Spontaneous Mutational Effects on Reproductive Traits of *Arabidopsis thaliana*

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ABSTRACT

A study of spontaneous mutation in *Arabidopsis thaliana* was initiated from a single inbred Columbia founder; 120 lines were established and advanced 17 generations by single-seed descent. Here, we report an assay of reproductive traits in a random set of 40 lines from generations 8 and 17, grown together at the same time with plants representing generation 0. For three reproductive traits, mean number of seeds per fruit, number of fruits, and dry mass of the infructescence, the means did not differ significantly among generations. Nevertheless, by generation 17, significant divergence among lines was detected for each trait, indicating accumulation of mutations in some lines. Standardized measures of mutational variance accord with those obtained for other organisms. These findings suggest that the distribution of mutational effects for these traits is approximately symmetric, in contrast to the usual assumption that mutations have predominantly negative effects on traits directly related to fitness. Because distinct generations were grown contemporaneously, each line was represented by three sublines, and seeds were equal in age, these estimates are free of potentially substantial sources of bias. The finding of an approximately symmetric distribution of mutational effects invalidates the standard approach for inferring properties of spontaneous mutation and necessitates further development of more general approaches that avoid restrictions on the distribution of mutational effects.

SPONTANEOUS mutation ceaselessly contributes new alleles to a population's pool of genetic variation. Potential evolutionary consequences of this process are increasingly well understood as a result of extensive theoretical study. Much of the standing genetic variance in quantitative traits could be due to a balance between the influx of variation through mutation and reduction of variation by selection (Lande 1976). Genetic load due to deleterious mutation can direct mating system evolution (*e.g.*, Charlesworth *et al.* 1990; Uyenoyama *et al.* 1992); it can also hasten extinction of small populations (Lande 1994; Lynch *et al.* 1995). Evolution of populations specialized to particular environments may also largely result from condition-dependent effects of newly arising deleterious mutations (Kawecki *et al.* 1997). Despite the emphasis on deleterious mutation, mutation also generates alleles that enhance fitness (Burch and Chao 1999), particularly in a new environment (*e.g.*, Bennett *et al.* 1992) or, temporarily, in a changing environment. Increase in frequency of fitness-enhancing mutations can indirectly cause mutation rate to evolve through increase in the frequency of linked modifiers of mutation (Gillespie 1981; Ishii *et al.* 1989; Johnson 1999), as demonstrated in *Escherichia coli* (Sniegowski *et al.* 1997). Moreover, rapid fixation of new alleles that increase fitness depletes variation at closely linked sites (Maynard Smith and Haigh 1974), as does selection against deleterious mutations (Charlesworth *et al.* 1993). Without exception, actual evolutionary outcomes of these processes depend critically on specifics of spontaneous mutation: rates of mutation (Turelli 1984), effects of mutations, especially on fitness (Caballero and Keightley 1994), and gene action of mutant alleles (Charlesworth *et al.* 1991; Caballero and Keightley 1994). To date, quantitative measures of all these aspects of spontaneous mutation remain uncertain.

The primary approach developed for study of influences of spontaneous mutation on quantitative traits involves establishing numerous lines from a single founder individual, such that the lines are as nearly genetically identical as possible at the outset (*i.e.*, they are in mutation-drift equilibrium; Lynch and Hill 1986). Each line is advanced through numerous generations, represented in every generation by one or few individuals so that fixation of newly arising mutations is dominated by genetic drift, rather than selection. Assays of a single advanced generation reveal the extent of phenotypic divergence among the lines as evidence of the accumulation of different mutations in different lines. Comparison of phenotypes expressed in advanced generation individuals with phenotypes observed in "control" populations, free of new mutations since the founder, provides evidence of the direction of cumula-

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tive effects of mutations. For such mutation-accumulation (M-A) experiments, Bateman (1959) developed a method that uses per-generation change in mean and increase in variance of a trait and assumes equal mutational effects to estimate mutation rate and the effect of mutations on that trait.

The M-A approach was pioneered by Mukai and his colleagues in massive studies of egg-to-adult viability in *Drosophila melanogaster* (Mukai 1964, 1969). Divergence among lines in viability was accompanied by substantial decline in viability. Analysis by Bateman's method indicated that mutations affecting viability arise at a rate of about one per diploid genome per generation and that these are predominantly of slight, deleterious effect, though a small fraction (*ca.* 1%) are severely deleterious (Simmons and Crow 1977).

