

Incidence of geitonogamy differs between two populations in the hawkmoth-pollinated *Platanthera bifolia* (Orchidaceae)

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Abstract: We estimated geitonogamy in individuals with different inflorescence sizes in a small (100–200 flowering individuals) and a large population (>700 flowering individuals) of the self-compatible, moth-pollinated orchid *Platanthera bifolia* (L.) L. C. Rich. (Orchidaceae). Geitonogamy was estimated as the percent reduction in pollen receipt by emasculated compared with control plants over seven nights. Geitonogamy in the small population was 23% and 38% during 2 years, respectively. In contrast, no geitonogamy was detected during a single flowering season in the large population. Geitonogamy did not vary with inflorescence size and emasculation had no impact on fruit set. The difference in geitonogamy between the populations in the present study may be related to pollinator abundance and behaviour. We suggest that incidence of geitonogamy will be higher if the pollinator carries smaller pollen loads when arriving at a plant because there will be a smaller fraction of cross-pollen carried after visiting one flower. Geitonogamy may be influenced by available number of mates, pollen load size, pollinator behaviour, and pollen carryover.

Key words: geitonogamy, population size, inflorescence size, pollen-limitation, pollen carryover, self-pollination.

Résumé : Les auteurs ont évalué la geitonogamie, chez des individus possédant des inflorescences de diverses dimensions dans une petite (100–200 individus en fleurs) et une grande population (>700 individus en fleur) du *Platanthera bifolia* (L.) L. C. Rich. (Orchidaceae), une orchidée auto-fertile pollinisée par des papillons nocturnes. Ils ont estimé la geitonogamie sous forme de réduction du pourcentage de réception du pollen chez des plantes émasculées, comparativement à des plants témoins, au cours de sept nuits. Dans la petite population, la geitonogamie a été de 23 % et de 38 % au cours de deux années, respectivement. Par opposition, on ne détecte aucune geitonogamie au cours d'une seule saison de floraison, dans la grande population. La geitonogamie ne varie pas selon la dimension de l'inflorescence, et l'émasculature n'a pas d'effet sur la grosseur du fruit. La différence de geitonogamie entre les populations, dans cette étude, pourrait être reliée à l'abondance des pollinisateurs et à leur comportement. Les auteurs suggèrent que l'incidence de la geitonogamie sera plus élevée si le pollinisateur transporte de plus faibles charges de pollens lorsqu'il arrive sur une plante, parce qu'il y aura une plus petite fraction de croisement de pollens après la visite d'une fleur. La geitonogamie pourrait être influencée par le nombre de partenaires disponibles, la charge pollinique, le comportement des pollinisateurs, et la rémanence pollinique.

Mots clés : geitonogamie, dimension des populations, dimension des inflorescences, limitation par le pollen, rémanence pollinique, auto-fécondation.

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Introduction

Geitonogamy, self-pollination between flowers on the same plant, has been proposed to be an important factor for floral evolution as well as the evolution of self-

incompatibility (Kalin de Arroyo 1976; Klinkhamer and de Jong 1993). This stems from the fact that geitonogamy, in contrast to other types of self-pollination, generally does not contribute to reproductive assurance but is a nonadaptive by-product of mechanisms for outcrossing (Lloyd 1992; Schoen and Lloyd 1992). The level of geitonogamy in an animal-pollinated plant depends on many factors, including the number of flowers available on the plant, structural mechanisms, reward production, pollinator behaviour, and pollen carryover (Johnson and Nilsson 1999). Plant fitness is often closely tied to inflorescence size (Maad 2000), but the plant reproductive output per flower (seeds produced/sired) may both increase and decrease with inflorescence size. A larger floral display may encourage pollinators to visit more frequently and (or) probe more flowers (Geber 1985) and may disproportionately increase number of seeds produced and seeds sired (Dudash 1991). However, if the number of flowers probed in succession on an individual increases there

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may be more pollen transfer within the inflorescence which will result in self-pollination, mainly from geitonogamy (Geber 1985; de Jong et al. 1992, 1993).

