

ACCUMULATION OF DELETERIOUS MUTATIONS: ADDITIONAL *DROSOPHILA MELANOGASTER* ESTIMATES AND A SIMULATION OF THE EFFECTS OF SELECTION

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Abstract.—We report an assay of egg-to-adult viability in full-sibling mutation accumulation (MA) lines derived from a completely homozygous population of *Drosophila melanogaster* and maintained for 210 generations. A simultaneous evaluation was also made of a large population derived from the same origin and maintained as a control for the same period. We also present computer simulations to explore the possible decline in viability of the control population due to mutation accumulation and the possible effect of selection within and between MA lines. For this purpose, we used two mutational models independent from the data analyzed and based on radically different assumptions. The first model implies a large number of mutations of small effect, whereas the second implies a much smaller number of mutations with much larger effects. The observed rate of decline in mean viability was very small but significant (0.077%). The rate of increase in among line variance (0.189×10^{-3}) was similar to those obtained previously in the same lines. The simulation results indicated that a model of many mutations of small effect is incompatible with the evolution of the mean viability of the control and MA lines over generations, the distribution of line means after 210 generations of mutation accumulation, and the pattern of line extinction over generations. Basically, this model predicted a large drop in viability, both in the control and particularly the MA lines, that is not observed empirically. It also predicted a rate of line extinction too low in the early generations and too high in the later ones. In contrast, the model based on few mutations of large effect was generally consistent with all the observations.

Key words.—Deleterious mutation, *Drosophila*, fitness, natural selection, viability.

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The rate of accumulation of deleterious mutations has important consequences for the survival of endangered species and many other evolutionary issues (e.g., Charlesworth and Charlesworth 1998; Lande 1998). Some of these can be directly evaluated through experimental procedures in which spontaneous mutations are allowed to accumulate under relaxed selection in lines derived from the same uniform genetic background (mutation accumulation, [MA] lines). From the decline in fitness or its components and the increase in among-line variance observed in such experiments, it is possible to obtain bound estimates of the rate of appearance of mutations and of their mean effect. Furthermore, the shape of the distribution of the line averages can provide additional information on these parameters and on the distribution of mutational deleterious effects (see reviews by García-Dorado et al. 1999; Keightley Eyre-Walker 1999; Lynch et al. 1999).

The first experiments of this type were carried out in *Drosophila*, using chromosome lines in which mutations accumulated, with homozygous viabilities being assayed by competition with a Cy marked chromosome (Mukai 1964; Mukai et al. 1972; Ohnishi 1977). These experiments suggested that the accumulation of deleterious mutations could be responsible for a decline in mean viability as large as 1% per generation, with important consequences in evolutionary biology and conservation issues. From these experiments it was also inferred that spontaneous mutations arise at an approximate rate of about $\lambda = 0.5$ new mutations per haploid genome and generation with an average deleterious effect of about $E(s) = 0.02$.

The results of Mukai and Ohnishi were widely accepted for about 20 years and it was only in the 1990s when experiments on other species provided additional light on the subject. Whereas one of these experiments, carried out with *Daphnia pulex*, seemed to confirm large rates of mutations both for deleterious and advantageous mutations (Lynch et al. 1998), all the others have challenged the classical view. In particular, experiments carried out on *Caenorhabditis elegans* (Keightley and Caballero 1997; Vassilieva and Lynch 1999; see also the reanalysis by Keightley and Bataillon 2000; Vassilieva et al. 2001), *Arabidopsis thaliana* (Schultz et al. 1999; Shaw et al. 2000), *Triticum durum* (Bataillon 2000), and *Escherichia coli* (Kibota and Lynch 1996) have indicated much smaller rates of mutations and, in general, larger mean effects, implying smaller rates of decline in fitness. This suggests, at least, that the previous results obtained in *Drosophila* are not a general feature of all species.

