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Lineages of *Silene nutans* developed rapid, strong, asymmetric postzygotic reproductive isolation in allopatry

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Reproductive isolation can rise either as a consequence of genomic divergence in allopatry or as a byproduct of divergent selection in parapatry. To determine whether reproductive isolation in gynodioecious *Silene nutans* results from allopatric divergence or from ecological adaptation following secondary contact, we investigated the pattern of postzygotic reproductive isolation and hybridization in natural populations using two phylogeographic lineages, western (W1) and eastern (E1). Experimental crosses between the lineages identified strong, asymmetric postzygotic isolation between the W1 and the E1 lineages, independent of geographic overlap. The proportion of ovules fertilized, seeds aborted, and seeds germinated revealed relatively little effect on the fitness of hybrids. In contrast, hybrid mortality was high and asymmetric: while half of the hybrid seedlings with western lineage mothers died, nearly all hybrid seedlings with E1 mothers died. This asymmetric mortality mirrored the proportion of chlorotic seedlings, and is congruent with cytonuclear incompatibility. We found no evidence of hybridization between the lineages in regions of co-occurrence using nuclear and plastid markers. Together, our results are consistent with the hypothesis that strong postzygotic reproductive isolation involving cytonuclear incompatibilities arose in allopatry. We argue that the dynamics of cytonuclear gynodioecy could facilitate the evolution of reproductive isolation.

KEY WORDS: **Asymmetric reproductive isolation, chlorosis, cytonuclear incompatibility, microsatellites, postzygotic reproductive isolation.**

Reproductive isolation is a key element in speciation: by reducing gene flow, it delimits species (Coyne and Orr 2004). Nevertheless, reproductive isolation may be incomplete, allowing gene flow even between well-defined species (Jacquemyn et al. 2012). In plants, hybridization between groups is prevented by multiple barriers that are often classified as being prezygotic or postzygotic (see reviews by Rieseberg and Willis 2007; Baack et al. 2015). Prezygotic barriers to reproduction can occur prior to mating, as a result of pollinator specialization or nonoverlapping flowering times. It can also occur post-pollination, such as when incompatibility between pollen and stigmas prevents fertilization. Postzygotic barriers are sometimes evident soon after fertilization (e.g., embryo/seed abortion), but can act much later as well, leading to the production

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of hybrid progeny that suffer from reduced viability and/or fertility.

Although prezygotic isolating barriers are generally thought to be stronger than postzygotic barriers in plants (Lowry et al. 2008a), this will depend on whether lineages are found in allopatry or in sympatry (Coyne and Orr 2004). Under the scenario of allopatric speciation (geographic isolation), postzygotic barriers are expected to occur as a result of the accumulation of genetic incompatibilities over time (Coyne and Orr 2004). Secondary contact between differentiated lineages can then reinforce the reproductive isolation process through premating isolating barriers (Noor 1999; Hopkins 2013).

Because intrinsic genetic incompatibilities accumulate via selective or neutral processes over time (Presgraves 2010; Lynch and Hagner 2015), postzygotic reproductive isolation is expected to increase with increasing genetic distance among taxa (Orr 1995; Moyle et al. 2004; Scopece et al. 2008). Genetic incompatibilities are thought to involve epistasis between two or more loci following the Bateson-Dobzhansky-Muller (BDM) model (Fraïsse et al. 2014) or a pathway-based model (Lindtke and Buerkle 2015). When genetic incompatibilities involve an interaction between the nuclear and uniparentally inherited cytoplasmic genomes (termed "cytonuclear"), the reproductive isolation is expected to be asymmetric between reciprocal crosses (Turelli and Moyle 2007).

Genetic incompatibilities are generally not expected to be involved in the process of adaptation. But in the framework of ecotypic differentiation or ecological speciation, the selection of adaptive alleles could directly (via pleiotropy) or indirectly (through linkage disequilibrium) drive the emergence of such incompatibilities (Wright et al. 2013). When multiple loci are under divergent selection, gene flow is reduced across the genome, allowing the rise of genetic incompatibilities, a stage called genome hitchhiking in the speciation-with-gene-flow model (Feder et al. 2012). Thus, postzygotic barriers can rise either as a consequence of genomic divergence in allopatry or as a byproduct of divergent selection in parapatry.

In the article we aim to identify the evolutionary mechanisms responsible for reproductive isolation by comparing divergent populations in allopatry and in parapatry (ecological divergence). For this purpose, we investigated intrinsic postzygotic barriers between phylogeographic lineages of the perennial herb *Silene nutans*. Discrete genetic lineages of this species have previously been identified based on nuclear microsatellite and plastid markers, in relation to past climatic events and postglacial migration history (Martin et al. 2016; Van Rossum et al. 2016; Van Rossum unpublished results). Lineages can be divided into two main genetically distant western and eastern groups. The western lineage, mainly located in western Europe, consists of several genetically differentiated sublineages. The most widespread sublineage (W1) occurs in England, France, and Belgium, whereas the most widespread eastern lineage (E1) primarily occurs in Eastern Europe, but has spread from the east, northward, and westward up into western Europe (Fig. 1). The W1 and E1 lineages form secondary contact zones in southern England, southern Belgium, and eastern France. In southern Belgium, they grow in close parapatry as separate ecotypes, as a result of edaphic evolution to different soils (De Bilde 1973; Van Rossum et al. 1999). Previous studies have shown that crosses between these two ecotypes from Belgium result in nearly complete postzygotic reproductive isolation (PRI), expressed as seedling chlorosis (Fig. S1) and reduced hybrid fitness (Van Rossum et al. 1996). In southern England and eastern France there is no evidence of ecotypic differentiation in relation to soil type (Van Rossum, unpublished results).

