The importance of autonomous selfing in preventing hybridization in three closely related plant species

Rein Brys[1,2]*, Jannick van Cauwenberghe[2] and Hans Jacquemyn[2]

[1]*Research Institute for Nature and Forest, Kliniekstraat 25, BE-1070 Brussels, Belgium; and* [2]*Biology Department, Plant Conservation and Population Biology, KU Leuven, Heverlee, Belgium*

Summary

1. In plants that are able to self-fertilize, autonomous selfing has been hypothesized to function as an effective mechanism that prevents heterospecific mating. However, there have only been few studies that have assessed the relative and absolute contribution of different modes of selfing to total reproductive isolation acting between species. Because selfing can be expected to increase fitness costs when offspring are sired from more outcrossing sister taxa, it can be hypothesized that the contribution of post-zygotic barriers to total reproductive isolation becomes stronger in selfing than in more outcrossing relatives.

2. To assess the efficiency of different modes of selfing against heterospecific mating, we conducted mixed-pollination experiments in three *Centaurium* species showing strong differences in mating system (i.e. delayed selfing in *Centaurium erythraea*, competing selfing in *C. littorale* and prior selfing in *C. pulchellum*). The contribution of selfing to total reproductive isolation was further examined by quantifying the strength of three pre-zygotic (flowering phenology, pollinator fidelity and pollen production) and four intrinsic post-zygotic (seed set and germination, survival and flowering capacity) barriers for each of the six species pair combinations.

3. Although autonomous selfing as a reproductive barrier was unable to completely impede gene flow, its efficiency as a mechanism to prevents heterospecific pollination strongly depended on the mode of selfing, with prior selfing being by far the most efficient mechanism and delayed selfing only offering a limited protection against hybrid fertilization at the end of a flower's lifetime. Apart from the prevention effect of selfing, differences in pollinator fidelity and pollen production also contributed substantially to total reproductive isolation.

4. Post-zygotic hybrid costs were high and most prevalent for seed production and germination success. Reductions in hybrid vigour were significantly associated with the mating system of the maternal species, with the highest reductions in the most selfing *C. pulchellum* and the lowest reductions in predominant outcrossing *C. erythraea*.

5. *Synthesis.* Our findings indicate that selfing may prevent species from interspecific gene flow and hybridization. The association between the capacity to self-autonomously and hybrid vigour further suggests that selfing may foster post-zygotic costs following hybridization.

Key-words: autonomous selfing, *Centaurium*, Gentianaceae, hybrid costs, plant–plant interactions, post-zygotic isolation, pre-zygotic isolation, speciation

Introduction

The maintenance of species integrity between sexually compatible species depends on the strength of reproductive barriers that together determine total reproductive isolation acting between them (Mayr 1942; Schluter 2001; Coyne & Orr 2004). Reproductive isolation (RI) is the crux of speciation (Dobzhansky 1937) and assessing the relative and absolute contribution of different types of reproductive barriers acting between species has become a central theme in speciation studies (Coyne & Orr 2004). In most cases, RI is the result of several isolating barriers that act sequentially. These barriers can function prior to mating (pre-mating), after mating but before zygote formation (post-mating pre-zygotic) and/or after zygote formation (post-zygotic) (Ramsey, Bradshaw & Schemske 2003; Lowry *et al.* 2008). In flowering plants, pre-zygotic isolation is generally more important and contributes more to total RI than post-zygotic isolation (Rieseberg &

*Correspondence author. E-mail: rein.brys@inbo.be

Willis 2007; Lowry *et al.* 2008; Widmer, Lexer & Cozzolino 2009).

In the absence of a geographic barrier to gene flow, floral isolation is regarded as one of the most important pre-zygotic barriers offering RI (Hodges & Arnold 1994; Dell'Olivo *et al.* 2011; Xu *et al.* 2011; Whitehead & Peakall 2014). Floral isolation can be mediated through differences in pollinator behaviour (i.e. ethological isolation) (Schemske & Bradshaw 1999; Kay & Schemske 2003; Aldridge & Campbell 2007; Xu *et al.* 2011; Whitehead & Peakall 2014) or differences in floral morphology (i.e. mechanical isolation) (Grossenbacher & Whittall 2011; Goldberg & Igic 2012; Wright, Kalisz & Slotte 2013). For the latter, floral adaptations that allow autonomous selfing are assumed to offer effective mechanical protection against heterospecific mating and thus to contribute to reproductive isolation (Wright, Kalisz & Slotte 2013). However, there is still little empirical evidence on the efficiency of autonomous selfing as an isolating barrier (but see Fishman & Wyatt 1999; Smith & Rausher 2006) and its relative contribution to total RI (but see Martin & Willis 2007; Brys *et al.* 2014). Moreover, because there is huge variation in the capacity and timing of autonomous selfing, the efficiency of selfing as a mechanism to suppress heterospecific mating is likely to vary as well.

Depending on the timing, three modes of autonomous selfing can roughly be defined with respect to the period of potential outcross pollen receipt (Lloyd 1979; Lloyd & Schoen 1992) (Fig. 1). At one end of the continuum, 'prior selfing'
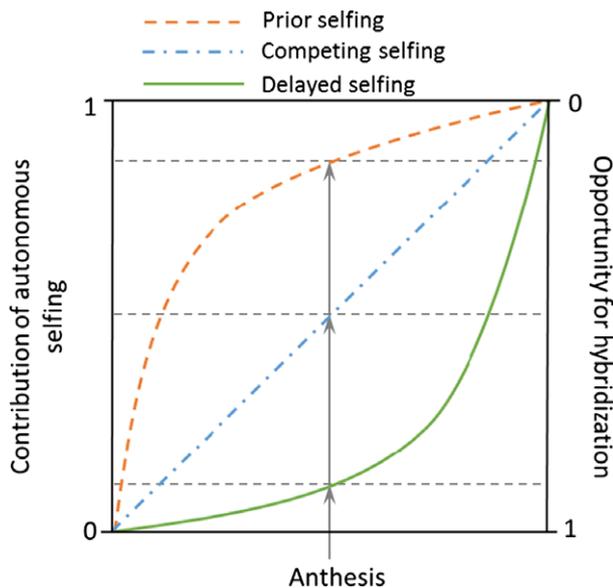

Fig. 1. Graphical model of the effect of autonomous selfing on the probability that heterospecific pollen can be successfully deposited on the stigmatic surface and hybrid fertilization can take place. The coloured curves depict three modes of autonomous selfing (*sensu* Lloyd 1979) depending on the timing when selfing is realized during floral development: prior selfing, competing selfing and delayed selfing. The solid grey arrows map the intervention of a hypothetical floral visitor carrying heterospecific pollen midway anthesis, whereas the three dotted grey lines map the probability that the stigmatic surface is already autonomously pollinated with self-pollen (left-hand *y*-axis), thereby diminishing the risk of being successfully pollinated with heterospecific pollen (right-hand *y*-axis).

