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RESEARCH PAPER

Influence of clonal growth on selfing rate in *Vaccinium myrtillus* L.

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ABSTRACT

Clonal growth, which allows the multiplication of flowering shoots of the same genet, can lead to a large floral display and may thus increase the rate of selfing through geitonogamy as a consequence of an increase in the number of successively visited flowers. The aim of the present research was to analyse the combined effect of the diversity and intermingling of clones on the rate of selfing in *Vaccinium myrtillus*. Four mother plants were selected within patches characterised by contrasting clonal structure (low *versus* high number and intermingling of clones). The selfing rate was significantly lower for plants situated within patches characterised by a high number of intermingled clones (3%) than for plants situated in patches with a low number of clones (50%). Therefore, for this species suffering from inbreeding depression, an increase in the number or the intermingling of the clones could reduce the rate and the cost of geitonogamy and allow a large floral display to attract pollinators. We also found that the main pollinators, bumblebee queens, presented a foraging behaviour favouring geitonogamy, as their successive visits to flowers were quite short (89% of flights were 40 cm or less).

INTRODUCTION

In hermaphrodite plant species, three modes of self-pollination can occur: autonomous, facilitated and geitonogamous. Facilitated and autonomous self-pollination consists of transfer of pollen within a flower with or without, respectively, the participation of an external agent. Geitonogamous self-pollination, defined as the transfer of pollen between flowers of the same genet, can occur in all hermaphrodite or monoecious plant species that produce a number of flowers at the same time at anthesis (Lloyd & Schoen 1992). In partially or fully self-compatible species, the pollen involved in self-pollination cannot be exported to other plants; this pollen discounting consequently reduces outcross male fertility, and thus male fitness (Harder & Barrett 1995; Barrett & Harder 1996; Barrett 1998). More obviously, geitonogamous self-pollination in self-compatible species can lead to geitonogamous selfing, so-called geitonogamy, and, if the species suffers from inbreeding depression, to a reduction in female fitness (de Jong *et al.* 1993; Harder & Barrett 1995; Barrett & Harder 1996; Barrett 1998). Therefore, geitonogamous self-pollination is usually considered as a negative but unavoidable consequence of adaptations for outcrossing (Lloyd 1992).

Because of the potential fitness costs involved in geitonogamous self-pollination through both sexual functions, traits that influence the incidence of this mode of self-pollination are expected to be subject to selection. These traits include functional attributes of both the breeding system and clonal growth, the latter determining the spatial organisation of reproductive units. Self-incompatibility or spatial separation of stamens and stigma are well-known examples of breeding system attributes. With regard to clonal growth, the multiplication of flowering shoots of the same genetic individual can lead to a large floral display and may thus increase the rate of selfing through geitonogamy (Charpentier 2002). However, the number of clones and their spatial distribution (*i.e.* clumped *versus* intermingled) within a patch are two factors that can modulate the impact of clonal growth on the rate of geitonogamy. Where single patches are composed of several clones, we can expect a negative correlation between the number of clones within a patch and the rate of geitonogamy. In the same way, Charpentier (2002) emphasised the importance of the large diversity of clonal architectures that determine the spatial distribution of clones within a patch. Indeed, we can expect the rate of geitonogamy within a patch to be lower if clones are more intermingled. Thus, an increase in the number of clones within a patch and/or an increase in the intermingling of clones could allow a plant species to grow large multiclonal patches, and thus large floral displays to attract pollinators, with a reduced selfing rate resulting from geitonogamous pollination.

Vaccinium myrtillus (bilberry) is a perennial and clonal shrub with an extensive rhizome system, found in a variety of temperate woodlands and heathlands throughout Europe (Ritchie 1956). The species also reproduces sexually and produces berries (Ritchie 1956). With an outcrossing rate ranging from 0.66 to 0.75 (Jacquemart *et al.* 1994), *V. myrtillus* is considered as a mixed-mating species (Jacquemart & Thompson 1996; Jacquemart 2003). High estimates of inbreeding depression measured at the level of fruit and seed set have been reported ($\delta = 0.84$, Guillaume & Jacquemart 1999; $\delta = 0.67$, Raspé *et al.* 2004). Multiple bilberry flowers are open at the same time on an individual, and bumblebee queens are their main pollinators (Jacquemart 1993). As bumblebees are known to cause high levels of within-plant pollen transfer in densely-flowered plants (Klinkhamer *et al.* 1994), geitonogamy is likely to be of significant importance to the selfing rate in *V. myrtillus* (Jacquemart & Thompson 1996).