Recently, however, Keightley (1996) has drawn attention to inconsistencies between the distribution of mutational effects implied by results of Mukai's studies of spontaneous mutation and the distribution arising from EMS mutagenesis. Among possible explanations, several originate in the inability to make direct, contemporaneous comparisons of late generations to flies free of new mutations. Contemporaneous rearing of individuals representing different generations is the preferred basis for comparisons between M-A generations, because uncontrolled aspects of environment may otherwise unaccountably influence the traits of interest. Moreover, Keightley (1996) has pointed out that the balancer stocks, presumed to be genetically static and for this reason used as a basis of comparison for the late M-A generations, could have evolved toward increased viability during the course of the study, giving the appearance that advanced generations of the M-A lines had declined in viability. Alternatively, Fry *et al.* (1999) suggest that improvement in an investigator's identification of Drosophila individuals bearing the *Cy* genetic marker would lead to an apparent decrease in viability of M-A lines. Keightley's (1996) and Garcia-Dorado's (1997) reanalyses of Mukai's data suggest that the mutation rate for viability may be two orders of magnitude lower than originally inferred. Yet doubts about biases in the data and resulting estimates persist, and these doubts motivate further efforts to characterize spontaneous mutation.

Empirical studies over the past five years have extended inference of mutational properties to a broader range of organisms, including the bacterium *E. coli* (Kibota and Lynch 1996), the nematode *Caenorhabditis elegans* (Keightley and Caballero 1997; Vassilieva and Lynch 1999), and the yeast *Saccharomyces cerevisiae* (C. Zeyl and J. A. G. M. DeVisser, unpublished results). No new consensus about the properties of spontaneous mutation has emerged from this approach or others (Crow 1997; Keightley *et al.* 1998; Lynch *et al.* 1999). Thus, uncertainty obscures aspects of the mutational process once widely accepted, and this impedes understanding of the actual evolutionary consequences of mutation.

We are studying properties of spontaneous mutation affecting quantitative traits of the annual crucifer, *Arabidopsis thaliana*. A set of 120 lines established from a single, highly inbred founder were advanced under minimal selection to generation 17. This article reports a contemporaneous comparison of lines sampled at generations 0, 8, and 17 of mutation accumulation. By generation 17, the lines had diverged significantly with respect to several reproductive traits, yet no overall change in the mean of these traits was detectable. We discuss implications of these results for inference of mutation rates and distributions of mutational effects.

MATERIALS AND METHODS

Experimental material: Aspects of *A. thaliana* that suit it particularly well to studies of spontaneous mutation include its short life cycle (10 wk) and its high fecundity (commonly over 500 seeds per plant). Of profound importance in ensuring accuracy of estimates of mutational variation is the lasting viability of seeds (see below). Simultaneous rearing of individuals sampled from distinct generations avoids confounding of temporal changes in environment with changes due to accumulated mutation. Stored seed may also be used for retrospective tracking of mutation events.

A. thaliana is highly self-pollinating. Anthers normally dehisce prior to anthesis, and flowers regularly self and set seeds in bud. In outdoor conditions designed to maximize the chance of outcrossing, Snape and Lawrence (1971) found outcrossing of *A. thaliana* at a frequency of 1.7%. Abbott and Gomes (1989) confirmed that *A. thaliana* outcrosses extremely rarely in nature. From seven natural populations found polymorphic at a peroxidase locus, a total of 2100 progeny were scored; not one was found to be heterozygous. Restriction fragment length polymorphism (RFLP) analysis of 11 natural populations also revealed no heterozygotes (Bergelson *et al.* 1998). Selfing in bud facilitates advancement of M-A lines by the method of single-seed descent. Although theoretical work suggests that selfing species might evolve mutation rates that are higher (Holsinger and Feldman 1983) or lower (Kondrashov 1995) than outcrossing species, deviation from mutation rates typical of other organisms is not obvious from screens for chlorophyll deficiency (2×10^{-4}) cited in Klekowski (1992). DNA sequence comparisons also suggest that mutation rates for *A. thaliana* are comparable to those for other organisms; sequence comparisons with close relatives of *A. thaliana*, together with divergence times inferred from the pollen record, have yielded estimates of 1.4×10^{-8} and 2.2×10^{-8} synonymous substitutions per site per year for chalcone synthase and alcohol dehydrogenase genes, respectively (M. A. Koch, B. Haubold and T. Mitchell-Olds, unpublished results). These values are within an order of magnitude of estimates for hominids (10^{-9}, Eyre-Walker and Keightley 1999).

In advance of M-A line initiation, 15 laboratory accessions of *A. thaliana* were screened to eliminate lines exhibiting visible segregation and poor fertility. A screen of molecular variation was also conducted to assess variablity within and among the source lines. On the basis of these assays, 3 accessions that showed no segregation but that differed from one another in height, flowering time, and RFLP phenotype were chosen as inbred sources for the M-A lines. These sources were main-

tained by selfing and single-seed descent for several generations and are therefore expected to have reached mutation-drift equilibrium, a condition that simplifies interpretation of the evolutionary dynamics of the change in mean phenotypes due to mutation (Lynch and Hill 1986). Each of these 3 accessions was used to establish a set of 120 M-A lines. However, the replication required for precise estimation of genotypic values and mutational parameters precluded simultaneous assessment of all three sets of M-A lines. Here, we present an assay of one of these sets of M-A lines, derived from an accession designated "Columbia," having no history of irradiation or other mutagenic treatment.