refers to early spatial and developmental overlap of the anthers and stigma within a flower (Fishman & Wyatt 1999). In this mode of selfing, autonomous selfing takes place just before or just after the beginning of anthesis, thereby strongly diminishing the possibility of outcross pollen receipt. 'Competing selfing', on the other hand, occurs concurrently with outcross pollen receipt and often results in substantial competition between deposited self- and outcross pollen (Leclerc-Potvin & Ritland 1994). Finally, 'delayed selfing' occurs at the end of a flowers lifetime after the opportunity for outcross pollen receipt has passed (Kalisz *et al.* 1999). Because self-pollination diminishes the possibility of outcross pollen deposition and fertilization, it can be hypothesized that differences in the timing of selfing have a big impact on the opportunity for both heterospecific pollen deposition and the chance of hybrid formation (Fig. 1). Moreover, because selfing species generally display much lower levels of heterozygosity (Wright, Kalisz & Slotte 2013), a long history of autogamy can theoretically be expected to fix underdominant alleles or chromosome rearrangements that have reduced fitness when exposed as heterozygotes (Charlesworth 1992). This may in turn lead to increasing post-zygotic costs when selfing species cross with more outcrossing sister taxa. It can thus be hypothesized that the contribution of post-zygotic barriers to total RI is stronger in selfing than in more outcrossing relatives.

To test these hypotheses, we assessed the efficiency and contribution of different modes of selfing to RI and post-zygotic hybrid costs in three closely related species of the genus *Centaurium* (Gentianaceae): *Centaurium erythraea*, *C. littorale* and *C. pulchellum*. Although these species show strong similarities in flower morphology and share numerous pollinators (mainly hoverflies), they show a clear differentiation in the mode of selfing (Brys & Jacquemyn 2011). *Centaurium pulchellum* shows by far the highest capacity of autonomous selfing and can be defined as a prior selfing species (Brys & Jacquemyn 2011). *Centaurium erythraea*, on the other hand, has the lowest selfing capacity that is realized at the end of a flower's lifetime and can be denoted as a delayed selfing species, whereas *C. littorale* realizes intermediate levels of competing selfing (Brys & Jacquemyn 2011). More specifically, we first assessed the efficiency of variable modes of selfing in avoiding hybrid formation by performing experimental pollinations using mixed pollen loads and calculated a generalized index of self-protection. Secondly, we assessed the strength of a suite of other pre- and post-zygotic barriers known to affect RI in *Centaurium* (flowering phenology, pollinator fidelity, pollen availability, interspecific seed set, germination, survival and flower production) and compared the absolute and relative contribution of these barriers to total RI with that generated by autonomous selfing.

Materials and methods

STUDY SPECIES AND POPULATIONS

Centaurium is a plant genus that belongs to the gentian family (Gentianaceae) and comprises 20 short-lived (annual or biennial),

monocarpic herbaceous species (Mansion & Struwe 2004). Most species occur in disturbed habitats, such as roadsides, stream banks, coastal dune areas and grazed grasslands. Here, we studied the biennials *C. erythraea* and *C. littorale* and the annual *C. pulchellum*. These species are widely distributed across Europe and show considerable overlap in distribution area, particularly in coastal habitats (Appendix S1). *Centaurium erythraea* is the largest of the three species and generally prefers dry soil conditions, varying from well-developed calcareous grasslands, over wood margins to coastal dunes and river banks. *Centaurium littorale* and *C. pulchellum*, on the other hand, generally develop under more dynamic and ruderal conditions within vegetation that is more open (van Tooren, Schat & ter Borg 1983; Schat & Scholten 1985). The latter two species also prefer wetter sites that are frequently exposed to intertidal influences (Schat & Scholten 1985).

The three study species produce very similarly looking, showy pink flowers that are hermaphroditic, self-compatible and typically characterized by coiling of the anthers during dehiscence (Brys & Jacquemyn 2011) (Fig. 2). Flowers do not produce any nectar and are mainly pollinated not only by pollen-gathering hoverflies (Diptera, Syrphidae), but also by small bees (Hymenoptera, Apidae) and flies (Empididae-Muscidae) (Brys & Jacquemyn 2011, 2012). The species show pronounced differences in their capacity to self-autonomously and in several floral traits associated with the selfing syndrome, including the level of herkogamy, floral size and pollen production (Brys & Jacquemyn 2011). *Centaurium erythraea* produces the largest flowers, with the highest level of herkogamy and amount of pollen, *C. pulchellum* develops the smallest flowers, with lack of herkogamy and lowest pollen production, and *C. littorale* shows intermediate levels for each of these floral traits (Brys & Jacquemyn 2011) (Fig. 2). Reproduction can only take place by means of seeds, which are very small (0.01 mg) and produced in large quantities (on average 241.1 ± 51.7, 206.5 ± 42.8 and 229.4 ± 25.2 seeds per fruit in *C. erythraea*, *C. littorale* and *C. pulchellum*, respectively, Brys & Jacquemyn 2011).

The study was conducted at three sites along the Belgian coast. At each site, a single population of two different species was found

(Groenpleinduinen: *C. erythraea* – *C. littorale*, Ter Yde: *C. erythraea* – *C. pulchellum*; Baai van Heist: *C. littorale* – *C. pulchellum*). For each studied population, the number of flowering plants was sufficiently large to perform the observations and experiments described below (>500 individuals).

DETERMINATION AND CALCULATION OF PRE-ZYGOTIC BARRIERS

Flowering phenology

In the summer of 2013, the flowering phenology of naturally occurring *C. erythraea*, *C. littorale* and *C. pulchellum* individuals was assessed. Within each of the study populations and at the start of the flowering season, five 2×2 m plots were established at patches were both species abundantly flowered (beginning of June). During the entire flowering period, we recorded for each species the total number of flowering plants that had at least one flower open in each plot. Flowering data were then used to assess the degree of synchronicity in flowering. The duration of flowering peak of a species was determined as the time period when more than 50% of the individuals in the sample (i.e. total amount of plants that flowered during the entire flowering season) was flowering.

The strength of RI due to asynchrony in flowering phenology can be calculated using the methods outlined in Sobel & Chen (2014), using the equation:

$$RI = 1 - \left(\frac{S}{S + U} \right), \qquad \text{(eqn 1)}$$

where S refers to the proportion of flowering time that is shared between the two species and U refers to the proportion of unshared flowering time. This metric of RI accounts for differences in flowering time and assesses deviations from random mating, where species flower simultaneously. The total probability of gene flow is thus weighted by the proportion of 'shared' and 'unshared' time of flowering.

