Selection and Floral Evolution in *Platanthera bifolia* and *P. chlorantha* (Orchidaceae)

BY

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Abstract


Natural selection mediated by pollinators has influenced the evolution of floral diversity of the flowering plants (angiosperms). The scope of this thesis was to study: 1) phenotypic selection, 2) mating systems, and 3) floral shifts involved in plant speciation. Model plant species were Platanthera bifolia and P. chlorantha (Orchidaceae). These orchids are moth-pollinated, strictly co-sexual (bisexual flowers), and produce a spike that displays 10-20 white flowers.

I explored the influence of characters on plant fitness by using multiple linear regressions. Pollen removal (male fitness) and fruit set (female fitness) increased with more flowers per plant in three P. bifolia populations. There was selection towards longer spurs in a dry year when average spur length was shorter than in normal-wet years. Female function was sensitive to drought, which enabled an application of the male function hypothesis of floral evolution (Bateman's principle). The results show that selection may vary between populations, years, and sex-functions.

I examined inbreeding by estimating levels of geitonogamy (self-pollination between flowers of an individual) with an emasculation method in two P. bifolia populations. Geitonogamy did not vary with inflorescence size. Levels of geitonogamy was 20-40% in the smaller, but non-significant in the larger population. This may relate to lower number of possible mates and pollinator activity in the smaller population.

Platanthera bifolia exhibits the ancestral character state of tongue-attachment of pollinia on the pollinator. Its close relative P. chlorantha attaches its pollinia onto the pollinator's eyes. To explore the mechanism of a floral shift, pollination efficiency and speed was compared between the two species. The results showed no differences in pollination efficiency, but P. chlorantha had faster pollen export and import. Efficiency of pollination in terms of speed may cause floral shifts, and thus speciation.

Key words: Evolution, phenotypic selection, male function hypothesis, mating systems, floral shifts, orchids, pollination, moth pollinators, Platanthera.

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Ingen blomma liknar denna
ingen blomma står så ensam.
Dold i djupa gräset
eller gömd i ljungen
lyfter den sitt vita ljus.
Rör den inte
bryt den inte!
På sin spåda stängel bär den
hela sommarnattens ljus.

(Ur Nattviol av Ebba Lindquist)
Preface

This thesis is based on the following four papers, which will be referred to in the text by their respective Roman numerals.


II  Maad, J. and Alexandersson, R. Patterns of selection vary with resources and differ between sex-functions in *Platanthera bifolia* (Orchidaceae). (Manuscript).

III  Maad J. and Reinhammar, L. G. Influence of population size and inflorescence display on geitonogamy in *Platanthera bifolia* (Orchidaceae). (Manuscript).


Paper I has been reproduced with the permission from the publisher.

In paper II, the co-author and I did the planning of the study together, while the fieldwork was carried out mainly by the co-author. Concerning paper III, I planned and carried out the fieldwork together with the co-author. The data collection in paper IV was done mainly by the co-author on *Platanthera chlorantha* and by me on *P. bifolia*. In all four studies, I was responsible for all of the statistical analyses as well as the writing, with continuous support from co-authors and my supervisor L. Anders Nilsson.
Sammanfattning på svenska


Hos en del växter kan selektion på blomegenskaper skilja sig mellan blommans hanliga och honliga könsfunktion. Det kan bero på att antalet avlade frön (hanlig framgång) begränsas främst av konkurrens mellan pollen, medan antalet producerade frön (honlig framgång) begränsas främst av mängden resurser som står till buds av till exempel vatten och näring. Denna konflikt mellan könen kallas ”Hanfunktionshypotesen” (Bateman’s princip) och kan leda till att en del av en individ blommor exporterar pollen, men inte sätter frön. Då kan selektion ske starkare genom växtens hanfunktion än honfunktion.

Blommans egenskaper och samspelet med pollinatören har betydelse även för växtens parningssystem. Hos många växter överförs en del pollen från ståndare till pistiller inom samma individ (självpollination). Frösättningen och frökvaliteten blir ofta sämre om växten självbefruktas jämfört med korsbefruktas, vilket har lett till att många växter har anpassat blommans egenskaper för att undvika självpollination.