Given the PRI between ecotypes in Belgium, we investigated whether PRI between these lineages exists on a broader scale or is localized, in other words, linked to edaphic differentiation. Two possibilities exist. First, any PRI that occurs between the western and eastern lineages does so because the lineages diverged in allopatry prior to the spread of the eastern lineage. Second, the PRI occurs because of local differentiation to edaphic conditions following secondary contact, in a speciation-with-gene-flow process (Smadja and Butlin 2011), and should thus be restricted to populations located in southern Belgium. We carried out a crossing experiment both within and between regions to investigate whether PRI results from allopatric versus local processes. Under the first scenario, we predict (1a) that PRI between the lineages will occur within the three studied regions, and (1b) similar degrees of PRI between the lineages regardless of whether the cross was within or between regions, as a consequence of the accumulation of genetic incompatibilities since the separation of the western and eastern lineages. Under the second scenario, (2a) the PRI between the lineages should vary depending on the region, with a higher PRI in Belgium if it is linked to edaphic adaptation, and (2b) as PRI is mainly a consequence of local divergence, within-region crosses should exhibit stronger PRI than between-region crosses.

PRI was estimated from hybrid progeny, ranging from the proportion of ovules fertilized to the mortality of five-week old seedlings, and the level of chlorosis. In parallel, we investigated the occurrence of hybrids between lineages in regions where they co-occur using population genetic analysis of nuclear and plastid markers to estimate net reproductive isolation—that is the overall effect of prezygotic and postzygotic barriers, between lineages in nature.

Material and methods

STUDY SPECIES AND SAMPLED POPULATIONS

Silene nutans L. (Caryophyllaceae) is a long-lived perennial plant species, occurring in dry habitats, on rock outcrops, sand, or shingle, with a continental distribution, extending from western and

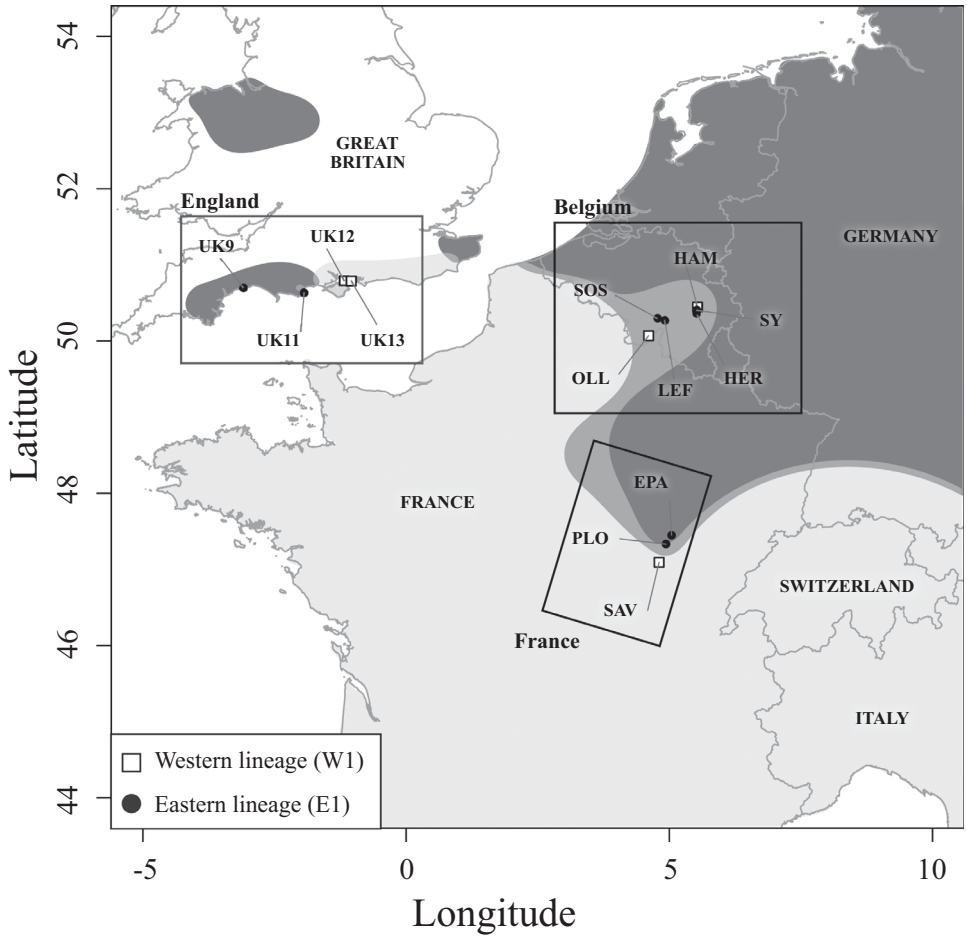

Figure 1. Geographic location of the 13 study populations in our crossing experiment from three regions—southern England, eastern France, and Belgium. Light and dark gray zones refer to the distribution area of the Western lineages and of the Eastern lineages, respectively, and the intermediate gray zone to the range overlap between Western and Eastern lineages.

northern Europe to central Siberia and the Southern Caucasus (Hepper 1956; Fitter 1978). Cytonuclear gynodioecy occurs in natural populations leading to the coexistence of hermaphrodites and females (Dufay et al. 2010; Garraud et al. 2011); in this study only hermaphrodite plants were used for the crosses. Flowers open at dusk and are protandrous, with one whorl of anthers dehiscing on the first evening of opening, followed by a second whorl the following evening. Stigmas become receptive on the third evening, and remain receptive for about two days (Hepper 1956). Several flowers are often simultaneously open on a plant, allowing geitonogamous self-fertilization that results in inbreeding depression (Hauser and Siegismund 2000; Dufay et al. 2010). Flowering occurs from mid-April to mid-July, with considerable among-population variation in the timing (De Bilde 1973; Hauser and Weidema 2000). *Silene nutans* is insect-pollinated, mainly by nocturnal moths, and by long-tongued diurnal bees (Jürgens et al. 1996).

