fluorescence dissecting microscope (10×). Presence of fluorescent dye particles and type of color were recorded on each stigma (Fig. S1b), and gave a clear indication of pollen transfer events between both study species (but not of pollen quantities that were deposited). Moreover, because we were not able to discriminate between single and multiple deposition events of the same color, this may have resulted in a slight underestimation of the real number of pollen transfers.

### *Pollen competition*

To determine the siring ability of deposited heterospecific pollen interfering with conspecific pollen, we examined seed set and the proportion of $F_1$ hybrid formation in a pollination experiment under controlled, pollinator-free conditions. We transferred 10 transplants per species from the sympatric population into pots and brought them to a pollinator-free greenhouse (May 2010). Once flowering, three pollination treatments were applied per plant: pollination with (1) pure intraspecific pollen on intact flowers, (2) a 50:50 mixture of pollen from both species on intact flowers, and (3) a 50:50 mixture of pollen from both species on flowers that were emasculated prior to anthesis. Treatment 2 mimics the natural situation as much as possible, whereas the comparison of treatment 2 and 3 allows uncoupling the impact of self-pollen interference realized via autonomous selfing as a shielding effect from that of potential interspecific differences in pollen germination and pollen tube growth in each of both reciprocal crosses. For all treatments, pollen loads from three male plants per species were used. The pollen mixture was obtained by using entire anthers that were collected into clean microcentrifuge tubes before dehiscence. Anthers of *C. erythraea* and *C. littorale* were combined in a 4:11 ratio corresponding to the ratio of pollen grain production per anther (20,390 and 7475 pollen grains in *C. erythraea* and *C. littorale*, respectively [data obtained from Brys and Jacquemyn 2011]). Shaking of the tubes ensured mixing of the pollen and distribution over the inner surface of the tube to which they adhered. For each pollination treatment, pollen was applied to stigmas in saturating amounts at the second day of anthesis, using a small paint brush. Three open flowers were randomly assigned to each of the treatments (in total nine flowers per plant). Fruits were harvested once seeds were ripe and seed production was determined per fruit and treatment.

To estimate the proportion of hybrid offspring produced following each of the mixed pollination treatments, per plant all seeds produced per treatment were pooled. Three subsamples of 50 seeds were then selected and sown in separate pots (8 × 8 × 15 cm) on moist potting soil. After growing for four months, we harvested one leaf per plant (summer of 2011). Based on several morphometric characteristics of the leaves (the density and height of papillae, leaf length:width ratio, leaf area, and color) (Fig. S2), $F_1$ hybrids and pure offspring could unambiguously be distinguished (Table S1).

## GREENHOUSE ESTIMATES OF INTERSPECIFIC SEED PRODUCTION AND HYBRID VERSUS PARENTAL FITNESS

The performance of $F_1$ hybrids and pure parentals was evaluated by measuring six components of fitness throughout the life cycle: seed set ($F_1$), percentage germination, survivorship, flower production, $F_1$ pollen viability, and female reproductive success. First, seed set was determined for fruits generated via pure intra- and interspecific hand-pollinations on emasculated flowers (three flowers per cross-type) of 10 *C. erythraea* and *C. littorale* maternal plants (July 2010). For both cross-types, flowers were emasculated prior to flowering to avoid any interference with intraspecific self-pollen. For each interspecific crossing, we used pollen from three pollen donors. When fruits were ripe, seed set was determined per fruit, and averaged per cross-type for each maternal plant.

Per maternal plant and cross-type, seeds were pooled and from this sample three replicates of 50 seeds were sown in 8 × 8 × 15 cm pots on a 3:1 mix of sand and potting soil. In December 2010, pots were put in a growth chamber (8:16 L:D cycle, 12–25°C). All pots were watered and checked for germination twice a week. After four months, total germination success and survivorship of all recruits were recorded, and progeny was reduced to five individuals per pot. Plants were then further grown outside and checked twice a month. During the second growing season (July 2012), total survivorship and flower production of the remaining plants was determined. To estimate pollen fertility, we harvested two flowers per plant and cross-type ($n = 10$ individuals per cross-type, 40 individuals in total) and for each flower three anthers were harvested. For each anther, pollen grains were germinated in Hoekstra germination medium (Hoekstra and Bruinsma 1975) at 20°C for 2.5 h. Per sample we took three subsamples of 3 μL in which the number of germinated pollen grains (pollen with pollen tubes) and nongerminated pollen grains was determined.

Estimates of $F_1$ female reproductive success were obtained by supplemental hand-pollinations on another subset of flowers (three per plant, $n = 10$ individuals per cross-type, 40 individuals in total). Pollen was used from three pollen donors that were obtained from the pure maternal $F_1$ plants. Once fruits were ripe, seed production was determined per fruit and averaged per plant.

## HYBRIDIZATION IN THE FIELD

To estimate hybridization rates in the field, we selected a plot of 10 × 30 m (in July 2012) in the sympatric study population,

where *C. erythraea* and *C. littorale* individuals grew and flowered in close proximity. Within this plot, leaf material was collected from all flowering individuals ($n = 203$). In addition, leaf samples were collected from 20 individuals of four allopatric populations (two per species). Finally, we also collected leaf samples from artificially obtained $F_1$ hybrids, both the *C. erythraea*♀ × *C. littorale*♂ and *C. littorale*♀ × *C. erythraea*♂ crossings ($n = 15$ per cross-type), which were used as reference material. The maternal plants for these experimental crossings were obtained from the allopatric reference populations.