Other recent studies carried out in *Drosophila* have also shown disagreement with the classical ones. Fernández and López-Fanjul (1996) performed an experiment of accumulation of mutations in full-sibling lines derived from an isogenic strain. This experiment indicated a rate of decline in viability of the order of 0.1% and suggested a rate of mutation of $\lambda \approx 0.02$ with mean effects of the order of $E(s) \approx 0.1$. More recently, Fry et al. (1999) performed an experiment similar to that carried out by Mukai and colleagues. Although the rate of decline in mean viability (0.6%) was about the same as that obtained by Mukai, the inferred mutation rate

was 10 times smaller ($\lambda = 0.04$) and the mean effect of mutations was much larger ($E[s] = 0.15$). The discrepancy may rest on differences between the *Drosophila* strains used in different experiments regarding their rates of transposition, but it has also been hypothesized that the estimates obtained by Mukai and Ohnishi are biased toward an excess of mutations of mild effect (Keightley 1996; García-Dorado 1997).

The results of the long-term experiment of Fernández and López-Fanjul are, so far, the main challenge to the classical *Drosophila* results of Mukai. However, Lynch et al. (1999) criticized their validity with the following arguments. First, the mean viability of the control in Fernández and López-Fanjul (1996) was not evaluated synchronously to the whole set of MA lines but to a few nonrandomly sampled lines. Second, the control population in this experiment consisted of about 800 individuals and could have accumulated mutations after a long period of time. Third, selection in the lines could have not been trivial, as shown by the loss of lines during the experiment. Finally, the assays performed by Fernández and López-Fanjul (1996) involved much more benign conditions than the competitive environment of the Mukai and Ohnishi experiments.

Chavarrías et al. (2001) assayed the same inbred lines and control at generations 250–255 for a competitive measure of viability. They found very similar results to those obtained in previous assays under noncompetitive conditions, showing that benign conditions are not responsible for the small rate of mutational viability decline observed in these lines. However, simultaneous evaluation of MA lines and control for noncompetitive viability remains to be obtained, and the effects of selection in the MA lines and mutation accumulation in the control remains to be explored. In this paper we have tried to address these issues using experimental and simulation approaches. First, we report a simultaneous assay of egg-to-adult viability in replicates of the control and the MA lines of Fernández and López-Fanjul at generation 210. Second, we present computer simulations addressing the possible evolution of the control and the possible effect of selection within and between MA lines.

MATERIALS AND METHODS

Experimental Procedure

From the initially isogenic line of *Drosophila melanogaster* obtained by Caballero et al. (1991) 200 MA inbred lines were started and maintained by full-sibling mating for 210 generations. Almost half of the lines were lost during this period, and only 111 lines were available for analysis. The isogenic line carried the recessive marker *sepia* (*se*) in chromosome III, as an indicator of possible contamination from exogenous flies. A large control population (about 800 parents per generation) was maintained in eight bottles with a circular mating scheme. More details about the experimental procedure for the maintenance of the lines and control can be obtained from Fernández and López-Fanjul (1996).

The present experiment was carried out in the University of Santiago de Compostela (Spain). Sixty MA lines were randomly sampled from the 111 surviving lines at generation 210. From each of these 60 MA lines, five males and five females were randomly taken and transferred to a plastic vial

(20-mm diameter, 100-mm height) with standard medium (Baker's yeast-agar-sucrose). Simultaneously, 40 control lines were set up in vials, each started with five males and five females taken from a pool to which all control bottles contributed equally. All lines (60 MA lines and 40 control lines) were simultaneously maintained at $25 \pm 1^\circ\text{C}$ in a chamber with no light, the position of the vials being randomized. The handling of flies was done at room temperature.

From each vial, six pupae of each sex were collected. Individuals of the same sex and line were kept in the same vial during four days. Then five replicates of each line were set up in fresh vials, each started by a single mating pair. On the third day after mating, each pair was transferred to a new vial containing a black plastic sheet covered by fresh medium. Flies were allowed to lay eggs on these sheets for one day. After this, the adults were discarded, the number of eggs laid was counted, and the plastic sheet was transferred to a new vial with fresh food to allow eggs to develop into adults. Egg-to-adult viability was measured at each vial as the proportion of adults emerged from the eggs laid.

