ORIGINAL ARTICLE

SPONTANEOUS MUTATION PARAMETERS FOR *ARABIDOPSIS THALIANA* MEASURED IN THE WILD

Matthew T. Rutter,[1,2,3] **Frank H. Shaw,**[4] **and Charles B. Fenster**[1]

[1]*Department of Biology, University of Maryland, College Park, Maryland 20742*

[2]*Department of Biology, College of Charleston, Charleston, South Carolina 29401*

[3]*E-mail: rutterm@cofc.edu*

[4]*Department of Mathematics, Hamline University, Saint Paul, Minnesota 55104*

Mutations are the ultimate source of genetic diversity and their contributions to evolutionary process depend critically on their rate and their effects on traits, notably fitness. Mutation rate and mutation effect can be measured simultaneously through the use of mutation accumulation lines, and previous mutation accumulation studies measuring these parameters have been performed in laboratory conditions. However, estimation of mutation parameters for fitness in wild populations requires assays in environments where mutations are exposed to natural selection and natural environmental variation. Here we quantify mutation parameters in both the wild and greenhouse environments using 100 25th generation *Arabidopsis thaliana* mutation accumulation lines. We found significantly greater mutational variance and a higher mutation rate for fitness under field conditions relative to greenhouse conditions. However, our field estimates were low when scaled to natural environmental variation. Many of the mutation accumulation lines have increased fitness, counter to the expectation that nearly all mutations decrease fitness. A high mutation rate and a low mutational contribution to phenotypic variation may explain observed levels of natural genetic variation. Our findings indicate that mutation parameters are not fixed, but are variables whose values may reflect the specific environment in which mutations are tested.

KEY WORDS: Beneficial mutations, mutation accumulation lines, mutation effect, mutation rate.

The rate of mutation and the effects of mutation on fitness can contribute enormously to the outcomes of many evolutionary processes, including adaptation (Fisher 1930; Martin and Lenormand 2006), the evolution of sex (Kondrashov 1988), extinction risks of small populations (Lande 1994; Lynch et al. 1995), and the frequency of polymorphism within populations (Johnson and Barton 2005). Despite the theoretical importance of mutations, we still have very little empirical information about the properties of spontaneous mutation especially with regard to fitness in natural populations (Bataillon 2003).

The principal approach for studying spontaneous mutations was pioneered by Mukai (1964), and involves initiating lines from a highly inbred individual or population, in which there is very little heterozygosity (mutation–drift equilibrium). The lines are propagated for a number of generations, and genetic differences that emerge among the lines must be due to spontaneous mutations that are unique to each lineage (Mukai 1964). The change in variance among the lines for a trait over time can be related to the spontaneous mutation rate for that trait given estimates of effect size (Bateman 1959; Shaw et al. 2002). The distribution of a trait among these mutation accumulation (MA) lines relative to the founder genotype reflects the distribution of mutational effects. MA lines have been created for a number of organisms, including *Drosophila melanogaster*, *Escherichia coli*, *Saccharomyces*

cerevisiae, *Caenorhabditus elegans*, *Daphnia pulex*, and *Arabidopsis thaliana* (reviewed in Lynch et al. 1999). In each case, the lines have been assayed for the effects of mutation in controlled laboratory or greenhouse conditions.

There are reasons to suspect that the fitness effects of mutations differ in natural conditions. It has been demonstrated that the fitness of mutant lines depends on the assay environment (Vassilieva et al. 2000; Kulheim et al. 2002). There is evidence that stressful conditions lead to a more deleterious average effect of mutations, and laboratory conditions are typically considered more benign than the wild (Kondrashov and Houle 1994). Furthermore, there may simply be more pathways active and more loci expressed in natural environments, allowing more mutations to have potential effects on fitness resulting in a higher measured value for the mutation rate for fitness traits. Consider that about 11.5% of the classified *Arabidopsis* proteome consists of proteins involved in plant defense (The Arabidopsis Genome Initiative 2000), yet laboratory or greenhouse grown plants are typically grown in a disease and insect free environment. Finally, we have no data on the scale of mutational variance parameters relative to environmental variance parameters in natural conditions. If environmental variance is large relative to mutational variance, then individual mutant alleles will be found in a greater range of phenotypic backgrounds and the effects of selection on the mutants will be weakened (Jaenike 1982; Lynch 1984; Johnson and Barton

2005). Thus, the scale of environmental variance to mutational variance establishes the predicted levels of genetic variation due to mutation–selection balance (Johnson and Barton 2005).

Although mutations are assumed to be nearly always deleterious (Keightley and Lynch 2003), there have been reports of the detection of beneficial mutations at higher rates than expected (e.g., Silander et al. 2007; Hall et al. 2008). In particular, there have been conflicting results from *A. thaliana* MA line experiments (Bataillon 2003) with one experiment reporting the mean effects of mutations as deleterious (Schultz et al. 1999) and other experiments (Shaw et al. 2000 and MacKenzie et al. 2005) reporting a symmetrical distribution of mutations with the number of beneficial mutations nearly equal to the number of deleterious mutations. If the documentation of a high frequency of beneficial mutations is accurate then this will have important implications for our understanding of the contribution of mutation to biological phenomena ranging from adaptation to mutational meltdown (Lynch et al. 1999).