Pollinator preferences

During flowering peak (July–August 2013), standardized surveys of flower visitors were conducted on warm and sunny days in each of the three study sites and within the above mentioned plots. During 18 observation intervals of 20 min for each combination of the studied *Centaurium* species, all potential pollinators visiting flowers of both focal species were identified per population over a 10 days period (between 10.00 and 14.00 h), and the number of visits, as well as the host-species on which they were foraging, were recorded. Based on these observations, for each insect species observed in each study population, we calculated the total number of floral visits for both *Centaurium* species and classified them as 'heterospecific' (i.e. shared) and 'conspecific' (i.e. unshared) pollinators. To estimate the degree of RI, and assuming that each pollinator has an equal efficiency in pollen uptake and stigmatic deposition for each of the three *Centaurium* species studied, we used the linear quantitative framework for estimating the strength of barriers presented in Sobel & Chen (2014):

$$RI = 1 - 2 \times \left(\frac{H}{H + C} \right), \qquad \text{(eqn 2)}$$

where H refers to the proportion of heterospecific pollinators and C to the proportion of conspecific pollinators foraging between each of the *Centaurium* species studied.
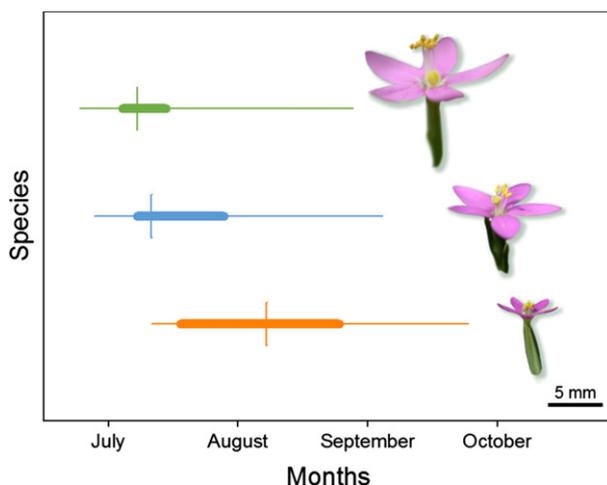

Fig. 2. Mean flowering phenologies with an illustration of the flowers of each of the three related *Centaurium* species (*C. erythraea. C. littorale* and *C. pulchellum*) obtained from the three sympatric study populations at the Belgian coast in summer of 2011. Thin lines indicate the average total flowering time (at least one plant in flower), thick lines indicate the average flowering peak period (50% of plants in flower), and vertical lines indicate the time when the maximum number of individuals were in flower.

Differential pollen production

After the potential action of flowering asynchrony and differences in pollinator preferences as reproductive barriers, differential pollen production can further act as a barrier because it alters the proportion of heterospecific/conspecific pollen in the pollen pool destined for the ovules of the species involved. To incorporate the effect of interspecific differences in pollen production as a potential barrier, we used pollen production data from Brys & Jacquemyn (2011). To calculate RI caused by differential pollen production and to incorporate deviations from expectations of random mating where pollen production is equal between each of the species studied, we used eqn (2) again (Sobel & Chen 2014), where *H* refers to the proportion of heterospecific pollen available in the total pollen pool and *C* to the proportion of conspecific pollen available.

Self-pollen interference

To quantify the efficiency of autonomous selfing as a mechanism to prevent hybridization, we examined proportional F_1 hybrid seed formation following mixed pollinations at different phases during flower development. Per study population, 10 plants were brought to a pollinator-free glasshouse for each species, 2 months before flowering (May 2013). Once plants started to flower, supplemental pollinations with a 50:50 mixture of intra- and interspecific pollen originating from plants of the same population were applied on each plant. Per plant, twelve flowers were then manually pollinated at different stages during floral development (further denoted as 'timing'): (i) the first day of anthesis, (ii) mid-way anthesis and (iii) the end of a flowers lifetime (these stages are further denoted as 'early', 'mid-' and 'late', respectively). Half of these flowers were left intact whereas the others were emasculated prior to flowering (further denoted as 'floral manipulation'). Flowers of *C. erythraea* and *C. littorale* have an average lifetime of 4 days, whereas those of *C. pulchellum* are generally open for only 3 days (Brys & Jacquemyn 2011). The 50:50 pollen mixes for each species combination were obtained using the same methodology as described in Brys *et al.* (2014). By adding these controlled pollen mixes on intact flowers, we mimicked the natural situation of interspecific pollen transfer without the action of any previous reproductive barrier. For each crossing, we then assessed the proportion of hybrid plants and compared proportional hybrid formation in intact flowers with that of the same pollination treatment on emasculated flowers. This allows to uncouple the impact of self-pollen interference realized via autonomous selfing from that of potential confounding effects due to species-specific differences in pollen–pistil interactions or other interspecific incompatibilities during the process of fertilization.

Once seeds were ripe, all seeds per plant were pooled for each treatment, and a sample of 50 seeds was sown to estimate the proportion of hybrid offspring after 4 months. At that time, F_1 hybrids and pure offspring can be unambiguously distinguished from each other based on several morphometric characteristics of the leaves (the density and height of papillae, leaf length: width ratio, leaf area and top of the leaf and colour) (see Appendix S2, and Brys *et al.* 2014). To test whether the proportion of hybrid seeds was significantly affected by pollination treatment (mixed pollination on intact vs. emasculated flowers), timing during floral development (early, mid and late pollination) and the interaction between both factors, a generalized linear mixed model (GLMM, proc GLIMMIX) was used for each species pair separately, with plant included in the model as a random factor.

To assess the importance of selfing in preventing hybrid formation (SP) at a certain moment (*t*) during floral development, we used the following formula:

$$SP_t = 1 - \left(\frac{\text{Proportion hybrid seeds in intact flowers}}{\text{Proportion hybrid seeds in emasculated flowers}} \right).$$
(eqn 3)

Assuming that the proportion of hybrid seeds in intact flowers is always lower than the proportion of hybrid seeds in emasculated flowers, this index ranges from 0 (i.e. no prevention of hybrid fertilization) to 1 (i.e. complete protection against hybrid fertilization) and allows comparing the exact contribution of autonomous selfing as a protection mechanism against hybridization for each species pair at a certain moment *t* during flower development. For each species pair, we used a GLMM (proc GLIMMIX) to test whether the maternal and paternal component in the mixed 50:50 crossings had a significant effect on the self-protection index and whether this was affected by the timing during floral development.

Based on the obtained indices of self-protection (SP_t) at different moments during floral development, a species-specific total index of self-protection (SP_{total}) against hybrid formation during the entire lifetime of a flower was calculated as follows:

$$SP_{total} = \frac{1}{T} \int_0^T f(t) \, dt.$$
(eqn 4)

In this equation, $f(t)$ describes changes in SP_t in function of time, with *T* referring to a flower's total lifetime. Since SP_{total} represents a proxy of the overall proportion of conspecific seeds sired (*C*) over a flowers entire lifetime, the proportion of heterospecific seeds sired is then $H = 1 - C$. Based on these estimates, RI resulting from autonomous selfing was calculated for each species pair using the same formula used above (eqn 2).