In a previous study, we investigated the clonal structure of *V. myrtillus* patches from different populations using both Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) markers (Albert *et al.* 2003; Albert 2004). RAPDs proved to be as efficient as AFLPs in detecting different clones (Albert *et al.* 2003). We observed high intra-population variability in the number of clones within patches. Moreover, even though most of the clones showed a clumped distribution within patches, some patches exhibited a more intermingled distribution of clones (Albert *et al.* 2004). The variability in the number of clones and their observed spatial distribution provided a good opportunity to test the incidence of these two factors on the selfing rate through geitonogamy in this species. To our knowledge, this has never been studied in any plant species. Consequently, the aim of the present study was to analyse the combined effects of the diversity and the spatial distribution of clones within *V. myrtillus* patches on the rate of geitonogamy by a direct estimation of the selfing rate. Because any effect of clonal diversity and structure on the level of geitonogamous pollination is dependant on pollinator behaviour (in particular the distance between successively visited flowers, Wilson *et al.* 2005), we also studied pollinator movements.

MATERIALS AND METHODS

Study sites and sampling

Sampled patches were selected in two previously studied *V. myrtillus* populations ('Sacrawé' and 'Le Poteau') situated on the Plateau des Tailles in Upper Ardenne, Belgium (Albert *et al.* 2004). The 'Sacrawé' population (N 50°15′00′, E 5°44′22′, alt. 652 m) is situated in a wet heathland. The vegetation consists of *Calluna vulgaris*, *Erica tetralix*, *Molinia caerulea*, *Trichophorum cespitosum*, *Vaccinium myrtillus* (10% cover), *V. oxycoccos* and *V. uliginosum*. The vegetation in the 'Le Poteau' site (N 50°13′34′, E 5°40′45′, alt. 600 m), situated in a beech grove, is composed of *Fagus sylvatica*, with a herbaceous cover containing *Agrostis tenuis*, *Deschampsia flexuosa*, *Luzula luzuloides* and *V. myrtillus* (15% cover). Some saplings of *Sorbus aucuparia* and *Picea abies* were also present in the latter site.

In April 1999, two 3 × 3-m sampling plots were selected within homogeneous patches of *V. myrtillus* of approximately 12 m^2, in both 'Sacrawé' (PH3 and PH4 plots) and 'Le Poteau' (BG3 and BG4 plots) populations (Albert *et al.* 2004). Within each plot, 49 twigs of *V. myrtillus* were collected at each node of a 0.5 × 0.5-m grid. An analysis of the clonal structure of these *V. myrtillus* patches, using RAPD markers, revealed a lower number of large clones with a clumped distribution in the BG4 and PH4 plots (three and two clones, respectively) compared to the BG3 and PH3 plots, where a higher number (14 and nine, respectively) of small and more intermingled clones were observed (Fig. 1; Albert *et al.* 2004). The size of these patches being similar (approximately 12 m^2 each), a potential influence of floral display size on the rate of geitonogamy (de Jong *et al.* 1992; Vrieling *et al.* 1999), and thus an interference with tested factors, *i.e.* the diversity and the spatial distribution of clones within a patch, was avoided.

In July 2003, one mother plant, previously genotyped with RAPD markers (Albert *et al.* 2004), was selected at or near the centre of each of the four plots (PH3, PH4, BG3 and BG4). The precise location of each mother plant was dependent on the availability of berries (Fig. 1). All ripe berries produced by the four mother plants (a total of 45 berries) were collected and dissected in a drop of water to isolate plump (viable) seeds. Plump seeds were air-dried before germination. Seeds were sterilised in 50% Glorix® (Unilever, Brussels, Belgium), rinsed three times in sterile water and sown on moist filter paper in Petri dishes (maximum 10 seeds per dish) and placed in a growth chamber (12-h daylength and 12:12 h alternating temperature regimes of 15:25 °C; Baskin *et al.* 2000). Once seedlings reached the four-leaf stage, they were stored at −80 °C until DNA isolation.