The discrepancy of the results in the different *A. thaliana* MA studies has been attributed in part to the environmental context in which fitness was measured (Bataillon 2003; Shaw et al. 2003). We conducted an assay of *A. thaliana* MA lines in field conditions (Fig. 1) and compared them with a companion experiment using the same MA lines grown in greenhouse conditions. Thus, we can examine whether the controversial finding of beneficial mutations
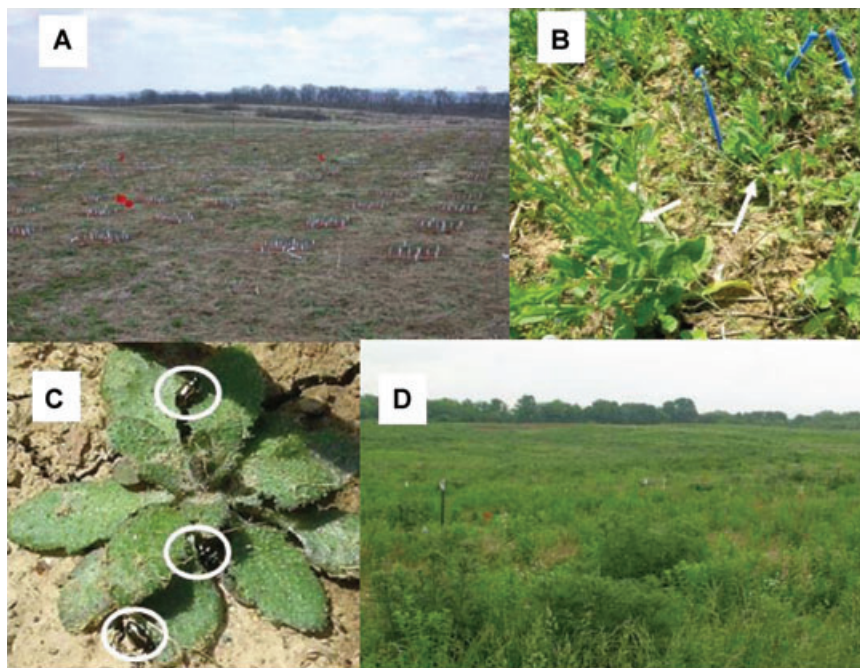
Figure 1. Site of the field mutation accumulation line experiment with representative *Arabidopsis thaliana* plants. **(A)** Site at time of planting in early spring. Block design is apparent by pattern of plant markers. **(B)** *Arabidopsis thaliana* plants flowering and growing in competition with the local community; two plants highlighted with white arrows (plastic stakes denote location of plants) and **(C)** an experimental plant being consumed by flea beetles (circled). **(D)** Site at time of harvest demonstrating that the MA lines had to compete with the surrounding vegetation consisting of forbs and grasses. Photo credits: M. Rutter.

is restricted to the benign conditions of the greenhouse. If so, we expect to observe a higher frequency of deleterious mutations under the harsher conditions of the field. In a broader context, we ask whether the properties of mutations affecting fitness differ in an environment where the phenotype of a mutation interacts with natural selection.

Materials and Methods
PLANT MATERIAL
We obtained 24th generation *A. thaliana* MA lines from R. Shaw (Shaw et al. 2000, 2002). The MA lines were derived from a single founder from the Columbia accession. We propagated the lines for an additional 25th generation. We randomly chose 100 of the 120 MA lines to use in this study. In addition, we obtained six lines from R. Shaw that were two generations removed (grand-progeny) from the progenitor individual and these lines were used to represent the premutation genotype. Each of the 100 MA lines was used to found five sublines to minimize biases due to maternal effects introduced by the specific location within the greenhouse. We founded six sublines from each of the six lines representing the premutation genotype. In 2003, subline plants were used to generate all seed utilized in all field experiments and the greenhouse experiment.

In March 2004, we cold stratified the experimental seed for 10 days at 4°C. Seed were then germinated on mist benches and transplanted from pots into 144-well plug trays that corresponded to the spatial position to which the seedlings would be transplanted in the field. From March 27 to 30 each approximately 15-day-old seedling was transferred from the plug trays into their randomized position in the field plot.

FIELD SITE AND FIELD PROTOCOLS
The field site is an old field at Blandy Experimental Farm, Virginia (39°03′45.1″N, 78°03′30.5″W). All plants were transplanted into the field very early in development, at the four-leaf stage. Planting occurred in early spring to mimic the phenology of local naturalized *A. thaliana*. At the time of planting, vegetation was scant but present. Differences within the site emerged as the native vegetation (mostly biennials and perennials) grew on the plots. By harvest, the *A. thaliana* plants were dwarfed by naturally occurring vegetation. We planted 7000 plants representing 100 lines of *A. thaliana* at the 25th generation of MA and 504 plants from lines representing the premutation genotype (Shaw et al. 2000, 2002). The plot was arranged in 14 spatial blocks, each of which included one seedling from each subline, for a total of 7504 plants. In a few cases not all five sublines produced enough seed from some of the MA lines, and in these cases other sublines from that line were overrepresented in some blocks to maintain the same overall number of plants per MA line. Plants that died within the first 3 days of transplant (about 50 plants) were considered to have died from transplant shock and were replaced with another plant from the same MA line.