Selektion förmedlad av pollinatörer kan ge upphov till nya växtarter med nya blomegenskaper. Förändringar i blomman kan till exempel ske genom anpassningar till nya pollinatörer. Selektion förmedlad av pollinatörer anses ha gett upphov till mycket av den stora variationen i blommans egenskaper (t. ex. färg, form och doft) som blomväxtena uppvisar.

I min avhandling har jag studerat selektion, parningssystem och förändringar blomman i samband med artbildning. Jag har använt nattviol (Platanthera bifolia) och grönvit nattviol (P. chlorantha) som modellväxter. Båda arterna är nattfjärils-pollinerade orkidéer som producerar två ovala blad och ett ax bestående av 10-20 vita blommor.

Selektion och hanfunktionshypotesen hos nattviol


Selektion och hanfunktionshypotesen hos nattviol


Självpollination inom individen


Förändringar i blomman i samband med artbildning


Eftersom blomevolution sker när blomman anpassas till pollinatörer, resonerade jag att skillnader i pollinationseffektivitet och -hastighet kan ge ledrådar till hur förändringar i blomman i samband med artbildning kan uppkomma. Pollinations-effektivitet är till exempel pollenexport och -import per insektsbesök. I min studie fann vi ingen skillnad pollinationseffektivitet, men P. chlorantha var snabbare att exportera...
och importera pollen än P. bifolia. Detta berodde främst på skillnader i pollinatör-aktivitet mellan studiepopulationerna, och det är oklart om det finns generella skillnader i pollinationshastighet mellan arterna. Ökad pollinationshastighet kan ge fördelar för både hanlig och honlig funktion, vilket kan resultera i ändringar i blommans morfologi som leder till artbildning.

Introduction

The interaction between flowering plants and pollinators, “the secret of nature” (Sprengel 1793), has fascinated biologists for more than 200 year. Flowers often offer nectar or other types of reward to attract insects or other pollinators. When visiting flowers a pollinator may transfer pollen from anthers to stigmas. If a stigma receives compatible pollen the plant may eventually produce seeds. Many flowering plants are dependent on pollinators for their reproduction, which has lead to adaptations of the floral traits to fit specific pollinators. The way different pollinators drive natural selection on plants has since the foundation of evolutionary biology been considered to have shaped the great diversity in floral variation among animal-pollinated angiosperms (Darwin 1862).

Selection in plants

Selection occurs when individuals with certain trait values have higher fitness (lifetime reproductive success) than others within a population. For example, plant individuals with large inflorescences tend to produce more seeds (female fitness) and sire more seeds (male fitness) than individuals with small inflorescences (Schemske 1980, Firmage and Cole 1988, Cruzan et al. 1988, Schmid-Hempel and Speiser 1988, Campbell 1989, Johnston 1991, Conner et al. 1996). For natural selection to operate on a character there has to be 1) variation in fitness and the character among individuals, 2) a relationship between fitness and the character values, and 3) heritability of the character. If the two first criteria are fulfilled but the heritability is unknown, which is usually the case in selection studies, there is at least phenotypic selection acting, but the response to selection in the next generation will be unknown (see Endler 1986, Willis 1996).