DNA was extracted from silica-dried leaf material using the QuickPick kit of Isogen. The AFLP protocol was similar to the protocol of Vos et al. (1995), but with restriction and ligation conducted in one single step. After preliminary tests, two fluorescently labeled primer pairs (E-ACC(NED)/M-CTG and ACC(NED)/M-CAA) were chosen, which resulted in clear bands of sufficient variability. PCR products from each primer pair were run separately for fragment length separation and detection on an ABI 3500 capillary sequencer (Applied Biosystems) with Genescan 600-Liz (PE Applied Biosystems) as an internal lane size standard. Raw data were sized with GeneMapper 4.1 (Applied Biosystems) with default parameters and a polynomial degree of three for peak recognition in the electropherograms. The quality of each individual AFLP profile was manually checked and individuals with low AFLP intensities or large peak height variation were discarded from the analysis. The scoring was performed using the automated RawGeno R CRAN package (Arrigo et al. 2004). Bands that were clearly not reproducible or that were not tested (i.e., not included in the replicates) were discarded from further analyses. AFLP runs were repeated two times for 45 selected samples, representing 10% of the total dataset, to estimate the error rates. Following the study of Bonin et al. (2004), error rates were estimated as proportion of phenotypic comparisons within individuals that were different. As recommended by Vekemans et al. (2002), the correlation between AFLP band size and frequency among samples was assessed to check for potential homoplasy.

# Data Analysis

## ESTIMATING THE STRENGTH OF THE PREZYGOTIC BARRIERS UNDER SYMPATRY

We used the approach of Martin and Willis (2007) to estimate the probability of $F_1$ hybrid formation ($q$) following the action of four prezygotic barriers: flowering asynchrony, differential pollen production, pollen transfer patterns, and pollen competition. The strength of each barrier, $w$, was calculated for each reciprocal cross separately by comparing potential $F_1$ hybrid offspring formation to that of pure offspring. Because these measures of the strength of prezygotic barriers are directly analogous to commonly used measures of postzygotic isolation (i.e., the fitness of hybrids relative to the fitness of pure species), reproductive isolation was defined for each reproductive component as $RI = 1 - w$.

### *Flowering asynchrony*

Because the average life span of a flower is similar in both species (on average four days), we assumed that all flowers are equally likely to mate. Assuming that both species produce similar amounts of pollen, we calculated for each species the strength of reproductive asynchrony as a barrier to $F_1$ formation as $w_{1,x} = (q_{1,x} / q_{0,x}) / [(1 - q_{1,x})/(1 - q_{0,x})]$, where $q_{1,x}$ represents the expected frequency of heterospecific pollen that can potentially fertilize the maternal species and $q_{0,x}$ the proportion of all heterospecific flowers produced throughout the season (in this case, the initial null expectation). For both species, $q_{1,x}$ is calculated as $\Sigma_i m_i p_i$ with $m_i$ the number of flowers of the maternal species open on day $i$ proportional to the total amount of flowers of that species that is produced throughout the flowering season, $p_i$ the fraction of flowers belonging to the paternal species proportional to the total number of flowers open on that particular day $i$, with $p_i + n_i = 1$, and $n_i$ the fraction of flowers belonging to the maternal species on day $i$ (for more details see Martin and Willis 2007).

### *Differential pollen production*

Differential pollen production may affect the frequency at which pollen are transferred by foraging pollinators and therefore alter the total amount of heterospecific pollen in the pollen pool. The strength of differential pollen production as a barrier to $F_1$ hybrid formation can be estimated as $w_{2,ery} = P_{litt}/P_{ery}$ and $w_{2,litt} = P_{ery}/P_{litt}$ for *C. erythraea* and *C. littorale*, respectively, with $P_{ery}$ and $P_{litt}$ being the mean number of pollen grains produced per flower in *C. erythraea* and *C. littorale*, respectively. Taking the weighted averages over the entire season into account and thus incorporating the impact of earlier acting barriers, the overall expected frequencies of heterospecific pollen can be calculated as $q_{2,x} = \Sigma_i m_i [(p_i w_{2,x}) / (p_i + (n_i w_{2,x})]$ (Martin and Willis 2007).

### *Pollen transfer patterns*

Using the total number of intraspecific crosses (*ee* and *ll*) and interspecific crosses (*el* and *le*) from the pollen transfer experiment, we estimated components of male and female assortative mating for both study species as $A_{\text{male}} = ee / (ee + el)$ and $A_{\text{female}} = ee / (ee + le)$ for *C. erythraea*, and $A_{\text{male}} = ll / (ll + le)$ and $A_{\text{female}} = ll / (ll + el)$ for *C. littorale* (Natalis and Wesselingh 2013). The overall strength of pollinator constancy was calculated as a barrier to $F_1$ hybrid formation for each study species as $w_{3,x} = [(\text{total observed interspecific crosses / expected interspecific crosses}) / (\text{total observed intraspecific crosses / expected intraspecific crosses})]$ (Aldridge and Campbell 2007; Martin and Willis 2007). Here, the expected number of inter- and intraspecific

crosses represents the number of "donor" flowers that were coated with fluorescent dye and is used to correct for differences in the total number of heterospecific and conspecific donor flowers, respectively. Finally, the formulas of Martin and Willis (2007) were used to estimate the expected frequency of heterospecific pollen in the pollen pool, $q_{3,x} = \Sigma_i m_i\ [(p_i w_{2,x} w_{3,x}) / (p_i + (n_i w_{2,x} w_{3,x})]$.

### *Pollen competition*

A two-way ANOVA was used to test whether seed set differed between the three different pollination treatments. Pollination treatment, species, and their interaction were incorporated as explanatory variables. A generalized linear mixed model was used to test whether autonomous self-pollen deposition in intact flowers significantly reduced the probability of hybrid seed formation following a 50:50 mixed pollination compared to the same pollination treatment on emasculated flowers. In this analysis, pollination treatment (mixed pollination on intact vs. emasculated flowers), species, and their interaction were incorporated as explaining variables, whereas the proportion of hybrid seeds was used as response variable.