Plants were censused weekly for survival. All plants were harvested from May 25 to 28, by which time they had senesced. Plants were oven dried, and all fruits produced by each plant were counted. Seedling survival and fruit number were combined for a measure of fitness, which is a good proxy of fitness for an annual selfing plant. Plants that died before fruiting were considered as contributing zero fitness to the performance of the line.

GREENHOUSE EXPERIMENT
In 2005 we planted the same 100 MA lines used in the field experiment in the greenhouse. Three sublines of each MA line were grown, with four replicates of each subline. One hundred premutation plants were grown, for a total experiment size of 1300 plants. Plants were grown in 9 cm square pots on benches in a temperature controlled greenhouse chamber and watered regularly during the course of the experiment. Plants were harvested at senescence, oven dried, and all fruits produced by the plant were counted. Plants were randomly divided across spatial blocks corresponding to greenhouse tables. As in the field experiment, fitness was measured as survival and fruit production of the seedlings.

Analyses
TESTING FOR MA LINE DIVERGENCE
We tested for differentiation among lines using a mixed-model approach that accounted for block and subline differences, and further tested whether the mean of the MA line performance differed from the premutation lines. To test whether MA lines had diverged from one another in fitness, we applied the mixed model

$$y_{ijs} = \text{line}_i + \text{subline}_j(\text{line}_i) + \text{block}_s + \text{error},$$

where y_{ijs} is the fitness value of a plant y grown in block s from MA line i and subline j within line i. Total fruit number was square root transformed to satisfy normality assumptions. All effects were treated as random. The MIXED procedure in SAS was used for all tests of significant variation between MA lines or between premutation lines (SAS 2007). Likelihood ratio tests were used for model comparison, applying a one-sided test as components of variance are by definition nonnegative. The generalized linear model (GLM) procedure was used to test for a difference between the fixed effect of MA in causing differences between the MA lines and the lines representing the premutation genotype. The GLM analysis included a fixed effect indicating whether lines were MA lines or premutation lines, and a random effect of block.

To assess whether plant survival differed among experimental units, each plant was coded with a binary variable indicating if it had survived to produce flowers. The effects of block and

MA line on this binary response variable were each modeled independently in PROC NLINMIXED in SAS, using a binomial response variable distribution with a logistic link function. One-sided likelihood ratio tests were used for model comparison.

QUANTIFYING ZYGOTIC MUTATION RATE

We used two methods to estimate mutation rate in terms of the number of spontaneous mutations in the diploid genome, both used in other *A. thaliana* MA studies: Markov Chain Monte Carlo Maximum Likelihood (MCMCML) (Shaw et al. 2002) and MLGENOMEU (Keightley and Ohnishi 1998). The two methods make different assumptions about the distribution of mutational effects and may yield different estimates of rate, thus comparisons across studies must use the same method. We estimated mutation rates and other mutation parameters from both the field and greenhouse experiments.

Our MCMCML algorithm has been described by Shaw et al. (2002). The statistical model used a Poisson distribution for the number of effects, a displaced gamma distribution for the size of the effects and a normal distribution for residuals. Estimates were simultaneously obtained for the parameters defining these distributions, based on the performance of the lines after removing subline effects. Umbrella sampling was used to ensure coverage of a broad range of mutation rates (0.001–0.5). Estimates of U using MCMCML were not made for greenhouse data because of the lack of significant mutational variance among lines.

Another Monte Carlo-based algorithm for estimating U is employed by MLGENOMEU (Keightley and Ohnishi 1998) and is also used in one of the greenhouse studies of *A. thaliana* MA lines (MacKenzie et al. 2005). This estimate does not incorporate subline effects. We derive different estimates of U with this algorithm, and so we report results from MLGENOMEU to compare our findings with those reported by MacKenzie et al. (2005). In order for the MLGENOMEU algorithm to compute successfully, we had to either make a simplifying assumption or reduce the dataset. First, we used a model that assumed mutations had equal effects (a reasonable assumption given the results below) and allowed mutation rate, the distribution scale parameter, and the proportion of beneficial effects to be determined by the algorithm. The assumption of equal effects was required for the MLGENOMEU program to run successfully on the complete dataset using each plant as the lowest level of replication ($n = 7504$). The assumption of equal effect size may result in a narrower confidence interval around rate estimates in MLGENOMEU. As an alternative to the assumption of an equal effects model, we performed an MLGENOMEU analysis on MA and control line means instead of the complete dataset. In this case, we did not constrain the model to equal effects, and determined the most likely model parameter values for a range of shape parameters of the gamma distribution. We evaluated shape parameters from 0.2 to 10, as

well as an assumption of equal effects of mutation in the line mean dataset.

