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FITNESS EFFECTS OF MUTATION ACCUMULATION IN A NATURAL OUTBRED POPULATION OF WILD RADISH (*RAPHANUS RAPHANISTRUM*): COMPARISON OF FIELD AND GREENHOUSE ENVIRONMENTS

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Spontaneous deleterious mutation has been measured in a handful of organisms, always under laboratory conditions and usually employing inbred species or genotypes. We report the results of a mutation accumulation experiment with an outbred annual plant, *Raphanus raphanistrum*, with lifetime fitness measured in both the field and the greenhouse. This is the first study to report the effects of spontaneous mutation measured under field conditions. Two large replicate populations ($N_e \approx 600$) were maintained with random mating in the greenhouse under relaxed selection for nine generations before the field assay was performed and ten generations before the greenhouse assay. Each generation, every individual was mated twice, once as a pollen donor and once as a pollen recipient, and a single seed from each plant was chosen randomly to create the next generation. The ancestral population was maintained as seeds at 4°C. Declines in lifetime fitness were observed in both the field (1.7% per generation; $P = 0.27$) and the greenhouse (0.6% per generation; $P = 0.07$). Significant increases in additive genetic variance for fitness were found for stems per day, flowers per stem, fruits per flower and seeds per fruit in the field as well as for fruits per flower in the greenhouse. Lack of significance of the fitness decline may be due to the short period of mutation accumulation, the use of outbred populations, or both. The percent declines in fitness are at the high end of the range observed in other mutation accumulation experiments and give some support to the idea that mutational effects may be magnified under harsher field conditions. Thus, measurement of mutational parameters under laboratory conditions may underestimate the effects of mutations in natural populations.

KEY WORDS: **Lifetime fitness, mutation accumulation, *Raphanus raphanistrum*, spontaneous mutation, wild radish.**

As the source of all new genetic variation, spontaneous mutation is one of the most fundamental processes in evolution. Theoretical predictions concerning the maintenance of genetic variation (Houle et al. 1996), the evolution of sex (Keightley and Eyre-Walker 2000), the evolution of aging (Rose 1991), and the persistence of small populations (Lande 1994; Lynch et al. 1995) depend on the rate and fitness effects of spontaneous mutation in nature. Spontaneous mutation is, however, very difficult to study empirically because mutations are rare and most are thought to have small effects on the phenotype.

Most spontaneous mutations are assumed to be deleterious, for two reasons. First, there are many more ways to dismantle an existing adaptation than there are ways to improve it. Second, data from molecular studies comparing the observed per-site rate of nonsynonymous amino acid substitutions (K_n; mutations which change the amino acid) to the per-site rate of synonymous amino acid substitutions (K_s; mutations which do not change the amino acid) indicate that the majority of nonsynonymous substitutions are deleterious (Keightley and Lynch 2003). The ratio of K_n/K_s averages 0.3 or less in all taxa for which estimates are available (Ohta 1995; Eyre-Walker et al. 2002), suggesting that at least 70% of all nonsynonymous mutations are eliminated by selection.

Mutation accumulation (MA) is the most common method used to study the rate and effects of spontaneous deleterious mutation on fitness. In this technique selection is reduced and often drift is maximized so that deleterious mutations are more likely to be fixed. This regime of reduced selection is repeated over multiple generations in independent lines to allow mutations to accumulate. After multiple generations of MA, fitness is estimated simultaneously in the MA lines and a control (ideally the ancestral state of no new mutations accumulated). The expectation is that fitness will be decreased in the MA lines relative to the ancestor due to the accumulation of spontaneous deleterious mutations.

Using MA experiments, the genomic spontaneous deleterious mutation rate for fitness has been estimated in a handful of model organisms including *Escherichia coli* (Kibota and Lynch 1996), yeast (Wloch et al. 2001; Zeyl and DeVisser 2001), *Caenorhabditis elegans* (e.g., Keightley and Caballero 1997; Vassilieva and Lynch 1999), *Daphnia pulex* (Lynch et al. 1998), *Drosophila melanogaster* (e.g., Shabalina et al. 1997; Fry et al. 1999), and *Arabidopsis thaliana* (Schultz et al. 1999; Shaw et al. 2000). Most of these studies have found decreased mean fitness after MA but a few have not. Shaw et al. (2000) and Keightley and Caballero (1997) did not report decreased mean fitness, although they did detect an increase in fitness variance, indicating that mutations had in fact accumulated. In yeast, Zeyl and DeVisser (2001) detected a significant decline in growth rate in DNA repair-deficient yeast but not in DNA repair-competent yeast.

A potential explanation for the finding of no decrease in mean fitness in some MA studies may be the environments in which fitness is estimated (Kondrashov 1998). Most studies of MA have used laboratory populations and all have assayed fitness in the laboratory or greenhouse, usually under benign conditions. Several studies of *D. melanogaster* that compared the effects of MA under stressful and benign environments have found greater declines under stress for some fitness components (e.g., Shabalina et al. 1997) and/or increases in among-line variance of MA lines (e.g., Fry and Heinsohn 2002). In these studies of MA in multiple environments the harsh or stressful environments are also often novel, that is, different from the environment under which mutations were accumulated. During accumulation, those mutations having particularly large deleterious effects in the environment of accumulation may be removed by selection. This will reduce the observed magnitude of fitness reduction due to new mutation when measured in the "benign" MA environment. This downward bias of estimates of mutational rates from MA experiments is expected (Lynch et al. 1999) due to the inability to completely remove selection, although the extent of the bias remains unknown. However, when MA lines or populations are assayed under other, novel, conditions, mutations that have a small effect in the accumulation environment may express larger deleterious effects in the novel environment, thus making it appear that the novel environment is "harsh" relative to the accumulation environment. Xu (2004) found support for this hypothesis in an MA study of the fungus *Cryptococcus neoformans* in which performance was better under the conditions experienced during MA than under novel conditions (altered temperature and growth medium). This suggests that mutations with large deleterious effects are removed during MA and some mutations that were neutral (or nearly so) in the MA environment were deleterious in a novel environment. Thus, both the environment experienced during MA and the environment of the ftness assay are important considerations in interpreting MA experiment results.

