

Effects of variation at the flower-colour *A* locus on mating system parameters in *Ipomoea purpurea*

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Abstract

Although alleles at both the *W* and *A* loci in the common morning glory, *Ipomoea purpurea*, produce similar white-flowered phenotypes, these alleles differ by over an order of magnitude in average frequency. In this initial attempt to determine the causes of this difference, we employed artificial arrays of plants to estimate mating system characteristics (total siring success, selfing rates and contribution to the outcross pollen pool) for the homozygous pigmented and white-flowered genotypes at the *A* locus. This experiment demonstrates that: (1) at both low and high frequencies, white-flowered plants were visited by pollinators at the same rate as plants with pigmented flowers; (2) at both frequencies, the *a* allele exhibited a greater total siring success (self and outcross pollen) than the *A* allele; (3) individuals of both genotypes contributed equally to the outcross pollen pool; and (4) *aa* plants may have a higher selfing rate than *AA* plants. Coupled with minimal inbreeding depression in *I. purpurea*, these observations indicate that the allele producing white flowers enjoys a transmission advantage that would tend to cause this allele to increase in frequency. This transmission advantage is very similar to that shown previously to be operating on the white-flowered allele at the *W* locus, although the specific causes of the advantage appear to differ between loci. The frequency difference between the two alleles is thus not likely to be due to differences in the effect of flower-colour variation on transmission. Rather, substantially greater deleterious pleiotropic effects associated with the white-flower *a* allele is likely to be the primary cause of the frequency difference.

Keywords: anthocyanin genes, male reproductive success, mating systems, pleiotropy, population genetics, selfing

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Introduction

Pleiotropy has long been recognized as a common characteristic of allelic variation (Wright 1968). Moreover, theoretical analyses have demonstrated that the magnitude of pleiotropy, manifested as genetic correlations among characters, or as costs associated with adaptation, can profoundly influence evolutionary trajectories (Lande 1979; Simms & Rausher 1987). Nevertheless, documented examples of the evolutionary consequences of pleiotropy are rare. Ideally, empirical analyses of the evolutionary effects of pleiotropy would compare the evolutionary trajectories of allelic variants (at the same or different loci) that differ in their degree of

pleiotropy, a situation that rarely presents itself in natural populations. Such a situation, however, is presented by allelic variation for genes affecting flower colour in the common morning glory, *Ipomoea purpurea*.

Natural populations of *I. purpurea* in southeastern North America are polymorphic at several flower-colour loci (Ennos & Clegg 1983; Epperson & Clegg 1987a). The most common flower colours are blue and pink. However, mutations at two loci, *A* and *W*, produce phenotypically similar white flowers when homozygous. The principal obvious phenotypical difference is that flowers of *ww* plants are white with pigmented rays, whereas flowers of *aa* plants lack such rays. Despite the similarity in phenotype, these alleles exist at very different frequencies in natural populations. The *w* allele, which appears to have been produced by a mutation in a transcription factor that activates anthocyanin structural genes (Tiffin *et al.* 1998), is commonly present in *I. purpurea* populations and may

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achieve allele frequencies up to 0.4–0.5 (Epperson & Clegg 1986; R. E. Miller pers. comm.). By contrast the *a* allele, which was produced by transpositional inactivation of Chalcone Synthase, the first structural gene in the anthocyanin pathway (Koes *et al.* 1994), is found only rarely in populations and seldom achieves a frequency of greater than 0.01. In particular, in a sample of 15 populations from Georgia, North Carolina and Virginia, the *a* allele was present in only one population, at a frequency of approximately 0.1 (Coberly 2003).

Two hypotheses can be offered to explain this difference in gene frequency between the *a* and *w* alleles: (1) the two loci differ in the magnitude of the direct effect of flower colour on pollinator transmission, despite the similarity in white-flowered phenotype produced by the two alleles; and (2) the loci differ in the magnitude of deleterious pleiotropic effects. It is unlikely that the *a* allele is at low frequency because it is advantageous but arisen very recently. In a sample of 15 copies of the *a* allele from North Carolina and Georgia, all copies were nearly identical at the molecular level, suggesting a single origin. In all copies, the inactivating transposon is located at the same position, and the sequences differ by at most one nucleotide substitution, although some copies exhibit a single deletion (Coberly 2003). Because this single *aa* phenotype has been present in eastern North America for at least 200 years (Coberly 2003), there has presumably been ample time for any positive selection to increase its frequency above that observed.