Based on nuclear and plastid sequences, *S. nutans* is split into genetically differentiated lineages in Europe, some of which co-occur in southern England, eastern France, and southern Belgium (Martin et al. 2016; Van Rossum et al. 2016; Van Rossum et al. unpublished results). As described above, the western lineage is composed of three sublineages, including the W1 lineage (found in southern England, France, and Belgium). Several lineages occur in Eastern Europe, but only the E1 lineage is found in the regions of focus (Martin et al. 2016; Van Rossum et al., unpublished results). Living rosettes were collected from 130 individuals growing in 13 natural populations in three regions containing multiple lineages (Fig. 1, Table S1), and grown to flowering in a greenhouse for controlled crosses.

CROSSING EXPERIMENT: DESIGN

To detect whether PRI between W1 and E1 lineages was restricted to Belgium, as an outcome of edaphic adaptation, or whether it occurred in other regions, as a result of allopatric processes, we conducted four types of reciprocal crosses within lineages (W1 × W1 and E1 × E1) and between lineages (W1 × E1 and E1 × W1, with maternal lineage listed first for each cross type)

within three different regions: England, eastern France, and southern Belgium.

In addition, to detect whether a regional effect on the degree of PRI existed, these four cross types were also performed between regions (for instance, an E1 Belgian dam crossed with an E1 British sire, see Table S2). If PRI followed secondary contact, mainly as a consequence of local evolutionary processes, between-lineage crosses should exhibit stronger PRI when crosses are performed within regions than between regions. As flowering periods in England and eastern France did not overlap, between-region crosses were only possible with individuals from Belgium. Depending on material availability, not all combinations between regions were possible.

CROSSING EXPERIMENT: PROCEDURES AND FITNESS MEASUREMENTS

Pollinations for crosses were performed from mid-April to June 2011 on a total of 550 flowers. These flowers were emasculated just before opening (prior to dehiscence of any stamens) and bagged to avoid pollen contamination. Once stigmas were receptive (three days after emasculation), they were hand-pollinated early in the morning on two consecutive days using freshly dehiscent anthers from three pollen donors and rebagged.

A total of 134 mature fruits were collected just before dehiscence, when seeds were ripe (about one month after pollination). Some pollinated flowers did not develop into a fruit. This can be caused by prezygotic reproductive barriers (Van Rossum et al. 1996), but can also be caused by ovary damage during emasculation. The number of unfertilized ovules, aborted seeds, and viable seeds were counted and summed to estimate the total number of ovules in each fruit. The proportion of ovules fertilized per fruit was calculated as the sum of aborted and viable seeds over the total number of ovules, while the proportion of seeds aborted per fruit was calculated as the number of aborted seeds over the sum of aborted and viable seeds.

In March 2012, up to 50 seeds per fruit (for a total of 3596 seeds) were germinated in two Petri dishes on moist filter paper (Whatman) in controlled conditions (20°C, 16 hour day length) for a total of 134 fruits. The proportion of seeds that germinated after two weeks was calculated. The fitness of each seedling was characterized by its chlorosis level according to its color (determined by eye, with a simultaneous comparison of all samples, by FVR and HM, on a total of 3434 seedlings): yellow-white (chlorotic), light green (partially chlorotic), and green (healthy, nonchlorotic) (Fig. S1). Two to 15 (mostly 11) randomly selected seedlings per fruit were transplanted individually into pots containing a soil mixture (3/4 compost and 1/4 perlite) and their growth was followed for five weeks for a total of 1245 seedlings. Each week, the diameter of the rosette at the widest point was measured, and the number of leaves was counted. The proportion of individuals that died within each family (i.e., from a single fruit) was recorded one week after transplantation and at the end of the five-week period.

CROSSING EXPERIMENT: STATISTICAL ANALYSES

The proportion variables (i.e., ovules fertilized, aborted seeds, germination, and mortality) were analysed using a logistic regression analysis (binomial distribution and log link function) using lme4 R package (Bates et al. 2015). For seedling chlorosis, a Chi-square test was performed. Because most of the interlineage hybrid progeny died (Figs. 2 and 3), we were not able to test for an effect of cross type on vegetative growth (number of leaves and rosette diameter). All statistical analyses were performed in the R environment (R Core Team Development 2014). Multiple comparisons of means using pairwise post-hoc Tukey contrast tests were performed when a significant effect was found.

To test for PRI between the W1 and E1 lineages, we analyzed the dataset restricted to crosses performed within regions. The models tested the effect of *Cross type* within regions (W1 × W1, E1 × E1, W1 × E1, and E1 × W1), the effect of *Region* (Belgium vs England + eastern France) and their interaction as fixed effect. Data for England and France were pooled because of a low number of fruits obtained from between-lineage crosses. *Maternal population* nested within *Region* was tested as a random effect. If PRI between lineages only occurs as an outcome of edaphic adaptation in Belgium, we should detect either no PRI between W1 and E1 lineages in England and eastern France or a higher PRI in between-lineage crosses (W1 × E1, E1 × W1) in Belgium than outside Belgium, and so a significant effect of the interaction between *Cross type* and *Region*. For seedling chlorosis, a Chi-square test was performed, first, to test for differences in the levels of seedling chlorosis (healthy, partially chlorotic, or fully chlorotic), (1) among *Cross types*, and (2) between Belgian and England + eastern France inter-lineage crosses, with W1 × E1 and E1 × W1 crosses tested separately.