The number and spatial distribution of individuals within a population as well as pollinator behaviour affect gene dispersal within populations (Murawski and Hamrick 1991; Morris 1993; Karron et al. 1995; Stout et al. 1998). Small populations of rewarding plant species are often less attractive to pollinators or receive lower proportions of compatible pollen than larger populations and their seed set is often more pollen limited (Waites and Ågren 2004). Moreover, the number of individuals with which a plant is likely to mate (i.e., neighbourhood size) decreases with decreasing population size, which may result in increased selfing (Murawski et al. 1990), as has been observed in some species (Oostermeijer et al. 1998; Paschke et al. 2002). The opportunity for geitonogamy can, additionally, vary with plant density and nectar availability because pollinators maximize their foraging efficiency and probe more flowers per visit in scarcely distributed plant individuals and in individuals with more nectar (Klinkhamer and de Jong 1990; de Jong et al. 1993; Hodges 1995; Mustajärvi et al. 2001).

The present study investigated the incidence of geitonogamy in a small and a large population and in plants with different inflorescence sizes. We chose the self-compatible *Platanthera bifolia* (L.) L. C. Rich. (Orchidaceae) as the study species because it is nectar-producing and lacks strong pollinarium bending to prevent geitonogamy.

Materials and methods

Plant species and study populations

Platanthera bifolia is a perennial terrestrial orchid with a wide Eurasian distribution (Hultén and Fries 1986). The flowering period of *Platanthera bifolia* in northern Europe is June–July, and the plant occurs in a variety of forest and meadow habitats (Pridgeon et al. 2001). The plant is polycarpic, but individuals do not flower each season (Maad 2000). *Platanthera bifolia* is generally pollinator limited within a season (Mattila and Kuitunen 2000; Maad and Alexandersson 2004) and self-compatible (Nilsson 1983; Brzosko 2003). Typically, a plant produces two oval leaves at the base of a single 20- to 60-cm-high flower-bearing stem. The inflorescence spike bears 10–20 white, nocturnally fragrant, approximately 1.5- to 2-cm-wide flowers. Each flower has a slender 2- to 4-cm-long nectar-containing spur as a backward extension of the lip petal. The column produces two pollinaria, each pollinarium comprising a 2-lobed, rather friable, pollinium attached by a short caudicle (stalk) to a sticky viscidium. The viscidium is exposed to fasten on the pollinator's tongue. The pollen in a pollinium is aggregated in hundreds of massulae, each massula containing 10–80 pollen tetrads (Nazarov and Gerlach 1997). The stigma, with two lateral lobes and one smaller median lobe, is situated around the entrance of the spur (see Nilsson 1983). When a pollinator moth inserts its proboscis deeply into the spur, parts of the carried pollinia become deposited on the stigma (Darwin 1862; Nilsson 1988). The pollinium caudicle is much shorter than that of the close relative *Platanthera chlorantha* (Cust.) Reich., and, accordingly, its

bending is hardly sufficient to avoid self-pollination (Nilsson 1983).

The study was carried out in two Swedish *Platanthera bifolia* populations. One relatively small population was located at Hammarskog (59°47'N, 18°35'E; hereafter referred to as “the small population”), approximately 10 km SW of Uppsala. During the study years of 1996 and 2000, it included approximately 200 and 100 flowering individuals, respectively. The individuals were distributed among two subpopulations separated by approximately 700 m, a distance well within the flight distances of hawkmoth pollinators (Nilsson et al. 1992). In 1996, all selected individuals were from one of the subpopulations, whereas individuals from both subpopulations had to be included in 2000, since the number of flowering individuals was lower. The vegetation at the Hammarskog site was a moist, relatively old, partly thinned *Picea abies* (L.) Karst. forest. The other population of *Platanthera bifolia* was larger and located on the hillside of Hönsarvsberget (60°31'N 15°27'E; hereafter referred to as “the large population”), approximately 6 km NE of Borlänge. During the year of study (1998), it consisted of more than 700 inflorescence-producing individuals. The vegetation in this study area was a thinned, relatively old *Picea abies* forest and, partly, a logged *Pinus sylvestris* L. forest (see Maad 2000). The density of flowering individuals varied within both populations from very dense (>5 individuals/m²) to sparse (>30 m to nearest neighbour) and did not differ considerably between populations. The weather during the flowering season of *Platanthera bifolia* was rainy and rather cold for all three years of study. The main pollinator in both study areas is the sphingid *Hyloicus pinastri* (L.) (J. Maad and L.G. Reinhammar, unpublished data).