The experiments reported here constitute an initial investigation into the validity of Hypothesis 1. In particular, these experiments were designed to determine whether these two alleles have similar direct effects on the mating system of *I. purpurea*. Previous investigations have shown that variation at the *W* locus influences mating system characteristics. Specifically, compared to plants with pigmented flowers (*WW* and *Ww* genotypes), white-flowered *ww* plants, when rare, are visited less frequently by pollinators (Brown & Clegg 1984; Rausher *et al.* 1993; Fry & Rausher 1997). Presumably due to this reduced visitation, *ww* plants also have a substantially higher selfing rate (Brown & Clegg 1984; Rausher *et al.* 1993; Fry & Rausher 1997) although, somewhat paradoxically, they exhibit no detectable pollen discounting (Rausher *et al.* 1993; Fry & Rausher 1997). This combination of effects, along with minimal inbreeding depression (Chang & Rausher 1999), is expected to cause the *w* allele to increase in frequency when rare because of this allele enjoys a transmission advantage through pollen (Fisher 1941; Lloyd 1979; Holsinger *et al.* 1984). Although the factors that prevent fixation of this allele are not understood completely, both the under-visitation of *ww* plants by pollinators and their elevated selfing rate disappear when the *w* allele reaches frequencies of around 0.5, thus eliminating the selection that acts to

increase the frequency of the *w* allele when it is rare (Epperson & Clegg 1987b; Rausher *et al.* 1993).

In contrast with the *w* allele, there have been no previous investigations of selection affecting *A*-locus variation in nature. In this investigation, we demonstrate that the effects of the *a* allele on transmission during mating are similar to those observed for the *w* allele. Differences between the frequencies of these two alleles thus cannot be explained by Hypothesis 1.

Methods

Experimental organisms

The tall morning glory, *Ipomoea purpurea* (L.) Roth (Convolvulaceae) is a weedy annual that is common in agricultural fields and other disturbed sites throughout southeastern North America. Plants typically germinate between late May and August, depending on the timing of soil disturbance and rainfall, and are killed by the first frost, which ranges from late September to early December in the piedmont of North Carolina. The showy flowers open at dawn and close and wither by late morning of the same day. The primary pollinators in this region are bumblebees (*Bombus pennsylvanicus*).

Experimental arrays

The overall design of this experiment was similar to that of Epperson & Clegg (1987b) and Rausher *et al.* (1993). On each of 17 days between 11 September and 25 October 1995 an experimental array was set up in a field near Durham, North Carolina. The field was mowed every 2 weeks during the experimental period to prevent flowering by native *I. purpurea* and pollen flow from such plants into the experimental arrays. Each array consisted of 20 *I. purpurea* plants. Experimental plants were grown in the Duke University biology greenhouse and transported to the experimental site 1 day prior to each experiment. All but one flower bud was removed from each plant, and half the plants of each genotype were emasculated. The plants were then placed on a 5 × 4 grid, with 1-m spacing between rows and columns, watered and left overnight. On the following day, plants were left in the field until all flowers had withered, whereupon all flowers were individually tagged and the plants were returned to the greenhouse to allow seeds to develop. All seeds produced (an average of 2.0 per plant) were grown to flowering to score flower-colour genotype, which are distinct for the three genotypes at the *A* locus (although pigmented, *Aa* plants have less intense pigmentation than *AA* plants). Counts of offspring genotypes were pooled over replicates after determining that there was no detectable heterogeneity of genotype proportions among days.

Two types of experimental arrays were established, corresponding to two experimental treatments: dark-majority and white-majority. In the dark-majority arrays, 16 of the experimental plants were *AA*, while four were *aa*. In the white-majority arrays, 16 plants were *aa* and four were *AA*. The positions of the 20 plants in each array were assigned randomly. These two treatments were established to determine whether the values of mating-system parameters depend on genotype frequency, as has been found for the *W* locus (see above).