Selection may be visualized as a curve or a surface describing the relationship between fitness and character values (Schluter 1988, Phillips and Arnold 1989, Schluter and Nychka 1994). Usually such relationships are estimated by using linear regressions, and the generated selection gradients (regression coefficients) are measures on the strength of selection (Lande and Arnold 1983). Selection may be directional when either lower or higher character values are associated with higher fitness; for example when plants with larger flowers produce more seeds than small-flowered plants, as has been documented in, e.g., Mimulus guttatus (Schrophulariaceae) and Ipomopsis aggregata (Polemoniaceae) (Campbell et al. 1991, Willis 1996). Curvature selection is when the selection curve or surface is not straight and includes stabilizing and disruptive selection sensu Endler (1986). Cresswell (2000) manipulated style length of Brassica napus flowers and detected stabilizing selection, i.e. flowers with medium style length had the highest fitness. Stabilizing selection is believed to maintain the architectural invariability of animal-pollinated flowers.
(Cresswell 2000). Disruptive selection (that tend to increase variation) is, however, as common as stabilizing selection in wild populations (Kingsolver et al. 2001). If more than one character is analysed, correlational selection may be estimated (Brodie et al. 1995). Correlational selection is when character combinations are associated with higher fitness. In a plant with variation in flower colour and size, e.g., large red flowers and small yellow flowers may have higher reproductive success than small red and large yellow ones. Correlational selection is believed to have importance in evolution of pollination syndromes but has hitherto only rarely been studied (see Armbruster et al. 2000, Herrera 2001). Selection surfaces may be complicated combinations of directional, curvature, and correlational selection components. The directional component is, however, easier to detect and often stronger than the other two types of selection components (Kingsolver et al. 2001).

**Male function hypothesis**

The relation between plant fitness (number of seeds sired and seeds produced) and reproductive characters, such as flower number and morphology, is believed to have impact on sex-allocation and evolution of mating systems (Zhang and Wang 1994, Elle 1999, Burd and Callahan 2000, Elle and Meagher 2000, Campbell 2000). The fact that reproductive traits such as flower number and size may influence male and female fitness in different ways has been acknowledged by the "male function hypothesis" (Bateman’s principle; Willson 1979, Burd 1994). This hypothesis rests on the assumption that the female function is generally more dependent on resources, such as water and nutrients, than the male function because the production of seeds and fruits is apparently more expensive than pollen production. The male function hypothesis predicts that some of the individual’s flowers will export pollen but not produce seeds in plants whose seed set is resource-limited but not pollen-limited (Burd and Callahan 2000). This may lead to stronger selection through male than female function on, e.g., flower number and size (Bell 1985, Campbell 1989). Conversely, in pollinator-limited plants selection may act stronger through female than male function on such reproductive characters (Wilson et al. 1994, Johnson 1996).

There is, however, seldom a simple dichotomy between pollinator and resource limitation of seed or fruit set (Calvo and Horvitz 1990, Burd 1994). Since pollinator activity and resource availability often vary within and between flowering seasons and populations, the degree of resource and pollen limitation may vary, as has been documented in the campion *Silene virginica* (Dudash and Fenster 1997). Varying selection with varying resource and pollinator availability has also been found through female function (seed set) in, for example, *Paeonia broteroi* (Herrera 2000) and *Drosera tracyi* (Wilson 1995). There are, however, few studies on how environmental factors affect selection through male fitness and the application of the male function hypothesis (but see Eckhart and Chapin III 1997).

**Mating systems**

The way pollinators move between flowers may have great impact on gene flow within and among plant populations (Ellstrand et al. 1989, Murawski and Hamrick 1991, Ellstrand 1992, Morris 1993). Pollinators may cause transfer of pollen within
individuals (self-pollination) or between individuals (cross-pollination). The number of individuals with which a plant is likely to mate decreases with less population size in isolated populations, which may result in increased selfing (Murawski et al. 1990).

Geitonogamy is self-pollination between flowers on the same plant. The level of geitonogamy in an animal-pollinated plant is dependent on a number of factors including number of flowers available on the plant, structural mechanisms, reward (e.g. nectar) production, and pollinator behaviour (Johnson and Nilsson 1999). A larger inflorescence may promote pollinator visitation (Geber 1985, Ohashi and Yahara 1999), and give the plant disproportionately more reproductive success, as has been observed in the gentian *Sabita angularis* (Dudash 1991). However, if the number of flowers probed in succession on an individual increases there may be more pollen transfer within the inflorescence causing geitonogamy (Geber 1985, de Jong et al. 1992, 1993).