We have assayed the effects of MA on fitness in two novel and harsh environments: in the field and under stress in the greenhouse in wild radish (*Raphanus raphanistrum*). Two replicate populations of 300 individuals collected from the same natural population were propagated under relaxed selection for nine (field assay) or ten (greenhouse assay) generations to allow mutations to accumulate. To our knowledge, this is the first study to examine the effects of spontaneous mutations on fitness under field conditions.

Methods

STUDY SPECIES

Wild radish, *R. raphanistrum* (Brassicaceae) is a self-incompatible annual weed that grows in highly disturbed habitats such as agricultural fields. *R. raphanistrum* is a model system in ecology and evolution, including many studies on plant–insect interactions (e.g., Agrawal 1998; Strauss et al. 2001), natural selection and genetic correlations (e.g., Stanton et al. 1986; Mazer 1987; Conner 2002) and adaptation to global climate change (e.g., Tevini et al. 1983; Kostkarick and Manning 1993; Case et al. 1998).

GENERATION AND PROPAGATION OF MUTATION ACCUMULATION POPULATIONS

Seeds for the experimental populations were collected from a natural population of wild radish in an alfalfa field near Binghamton, NY in 1988 as described in Conner and Via (1993) and stored as seeds at 5°C. Two populations (designated MA1 and MA2) of 300 individuals each were created in 1991 and maintained in the

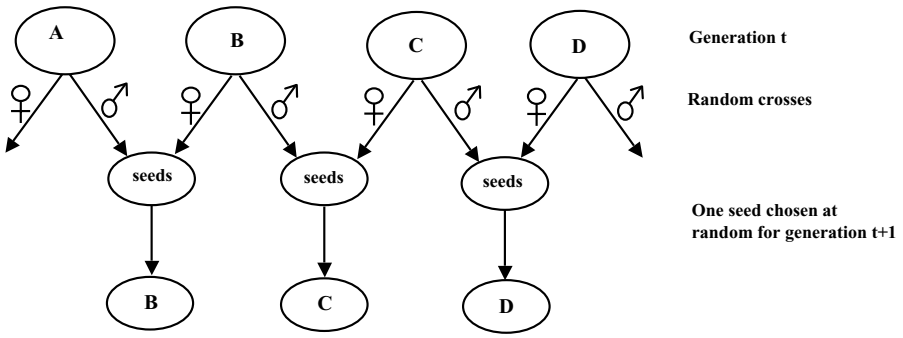

Figure 1. Hermaphrodite middle-class neighborhood crossing design. Each individual is used as a male once and a female once, contributing two offspring to the next generation.

greenhouse for nine generations under relaxed selection, using a middle-class neighborhood (MCN) crossing design modified for use in hermaphrodites. In this design, each individual is mated twice, once as a male and once as a female (Fig. 1), that is, each individual contributed two offspring to the next generation, one through seed and the other through pollen. This design maintained a large effective population size ($N_e \approx 600$) because there was no variation in family size (Crow and Kimura 1970). A single seed produced by each individual was chosen randomly to be planted for the next generation. An MCN design minimizes the opportunity for selection and thus allows mutations to accumulate. This also minimizes adaptation to the greenhouse environment. The large effective population size used in this study also minimizes the effects of genetic drift. There may have been some selection on germination, as about 3% of mothers had no offspring germinate in the next generation, but this was likely mainly due to pests and diseases in the greenhouse rather than genetic differences (Conner 2002). Still, mutations that affected germination success may have experienced some selection. To eliminate selection for early germination and flowering, we assigned crosses randomly in advance and waited for plants to germinate and flower to perform crosses. Although most wild radish plants in this population germinate quickly and flower within 4–6 weeks, some plants take much longer, so that the nine generations took about 9 years to complete. Ancestral populations were maintained as seeds at 4°C. For further details see Conner (2002).

FIELD ASSAY OF MA

Experimental design

All three populations (Ancestor, MA1, MA2) were germinated, grown, and crossed in the greenhouse for one generation prior to planting in the field (common garden generation ten). The field generation corresponds to nine generations of MA, because the common garden generation included the ancestor and therefore the MA populations did not accumulate additional mutations relative to the ancestor in this generation. Populations were regularly interspersed on the greenhouse benches to eliminate average maternal environmental differences among populations. Families were created using a nested half-sibling mating design with 75 sires and three dams per sire creating 225 full-sibling families. Due to failed germination and crosses the final numbers were 75 ancestral sires (207 dams), 70 MA1 sires (201 dams), and 68 MA2 sires (193 dams). Two MA1 sires were only crossed with two dams but all others were crossed with three. One offspring from each full-sibling family was grown in the field trial.

Seeds from the common garden generation were germinated in the greenhouse to ensure high germination success; germination was recorded daily. All seeds were weighed prior to planting. Initially one haphazardly chosen seed per dam was planted. If the first seed did not germinate within one week, a second seed was planted. If the second seed did not germinate, two more were planted and this was continued until a seed germinated or no more seeds were available. A maximum of seven seeds were planted for a single dam, and 25 dams failed to have any offspring germinate, leaving germinated offspring from 201 ancestral, 193 MA1, and 182 MA2 dams (576 total). The first seed planted germinated for 90% of the dams and more than four seeds were planted for only 2.5% of dams.

Seedlings were planted in May 2001 at Kellogg Biological Station (KBS) in southwestern Michigan. Planting took place before seedlings had their first true leaves, within five days of germination. Individuals from each population were randomly assigned to one of seven blocks in the field with 90 individuals per block (30 from each population). The populations were regularly interspersed in the field within blocks, in 10 rows by 9 columns, with 1-m spacing. Mortality and date of first flowering were recorded. A large number of plants (227 out of 576) were lost early in the experiment to rabbit herbivory due to a hole in the fence surrounding the field. Once the hole was repaired, mortality was low until the end of the season (90% survived to flowering). All stems, flowers, and fruits were collected and counted for each plant. The number of seeds in each fruit was also counted on all fruits allowing the calculation of seeds per fruit and total number of seeds (lifetime female fitness) for each plant.

Study site

The field used in this study is located at the Plant Ecology Field Lab of KBS. This site was an agricultural field before its addition to the field station, thus it represents the type of habitat in which wild radish might be found naturally. To further simulate the natural agricultural habitat we tilled the field before planting.