To assess whether mutation rates differed between the assay environments, we compared the summed maximum likelihoods when parameters were allowed to vary independently for each dataset with the maximum likelihood when the mutations rates were constrained to be the same for both datasets. To determine the 95% confidence intervals for the mutation rates, we created profile likelihoods by estimating likelihood while varying mutation rate and constraining the other parameters. Both here and below we present mutation rates and associated parameters on a per-generation basis.

QUANTIFYING THE CONTRIBUTION OF MUTATION TO PHENOTYPIC VARIATION

Another common way to measure mutation rate is in terms of the additive genetic variance introduced to a population by mutation, that is, h^2m. Thus, h^2m describes the contribution of mutation to heritable variation and is scaled to the environmental variance. We used the MCMCML program to generate estimates of the mutational and environmental variance. Subline effects (random) were estimated simultaneously with the other parameters. Fixed block effects were accounted for prior to the analysis. Consequently, we are examining environmental variance at a finer scale than block-level variation. Including block in the environmental variance would decrease values of h^2m by approximately 10%. The mutational coefficient of genetic variation scales the mutational variance to the mean of the trait and is computed as $100\frac{(\sqrt{V_m})}{\bar{x}}$ where V_m is the mutational variance and $\bar{x}$ is the mean of the trait (SAS 2007). We computed 95% confidence intervals for the mutational coefficient of genetic variation for both the greenhouse and field using the "exact" approach (Johnson and Welch 1940; Verrill 2003). For this measure, we used square-root transformed data.

Results
DIVERGENCE OF MA LINES

Over the course of the experiment in the field, the plot transformed from a nearly barren condition to being thick with vegetation (Fig. 1). Experimental plants in the field experienced shading, crowding, herbivory, and other complex environmental effects characteristic of the natural environment. Approximately 79% of field experimental plants survived to produce mature fruit, with the average surviving plant producing 22.2 fruit. MA lines diverged significantly for both survival and fitness, thus demonstrating that the fixation of different spontaneous mutations within the lines contributed to differentiation between the lines (Table 1). Block effects on survival and fitness were significant, indicating an important role of microgeographic variation at the planting site. Subline effects were also significant, indicating that maternal

Table 1. Mixed-model analysis of variance of field planting results of the *Arabidopsis thaliana* mutation accumulation lines. The analysis for fitness included effects of block, mutation accumulation line, and subline (nested within mutation accumulation line). Additionally, we report the result of a comparison between the mean of the MA lines and the mean of the premutation lines for fitness (degrees of freedom=1 for this comparison).

Trait	Effect	Covariance parameter estimate	*P*-value
Survival	Block	0.003218	<0.001
	MA line	0.001213	0.0031
Fitness	Block	0.7213	<0.0001
	MA line	0.04436	0.0325
	Subline (MA line)	0.1106	0.0043
	MA versus premutation comparison		0.8650

effects contributed to lifetime fitness. The mean fitness of the individual MA lines were approximately normally distributed (Fig. 2). The fitness of the lines representing the premutation genotype was very near the center of the distribution of the mean fitness of the MA lines, indicating that an equal number of MA lines had higher fitness than the premutation genotype as had lower fitness. The among-line variance component for the premutation lines was zero. There was no significant difference between the mean MA line fitness and the mean fitness of the premutation lines.

In contrast to the results from the field assay, nearly all the greenhouse grown plants survived (99.2%) and the average surviving plant produced 42.8 fruits. We detected no significant variation
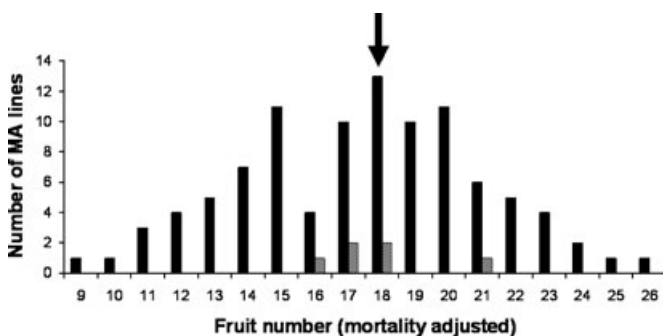
Figure 2. The black bars represent the distribution of fitness means of 100 *Arabidopsis thaliana* MA lines grown in field conditions. Mean fitness for a MA line is calculated as the average number of fruit produced by the 70 individuals assayed, with individual plants that died before producing fruit included as producing zero fruit. The grey bars represent the distribution of fitness of premutation lines, the arrow marks the mean performance of the premutation line.

among MA lines. We detected no significant difference between the performance of the MA lines and the premutation genotype. As in the field experiment, the among-line variance component for the premutation lines was zero.