Most of the flowering plants exhibit hermaphroditic flowers. Many of such bisexual plants have become adapted in their floral traits to avoid self-pollination (Darwin 1877, Lloyd and Webb 1986, Webb and Lloyd 1986). They may for example separate male and female function in time like the balsam *Impatiens walleriana* that in each flower presents pollen before stigma (Lloyd and Webb 1986). Most of the species in the Orchidaceae, however, are perfect-flowered hermaphrodites presenting the two sex functions at the same time in each flower (Dressler 1993). Many orchids produce flowers that lack nectar. It has been suggested that this is in order to promote pollinators to leave the plant after visiting one or a few flowers — an adaptation to avoid geitonogamy (see Johnson and Nilsson 1999). Most orchids have pollinia, i.e. packages of pollen, which are transferred by pollinators as whole units (see Johnson and Edwards 2000). This has led to other specialisations in this plant family. Some orchids have evolved pollinarium bending mechanisms — a removed pollinium is not in position to reach a stigma until it has finished its bending and the pollinator by then often has left for another plant individual (Darwin 1862, Johnson and Nilsson 1999, Johnson and Edwards 2000).

*Floral evolution*

The role of pollinators in plant speciation processes has attracted interest since the 19th century (Darwin 1862, Grant 1948, 1994, Grant and Grant 1965, Stebbins 1970, Johnson 1996). Floral evolution is driven according to the simple principle of the locally "most effective pollinator" (Stebbins 1970, Johnson et al. 1998). Therefore, changes in floral morphology do not occur by selection of traits for reproductive isolation, but rather for increased pollination efficiency, speciation being a by-product from differentiation (Grant 1994, Johnson 1996). Pollination is export, dispersal, and import of pollen. Morphological traits in hermaphroditic plants are known to influence efficiency of pollen export and import, measured as reproductive output per flower or individual (Bell 1985, Nilsson 1988, 1992a, Campbell 1989). The plant characters may have impact on total number of pollinator visits or the output per visit (Fulton and Hodges 1999). Especially in plants whose fruit set is not pollen-limited there may be competition among pollen grains to reach stigmas and fertilize ovules (Spira et al. 1996), and fast export of pollen may be advantageous (Stanton 1994). Fast import of
pollen to stigmas may also be advantageous if flowers are costly to keep attractive (Schoen and Ashman 1995, Galen et al. 1999, Luyt and Johnson 2001).

Orchidaceae are a family that is florally highly radiated, apparently due to interactions with pollinators (Darwin 1862, van der Pijl and Dodson 1966, Ornduff 1969, Nilsson 1992b, Dressler 1993, Johnson 1996, Johnson and Steiner 2000). Orchid flowers generally have their single fertile anther and stigma fused together into a column. In Platanthera (see Fig. 1) and many other orchid genera the anther contains two pollinia (pollen packages). Each pollinium is attached to a viscidium, thus forming a pollinarium, that is adapted to fasten to the pollinator; a pollinarium is removed and transported as a whole unit. Orchids adapted to moth- and butterfly-pollination usually place their pollinaria on the proboscis or the eyes (rarely the legs) of the pollinator, since these are the only sites without scales (Nilsson 1983, Johnson and Bond 1994, Johnson and Edwards 2000).

Some floral characters have been shown to be evolutionary labile and easily shifted when subjected to selection (Goldblatt et al. 1995, Barrett et al. 1996, Sakai et al. 1997, Johnson et al. 1998), and column morphology seems to be such a character in

Fig. 1. A) and B) Platanthera bifolia (Orchidaceae), C) Platanthera chlorantha
Platanthera (Hapeman and Inoue 1997). Within Platanthera a floral construction that places the pollinaria on the eyes of the pollinator is usually a derived character state and has arisen from tongue-attachment at least seven times (Hapeman and Inoue 1997). As has been documented by Darwin (1862) and Nilsson (1983), P. bifolia with a narrow column and short distance between the viscidia is adapted for tongue-attachment of pollinia on pollinators (Fig. 2). The column of P. chlorantha, in contrast, is wide and the viscidia are situated relatively distant apart — an adaptation for eye-attachment of pollinia on the pollinator (Fig. 2).