Analysis

The measured traits were divided into parental and offspring groups. Each group was analyzed first with a multivariate analysis of variance (MANOVA) and then with individual analysis of variance (ANOVA) for each trait using SAS (SAS Institute, Cary, NC). Parental traits were average seed weight, germination success (proportion of seeds germinated), and average days from planting to germination. The parental traits, including germination success, represent averages of all of the multiple seeds planted per dam; therefore, they represent traits of the parents not traits of any single offspring. Traits of individual offspring were partitioned into multiplicative fitness components (survival to flowering, reproductive life span [number of days in flower], flowering stems produced per day, flowers produced per stem, the proportion of flowers that set fruit, and the average number of seeds per fruit); their product is total lifetime female fitness (number of seeds). The plants that died before flowering (those eaten by rabbits plus 34 that died for unknown reasons) were not included in the last four fitness components but were included in total fitness. The multiplicative fitness components are largely independent ($r \leq 0.19$), whereas the raw variables are highly correlated. Population was a fixed effect in the model. Block and sire (nested within population) were modeled as random effects. A planned contrast comparing the mean of the two MA populations to the ancestral mean was performed for each trait. Residual plots showed no signs of serious heteroscedasticity.

This portion of the study was designed to test for a change in mean, but the inclusion of multiple offspring per sire allows us to test for significant additive genetic variance as well. Additive genetic variance within each population was estimated as four times the sire variance component in a model estimating separate variances for each population. The presence of significant sire variance was tested by performing a one-tailed chi-square test comparing the two-times log-likelihood of the model including sire to that of the model without sire (Littell et al 1996). The hypothesis of greater variance in the MA populations was tested by comparing a model estimating one sire variance across all three populations (equal variance model) to one estimating separate (unequal) variances for each population. Significance was tested by performing a one-tailed chi-square test comparing the two-times log likelihood of the full model (unequal variance) to that of the reduced model (equal variance). This test has two degrees of freedom because the models differ by two parameters (the equal model estimates one variance and the unequal model estimates three variances). Significance of this test indicates different variances among the three groups but does not specifically indicate significantly greater variance in the MA groups relative to the Ancestor.

GREENHOUSE ASSAY OF MA

Experimental design

The fitness assay was repeated in the greenhouse using a larger half-sibling mating design to more powerfully test for increases in additive genetic variance (V_A) as well as declines in mean fitness due to MA. Stored seeds were planted to create the ancestor half-sibling families. For the MA populations, seeds from the common garden generation ten (prior to the field assay) were planted to create the half-sibling families for the greenhouse assay. This planting represents ten generations of MA for the MA populations because new ancestral seeds were chosen; thus, the common garden generation was an additional generation of MA for the MA populations relative to the ancestor. In each population, 50 plants were chosen randomly to be sires and three unique dams were assigned randomly to each sire to generate 150 full-sibling families nested within 50 paternal half-sibling families. Due to space constraints, the 50 half-sibling families were split into two blocks of 25 that were grown and crossed at different times. Due to failure to set seed, six full-sibling families were lost resulting in 148 ancestral full-sibling families, 149 MA1 full-sibling families, and 147 MA2 full-sibling families.

Seeds from these families were grown in the greenhouse under water and nutrient stress. For the ancestor, four seedlings per dam were grown (592 seedlings; 577 survived to produce fruit). Two seedlings per dam were grown for each MA population (298 MA1, 297 survived to fruiting; 294 MA2, 290 survived to fruiting). This design uses the same number of ancestral plants as MA plants, increasing the power to detect differences in mean and variance between the ancestor and the MA populations. Two offspring per dam provided reasonable power to detect changes in additive genetic variance for two reasons. First, the number of sires is the primary determinant of the power to detect additive variance. Second, environmental variance was minimized by periodically rotating plant location in the greenhouse (see below).

Seeds were planted in 3-inch pots (to produce water stress) with Metro-mix 360 and fertilized with a total of 5 g Osmocote Plus 15–9–12 (NPK) controlled release pellets (nutrient stress). Fertilizer was applied gradually, 1.25 g was applied just after planting, 1.25 g applied just before flowering, and 2.5 g applied during peak flowering. A regular dose is 5 g of Osmocote pellets applied at planting rather than gradually over the life of the plant. To compensate for failures in germination, six seeds were planted for each ancestral dam and four seeds for each MA1 and

MA2 dam. Extra seedlings were thinned or transplanted to pots from the same dam that did not have seedlings. When necessary, additional seeds were planted until the desired numbers of offspring were achieved. During germination, pots from the three populations were regularly interspersed in flats of 33 plants (11 from each population) to eliminate average environmental differences between populations.

Once plants had germinated they were transferred to new flats containing individuals from one population only, with one offspring from each of the 25 sires in a time block (25 plants from different families per flat). There were 24 ancestor flats, 12 MA1 flats and 12 MA2 flats. Flats were spaced at least 29 cm apart to minimize accidental cross-pollination between groups. Flat order on the greenhouse bench was randomly assigned within each population and each flat bordered two other flats, one from each of the other two populations. Flat position was re-randomized twice a week prior to flowering to minimize environmental differences among flats. Within-flat pot position was randomized initially. Two greenhouse rooms were used and assignment of flats to rooms was random. Germination and first day of flowering were recorded daily. Once flowering began, mass pollination was performed within each population (primarily within a flat) two to three times per week until flowering ceased. Flowers were pollinated by sweeping a paintbrush haphazardly across the tops of all open flowers in a flat for 5–7 min per flat.

Pollen viability was assayed on one newly opened flower of two haphazardly chosen offspring per sire for the ancestral population ($n = 100$) and on one offspring per sire for MA1 ($n = 50$) and MA2 ($n = 50$). Viability was assessed by the Heslop-Harrison fluorochromatic reaction (FCR) test (modified from Kearns and Inouye 1993 and Thomson et al. 1994) as follows. A single newly opened flower was collected from the focal plant and the cut pedicel placed into 10% sucrose for a maximum of 3 h. A sample of pollen (about 100 grains) was removed from one anther with a pin and placed in a single drop of Fluoroscein diacetate (FDA) solution on a glass slide. The sample was incubated for 5–10 min at room temperature in the dark before a cover slip was added. All strongly fluorescent (viable) grains were counted under epifluorescent illumination. A count of all grains was then performed under visible-light illumination.

Ovule number was counted for one newly opened flower from two offspring for each ancestral dam ($n = 296$) and one offspring for each MA1 dam ($n = 149$) and MA2 dam ($n = 144$, 3 not collected). Fruits were collected as they ripened and all seeds were counted. Once plants senesced all shoots were collected and all flowers and fruits were counted.