Analysis of Data

Nonsevere deleterious mutations were assumed to randomly drift in the MA lines due to their small size ($N = 2$ breeding individuals per generation). Thus, the probability that a randomly sampled individual from a given line at generation t is homozygous for a new mutation occurred at a previous generation x is $(1/2N)F_{xt}$, where $(1/2N)$ is the frequency of the mutation at generation x and F_{xt} is the probability of identity by descent (i.e., the inbreeding coefficient) at generation t , taking x as the reference noninbred generation. Let λ be the expected number of viability mutations occurring per gamete and generation, so that $2N\lambda$ new deleterious mutations are expected to occur per generation and line. Therefore, MA individuals sampled at generation t are expected to be homozygous for λF_{xt} mutations that arose at generation x . Thus, considering mutations from all previous generations, MA individuals are expected to be homozygous for $\lambda \sum_{x<t} F_{xt}$ new mutations. Because mutation is assumed to not recur, individuals from different MA lines will be homozygous for different mutations, and $\lambda \sum_{x<t} F_{xt}$ is interpreted as the expected number of mutations accumulated per MA line at generation t . We refer to $F_t^c = \sum_{x<t} F_{xt}$ as the “forward cumulated inbreeding coefficient”, which is roughly equivalent to generation number after long MA periods.

Viability data were analyzed in the arcsine scale, given in radians, to normalize the distribution. The rate of decline in mean relative viability was estimated as $\Delta M = (M_c - M_l) / (M_c F_{210}^c)$, where M_c and M_l are the mean viability of control and MA lines, respectively, and $F_{210}^c = 203$ after 210 generations of full-sibling mating. The corresponding rate of increase in variance was computed as $\Delta V = \sigma_l^2 / (M_c^2 F_{210}^c)$, where σ_l^2 is the between-line component of variance estimated for the MA lines by one-way ANOVA.

In the absence of selection, the expected rates of mean decline and increase in variance are $E[\Delta M] = \lambda E(s)$, $E[\Delta V] = \lambda E(s^2)$, respectively, where λ is the viability mutation rate and s is the homozygous deleterious effect of a new mutation.

Thus, bound estimates for the mutational parameters were computed as

$$\lambda \geq \Delta M^2 / \Delta V \quad \text{and} \quad (1a)$$

$$E(s) \leq \Delta V / \Delta M, \quad (1b)$$

which are known as Bateman-Mukai (BM) estimates. These would provide unbiased estimates if the deleterious effects were the same for all mutations (Bateman 1959; Mukai 1964).

For comparison with the above estimates, we also obtained minimum distance estimates (MD; for details see García-Dorado 1997) using the data from a viability evaluation performed at generations 208–209 in Madrid (García-Dorado et al. 2000), where MA lines (but not the control) were more extensively evaluated (111 lines evaluated in two consecutive generations). The MD method has often been shown to be more robust than maximum likelihood to departures from underlying assumptions (Woodward et al. 1984). This method uses the information contained in the empirical distribution function F_n of the mean viability, \bar{v} , of n lines. Basically, a mutational model is assumed (Poisson distributed number of accumulated mutations with additive, reflected-gamma distributed effects) and the theoretical distribution functions F_θ of \bar{v} for different vector parameters θ are derived. The empirical and theoretical distributions are compared using the Cramér-von Mises distance, defined as

$$W^2(F_n, F_\theta) = \int_{-\infty}^{\infty} [F_n(\bar{v}) - F_\theta(\bar{v})]^2 dF_\theta(\bar{v}), \quad (2)$$

which is a quadratic measure of the distance between the empirical and the theoretical distribution functions. Then the MD estimator of θ is defined as the value of this parameter which minimizes $W^2(F_n, F_\theta)$. The basic idea is to estimate the true value of θ as the value that makes the assumed model closer to the sampling information given by the empirical distribution (a general introduction to MD estimation can be found in Titterton et al. 1985).