Bagging and emasculation of individuals

We performed bagging of all study plants before flowering. In the study sites, we chose individuals with one single inflorescence (very few individuals produced more than one spike). In the small population (Hammarskog) we bagged 40 and 73 individuals in 1996 and 2000, respectively. In the large population (Hönsarvsberget) 80 individuals were bagged during 1998. Experimental design differed between population and year. In the small population during 1996 the individuals were arranged in pairs consisting of two individuals of approximately the same size and flowering phenology and located near each other. During 2000, the individuals were divided in two exposure groups depending on flowering phenology. In the large population, inflorescences were of two different sizes (see below). It was randomly assigned, within pair (1996), size group (1998), or exposure group (2000), which individuals were to be emasculated and which ones were to be controls.

All flowers on the plants assigned to emasculation treatment were emasculated by pollinium removal. Emasculation probably does not alter the function of the flower since only the pollinaria are removed and all other parts of the flower, including the anther caps that envelope the pollinaria, are left intact. During 1996, 1998, and 2000, the numbers of emasculated individuals were 20, 40, and 36, respectively. These numbers corresponds to approximately 10%, 5%, and 30% of all flowering individuals in the population during

1996, 1998, and 2000, respectively. In the small population individuals were exposed to pollinators simultaneously within pair (1996) or within exposure group (2000). In the large population all bags were removed simultaneously for all 80 individuals.

To investigate geitonogamy in relation to inflorescence size, the plants were manipulated in the large population (1998): the 40 largest inflorescences were trimmed to 15 flowers and the rest to 7 flowers. In 2000, we also investigated the effect of inflorescence size, but instead of manipulating inflorescence sizes, all the open flowers at the start of experiment were kept (excess buds were removed). In 1996, all chosen individuals had 12 or more flower buds, and at the start of the experiment, plants were trimmed to 12 flowers by cutting off excess flowers.

Census of flowers

In the small population (1996 and 2000), all flowers were censused (checked for pollinium removals and pollen depositions on stigmas) after the first, third, and seventh night after exposing plants to pollinators. In addition, all flowers were censused each day until they received pollen. In the large population (1998), all flowers were censused after the third night and when all flowers had wilted. During each census, the number of pollinia removed (0, 1, or 2) from flowers was counted. The amount of pollen deposited on stigmas was classified as one of five pollen import classes (PIC 0–4): 0, no pollen deposited; 1, 1–5 massulae; 2, 6–20 massulae; 3, >20 massulae and <1/2 stigmatic surface covered; and 4, >1/2 stigmatic surface covered. A flower was considered visited when the stigma had massulae or traces from a pollinator (scales or hairs). Additionally, 1 month after wilting, fruit set was recorded in the small and large population during 2000 and 1998, respectively.

Data analysis

To compare pollen import between treatments, we derived mean values of individuals. Pollen import classes (PIC) have an ordinal scale from 0 to 4 (see above), so they cannot be used directly to calculate individual means. However, the PIC (0, 1, 2, 3, or 4) of each flower approximates to the degree to which pollen covered (PC) a stigma (midpoints of 0, 5, 15, 35, and 75%) based on $PC = 5 \times 2^{PIC} - 5$. This measure was used to calculate mean pollen import.

The incidence of geitonogamy was estimated as the proportional decrease of pollen import in emasculated compared with control individuals (see de Jong et al. 1992; Bosch and Waser 2001). On the data set from the small population 1996, we used paired *t* tests to compare the pollination (mean PC and timing of pollination) of emasculated and control plants. For the 2000 data from the small population, we performed a 3-way ANCOVA to evaluate whether flower number (covariate), exposure group, subpopulation, or treatment affected the number of flowers visited during first visit-night, timing of first efficient visit, PC (after three and seven nights), and fruit set. Nonsignificant interaction terms ($P > 0.05$) were excluded from the final analyses. We performed a 2-way ANOVA on the 1998 data from the large population to see if either inflorescence size or treatment or their interaction affected PC and fruit set. A significant main effect of emasculation treatment on PC indicates geito-

nogamy, provided that emasculated plants received less pollen.