Analysis

Results for pollen viability, ovules per flower, flowers produced, fruits per flower, seeds per fruit, and total seeds produced were analyzed using the MIXED procedure in SAS (SAS Institute 2004). Population and block were fixed effects and sire (nested within population and block) and dam (nested within sire, population, and block) were random effects. A priori contrasts were constructed comparing the ancestor mean to the mean of the two MA lines to test for decreased fitness of the MA lines. The multiplicative fitness components, pollen viability, and ovules per flower were log(Y+1) transformed to reduce differences in scale between variables and analyzed with MANOVA. The untransformed data, including total seeds produced, were also analyzed with univariate ANOVAs. Residual plots showed no signs of serious heteroscedasticity. Additive genetic variance was estimated from untransformed data as previously described for the field study.

Results

FIELD ASSAY OF MUTATION ACCUMULATION

The means did not differ significantly between populations for any trait (Fig. 2; MANOVA parental traits $F_{2,1700} = 0.38$, $P = 0.7$; MANOVA offspring traits $F_{2,2245} = 1.83$, $P = 0.16$), and although lifetime fitness (number of seeds produced) declined in the MA populations relative to the ancestor, this decline was not statistically significant (Fig. 2J). The estimated fitness difference between the Ancestor and the MA lines was 7.4 seeds (SE = 5.7), which represents a 17.9% fitness decline due to the nine generations of MA. The fitness component that declined the most was survival to flowering (Fig. 2C). Germination success was high and similar across populations (Fig. 2B), suggesting that little or no inadvertent selection on this trait occurred.

There was evidence for increases in V_A in the MA populations. Significant sire variance was found for survival to flowering, stems per day, and fruits per flower in population MA1, for flowers per stem and seeds per fruit in population MA2, but not for any trait in the Ancestor (Fig. 3). All traits with significant sire variance also showed evidence of significant differences in sire variance among populations (Fig. 3).

GREENHOUSE ASSAY OF MUTATION ACCUMULATION

The MANOVA was significant ($F_{2,141} = 3.49$, $P = 0.03$), indicating differences in fitness between the populations. The planned contrast was also significant ($F_{1,115} = 3.78$, $P = 0.05$), demonstrating that the significance of the MANOVA is due to a decrease in mean fitness of the MA populations relative to the ancestor. The estimate of difference of the MA lines from the Ancestor was 5.1 (SE = 2.9), that is, fitness of the MA lines is 6.6% lower than that of the Ancestor. Univariate ANOVAs showed that the significance of the MANOVA contrast was likely due to two individual traits: fruits per flower showed a significant decrease in the MA populations relative to the ancestor (Fig. 4D), and the decrease in pollen viability approached significance (Fig. 4A). The univariate

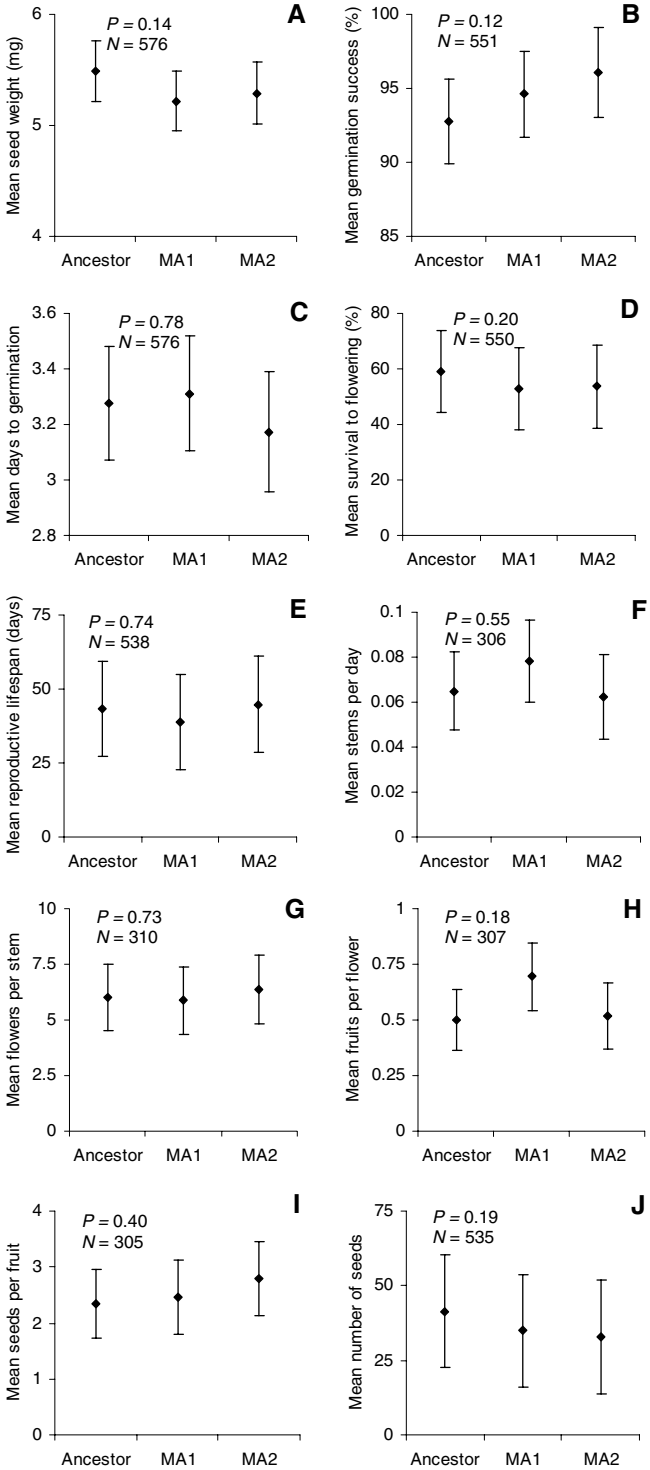

Figure 2. Field assay least-square (LS) means (± two standard errors) of fitness components from individual ANOVAs. (A) seed weight, (B) germination success, (C) days from planting to germination, (D) survival to flowering, (E) reproductive life span (number of days in flower), (F) flowering stems produced per day, (G) flowers per stem, (H) proportion of flowers setting fruit, (I) average number of seeds per fruit, (J) total lifetime seed production. *P* values are from planned contrasts of the ancestor to the mean of the two MA populations.