The M-A lines were established in 1991 by planting 120 seeds from a single founder individual, each seed establishing a single line. Each line was propagated each generation by a single individual randomly chosen from 5 seeds sown, with remaining seeds from each line stored in microfuge tubes. This single-seed descent method of advancing lines limits effective population size (N_e) to 1 and thus allows genetic drift to dominate changes in allele frequencies, minimizing selection. In this respect, the single-seed descent method of M-A is intermediate between using balancer stocks to allow mutations to accumulate on a single chromosome (*e.g.*, Mukai 1964, 1969; Houle *et al.* 1992, 1994) and employing sib or cousin mating with few individuals per subline (*e.g.*, Mackay *et al.* 1992; Fernandez and Lopez-Fanjul 1996). Under this scheme, strictly neutral mutations are expected to be fixed with probability of 1/2, while even strongly deleterious mutations, $s = 0.5$, are expected to be fixed with probability of 1/3 (see Figure 1 in Keightley and Caballero 1997). Lethal mutations are not recovered. This deficiency does not seriously compromise inference of evolutionary consequences of spontaneous mutation, because lethal mutations, and even far less deleterious ones, are expected to be eliminated quickly and therefore to contribute little to standing genetic variance in large populations at equilibrium (Caballero and Keightley 1994). Of the original 120 lines, 117 (97.5%) were advanced to generation (Gen) 17 by 1995. Thus, although selection or accidental loss can never be entirely eliminated from M-A studies, an unusually large population (in absolute numbers of lines and in the fraction of lines advanced to the latest generation) has been retained for these M-A studies.

In preparation for a series of assays of generations 0, 8, and 17, sublines were developed for each line in each generation so that environmentally induced maternal effects would not be confounded with differences among lines. Among-line genetic differences that are maternally inherited, for example, mutations in organelle genomes, would contribute to our estimates of mutational effects, as is true of other M-A studies (*e.g.*, of *C. elegans*, Keightley and Caballero 1997; Vassilieva and Lynch 1999). We are conducting crossing studies that would distinguish maternal genetic effects from direct effects of mutations. To eliminate differences in seed age or conditions of seed maturation, the sublines for all generations were established at one time in a large growth chamber. Four sublines for each available line from Gens 8 and 17 were established in one generation by growing four offspring for each line and collecting seed from each. For Gen 0, the source seed was from the same parent that gave rise to Gen 1 of the original 120 M-A lines. We developed sublines to represent Gen 0 in two generations. In the first, we grew 17 plants from the seeds retained from the original founder. From each of these 17 plants, we grew seven progeny and collected seed from each individually.

Experimental design of assay: Forty M-A lines were chosen at random for this assay of reproductive traits. The same 40 lines were grown for Gens 8 and 17, and each was represented by three sublines in each generation. One representative for each line-generation (line-gen) subline was grown in each of four blocks for generations 8 and 17. The founder generation (Gen 0) was represented by 13 lines with two replicate individuals from each of four sublines within each of the four blocks. The plants were grown in $6 \times 6 \times 8.5$-cm pots two-thirds filled with coarse vermiculite and topped with a mixture of African violet soil and fine vermiculite. Four seeds were sown per pot and thinned to a single plant nearest the center. Within each block, plants representing all line-gens were randomized together.

Of the planned design, 93–95% of plants were available for measurement of reproductive traits (Gen 0: 388 out of 416 planned; Gen 8: 459 of 480; Gen 17: 467 of 480). We determined the number of seeds per fruit, the total number of fruits produced, and dry mass of the infructescence for each plant. The seeds of four fruits were counted for each plant. Usually the third, fifth, seventh, and ninth fruits produced were collected for counting unless very few fruits were produced. Counting the large number of tiny seeds was facilitated by digital imaging. Seeds were taped to file cards and scanned (NIH Image). Fruits were counted when the plants had fully senesced. Thereafter, the infructescence was oven-dried and weighed to $10^{-5} \times g$.

Analysis: Composite effects of mutations that have accumulated within lines are evidenced by comparison of overall trait means expressed in different M-A generations and by variance accruing among lines with advancing generations. In particular, a trend in trait means with generations indicates a bias in the direction of mutational effects. Directional trends over generations were quantified as the regression coefficient, β, in a linear regression of trait values on generation number. This regression model also included the design factors, planting flat, and maternal parent, as well as a covariate, germination date. None of the traits were transformed for this or any of the analyses outlined below, since each trait satisfied normality assumptions.