The strength of the combined impact of heterospecific pollen that interfere with conspecific pollen deposition and pollen precedence as a barrier to $F_1$ hybrid formation was estimated as $w_4$ = [(seed production following mixed pollination) / (seed production following intraspecific pollination)] × [($F_{1,hybrid\ observed}$ / $F_{1,hybrid\ expected}$) / ($F_{1,parental\ observed}$ / $F_{1,parental\ expected}$)], where $F_{1,hybrid\ observed}$ is the proportion of hybrids and $F_{1,parental\ observed}$ the proportion of parentals following mixed 50:50 pollination for both reciprocal crosses (Ramsey et al. 2003; Martin and Willis 2007). $F_{1,hybrid\ expected}$ and $F_{1,parental\ expected}$ are based on the proportion pollen that is used in the pollen mix. The expected frequency of heterospecific pollen arriving at the two species' ovules is calculated as $q_{4,x} = \Sigma_i m_i\ [(p_i w_{2,x} w_{3,x} w_{4,x}) / (p_i + (n_i w_{2,x} w_{3,x} w_{4,x})]$ (Martin and Willis 2007).

### COMPARING HYBRID VERSUS PARENTAL FITNESS AND ESTIMATING THEIR STRENGTH AS POSTZYGOTIC BARRIERS

For each of the six fitness components measured, we calculated the mean values per cross-type and maternal parent. First, seed set following intraspecific and reciprocal interspecific crosses, and the resulting $F_1$ offspring values of germination success, flower production, pollen viability, and seed production were compared with each other using a one-way ANOVA. A logistic regression analysis using PROC GLIMMIX in SAS (SAS Institute 2005) was used to test whether survivorship of pure and hybrid $F_1$ offspring differed significantly from each other. Finally, reproductive isolation due to sequential action of each of the above-mentioned postzygotic barriers is computed as: $RI_{\text{postzygotic}} = 1 - [\text{fitness of } F_{1,hybrids} / \text{fitness of } F_{1,parentals}]$ (Ramsey et al. 2003).

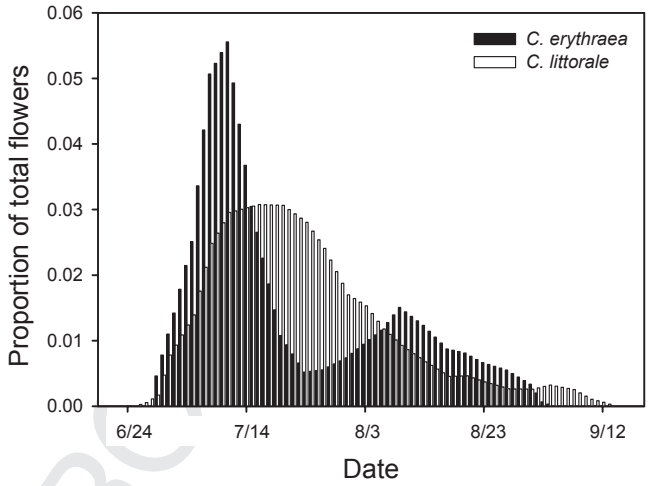


**Figure 2. Flowering phenology for naturally occurring *Centaurium littorale* and *C. erythraea* assessed on a daily basis in one of the studied sympatric populations (S1). The census number is expressed as a percentage of the total number of flowers open throughout the entire period (separately for each species). A total number of 3371 *C. littorale* flowers and 8516 *C. erythraea* flowers were surveyed throughout the entire flowering period.**

### GENETIC STRUCTURE

A matrix of genetic distances among individuals based on Jaccard's similarity was calculated from the AFLP genetic marker data. Genetic structure was assessed using principal coordinates analysis (PCoA) with the program GENALEX (Peakall and Smouse 2006). In this analysis, data obtained from the sympatric population, the allopatric populations, and the experimentally $F_1$ hybrids were included.

## *Results*

### FLOWERING PHENOLOGY AND ISOLATION DUE TO ASYNCHRONY

Although *C. erythraea* was present in higher frequencies (62.7%), and produced more than twice as many flowers than *C. littorale* (on average 27.8 ± 2.6 and 12.3 ± 1.3 flowers per plant for *C. erythraea* and *C. littorale*, respectively), the overall period of flowering and the mean flowering date largely coincided (67 and 80 days of flowering and mean flowering date at the first and third of August for *C. erythraea* and *C. littorale*, respectively) (Fig. 2). Without any prezygotic barrier and assuming random mating, this would hypothetically result in a probability of hybrid formation that equals frequencies in flower production. Because *C. erythraea* produced on average 79% of all flowers in the population, the species would sire 79.2% of all seeds produced either by itself

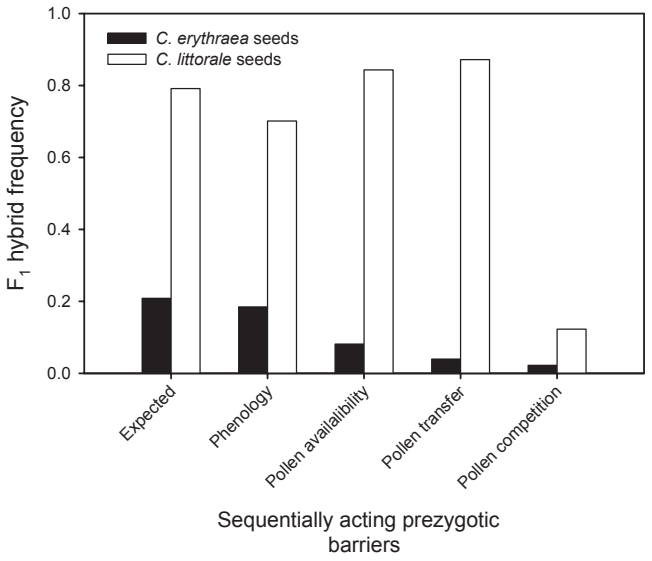


**Figure 3. Estimated frequencies of $F_1$ hybrid seed production in *C. erythraea* and *C. littorale* in a natural sympatric population, expected after the action of four sequentially acting prezygotic barriers.**