A simple expression to evaluate the Cramér-von Mises distance from the empirical to the theoretical distribution is given by Woodward et al. (1984),

$$W_n^2 = \frac{1}{12n} + \sum_{i=1}^n \left[F_\theta(\bar{v}_i) - \frac{i - 0.5}{n} \right]^2, \quad (3)$$

where n is the sample size (the number of MA lines in our case), \bar{v}_i is the i th order statistic in the sample of $n\bar{v}$ values, and θ includes the deleterious mutation rate and the parameters of the reflected-gamma distribution for the mutational effects. This distance has been found to perform well in a variety of settings (Parr and Shucany 1980). In the computation of the MD estimator we only consider θ values producing the ΔV that had been estimated in the ANOVA analysis but, in the present analysis, we do not constrain θ to any external estimate of ΔM . Thus, a MD prediction for ΔM is obtained. Because our BM and MD estimates are based on different assays, they provide statistically independent estimates of the properties of deleterious mutations of our MA lines.

Simulation Procedure

Simulations were run to evaluate the expected behavior of the large control population and the MA lines over the experiment under each assumed model of mutations. Populations with $N = 100$ or 1000 (control) or $N = 2$ (MA lines) breeding individuals were simulated. A multilocus model with multiplicative fitness action among loci was assumed. For each locus, the genotypic fitnesses are 1, $1 - sh$, $1 - s$, for the *AA*, *Aa*, *aa* genotypes, respectively.

At generation 0 there were no mutations in the population (control and MA lines). Every generation, an average number (Poisson distributed) of λ new deleterious mutations per haploid genome arose and were randomly assigned to nonsegregating positions of the genome. The simulation of genes was made by the use of binary masks and bit-step operators. Two different mutational models were used. To obtain simulation results independent of our empirical data, both models were based on estimates obtained from different analyses of Mukai's et al. (1972) data.

The first model was based on the original BM estimates from the quasi-normal chromosomes given by Mukai et al. (1972, p. 350), that is, $\lambda = 0.43$ per haploid genome and generation, $E(s) = 0.026$. To account for the whole rate of increase in variance for nonlethal lines (0.00086), the shape parameter for the gamma distribution of homozygous effects was set to 0.51. We will refer to this set of parameters as the BM-Mukai model. To simulate this model of mutations, an initial (generation 0) mean viability of one was assumed. This is a highly unrealistically large value but, as will be shown below, it is the only way to predict, under this model, a control viability close to that observed at generation 210 and mean line viabilities not too close to zero after 210 generations.

The second model, which we will refer to as the MD-Mukai model, was based on analysis performed on the same data (García-Dorado et al. 1998) and implies that a part of the fitness reduction observed by Mukai et al. (1972) was not mutational. For this model, $\lambda = 0.011$, $E(s) = 0.191$, and the gamma shape parameter is 3.12. Thus, the main difference between the models is that the BM-Mukai model assumes a large number of mutations, a large fraction of them having effects of a few percent, whereas the MD-Mukai model implies a much smaller number of mutations, with an effect close to 20%. For this model, we assumed a more realistic initial mean value of 0.65. This was computed by adding to the mean viability observed at generations 104–106 for the MA lines (0.56) the MD estimate of the viability decline (0.00093×98), where 0.00093 is the MD estimate of the rate of viability decline and 98 is the F_{105} -value (García-Dorado 1997). No beneficial mutations were simulated under any model.

The dominance coefficient of mutations was assumed to be related to the selection coefficient by using an exponential function, as proposed by Caballero and Keightley (1994). Thus, the dominance coefficient of a mutation is taken from a uniform distribution between zero and $\exp(-ks)$, where k is a constant allowing the mean dominance coefficient to be the desired one. For the Mukai model the average coefficient of dominance used was 0.36, a value widely assumed for

TABLE 1. Estimated mutational parameters.