ASSESSING THE STRENGTH OF POST-ZYGOTIC BARRIERS

Interspecific seed production and hybrid vs. parental fitness

For each species pair, the performance of F_1 hybrids was compared with that of offspring resulting from pure parental crossings at four different stages during the life cycle of the plants. These included seed set, germination, survivorship and flower production. In May 2012, 2 months before flowering, we transferred 10 transplants per species from each of the sympatric populations into pots and brought them to a pollinator-free glasshouse. On these transplants, intra- and interspecific hand pollinations were conducted to assess hybrid viability and estimate post-zygotic isolation. During flowering (July 2012), three flowers per plant were selected per pollination treatment on each maternal plant per species and flowers were emasculated prior to flowering to avoid any interference with intraspecific self-pollen. In total, three pollination treatments (two interspecific and one intraspecific pollination) were conducted on each plant. When fruits were ripe, seed set was quantified per fruit for each pollination treatment as the amount of seeds produced proportional to the total amount of ovules.

Per maternal plant and species pair, seeds were pooled, and from this sample, three replicates of 50 seeds were sown in $8 \times 8 \times 15$ cm pots on a 3:1 mix of sand and potting soil. In December 2012, all pots were put in a growth chamber (8:16 L:D cycle, 12–25°C) and watered and checked for germination twice a week. After 4 months (at the end of March), total germination success

and survivorship of all recruits were recorded and progeny was reduced to the five most viable individuals that were transplanted into separate pots. These plants were further grown in the botanical garden of the university and checked twice a month. In the species pairs where *C. pulchellum* functioned as the mother plant, the F_1 plants started flowering the first summer following germination (July 2013), whereas offspring resulting from the other species pair crossings started to flower the second growing season (July 2014). At the moment of flowering, total survivorship (i.e. mean survivorship of seedlings × mean survivorship from transplantation until flowering) and flower production of each of the remaining F_1 plants were determined.

For each of the four fitness components measured (seed set, germination, survivorship and flower production), we calculated the mean values per maternal parent and species pair. General and generalized linear mixed models were used to test whether seed set, germination success and flower production of F_1 offspring depended significantly on the maternal and paternal plant used in the reciprocal crossings and their interaction. Proc GLIMMIX with logit-link function was used for dependent variables with a binomial distribution (values of proportional seed set and germination), whereas proc MIXED was used to analyse the log-transformed flower production data. To test whether survivorship of hybrid F_1 offspring differed significantly with that of pure F_1 offspring, we used a chi-squared test. Finally, barrier strengths at each of these four post-zygotic stages were calculated using eqn (2) described above (Sobel & Chen 2014), with H referring to interspecific offspring fitness and C referring to intraspecific offspring fitness.

ESTIMATING TOTAL ISOLATION AND RELATIVE CONTRIBUTIONS OF BARRIER STRENGTHS

Total RI acting between each species pair was calculated as follows:

$$RI_{total} = 1 - 2 \times \left(\frac{S \times H_S + U \times H_U}{(S \times H_S + U \times H_U) + (S \times C_S + U \times C_U)} \right),$$
(eqn 5)

where S refers to the extent of shared period of flowering, and U refers to the unshared period of flowering. H and C represent heterospecific and conspecific effects, but are multiplied across all components of RI and are considered both within the shared (H_S, C_S) and the unshared (H_U, C_U) period of flowering (Sobel & Chen 2014). To calculate the absolute contribution (AC_i) of each of the studied barriers to total isolation, the individual strength of a barrier was discounted by the impact of previously acting barriers as follows:

$$AC_i = RI_{[1,i]} - RI_{[1,i-1]}.$$
(eqn 6)

The relative contribution (RC_i) of each barrier to total isolation was calculated using the general equation from Ramsey, Bradshaw & Schemske (2003):

$$RC_i = \frac{AC_i}{RI_{total}}.$$
(eqn 7)

Results

PRE-ZYGOTIC BARRIERS

Flowering phenology

Flowering of the three *Centaurium* species occurred from the end of June until the end of September (Fig. 2). *Centaurium*

erythraea was the first species to flower and showed the shortest flowering time (on average 67 flowering days, with flowering peak on the 8th of July) compared to *C. littorale* (on average 73 flowering days, flowering peak on the 11th of July) and *C. pulchellum* (on average 74 flowering days, flowering peak on the 9th of August). Overall, the flowering phenology of the three *Centaurium* species was highly overlapping, and consequently resulted in relatively low barrier strengths (ranging from 0.030 for the $E^{\female}L^{\male}$ species combination to 0.284 for the $P^{\female}E^{\male}$ species combination) (Appendix S3).

Pollinator preferences

In total, 519 insect visits were recorded on flowers of the studied *Centaurium* species during all the observations made (Appendix S4). Most of them (68.8%) were observed on flowers of *C. erythraea*, whereas 24.9% and only 7.3% of the visiting insects were recorded on *C. littorale* and *C. pulchellum,* respectively. In total, 25 different insect species were recorded, the majority (87.5%) of them being pollen-gathering hoverflies (Diptera, Syrphidae), most often *Episyrphus balteatus*, *Sphaerophoria* spp., or *Scaeva* spp. Other flower visitors were observed at much lower frequencies and were mostly small flies (Empididae – Muscidae; <7%), some bees and bumblebees (Hymenoptera, Apidae; <4%), and occasionally a butterfly (Lepidoptera; <2%). The resulting barrier strengths varied between 0 (complete overlap in pollinator assemblages) for the $P^{\female}E^{\male}$ combination and 0.545 for the $E^{\female}P^{\male}$ combination (Appendix S3).

Differential pollen production

Due to significantly different numbers of pollen that are produced per flower (on average $101\,950 \pm 7179$, $37\,375 \pm 5835$ and $20\,026 \pm 3518$ pollen per flower in *C. erythraea*, *C. littorale* and *C. pulchellum,* respectively (data obtained from Brys & Jacquemyn 2011), barrier strengths at this stage were highly variable and symmetric for each species combination, ranging from −0.672 to 0.672 (see Appendix S3).

Self-pollen interference

Overall, the proportion of hybrid seeds sired following mixed 50:50 pollinations in intact flowers was 62.5% lower than in emasculated flowers. The strongest reductions were found in *C. pulchellum* (85.3% reduction), *C. littorale* showed an average reduction of 65.9% and *C. erythraea* had the lowest overall reduction (on average 36.2%). In experiments where *C. pulchellum* acted as the maternal plant, the proportion of hybrid seeds was significantly affected by pollination treatment ($F_{1,54} > 7.54$; $P < 0.008$; Appendix S5). In experiments where *C. erythraea* and *C. littorale* acted as maternal species, both pollination treatment and timing had a significant impact on hybrid formation ($F_{2,54} > 4.76$; $P < 0.033$). Moreover, the efficiency with which hybrid formation was prevented

significantly increased with floral age, as indicated by the significant interaction effect between timing and floral manipulation ($F_{2,54} > 4.58$; $P < 0.037$) (Appendix S5). The resulting self-protection indices depended significantly on maternal species ($F_{2,162} > 15.27$; $P < 0.0001$), with *C. pulchellum* showing the highest and *C. erythraea* the lowest capacity to prevent hybridization by means of autonomous selfing (Fig. 3). Pollen donor, on the other hand, had no significant effect on SPtotal ($F_{2,162} = 0.63$; $P = 0.533$). Timing of pollination had a significant and positive impact on SPtotal ($F_{2,162} = 62.07$; $P < 0.001$; Fig. 3) and showed a significant interaction effect with maternal species ($F_{5,162} = 3.65$; $P = 0.028$). RI resulting from autonomous selfing in both species combinations per maternal plant was on average -0.325 in *C. erythraea*, 0.342 in *C. littorale* and 0.709 in *C. pulchellum* (Appendix S3).