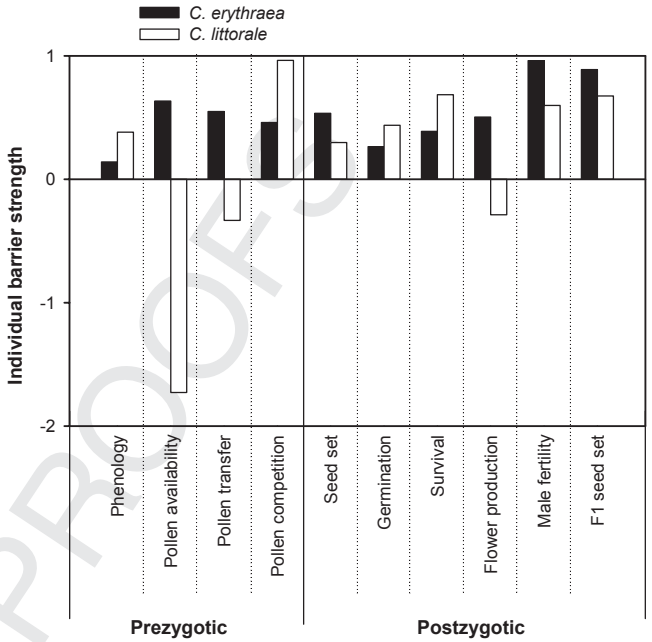


**Figure 4. Components of reproductive isolation for four prezygotic barriers (i.e., phenology, pollen availability, pollen transfer, and stigmatic pollen competition) and five postzygotic barriers (i.e., seed germination, survivorship, total flower production per plant, pollen viability, and seed set). For each individual barrier, the level of asymmetry is calculated as the absolute value of the difference between barriers (see Sanchez-Guillén et al. 2011).**

or in *C. littorale*. Conversely, *C. littorale* produced only 20.8% of all the flowers throughout the flowering season, resulting in 20.8% seeds produced by both species sired by *C. littorale*. However, the proportional number of flowers that were open in synchrony differed between the two species, which substantially affected the availability of heterospecific pollen in the pollen pool and reduced the probability of $F_1$ hybrid formation relative to random-mating expectations. In *C. erythraea* there were two flowering peaks, whereas *C. littorale* only showed one flowering peak lying more or less in between those of *C. erythraea* (Fig. 2). This resulted in a decrease of the proportion of *C. erythraea* seed progeny that can be expected to be $F_1$ hybrids from $q_{0,ery} = 0.208$ to $q_{1,ery} = 0.185$ (Fig. 3). For *C. littorale* seed progeny, the expected proportion of $F_1$ hybrids decreased from $q_{0,litt} = 0.792$ to $q_{1,litt} = 0.701$ (Fig. 3). The resulting effect of flowering asynchrony as a reproductive barrier was more than two times lower for *C. erythraea* ($w_{1,ery} = 0.860$ and $RI_{1,ery} = 0.140$) than for *C. littorale* ($w_{1,litt} = 0.618$ and $RI_{1,litt} = 0.382$) (Fig. 4).

## ISOLATION DUE TO DIFFERENTIAL POLLEN PRODUCTION

Overall, *C. erythraea* produced 2.7 times more pollen per flower than *C. littorale*. Assuming that the chance for successful deposition does not differ between both species and for each reciprocal cross, this resulted in a 2.7 times higher expected formation of $F_{1,litt}$ hybrids compared to the formation of pure *C. littorale* individuals ($w_{2,litt} = 2.728$ and $RI_{2,litt} = -1.728$) (Fig. 4). In *C. erythraea*, differences in pollen production reduce $F_{1,ery}$ formation relative to the formation of pure *C. erythraea* progeny, resulting in a barrier strength of $w_{2,ery} = 0.367$ and $RI_{2,ery} = 0.633$ (Fig. 4). The observed differences in pollen production have marked effects on the expected rates of hybrid formation, increasing the expected $F_1$ hybrid formation in *C. littorale* by ca. 20% ($q_{2,litt} = 0.844$; Fig. 3). In *C. erythraea*, on the other hand, the expected frequency of $F_1$ hybrid production decreased by 56% ($q_{2,ery} = 0.081$) compared to the expected proportion of $F_1$ hybrid formation after the action of the previous prezygotic stage (Fig. 3).

## RATES OF INTRA- AND INTERSPECIFIC POLLEN TRANSFER

Of the 279 pollen dye transitions recorded, there were 180 intraspecific (151 within *C. erythraea* and only 29 within *C. littorale*) and 99 interspecific pollinator movements (50 from *C. erythraea* to *C. littorale* and 49 from *C. littorale* to *C. erythraea*). Male and female assortative mating components differed between the two species. In *C. erythraea*, on average 75.5% of pollen export transitions and 75.1% of pollen import transitions were conspecific, whereas *C. littorale* conspecific pollen export and import transitions were substantially lower (36.7% and 37.2%, respectively). Overall, the assortative behavior of pollinators in *C. erythraea* resulted in a relatively strong barrier to $F_1$ hybrid formation ($w_{3,ery} = 0.451$ and $RI_{3,ery} = 0.549$), whereas in *C.*

