

BALANCING SELECTION ON A FLORAL POLYMORPHISM

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Abstract.—The common morning glory, *Ipomoea purpurea*, exhibits a flower color polymorphism at the *W* locus throughout the southeastern North America. The *W* locus controls whether flowers will be darkly pigmented (*WW*), lightly pigmented (*Ww*), or white with pigmented rays (*ww*). In this report, we describe results of a perturbation, or convergence, experiment using five plots designed to determine whether balancing selection operates on the *W* locus. The pattern of gene frequency changes obtained are indicative of balancing selection operating at the *W* locus, providing direct evidence that both the alleles are actively maintained by selection.

Key words.—Balancing selection, frequency-dependent selection, *Ipomoea*, morning glory, polymorphism.

Received November 24, 1998. Accepted August 24, 1999.

A major focus of evolutionary biology is to account for the great genetic diversity exhibited by natural populations of most organisms, both at the molecular level and in ecologically important characters. Four general explanations can account for genetic variation in a particular trait: (1) variation reflects passive accumulation of neutral mutations due to genetic drift; (2) it reflects some form of mutation-selection balance; (3) it reflects a transient phase in which natural selection is causing certain genetic variants to replace others; or (4) it reflects some form of balancing selection. At the molecular level, explanations (1) and (4) seem consistent with much DNA and protein sequence variation (Li 1997). For some ecologically important traits, including sex ratio (Uyenoyama and Bengtsson 1979), self-incompatibility systems (Charlesworth and Charlesworth 1979; Clark 1993; Veckmans and Slatkin 1994; Richman et al. 1995), heterostyly (Eckert et al. 1996), and possibly disease resistance (Ennos 1983; Antonovics 1994), selection is believed to be inherently frequency dependent, resulting in balancing selection. For the vast majority of variable ecological traits, however, inherent frequency dependence is not expected and there is little information regarding which explanation accounts for observed variation. In particular, the general importance of

balancing selection in maintaining genetic variation in ecologically important characters is unclear.

In this report, we describe results of a perturbation, or convergence, experiment designed to determine whether balancing selection operates on a locus influencing floral pigmentation in the morning glory, *Ipomoea purpurea*. The *W* locus, which controls whether flowers will be darkly pigmented (*WW*), lightly pigmented (*Ww*), or white with pigmented rays (*ww*), is often polymorphic in populations in southeastern North America (Ennos 1981; Ennos and Clegg 1983; Epperson and Clegg 1986). The frequency of the white (*w*) allele typically ranges from 0.0 to 0.4 (Epperson and Clegg 1986). The primary pollinators, bumblebees, discriminate among the color morphs and undervisit whites when they are in the minority, which results in increased selfing by whites, but no detectable pollen discounting (Brown and Clegg 1984; Epperson and Clegg 1987; Rausher et al. 1993; Fry and Rausher 1997). Flower color is thus ecologically important in this species in that it modulates interactions with symbionts and influences the mating system.

METHODS

We performed a convergence experiment in which we established five experimental populations with initial allele frequencies perturbed from their typical values. We then monitored changes in gene frequency over one generation to determine whether gene frequencies tended to converge toward typical, intermediate values, as would be expected if bal-

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ancing selection were operating. Two of the populations (populations 1 and 2) were begun with low frequencies of the white allele ($p_w = 0.13$). This frequency is approximately equal to the mean frequency of the white allele ($p_w = 0.11$) in 19 polymorphic populations reported in a gene frequency survey by Epperson and Clegg (1986). Although a lower initial frequency in these two experimental populations would have been desirable, the value chosen reflected a necessary compromise between the need to begin at a frequency as low as possible and the need to have a sufficient number of plants of the least common (white) genotype to provide sufficient statistical power for detecting selection and a change in gene frequency. A third population (population 5) was begun with equal frequencies of the two alleles, which represents a higher frequency of the white allele than has been recorded in natural populations. Finally, two experimental populations (populations 3 and 4) were begun with high frequencies of the white allele ($p_w = 0.81$). Our a priori expectation, based on gene frequencies observed in natural populations and the assumption that balancing selection operates, was that p_w in populations 3–5 would decrease from high to intermediate values, whereas p_w populations 1 and 2 would increase from low to intermediate values.