POST-ZYGOTIC BARRIERS

Estimates of hybrid vs. parental fitness and their contribution to post-zygotic isolation

For all species pairs, seed production depended significantly on maternal species, pollen donor and the interaction between them (Table 1). Interspecific crossings generated significantly lower seed set compared to intraspecific crossings (average reduction of 40.9%; Appendix S6). The strongest reduction occurred when *C. pulchellum* functioned as the mother plant (reduction > 55%), whereas the lowest reductions were observed when *C. littorale* was the mother plant (reduction < 14%). These differences in seed set resulted in barrier strengths that varied between 0.027 and 0.500 (Appendix S3). Percentage germination depended significantly on the mater-
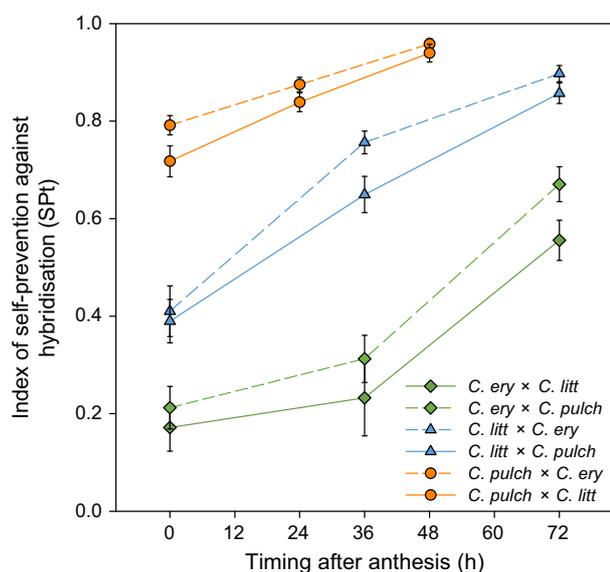
Table 1. Results of two-way anovas testing for the effects of maternal and paternal species involved in the intra- and interspecific artificial crossings on seed set, germination capacity and flower production, with *Centaurium erythraea*, *C. littorale* and *C. pulchellum* as the three study species used

Source	d.f.	F	P
Proportional seed set			
Maternal	2,81	69.61	<0.001
Paternal	2,81	22.78	<0.001
Maternal × Paternal	4,81	104.72	<0.001
Germination success			
Maternal	2,81	77.93	<0.001
Paternal	2,81	1.24	0.294
Maternal × Paternal	4,81	16.28	<0.001
Flower production			
Maternal	2,81	9.07	<0.001
Paternal	2,81	5.30	0.007
Maternal × Paternal	4,81	1.64	0.173

nal species and the interaction between maternal and paternal species, but not on the paternal component (Table 1). Overall, F_1 hybrid seeds showed a significantly lower germination success compared to their pure parental congeners (Appendix S6). Germination capacity of hybrid seeds was most strongly reduced when *C. pulchellum* was the mother plant (reduction > 83%), whereas the lowest reductions in germination capacity of hybrid seeds were observed when they originated from *C. erythraea* as the mother plant (reduction < 40%). Overall, the much lower germination capacity of hybrid seeds resulted in barrier strengths varying between 0.152 and 0.792 (Appendix S3). Once successfully germinated survivorship of pure and hybrid F_1 offspring differed significantly between crosses ($\chi^2 = 25.99$; $P = 0.001$), with F_1 hybrids tending to have lower mortality rates than pure F_1 offspring, except for hybrids resulting from the $E^♀P^♂$ crossing. Hybrids resulting from the $P^♀E^♂$ crossings showed the highest survivorship whereas those resulting from the $L^♀E^♂$ crossings had the lowest chance to survive until flowering (Appendix S6). The resulting barrier strengths ranged from -0.011 to 0.685 (Appendix S3). Finally, flower production of F_1 offspring depended significantly on both the maternal and paternal species (Table 1), with hybrids generally showing significantly smaller numbers of flowers produced compared to their pure congeners, except in the $L^♀E^♂$ and $P^♀E^♂$ cross-direction (Appendix S6). Altogether, this resulted in relatively low barrier strengths (average: 0.024) (Appendix S3).

CONTRIBUTION TO TOTAL ISOLATION

Total RI acting between the studied *Centaurium* species was on average 0.918, and was lowest for the species combination $L^♀E^♂$ (RI = 0.788) and highest for the species combination $P^♀L^♂$ (RI = 0.983) (Fig. 4). The relative contribution of each of the individual barriers to total RI varied between -0.507 up to 0.627. The highest contributions were found at the stage of pollinator fidelity and autonomous selfing (Fig. 4; Appendix S3).

Fig. 3. Interspecific differences in the mean (±SE) index of self-defence resulting from autonomous selfing, at three different stages of floral development (i.e. early, mid- and late anthesis) for each of the six species pairs between *Centaurium erythraea*, *C. littorale* and *C. pulchellum*.

Discussion

Our mixed-pollination experiments clearly demonstrated that autonomous selfing, which is generally considered as a strategy to decouple reproduction from the pollination environment and to assure reproductive success under severe pollen limitation, can shelter stigmas from heterospecific pollen deposition. However, the efficiency of protecting stigmas from heterospecific pollen strongly depended on the mode of autonomous selfing, with prior selfing being by far the most efficient mechanism and delayed selfing only offering a limited prevention of hybrid fertilization at the end of a flowers lifetime. The observed high levels of self-protection, especially in the prior selfing *C. pulchellum*, but also in the competing selfing *C. littorale*, can be explained by pre-emptive self-pollination and/or stigma clogging. In contrast to *C. erythraea*, the close positioning of anthers around the stigmatic surface in both selfing species may prevent pollinators to come into direct contact with the stigma, thereby limiting the amount of cross-pollen that can be deposited on the stigma (Webb & Lloyd 1986). Our findings are in accordance with results of Smith & Rausher (2006), who showed that close stigma–anther proximity in *Ipomoea hederacea* also functioned as an important pre-zygotic isolation barrier when this species was exposed to heterospecific pollen flow from *I. purpurea*. In the studied *Centaurium* species, the relative contribution of autonomous selfing to total RI significantly increased from −0.147 in the predominantly outcrossing *C. erythraea*, 0.234 in the mixed-mating *C. littorale* to 0.568 in the predominantly selfing *C. pulchellum*. For the latter species, autonomous selfing was even the most important barrier overall.