Scope of this thesis

Paper I and II document phenotypic selection through male and female function in natural populations of the hawkmoth-pollinated Platanthera bifolia. In paper I, I explored fitness surfaces, i.e. investigate the complex relationship between plant fitness (fruit set and pollinium removal) and four plant characters, by using standard and new statistical methods. In paper II, I investigated how resource limitation may alter selection pressures on pollinator attraction characters and discuss how the male function hypothesis of floral evolution (Bateman’s principle) may be applicable.

In paper III, I investigated how geitonogamy, i.e. self-pollination between flowers of the same individual, relates to population size and inflorescence display in Platanthera bifolia. Geitonogamy has shown to be higher in larger than smaller
inflorescences of some plants (de Jong et al. 1992, 1993). There are, however, no studies that have investigated geitonogamy in relation to population size before.

The scope of paper IV is the mechanism of floral shifts involved in speciation. I studied the closely related Platanthera bifolia and P. chlorantha. Platanthera bifolia exhibits tongue-attachment of pollinia on pollinators, a character state that previously has been found to be the ancestral state (Hapeman and Inoue 1997). P. chlorantha is adapted to eye-attachment of pollinia, the derived character state. I reasoned that differences in pollination efficiency between the two species could give clues how such a divergent floral morphology of two sister taxa may evolve.

**Methods**

The present investigations were based on fieldwork in populations of the two nocturnally moth-pollinated species Platanthera bifolia (L.) L. C. Rich. and P. chlorantha (Custer) Reichb. (Table 1). Both study species are terrestrial orchids that produce inflorescence spikes with white, nectariferous, spurred, nocturnally fragrant flowers (Fig. 1). The two species differ in the column morphology: P. bifolia having the viscidia close to each other and a small stigma, and P. chlorantha having the viscidia distant apart and a large stigma (Fig 2).

In paper I and II, which both focus on phenotypic selection in Platanthera bifolia, characters and fitness were measured on individual plants. Male fitness was estimated as number of pollinia (pollen packages) removed and female fitness as number of fruits produced. The characters measured in paper I was mid-flowering date (i.e. the day in the middle of an individual’s flowering time), number of flowers, stalk length (plant height minus spike length), and spur length. In paper II, the aim of which

<table>
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<tr>
<th>Species</th>
<th>Population and province</th>
<th>Position</th>
<th>Year and paper</th>
<th>Vegetation description</th>
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<tr>
<td>P. b.</td>
<td>Lillberget, Västerbotten</td>
<td>N 64°00'; O 19°27'</td>
<td>1994–1995 (II)</td>
<td>Logged pine forest</td>
</tr>
<tr>
<td>P. b.</td>
<td>Yttre Hemberget, Västerbotten</td>
<td>N 63°54'; O 19°54'</td>
<td>1995 (II)</td>
<td>Coniferous forest rich in herbs</td>
</tr>
<tr>
<td>P. c.</td>
<td>Skogsby, Öland</td>
<td>N 56°38'; O 16°20'</td>
<td>1992 (IV)</td>
<td>Open alvar grassland</td>
</tr>
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was to explore differences in selection pressures through male and female function under resource limitation, I measured the same characters except mid-flowering date. To evaluate which characters were subjected to selection multiple linear regression was used — a method which disentangles direct from indirect effects of variables (see Lande and Arnold 1983). Directional, curvature (e.g., stabilizing/disruptive), and correlational (i.e. favouring combinations of traits) selection was investigated through male and female fitness in natural populations in the provinces of Dalarna (I) and Västerbotten (II). To be able to compare selection gradients between characters, populations, and years, the regression analyses was done on relative fitness (absolute fitness divided by mean absolute fitness) and plant characters that had been standardized to unit variance (see I and Kingsolver et al. 2001). In paper I, which focused on selection surfaces, differences in selection patterns between years were evaluated by using ANCOVAs for male and female function. In addition to these parametric methods, the selection surface was explored (paper I) by using projection pursuit regression – a nonparametric surface fitting method outlined for evolutionary studies by Schluter and Nychka (1994).