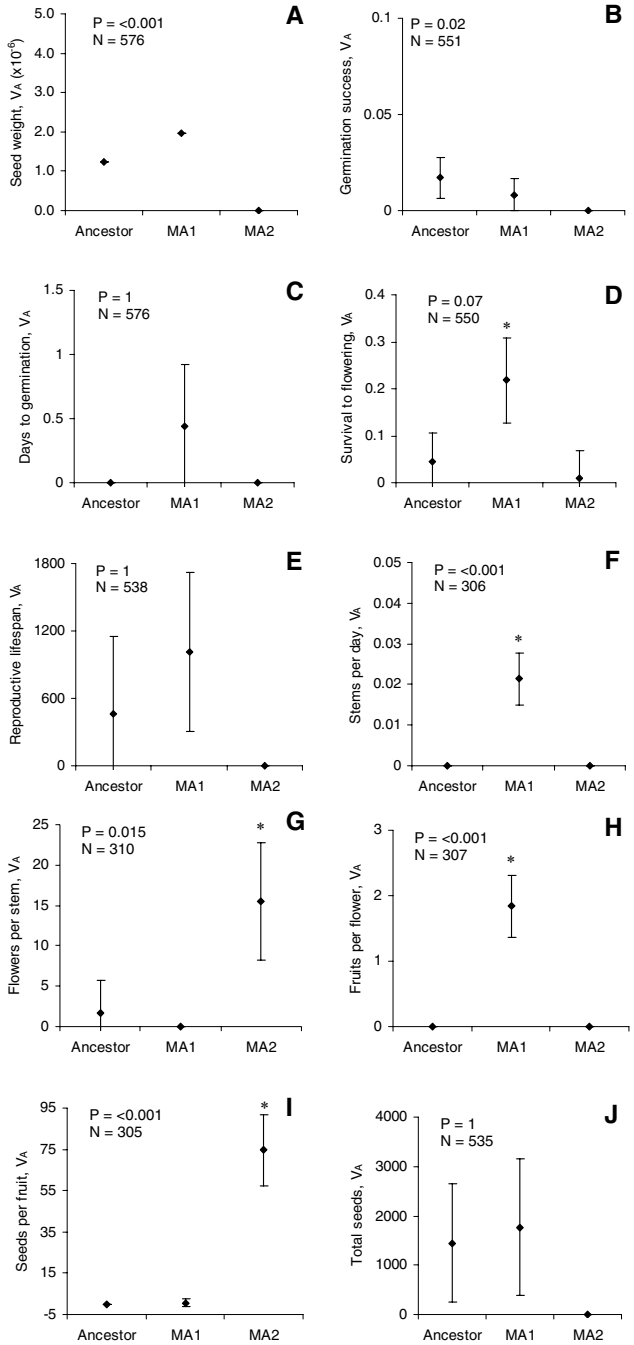

Figure 3. Field assay additive genetic variances (V_A; ± one standard error) for fitness characters. Traits as in Figure 2. *P* values are from the test for differences in V_A among the three groups (see Methods). Asterisks indicate individual variances that are significantly greater than zero ($P < 0.05$). Variances estimated to be zero have no standard error.

ANOVA for number of seeds produced showed a nearly significant decrease (Fig. 4F) in the MA populations relative to the ancestor.

There was evidence for increased V_A due to MA for fruits per flower in population MA2 (Fig. 5B). There were no statistically significant differences in additive variance among populations for

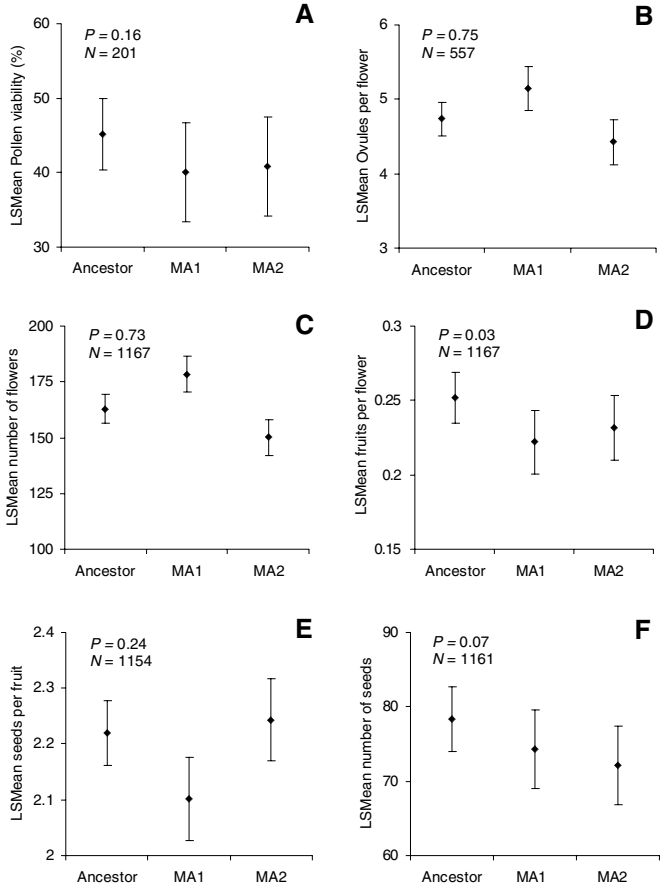

Figure 4. **Greenhouse assay least-square (LS) means (± two standard errors) of fitness characters. (A) pollen viability, (B) ovule number, (C) flower number, (D) fruits per flower, (E) seeds per fruit, (F) seed number. Note that the y-axes do not start at zero. *P* values are from contrasts of the ancestor to the mean of the MA populations.**