Apart from the effect of autonomous selfing to suppress heterospecific mating, differences in pollinator fidelity and pollen production also contributed to RI, confirming the general assumption that early acting pre-zygotic barriers generally contribute more to total isolation than barriers occurring later in a plant's life cycle (Schemske 2000; Ramsey, Bradshaw & Schemske 2003). Although not explicitly studied here, shifts in other floral traits associated with the selfing syndrome, such as reductions in flower size or floral display, can also be expected to affect the probability of interspecific pollen transfer, thereby amplifying the relative importance of the selfing mechanism as a barrier against hybridization. Variable levels of attractiveness between selfing and outcrossing species may cause asymmetries in pollinator mediated pollen flow and can result into higher risks of heterospecific pollen deposition in the more selfing species (Briscoe Runquist *et al.* 2014; Brys *et al.* 2014). Because different pollinators may show differences in pollen uptake and pollen deposition and because these differences may depend on the floral morphology of the plant, it can be assumed that this further affects the probability of hybrid mating in the field (Adler & Irwin 2006; Natalis & Wesselingh 2012). However, this was not considered in the present study, but merits further investigation.

Apart from the strong cumulative impact of pre-zygotic barriers on the probability of interspecific gene flow, we also found substantial post-zygotic costs that were most prevalent for seed production and germination success. These findings confirm previous results reported for other closely related species pairs, although in most cases the relative contribution of post-zygotic barriers to total RI was very small (Ramsey, Bradshaw & Schemske 2003; Kay 2006; Martin & Willis 2007). In theory, a long history of autonomous selfing may increase post-zygotic fitness costs due to higher homozygosity and the resulting fixation of underdominant alleles, or chromosome rearrangements (Charlesworth 1992). Stronger overall reductions of hybrid vigour in more selfing species can thus be expected to occur when reciprocally crossed with more outcrossing, and thus more heterozygous, related sister taxa (Skrede *et al.* 2008). In the studied *Centaurium* species, the strongest reductions in hybrid vigour were found when the most selfing *C. pulchellum* acted as the mother plant, and hybrid vigour was highest when the outcrossing *C. erythraea* functioned as the maternal species. However, increased genetic divergence over evolutionary time may have contributed to the observed post-zygotic costs as well due to a 'clocklike' accumulation of reproductive isolation (Coyne & Orr 2004). Several studies have detected positive correlations between the genetic distance separating taxa and the strength of post-zygotic reproductive isolation acting between them, both in animals (Sasa, Chippindale & Johnson 1998; Presgraves 2002) and in plants (Jewell *et al.* 2011). In case of our *Centaurium* species, *C. pulchellum* differentiated about 14.2 million years ago from the clade where the closely related *C. erythraea* and *C. littorale* belong to (Mansion & Struwe 2004). The latter thus suggests that genetic divergence may also have contributed to the observed patterns of post-zygotic costs.

Much of our knowledge of isolating mechanisms comes from situations where related taxa are currently found growing in sympatry. Under such conditions, it is possible to examine the effectiveness of different barriers in limiting gene flow between related taxa. Nevertheless, several studies have incorporated the importance of geographic isolation as a part of total reproductive isolation (Ramsey, Bradshaw & Schemske 2003; Kay 2006; Sobel 2014). In the case of the studied *Centaurium* species and based on rough estimates of the species total distribution ranges (Appendix S1), barrier strengths due to geographic isolation appeared to be strong (on average 0.503) and highly variable depending on the species combination (ranging between 0.139 and 0.987, data not incorporated). Given that isolating barriers act sequentially and that geographic isolation is the first barrier that limits gene flow and prevents hybridization, this barrier often shows a disproportionate impact on total reproductive isolation (Ramsey, Bradshaw & Schemske 2003). Therefore, studies that examined the contribution of different reproductive barriers often assessed total reproductive isolation with and without the action of geographic isolation (Ramsey, Bradshaw & Schemske 2003; Kay 2006). Moreover, current patterns of geographic isolation may represent a holdover from historical allopatric distributions, making it in some cases disputable to incorporate it as an ecological isolating mechanism (Kay 2006). Because the aim of this study was to examine the
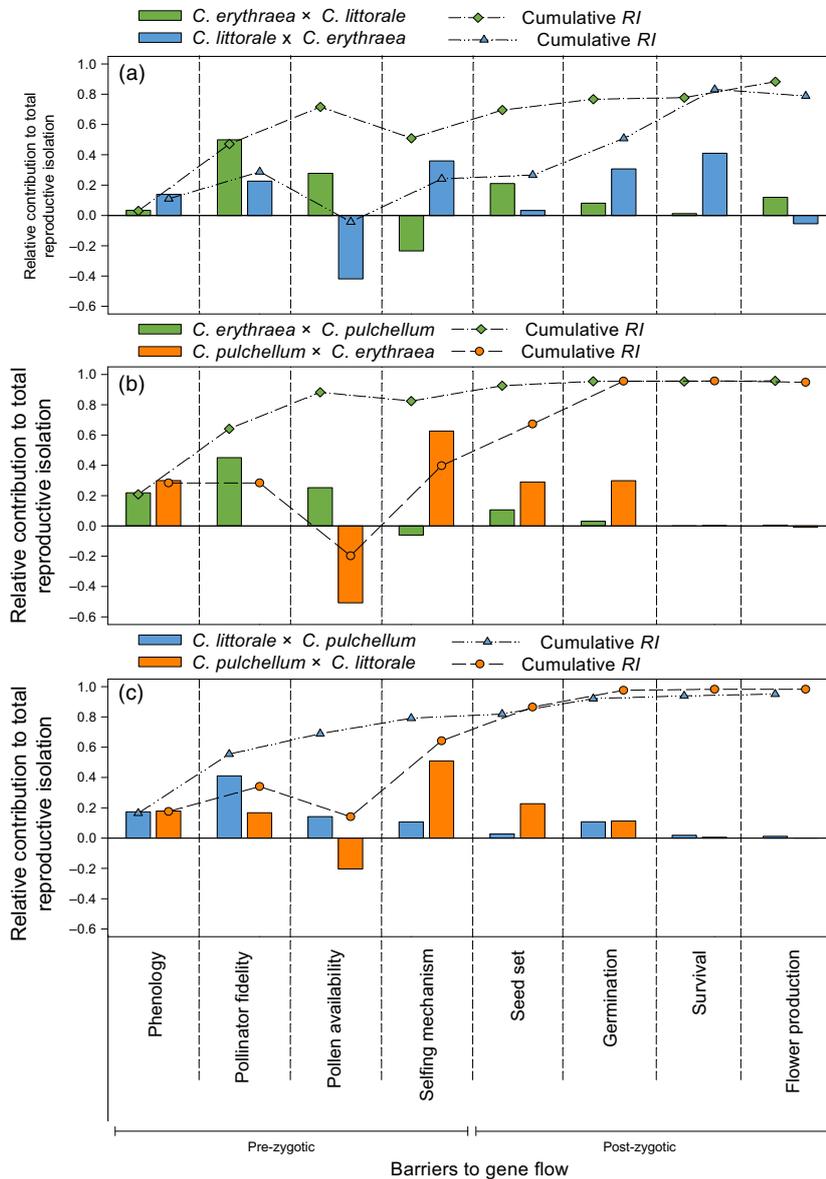
Fig. 4. Relative contributions of eight sympatric barriers to total isolation in reciprocal crosses between each of the three species pairs: a) *Centaurium erythraea - C. littorale*, b) *C. erythraea - C. pulchellum* and c) *C. littorale - C. pulchellum* following the method described in Sobel & Chen (2014). The line graph represents the cumulative contribution to RI of a mechanism after accounting for each of the investigated previous mechanisms.