To test for a regional effect on the degree of PRI by comparing crosses performed within and between regions, we analyzed the complete dataset, but considering different factors compared to previous analyses. The models tested the effect of *Cross type* with the same four levels previously described, the effect of *Cross location* (within regions vs between regions), and their interaction as fixed effects. *Maternal regions* and *Maternal population* nested within *Maternal region* were tested as a random effect. If PRI between lineages occurs at a local scale, we should detect a significant effect of the interaction between *Cross type* and *Cross location* due to a higher PRI in between-lineages crosses (W1 × E1, E1 × W1) within regions than between regions. For seedling chlorosis, a Chi-square test was performed, first, to test for differences in the levels of seedling chlorosis (healthy, partially chlorotic, or fully chlorotic), (1) among *Cross types*, and (2)

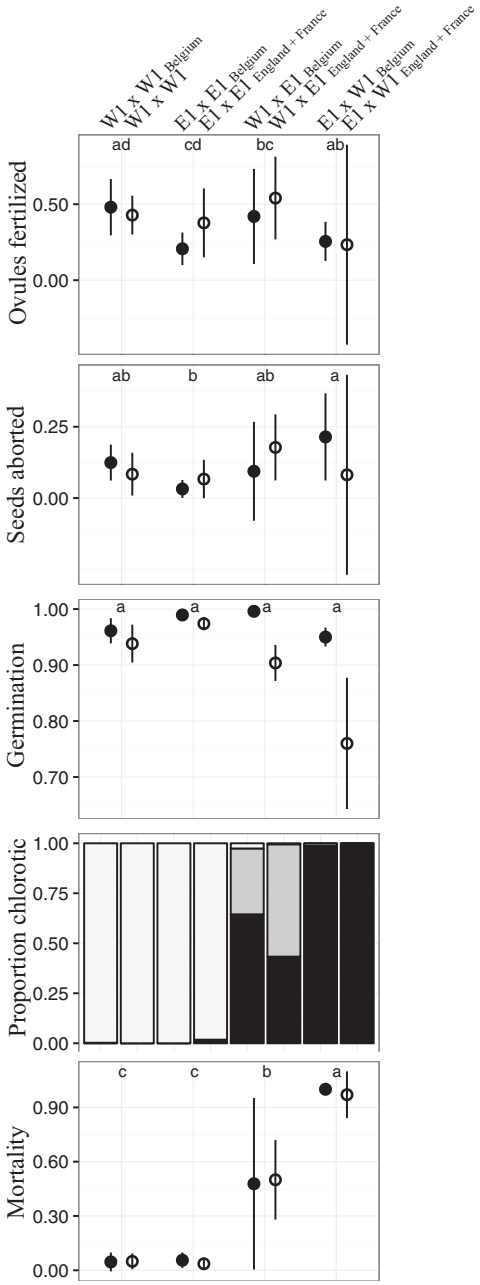

Figure 2. Fitness measurements of the progeny from crosses between the W1 and E1 lineages within regions for Belgium and England + eastern France. For each cross type, the mean of the proportion of ovules fertilized, proportion of seeds aborted, germination (in proportion), proportion of one-week old seedlings that were chlorotic and the degree of their chlorosis (light bars—no chlorosis, gray bars—partial chlorosis, dark bars—complete chlorosis, Fig. S1) and mortality (in proportion) one week following transplantation of within- and between-lineage crosses (dam × sire) are plotted. The 95% CI are plotted for the proportion of ovules fertilized, proportion of seeds aborted, germination (in proportion), and mortality (in proportion). Small letters a, b, c, and d show the results of post-hoc Tukey tests on pairwise comparison between *Cross types* (when significant). Different letters indicate a significant difference ($P < 0.05$).

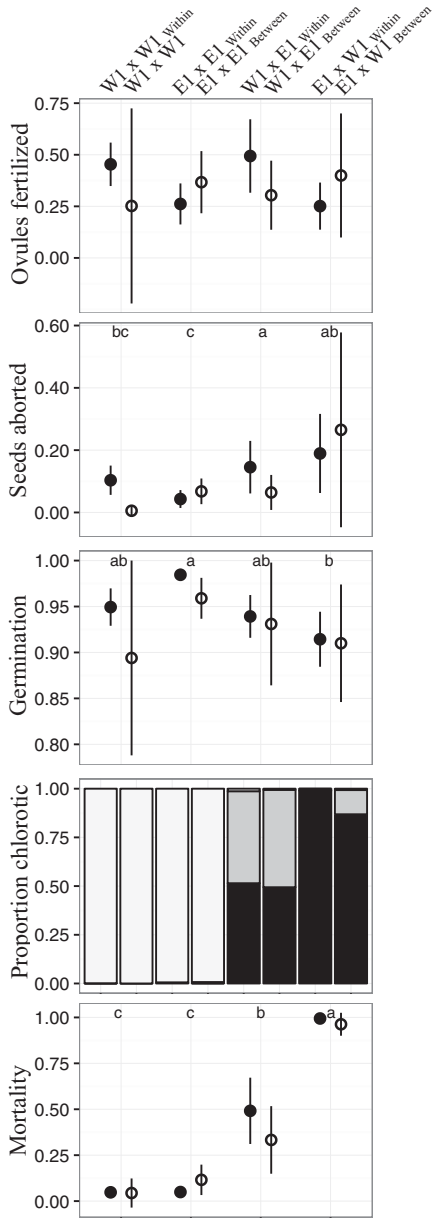

Figure 3. Fitness measurements of the progeny from crosses between the W1 and E1 lineages within three regions (England, France, and Belgium) and between regions (Belgium crossed with England or eastern France). For each cross type, the mean of the proportion of ovules fertilized, proportion of seeds aborted, germination (in proportion), proportion of one-week old seedlings that were chlorotic and the degree of their chlorosis (light bars—no chlorosis, gray bars—partial chlorosis, dark bars—complete chlorosis, Fig. S1) and mortality (in proportion) one week following transplantation of within- and between-lineage crosses (dam × sire) are plotted. The 95% CI are plotted for the proportion of ovules fertilized, proportion of seeds aborted, germination (in proportion) and mortality (in proportion). $_{\text{Within}}$ = within-region crosses, $_{\text{Between}}$ = between-region crosses. Small letters a, b, c, and d show the results of post-hoc Tukey tests on pairwise comparison between *Cross types* (when significant). Different letters indicate a significant difference ($P < 0.05$).

between within- and between-region interlineage crosses, with W1 × E1 and E1 × W1 crosses tested separately.