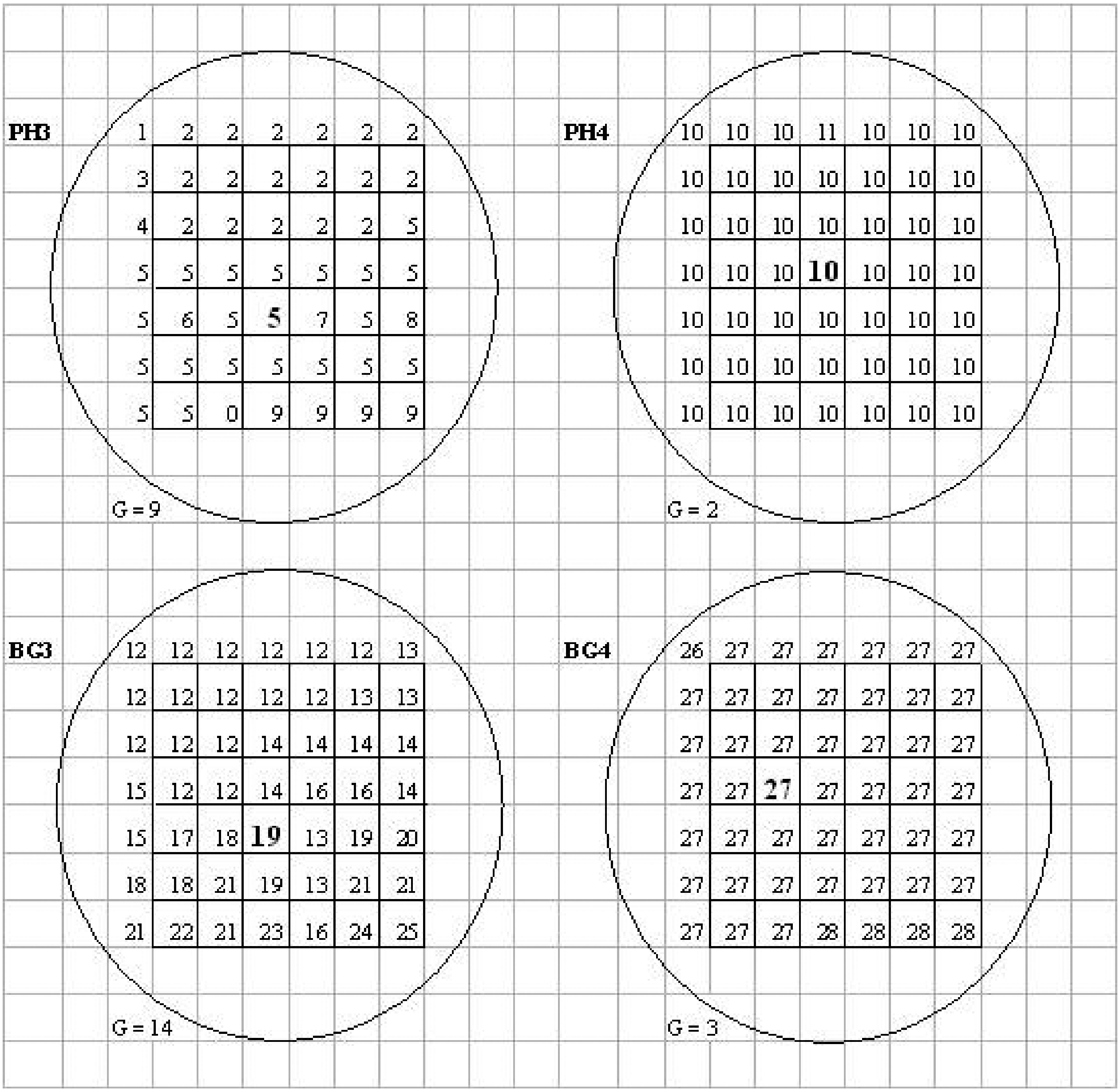

Fig. 1. Spatial distribution of clones in four *Vaccinium myrtillus* patches: PH3, PH4 from 'Sacrawé' population, and BG3, BG4 from 'Le Poteau' population (Albert *et al.* 2004). In each patch, samples were collected in a 3 × 3-m sampling plot at each point of intersection of a 0.5 × 0.5-m grid. Samples belonging to the same clone are represented by the same number. In each plot, the mother plant is in bold type. The absence of data is indicated by 0. G indicates the number of clones observed in a plot.