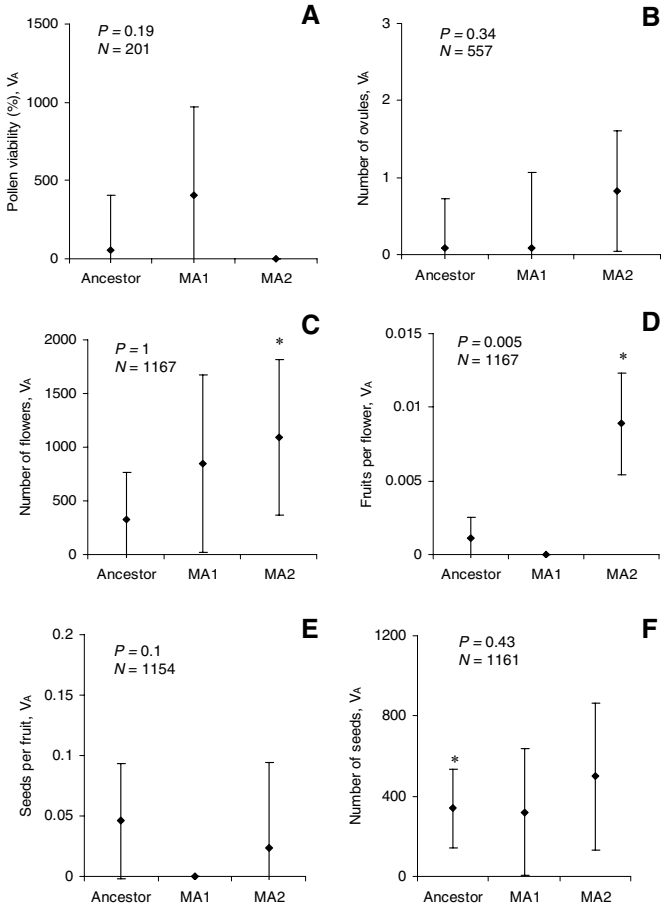

Figure 5. **Greenhouse assay additive genetic variances (V_A; ± one standard error) for fitness characters. Traits as in Figure 4. *P* values are from the test for differences in V_A among the three groups (see Methods). Asterisks indicate individual variances that are significantly greater than zero ($P < 0.05$). Variances estimated to be zero have no standard error.**

any other trait. However, there were nonsignificant trends for at least one MA population to have a larger estimate of V_A than the ancestor for all traits except seeds per fruit. Significant V_A was found for only three traits: flowers and fruits per flower in MA2 (Fig. 5A, D) and number of seeds in the ancestor (Fig. 5F). All other populations and traits had estimates of V_A that were not significantly different from zero.

Discussion

No mean differences in fitness were detected in the field, although a trend for decreased fitness in the MA populations relative to the ancestor was present for four of ten traits (seed weight, survival to flowering, flowers per stem, and lifetime fitness). The greenhouse assay did reveal significant decreases due to MA in the overall MANOVA, for fruits per flower, and a marginally significant decline in lifetime female fitness, number of seeds. However, the percent decline in fitness was almost three times greater in the field than in the greenhouse (1.7% vs. 0.66% per generation); the higher statistical significance in the greenhouse results from lower variance in the fitness estimates due to larger sample sizes and lower environmental variance. These estimates of fitness decline are for heterozygous mutations and are similar to the highest heterozygous estimates of 2% per generation in *Drosophila* (Lynch et al. 1999). Assuming $h \sim 0.15$–0.3 for mildly deleterious mutations (Lynch and Walsh 1998), our estimates correspond to a homozygous decline of ~6–11% per generation in the field and ~2–4% per generation in the greenhouse. Declines such as these could present a substantial challenge to a small population in nature. Alternatively, they could be biased upward by selection or recombination (see below).

One possible explanation for not observing stronger statistical support for a decline in fitness is an inherently low rate of mutation combined with a relatively short period of MA (nine or ten generations). Of the MA studies that assayed fitness at or near ten generations, three found a decline in mean under some

conditions (Shabalina et al. 1997; Schultz et al. 1999; Schoen 2005) whereas two found no difference from the control (Vassilieva and Lynch 1999; Shaw et al. 2000). These latter two studies continued and were assayed at later times—Shaw et al. (2000) still reported no significant decrease in fitness in *A. thaliana* after 17 generations, but Vassilieva and Lynch (1999) found a significant decrease for some traits in *C. elegans* after 50 generations. These studies demonstrate that it is possible to statistically detect the effects of MA after ten generations, although it is not guaranteed.

POTENTIAL PROBLEMS WITH MCN DESIGNS

Shabalina et al. (1997) used a breeding design similar to ours with two large populations ($N_e \approx 400$ per population, smaller than ours) of *D. melanogaster* and detected a significant decrease in fitness after ten generations. Several concerns that were raised by Keightley et al. (1998) about the interpretation of the MCN experiment of Shabalina et al. (1997) could potentially apply to our experiment. First, adaptation in the MA lines to the greenhouse environment is possible. Within-family selection, due to inviable seeds, would result in adaptation to the greenhouse during MA. We minimized this source of selection by randomly choosing a single seed per individual for the next generation, thus allowing genetic drift to dominate within-family selection. Adaptation to the MA environment could have operated in Shabalina et al.'s (1997) experiment, where 20% mortality was experienced each generation in the MA populations. In contrast, our populations experienced much lower mortality, only losing 3% of dams per generation. There was a large decline in fitness per generation in the greenhouse, and no significant difference between Ancestor and MA lines in germination success in the greenhouse, which argue against a major effect of selection during MA, because adaptation to the greenhouse should make the decline in fitness in the MA lines relative to the ancestor smaller. Thus, the greenhouse comparisons are conservative in the event of selection during MA.

Second, if selection occurs in the seeds of the Ancestor population during storage, then lower fitness of MA populations relative to the Ancestor could be due to increased Ancestor fitness rather than decreased MA fitness. However, in our study germination success was high (93–96%) and not significantly different between Ancestor and MA lines. This is in contrast to the low recovery from cryopreservation of the controls (8–18%) in Shabalina et al. (1997). Thus, adaptation of the control is unlikely to be a problem in this study due to low opportunity for selection in the Ancestor.

Finally, Keightley et al. (1998) suggest that inbreeding depression could cause a decrease in fitness similar in magnitude to that observed by Shabalina et al. (1997). Shabalina et al. (1997) maintained populations of $N_e \approx 400$, leading to an increase in the inbreeding coefficient of 1/800 per generation or 4% by the end of the experiment (Crow and Kimura 1970). Our populations were maintained with $N_e \approx 600$ resulting in an increase in the inbreeding coefficient of 1/1200 per generation or 0.8% by the end of the experiment. A previous study of inbreeding depression in the closely related *R. sativus* found no reduction in total fitness after inbreeding at $F = 0.0315$ (3.15%) in a natural population (Nason and Ellstrand 1995). These data suggest that our level of inbreeding would not produce effects on fitness similar to those we have observed.