In order to examine if geitonogamy level is related to inflorescence size and population size, I used an emasculation method, i.e. artificial removal of pollinia (paper III). The study was carried out during two years in the small population and one year in the large population (Table 1). A subset of P. bifolia individuals was isolated until most flowers had opened. Then half of the individuals were chosen randomly for the emasculation treatment. To estimate the degree of geitonogamy, the amount of pollen imported to stigmas was compared between emasculated and control individuals by using ANOVA/ANCOVA and Sign rank test for paired comparisons. To examine if geitonogamy was higher in larger inflorescences, cross products of inflorescence size and emasculation treatments were included in the ANOVAs/ANCOVAs.

In paper IV, which aimed at exploring the mechanisms of floral shift from tongue- to eye-attachment of pollinia, I studied individual flowers of Platanthera bifolia and P. chlorantha and compared the pollination efficiency of the both sex functions. Each flower was checked daily for previous-night pollinium removals and pollen depositions on stigmas. The pollination efficiency was calculated on a flower basis, as pollen export and import per visit-night until the eventual pollen export/import was reached. A visit-night was a night when a flower experienced pollen export and/or import. If, e.g., a flower exported the two pollinia at different nights and had pollen imported during a third night, the pollen export efficiency per visit-night was 1 (2 pollinia /2 nights) and pollen import efficiency per visit-night was 1/3. Additionally, the speed of pollination was estimated. Speed of pollen export was 0 if no pollinium was removed or the number of pollinia removed (1 or 2) divided by age of the flower at the last removal. Speed of pollen import was 0 if no pollen import or 1 divided by the age of the flower at pollen import.
Results and Discussion

Phenotypic selection in *Platanthera bifolia*

General patterns of selection

The major target of selection in *Platanthera bifolia* was on plant size. In all three years of study in Dalarna (I) and in one of two years of study in Västerbotten (II), there was significant strong selection towards larger inflorescences through both male and female fitness (Fig. 3). Such selection is hardly surprising and has also been documented for a number of other plant species (Schemske 1980, Firmage and Cole 1988, Cruzan et al. 1988, Schmid-Hempel and Speiser 1988, Campbell 1989, Johnston 1991, Fritz and Nilsson 1996). In *P. bifolia* and other plants, such a relationship between flower number and fitness occurs, partly, because flower number sets a limit to the amount of pollen removed and fruits produced, but also because large inflorescences attract more insect visitors than small ones.

Selection varies between years

Interestingly, increasing spur length had positive effect on male and female fitness in Dalarna (I) and on female fitness in Västerbotten (II) during the season of 1994 (Fig. 4), a relatively dry year when average spur length was shorter than in other study years. This selection may have occurred because the spur of short-spurred plants was too
short relative to the tongue length of the major hawkmoth pollinator (*Hyloicus pinastri* [L.]) for optimal pollen export and import. In Västerbotten, the impact of the drought was so severe for female fitness in 1994, that 86% of the individuals failed to set fruits (II). An indirect relation between spur length and fitness may thus have been generated via lack of water because spur length and fruit maturation are both dependent on turgor.

The fact that selection on spur length was only detected in the dry year of study (I, II) raises an interesting general issue. If selection is present only in stressful years when phenotype is skewed towards small size, then evolution may proceed in "unexpected ways" in terms of observations made in an average year. Nectar spurs may actually be longer than necessary, because selection is operating only when they are dwarfed (for theoretic models, see Gomulkiewicz and Kirkpatrick 1992). This may result in absence of selection for longer spurs in most years, and may be one reason why such selection is rare in natural populations (see Alexandersson and Johnson 2002).

The extreme weather of 1994 additionally caused correlational selection in Dalarna, which was not found in the other years of study (I). Among small individuals (apparently more sensitive to drought than large ones), early-flowering plants had higher male and female fitness. There is an indication that small plants in Västerbotten may also be more resource-limited than large ones, as there was a strong (but non-significant) accelerated gain in fruit set with extra flowers (II). Mattila and Kuitunen (2000) have, similarly, found that small but not large plants are nutrient-limited within a single season of a *P. bifolia* population, but effect of added water was not tested.