To compare geitonogamy between the two populations, we first standardized PC (in controls and treated individuals) by dividing it with the yearly mean PC of controls and multiplying by 100. This was done to be able to compare levels of geitonogamy (i.e., percentage decrease in PC of emasculated compared with controls) directly. We then investigated the effects of year, emasculation, and year \times emasculation on standardized PC by ANOVA. Then we calculated contrasts using the ESTIMATE option in the GLM procedure of SAS (SAS Institute Inc. 1996a): $D = (\mu_{1996C} - \mu_{1996E} + \mu_{2000C} - \mu_{2000E})/2 - (\mu_{1998C} - \mu_{1998E})$, where μ is the mean standardized PC of control (C) or emasculated (E) plants in the denoted year. To compare natural levels of pollen export and import (nonstandardized PC) between years, an ANOVA was performed on control individuals to test the effect of year. Then we calculated contrasts to compare the two populations: $D = (\mu_{1996C} + \mu_{2000C})/2 - \mu_{1998C}$, where μ is the mean of controls in the denoted year.

Variances were approximately homogenous, but the errors of most dependent variables deviated from a normal distribution, so we estimated probabilities of *F* values by randomization. We generated 5000 permutation samples without replacement. The dependent variable was separated from the independent variables, and for each permutation sample, the values of the dependent variable were assigned randomly to the independent variable values. Each permutation sample had the same number of observations as the original dataset. The *F* statistics were estimated for each permutation sample. Finally, each *P* value was estimated as the proportion of permutation samples with an *F* value equal to or greater than the original *F* value. *P* values from such a method are more accurate than those from the corresponding parametric test when data are from non-normal distributions and relate to probabilities of getting similar or higher *F* values by chance (see Crowley 1992).

Significances of differences between treatments (1996 data) and contrasts (combined data) were determined by generating 5000 bootstraps from the original data set. Each bootstrap sample was obtained by resampling with replacement and had the same number of observations as the original data set. The statistic (difference or contrast) was estimated for each bootstrap sample to generate bootstrap distributions. The significances of the original statistics were estimated by calculating confidence intervals as the mid 95, 99, and 99.9 percentiles of the bootstrap distributions (see Dixon 1993). A statistic was considered significant, at the given level, if the confidence interval excluded zero.

Damaged individuals and individuals with floral malformations (e.g., with pollinaria lacking caudicle) were not included in the analyses. All independent variables of the ANCOVAs and ANOVAs were considered as fixed effects in our analyses. All statistics and graphics were made with the SAS package for PC (SAS Institute Inc. 1996b).

Results

In the small population, flowers waited about 3 d, on average, for a visit resulting in pollen import during 1996 and 2000 (Table 1). This waiting time did not differ between

Table 1. Means and standard errors (SE) of two variables related to insect visitation frequency in the small population of *Platanthera bifolia* (Hammaraskog) in 1996 and 2000.

Variable	1996		2000	
	Control	Emasculated	Control	Emasculated
Timing of first visit to a flower	2.9±0.27	3.3±0.33	3.0±0.28	3.0±0.21
Number of flowers visited at first visit-night	6.5±0.86	3.5±0.81	6.6±0.64	4.1±0.49

treatments in either year (Tables 2 and 3). Number of flowers recorded as visited after the first visit-night differed significantly between control and emasculated plants during 1996 but not during 2000 (Tables 1–3). Number of flowers visited during the first visit-night varied positively with inflorescence size (measured as flower number) in the small population during 2000 (Table 3). Control individuals had more pollinia removed in the large than in the small population (Table 4, Fig. 1). Amount of pollen imported to stigmas was higher in the large population compared with the small population after three nights but not after seven nights (Table 4, Fig. 2).

Emasculated *Platanthera bifolia* individuals in the small population (Hammaraskog) 1996 had 17% and 23% less pollen on stigmas (PC) than control individuals after three and seven nights, respectively (Fig. 2). These percentage differences correspond to levels of geitonogamy experienced by control plants. Geitonogamy differed significantly from zero after 7 d but not after 3 d (Table 2). In the same population during 2000, emasculated individuals received 33% and 37% less pollen after three and seven nights, respectively (Fig. 2). Both of these estimates on geitonogamy differed significantly from zero (Table 3). In the large population during 1998, the mean PC after three nights and wilting was 6% and 1% less, respectively, in emasculated compared with control individuals (Fig. 2). These estimates of geitonogamy were nonsignificant (Table 5). Combined analysis with data from both populations and all years showed that emasculation significantly reduced pollen receipt after 7 d (Table 6). Plants in the small population experienced slightly more geitonogamy ($P = 0.06$) than those in the large population.