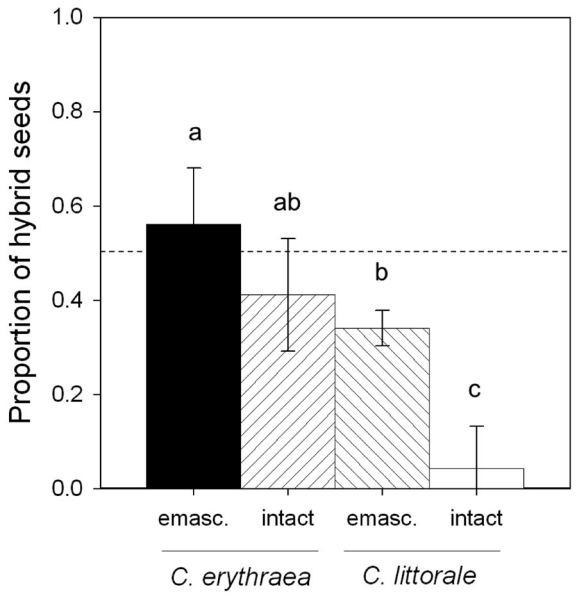


**Figure 5. Hybrid percentage in seeds produced following pollination with 50:50 pollen mixture on intact and emasculated flowers in the two sister species *C. erythraea* and *C. littorale*. For equal siring success of the two species, 50% hybrids would be expected (dashed line).**

*littorale* low levels of assortative pollination resulted in barrier strengths and values of reproductive isolation of $w_{3,litt} = 1.333$ and $RI_{3,litt} = -0.333$, respectively. This resulted in expected proportions of $F_1$ hybrid formation of $q_{3,ery} = 0.040$ and $q_{3,litt} = 0.872$ for *C. erythraea* and *C. littorale*, respectively (Fig. 3).

## ISOLATION DUE TO POLLEN COMPETITION

Pollination with mixed 50:50 pollen loads resulted in significantly lower seed production per fruit compared to intraspecific pollinations, especially in emasculated flowers ($F_{2,54} = 23.57$; $P < 0.0001$). Seed set was also significantly higher in *C. erythraea* than in *C. littorale* ($F_{1,54} = 8.91$; $P = 0.004$), but the interaction was not significant ($F_{2,54} = 0.98$; $P = 0.381$). In *C. erythraea*, mean seed set per fruit was on average $259.7 \pm 19.1$, $200.9 \pm 12.3$, and $186.1 \pm 11.9$ following intraspecific pollination, and mixed 50:50 pollination on intact and emasculated flowers, respectively. In *C. littorale*, average seed production per fruit following intraspecific and mixed pollination on intact and emasculated flowers, was on average $201.0 \pm 7.9$, $164.8 \pm 20.6$, and $158.9 \pm 19.4$, respectively. Pollination with mixed 50:50 pollen loads on *C. erythraea* stigmas caused a significantly higher hybrid seed formation than in *C. littorale* ($F_{1,36} = 360.59$; $P < 0.0001$; Fig. 5). In addition, self-pollen interference in intact flowers strongly reduced the proportion of hybrid seed production compared to emasculated flowers ($F_{1,36} = 251.25$; $P < 0.0001$), and this reduction was significantly larger in *C. littorale* than in *C. erythraea*, as indicated by the significant interaction term ($F_{1,36} = 91.51$; $P < 0.0001$; Fig. 5). The associated barrier strengths and values of reproductive isolation were $w_{4,ery} = 0.540$ and $RI_{4,ery} = 0.460$, and $w_{4,litt} = 0.037$ and $RI_{4,litt} = 0.963$ (Fig. 4). $F_1$ hybrid formation was thus severely restricted by this barrier, especially in *C. littorale* where it decreased the chance of $F_{1,litt}$ hybrid formation from 0.872 to 0.123 (85.9% reduction). In *C. erythraea*, on the other hand, the expected frequency of $F_{1,ery}$ hybrids was reduced by 44.2%, from 0.040 to 0.022 (Fig. 3).

## ESTIMATES OF HYBRID VERSUS PARENTAL FITNESS AND THEIR CONTRIBUTION TO POSTZYGOTIC ISOLATION

Mean seed production per fruit differed significantly between intraspecific and reciprocal interspecific crosses ($F_{3,36} = 20.98$; $P < 0.0001$). The largest amounts of seeds produced per fruit were observed in *C. erythraea*, intermediate levels of seed production in *C. littorale*, and the lowest numbers of seeds produced following interspecific crossing on both maternal species (Table 1). The fraction of hybrid seeds germinating differed significantly between cross-types ($F_{3,36} = 5.26$; $P = 0.004$), and was generally lower than that of pure parental seeds (see Table 1). Survivorship of pure and hybrid offspring also differed significantly between cross-types ($F_{3,405} = 15.54$; $P < 0.0001$), with $F_1$ hybrids showing a lower survivorship compared to their pure parental species, especially when *C. littorale* was the maternal plant (Table 1). Plants originating from the different cross-types produced significantly different numbers of flowers per plant ($F_{3,76} = 4.15$; $P = 0.009$), with $F_{1,ery}$ hybrids producing the lowest amounts of flowers, and $F_{1,litt}$ hybrids producing intermediate numbers of flowers per plant compared to their pure parental species (Table 1). Finally, cross-type had a strong impact on $F_1$ pollen viability ($F_{3,36} = 63.06$; $P < 0.0001$) and seed production ($F_{3,36} = 25.11$; $P < 0.0001$). Both fitness components did not differ significantly between pure *C. erythraea* and *C. littorale* offspring, but were largely reduced in their $F_1$ hybrids (Table 1). Each of the above-mentioned stages, that is, seed production, germination, survivorship, flower production, and $F_1$ male and female fitness, resulted in the following values of reproductive isolation 0.535, 0.264, 0.389, 0.504, 0.961, and 0.888, respectively, for *C. erythraea*, and 0.298, 0.438, 0.686, −0.288, 0.598, and 0.675, respectively, for *C. littorale* (Fig. 4).