Finally, following Lowry et al. (2008a,b) we determined the strength of the F1 survival barrier as $RI_{postzygotic} = 1 - [(1—mean\ interlineage\ progeny\ mortality)/(1—mean\ intralineage\ progeny\ mortality)]$ for each reciprocal cross, using the proportion of seeds that had suffered mortality when measured five weeks after transplantation. The degree of asymmetry of PRI was calculated as the absolute value of the difference for the strength of the $RI_{postzygotic}$ value between reciprocal crosses.

MOLECULAR MARKERS

DNA was extracted from 15–20 mg of dried leaf tissue using Macherey-Nagel (Düren, Germany) NucleoSpin® 96 Plant II kits following the standard protocol outlined in the manufacturer's handbook. An individual's assignment to W1 or E1 plastid groups was based on a combination of six plastid SNPs developed from the intergenic spacer sequences *psbA-trnH* and the *matK* gene fragment of *S. nutans* representative samples (Lahiani et al. 2013) and described in Martin et al. (2016). To investigate the possibility of hybridization events in the regions of focus, we used a set of 24 nuclear microsatellites (Table S3). Amplification procedures, multiplexing, and genotyping were carried out following the standard protocols described in Godé et al. (2014) and Martin et al. (2016).

The populations of England, eastern France, and four Belgian populations (HAM, LEFF, OLL, and HER) were previously genotyped using six plastid SNPs and 13 nuclear microsatellites (B09, E08, G01, H07, D10, Sil16, Sil19, Sil24, Sil31, Sil35, Sil36, Sil37, and Sil42) in Martin et al. (2016). For this study, we genotyped 11 additional microsatellites (Sil01, Sil03, Sil05, Sil08, Sil15, Sil18, Sil26, Sil27, Sil29, Sil30, Sil41) to increase the accuracy of the genetic analyses. Two additional Belgian populations (SOS and SY, with $n = 20$ and 16 individuals, respectively) were sampled and genotyped using six plastid SNPs and the 24 nuclear microsatellites.

POPULATION GENETIC VARIATION AND HYBRID IDENTIFICATION

The following measures of genetic variation based on 24 nuclear microsatellite loci were calculated within and over all populations: observed heterozygosity (H_O) and expected heterozygosity corrected for sample size (H_E) using GENECLASS2 (Piry et al. 2004), and the mean number of alleles (*An*) and allelic richness (*Ar*) based on a minimum sample size of eight individuals (El Mousadik and Petit 1996) using FSTAT version 2.9.3.2 (Goudet 1995). For each population, we estimated selfing rate (*s*) derived from the multilocus correlation structure of population samples, following a maximum likelihood based on the observed distribution of multilocus heterozygosity, described in David et al. (2007) and implemented in SPAGeDI version 1.5 (Hardy and Vekemans 2002). Using GENEPOP version 4.3 (Rousset 2008), deviations from Hardy–Weinberg equilibrium were also tested by estimating the intrapopulation fixation index (F_{IS}) following Weir and Cockerham (1984). Significance of F_{IS} estimates was tested using exact probability tests across loci and populations. Markov chain method provided unbiased estimates of the Fisher's exact test probability using the following parameters: 100,000 dememorizations, 1000 batches, and 100,000 iterations per batch. Over all populations, mean fixation indices (F_{IS}, F_{IT}, F_{ST}, following Weir and Cockerham 1984) were estimated and their significance was tested with 20,000 permutations using SPAGeDI.

To infer population structure and hybrid detection within each region, a nonspatially explicit Bayesian clustering was carried out using STRUCTURE (Pritchard et al. 2000; Vähä and Primmer 2006). The number of potential *K* clusters was assessed from 10 different runs of *K* ranging from 1 to 8. To ascertain adequate convergence of the Markov Chain Monte Carlo (MCMC) model, we allowed a burn-in of 100,000 iterations, followed by 2×10^6 MCMC replications without any prior geographic information on the putative affiliation of individuals. To identify the most likely number of *K* clusters, the ad hoc ΔK was calculated as described in Evanno et al. (2005). CLUMP version 1.1.2 (Jakobsson and Rosenberg 2007) was used to find the optimal alignment of independent runs by averaging the top runs. The resulting most likely grouping of populations was plotted using DISTRUCT version 1.1 (Rosenberg 2004). However, the assumption of panmixia implied in such Bayesian clustering does not necessarily hold in *S. nutans*, which can exhibit a mixed-mating system and female individuals (Lahiani et al. 2015). To avoid this potential bias, we performed a Principal Component Analysis (PCA) using *adegenet* R package (Jombart 2008; R Core Team Development 2014), which does not require any genetic assumptions, within each region.

Results

PRI BETWEEN EASTERN AND WESTERN LINEAGES IS ASYMMETRIC AND NOT RESTRICTED TO BELGIUM

Although a significant effect of the interaction between *Cross type* and *Region* was found for all variables except for mortality (Table 1A), the results were overall not consistent with the hypothesis of PRI restricted to Belgium. Regarding the proportions of ovules fertilized and the proportion of seeds aborted, the significant interaction effect was not due to a lower proportion of ovules fertilized or a higher proportion of seeds aborted in between-lineage crosses in Belgium compared to England and eastern France (Fig. 2). Moreover, although a significant effect of *Cross type* was detected for these two variables (Table 1A), hybrid crosses did not clearly suffer from a lower proportion

Table 1. Results of logistic regression analyses.