COMPARISON OF MCN DESIGNS TO SINGLE-OFFSPRING DESCENT

The inability to reproduce by selfing of many sexual organisms presents a challenge for MA experiments. The time (20 generations or more) required to create genetic homogeneity through inbreeding before the start of an MA experiment is impractical in most cases, and would require strong selection against deleterious recessives in an obligate outcrosser like *Raphanus*, so that the lines would no longer be representative of natural populations. One method used in *Drosophila* (but not available in *Raphanus*) is balancer chromosomes (where crossing-over is reduced), which protects one chromosome from selection (Mukai 1964; Mukai et al. 1972). Another approach is the MCN design, which was used by Shabalina et al. (1997) and in this experiment. The main difference between the single-offspring descent selfing method and MCN is the probability of fixation of a new mutation. In selfing lines, 75% of the offspring will carry a new mutation, with one-quarter homozygous for it. So there is a 25% probability of fixation and a 25% probability of loss of a new mutation each generation due to chance. In our MCN design, each individual has two offspring so the genetic contribution is the same as in a selfing experiment (two genomic complements). Half of the offspring will carry the new mutation but never in a homozygous form. However, there is a 25% probability that both offspring will carry a copy of the mutation and thus two copies will be passed on to the next generation. Similarly, there is a 25% probability that neither offspring will carry the mutation (it will be lost from the population). Although fixation of a new deleterious mutant is unlikely in an MCN design, the probabilities of loss and transmittal of new mutations are the same in inbred lines and MCN designs. An advantage of outbred MCN designs is that they allow the accumulation of more deleterious recessive mutations, such as homozygous lethals. These mutations will be eliminated from an inbred design but are much more likely to accumulate in heterozygotes in an MCN design.

Thus, the main difference between inbred and MCN MA experiments is in the expression of new recessive mutants. If most new mutations are partially recessive, their effects will be more difficult to observe in MCN populations, where nearly all mutations will be heterozygous. Partially recessive mutations are likely—the dominance of deleterious alleles has been estimated as $h \sim 0.1$

overall and $h \sim 0.15$ to 0.3 for mildly deleterious alleles excluding lethal alleles ($h = 1$ is completely dominant and $h = 0$ completely recessive; Lynch and Walsh 1998). Note that partially recessive mutations will have an effect on fitness in a heterozygous form, so although new mutations will have stronger phenotypic effects in an inbred design they will still affect fitness in an MCN design.

There are two additional disadvantages of the MCN design compared to inbred line studies. First, declines in fitness could be due to recombination breaking up adaptive gene complexes rather than the accumulation of mutations; this problem is difficult to deal with. Second, declines in fitness could be due to rare deleterious mutations that were present in the natural population and that increase in frequency due to reduced natural selection in the laboratory. This is not a problem in the inbred line studies that are initiated with isogenic base populations. However, using large, outcrossed populations as in our experiment will minimize the effects of genetic drift and inbreeding in increasing the frequency of these preexisting mutations when selection is relaxed. Thus, we assume that the frequency of preexisting deleterious mutations in the Ancestor approximated their frequency in the MA populations at the end of our experiment.

MEASUREMENTS OF MA IN MULTIPLE ENVIRONMENTS

Dependence of mutational effects on the assay environment has been studied for several systems. Studies in *Drosophila* illustrate the variety of results of such studies. Fry and Heinsohn (2002) found no interaction of mutations and environment on viability in *Drosophila* measured under high and low densities, normal and low temperatures, and presence/absence of ethanol. Two other studies have found an effect of environment on mean decline in fitness. Shabalina et al. (1997) measured competitive ability (primarily survival), motility, and longevity under benign and harsh competitive conditions (low and high density), and found little to no decline in mean under benign conditions but did find a decline in mean viability under competitive conditions. Kondrashov and Houle (1994) studied genotype–environment interactions for accumulated mutations in *Drosophila* with three factors: parental density, dilution of the growth medium, and temperature (crossed with medium dilution), and found a larger decline in mean fitness (productivity) under harsh conditions. In addition to effects on the mean, the harsh environment might also affect estimates of V_M, mutational variance (Kondrashov and Houle 1994). However, in *Drosophila* Fernandez and Lopez-Fanjul (1997) and Fry et al. (1996) failed to find any increase in V_M under harsh conditions. Our results are similar to the Kondrashov and Houle (1994) study because we found a trend toward decreased fitness that was proportionally larger under harsher field conditions compared to more benign greenhouse conditions (overall seed production was greater in the greenhouse: compare Figs. 2J and 3F). Thus, our study does provide some support for the magnification of mutational effects under stress. However, some of this magnification could be due to the fact that the field was not the environment under which mutations accumulated (but it is the recent natural environment of this species).

CONCLUSIONS

The low rate of mutation and small average mutational effect on fitness require large sample sizes and controlled conditions for the estimation of mutational parameters, making studies of fitness effects of spontaneous mutations challenging. Although it is more difficult, we have shown that the effects of mutation can be detected with outbred populations (also demonstrated by Shabalina et al. 1997 and Schoen 2005) and, for the first time, under field conditions. We found a trend for decreased fitness and evidence of increased additive genetic variance after nine or ten generations of MA. In addition, we found support for the idea that harsher environments exacerbate the deleterious effects of mutations, resulting in a decline in fitness in the field that is nearly three times the decline found in the less stressful greenhouse environment.

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LITERATURE CITED

Agrawal, A. A. 1998. Induced responses to herbivory and increased plant performance. Science (Wash. D. C.) 279:1201–1202.

Case, A. L., P. S. Curtis, and A. A. Snow. 1998. Heritable variation in stomatal responses to elevated CO2 in wild radish, *Raphanus raphanistrum* (Brassicaceae). Am. J. Bot. 85:253–258.

Conner, J. K. 2002. Genetic mechanisms of floral trait correlations in a natural population. Nature (Lond.) 420:407–410.

Conner, J., and S. Via. 1993. Patterns of phenotypic and genetic correlations among morphological and life-history traits in wild radish, *Raphanus raphanistrum*. Evolution 47:704–711.

Crow, J. F., and M. Kimura. 1970. An introduction to population genetics theory. Harper and Row, New York.