impact of autonomous selfing as a mechanism to suppress hybridization and due to the overwhelming impact of geographic isolation on later acting barriers, the rather rough estimates of distribution ranges obtained here were not used in calculations of total reproductive isolation, but it is clear that geographic isolation will further increase reproductive isolation in the studied species, leading to estimates of total reproductive isolation that approach one.

Previous studies have indicated that hybridization between *C. erythraea* and *C. littorale* and between *C. erythraea* and *C. pulchellum* has been repeatedly observed at several locations in Britain and on the European mainland, but hybridization between *C. littorale* and *C. pulchellum* appears to be much rarer (Melderis 1931; Zeltner 1970; Moore & Ubsdell 1975; Ubsdell 1976; Mansion & Struwe 2004; Mansion, Zeltner & Bretagnolle 2005; Brys *et al.* 2014; Stace, Preston & Pearman 2015). When hybridization occurs, but is costly, for instance, due to reduced hybrid viability or fertility fol-

lowing hybridization (Levin & Kerster 1967; Whalen 1978), selection for enhanced pre-mating isolation may occur (Dobzhansky 1937; Smith & Rausher 2008; Hopkins 2013). In our study system, however, it is likely that the evolution to selfing is for a large part the result of reproductive assurance under pollen-limited conditions (see for instance Brys & Jacquemyn 2011, 2012). Nonetheless, given that post-zygotic fitness costs were high, it is not unlikely that reinforcement has further contributed to the observed differences in mating system in these species. Preliminary research has shown significant differences in floral morphology between allopatric and sympatric populations of *C. erythraea* and *C. littorale* over a large geographic range (D. Schouppe, R. Brys & H. Jacquemyn, unpubl. results). Moreover, floral traits appear to be very plastic and a reversal in floral morphology has been observed between plants from the Continent and the UK. To unequivocally show that post-mating hybrid costs have reinforced the evolution to selfing, future studies should therefore

investigate whether the observed differences in floral morphology between allopatric and sympatric populations is simply a by-product of adaptation to pollination environments that differ between the allopatric and sympatric portions of the subspecies' range or is the result of interactions with congenerics in sympatry. Whatever the driving force(s) is responsible for the observed differentiation in floral morphology and mating system, our study clearly showed that autonomous selfing is an effective mechanism to avoid hybrid fertilization and that can be subject to selection under sympatric conditions due to high post-zygotic costs.

Acknowledgements

Data accessibility

All data used in this manuscript are available in figures and tables within the article or in the supporting information.

References

Adler, L.S. & Irwin, R.E. (2006) Comparison of pollen transfer dynamics by multiple floral visitors: experiments with pollen and fluorescent dye. *Annals of Botany*, **97**, 141–150.

Aldridge, G. & Campbell, D.R. (2007) Variation in pollinator preference between two *Ipomopsis* contact sites that differ in hybridization rate. *Evolution*, **61**, 99–110.

Briscoe Runquist, R.D., Justin, E.C., Iverson, L., Kopp, J.C. & Moeller, D.A. (2014) Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution*, **68**, 2885–2900.

Brys, R. & Jacquemyn, H. (2011) Variation in the functioning of autonomous self-pollination, pollinator services and floral traits in three *Centaurium* species. *Annals of Botany*, **107**, 917–925.

Brys, R. & Jacquemyn, H. (2012) Effects of human-mediated pollinator impoverishment on floral traits and mating patterns in a short-lived herb: an experimental approach. *Functional Ecology*, **26**, 189–197.

Brys, R., Vanden Broeck, A., Mergeay, J. & Jacquemyn, H. (2014) The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. *Evolution*, **68**, 1281–1293.

Charlesworth, B. (1992) Evolutionary rates in partially self-fertilizing species. *The American Naturalist*, **140**, 126–148.

Coyne, J.A. & Orr, H.A. (2004) *Speciation*. Sinauer Associations, Sunderland, MA.

Dell'Olivo, A., Hoballah, M.E., Gübitz, T. & Kuhlemeier, C. (2011) Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution*, **65**, 1979–1991.

Dobzhansky, T. (1937) *Genetics and the Origin of Species*. Columbia University Press, New York, NY, USA.

Fishman, L. & Wyatt, R. (1999) Pollinator-mediated competition, reproductive character displacement, and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution*, **53**, 1723–1733.

Goldberg, E.E. & Igic, B. (2012) Tempo and mode in plant breeding system evolution. *Evolution*, **66**, 3701–3719.

Grossenbacher, D.L. & Whittall, J.B. (2011) Increased floral divergence in sympatric monkeyflowers. *Evolution*, **65**, 2712–2718.

Hodges, S.A. & Arnold, M.L. (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 2493–2496.

Hopkins, R. (2013) Reinforcement in plants. *New Phytologist*, **197**, 1095–1103.

Hultén, E. & Fries, M. (1986) *Atlas of North European Vascular Plants North of the Tropic of Cancer*. Koeltz Scientific Books, Königstein, Germany.

Jewell, C., Papineau, A.D., Freyre, R. & Moyle, L.C. (2011) Patterns of reproductive isolation in *Nolana* (Chilean bellflower). *Evolution*, **66**, 2628–2636.

Kalisz, S., Vogler, D., Fails, B., Finer, M., Shepard, E., Herman, T. & Gonzales, R. (1999) The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). *American Journal of Botany*, **86**, 1239–1247.

Kay, K.M. (2006) Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution*, **60**, 538–552.

Kay, K.M. & Schemske, D.W. (2003) Pollinator assemblages and visitation rates for 11 species of neotropical *Costus* (Costaceae). *Biotropica*, **35**, 198–207.

Leclerc-Potvin, C. & Ritland, K. (1994) Modes of self-fertilization in *Mimulus guttatus* (Scrophulariaceae). A field experiment. *American Journal of Botany*, **81**, 199–205.