A—Crosses between the W1 and E1 lineages within regions, comparing Belgium vs England + eastern France

	Cross type			*Region*			*Cross type* × *Region*			*Maternal population* (nested in *Region*)	
Variable	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	Variance	SD
Ovules fertilized	3	9.839	0.020	1	0.317	0.574	3	47.647	< 0.001	0.598	0.774
Seeds aborted	3	92.069	< 0.001	1	0.021	0.884	3	7.794	0.050	0.993	0.997
Germination	3	11.119	0.011	1	3.175	0.075	3	10.929	0.012	0.377	0.614
Mortality	3	113.022	< 0.001	1	0.068	0.794	3	0.534	0.911	0.181	0.425

B—Crosses between the W1 and E1 lineages, comparing within regions vs between regions

	Cross type			*Cross location*			*Cross type* × *Cross location*			*Maternal population* (nested in *Maternal region*)		*Maternal region*	
Variable	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	Variance	SD	Variance	SD
Ovules fertilized	3	7.501	0.057	1	21.990	< 0.001	3	273.903	< 0.001	0.539	0.734	< 0.001	< 0.001
Seeds aborted	3	222.429	< 0.001	1	0.004	0.952	3	86.307	< 0.001	1.839	1.356	< 0.001	< 0.001
Germination	3	25.619	< 0.001	1	2.724	0.099	3	25.873	< 0.001	0.412	0.642	0.366	0.605
Mortality	3	182.283	< 0.001	1	0.085	0.771	3	12.231	0.007	0.212	0.460	0.004	0.063

Cross type includes crosses within W1 and E1 lineages and the two reciprocal crosses between lineages. Mortality refers to mortality one week after transplantation.

of ovules fertilized and a higher proportion of seeds aborted compared to within-lineage crosses (see post-hoc Tukey tests in Fig. 2). Regarding seed germination, the significant effect of the interaction (Table 1A) were explained by lower values in E1 × W1 crosses in England and eastern France than in Belgium ($\chi^2_{(df = 1)} = 4.000$, $P = 0.045$, Fig. 2). Although a significant effect of *Cross type* was also detected for this variable, hybrid crosses did not suffer from a lower proportion of seeds that germinated compared to within-lineage crosses (see post-hoc Tukey tests in Fig. 2).

There was a significant association between the type of cross and the chlorosis levels of the seedlings ($\chi^2_{(df = 6)} = 2921.854$, $P < 0.001$, Fig. 2). While intralineage progeny were mostly green and healthy, half of the W1 × E1 progeny were partially chlorotic and half were fully chlorotic; most E1 × W1 progeny were fully chlorotic. For W1 × E1 cross type, there was a significant association between the seedling chlorosis levels and whether crosses were from Belgium or outside Belgium ($\chi^2_{(df = 2)} = 40.920$, $P < 0.001$). In Belgium, more of the W1 × E1 progeny were fully chlorotic than were partially chlorotic, while outside of Belgium, more of the progeny were partially chlorotic than fully chlorotic. On the contrary, for E1 × W1 crosses, there was no difference between regions (Belgium or in England-eastern France) in seedling chlorosis level ($\chi^2_{(df = 1)} = 0.469$, $P = 0.493$). Only *Cross type* significantly affected mortality (Table 1A), with a much higher level of mortality in between-lineage crosses than in within-lineage crosses (Fig. 2). In the first week of the survey, half of W1 × E1 and most of E1 × W1 progeny were dead while most of the intralineage progeny survived. At the end of five weeks, the mortality of hybrid progeny increased: on average 72% of W1 × E1 progeny and all E1 × W1 progeny were dead. No significant effect of *Region* was found for any variables, except for chlorosis.

THE DEGREE OF PRI DOES NOT DIFFER FOR WITHIN- VS BETWEEN-REGION CROSSES

When comparing crosses between and within regions, we detected a significant effect of the interaction between *Cross type* and *Cross location* (within vs between regions) for all variables (Table 1B). However, regarding the proportion of ovules fertilized, seeds aborted, and seeds germinated, the interaction was not due to lower hybrid fitness in within-region crosses than between-region crosses (Fig. 3). For these three variables, no clear pattern of PRI was detected: the significant effect of *Cross type* was not due to lower fitness of hybrid progeny compared to within-lineage progeny (see post-hoc Tukey tests in Fig. 3). A significant effect of *Cross location* was only found for the proportion of ovules fertilized: on average, between-region crosses had a higher proportion of ovules fertilized than within-region crosses.

There was a significant association between *Cross type* and the seedling chlorosis levels ($\chi^2_{(df = 6)} = 4070.146$, $P < 0.001$) (Fig. 3). While > 99 % of intralineage progeny were green and healthy, half of the W1 × E1 progeny were partially chlorotic and half fully chlorotic, and most (98%) of the E1 × W1 progeny were fully chlorotic. For both W1 × E1 and E1 × W1 cross types, there was no significant association between the seedling chlorosis levels and *Cross location* ($\chi^2_{(df = 2)} = 0.993$, $P = 0.609$, and $\chi^2_{(df = 2)} = 4.900$, $P = 0.087$). Mortality mirrored seedling chlorosis levels: in the first week of the survey, hybrid progeny suffered from high mortality: on average 42% of W1 × E1 progeny and 98% of E1 × W1 progeny died, while most intralineage progeny survived, revealing a significant effect of *Cross type* on mortality (Table 1B, Fig. 3). Although we found this same pattern for within- and between-region crosses (i.e., higher mortality when E1 was the maternal parent in a between-lineage cross), we found slightly higher mortality of W1 × E1 progeny in within-region crosses compared to between-region crosses, leading to a significant interaction between *Cross type* and *Cross location* (Table 1B). We noted that the few surviving hybrids were markedly smaller than intralineage progeny regardless of whether the cross was within or between regions (data not shown).

At the end of five weeks, on average 67.7% of W1 × E1 progeny and 99.6% of E1 × W1 progeny were dead, while most intralineage progeny survived (5.4% and 8.9% of the progeny died in W1 × W1 and E1 × E1 crosses, respectively). Grouping within- and between-region crosses, the strength of the F1 survival barrier was $RI_{postzygotic\ (E1\ x\ W1)} = 0.996$ and $RI_{postzygotic\ (W1\ x\ E1)} = 0.645$, resulting in a degree of asymmetry of 0.351.