Inflorescence size did not influence geitonogamy because emasculation and inflorescence did not interact significantly in any ANOVA or ANCOVA of pollen PC on stigmas in the small (2000) and large (1998) populations (Tables 3 and 5). Pollen import differed between subpopulations during 2000 (Table 3): plants in the small subpopulation received less pollen than those in the large subpopulation. However, the incidence of geitonogamy after 3 and 7 nights did not differ between subpopulations (effect of emasculation \times subpopulation on PC after 3 nights: $F_{1,56} = 0.03$, $P > 0.5$; after 7 nights: $F_{1,56} = 0.00$, $P > 0.5$; removed from the final model).

Emasculation did not significantly affect fruit set in the small population (2000) or in the large population (1998) (Tables 3 and 5). Fruit set (%), mean \pm SE was 65 ± 2.3 and 65 ± 2.3 in control and emasculated individuals, respectively, in the small population, and 82 ± 3.4 and 78 ± 4.7 in control and emasculated individuals, respectively, in the large population (see Fig. 1 for sample sizes). The only significant effect on fruit production in the small population during 2000 was subpopulation, with lower fruit set in the smaller subpopulation.

Table 2. Paired *t* test comparing pollination of emasculated and control *Platanthera bifolia* individuals in the small population (Hammaraskog) during 1996.

Response variable	df	<i>t</i>
Timing of first visit to a flower (days)	13	0.27
Number of flowers visited at first visit-night	13	2.62*
PC after three nights (%)	15	1.28
PC after one week (%)	15	1.93

Note: Significances were estimated with a bootstrap technique (see Materials and methods). Mean values of variables are given in Fig. 1 and Table 4. PC, percentage of the stigmatic surface covered with pollen; *, statistical significance at $P < 0.05$.

Discussion

Plant population size and geitonogamy

In theory, increased selfing would be expected in smaller populations of self-compatible plants (Murawski et al. 1990). Outcrossing rate has been found to be positively correlated with population size in some plant species (Oostermeijer et al. 1998; Paschke et al. 2002; Vergeer et al. 2003; but see, e.g., Luijten et al. 2000; Lienert et al. 2002; Wallace 2002). However, low outcrossing rates in small populations are not always caused directly by population size. For example, Vergeer et al. (2003) concluded that the effect of population size on mating system in *Succisa pratensis* (Dipsacaceae) was indirect via habitat quality that was correlated with population size. The results of the present study indicate that the incidence of geitonogamy varies between populations in *Platanthera bifolia*. In the smaller population (with 100–200 flowering individuals) of Hammaraskog, control individuals had 23%–38% geitonogamy after 1 week (measured as a decrease in pollen import in emasculated individuals), which is in the range of levels of geitonogamy (22%–85%) found in other rewarding orchids without pollinarium bending (Peakall 1989; Peakall and Beattie 1991; Nilsson et al. 1992; Salguero-Faria and Ackerman 1999). However, in the larger population (with more than 700 flowering individuals) of Hönsarvsberget, no geitonogamy was detected.

Since we have not controlled for factors like pollinator abundance, weather conditions, habitat quality, etc., and only investigated one large and one small population, we can only hypothesize on what factors caused this difference in geitonogamy. Pollinator behaviour generally has a large impact on mating in animal-pollinated plants (Johnson et al. 2004). Pollinators respond to the distribution of rewards, foraging to optimize energy intake (see Charnov 1976). Hawkmoths visit more flowers in succession at larger inflorescences and inflorescences with higher standing crops of nectar (Dreisig 1985; Hodges 1995). In the same way, plant density can in-

Table 3. Results from ANCOVA on reproductive measurements of *Platanthera bifolia* individuals in the small population (Hammarskog 2000).