In inferring the variance among M-A lines for each trait, V_L, influences on the expression of each trait were formulated according to a mixed model. Specifically, each observation was modeled as the sum of random effects of the *i*th M-A line in the *j*th generation of assay (l_{ij}) and the *k*th maternal parent within M-A line-generation ($m_k(l_{ij})$), as well as fixed effects of the flat in which the plant grew (f_s) and of a linear covariate, germination date (g_t):

$$y_{ijkst} = l_{ij} + m_k(l_{ij}) + f_s + g_t + e$$

The distributional assumption required for the maximum-likelihood analysis was that the effects of a given line in the two assayed generations, l_{i8}, l_{i17}, are drawn from a bivariate normal distribution, with mean zero and variance-covariance matrix

$$\begin{matrix} V_{L8} & \mathrm{cov}_{L8,17} \\ \mathrm{cov}_{L8,17} & V_{L17} \end{matrix}$$

Components of this model were estimated by restricted maximum likelihood (REML) in a bivariate analysis treating the trait in generation 8 as trait 1 and the same trait in generation 17 as trait 2. This analysis provides an estimate of a component of variance of line effects for each trait in generations 8 and 17, V_{L8}, and V_{L17}. Because observations are available for the same lines sampled in generations 8 and 17, the covariance between trait values expressed in a particular line in Gen 8 and that line in Gen 17, $\mathrm{cov}_{L8,17}$, can also be obtained. Components of variance due to maternal parent, V_{Mat}, and to environmental effects unique to individuals, V_E, were also estimated in this analysis. Because a single maternal plant is represented in only one generation, the maternal covariance between gen-

erations is zero; the environmental covariance is also zero, because each individual represents a single generation.

For M-A lines advanced by a single diploid individual each generation, V_L, has the expectation, $2tV_M$, where t is the number of generations since the common founder (Lynch and Hill 1986). The covariance between individuals in the same line but different generations, t_1 and t_2, has the expectation $2t_1V_M$, (Wray 1990). Thus, the estimates of V_{L8}, V_{L17}, and $\mathrm{cov}_{L8,17}$ indirectly provide estimates of V_M.

A second joint analysis of the observations from generations 8 and 17 was developed to estimate V_M for each trait directly and, therefore, more precisely. In this case, each observation was modeled as above, with the exception that the variance-covariance matrix of l_{i8}, l_{i17}, was parameterized in terms of V_M:

$$\begin{matrix} 16V_M & 16V_M \\ 16V_M & 34V_M. \end{matrix}$$

To assess the contribution of mutation to the covariances between the reproductive traits, a multivariate REML analysis was used to obtain estimates of among-line variance and covariance components, as well as estimates of environmental components. This analysis was restricted to Gen 17, because significant variation among lines was not detected for Gen 8.

For all analyses, likelihood ratio tests (Shaw 1987) were used to test hypotheses. Because components of variance are defined to be nonnegative, a one-sided testing procedure is appropriate. In a univariate analysis, this is accomplished by dividing the tabled P value by 2 (Self and Liang 1987). Asymptotic standard errors of estimates were also obtained (Table 1), and these provide a second, approximate test. In view of the nonnegativity of variance components, these are considered significantly greater than zero at $P < 0.05$ when the estimate exceeds 1.645 times its standard error. Agreement between the two tests is expected asymptotically, but discrepancies in the significance levels are apparent in some cases reported here.

RESULTS

In over 96% of the pots planted, at least one plant germinated from the four seeds sown, and the plant that was retained after thinning survived to reproduction. The three reproductive traits showed sensitivity to environmental variation; each varied significantly among the planting flats. Moreover, germination date was a significant predictor of number of seeds per fruit ($\beta = -0.96$; $P < 0.0001$) and the number of fruits per plant ($\beta = 1.67$; $P < 0.03$). Because germination date did not vary significantly among the lines in any generation (Gen 0, $P = 0.94$; Gen 8, $P = 0.13$; Gen 17, $P = 0.64$), it likely also reflects environmental variation in the greenhouse. The factors flat and germination date were included in all further analyses.

Generation means: Means for each reproductive trait differed negligibly among the three assayed generations (Table 1; Figures 1 and 2). For seed number per fruit, though the regression estimate of per generation change in the trait was negative (estimate: −0.004, $P > 0.7$, −0.01%; 95% confidence interval (C.I.) lower bound, −0.25%), the mean was slightly higher in generation 8 than in generation 0 or 17. Both the mean number of fruits per plant and reproductive biomass (not shown) declined very weakly but consistently [estimates of per-generation change in the mean: −0.11 fruit

TABLE 1

Estimates of means and (co)variance components among lines for three reproductive traits in contemporaneously assayed plants of *A. thaliana* representing generations 8 and 17 of mutation accumulation

	Seed number per fruit	Fruit number	Reproductive mass (mg)
Mean_0	32.6	59.7	45.3
	(0.4)	(1.8)	(1.5)
Mean_{17}	32.6	57.6	44.5
	(0.6)	(1.7)	(1.3)
V_{L8}	1.2	8.2	3.0
	(1.9)	(44.7)	(28.0)
$\mathrm{Cov}_L(8,17)$	1.2	22.1	15.0
	(1.3)	(30.2)	(19.5)
$r_L(8,17)$	0.64	0.81	1
V_{L17}	2.9	90.5	71.0
	(1.9)	(49.1)	(32.9)
V_{E8}	45.4	1325.2	713.0
	(4.0)	(116.9)	(63.3)
V_{E17}	51.8	1137.2	682.0
	(4.0)	(87.2)	(52.4)
V_{Mat8}	3.29	44.8	66.0
	(3.1)	(81.3)	(51.0)
V_{Mat17}	0	0	0

There is no environmental or maternal covariance between traits expressed in different generations because different generations are represented by distinct sets of individuals and maternal sublines. Standard errors are given in parentheses.