NO EVIDENCE OF HYBRIDIZATION BETWEEN EASTERN AND WESTERN LINEAGES IN NATURAL POPULATIONS

At the population level, F_{IS} values ranged from 0.01 to 0.17 and mirrored low estimates of selfing ($s = 0.00$–0.11; Table S1). The combination of six plastid SNPs assigned individuals to two groups corresponding to the lineages, and no intrapopulation polymorphism was found. Within each region, the highest likelihood found using the ΔK statistic based on 24 nuclear microsatellite loci revealed clustering of individuals into two groups corresponding to the two lineages based on plastid SNPs, with no hybrids identified (Fig. 4). The first axis of the PCA using the nuclear data also clearly differentiated the lineages without intermediate individuals and explained from 7.8% of the variance in Belgium to 16.3% in England (Fig. 4). The second axis split E1 populations, while the two W1 populations in England and Belgium stayed grouped together and explained from 3% of the variance in Belgium to 7.7% in England (Fig. 4).

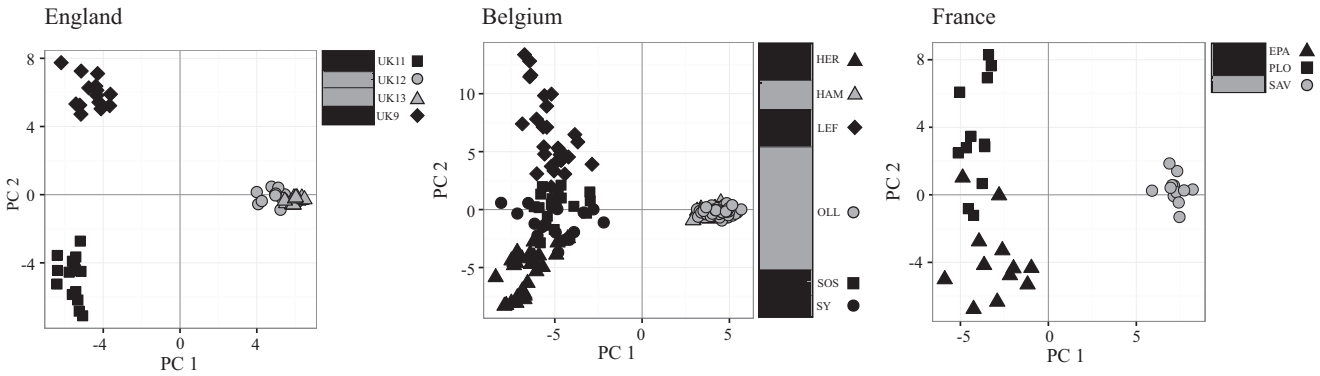

Figure 4. **Genetic assignment of individuals sampled from natural populations from which parents of the crosses originated. For each region (a) England, (b) Belgium, and (c) eastern France, the plot of the first and second axes of the Principal Component Analysis (PCA) and bar-plot of assignment probabilities of an individual's membership into $K = 2$ clusters based on their genotype at 24 microsatellite loci are given. Each individual is represented by a thin line partitioned into two colored segments displaying the individual's estimated membership fraction in the two clusters. Label colors in the bar-plot refer the two lineages based on plastid SNP combinations (gray = W1; black = E1).**

Discussion

Western and eastern lineages of *S. nutans* have come into secondary contact following postglaciation spread (Martin et al. 2016). We took advantage of the presence of regions of contact to test whether reproductive isolation between lineages was a consequence of the accumulation of incompatibilities that arose when the lineages were allopatric, or whether this isolation was a result of local divergence in sympatry following the spread. We found strong PRI between the western W1 lineage and the eastern E1 lineage across all study regions regardless of whether the crosses were within or between regions. The PRI was expressed mainly in the form of seedling chlorosis that resulted in mortality. Mortality among cross types was asymmetric and dependent on which lineage acted as the maternal parent, consistent with cytonuclear incompatibility. Furthermore, no hybrids were found in nature. The high seedling mortality between W1 and E1 observed both in within- and between-region crosses led to estimates of barriers to F1 survival that are relatively high ($RI_{postzygotic\ (E1\ x\ W1)} = 0.996$ and $RI_{postzygotic\ (W1\ x\ E1)} = 0.645$) compared to those reported in a review by Lowry et al. (2008a). Using 19 plant systems, they compiled the strength of prezygotic and postzygotic reproductive isolating mechanisms. In five plant systems, they report hybrid vigor leading to negative values of the F1 viability barriers, and when positive, these values did not exceed 0.190. Together, our findings reveal strong barriers between the western and eastern lineages, which arose in allopatry, prior to subsequent postglaciation spread and secondary contact, and prior to local ecotypic differentiation, and indicate that these lineages should be considered separate species.

Our results raise questions regarding the tempo of the accumulation of genetic incompatibilities that led to the complete reproductive isolation of these phylogenetic lineages, which until now have been assumed to constitute a single species. This single-species designation was based on the lack of any discernable geographic pattern of variation in morphological tendencies, mainly based on vegetative traits (Jeanmonod and Bocquet 1983). However, the edaphic ecotypes in Belgium can be clearly differentiated based on several reproductive traits (e.g., De Bilde 1973), and two varieties, corresponding to the eastern and western lineages (Martin et al. 2016) had been previously described in UK (Hepper 1951), indicating the need for further morphological investigation. We now know that the geographical distribution of the genetically differentiated lineages exhibits a clear pattern that can be assigned to Quaternary climate oscillations, that is fewer than 1 My ago (Martin et al. 2016). When compared to other species pairs, the rise of reproductive isolating barriers seems to be fast. In the same genus, the dioecious species *S. latifolia* and *S. dioica*, which diverged more than 1.5 My ago (Slancarova et al. 2013), have been described as fully cross fertile (Karrenberg and Favre 2008), even though postzygotic isolation that follows Haldane's rule, lower fitness of the hybrids of the heterogametic sex, has been documented (Brothers and Delph 2010). *Helianthus annus* and *H. petiolaris*, which diverged approximately 1 My ago, hybridize naturally (Strasburg and Rieseberg 2008), even though postzygotic isolation is expressed in the form of hybrid sterility (Lowry et al. 2008a). *Arabidopsis arenosa* and *A. lyrata*, which diverged 1–2 My ago, also naturally hybridize (Muir et al. 2015). Even animal taxa, for example *Mytilus edulis* and *M. galloprovincialis* (diverged 2.5 My ago), exhibit strong but incomplete reproductive isolation, such that hybridization occurs in nature after secondary contact (Bierne et al. 2003; Roux et al. 2014).