Source of variation	Number of flowers visited during first visit-night	Timing of first visit to a flower	PC after three nights	PC after seven nights	% fruit set
Flower number	$F_{1,62}=30.26^{***}$	$F_{1,59}=1.91$	$F_{1,62}=1.38$	$F_{1,62}=0.58$	$F_{1,62}=1.01$
Emasculation	$F_{1,62}=0.03$	$F_{1,59}=0.19$	$F_{1,62}=4.14^*$	$F_{1,62}=7.50^{**}$	$F_{1,62}=0.00$
Start	$F_{1,62}=5.33^*$	$F_{1,59}=7.63^{**}$	$F_{1,62}=1.45$	$F_{1,62}=2.30$	$F_{1,62}=2.77$
Subpopulation	$F_{1,62}=15.89^{***}$	$F_{1,59}=0.65$	$F_{1,62}=2.39$	$F_{1,62}=3.98^*$	$F_{1,62}=12.14^{**}$

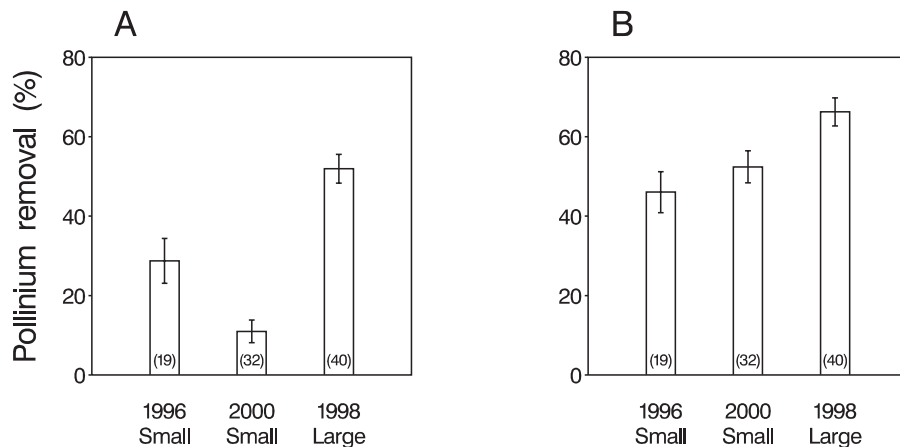
Note: Significances were estimated with a randomisation method (see Materials and methods). Nonsignificant ($P > 0.05$) interactions were excluded from final analyses. Mean values of variables are given in Fig. 1, Table 4, and Results. PC, percentage of the stigmatic surface covered with pollen; *, statistical significance at $P < 0.05$; **, statistical significance at $P < 0.01$; ***, statistical significance at $P < 0.001$.

Table 4. ANOVA on control individuals in the small (Hammarskog 1996 and 2000) and the large (Hönsarvsberget 1998) *Platanthera bifolia* populations.

Source of variation	Pollinium removal after 3 nights	Pollinium removal after 7 nights ^a	PC after three nights	PC after seven nights ^a
Year	$F_{2,88}=33.88^{***}$ $D=-32.1^{***}$	$F_{2,88}=6.26^{**}$ $D=-17.0^{***}$	$F_{2,88}=4.96$ $D=-11.5^{**}$	$F_{2,88}=0.14$ $D=-2.0$

Note: Probabilities of the F statistics were estimated using a randomisation method. Significances of contrasts ($D = (\mu_{1996C} + \mu_{2000C})/2 - \mu_{1998C}$; testing for differences between the small and the large population) were estimated using a bootstrap technique (see Materials and methods). PC, percentage of the stigmatic surface covered with pollen; **, statistical significance at $P < 0.01$; ***, statistical significance at $P < 0.001$.

^aMeasured after wilting in the large population in 1998.

Fig. 1. Pollinium removal (%) of control *Platanthera bifolia* individuals in the small (Hammarskog) and large (Hönsarvsberget) populations at three (A) and seven nights (B) after the initiation of the experiment (measurements in Fig. 1B were taken after wilting at Hönsarvsberget). Values are means of pollinium removal; error bars show standard errors. Numbers of observations are given in parentheses. For among population comparisons, see Table 4.

fluence pollinator behaviour (Klinkhamer and de Jong 1990; but see Bosch and Waser 2001). In the present study, plant density did not differ between populations, but the pollinator visitation frequencies probably differed. If lower visitation frequencies in the small population caused higher standing crops of nectar than in the large population, the pollinators may have had divergent behaviour in the populations, visiting more flowers in sequence in the small population. Such a difference in behaviour would cause more geitonogamy in the small than the large population. Lower visitation frequencies may additionally have caused smaller pollen loads

on pollinators, a situation that also may generate more geitonogamy (see below).