## HYBRIDIZATION RATES IN THE FIELD

We successfully scored 180 polymorphic AFLPs, and each genotyped individual ($N = 282$) displayed a unique banding pattern. The mean error rate was 2.5% and no indications for size homoplasy were found. Thirty-one samples, all from the sympatric population, failed to amplify. The four allopatric populations and the artificially obtained $F_1$ hybrids clustered in three distinctive groups: (1) pure *C. erythraea* individuals, (2) pure *C. littorale* individuals, and (3) an intermediate cluster of $F_1$ hybrids

**Table 1.** Means (± SE) for each of the six fitness components following experimental conspecific and heterospecific pollinations ($n$ = 10 per cross-type) within and between *C. erythraea* and *C. littorale*: seed set, seed germination, survivorship, flower production, pollen viability, and seed set of $F_1$.

| Fitness component | *C. ery*♀ × *C. ery*♂ | *C. ery*♀ × *C. litt*♂ | *C. litt*♀ × *C. litt*♂ | *C. litt*♀ × *C. ery*♂ |
|---|---|---|---|---|
| Seed set per fruit | 259.7 ± 19.1$^{a}$ | 120.7 ± 15.9$^{c}$ | 201.0 ± 7.9$^{b}$ | 141.2 ± 8.3$^{c}$ |
| Germination (%) | 56.1 ± 4.2$^{a}$ | 41.3 ± 4.7$^{ab}$ | 59.4 ± 5.2$^{a}$ | 33.4 ± 4.2$^{b}$ |
| Survivorship (%) | 65.2$^{a}$ | 39.9$^{b}$ | 58.4$^{ab}$ | 18.4$^{c}$ |
| Flower number | 25.8 ± 4.8$^{a}$ | 12.8 ± 1.0$^{b}$ | 13.9 ± 2.0$^{b}$ | 17.9 ± 2.4$^{ab}$ |
| Viable pollen (%) | 60.1 ± 3.70$^{a}$ | 2.3 ± 0.6$^{c}$ | 49.3 ± 3.4$^{a}$ | 19.8 ± 3.4$^{b}$ |
| Seeds per fruit of $F_1$ | 240.3 ± 20.2$^{a}$ | 26.8 ± 13.7$^{b}$ | 181.0 ± 14.7$^{a}$ | 58.8 ± 28.3$^{b}$ |

Means followed by the same letter do not differ from each other (Tukey's test, $P > 0.05$).

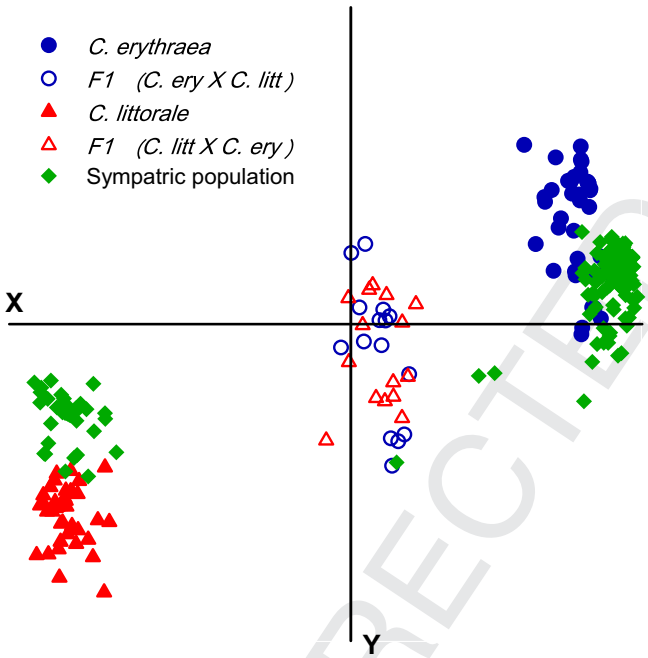


**Figure 6.** Plot of genetic structure (PCoA) based on variation at 180 AFLP markers, of the sympatric study population, two allopatric reference populations of *C. erythraea* and *C. littorale*, and $F_1$ individuals from controlled crosses. Axes *x* and *y* representing 88.1% and 3.2% of the variance in genetic structure, respectively.

resulting from both *C. erythraea*♀ × *C. littorale*♂ and *C. littorale*♀ × *C. erythraea*♂ crossings (Fig. 6). Individuals from the sympatric population mainly clustered around the two well-defined groups of pure *C. erythraea* ($n$ = 130 individuals) and pure *C. littorale* ($n$ = 39 individuals) populations. Only three individuals lay between both clusters and can be considered as putative hybrids or even backcrosses (Fig. 6).

## Discussion

The evolution of selfing is one of the most common transitions in flowering plants (Stebbins 1957; Baker 1959). This change in mating pattern has important systematic and ecological consequences because the evolution of selfing often initiates reproductive isolation and speciation (Baker 1961; Barrett 1989). The importance of mating system evolution in contributing to reproductive isolation acting between co-occurring species has recently received considerable attention (e.g., Fishman and Wyatt 1999; Fishman 2000; Lowe and Abbott 2004; Willis and Martin 2007; Ruhsam et al. 2011, 2013). However, most of these studies were concerned with species at the extreme ends of the mating system variation (i.e., obligately selfing vs. outcrossing species). Here we have shown that floral trait differences between two mixed mating, generalist pollinated species can have a profound impact on the strength of reproductive barriers and the direction of hybridization.