	$\Delta V \times 10^3$	ΔM (%)	λ	E(s)
BM ¹	0.189 \pm 0.139	0.077 \pm 0.037	0.0031	0.246
MD ²	0.179 \pm 0.027	0.081 \pm 0.041	0.0050 \pm 0.0047	0.162 \pm 0.125

¹ ΔM and ΔV observed at generation 210 with the corresponding Bateman-Mukai estimates.

² ΔV observed at generations 208–209 together with the corresponding MD estimates for viability relative to an initial average 0.65 (the MD estimate of the shape parameter was 2.7 and the estimated proportion of mutations increasing viability was zero). Rough bootstrap errors, based on 20 bootstrapped samples, are given.

mild viability mutations (e.g., Lynch et al. 1995). For the new model, a value of 0.20 was used. This has been inferred from a reanalysis of the MA experiment of Ohnishi (1977; see García-Dorado and Caballero 2000).

Selection within and between MA lines was simulated as follows. In each case, two individuals were mated to produce offspring. Free recombination among loci was assumed in the production of gametes from each parent. A model with restricted recombination (a total genome length of 1.25 Morgans) was also simulated but did not have any substantial effect on the results (not shown). The viability of an offspring was evaluated and compared to a random number between zero and one. The individual survived if this number was smaller or equal to its viability value and died otherwise. Thus, the effects of the environmental variance for viability are incorporated into the MA process through the stochastic component of survival. The process was repeated until two surviving progeny were obtained or a number R of attempts (the reproductive capacity) to produce surviving offspring had been made. If, after this number of attempts, no surviving couple was obtained, the line was considered to be extinct.

Under each model, cases were run with different R -values, giving different proportions of lines lost after 210 generations. It was found that $R = 45$ for the BM-Mukai model and $R = 11.5$ for the MD-Mukai model gave at generation 210 the proportion of line extinction experimentally observed (45%). Results will be focused on these simulations. Cases were also simulated in which selection was completely absent (random fixation of all mutations) or occurred only within lines ($R \rightarrow \infty$). This latter case would simulate competition among progeny in the same line as well as the use of backups, but not extinction of lines. The above model is a conservative one, as a number of lines were likely to be lost because of accidents and mutations affecting other fitness components, such as fecundity.

For the large control population, random mating excluding self-fertilization was assumed. Two parents were randomly taken to produce one offspring. If this individual died due to its genotypic value, a new random pair of parents was sampled (with replacement), and the process was repeated until the total number of offspring required (N) was obtained.

Each simulated case consisted of 200 replicates of (initially) 200 MA lines ($N = 2$) or one control line ($N = 100$ or 1000), and results were averaged over replicates. From the 200 replicates, 95% confidence limits were obtained determining the 2.5 and 97.5 percentiles. For the comparison between observed and simulated line's distribution at generation 210, the viabilities of the simulated lines were arcsine transformed and sampling error was added. The mean square error obtained in the experiment in arcsine scale was 0.127,

and dividing by the mean number of replicates per line (3.98) gives an error variance for MA line means of 0.0319. Thus, random normal deviations with mean zero and variance 0.0319 were added in the simulations to the arcsine means of the lines. The arcsine transformation and the addition of the arcsin error deviation were made only for the purpose of line's distribution drawing. Analogously, for the comparison between observed and simulated mean viabilities at different generations, standard errors of observed mean viabilities were calculated from the corresponding observed within- and among-generation environmental sampling variance components, as these sources of variation were not included in the simulations. In the case of generation 210, the among-generation variance component used was that of generations 208–209.