Inflorescence size and geitonogamy

Hawkmoths visit more flowers in succession at larger inflorescences and inflorescences with higher standing crops of nectar (Dreisig 1985; Hodges 1995). Increasing opportunity for geitonogamy with plant size has also been reported in various bee- and hummingbird-pollinated species (see, e.g., Webb and Bawa 1983; Klinkhamer and de Jong 1990; Dudash 1991; de Jong et al. 1992). In the present study of

Fig. 2. Degree of pollen covering on stigmas (PC) of control (empty bars) and emasculated (filled bars) individuals and degree of geitonogamy (%) in the small (Hammarskog) and large (Hönsarvsberget) populations of *Platanthera bifolia* at three (A) and seven nights (B) after the initiation of the experiment (measurements in Fig. 1B were taken after wilting at Hönsarvsberget). Values are means of PC; error bars show standard errors. Numbers of observations are given in parentheses. Estimated degree of geitonogamy is given above the bars and is the proportional decrease in PC of emasculated compared with control individuals. An asterisk indicates significant geitonogamy (see Results and Tables 2, 3, and 5). For among population comparisons, see Table 6.

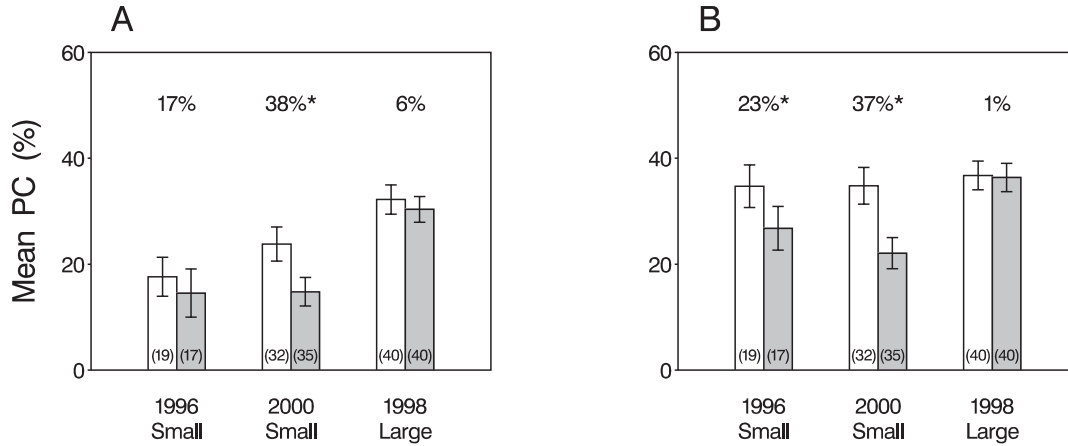


Table 5. Results from 2-way ANOVA on reproductive characters of *Platanthera bifolia* emasculated and control individuals in the large population (Hönsarvsberget 1998) with two classes of inflorescence sizes.

Source of variation	PC after three nights	PC after wilting	% fruit set
Emasculaton	$F_{1,76}=0.22$	$F_{1,76}=0.00$	$F_{1,76}=0.50$
Size	$F_{1,76}=1.39$	$F_{1,76}=2.63$	$F_{1,76}=0.11$
Emasculaton × size	$F_{1,76}=0.21$	$F_{1,76}=0.03$	$F_{1,76}=1.22$

Note: *P* values (not listed) were estimated with a randomisation method (see Materials and methods) and were not significant in any case. Mean values of variables are given in Fig. 1 and Results. PC, percentage of the stigmatic surface covered with pollen.

Table 6. ANOVA on emasculated and control individuals from the small (Hammarskog 1996 and 2000) and the large (Hönsarvsberget 1998) *Platanthera bifolia* populations.