MUTATION RATES

A MCMCML likelihood profile for the mutation rate parameter quantified from the field planting has a peak at $U = 0.228$ (Table 2). The distribution of the mutational effects is described by a displaced gamma distribution with displacement parameter $\rho = 0.1214$ (95% CI: 0.1108–0.1320), shape parameter $\alpha = 1.4608$ (95% CI: 1.0552–1.8664), and scale parameter $\lambda = 11.6427$ (95% CI: 8.2733–15.0121) (Shaw et al. 2002). The mean mutation effect is -0.0044 with a standard error of 0.348 as calculated by the delta method. The very limited MA line variation in the greenhouse prevented an estimate of U from the MCMCML algorithm. The MLGENOMEU program, using a model that assumed equal effects of mutations, provided estimates of $U = 1.06$ and 0.10 from the field and greenhouse data respectively. Both estimates were significantly different from zero, and although neither estimate overlapped with the others 95% CI, the estimates were not significantly different from one another when the scale parameter was allowed to vary. A nonzero estimate of mutation rate in the greenhouse by the MLGENOMEU approach may indicate variation among lines, because MLGENOMEU makes more efficient use of data than methods based on among-line variances (Keightley 1994). The sizes of the confidence interval generated by the MLGENOMEU program may be influenced by our computationally necessary assumption of mutations of equal effects. However, using line means for the field data instead of the complete dataset to relax that assumption did not provide a clear estimate of U. Depending on the value of the shape parameter (β in MLGENOMEU), the most likely value of U varied from 0 ($\beta = 10$) to 22 (equal-effects model). The equal-effects model had the highest likelihood for the line mean dataset, and differences between the likelihood of equal-effects and shape parameters with $\beta > 5$ were nearly significant ($P = 0.07$). However, no choice of shape parameter value was more significantly likely than another. Although the approach of using MA line means has been employed by a number of other authors to ameliorate computational difficulties (Keightley and Bataillon 2000; Joseph and Hall 2004), for our dataset it did not provide clearer guidance about values of U. However, the analyses of the line mean dataset do suggest that an equal-effects assumption may not be unreasonable.

Finally, subline effects are not included in the MLGENOMEU analysis, which may have influenced our detection of a mutation rate in the greenhouse which was not found in the mixed-model analysis or tests for variance among lines.

Table 2. Mutation parameters from field and greenhouse assays of fitness (95% confidence intervals in parentheses). The trait for which parameters are estimated is the square root of total fruit production, accounting for survival. In addition, untransformed means for this trait are reported. We used two methods of estimation of parameters, MCMCML and MLGENOMEU. [1]MCMCML result [2]MLGENOMEU result *, No estimate from MCMCML. [†], We report mutation rate with highest likelihood, but near zero mutation effects on variance in the greenhouse make the greenhouse mutation rate an imprecise estimate.

Mutation parameters	Field assay	Greenhouse assay
V_M	0.00184461	0.00036
	(0.00068–0.00984)	(0–0.00124)
V_E	7.5693 (7.336–7.857)	0.8003 (0.7317–0.8689)
h^2m (transformed fitness)	0.00024 (0–0.0005)	0.00047 (0–0.00133)
CV_m	1.416 (1.393–1.44)	0.298 (0.287–0.31)
U	0.228033 (0.1654–0.2659)[1]	*
	0.95692 (0.29–1.95)[2]	0.10161 (0.06–0.15)[2]
Proportion beneficial mutations	0.405 (0.352–0.458)[1]	*
	0.49079 (0.45–0.52)[2]	0.99224 (0.78–1)[2]
$\bar{x}$	17.4455 (untransformed)	42.458 (untransformed)
	3.03 (transformed)	6.36 (transformed)

CONTRIBUTION OF MUTATION TO PHENOTYPIC VARIATION

For untransformed fitness in our field study, h^2m $= 0.000107$. Furthermore, our estimate of h^2m from field data was half the estimate from our greenhouse data, based on square-root transformed data, although the confidence intervals of the two parameters overlapped (Table 2).

Although h^2m is low from the field plantings, we find evidence for a 4.5-fold greater mutational contribution to fitness variation among lines (V_M) in the natural environment than the controlled environment of the greenhouse, 0.0018 versus 0.0004, respectively ($P = 0.0085$). Only the mutational variance measured in the field was significantly different from zero. In addition, larger genetic differences among the MA lines detected in the field correspond to significantly higher estimates of the mutational coefficient of genetic variation (CV_m) in the field versus the greenhouse: 1.416 and 0.298, respectively, nearly a fivefold difference (Table 2; $P < 0.0001$). Finally, the environmental contribution to variation in fitness (V_E) was approximately an order of magnitude larger in the field than in the greenhouse assay (Table 2: $P < 0.0001$).

Discussion

We consistently detected a stronger signal of mutation in the field assay. Only in the field assessments of performance did we detect significant variation in fitness due to MA line effect using a mixed-model analysis. In the field, MA lines diverged significantly for both survival and fitness, thus demonstrating that the fixation of different spontaneous mutations within the lines contributed to differentiation between the lines (Table 1). MCM-CML and MLGENOMEU approaches using data from the field both provided relatively high estimates of the whole genomic mutation rate for fitness U in comparison with other previously reported MA line experiments (Lynch and Walsh 1998). Furthermore, consistent with two other *A. thaliana* MA line experiments (Shaw et al. 2000; MacKenzie et al. 2005), the fitness of the lines representing the premutation genotype was very near the mean fitness of the MA lines, indicating that a large proportion of spontaneous mutations are beneficial. However, the detection of a relatively high U and a large proportion of beneficial mutations occurred when environmental variation contributed substantially to the performance of the lines. We discuss the implications of these findings in turn.