(−0.2%; 95% C.I. lower bound, −0.68%) and −0.06 mg (−0.1%; 95% C.I. lower bound, −0.61%), respectively, both $P > 0.45$]. Total reproductive fitness for each plant was estimated as the product of number of fruits per plant and the mean number of seeds per fruit. Mean reproductive fitness was slightly greater for generations 8 and 17 than for generation 0 (not shown); these differences were not statistically significant ($P > 0.5$). Thus, these data do not reject the null hypothesis that there is no composite effect of accumulating mutations on overall means of these reproductive traits. This outcome would arise if mutations affecting these traits had not accumulated in the 17 generations of this study or if the mutations that accumulated were not consistent in the direction of their effects.

Variance partitioning: For all three reproductive traits, divergence among the lines was evident in analyses of generation 17 alone. Analysis of variance (not shown) demonstrated significant variance among lines for each trait considered singly (for each, $P < 0.03$), as well as in the multivariate sense ($P < 0.04$).

Bivariate analysis of the two M-A generations (Table 1) confirmed that variance among lines, V_L, was greater at generation 17 than at generation 8, as expected for divergence with accumulating mutations. For each of the three traits, V_L was significantly greater than zero only at generation 17. For the trait seed number per

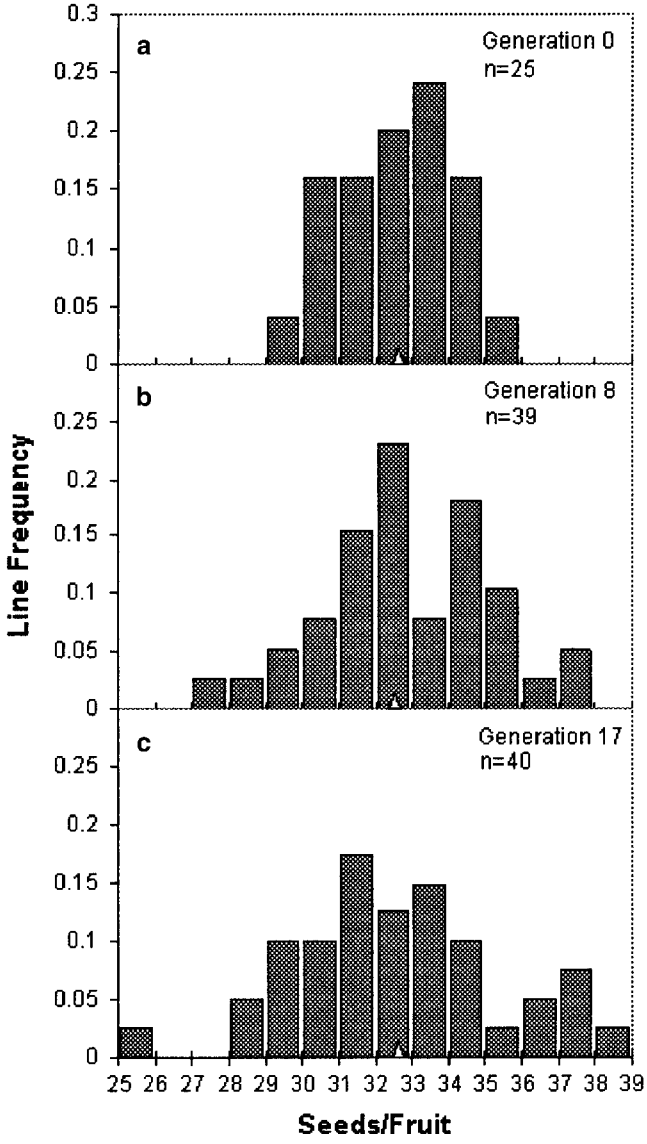

Figure 1.—Frequency distributions of least squares means of the trait, mean number of seeds per fruit for each line. (a) Generation 0, means obtained for sublines, N = 9–16 plants per subline mean. (b) Generation 8, means obtained for each of 40 randomly chosen M-A lines, N = 9–12 plants per line. (c) Generation 17, means obtained for the same 40 M-A lines as in generation 8, N = 10–12 plants per line.