RESULTS

Experimental Results

Only replicate vials with more than two eggs were considered in the analysis of egg-to-adult viability. Thus, the number of control and MA lines available for analysis was 37 and 55, with a mean number of replicates per line of 3.92 and 3.98, respectively. Analyses discarding a larger number of replicate vials (those with less than four eggs, five eggs, etc.) did not change the results substantially but gave larger error variances.

The mean proportion of emerging adults (raw untransformed data) for the control lines was 0.625 ± 0.029 . That for the MA lines was 0.530 ± 0.028 , in close agreement with the value (0.539 ± 0.015) obtained for the whole set of 111 surviving lines at generations 208–209 at a different laboratory in Madrid (García-Dorado et al. 2000), although in this latter case a simultaneous evaluation of the control was not available.

The rate of mean decline and that of increase in variance for relative viability obtained from arcsine-transformed data, as well as the corresponding BM estimates, are given in Table 1. The rate of viability decline was very small but significantly larger than zero ($P < 0.024$). This was about half the ΔM estimated at generations 104–106, either by comparison to a nonsimultaneous evaluation of the control (corrected for generation environmental effects) or by MD estimation (García-Dorado et al. 1998). It was about 7% the ΔM estimated in Mukai's experiments.

Assuming between-loci multiplicative viability, the rate of viability decline, as well as the BM or MD estimates, would be more adequately computed from log-transformed data. However, due to the small overall viability reduction observed in this experiment, arcsine-transformed, real-scale,

and log-transformed data (not shown) produced similar estimates. Because our viability measure is a proportion, we chose arcsine transformation to normalize the variable as required for the statistical analysis.

The estimate of the rate of increase in variance was small and nearly significant ($P < 0.057$). ΔV can also be computed from the difference between the observed variance of mean viability in the MA and the control lines, which gives a similar but nonsignificant value ($\Delta V = 0.175 \times 10^{-3}$). An accurate estimate of the between-line variance had been obtained at generations 208–209 (García-Dorado et al. 2000). To compute a ΔV estimate using this between-line variance, which is statistically independent of that obtained at generation 210, we need an independent estimate of the control or the original mean viability. Using the value $M_c = 0.65$ estimated by MD, together with the between-line variance at generations 208–209, gives a very similar ΔV (see Table 1; MD estimate), which is significantly larger than zero with $P < 10^{-6}$. In García-Dorado et al. (1998, 2000), viability was relative to the mean of the MA lines (0.56), although in García-Dorado et al. (1998) it was erroneously said to be relative to the control mean. In turn, all these alternative estimates of ΔV are only slightly smaller than the value estimated at generations 105–106 ($\Delta V = 0.199 \times 10^{-3}$ after scaling it for an original average of 0.65 instead of 0.56; García-Dorado 1997). Therefore, all independent estimates of ΔV obtained at generations 104–106 (García-Dorado et al. 1998), 208–209 (García-Dorado et al. 2000) and 210 (this experiment) are markedly similar.

The BM estimate of λ (Table 1) was very small, about five times smaller than the corresponding estimates obtained at generations 104–106 either by BM or by MD methods. This suggests that selection could have been more efficient to slow down the accumulation of mutations in the second half of the experiment, which is in agreement with simulation results given below. The BM estimate for $E(s)$ was quite large, about twice that obtained at generations 104–106. Independent MD estimates obtained at generation 208–209 are also given in Table 1 and are in good agreement with the BM ones. As expected, the BM lower bound estimate for λ was smaller than the MD estimate, and the BM upper bound estimate for $E(s)$ was larger than the MD estimate. The difference between BM and MD estimates was larger than that observed at generations 104–106. This indicates that the distribution of deleterious effects expressed by the later stages had more variance than at earlier stages. A possible explanation is reinforcing epistasis reducing viability in lines carrying several deleterious mutations.

Simulation Results

The evolution of the mean relative viability from the simulations is plotted in Figure 1. For comparison, the figure also shows the empirical estimates for MA lines and control available from different assays. Statistical comparisons between observed and simulated results are given in Table 2. There was no experimental suggestion of decline in the control viability between the two available estimates (generations 105 and 210), and the differences in mean viability between control and MA lines were very small.