LARGER ESTIMATE OF *U* IN THE FIELD

The estimate of U from the field experiment is higher than the estimate from our greenhouse study and 2–200-fold higher than previous studies of MA lines from this accession of *A. thaliana* (Shaw et al. 2002; MacKenzie et al. 2005). Furthermore, we quantified a significantly greater genetic contribution to fitness variation among lines in the natural environment than in the controlled environment of the greenhouse. Only in the field was the variation among MA lines significant and V_M significantly different from zero, despite the presence of much more environmental variation in the field. In addition, larger genetic differences among the MA lines detected in the field correspond to significantly higher estimates of the mutational coefficient of genetic variation in the field versus the greenhouse. It should be noted that previous greenhouse assays of these MA lines in previous generations have detected mutational variance for fitness (Shaw et al. 2000; MacKenzie et al. 2005). It is possible that a subtle difference between greenhouse conditions minimized mutational variance in our study. For example, our greenhouse environment may have been less stressful,

although our mean fruit production was actually slightly lower than reported by Shaw et al. (2000). Mutations in the intervening generations since previous assays may have obscured some of the variance if they had effects opposite in sign from previous generations.

Mutations may contribute more to line variation in the field experiment than in greenhouse or laboratory settings if greater stress is imposed upon plants in the field. Stress has been implicated in increasing values of three different mutational parameters: the variance of mutation effects, the average effect of mutations, or the number of expressed mutations (Martin and Lenormand 2006). Mutation parameters are typically quantified where mutations are shielded from environmental challenges, potentially disguising the effects of mutation in natural populations. Evidence exits that stress may affect estimates of mutation parameters. In *Drosophila*, the magnitude of mutational decline in trait means is greater under harsher lab conditions (Kondrashov & Houle 1994; Shabalina et al. 1997; Yang et al. 2001, but see Chang & Shaw 2003 and Kavanaugh & Shaw 2005 for no mutational decline with stress in *A. thaliana*). In *C. elegans*, only 4% of deleterious mutations are detected in benign laboratory conditions (Davies et al. 1999). Martin and Lenormand (2006) argue that the increase in V_M with stress is consistent with a model of Gaussian fitness with steeper slope of fitness on phenotype the further a genotype is from its adaptive optimum (or, in their context, more stressful conditions).

At present, we do not have experimental replication across environments to quantitatively determine whether our results are consistent with the more general findings of Martin and Lenormand (2006), that is, if our higher estimate of U in the field is due to a greater variance of mutational effects. However, we have reason to believe that our results are at least partially consistent with an explanation that includes a larger number of mutations expressed in the field environment. For example, we only see MA line effects on survivorship in the field. Typical plant MA line experiments from greenhouse studies record nearly 100% survivorship, similar to what we found in our own greenhouse experiment and in contrast to the 79% survivorship in the field. In addition, overall fitness was much lower in the field than in the greenhouse. For most species, there are many characters important in the natural environment but not expressed in typical controlled conditions. For example, a mutation affecting light harvesting under fluctuating light intensities (e.g., sporadic cloud cover or intermittent shading as the inclination of the sun changes across the day) in *A. thaliana* had no effect on fitness under greenhouse conditions but had enormous effects in the field (Kulheim et al. 2002). In our field plots we observed evidence of herbivory by slugs and insects, and our field experimental plants grew in a competitive environment typical of a secondary successional plant community. *Arabidopsis thaliana* populations are geneti-

cally adapted to a range of light and climatic conditions including varying degrees of diurnal fluctuation in temperature and precipitation (Rutter and Fenster 2007). However, these fluctuations are typically eliminated or tempered in a greenhouse environment. Thus, a higher estimate of U in the field may reflect the fixation of different spontaneous mutations in loci whose expression affects performance under these natural conditions but not in the greenhouse.

Using direct sequencing techniques the diploid whole genomic mutation rate has been measured as 1.2 and 4.2 in *Drosophila* and *C. elegans*, respectively (Denver et al. 2004; Haag-Liautard et al. 2007). If we standardize these estimates based on the number of base pairs in *A. thaliana*, then our estimate of the mutation rate from MLGENOMEU indicates that between 14 and 67% of mutations affect fitness in natural conditions. These results are consistent with the hypothesis that many mutations are cryptic under benign conditions, as discussed above. Our estimate from the MCMCML algorithm suggests that a smaller fraction, between 3 and 15%, of mutations affect fitness. The MCMCML approach incorporates subline effects and allowed variable mutation effect size and thus may be a more accurate estimate of mutation rate. Whether many mutations do or do not lead to fitness effects may best be determined when the effects of individual spontaneous mutations can be mapped to fitness. Our experiments were designed to describe the distribution of effects of mutations, not to discern exactly which lines differed from the premutation genotype. In fact, our inclusion of 100 lines makes it more difficult to be certain with post hoc tests if a specific line differs from the premutation line. A first step in mapping mutations to fitness effects would be to determine which lines do in fact differ in fitness from the premutation genotype. Such a result could be obtained by testing a subset of the MA lines against the premutation control.