RAPD analysis

A preliminary test showed the efficiency of RAPD markers to estimate the selfing rate in *V. myrtillus* (Albert 2004). Estimation of selfing rate by RAPD markers has also been successfully done in several other plant species (*e.g.* Trame & Coddington 1995; Gaiotto *et al.* 1997; Kobayashi *et al.* 2000).

Among the sampled berries that provided a minimum of 10 seedlings, five different berries (10 selected seedlings per berry for a total of 50 seedlings) were selected per mother plant in the PH3, PH4 and BG4 patches. For the mother plant in the BG3 patch, only 30 seedlings were available because only three berries provided a minimum of 10 seedlings. DNA was isolated from these 180 seedlings using a modification of the CTAB procedure of Doyle & Doyle (1990) (see Albert *et al.* 2003). The previously isolated DNA samples of the four mother plants were used for PCR amplification. The DNA concentration of each sample was estimated using a VersaFluor

Fluorometer (Bio-Rad Laboratories, Hercules, CA, USA) and samples were diluted to 5 ng·μl^{-1} before amplification by PCR. All details of the RAPD procedure are given in Albert *et al.* (2004). The six primers used in this study were the same as those used in Albert *et al.* (2003, 2004). PCR products were separated on 1.6% agarose gels (Tris-borate-EDTA buffer), run at 100 V for 195 min, stained with ethidium bromide, and visualised under UV transillumination. The PCR products of each offspring was loaded on a gel, together with a sample of the mother plant, to allow identification of non-maternal bands. The presence or absence of DNA fragments was scored using the MOLECULAR ANALYST SOFTWARE v. 1.12 (Bio-Rad Laboratories 1994).

Pollinator behaviour

In each of the study plots, pollinator behaviour was monitored every day from 8 to 10 May 2003 (suitable sunny days with temperature >15 °C) from approximately 10.00 till 17.00. We restricted our records to visits of the main pollinator of *V. myrtillus*, *i.e.* bumblebee queens (Jacquemart 1993). A single incoming pollinator was followed at a time, until it left the study plot. For each incoming pollinator, the number of visited flowers and distances between sequentially visited flowers were recorded.

Data analysis

Estimates of selfing rate were obtained using the method developed by Vrieling *et al.* (1997), which only requires the comparison of band profiles between single offspring arrays and their mother plant, on the one hand, and between mother plants and potential siring plants, on the other hand. The latter comparison was made previously (Albert *et al.* 2004). In this method, an offspring is considered outcrossed if it shows one or more non-maternal bands, assuming that non-maternal bands are not caused by mutation. If only maternal bands are shown by an individual offspring, the latter can be either selfed or the result of fertilisation by a non-self gamete whose banding pattern was indiscernible from that of a self gamete (Vrieling *et al.* 1997). The apparent selfing rate (proportion of offspring showing no non-maternal band) is therefore corrected by multiplying by Os, the probability of selfing given that no non-maternal bands were observed in the offspring (for details of the method, see Vrieling *et al.* 1997).

RESULTS

A total of 19, 8, 13 and 5 non-maternal bands were observed in offspring families from the mother plants situated in the PH3, PH4, BG3 and BG4 plots, respectively. The estimated selfing rate was largely and significantly higher in plots characterised by a low number and intermingling of clones (S = 0.535 ± 0.037 and 0.416 ± 0.039 in PH4 and BG4, respectively; Table 1) than in plots with a high number and intermingling of clones (S = 0.056 ± 0.037 and 0.000 ± 0.063 in PH3 and BG3, respectively; Table 1). The probability of selfing given that no non-maternal bands are present in the offspring, Os, varied between 0.871 and 0.992 (Table 1), which means that the polymorphism of the genetic markers scored was sufficient to detect nearly all outcrossing events.

Table 1. Selfing rate estimates for *Vaccinium myrtillus* mother plants sampled in patches with contrasted clonal structure.

patch	clonal structure*	n offspring	Fnnm	Os	S (95% CI)
PH3	high	50	0.06	0.933	0.056 ± 0.037
PH4	low	50	0.58	0.922	0.535 ± 0.037
BG3	high	30	0.00	0.871	0.000 ± 0.063
BG4	low	50	0.42	0.992	0.416 ± 0.039

*Both number and intermingling of clones. Fnnm = fraction of offspring showing no non-maternal bands; Os = probability of selfing given that no non-maternal bands are observed in the offspring RAPD profile; S = estimated selfing rate (95% CI).