Source of variation	PC after three nights	PC after seven nights ^a
Year	$F_{2,177}=0.95$	$F_{2,177}=2.39$
Emasculaton	$F_{1,177}=3.43$	$F_{1,177}=6.78^*$
Year × emasculaton	$F_{2,177}=0.95$ $D=28.6$	$F_{2,177}=2.39$ $D=21.8^\dagger$

Note: PC (percentage of the stigmatic surface covered with pollen) was standardised by dividing with yearly mean PC of controls and multiplying by 100. Probabilities of the *F*-statistics were estimated using a randomisation method. Significances of contrasts ($D = (\mu_{1996C} - \mu_{1996E} + \mu_{2000C} - \mu_{2000E})/2 - (\mu_{1998C} - \mu_{1998E})$; testing for differences in level of geitonogamy between the small and the large population) were estimated using a bootstrap technique (see Materials and methods). *, statistical significance at $P < 0.05$; †, statistical significance at $P < 0.06$.

^aMeasured after wilting in the large population in 1998.

the hawkmoth-pollinated *Platanthera bifolia*, plant size did not influence the degree of overall geitonogamy. The number of flowers visited during the first visit-night in 2000 was positively related to flower number. This may reflect a higher number of flowers visited in succession, but may also be due to a higher probability of having two visits per inflorescence (by potentially different pollinator individuals). The presence of a relationship between plant size and geitonogamy depends highly on behaviour of pollinators, which generally deserves much more study.

Emasculaton and exposure of virgin flowers

The time that flowers waited for the first visit did not differ between treatments in the small population, but the number of flowers visited after the first visit-night differed between emasculated and control plants in 1996, indicating that pollinators visited more flowers in sequence on control plants. However, this may have been an artefact through the way flowers were considered visited and not due to pollinator discrimination of treatments: Emasculated flowers were probably visited without importing pollen (or traces from the pollinator such as scales and hairs) more often than control plants and emasculaton treatment disabled classifying such flowers as visited by looking at pollen export. We do not know if pollinators can discriminate between emasculated and control plants. We have observed that floral longevity does not change with emasculaton treatment (J. Maad and L.G. Reinhammar, unpublished data). Whether emasculaton changes the olfactory and (or) visual signals in *Platanthera* has not been investigated (but see Tollsten and Bergström 1989; Stpiczynska 2003 for floral changes after pollen depositions).

We exposed inflorescences with virgin (unvisited) flowers that had previously been bagged for several days, which may affect pollinator behaviour and pollen carryover. Rademaker et al. (1999) found higher geitonogamy when using whole inflorescences of virgin flowers than in naturally open pollinated individuals. Experimental plants in the present study

probably had more flowers open and higher standing crops of nectar than nonmanipulated plants during their first pollinator visit. Higher nectar availability promotes longer visitation sequences, which in turn may increase the opportunity for geitonogamy (Hodges 1995).

Visitation frequencies and pollen load size

Our method may overestimate geitonogamy in small populations (such as Hammarskog 2000) if it increases pollen limitation when a large proportion of flowering individuals are bagged and (or) emasculated. However, the relatively lower level of pollen export from control individuals in the small population (Hammarskog) indicates that pollinator activity was lower than in the large population (Hönsarvsberget), a situation that may also cause pollen limitation (see Waites and Ågren 2004). Moreover, pollen deposition in control plants after three nights was higher in the large than in the small population, indicating that pollinators in the large population carried larger pollen loads when arriving at a plant individual. Larger pollen loads on pollinators before plant arrival decrease level of geitonogamy (Rademaker et al. 1999).

Although we did not find significant geitonogamy in the large population, it was probably nonzero. Stigmas may have been saturated with pollen quickly and a difference in quantity of pollen import between control and emasculated individuals may have been detectable only after one or a few visits. The size of a pollen deposition depends on pollen load size as well as stigma size and the amount of pollen already deposited on the stigma (Rademaker et al. 1999).

Conclusions

In the present study, level of geitonogamy was higher in the small population than in the large one. This pattern may well have general relevance in animal-pollinated plants. Pollen load size on pollinators probably had an impact on level of geitonogamy, as did pollinator behaviour and pollen carryover. Such factors are probably not independent of population size, but all remain inevitable topics for future basic research in plant population ecology.

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