MANY BENEFICIAL MUTATIONS

The distribution of MA line fitness found in the field environment indicates a very high proportion of beneficial mutations, 40 or 49% (MCMCML and MLGENOMEU algorithms, respectively). Note that in the greenhouse, MLGENOMEU estimated that nearly all mutations were beneficial. This finding is likely an artifact. The mean fitness of the MA lines in the greenhouse was slightly higher than the premutation lines, although this difference was not significant. The extremely high proportion of beneficial mutations estimated by MLGENOMEU may be due to three reasons: the low mutation rate, resultant small number of mutations affecting fitness, and a slightly higher mean effect of mutations.

Although our finding of frequent beneficial mutations in the field is surprising in light of evidence for predominantly deleterious mutations in many studies (Eyre-Walker and Keightley 2007), ours is the third study of MA lines in *A. thaliana* to report

this result, suggesting that the fitness effects may include scenarios where many mutations are beneficial (Shaw et al. 2000; MacKenzie et al. 2005). We found a proportion of beneficial mutations in the field similar to greenhouse studies despite the potential for stressful mutations to increase the proportion of deleterious mutations. Furthermore, in four additional plantings of our MA lines, (1 additional spring and 3 fall plantings) we consistently observed that 50% of the MA lines performed as well or better than the premutation line (M. T. Rutter and C. B. Fenster, unpubl. data). The recovery of high fitness MA lines suggests that the input of spontaneous mutations to populations may not necessarily result in the extinction of small populations (Lande 1994; Poon and Otto 2000).

Criticism of previous findings of high rates of beneficial mutation in *A. thaliana* has included concerns about greenhouse fitness measures (Shaw et al. 2003). However, we measured fitness in the field, where lower survival and fruit production than in greenhouse conditions suggest a more challenging environment for the plant. Our measure of fitness, total fruit production accounting for survival, is an approximation of the total fitness of the plant. One explanation for our finding of beneficial mutations is that there are other components of fitness that we did not measure that are negatively correlated with fruit production and survival. Such traits could include germination success, temperature or desiccation tolerance in the seed, survival at the cotyledon stage, or survival in other more relevant environments. Note that we ensured that all MA lines were represented by 700 plants at transplantation in to the field at the 15-day stage. However, the inclusion of greenhouse germination rates of MA lines for the seedlings used in the field and greenhouse studies did not alter our findings that many of the MA lines performed better than the founder.

It is also possible that selection removes deleterious mutations during development in organisms without an isolated germ line, such as plants, increasing the ratio of beneficial to deleterious mutations being passed to the next generation (Otto and Orive 1995). In plants, many loci are expressed in pollen, allowing an opportunity for selection at the gamete stage that is less important in organisms such as animals in which fewer genes are expressed by sperm (Joseph and Kirkpatrick 2004). In addition, it is possible that plants such as *A. thaliana* possess functional and genetic redundancies that buffer against deleterious effects of mutation. Such buffering might diminish the number of mutations with deleterious phenotypes while allowing beneficial mutations to occur. It has been suggested that these redundancies might explain why so few knockout mutations have observable phenotypes in *A. thaliana* (Bouché and Bouchez 2001; Briggs et al. 2006).

However, studies in organisms other than plants have also quantified relatively high beneficial mutation rates. In a MA study in yeast lasting over 2000 generations, 13% of the mutations were beneficial (Hall et al. 2008), whereas 15% of mutations were found to be beneficial in an experimental virus population (Silander et al. 2007). In both of these studies, the authors suggest that the beneficial mutation rate may have been increased because of the low fitness of the ancestor. The Columbia accession has been removed from the natural environment for more than 50 years and propagated for an unknown number of generations in likely varied selective regimes. The Columbia accession may be currently poorly adapted in both field and greenhouse, thus a higher proportion of mutations would be expected to be beneficial reflecting compensatory mutations and a genotype far from the optimum for the field environment (Fisher 1930; Poon and Chao 2005; Martin and Lenormand 2006; Silander et al. 2007). Alternatively, the Columbia genotype may have been selected in the laboratory for rapid reproduction without concern for total fruit production or survival, yielding an organism with a low fitness measure with the methods we have used. It is perhaps noteworthy that all three experiments finding a high proportion of MA lines with increased fitness were conducted using the Columbia accession, as opposed to another *A. thaliana* MA study that found a mean deleterious effect of mutation using the Landsberg accession to found the MA lines (Schultz et al. 1999). The Fisher model predicts a maximum of 50% beneficial mutations at infinite distance from the optimum (or for infinitesimally small mutations). However, it is less clear what mutation effect size or distance from the optimum would have to be to generate a pattern of nearly 50% beneficial mutations (Martin and Lenormand 2006).

In MA experiments such as ours, the mutations with very small effects may be obscured because of the variance of mutation effect size. Such small effect mutations may be very abundant and may play a critical role in long-term evolutionary processes. Quantifying the effects of such mutations will likely require assaying the effects of many individual mutations with experiments large enough to detect very subtle phenotypic differences between genotypes.