A total of 15 and 13 pollinator visit sequences were recorded in 'Sacrawé' and 'Le Poteau', respectively (with 550 and 187 bumblebee queen flight events between flowers, respectively). In 'Sacrawé' and 'Le Poteau', the mean number of visited flowers per incoming pollinator was 40 (SE = 20) and 18 (SE = 7), respectively, while the spatial distance between the two most distant flowers visited during a foraging trip averaged 1.7 m (SE = 0.4) and 1.4 m (SE = 0.4), with 90.5% and 88.3% of flights being a distance of 40 cm or less (Fig. 2).

DISCUSSION

As predicted, the selfing rate was largely and significantly lower in *V. myrtillus* patches characterised by a higher number and a more intermingled distribution of clones (PH3 and BG3 patches; Fig. 1). Eckert (2000) also observed a negative correlation between clonal diversity and selfing rate in *Decodon verticillatus*, although information about the size and the spatial structure of clones was totally lacking. Since variation in the clonal structure among patches is not expected to influence the proportion of autonomous and facilitated within-flower self-pollination, the observed difference in selfing rate in *V. myrtillus* is likely due to a difference in geitonogamous self-pollination rate. Although it is impossible to distinguish the relative contribution of these three self-pollination modes to the observed selfing rate, it is highly likely that, in this species, geitonogamy contributes significantly more than the other two self-pollination modes. Indeed, as observed in the present and in a previous field study (Jacquemart 1993), bumblebee queens, the main pollinators, visit many flowers per patch sequentially. Moreover, as indicated by low autofertility indices, autonomous self-pollination is likely to be rare (<10% fruit set) in *V. myrtillus* (Jacquemart & Thompson 1996). Although

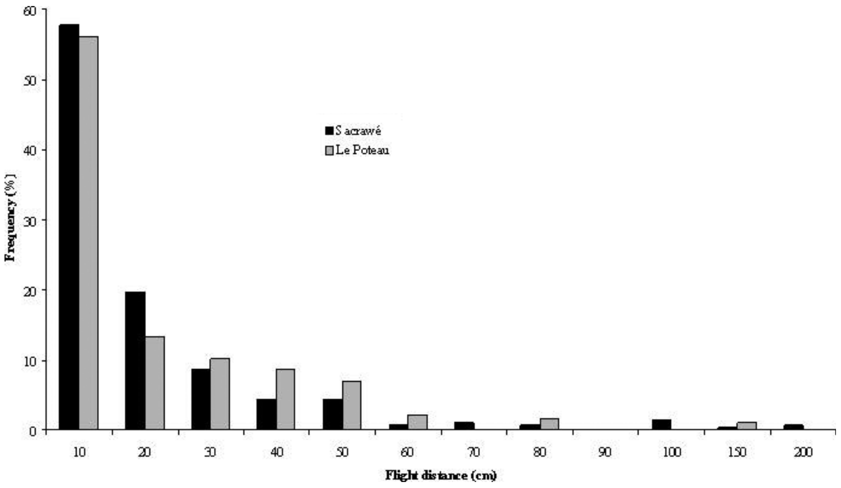

Fig. 2. Frequency distribution of distances between sequentially visited flowers in *Vaccinium myrtillus* patches from 'Sacrawé' population (550 bumblebee queen flights) and 'Le Poteau' population (187 bumblebee queen flights).

geitonogamy level has rarely been measured, it was shown to dominate in other hermaphrodite mass flowering species like *Decodon verticillatus* (Eckert 2000), *Impatiens pallida* (Schoen & Lloyd 1992) and *Mimulus guttatus* (Leclerc-Potvin & Ritland 1994). Geitonogamy could be important in many other plant species with numerous simultaneously receptive flowers (Eckert 2000).

The lower selfing rates in the PH3 and the BG3 patches are in accordance with the observed foraging behaviour of bumblebee queens. Indeed, the majority of flights (90.5% and 88.3% in 'Sacrawé' and 'Le Poteau' populations, respectively) were separated by 40 cm or less and, therefore, fall well within the scale of the 3 × 3-m patches studied. Nuortila *et al.* (2002) also observed that the majority (76.1%) of bumblebee flights were at distances of 40 cm or less within *V. myrtillus* patches in northern Finland. Therefore, considering the size and the spatial distribution of clones within the studied patches (Fig. 1), and the foraging behaviour of bumblebee queens, the probability of transfer of self-pollen is lower in patches characterised by a higher number of clones and a more intermingled distribution of these clones.