Experimental Procedures

Experimental procedures followed those of previous studies (Rausher and Simms 1989; Simms and Rausher 1989; Rausher and Fry 1993). Each experimental population was established by planting a sample of seeds of known genotypic composition at the *W* locus. The seeds were planted in a rectangular grid with one-foot spacing between rows and columns. Individual seeds were randomly assigned to positions in the grid and were allowed to grow and reproduce naturally. After capsules began to mature, plants were visited twice weekly to collect all seeds produced. For each population, a random sample of offspring seeds were scored for genotype at the *W* locus to estimate the change in gene frequency after one generation. Locations of experimental populations were at open fields within the Duke Forest at Duke University, Durham, North Carolina. These sites were isolated from each other and from all other localities with *I. purpurea* present by at least 3 km, thus ensuring that gene flow into the sites was probably nonexistent. In addition, all nonexperimental *I. purpurea* were eradicated to prevent pollen contamination from native plants.

Experimental population 5 was established in early July 1989 by planting 1968 experimental seeds (656 *WW*, 640 *Ww*, and 672 *ww*). These seeds were generated by a crossing design similar to that reported by Rausher and Fry (1993), which randomizes the genetic background for genes unlinked to the *W* locus. This design involves three generations of crosses. In generation 1, six pairs of dark \times white crosses were performed, with each pair constituting a "unit." In generation 2, one light (heterozygous) offspring from each unit was selfed to produce numerous offspring. In generation 3, self-sibs (offspring within the same unit) were paired in three ways (darks with darks, darks with whites, and whites with whites) to produce the experimental seed (dark, light, and white, respectively). Within each unit each cross type was

replicated with three or four pairs, and within each pair reciprocal crosses were performed.

Experimental populations 1–4 were established in early July 1992 using seed produced in 1989 by population 5. To ensure that the frequency of the white allele was low in populations 1 and 2, these populations were established by planting 350, 40, and 20 seeds chosen randomly from those produced by dark, light, and white plants respectively in population 5. Similarly, to ensure that the frequency of the white allele was high in populations 3 and 4, these populations were established by planting 350, 40, and 20 seeds chosen randomly from those produced by white, light, and dark plants respectively in population 5. The initial gene frequencies in these samples were determined by planting a "companion" sample of seeds in the greenhouse at the same time and scoring the color of the flowers they produced, as described below.

Statistical Analyses

All statistical analyses of changes in gene frequencies are based on two sets of seed samples. One set, the greenhouse companion sample, consists of approximately 1500 seeds produced by the experimental plants in plot 5. In particular, random samples of 523, 435, and 534 seeds were selected from the seeds produced by darks, lights, and whites, respectively, in the Plot 5 experiment. These seeds were germinated in the greenhouse, allowed to grow to flowering, and their genotype determined by inspection. This sample of seeds was used for two purposes: (1) to estimate the final gene frequency in population 5 by comparing offspring gene frequency with the initial gene frequency in that population; and (2) to estimate the initial gene and genotype frequencies in plots 1–4 for comparison with final (offspring) gene frequencies in these plots. The second set of samples, the "offspring" samples, consisted of 545, 366, 680, and 444 randomly chosen seeds produced by the plants in plots 1–4 respectively. The seeds of these samples were also germinated and grown in the greenhouse to estimate final genotype and gene frequencies in each plot. Germination success of all samples was greater than 95%, thus providing estimates of genotype frequencies that are relatively free of potential bias introduced by natural selection operating in the greenhouse. In particular, this high germination rate makes it unlikely that any observed change in gene frequency in population 5 could be ascribed to differential seed mortality during the almost three years between collection and scoring the offspring seeds of that population.