Nonetheless, the surprisingly high estimate of the proportion of beneficial mutations combined with a higher estimate of *U* suggests that in some natural populations newly arising mutations can contribute more to the adaptive response to selection than previously thought. The existence of conditions in which beneficial mutations are relatively frequent has implications for evolutionary models ranging from the evolution of sex to the role of mutations in successful biological invasions (Lynch et al. 1999).

ENVIRONMENTAL VARIATION

Natural populations of plants often experience microgeographic and temporal environmental variation, such that "there is no scale at which plants live where the environment can be realistically represented as uniform." (Bell and Lechowicz 1991). However, the goal of many MA line experiments is the reduction

of environmental variation so as to better detect genetic sources of variation. In contrast, our field experiment was designed with the explicit intention of quantifying mutational effects (through the performance of MA lines) in an environmental context approximating the range of environmental variables normally experienced by a spontaneous mutation appearing in an *A. thaliana* genotype. The detection of significant block effects indicates an important role of microgeographic variation at the planting site. The large contribution of environment to the performance of any given replicate in our experiment is reflected in the low estimate of h^2m, near the lower bound of reported h^2m values for untransformed fitness across a range of organisms and traits, and an order of magnitude lower than the value reported for the same trait in a greenhouse study of the same *A. thaliana* MA lines (Lynch and Walsh 1998; Shaw et al. 2000).

Although our finding in the field of greater mutational variance and lower contribution of mutation to heritable variation may appear to be contradictory, it can be explained by higher environmental variance in the field that overwhelms the increased effect of mutation on fitness. Thus in the natural environment there is a lower contribution, on average, of a mutation to phenotypic variation. Another study quantified the effects of spontaneous mutations in contrasting field and greenhouse conditions using an outbred design in which two populations of *Raphanus raphanistrum* under relaxed selection were compared to an ancestral population (Roles and Conner 2008). They observed a significant effect of mutations in increasing among-line variances in the greenhouse but not in the field, although the mutations appear to have larger effect in the field. Although not directly comparable to our study, which examines the performance of 100 lines relative to an ancestral population, their results do suggest that environmental effects can obscure the effect of mutations on fitness. The decreased effect of mutation on phenotypic variance results in a diminished ability of selection to influence mutation frequencies (Houle et al. 1996). That we detect MA line differences in the field and not in the greenhouse combined with our findings from other studies in which the performance of MA lines is not correlated across different plantings (M. T. Rutter and C. B. Fenster, unpubl data; A. Roles, M. Rutter, C. Fenster, and J. Conner, unpubl. data) indicate that mutational effects are dependent on specific field environments (a genotype by environment interaction). In our greenhouse-field study we observed a specific form of genotype–environment interaction, variance genotype–environment interaction, in which genetic variance is expressed in one environment and not in another. The pattern of variance genotype–environment interaction would be consistent with a larger average effect of mutations in the field conditions. If context dependency of the type we observe between the greenhouse and field is also present between natural populations, then it may facilitate the accumulation of standing genetic variation

as well as contribute to ecological specialization (Martin and Lenormand 2006). The high *U* value combined with low contribution of mutations to phenotypic variation in the field also suggests that mutations may rapidly affect genetic load and consequently contribute to mating system evolution (Lynch et al. 1999). If, as in our study, laboratory conditions underestimate both the mutation rate for a trait and the environmental variance, the amount of genetic variation at mutation–selection balance in natural populations would be higher than predicted from laboratory studies (Johnson and Barton 2005).

CAVEATS AND CONCLUSIONS

Estimates of *U* and the distribution of mutation effects are likely to be more accurate when tested in the environment where the premutation genotype has evolved. When tested in the wrong environment, mutational effects may change leading to either over- or underestimation of *U* (Martin and Lenormand 2006) and as previously discussed the proportion of beneficial mutations may be dependent on the original fitness of the founder. Note that while ours is the first attempt to quantify mutation rate and the distribution of mutation effects in a setting approximating an environment where natural selection acts, it is, nonetheless only an approximation of the environment where a mutation affecting a Columbia accession genotype would experience. The Columbia accession is derived from the Landsberg accession, originally collected in northern Germany (48°11′N, 10°52′E) whereas we assessed the effect of mutations on the Columbia phenotype in the northern Blue Ridge of Virginia (39°03′N, 78°03′W). Thus our estimates of mutation parameters should be treated with caution in that the field assessment of performance may be very different in an environment more similar to the actual locale from where Columbia was first collected.

Overall, our study provides evidence that mutation parameters for phenotypic traits are best considered as variables dependent upon the environment and local patterns of selection rather than as fixed constants. Our documentation of relatively high *U* and low h^2m in the field assay suggests that most mutations have effects well below the scale of detection by natural selection. Consequently, our results indicate that mutation can substantially contribute to standing genetic variation. However, to improve the accuracy of our predictions about the contribution of mutation to evolutionary processes such as adaptation, we will need much more study of the effects of mutation in environments that are both natural and variable.

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Associate Editor: T. Juenger