Levin, D.A. & Kerster, H.W. (1967) Natural selection for reproductive isolation in *Phlox*. *Evolution*, **21**, 679–687.

Lloyd, D.G. (1979) Some reproductive factors affecting the selection of self-fertilization in plants. *The American Naturalist*, **113**, 67–79.

Lloyd, D.G. & Schoen, D.J. (1992) Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences*, **153**, 358–369.

Lowry, D., Modliszewski, J., Wright, H., Wu, C. & Willis, J. (2008) The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **363**, 3009–3021.

Mansion, G. & Struwe, L. (2004) Generic delimitation and phylogenetic relationships within the subtribe *Chironiinae* (Chironieae: Gentianaceae) with special reference to *Centaurium*: evidence from nrDNA and cpDNA sequences. *Molecular Phylogenetics and Evolution*, **32**, 951–977.

Mansion, G., Zeltner, L. & Bretagnolle, F. (2005) Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae–Chironieae). *Taxon*, **54**, 931–950.

Martin, N.H. & Willis, J.H. (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**, 68–82.

Mayr, E. (1942) *Systematics and the Origin of Species*. Columbia University Press, New York, NY, USA.

Melderis, A. (1931) Genetical and taxonomical studies in the genus *Erythraea* Rich. *Acta Horti Botanici Universitatis Latviensis*, **6**, 123–156.

Moore, D.M. & Ubsdell, R. (1975) *Centaurium* Hill. *Hybridisation and the Flora of the British Isles* (ed. C.A. Stace), pp. 349–351. Academic Press, London.

Natalis, L.C. & Wesselingh, R.A. (2012) Shared pollinators and pollen transfer dynamics in two hybridizing species *Rhinanthus minor* and *R. angustifolius*. *Oecologia*, **170**, 709–721.

Presgraves, D.C. (2002) Patterns of postzygotic isolation in Lepidoptera. *Evolution*, **56**, 1168–1183.

Ramsey, J., Bradshaw, H.D. & Schemske, D.W. (2003) Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution*, **57**, 1520–1534.

Rieseberg, L.H. & Willis, J.H. (2007) Plant speciation. *Science*, **317**, 910–914.

Sasa, M.M., Chippindale, P.T.T. & Johnson, N.A. (1998) Patterns of postzygotic isolation in frogs. *Evolution*, **52**, 1811–1820.

Schat, H. & Scholten, M. (1985) Comparative population ecology of dune slack species: the relation between population stability and germination behaviour in brackish environments. *Vegetatio*, **61**, 189–195.

Schemske, D.W. (2000) Understanding the origin of species. *Evolution*, **54**, 1069–1073.

Schemske, D.W. & Bradshaw, H.D. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11910–11915.

Schluter, D. (2001) Ecology and origin of species. *Trends in Ecology and Evolution*, **16**, 372–380.

Skrede, I., Brochmann, C., Borgen, L. & Rieseberg, L.H. (2008) Genetics of intrinsic postzygotic isolation in a circumpolar plant species, *Draba nivalis* (Brassicaceae). *Evolution*, **62**, 1840–1851.

Smith, R.A. & Rausher, M.D. (2006) Close clustering of anthers and stigma in *Ipomoea hederacea* enhances prezygotic isolation from *Ipomoea purpurea*. *New Phytologist*, **173**, 641–647.

Smith, R.A. & Rausher, M.D. (2008) Experimental evidence that selection favors character displacement in the ivyleaf morning glory. *The American Naturalist*, **171**, 1–9.

Sobel, J.M. (2014) Ecogeographic isolation and speciation in the genus *Mimulus*. *The American Naturalist*, **184**, 565–579.

Sobel, J.M. & Chen, G.F. (2014) Unification of methods for estimating the strength of reproductive isolation. *Evolution*, **68**, 1511–1522.

Stace, C.A., Preston, C.D. & Pearman, D.A. (2015) *Hybrid Flora of the British Isles*. Botanical Society of Britain & Ireland, Bristol, UK.

Ubsdell, R.A.E. (1976) Studies on variation and evolution in Centaurium erythraea Rafn and C. littorale (D. Turner) Gilmour in the British Isles. *Watsonia*, **11**, 33–43.

van Tooren, B.F., Schat, H. & ter Borg, S.J. (1983) Succession and fluctuation in the vegetation of a Dutch beach plain. *Vegetatio*, **53**, 139–151.

Webb, C.J. & Lloyd, D.G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany*, **24**, 163–178.

Whalen, M.D. (1978) Reproductive character displacement and floral diversity in *Solanum* section *Androceras*. *Systematic Botany*, **3**, 77–86.

Whitehead, M.R. & Peakall, R. (2014) Pollinator specificity drives strong pre-pollination reproductive isolation in sympatric deceptive orchids. *Evolution*, **68**, 1561–1575.

Widmer, A., Lexer, C. & Cozzolino, S. (2009) Evolution of reproductive isolation in plants. *Heredity*, **102**, 31–38.

Wright, S.I., Kalisz, S. & Slotte, T. (2013) Evolutionary consequences of self-fertilization in plants. *Proceedings of the Royal Society of London B: Biological Sciences*, **280**, 20130133.

Xu, S.Q., Schluter, P.M., Scopece, G., Breitkopf, H., Gross, K., Cozzolino, S. & Schiestl, F.P. (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution*, **65**, 2606–2620.

Zeltner, L. (1970) Recherches de biosystématique sur les genres *Blackstonia* Huds. et *Centaurium* Hill (Gentianacées). *Bulletin de la Société des science naturelles de Neuchâtel*, **93**, 1–164.

Supporting Information

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

Appendix S1. Distribution areas of *Centaurium erythraea*, *C. littorale* and *C. pulchellum* adapted from Hultén & Fries (1986).

Appendix S2. Typical examples of rosette leaves of the three parental sister species (*C. erythraea*, *C. littorale* and *C. pulchellum*) and their resulting hybrids obtained from controlled crosses.

Appendix S3. Measures of barrier strength and contributions to total RI calculated for eight sequential reproductive barriers.

Appendix S4. Frequencies of all floral visitors observed on *Centaurium erythraea*, *C. littorale* and *C. pulchellum* within the sympatric study populations and recorded during 36 intervals of 20-min observation time within 2×2 m plots during flowering peak.

Appendix S5. Results of a generalized linear mixed model (GLMM) for the effect of pollination treatment (mixed pollination on intact vs. emasculated flowers), timing during floral development (early, mid and late pollination), and the interaction between both factors on the proportion of hybrid seed produced following supplemental pollination with 50:50 mixed pollen loads on each of the six species pairs.

Appendix S6. Means ($\pm$SE) of the four fitness components measured: proportional seed set, seed germination, survivorship and flower production following intra- and interspecific crosses between E = *C. erythraea*, L = *C. littorale*, and P = *C. pulchellum* ($n = 10$ per cross type).