### HETEROSPECIFIC POLLEN FLOW DUE TO DIFFERENCES IN PHENOLOGY, POLLEN PRODUCTION, AND POLLEN TRANSFER

In closely related species, reproductive isolation is rarely achieved by the action of a single reproductive barrier (Lowry et al. 2008) and the strength of barriers often appears to be asymmetric among reciprocal crosses (Tiffin et al. 2001; Rieseberg and Willis 2007). Compared to the hypothetical situation of random mating, the joint action of four prezygotic barriers (i.e., flowering asynchrony, differential pollen production, pollination patterns, and pollen competition) decreased the expected frequency of hybrid seed production in *C. erythraea* from 0.208 to 0.022 (reduction of 89.4%) and in the more selfing *C. littorale* from 0.792 to 0.123 (reduction of 84.5%). In *C. erythraea*, we found that each of the studied prezygotic barriers showed a cumulative and gradual contribution to the prevention of hybrid seed formation, whereas in *C. littorale* the prevention of hybrid formation was almost entirely due to one single reproductive barrier. Nearly 81% of the total prezygotic isolation in *C. erythraea* was due to the twofold larger floral display and the three times higher pollen production rates per flower, which led to more constant pollinator services in this species. In *C. littorale*, on the other hand, the smaller floral display size and overall reduction in pollen reward strongly reduced the species'

contribution to the total pollen pool and its attractiveness to pollinators. As a result, *C. littorale* received less pollinator visits than *C. erythraea* (reduction of ~60%) and most pollinator-mediated pollen depositions resulted from interspecific pollinator movements, making *C. littorale* more prone to heterospecific pollen deposition. This is also confirmed by our estimates of potential hybrid seed production following the sequential action of these prezygotic components, in which the probability of hybrid production in *C. littorale* increased by ~10%. Martin and Willis (2007) also found that the higher pollen production rates in the outcrossing *M. guttatus* compared to the predominantly selfing *M. nasutus* increased the probability of $F_1$ hybrid formation in *M. nasutus* (increase of 90%). On the other hand, Natalis and Wesselingh (2012) recently showed that pollen loads on bumblebees visiting two related *Rhinanthus* species were inversely related to pollen production of these species. They attributed this observation to the grooming behavior of the main pollinators (i.e., bumblebees) after being dusted by a heavy load of pollen, a behavior that is not observed in the pollinators (i.e., hoverflies and small flies) of our study species. Higher risks of heterospecific pollen deposition in the more selfing species can be exacerbated when the species occurs in minority compared to its more outcrossing relative, which was the case in our study population (relative frequency of flowering *C. littorale* vs. *C. erythraea* individuals was 3:7).

## ANTHER–STIGMA CLUSTERING: AN EFFICIENT BARRIER

The high levels of heterospecific pollen flow and expected frequency of hybrid formation in *C. littorale* are counterbalanced at the stage where interspecific deposited pollen interferes with intraspecific self-pollen. At this stage, the expected frequency of hybrid formation in *C. littorale* is reduced by 85.9% (compared to only 44.2% in *C. erythraea*) in comparison to the previous prezygotic stage (i.e., pollen transfer). Although the exact reasons for the lower probability of hybrid formation in *C. littorale* are not clear, the results of the mixed pollination versus emasculation experiment suggest that this is primarily related to the species' capacity for competing selfing, most likely via pre-emptive self-pollination and/or stigma clogging. In addition, the close positioning of anthers around the stigmatic surface (see Fig. 1b) mechanically prevents pollinators from coming into direct contact with the stigma and maximizes the number of self-pollen grains deposited on the stigma (Webb and Lloyd 1986). Similar results have been reported by Smith and Rausher (2006), who showed that close stigma–anther proximity in the morning glory (*Ipomoea hederacea*) was an important prezygotic isolation barrier when these flowers were exposed to heterospecific pollen flow from *I. purpurea*.

Selfing species generally also have short styles and pollen with less rapid pollen tube growth and lower competitive abilities, which may cause a disadvantage once mixed pollen loads have been deposited (e.g., Diaz and MacNair 1999; Montgomery et al. 2010; Runquist 2012, but see Natalis and Wesselingh 2012). In case of the studied *Centaurium* species, the styles are significantly shorter ($F_{1,119} = 20.5$; $P = 0.011$) and pollen tube growth is significantly slower ($F_{1,59} = 23.4$; $P < 0.0001$) in *C. littorale* than in *C. erythraea* (R. Brys and H. Jacquemyn, unpubl. data). However, it remains unclear to what extent these differences in pollen–stigma and pistil interactions have contributed to the observed asymmetries in $F_1$ hybrid formation.

## TOTAL INTERSPECIFIC BARRIER STRENGTH AND HYBRID OCCURRENCE IN THE FIELD

Despite the large number of $F_1$ hybrid progeny that was obtained after artificially crossing emasculated *C. erythraea* and *C. littorale* flowers the probabilities of $F_1$ hybrid seed formation following the sequential action of the four studied prezygotic barriers were small (2.2% and 12.3% in *C. erythraea* and *C. littorale*, respectively). Nonetheless, the obtained values of barrier strength suggest that there still is an opportunity for potential hybrid seed production. Using the multiplicative formula proposed by Ramsey et al. (2003), this resulted in a total prezygotic isolation of 0.923 and 0.918 for *C. erythraea* and *C. littorale*, respectively.

On the other hand, when $F_1$ hybrid seeds were successfully produced, the chance for hybrid establishment and potential introgression was further diminished by the action of several postzygotic barriers. Hybrid seeds showed a much lower germination and poorer survival than pure seeds. In addition, experimentally obtained F1 hybrids also showed low pollen fertility (mean reduction: ~60%) and strongly reduced seed production (mean reduction: ~68%), which may have arisen from meiotic irregularities or other genetic factors (Stebbins 1958; Rieseberg 2001). The observed levels of hybrid infertility are in line with artificially obtained $F_1$ hybrids originating from populations in the United Kingdom, Denmark, Sweden, Finland, and Germany, which also showed reduced fitness, infertile pollen, and limited female reproductive output (Zeltner 1970; Ubsdell 1976, 1979).