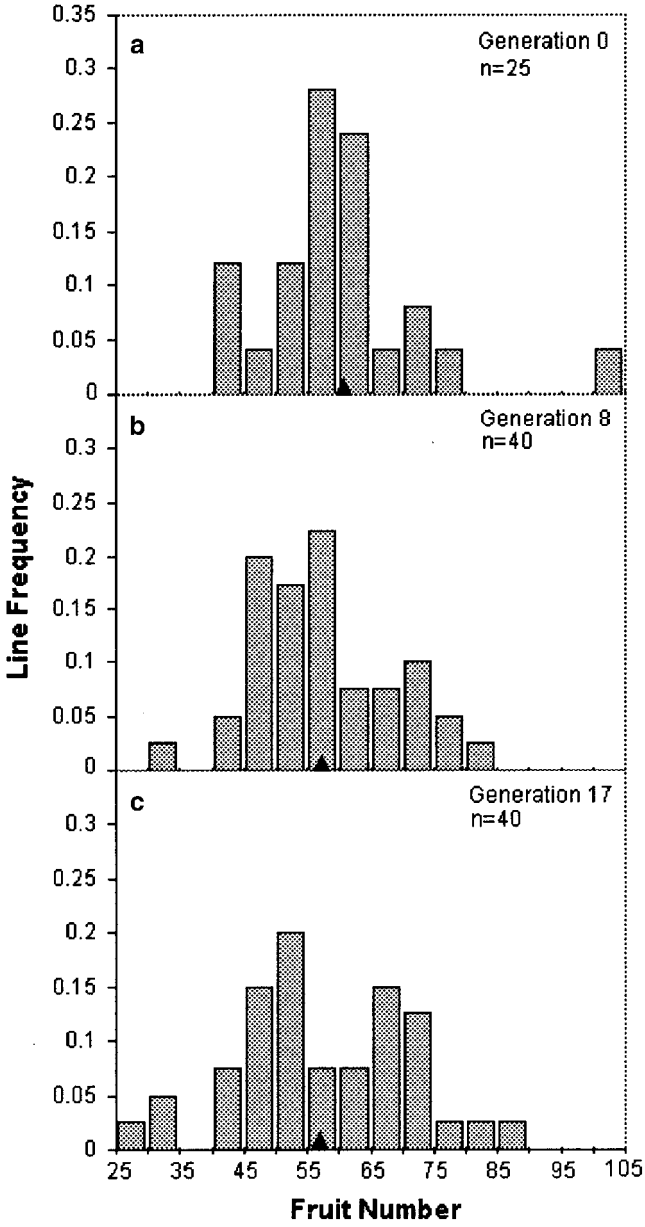

Figure 2.—Frequency distributions of line means for the trait, mean number of fruits per plant. a–c as in Figure 1.

fruit the increase in V_L appeared to be approximately constant, whereas V_L for fruit number and reproductive mass appeared to increase faster between generations 8 and 17 than in the first eight generations. For seed number per fruit, the correlation among lines between traits expressed in generations 8 and 17 closely approximated the expectation of 0.68 [*i.e.*, $t_1/(t_1t_2)^{1/2}$] based on considering divergence of a line from an ancestral, genetically identical sample (Wray 1990).

This analysis also revealed substantial contributions of environmentally induced maternal effects to variance in generations 0 and 8. For generation 8, the maternal component of variance, V_{Mat8}, dwarfed V_{L8} for each trait, although none of the V_{Mat8} differed significantly from zero. Analyses eliminating this component from the model led to estimates of V_{L8} (not shown) that were higher by factors of 1.7 (for number of seeds per fruit) and 5.7 (for reproductive mass). In contrast, no evidence of maternal effects was found for generation 17. Negative values for V_{Mat17} were obtained initially for each trait, but in no case were these values as large as their asymptotic standard errors. They were judged to be due to sampling error and were set to zero in subsequent analyses.

Mutational variance, V_M, was estimated directly for each trait by REML analyses of generations 8 and 17 jointly (Table 2). For all three traits, V_M differed significantly from zero by likelihood ratio tests. Estimates of V_M scaled by V_E (h^2_M) fall well within range of estimates for numerous traits, particularly life history characters, in other organisms (Houle *et al.* 1996). Estimates of V_M, standardized as the mutational coefficient of variation, CV_M, were also approximately consistent with the range documented by Houle *et al.* (1996).

Covariances between traits: Multivariate analysis of the three traits measured on plants representing generation 17 demonstrated strong positive among-line components of covariance for each pair of reproductive traits (Table 3); among-line correlations (r_L) exceeded 0.7 in each case, suggesting that individual mutations tend either to increase all three traits or to decrease all three. Environmental contributions to correlations between traits (r_E) were also positive and statistically significant, although r_E of seed number per fruit with each of the remaining reproductive traits was substantially weaker than the corresponding r_L. This analysis confirmed the finding of significant divergence among the lines; the likelihood ratio test strongly rejected ($P < 0.0001$) the null hypothesis that all of the among-line components of (co)variance are zero.

TABLE 2

Estimates of variance introduced each generation by spontaneous mutation in *A. thaliana*, based on joint analysis of generations 8 and 17

Trait	V_M	$V_M/V_E \times 10^3$	CV_M
Seeds/fruit	0.07*	1.34	0.64
	(0.04)		
Fruits	2.01**	1.68	5.1
	(1.11)		
Reproductive mass (mg)	1.45***	2.1	4.2
	(0.72)		

Standard errors are given in parentheses. Because variance components are necessarily positive, a one-sided test is appropriate; thus, an estimate of a variance component is significantly greater than zero ($P < 0.05$) if it exceeds its standard error by a factor of 1.645. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$, based on likelihood ratio tests of H_0:$V_M = 0$.