![Fig. 4. Strength of directional selection on spur length through A) male function and B) female function in three Swedish populations of *Platanthera bifolia*, as indicated by standardized selection gradients (solid bars) and standard errors (thin bars). See also description of Fig. 3.](image)
Availability of resources and pollinators are factors that may vary spatially and temporally (Burd 1994), and thus cause variable selection patterns.

Sex-differential selection

According to the male function hypothesis (Bateman’s principle), selection on flower number should be stronger through male than female function when fruit set is resource-limited (Burd and Callahan 2000). I observed sex-differential selection on flower number in Västerbotten in an exposed *Platanthera bifolia* population during the unusually dry year of 1994 when fruit set probably was limited by water (II). Selection on flower number was significant through male but not female function, indicating an application of the male function hypothesis. However, these selection gradients did not differ in magnitude (Fig. 3). In the same population and year, there was also sex-differential selection on spur length (Fig. 4), selection acting strongly and significantly through female function only. There are additional results from the ANCOVAs and projection pursuit regressions that indicate a sex-differential selection (I): Generally, male function was influenced by flower number only, but all measured characters, flower number, spur length, stalk length, and flowering time, had some influence on female function. It seems that male function improves its efficiency through enhanced floral attraction, while female function additionally improves its efficiency by adjusting floral morphology, for example spur length, to increase efficiency per pollinator visit. The results indicate that the male function hypothesis may be valid even at intermediate pollination levels, as has been found by modelling (Stanton 1994).

Influence of population size and inflorescence display on geitonogamy

On theoretical grounds, increased selfing would be expected with decreased population size of self-compatible plants (Murawski et al. 1990). I found different levels of geitonogamy (self-pollination between flowers of the same individual) in the two *Platanthera bifolia* populations (III) (Fig. 5). In the smaller population, the import levels of pollen after one week was 22 and 38% lower in emasculated plants, in two different years, percentages that correspond to geitonogamy levels. In the larger population, however, there was no significant geitonogamy. I found no relation between level of geitonogamy and inflorescence size in either of the populations. Geitonogamy has been observed to increase with inflorescence size in other plants (Dudash 1991, de Jong et al. 1992, Harder and Barrett 1995).

There are several factors that may have impact on levels of inbreeding in animal-pollinated plants. Such factors are, for example, pollen carryover, pollinator abundance, pollinator behaviour, and number of possible mates. Pollen limitation may be generated in populations with low pollinator activity, which seems to be the case in the smaller population of the present study (III). Pollen limitation may result in small pollen loads on the pollinators, a situation that may increase level of geitonogamy (Rademaker et al. 1999). Additionally, pollen carryover may vary with amount of pollen already deposited on the stigma, which may lead to lower levels of geitonogamy in populations with high pollinator activity, as in the large population of study. The observed difference in level of geitonogamy between the populations in the present study was probably related to differences in levels of pollen removal and deposition.
that were higher in the large population.

The harmful effects of close inbreeding (inbreeding depression) have been observed in both animals and plants (Charlesworth and Charlesworth 1987). Inbred plant individuals may have reduced size or survival at various life stages, or reduced fertility (Schoen 1983, Dudash 1990, Fenster 1991, Dudash et al 1997). I have observed higher frequencies of floral malformations in the smaller than the larger population of the present study (J. Maad, unpublished data), malformations that may reduce male and/or female reproductive success. Developmental instability caused by inbreeding has been suggested to cause novel options for floral function, which may result in new modes of pollination (Levin 1970).

Floral shifts from tongue- to eye-attachment of pollinia

Efficiency of pollination

The *Platanthera bifolia* and *P. chlorantha* populations did not differ in efficiency of pollen export (male function) or pollen import (female function) per visit-night (IV). However, *P. bifolia* flowers exported pollinia during the same visit-night to a higher extent than *P. chlorantha*, apparently because it presents the viscidia close together as single target-surface for firm attachment on the pollinator’s tongue. The viscidia of *P. chlorantha* are, in contrast, positioned relatively distant apart from each other and often
only one eye of the pollinator seems to effectively touch a viscidium, which lead to higher levels of one by one removals of pollinia, as has been observed previously by Darwin (1862) and Nilsson (1983). Higher pollen import efficiency per pollinator visit is predicted for *P. chlorantha* because of its larger stigma. Nazarov and Gerlach (1997) documented that *P. chlorantha* had larger size of pollen loads deposited on stigmas than *P. bifolia*.