Despite the action of the 10 pre- and postzygotic barriers studied, total reproductive isolation still remained incomplete ($RI_{total} \sim 0.999$). The fact that three putative hybrids were observed in the field is in line with these findings. Several studies have described, based on morphological characteristics alone, the occurrence of putative hybrids between *C. erythraea* and *C. littorale*, and some of them even suggested prevalence of backcrosses with *C. erythraea* in the field (Wheldon 1897; Salmon and Thompson 1902; Ubsdel 1976, 1979). Based on our molecular data, it seems likely that two of the three putative hybrids observed were also backcrosses with *C. erythraea* (see Fig. 6). The occurrence of

backcrosses may be surprising, given the low numbers of hybrids and their strongly reduced fertility. However, given that *C. erythraea* was more abundant than *C. littorale*, backcrossing with *C. erythraea* is the most probable direction of introgression (Ducarme et al. 2010; Jacquemyn et al. 2012). Furthermore, when $F_1$ hybrid fertility is low (especially pollen viability) and assuming intermediate characteristics in mating system and floral traits, $F_1$ hybrids most likely contribute offspring as an outcrossing maternal plant (Willis and Martin 2007; Ruhsam et al. 2011). As a result, backcrossing is most likely the result of $F_1$ hybrid fertilization sired by the outcrossing parental species, resulting in introgression from the more selfing *C. littorale* to the more outcrossing *C. erythraea*.

## CONCLUDING REMARKS

In sympatric species that share a generalized floral morphology and pollinator community, intense competition for pollination and severe costs related to hybridization may select for floral traits that contribute to prezygotic isolation (Servedio and Noor 2003). In the studied *Centaurium* species, our observations clearly showed that heterospecific pollen transfer and hybrid fertilization were costly, both in terms of seed production and progeny fitness. Under such conditions, morphological features that mechanically prevent interspecific pollen transfer and favor selfing (e.g., clustering of anthers and stigmas) may be favored to reinforce isolation (Dobzhansky 1937; Hopkins 2013).

Preliminary investigations of some sympatric and allopatric populations of both *Centaurium* species along the Belgian coast showed significant differences in stigma–anther separation in *C. littorale*, depending on whether the species co-occurred with *C. erythraea* or not. In sympatric populations, the mean anther–stigma separation in *C. littorale* was about seven times smaller compared to that of allopatric populations (mean: $0.09 \pm 0.07$ mm and $0.71 \pm 0.03$ mm, respectively; $F_{1,114} = 37.77$; $P < 0.001$), whereas the level of herkogamy in *C. erythraea* did not differ significantly between sympatric (mean: $0.99 \pm 0.04$ mm) and allopatric populations (mean: $0.94 \pm 0.05$ mm) ($F_{1,243} = 0.50$; $P = 0.481$) (Brys and Jacquemyn, unpubl. data). Similarly, *C. littorale* showed substantial herkogamy in some allopatric populations in the United Kingdom (Ubsdell 1979). These results are compatible with results of Fishman and Wyatt (1999) who showed that prior selfing in the polymorphic annual plant *Arenaria uniflora* (Caryophyllaceae) functioned as a reproductive barrier against heterospecific pollen transfer, consistent with increased autogamy in areas of range overlap with that of its congener *A. glabra*.

However, our findings that anther–stigma clustering substantially enhanced prezygotic isolation in *C. littorale* do not necessarily imply that selection against maladaptive hybridization is the primary factor explaining the observed differences in anther–stigma clustering. The observed shift in herkogamy may also have resulted from interspecific differences in pollinator and/or mate limitation. In this case, the evolution of close anther–stigma clustering would result in reproductive assurance in the species suffering from severe pollen limitation (Eckert et al. 2010). The fact that *C. littorale* suffers less from inbreeding depression than *C. erythraea* (on average $\delta = 0.11$ and 0.57 in *C. littorale* and *C. erythraea*, respectively; R. Brys and H. Jacquemyn, unpubl. data) may support this hypothesis.

Further experiments are therefore needed to unequivocally show that the higher capacity for autonomous selfing in *C. littorale* established as a response to maladaptive hybridization. Future work should focus on the role of floral morphology in determining reproductive isolation and assess the total strength of pre- and postzygotic barriers over a large ecogeographic range in allopatric versus sympatric populations. In addition, selection experiments may offer a convincing tool to provide clear empirical evidence that prezygotic isolation is enhanced in response to selection against hybridization. Such experiments also allow demonstrating whether modest shifts in breeding system and associated changes in floral traits enhance asymmetries in pollen flow, resulting in further differentiation of the breeding system.

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## *Supporting Information*

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** (a) Experimental setup of the arrays (6 × 6), containing 18 *C. erythraea* plants interspersed checkerboard fashion with 18 *C. littorale* plants (separation of 0.5 m among plants).

**Figure S2.** Plot of leaf characteristics (PCoA) based on the variation in the ratio of leaf length versus width, leaf area ($mm^2$), leaf color (mean component of red, green, and blue), number and height of papilla in the two parental sister species (*C. erythraea* and *C. littorale*), and their reciprocal hybrids (*C. erythraea* × *C. littorale* and *C. littorale* × *C. erythraea*) obtained from controlled crosses.

**Table S1.** Mean values of six leaf characteristics measured on experimentally obtained $F_1$ individuals of two sister species (*C. erythraea* and *C. littorale*) and their reciprocal hybrids (*C. erythraea* × *C. littorale* and *C. littorale* × *C. erythraea*) obtained via pure and interspecific hand-pollinations.

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