DISCUSSION

For each of the three reproductive traits, the *A. thaliana* M-A lines diverged rapidly; the lines differed significantly for each trait by Gen 17. The mutational variance V_M was comparable to that found in studies of diverse traits in other organisms (Houle *et al.* 1996). In contrast to the common expectation (Lynch 1994), however, plants from Gens 0, 8, and 17 of mutation accumulation did not differ detectably in mean phenotype; any decline in trait means was significantly less than 1% per generation. Thus, this study does not support the view that the overwhelming majority of muta-

TABLE 3

Estimates of variance and covariance components for three reproductive traits assayed in plants representing generation 17

	Seed/fruit	Fruits	Reproductive mass
		Among lines	
Seed/fruit	3.1	11.9	11.7
	(2.0)	(7.5)	(6.5)
		[0.71]	[0.77]
Fruits		92.5	85.7
		(48.4)	(39.6)
			[1.0]
Reproductive mass			75.5
			(33.7)
		Environmental	
Seed/fruit	51.8	63.9	58.4
	(4.0)	(13.6)	(10.7)
		[0.26]	[0.31]
Fruits		1131.3	797.9
		(86.8)	(64.3)
			[0.91]
Reproductive mass			683.3
			(52.3)

Standard errors are given in parentheses and component correlations in brackets. On the basis of results in Table 1, we did not include maternal (co)variances in the model.

tions are negative in their effect on these components of fitness. Frequency distributions of the line means for each generation (Figures 1 and 2) were approximately symmetric around the mean of Gen 0, suggesting that mutations increasing trait values approximately balance those of decreasing effect. This study avoided potentially serious biases in inference of the mutation-induced changes in the means and among-line variances of traits by means of contemporaneous assaying of generations, with all M-A lines represented by seeds of the same age from multiple sublines. These aspects of the design eliminate direct and maternal environmental contributions to differences among generations and among lines.

Our study is most directly comparable to a recent mutation accumulation study of the Landsberg erecta strain of *A. thaliana* (Schultz *et al.* 1999). After 10 M-A generations, Schultz *et al.* did not detect significant variation among the lines for any character, as we also did not for our Gen 8. For germination fraction or fruit set, mean differences between Gen 0 and Gen 10, grown contemporaneously, were not significant, whereas a significant ($P < 0.05$) 0.4% per generation decline in seed number per fruit was found.

Recent M-A studies of other organisms have generally demonstrated significant divergence among M-A lines, whereas mutation-induced declines in means of fitness characters have not been detected in several instances. In the nematode *C. elegans*, the overall mean intrinsic growth rate, *r*, did not demonstrably decline even with up to 60 M-A generations (Keightley and Caballero 1997; Vassilieva and Lynch 1999), though downward skewness in the distribution of line means was evident (Keightley and Eyre-Walker 1999). Keightley and Caballero (1997) did not detect a significant decline in mean longevity, whereas Vassilieva and Lynch (1999) did. In the microcrustacean *Daphnia pulex*, size at birth and maturity, as well as survival, declined through M-A generations, while development rate and the size of late clutches increased (Lynch *et al.* 1998). Pletcher *et al.* (1998), studying 29 M-A lines of *D. melanogaster*, identified several lines in which mortality rate was reduced, as well as lines showing increased mortality, relative to control lines. In a separate study of *D. melanogaster* (Fry *et al.* 1999), mean viability declined significantly, though significantly less than in the studies of Mukai (*e.g.*, Mukai 1964). Average fitness declines through M-A have also been found to be small for the microorganisms *E. coli* (Kibota and Lynch 1996) and *S. cerevisiae* (C. Zeyl and J. A. G. M. deVisser, unpublished results). In contrast, *D. melanogaster* cultured in "middle class neighborhoods" (*i.e.*, populations of 100 mating pairs of flies in which selection was minimized by equalizing the contributions of each pair to the next generation) showed a significant decline in viability after 30 generations (Shabalina *et al.* 1997). It is not clear, however, that this decline is strictly due to accumulation of new mutations that reduce viability (Keightley *et al.* 1998).

Among possible explanations for our failure to detect a change in the mean of several fitness traits, four seem particularly noteworthy.