The plants used in these experiments were from inbred lines descended from a single population in Orange County, North Carolina. Three *aa* (white-flowered) lines and 13 *AA* (pigmented) lines were used. Approximately 100 seeds from each *aa* line and 40 seeds from each *AA* line were planted in the greenhouse to form a pool of experimental plants. Plants for each array were chosen randomly from plants in the pool that were of the appropriate genotype and that had at least one flower bud that would open the next day.

Pollinator observations

On days when the morning temperature exceeded 7 °C, pollinator visitation was observed in the arrays beginning when flowers opened (between 6:30 and 10:30 a.m. depending on temperature) and ending 4 h later. Each pollinator was observed for 10 min or until it left the array, whichever occurred first. During observation, each flower visited and the time of visit was recorded.

Estimation of net siring success

For each flower-colour genotype in each array type we estimated the net siring success, which is the total number of seeds (outcrossed and selfed) sired by all plants of that genotype. Specifically, for unemasculated plants we estimated the parameters ω_A and ω_a , which are, respectively, the proportions of seeds from *AA* and *aa* maternal plants that were sired by *A* pollen. The maximum likelihood estimates of these parameters are simply:

$$\hat{\omega}_A = \frac{\text{Number of } AA \text{ offspring of } AA \text{ maternal plants}}{\text{Total number of offspring of } AA \text{ maternal plants}}$$

and

$$\hat{\omega}_a = \frac{\text{Number of } Aa \text{ offspring of } aa \text{ maternal plants}}{\text{Total number of offspring of } aa \text{ maternal plants}}.$$

For a population in which the proportions of *AA* and *aa* individuals are ψ and $1 - \psi$, respectively, the expected proportion of seeds sired by *AA* individuals, averaged over *AA* and *aa* maternal parents, is:

$$\bar{\omega} = \psi\omega_A + (1 - \psi)\omega_a.$$

Under the null hypothesis that individual *AA* and *aa* plants are equally successful at siring seeds, it is expected that:

$$\bar{\omega} = \psi \quad (1).$$

To test statistically for departure from this null hypothesis, we first calculated the unrestricted likelihood of the data, \hat{L}_U , assuming no constraint on ω_A and ω_a . This likelihood corresponds to the maximum of the likelihood equation:

$$L_U = C(\omega_A)^{N_{AA}}(1 - \omega_A)^{N_{Aa}}(\omega_a)^{N_{aA}}(1 - \omega_a)^{N_{aa}},$$

where N_{ij} is the total number of seeds produced by *ii* mothers that were sired by pollen carrying the *j* allele, and *C* is a combinatorial constant. Under the null hypothesis given by eqn 1, the restricted likelihood, \hat{L}_R , is the maximum of the likelihood equation:

$$L_R = C(\omega_A)^{N_{AA}}(1 - \omega_A)^{N_{Aa}} \left(\frac{\psi}{1 - \psi} (1 - \omega_A) \right)^{N_{aA}} \left(\frac{1 - 2\psi + \psi\omega_A}{1 - \psi} \right)^{N_{aa}}.$$

The standard test statistic:

$$\Lambda = -2 \ln \frac{L_R}{L_U},$$

which has a χ^2 distribution with 1 degree of freedom, was used to determine whether the null hypothesis should be rejected (Hocking 1985).

Estimation of mating system parameters

For each flower-colour genotype in a given array type we estimated and compared two mating-system parameters: (1) outcrossing rate, *t*, the proportion of seeds produced by a maternal plant by outcrossing; and (2) the proportion of outcrossed seeds on a maternal plant that were pollinated by *AA* (pigmented) plants, θ . The parameter θ reflects the relative contribution of *AA* and *aa* plants to outcross pollinations: if θ is greater than the proportion of *AA* plants in the array, those plants contribute more pollen on a per capita basis than *aa* plants to the outcross pollen pool and vice versa.