The statistical significance of the change in gene frequency in each population was assessed using likelihood methods (Edwards 1972; Weir 1990). In each of the four populations with extreme allele frequencies (populations 1–4), the unconstrained joint likelihood of the observed genotype counts in the greenhouse samples and in a random sample of offspring seed produced by that population is given by

$$L = \left(\prod_{i,j} p_{ij}^{N_{ij}} \right) \left(\prod_i p_i^{N_i} \right),$$

where i and j can take on the values D, L, and W, corre-

sponding to dark, light and white genotypes; p_{ij} is the probability that a parent plant of genotype i in population 5 produced an offspring seed of genotype j ; N_{ij} is the number of offspring of genotype j produced by a maternal parent of genotype i in the greenhouse sample; p_i is the probability that an offspring seed produced by the population is genotype i , and N_i is the number of offspring in a random sample of seeds produced by the population that has genotype i . The null hypothesis that the allele frequencies in the offspring seed produced by one of these populations is equal to the initial allele frequencies, that is, that there is no change in gene frequency from one generation to the next, is represented by the constraint

$$\begin{aligned} & \left(\frac{K_D}{410}\right)\left(p_{DD} + \frac{1}{2}p_{DL}\right) + \left(\frac{K_L}{410}\right)\left(p_{LD} + \frac{1}{2}p_{LL}\right) + \left(\frac{K_W}{410}\right)\left(\frac{1}{2}p_{WL}\right) \\ & = p_D + \frac{1}{2}p_L, \end{aligned}$$

where K_i is the number of offspring seed produced by genotype i planted (note that $\sum_i K_i = 410$, the total number of seeds planted in each experiment). The left side of this constraint represents the initial gene frequency as a weighted average of the gene frequencies in the three greenhouse samples derived from maternal parents of different W -locus genotype, where the weights are the fractions of planted seeds that are offspring from these genotypes. This null hypothesis is tested using the standard likelihood-ratio statistic $\Lambda = -2 \ln$ where L_0 is the maximum likelihood corresponding to L with the constraint incorporated and L_1 is the maximum likelihood corresponding to the unconstrained version of L . Under the null hypothesis of no change in gene frequency, the statistic Λ has an approximate χ^2 distribution with one degree of freedom.

A similar analysis was performed for population 5. In this case, the initial gene and genotype frequencies were known exactly. The likelihood function for the offspring seeds is

$$L = \left(\prod_{i,j} p_{ij}^{N_{ij}}\right),$$

and the constraint corresponding to no gene frequency change over one generation is given by

$$\begin{aligned} & \left(\frac{S_D}{S_T}\right)\left(p_{DD} + \frac{1}{2}p_{DL}\right) + \left(\frac{S_L}{S_T}\right)\left(p_{LD} + \frac{1}{2}p_{LL}\right) + \left(\frac{S_W}{S_T}\right)\left(\frac{1}{2}p_{WL}\right) \\ & = \text{initial frequency of dark allele,} \end{aligned}$$

where S_i [$i \in (D, L, W)$] is the total number of seeds produced by plants of genotype i and $S_T = \sum_i S_i$.

For all analyses, likelihoods were assessed by an iterative grid-search method. Likelihoods were first calculated for all combinations of the parameters p_{ij} at intervals of 0.1. (For example, for the unconstrained models, there are five free parameters, each with the eleven possible values 0.0, 0.1, . . . 0.9, 1.0. This yielded a grid of 11^5 points. The likelihood was evaluated at each of these points.) Inspection of the resulting likelihood surface indicated that in all cases the surface had a single, global maximum. Using the grid point with the maximum likelihood as an approximation of the global

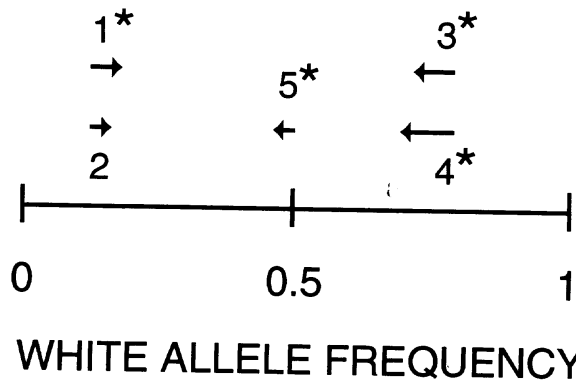


FIG. 1. Change in white allele frequency in experimental populations 1–5. Arrows indicate direction and magnitude of change in frequency. Base of arrow indicates initial allele frequency. Numbers correspond to populations. Asterisks indicate significant changes in gene frequency.

maximum, a second grid search was then performed. For this search, the grid was centered on the maximum of the first search and a new grid with intervals of 0.02 was searched for the grid point with the maximum likelihood. A third search was then performed using a grid with intervals of 0.002 for each parameter. The maximum likelihood found in the third search was taken as a good estimate of the true maximum because the difference in likelihood value between that grid point and adjacent points was always less than 2%.