Eyre-Walker, A., P. D. Keightley, N. G. C. Smith, and D. Gaffney. 2002. Quantifying slightly deleterious mutation model of molecular evolution. Mol. Biol. Evol. 19:2142–2149.

Fernandez, J., and C. Lopez-Fanjul. 1997. Spontaneous mutational genotype-environment interaction for fitness-related traits in *Drosophila melanogaster*. Evolution 51:856–864,

Fry, J. D., and S. L. Heinsohn. 2002. Environment dependence of mutational parameters for viability in *Drosophila melanogaster*. Genetics 161:1155–1167.

Fry, J. D., S. L. Heinsohn, and T. F. C. Mackay. 1996. The contribution of new mutations to genotype-environment interaction for fitness in *Drosophila melanogaster*. Evolution 50:2316–2327.

Fry, J. D., P. D. Keightley, S. L. Heinsohn, and S. Nuzhdin. 1999. New estimates of the rates and effects of mildly deleterious mutation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 96:574–579.

Houle, D., B. Morikawa, and M. Lynch. 1996. Comparing mutational variabilities. Genetics 143:1467–1483.

Kearns, C.A., and D.W. Inouye. 1993. Techniques for Pollination Biologists. University Press of Colorado, Niwot, CO.

Keightley, P.D., and A. Caballero. 1997. Genomic mutation rates for lifetime reproductive output and lifespan in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 94:3823–3827.

Keightley, P. D., and A. Eyre-Walker. 2000. Deleterious mutations and the evolution of sex. Science (Wash. D. C.) 290:331–333.

Keightley, P. D., and M. Lynch. 2003. Toward a realistic model of mutations affecting fitness. Evolution 57:683–685.

Keightley, P.D., A. Caballero, and A. Garcia-Dorado. 1998. Population genetics: surviving under mutation pressure. Curr. Biol. 8:R235–R237.

Kibota, T., and M. Lynch. 1996. Estimate of the genomic mutation rate deleterious to overall fitness in *E. coli*. Nature (Lond.) 381:694–696.

Kondrashov, A. S. 1998. Measuring spontaneous deleterious mutation process. Genetica 102/103;183–197.

Kondrashov, A. S., and D. Houle. 1994. Genotype-environment interactions and the estimation of the genomic mutation rate in *Drosophila melanogaster*. Proc. R. Soc. Lond. B 258:221–227.

Kostkarick, R., and W. J. Manning. 1993. Radish (*Raphanus sativus* L)—a model for studying plant responses to air pollutants and other environmental stresses. Environ. Pollut. 82:107–138.

Lande, R. 1994. Risk of population extinction from fixation of new deleterious mutations. Evolution 48:1460–1469.

Littell, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. SAS system for mixed models. SAS Institute Inc., Cary, NC.

Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland, MA.

Lynch, M., J. Conery, and R. Burger. 1995. Mutation accumulation and the extinction of small populations. Am. Nat. 146:489–518.

Lynch, M., L. Latta, J. Hicks, and M. Giorgianni. 1998. Mutation, selection, and the maintenance of life-history variation in natural populations. Evolution 52:727–733.

Lynch, M., J. Blanchard, D. Houle, T. Kibota, S. T. Schultz, L. L. Vassilieva, and J. H. Willis. 1999. Perspective: spontaneous deleterious mutation. Evolution 53:645–663.

Mazer, S. J. 1987. The quantitative genetics of life-history and fitness components in *Raphanus raphanistrum* L (Brassicaceae)—ecological and evolutionary consequences of seed-weight variation. Am. Nat. 130:891–914.

Nason, J. D., and N. C. Ellstrand. 1995. Lifetime estimates of biparental inbreeding depression in the self-incompatible annual plant *Raphanus sativus*. Evolution 49:307–316.

Ohta, T. 1995. Synonymous and nonsynonymous substitutions in mammalian genes and the nearly neutral theory. J. Mol. Evol. 40:56–63.

Rose, M. R. 1991. Evolutionary biology of aging. Oxford Univ. Press, New York.

Schultz, S. T., M. Lynch, and J. H. Willis. 1999. Spontaneous deleterious mutation in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 96:11393–11398.

Schoen, D. J. 2005. Deleterious mutation in related species of the plant genus *Amsinckia* with contrasting mating systems. Evolution 59:2370–2377.

Shabalina, S. A., L. Y. Yampolsky, and A. S. Kondrashov. 1997. Rapid decline of fitness in panmictic populations of *Drosophila melanogaster* maintained under relaxed natural selection. Proc. Natl. Acad. Sci. USA 94:13034–13039.

Shaw, R. G., D. L. Byers, and E. Darmo. 2000. Spontaneous mutational effects on reproductive traits of *Arabidopsis thaliana*. Genetics 155:369–378.

Stanton, M. L., A. A. Snow, and S. N. Handel. 1986. Floral evolution—attractiveness to pollinators increases male fitness. Science (Wash. D. C.) 232:1625–1627.

Strauss, S. Y., J. K. Conner, and K. P. Lehtila. 2001. Effects of foliar herbivory by insects on the fitness of Raphanus raphanistrum: Damage can increase male fitness. Am. Nat. 158:496–504.

Tevini, M., W. Iwanzik, and A. H. Teramura. 1983. Effects of UV-B radiation on plants during mild water stress. II. Effects on growth, protein and flavonoid content. Z. Pflanzenphysiol. Bd. 110:459–467.

Thomson, J. D., L. P. Rigney, K. M. Karoly, and B. A. Thomson. 1994. Pollen viability, vigor, and competitive ability in *Erythronium grandiflorum* (Liliaceae). Am. J. Bot. 81:1257–1266.

Vassilieva, L. L., and M. Lynch. 1999. The rate of spontaneous mutation for life-history traits in *Caenorhabditis elegans*. Genetics 151:119–129.

Wloch, D., K. Szafraniec, R. Borts, and R. Korona. 2001. Direct estimate of the mutation rate and the distribution of fitness effects in the yeast *Saccharomyces cerevisiae*. Genetics 159:441–452.

Xu, J. 2004. Genotype-environment interactions of spontaneous mutations for vegetative fitness in the human pathogenic fungus *Cryptococcus neoformans*. Genetics 168:1177–1188.

Zeyl, C., and J. A. G. M. DeVisser. 2001. Estimates of the rate and distribution of fitness effects of spontaneous mutation in *Saccharomyces cerevisiae*. Genetics 157:53–61.

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