Parameters were estimated and compared between treatments within an array type using a standard likelihood approach (Rausher *et al.* 1993; Fry & Rausher 1997). In the most general likelihood model, the probabilities, γ_{ij} , that maternal plants of genotype *i* produce offspring of genotype *j* are expressed in terms of the parameters *t*

Table 1 Assumed probabilities, λ_{ij} , of observing offspring genotype j given maternal genotype i

Maternal genotype (i)	Offspring genotype (j)		
	AA	Aa	aa
AA emasculated	θ_A^E	$1 - \theta_A^E$	0
AA not emasculated	$1 - t_A + t_A\theta_A^N$	$t_A(1 - \theta_A^N)$	0
aa emasculated	0	θ_a^E	$1 - \theta_a^E$
aa not emasculated	0	$t_a\theta_a^N$	$1 - t_a + t_a\theta_a^N$

t_i , where $i \in (A, a)$, is the probability an ovule on a maternal plant of genotype i is outcrossed. θ_i is probability that an outcrossed ovule on a maternal plant of genotype m carries A allele. Subscripts: A = genotype AA , a = genotype aa . Superscripts: E = emasculated plant, N = non-emasculated plant. For dark-majority arrays, it is assumed that $\theta_A^N = 7\theta_A^E / (8 - \theta_A^E)$ and $\theta_a^N = 2\theta_a^E / (1 + \theta_a^E)$; for white-majority arrays, it is assumed that $\theta_A^N = 2\theta_A^E / (1 + \theta_A^E)$ and $\theta_a^N = 7\theta_a^E / (8 - \theta_a^E)$ (see text).

Table 2 Results of log-likelihood-ratio tests of hypotheses regarding mating system parameters in white-majority arrays

Comparison	Hypothesis	Restriction on unrestricted model	Additional restriction on restricted model	Λ
1	Equal AA male outcross success to AA and aa stigmas	None	$\theta_A^E = \theta_a^E$	23.14***
2a	Equal male outcross success to AA stigmas	None	$\theta_A^E = 0.2$	15.18***
2b	Equal male outcross success to aa stigmas	None	$\theta_a^E = 0.2$	8.00**
2c	Equal male outcross success to all stigmas combined	None	$0.8\theta_a^E + 0.2\theta_A^E = 0.2$	1.26
3	Equal selfing rates	None	$t_A = t_a$	2.64*

* $P < 0.1$; ** $P < 0.005$; *** $P < 0.0001$.

and θ as specified in Table 1. Although ideally θ should be measured separately for emasculated and non-emasculated plants, this estimation is not possible for non-emasculated plants because θ cannot be estimated independently of t . For this reason, θ was estimated independently for emasculated plants and was used to infer θ for non-emasculated plants. In the model specified in Table 1, we do not assume that $\theta_A^N = \theta_A^E$ for the dark-majority arrays, but rather than $\theta_A^N = 7\theta_A^E / (8 - \theta_A^E)$. (The superscripts N and E refer to non-emasculated and emasculated plants, respectively.) This corrected assumption is motivated by the fact that while there are eight AA pollen donors for each emasculated plant, there are only seven non-self AA pollen donors for each dark, non-emasculated plant. The justification for this correction is as follows: because for AA maternal plants there are eight AA and two aa pollen donors, the per-capita proportional contributions of these pollen donors to outcrossed ovules on emasculated AA plants are $\frac{\theta_A^E}{8}$ and $\frac{(1 - \theta_A^E)}{2}$ for AA and aa donors, respectively. Assuming individuals of both genotypes contribute the same relative per-capita fractions to outcrossed seeds produced by non-emasculated plants, the proportion of such seeds fertilized by AA individuals is

$$\frac{7\theta_A^E}{8} \bigg/ \left(\frac{7\theta_A^E}{8} + \frac{2(1 - \theta_A^E)}{2} \right) = 7\theta_A^E / (8 - \theta_A^E).$$

Similar corrections for θ_a^N in dark-majority arrays and for θ_A^N and θ_a^N in white-majority arrays are listed in Table 1.

The overall likelihood of observing the data for a particular array is then:

$$L = C \prod_{ij} (\gamma_{ij})^{n_{ij}},$$

where C is a combinatorial constant and n_{ij} is the number of seeds of genotype j produced by maternal plants of genotype i . Estimates of the parameters t and θ were obtained either by analytical solution or numerical iteration using standard likelihood techniques (Edwards 1992) implemented on MATHEMATICA software (Wolfram 1991).