The method used to estimate selfing rates is based on some assumptions that may not be completely met by the studied system; deviations from these assumptions may introduce error in the selfing rate estimates. In particular, it is assumed that all bands present in the profile of potential siring plants correspond to a heterozygous state. It is likely that at least some of the bands observed were homozygous. This would increase the power of detection of outcrossed progeny and, consequently, the probability of selfing, given that non-maternal bands are present in the offspring profile, Os. However, the fraction of offspring showing non-maternal bands is already so high for mother plants from high-clonal diversity patches (100% and 94%) that even a much higher homozygosity in potential siring plants of these mothers would not noticeably increase the selfing rate estimate. On the other hand, if homozygosity was higher in potential siring plants of mother plants from low-diversity patches, and the selfing rate in these patches was underestimated, this would only reinforce the difference in selfing rate between high- and low-clonal diversity patches. In any case, the underestimation would be very limited, given the already high values of Os. The difference in selfing rate estimates between high- and low-clonal diversity patches is therefore strong evidence for an effect of clonal diversity and architecture on the selfing rate.

Since the selfing rate through geitonogamy was lower within patches characterised by higher clonal diversity and more intermingled distribution of clones, an increase of these two factors can limit inbreeding depression resulting from geitonogamy. This may allow *V. myrtillus* to have a large patch size, and thus a large floral display to attract pollinators, with a reduced fitness cost through inbreeding depression. This is in accordance with results of two previous studies on *V. myrtillus* where all sampled patches consisted of several clones (Albert *et al.* 2003, 2004). Because clonal diversity and the spatial distribution of clones within patches can reduce the fitness costs due to geitonogamous self-pollination in *V. myrtillus*, both of these traits are expected to be under selection. Therefore, architectural parameters, such as internode length and branching angle, which determine the spatial structure of clones (McLellan *et al.* 1997), can be selected to enhance the level of intermingling of clones within a patch. In the same way, traits that promote the establishment of a high number of clones within a patch are expected to be positively selected. The high production of well-dispersed seeds, despite rare seedling recruitment in *V. myrtillus* (Ritchie 1956; Eriksson & Fröborg 1996; Jacquemart *et al.* 2003), may be considered as such a trait. Indeed, Eriksson & Fröborg (1996) observed that in *V. myrtillus* the recruitment within stands of established conspecific adults is limited spatially and temporally by a combination of seed and microsite availability (hypothesis of recruitment at windows of opportunity). In this context, the high production of seeds is a trait that enhances the ability to find these windows, and thus increases the clonal diversity within *V. myrtillus* patches (Eriksson & Fröborg 1996).

Selection may also have led different species with different breeding or mating systems to evolve contrasting clonal structures (or *vice-versa*), as shown in two co-occurring marine angiosperms (Ruggiero *et al.* 2005). In the genus *Vaccinium*, most of the patches of *V. uliginosum*, which occur together with *V. myrtillus* in wet heathlands, consist of a single clone (Albert *et al.* 2005). Moreover, *V. uliginosum* patches show a more typically clumped distribution of clones than *V. myrtillus* patches (Albert *et al.* 2005). This dissimilarity in clonal structure between *V. myrtillus* and *V. uliginosum* can be related to the mating system of these species. Indeed, as suggested by floral traits and selfing ability, *V. uliginosum* is a more highly selfing species than *V. myrtillus* that does not suffer from inbreeding depression, at least for fruit and seed production (Jacquemart & Thompson 1996; Jacquemart 2003; Albert *et al.* 2005) and can therefore have larger monoclonal patches without or with only limited negative consequences on fitness.

In clonal plant species that are not fully self-fertile, such as *V. myrtillus*, clonal growth, which allows the multiplication of flowering units of the same genetic individual, unavoidably induces fitness costs through geitonogamy. Nevertheless, it is highly likely that the advantages of clonal growth, *e.g.* increased resource acquisition and storage, reduced risk of individual extinction, are more than sufficient to compensate fitness costs of geitonogamy (Eckert 2000). Additionally, as shown in this study, the negative effects of geitonogamy could be limited by an increase in the clonal diversity and the intermingling of clones within patches.

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