Viability and Seed Production in Population 5

In addition to estimating changes in gene frequency in population 5, we also compared the viability and fecundity of plants of the three genotypes to obtain information about the causes of any observed change in gene frequency. Viability, which was estimated as the proportion of plants surviving to produce at least one offspring seed, was compared among genotypes with a standard G -test (Sokal and Rohlf 1969). Total seed production was determined for each plant by collecting and counting seeds three times a week (Rausher and Fry 1993). Comparison of seed production among genotypes was performed using analysis of variance (ANOVA).

RESULTS

The pattern of gene frequency changes in the five populations are indicative of balancing selection operating at the W locus (Fig. 1). The three populations with initial frequencies of the white allele greater than normally observed (populations 3–5) all exhibited a decrease in the frequency of the white allele (Table 1). In contrast, the two populations (1 and 2) with initially low frequencies of the white allele each exhibited an increase in its frequency. In all populations except population 2, these changes were highly significant (Table 1). Overall, the direction of gene frequency change in each of the five populations was as expected under the hypothesis that selection is balancing. The probability of this result under the null hypothesis of neutrality (equal likelihood of increase or decrease in frequency in a given experimental population) is $P = 0.034$. This pattern indicates that regardless of initial allele frequencies, selection causes frequencies

TABLE 1. Genotype and gene frequencies among seed samples used to estimate initial and final gene frequencies. Greenhouse samples were obtained from the offspring of population 5, whereas offspring samples were obtained from the offspring of populations 1–4 (see text). p_w is the frequency of the white allele in the sample. Δp_w is the estimated change in gene frequency in the indicated experimental population during the experiment. χ^2 and P are the chi-square value and associated probability corresponding to the likelihood-ratio test for change in gene frequency (see text). Genotype frequencies for population 5 were estimated indirectly rather than by direct count, but this does not affect the validity of the likelihood-ratio test.

Sample	Genotype			p_w	Δp_w	χ^2	P
	WW	Ww	ww				
Greenhouse: WW parents	0.87	0.13	—				
	456	67	—				
Greenhouse: Ww parents	0.37	0.37	0.26				
	160	162	113				
Greenhouse: ww parents	—	0.21	0.79				
	—	114	420				
Offspring: population 1	0.70	0.21	0.09	0.19	+0.06	11.37	0.001
	384	114	47				
Offspring: population 2	0.75	0.18	0.08	0.17	+0.04	1.75	0.02
	274	64	28				
Offspring: population 3	0.17	0.19	0.64	0.73	-0.08	20.70	0.001
	114	131	435				
Offspring: population 4	0.13	0.33	0.54	0.70	-0.10	24.09	0.001
	57	146	241				
Offspring: population 5	0.41	0.24	0.35	0.47	-0.04	441.1	0.0001
	523	435	534				

to converge to a region in which the frequency of the white allele is between approximately 0.25 and 0.45, which corresponds quite well to the range of frequencies commonly observed in natural populations.

In population 5, genotypes exhibited no detectably significant differences in either viability or seed production (Table 2). Moreover, the estimates of these fitness components for the two homozygote genotypes were remarkably similar. Whereas the viability estimate was slightly higher for the dark genotype, the opposite was the case for seed number. These differences compensated almost exactly to produce an estimate of expected seed production (viability \times seed number) that differed by less than 0.05% (Table 2). Mean seed number exhibited a slight but significant degree of overdominance (Table 2).