Comparison of parameters between genotypes was accomplished by formulating specific hypotheses (Table 2). To each hypothesis there corresponds a restriction on one or more of the parameters. For example, to test the hypothesis that selfing rates of AA and aa plants are equal, the following restriction is used: $t_A = t_a$. To test this null hypothesis statistically, the maximum likelihood of a model, $L_{R'}$ with this restriction ('more-restricted model') is

Table 3 Bumblebee visitation to white and dark flowers in experimental arrays

Date	Total visits	Visits to white flowers			χ^2
		Expected	Observed	Excess	
Dark-majority arrays					
9/11	28	5.6	4	-	0.57
9/17	38	7.6	6	-	0.42
9/20	38	7.6	8	+	0.03
9/29	58	11.6	11	-	0.04
10/2	44	8.8	11	+	0.69
10/16	5	1.0	1	-	0.00
10/24	9	1.8	0	-	2.25
Pooled	220	44.0	41	-	0.26
White-majority arrays					
9/13	69	55.2	56	+	0.06
9/15	35	28.0	29	+	0.18
9/21	52	41.6	39	-	0.81
10/9	23	18.4	16	-	1.57
10/15	8	6.4	7	+	0.28
10/21	11	8.8	6	-	4.45*
Pooled	198	158.4	153	-	0.93

Total visits is number of visits to both types of flowers. Expected visits to white flowers is total number of visits times proportion of white flowers in array. Excess indicates whether whites were undervisited (-) or overvisited (+) relative to their frequency in array. * $P < 0.05$.

compared to the maximum likelihood, L_U , of an identical model without this restriction ('less-restricted model') by calculating the log-likelihood-ratio statistic:

$$\Lambda = -2 \ln \left(\frac{L_R}{L_U} \right),$$

which has an approximately χ^2 distribution with degrees of freedom equal to the number of restricted parameters (Hocking 1985).

Results

Pollinator visitation

As has been found in previous studies, bumblebees constituted the vast majority of insect visitors to flowers in the experimental arrays: 90% of all visits were by bumblebees. Unlike in previous investigations of the *W* locus, however, there is little indication that bumblebees preferentially visited dark flowers (Table 3). In the dark-majority arrays, on only 4 of the 7 days on which visits were recorded did undervisitation occur. On 2 of the days, whites were visited slightly more often than expected from their frequency. None of these deviations from expectation were statistically significant; nor, when the observations were pooled over days, was the slight undervisitation of whites (41 visits vs. 44 expected) statistically significant. In the white-majority arrays, there was nominally significant

undervisitation of whites on 1 day, but this significance disappears when a Bonferroni correction for multiple comparisons (Rice 1989) is applied. On other days, there was no significant discrimination, and on 3 of 6 days over-visititation of whites occurred. When visits were pooled over days there was very slight, but not significant, undervisitation (153 visits to whites vs. 158 expected).

Net siring success

To the extent that selfing and pollinator constancy occurs, it is expected that ω_A , the proportion of seeds produced by dark individuals that are sired by dark plants, should be greater than ω_a , the analogous proportion for seeds produced by white plants. This expectation is realized in both arrays (Table 4), the differences between ω_A and ω_a being highly significant (Table 5). However, if the *A* locus does not experience selection due to differential transmission through pollen, the overall proportion of seeds in the experimental arrays pollinated by dark plants, $\bar{\omega}$, should equal the proportion of dark plants in the arrays. This expectation was not realized in either array type. In the dark-majority arrays the expected value of $\bar{\omega}$ was 0.8, whereas the maximum likelihood estimate of $\bar{\omega}$ was 0.68. In the white-majority arrays the expected value of $\bar{\omega}$ was 0.2, whereas the maximum likelihood estimate of $\bar{\omega}$ was 0.15. Both these deviations from expectation are significant (Table 5), indicating that net siring success is higher for white plants than for dark plants.