Speed of pollination may be under selection if there is competition among males to reach the stigmas first or if it is costly to keep flowers attractive to pollinators (Stanton 1994, Schoen and Ashman 1995, Snow et al. 2000). *Platanthera chlorantha* was two and three times more efficient than *P. bifolia* to export and import pollen, respectively, in terms of speed (IV). This was mainly because of differences in pollinator visitation frequencies, which may vary with a number of factors including attractiveness of the plants and pollinator abundance.

**Floral evolution**

Johnson (1996) stated the hypotheses that there should primarily be selection through efficiency of male function if the plant population is pollinator-saturated and primarily through efficiency of female function if the species is pollinator-limited. If species with the derived character of eye-attachment generally are more pollen-limited than tongue-attached adapted species has, however, never been tested.

The level of geitonogamy is probably generally higher in *P. bifolia* than in *P. chlorantha* because of a stronger pollinarium bending mechanism in the latter species (see III, Johnson and Nilsson 1999). *Platanthera bifolia* has probably a higher pollen carryover fraction than *P. chlorantha*, which may be one reason (in addition to constraints such as pollinarium morphology) why *P. bifolia* has not evolved an efficient pollinarium bending mechanism (III).

It has been suggested that *Platanthera bifolia* and *P. chlorantha* as well as two other *Platanthera* species-pairs evolved sympatrically from ancestors with intermediate or polymorphic column morphology (Hapeman and Inoue 1997). Such an evolutionary history is however unlikely, because of the delimited solid surfaces for firm placement on the moth’s head (virtually only tongue or eyes) make any such intermediate ancestor species improbable (Nilsson 1983). No species or population with polymorphic column has yet been reported from the Orchidaceae. One possible explanation for shift to eye-attachment, which seems to be the direction of a floral shift causing the divergence of *P. bifolia* and *P. chlorantha* (see Hapeman and Inoue 1997), is that a tongue-attaching population has been exposed to a new pollinator, for instance at the margin of its distribution or when colonizing (see, e.g., Johnson 1997).

**Conclusions**

In this thesis I have studied selection mediated by pollinators, mating systems, and mechanisms for floral shifts caused by adaptation to pollinators. I found that the major target of selection in *Platanthera bifolia* is inflorescence size through both male and female fitness, which probably is general for many plants (I, II). The results support that the male function hypothesis may have some relevance even at intermediate levels of pollen limitation, as has been proposed by Stanton (1994). The results show that
there was variation in selection between years, populations, and sex functions, which illustrates the importance of carrying out selection studies for more than one year and to estimate selection through both male and female function.

I found higher levels of self-pollination in a small than a large population (III). There are many factors that may be related to plant population size, including pollinator abundance and behaviour, which all deserves to be investigated in relation to level of inbreeding in plants. The consequence of high levels of self-pollination in a self-compatible, outcrossing plant population may be higher inbreeding depression at various life stages (see Charlesworth and Charlesworth 1987).

In paper IV I suggest that pollinator-mediated differences in efficiencies of sex functions may be the actual mechanisms of floral shifts in Platanthera. There are several differences in pollination efficiency between the moth-pollinated Platanthera bifolia and P. chlorantha that enlighten the mechanism of floral shifts and thereby speciation. However, it is uncertain if these differences are general at the species level. Geitonogamy may have played a role in floral evolution of P. bifolia and P. chlorantha since P. chlorantha has evolved the mechanism of pollinarium bending that prevents geitonogamy (III). Pollinator-mediated improvement in efficiencies of sex functions is probably the actual mechanism of floral shifts in Platanthera as well as in other plant groups subject to pollination limitation (see Johnson 1996).

I have studied phenotypic selection, mating systems, and mechanisms of floral shifts, which is fundamental if we are to understand the mechanisms maintaining and increasing diversity of plants.

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