1. The conditions in which the plants were grown may have been too benign to reveal negative effects of mutations. The magnitude of mutational decline in means has been shown to depend on environmental conditions (Kondrashov and Houle 1994; Shabalina *et al.* 1997). We are currently determining effects of two environmental factors on our assessment of the change in trait means.
2. Whereas the evidence does not clearly demonstrate decline with respect to individual traits contributing to fitness, accumulating mutations may nevertheless substantially reduce overall fitness through pleiotropic effects on multiple traits. Pleiotropy is suggested by the strong positive among-line correlation ($r = 0.77$) between the number of seeds per fruit and the number of fruits per plant. In an effort to account for the effect of joint change in these traits on overall fitness, we estimated seed number per plant as the product of the two reproductive traits. The change in mean of this composite trait does not support the interpretation that mutations reduce fitness through pleiotropic effects; we found a very slight relative increase from generation 0 to generation 17 of 0.03% per generation, rather than a more substantial decline than for each trait singly. Given currently available data, we cannot assess pleiotropy involving other components of fitness.
3. Selection can, in principle, operate within individuals; in plants and other organisms lacking a distinct germ line, intra-individual selection is expected to be especially effective, because of the potential for differential growth of cell lines. This phenomenon has received limited theoretical attention (Klekowski and Kazarinova-Fukshansky 1984; Antolin and Strobeck 1985; Kondrashov 1994; Otto and Orive 1995), yet it is clear that intra-individual selection can greatly affect observed mutation rates by, for example, reducing them by as much as a half with mutational reduction in cell lineage growth rate of 10%, as shown by Otto and Orive (1995). If mutations that enhance cell growth rates also enhance fitness or its components, then intra-individual selection would also increase the apparent rate of occurrence of beneficial mutations. Unfortunately, quantitative information on mitotic mutation and differential cell lineage growth does not seem to be available (Gill *et al.* 1995). If this phenomenon has an appreciable effect in *A. thaliana*, it seems likely that it would in other organisms lacking a germ line. This would imply that evolutionarily effective deleterious mutation rates for such organisms would be

lower, and rates of beneficial mutation higher, than for organisms such as fruit flies, nematodes, and humans, in which the germ line is isolated.

4. Finally, it is possible that new mutations of positive effect on fitness arise at a detectable, evolutionarily significant rate. In comparing five models of molecular evolution, Gillespie (1994) found support for two, for which equal numbers of advantageous and deleterious substitutions are expected. Further, he "[went] so far as to claim that there are no biologically realistic models in which most of the substitutions of mutations of very small effect are deleterious."

A major difficulty in assessing mutation rates and effects of mutations is that the estimation method originally devised and still in common use assumes that all mutations have the same effect on the trait under consideration (Bateman 1959). This assumption necessarily also entails that mutations are consistent in the direction of their effects. Mutations are generally assumed to be predominantly deleterious in their effects, and a consistent decline over M-A generations in the mean of fitness traits is expected for this reason. Bateman noted that violation of this assumption downwardly biases estimates of mutation rates, while upwardly biasing estimates of (mean) mutational effect. These biases are extreme if the distribution of mutational effects is symmetric. In this case, the trait mean would not change during line advancement, because the effect of mutations increasing the trait value would tend to be negated by mutations of opposite effect. With precise balancing of mutational effects, the consequent Bateman (1959) estimate of genomic mutation rate,

$$U = R^2/V_M,$$

where R is the per-generation change in trait mean and V_M is the per-generation mutational contribution to variance, would be zero. The corresponding estimate of mean mutational effect,

$$a = V_M/R,$$

is infinitely large. With our results, the Bateman method leads to the following minimum estimates of mutation rate per diploid genome per generation: 3×10^{-4} for seed number per fruit, 8×10^{-3} for fruit number, and 2×10^{-3} for reproductive mass. The corresponding estimates for maximum mean mutational effect are 14.9 (46%), 16.3 (27%), and 30.8 (68%).

Keightley (1994, 1996, 1998) developed a maximum likelihood approach to estimating mutational parameters. His approach avoids the assumption of equal mutational effects and assumes a Γ-distribution for the mutational effects. The Γ-encompasses a wide range of distributional shapes. Under the conditions that the mutation rate is low and the distribution of mutational effects is not leptokurtic, no clear indication of bias is found (Keightley 1998). The Γ-distribution is unidirectional and undefined at zero, however; this singularity leads to upwardly biased estimates of mutation rate if the true distribution of effects is leptokurtic, with most mutations having effects very near zero (Keightley 1998). If effects of mutations can be either positive or negative, then this problem would become even more severe. In this case, a "reflected gamma" distribution, with a portion of the distribution on either side of zero (see Keightley and Hill 1988), can be used to model mutational effects, but because this compound distribution also is not continuous at zero, biases in estimates of mutational parameters would remain. Accurate inference of mutational properties will require further statistical development to address these limitations. Further, a full understanding of evolutionary consequences of spontaneous mutation must take into account the full range of mutational effects, rather than solely mutations of negative effect on fitness components. Even once estimation methods are developed that allow for mutations of positive effect, it remains possible that M-A experiments will underestimate the genomic mutation rate if there is a very large class of mutations of such slight effect that they go undetected, as suggested by the study of Davies *et al.* (1999).

In summary, even at Gen 17, early in M-A compared to other studies, the statistical power of our study is sufficient to detect V_M. We, like some others in recent M-A work, have not detected systematic changes in trait means over generations. Instead, we have found approximately symmetric spread of M-A line means about the mean of the founding generation. This result challenges the assumption that mutations reducing components of fitness overwhelmingly outnumber mutations that increase these traits. Precise and general estimation of the fundamental mutational properties thus requires an approach that allows for bidirectional mutational effects.

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