Table 4 Numbers of offspring seeds pollinated by indicated pollen genotype. Numbers are pooled over all replicate for a given array type, maternal genotype and treatment (emasculated vs. unemasculated)

	Array type	White-majority		Dark-majority	
		Maternal genotype	<i>AA</i>	<i>aa</i>	<i>AA</i>
Unemasculated plants	Pollen genotype				
	<i>A</i>	26	9	156	8
	<i>a</i>	21	174	36	42
	proportion <i>a</i>	0.45	0.95	0.19	0.84
Emasculated plants	<i>A</i>	19	14	96	4
	<i>a</i>	21	116	29	3
	proportion <i>a</i>	0.53	0.89	0.23	0.43

Table 5 Estimates of net siring success. $\hat{\omega}_A$ and $\hat{\omega}_a$ are proportions of seeds of *AA* and *aa* individuals, respectively, that were pollinated by *A* pollen. P_1 is probability that $\hat{\omega}_A = \hat{\omega}_a$. ψ is proportion of plants in array that are *AA*. $\hat{\omega}$ is estimated proportion of all seeds in an array pollinated by *A* pollen. χ_1^2 and P_1 are χ^2 value and corresponding probability associated with hypothesis that that $\hat{\omega} = \psi$. s is estimated selection coefficient for transmission advantage associated with *a* allele. All estimates derived from unemasculated plants

Array type	$\hat{\omega}_A$	$\hat{\omega}_a$	P_1	ψ	$\hat{\omega}$	χ_1^2	P_1	s
<i>AA</i> majority	0.81	0.16	<0.001	0.8	0.68	26.9	<0.001	0.47
<i>aa</i> majority	0.55	0.05	<0.001	0.2	0.15	5.8	<0.02	0.31

A rough estimate of the magnitude of selection caused by these differences in net siring success can be obtained by assuming that the relative siring successes for *aa* and *AA* plants are given by 1 and $1 - s$, respectively, where s is the selection coefficient acting against *AA*. Then the proportion of seeds from the experimental array sired by *AA* individuals is related to the selection coefficient in the following way:

$$\bar{\omega} = \frac{\psi(1-s)}{\psi(1-s) + (1-\psi)},$$

which, upon rearrangement, yields:

$$s = \frac{\psi - \bar{\omega}}{\psi(1 - \bar{\omega})}.$$

Inserting estimated values of $\bar{\omega}$ yields estimated selection coefficients of 0.47 and 0.31 for the dark- and white-majority arrays, respectively (Table 5).

Mating system parameters

The greater net siring success for white plants could be due either to a higher selfing rate, a higher outcross success, or both. To determine the relative contribution of each of these factors, we estimated the mating system parameters t and θ for dark and white plants. Unfortunately, we could

not carry out this analysis reliably for the dark-majority arrays because in these arrays, very few seeds (7) were produced by emasculated white plants (Table 4), which means that our estimate of θ parameters would be very unreliable. Consequently, we present this analysis for white-majority arrays only.

We first examined the hypothesis that the frequency of allele *A* in pollen fertilizing outcrossed ovules was the same for both *AA* and *aa* maternal plants, i.e. $\theta_A^E = \theta_a^E$. The estimated frequency of allele *A* in the outcross pollen was approximately four times greater for pollen fertilizing *AA* ovules than for pollen fertilizing *aa* ovules ($\hat{\theta}_A^E = 0.43$ vs. $\hat{\theta}_a^E = 0.11$). The log-likelihood-ratio statistic associated with this difference was highly significant (Table 2, comparison 1). Consequently, when testing subsequent hypotheses, the restriction $\theta_A^E = \theta_a^E$ was not included in either the restricted or unrestricted model.

The second hypothesis we examined is that *AA* and *aa* plants have equal outcross male fitness, i.e. *AA* and *aa* plants contribute equally, on a per capita basis to all the outcross ovules in the experimental array. Because $\theta_A^E \neq \theta_a^E$, we tested this hypothesis separately for each maternal genotype. We thus asked first whether the proportions of outcross pollen carrying the *A* allele that fertilized *AA* and *aa* ovules (θ_A^E and θ_a^E , respectively) differed individually from 0.2, the frequency of allele *A* in the arrays. In both cases, the null hypothesis of equality is rejected with high significance (Table 2, comparisons 2a and 2b). Outcrossed

AA ovules receive a higher frequency of *A* pollen than expected by chance, whereas outcrossed *aa* ovules receive a lower frequency. Despite these effects it is still possible that, averaged over all maternal plants, the per capita contribution to the outcross pollen pool is similar for the two genotypes. The log-likelihood-ratio statistic for this hypothesis, which corresponds to the restriction $0.2\hat{\theta}_A^E + 0.8\hat{\theta}_a^E = 0.2$, is not significant (Table 2, comparison 2c). Thus, although *AA* individuals contribute proportionally more to outcrossed *AA* ovules and proportionally less to outcrossed *aa* ovules, these two effects cancel each other, resulting in no detectable differential contribution to the total outcross pollen pool ($\hat{\theta}^E = 0.2\hat{\theta}_A^E + 0.8\hat{\theta}_a^E = 0.17$).