DISCUSSION

The principle result of this study is that the *W* locus in *I. purpurea* appears to be subject to balancing selection, at least in the area in which this investigation was conducted. We

TABLE 2. Fitness components of *W*-locus genotypes in population 5. P is probability fitness component differs among genotypes. For viability, P was determined by a G -test with two degrees of freedom ($G = 1.02$). For ln seed number, P was determined by ANOVA ($F_{2,105} = 0.09$), which contained other sources of variation in addition to genotype. Numbers in parentheses are standard errors.

Fitness component	Genotype			P
	WW	Ww	ww	
Viability	0.83 (0.04)	0.73 (0.05)	0.79 (0.04)	> 0.90
ln seed number	3.34 (0.99)	3.35 (1.03)	3.39 (0.96)	> 0.87
Viability \times seed number	23.42	20.81	23.43	
ln mean seed size	2.89 (0.21)	2.93 (0.19)	2.91 (0.18)	< 0.008

infer balancing selection from the observation that both alleles seem to have increased in frequency when rare. Previous investigations have suggested the operation of a mechanism that likely causes the white allele to increase in frequency when rare. When this allele is at low frequency, white (*ww*) plants exhibit a higher selfing rate than darks or lights, apparently because bumblebees, the primary pollinator, visit whites less frequently (Brown and Clegg 1984; Fry and Rausher 1997). Coupled with an apparent lack of pollen discounting (Rausher et al. 1993) and minimal inbreeding depression (Pear 1983; S.-M. Chang and M. D. Rausher, unpubl. ms.), this increased selfing is expected to contribute to a net advantage to the white allele (Fisher 1941; Lloyd 1979; Wells 1979; Holsinger et al. 1984). The increase in the gene frequency of the *w* allele reported here for populations 1 and 2 lends credence to this explanation and provides the first direct evidence indicating that selection acts to prevent the elimination of the white allele.

When the white allele is not at low frequency, pollinators visit all color variants at the same rate, selfing rates are similar for the three genotypes (Epperson and Clegg 1987; Rausher et al. 1993; Fry and Rausher 1997), and there is thus not expected to be an analogous increase in the frequency of the white allele. If no other evolutionary forces were operating on the *W* locus, the white allele would be expected to eventually drift to fixation. Our observation that the frequency of the white allele decreases when initially at high and intermediate frequencies (populations 3–5) indicates that such fixation is not occurring and that therefore some other process must cause the dark allele to increase in frequency when rare.

Previous experiments have provided inconclusive evidence regarding the nature of this additional selective force. No dark-allele advantage was detected by Rausher and Fry (1993) in either survival or seed production. Overdominance in seed size does seem to occur (Rausher and Fry 1993; Mojonnier and Rausher 1997; Table 2), but its effects on

offspring fitness appear not to be of sufficient magnitude to maintain high frequencies of the dark allele (Mojonnier and Rausher 1997). In an experimental population, Fry and Rausher (1997) detected biased inheritance favoring the dark allele in pollen produced by heterozygotes, which could act to prevent the dark allele from being eliminated when rare. However, subsequent experiments (S. Paulsen and M. D. Rausher, in review) indicate that this mechanism does not operate in all, or even most, polymorphic *I. purpurea* populations.

Regardless of mechanism, our results provide direct evidence that the dark allele is actively maintained by selection. In addition, our estimates of viability and female fitness components in population 5 provide additional support for some of the conclusions derived from previous experiments. In particular, the lack of differences among genotypes in these fitness components confirms Rausher and Fry's (1993) results and means that a dark-allele advantage in these fitness components cannot explain the decrease in frequency of the white allele observed in this population. The increase in frequency of the dark allele in this population must therefore have been due to a fitness advantage of that allele either through male fitness or during the gametic stage, a conclusion that is consistent with Fry and Rausher's (1997) observation of non-Mendelian inheritance in pollen from heterozygotes. Our results thus reinforce the notion that in some polymorphic populations, but not all, some sort of biased inheritance may contribute to maintenance of the dark allele. As Fry and Rausher (1997) have shown, an interaction of this type of force with the frequency-dependent transmission advantage of whites associated with increased selfing is sufficient to maintain gene frequencies by balancing selection at roughly the values typically seen in nature.

ACKNOWLEDGMENTS

This research was supported in part by NSF grant DEB 9318919 to MDR.

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