The relatively rapid rate of complete reproductive isolation between lineages of *S. nutans* may be a result of cytonuclear incompatibility. Interestingly, interlineage seedling chlorosis and

hybrid mortality were asymmetric in crosses involving W1 and E1 lineages, with higher mortality when the maternal parent was from the E1 lineage. Asymmetry in reproductive isolation is often seen when reciprocal crosses are conducted between species (Orr 1995). Several genetic causes have been proposed to explain this asymmetry, including cytonuclear incompatibilities (Levin 2003; Turelli and Moyle 2007). This mechanism can also lead to hybrid chlorosis (or bleaching, Greiner et al. 2011) that can involve not only chloroplastic genes but also mitochondrial ones (Kühn et al. 2015). The involvement of such incompatibilities is gaining support as an important component of the speciation process (Greiner et al. 2011; Gagnaire et al. 2012; Barnard-Kubow et al. 2016; Simon et al. 2016), and as seen here, may be involved in rapid development of reproductive isolation. In the case of *Campanulastrum americanum*, biparental chloroplast inheritance has led to rescue from cytonuclear incompatibility limiting the reproductive isolation between genetically divergent plastid clades (Barnard-Kubow et al. 2017). Even though a preliminary study suggests a possible paternal leakage in *S. nutans* (Garraud 2011), which could theoretically be selected for in a gynodioecious species (Wade and McCauley 2005), the absence of any detected hybrid in natural populations suggests that paternal leakage might be scarce and/or that additional premating isolating barriers are in action. Whether the lineages, which occur both on calcareous and acidic soils outside Belgium (Van Rossum et al., unpublished results), show variation in substrate adaptation across their range also merits investigation.

Silene nutans is gynodioecious, with a cytonuclear control of sex. This breeding system gives rise to genomic conflict between cytoplasmic male sterility factors and nuclear restorers of fertility (Saumitou-Laprade et al. 1994; Garraud et al. 2011). The "arms-race" between a selfish element and a counteracting restorer can drive the evolution of genetic incompatibilities (Phadnis and Orr 2009; Chou and Leu 2010; Case et al. 2016). But genetic incompatibilities should not be necessarily directly involved in male sterility. Previous studies on *S. nutans* have suggested that gynodioecy is under balancing selection, thus enabling the maintenance of cytoplasmic genomes for a long period of time that could ultimately accumulate mutations genetically linked to the sterilizing gene (Touzet and Delph 2009; Lahiani et al. 2013). In addition, comparative genomic studies of sterilizing and nonsterilizing mitochondrial genomes that co-occur in a given species have shown that sterilizing genomes exhibit a higher rate of evolution (Darracq et al. 2010, 2011). This phenomenon could be due to the relaxation of purifying selection and/or to the mutagenic effect of reactive oxygen species in a defective mitochondrion, which is nevertheless advantageous for the females (Touzet 2012). Therefore, we expect that the accumulation of mutations in the mitochondrial genome occurs independently in the two *S. nutans* lineages and would ultimately induce reproductive isolation through cytonuclear genetic incompatibilities. The investigation of cytonuclear genetic polymorphism at the whole genome scale in the two lineages is under way to find possible signatures of coevolution between cytoplasmic and nuclear genes that interact. A similar approach has been successfully applied to other *Silene* species exhibiting high mitochondrial mutation rates (Havird et al. 2015). As dioecy and sex-chromosomes have been suggested to drive speciation rates (Käfer et al. 2014; Graves 2016), gynodioecy could reveal itself to be another reproductive system that facilitates reproductive isolation and thus speciation in a very effective way, in a tempo faster than phenotypic diversification (Orr 1995), resulting in cryptic species.

AUTHOR CONTRIBUTIONS

F.V.R. and P.T. conceived the study, F.V.R. performed the crosses, F.V.R., H.M., and E.L. followed the growth of siblings, E.S., H.M., and E.L. took care of the plants in the green house, H.M. and M.D. performed the analyses, H.M., P.T., F.V.R., and M.D. made interpretation of the results, H.M. drafted the article, and H.M., P.T., F.V.R., L.D., and M.D. brought critical revision of the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

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Supporting Information

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

Table S1. Populations details and within-population genetic-variation estimates from collections of individual plants in the wild: geographic coordinates, sample size (n), lineages (eastern, E1 and western, W1), allelic richness (Ar), observed heterozygosity (H_O), expected heterozygosity (H_E), intra-population fixation index (F_{IS}) and selfing rate (s).

Table S2. Summary of the crosses from the crossing experiment: cross type, regions used for the crosses (UK = England, Fr = France, and Be = Belgium), number of crosses made, mean ±SD of the number of seeds per cross (fruit), the proportion of ovules fertilized, the proportion of seeds that were aborted and germinated, the number of seedlings transplanted per cross, and the mortality (in proportion) following the first and fifth week of transplantation. $_W$ = within region, $_B$ = between regions.

Table S3 Estimates of nuclear genetic variation for 24 microsatellite loci over 13 populations of *Silene nutans* for each locus and over all loci: the mean fixation indices (F_{IT}, F_{IS}, F_{ST}, *** $P<0.001$, ** $P<0.010$, * $P<0.050$, ^{NS} not significant), total number of alleles (An), observed heterozygosity (H_O), and expected heterozygosity (H_E).

Figure S1. Categories of the degree of chlorosis seen in the progeny.