The third hypothesis we examined is that *AA* and *aa* maternal plants have equal outcrossing rates, to which corresponds the restriction $t_A = t_a$. The maximum-likelihood estimate of outcrossing rate for the *AA* individuals is twice that for *aa* individuals ($t_A = 1.0$ vs. $t_a = 0.52$), and this difference approaches statistical significance (Table 2, comparison 3, $P = 0.1$).

Discussion

Differential pleiotropy as a cause of gene frequency differences

The results presented here indicate that, compared to *AA* plants with pigmented flowers, white-flowered (*aa*) plants have a higher net siring success. Because inbreeding depression is low compared to the estimated selection coefficients associated with pollination success (Chang & Rausher 1999) these results indicate that the *a* allele, like the *w* allele that produces phenotypically similar white flowers, has a net transmission advantage. In other words, at both loci the component of selection operating through the mating system strongly favours alleles that produce white flowers, although the exact cause of this advantage appears to differ for the two loci (see below). It thus appears that the consistently low frequency of the *a* allele in natural populations, compared to the *w* allele, cannot be ascribed to differences in the transmission component of selection (Hypothesis 1). Moreover, it seems a priori unlikely that the transmission advantage of the *a* allele is exactly compensated for by selection operating at other stages of the life cycle, resulting in evolutionary dynamics governed by genetic drift. Rather, the failure of the *a* allele to increase in frequency when rare seems due more probably to an additional component of selection that opposes this allele's transmission advantage. Such a component of selection, which is apparently weaker or absent on the *W* locus, is probably produced by pleiotropic effects.

This inference implies that the magnitude of negative pleiotropic effects on fitness is greater for the *a* allele than for the *w* allele, an implication that is supported by available

information. Previous investigations that have attempted to detect selection acting on the *W* locus at various phases of the life cycle have found no components of selection opposing the transmission advantage of the *w* allele when rare (Rausher & Fry 1993; Mojonner & Rausher 1997). By contrast, growth chamber experiments have demonstrated that *aa* plants have reduced male and female fertility at high (although naturally occurring) temperatures and produce fewer flowers, compared to *AA* plants (Coberly & Rausher 2003). In addition, field experiments have demonstrated a reduction of seed germination and seedling survival in *aa* plants (Coberly 2003). The magnitude of these pleiotropic disadvantages appear to be large enough to offset the transmission advantage of the *a* allele (Coberly 2003; Coberly & Rausher 2003).

This difference in degree of pleiotropy is also consistent with the nature of the genes corresponding to the *A* and *W* loci. The *A* locus corresponds to the gene coding for the enzyme Chalcone Synthase D (several duplicate copies of chalcone synthase occur in *I. purpurea*; Durbin *et al.* 1995; Fukuda-Tanaka *et al.* 1997). The *a* allele has been rendered nonfunctional due to an insertion of the *Ac/Ds* transposon TIP100 into the sole intron (Habu *et al.* 1998). Because Chalcone Synthase D is the first enzyme in the flavonoid pathway, and because it is the primary copy of chalcone synthase expressed in vegetative tissues as well as the corolla limb (Durbin *et al.* 2000), its inactivation not only eliminates anthocyanin pigments in flowers, but also probably prevents the production of various flavonoids in other tissues (Koes *et al.* 1994). Because flavonoids perform a variety of functions in plants, including providing protection from UV radiation and natural enemies, mediating interactions between plants and mycorrhizal symbionts, and facilitating pollen–pistil interactions (Koes *et al.* 1994), loss of Chalcone Synthase function is expected to cause detrimental fitness effects of the type observed at the *A* locus.

In contrast, the gene corresponding to the *W* locus is believed to be a transcriptional activator of anthocyanin pathway structural genes (Tiffin *et al.* 1998). The *a* allele appears to be nonfunctional because in homozygous individuals, expression of six anthocyanin genes is markedly reduced. Although the expression pattern of the *W* locus itself is unknown, many anthocyanin transcription activators are highly tissue-specific, with different duplicate copies being expressed in different tissues (van der Meer *et al.* 1993; Holton & Cornish 1995). Such specificity is supported for the *W* locus by the fact that although *ww* plants lack pigmentation in the most of the corolla limb, anthocyanins and presumably other flavonoids, are still present in stems, leaves and floral rays. This spatially limited expression pattern of the *W* locus suggests that adverse pleiotropic effects associated with lack of flavonoid expression are likely to be minor, consistent with the prior failure to detect such effects for the *w* allele.

Differences among loci in cause of transmission advantage

Although both the *a* allele and the *w* allele appear to enjoy a transmission advantage, the causes of this advantage appear to differ for the two alleles. A transmission advantage can arise in three different ways: through an increased selfing rate of white flowers, through a greater outcross siring success of white flowers or through a competitive advantage associated with pollen carrying the white allele. Previous investigations have demonstrated that the transmission advantage associated with the *w* allele arises because pollinators visit *ww* white flowers less often than is expected from their frequency in the population, which results in an increased selfing rate for these white flowers (Brown & Clegg 1984; Epperson & Clegg 1987b; Rausher *et al.* 1993; Fry & Rausher 1997). Neither differential outcross siring success (Rausher *et al.* 1993; Fry & Rausher 1997) nor competitive differences among pollen genotypes (Paulsen & Rausher 2001) appear to operate.

By contrast, in this study we observed no undervisitation of *aa* white flowers. We note that our analysis of visitation rates to *A*-locus genotypes is based on fewer visits than in previous analyses of visits to *W*-locus genotypes, and that therefore our failure to detect undervisitation may be due to lack of power rather than to a true lack of undervisitation. However, in an analysis involving more than 4000 pollinator visits (more than in any previous study), Coberly (2003) also found no evidence for undervisitation of *aa* whites, suggesting that our failure to detect it was not due to lack of power. Nevertheless, our data suggest that *aa* plants may still experience higher selfing rates, as the observed difference in outcross rates approached statistical significance. If this difference is real it can explain the higher transmission rates of *aa* plants, but its cause must be something other than reduced visitation rates. Alternatively, we cannot rule out the possibility that pollen competition contributes to the transmission advantage, although differential outcross siring success does not seem to be a factor.

A second difference between the *W* and *A* loci is that the transmission advantage of the *w* allele appears to be frequency dependent, whereas that of the *a* allele does not. Several experiments have shown that undervisitation and higher selfing rates, and thus the transmission advantage, experienced by *ww* whites at low frequencies both disappear as the frequency of whites increases (Epperson & Clegg 1987b; Rausher *et al.* 1993). Thus, only very small, probably undetectable, negative selection on the *w* allele due to pleiotropy is required to explain maintenance of the *W*-locus polymorphism at an equilibrium at which the frequency of the *w* locus is relatively high. In the experiments with the *A* locus reported here, however, there is no frequency dependence of visitation rates. Moreover, although the net transmission advantage, reflected by the selection coefficient, *s*, was slightly lower in the white-majority

arrays, it was still large. It thus seems that large deleterious pleiotropic effects associated with the *a* allele are required to prevent that allele from sweeping to fixation. As argued above, such effects are probably large enough to prevent the *a* allele from increasing above negligible frequencies.

Conclusions

Evidence from this and previous investigations point to the importance of differences in the magnitude of deleterious pleiotropic effects as the primary cause for differences in the evolutionary dynamics at two loci at which phenotypically similar variation in flower colour exists. A lack of measurable deleterious pleiotropy at the *W* locus appears to allow selection caused by increased selfing to maintain the *w* allele at relatively high frequencies. By contrast, although similar selection acts to favour the *a* allele at all frequencies, strongly deleterious pleiotropic effects associated with that allele appear to more than offset this transmission advantage. The observed patterns are thus consistent with net purifying selection against the *a* allele, and maintenance of this allele at a very low frequency due to mutation-selection balance.

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