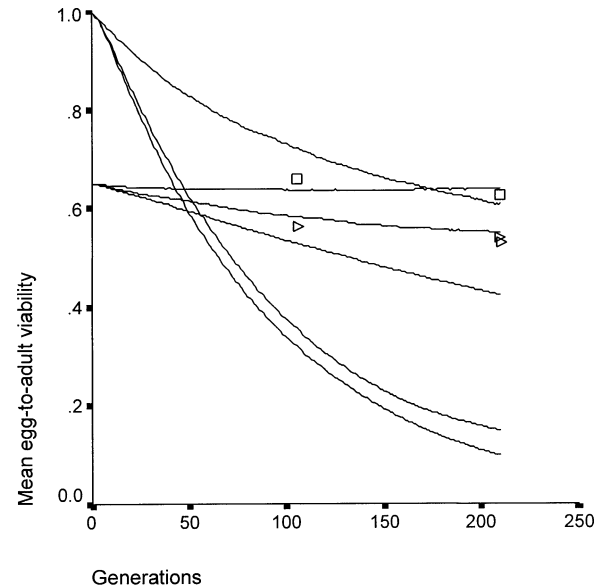


FIG. 1. Evolution of mean egg-to-adult viability from simulations (lines) and experimental data (squares and triangles). The two squares are the available means for the control in two different experiments (estimate of generation 105 from Fernández and López-Fanjul [1996], correcting for generation effects as explained by García-Dorado [1997]; and generation 210 from this experiment). The three triangles are the mean for the MA lines in three experiments (average of generations 104, 105, and 106 from Fernández and López-Fanjul [1996]; average of generations 208 and 209 from García-Dorado et al. [2000]; and generation 210 from this experiment). Lines starting with mean 1.00 refer to the BM-Mukai model and those starting with mean 0.65 refer to the MD-Mukai model (see text). In each case, the upper line refers to the control population of $N = 100$, the middle line refers to the MA lines ($N = 2$) assuming selection within and between lines, and the lower line refers to the MA lines without selection.

Simulations using the BM-Mukai model (classical estimates with many mutations of small effect) start at an initial viability of one. Those from the MD-Mukai model (fewer mutations of larger effect) start at a value of 0.65, which was the original mean estimated by MD. The upper line in both cases corresponds to the control population of size $N = 100$ (results for $N = 1000$ were quite similar and are not shown). The other two lines refer to MA lines ($N = 2$) assuming selection (middle line) or not (lower line). In the case of no selection, all new mutations were fixed at random in the lines. In the case of selection, this operated within and between lines, and the reproductive capacity was adjusted for each model so that 45% of the lines were lost by generation 210.

The evolution of the control for the BM-Mukai model was made consistent with the experimental observation at generation 210 by using an initial mean viability of one. The MD-Mukai model predicted an imperceptible change in the control mean, which agrees with the experimental observations over time. Table 3 shows some details of the simulated controls at generation 210. The inbreeding depression predicted by the MD-Mukai model was a 0.044% (with confidence limits of 0.005% and 0.099%) reduction in mean per 1% increase in inbreeding coefficient. This value is in agreement with the observed inbreeding depression (0.063%) shown by the control population in an evaluation of the lines

TABLE 2. Statistical comparison between observed and simulated mean viabilities plotted in Figure 1.

	Generation	Observed		Simulations MB-Mukai		Simulations MD-Mukai	
		Mean \pm SE ¹	Confidence interval ²	No selection	Selection ³	No selection	Selection ³
				Confidence interval ⁴	Confidence interval ⁴	Confidence interval ⁴	Confidence interval ⁴
Control	105	0.659 \pm 0.017	(0.626–0.692)		(0.677–0.775)		(0.626–0.646)
	210	0.625 \pm 0.045	(0.537–0.713)		(0.565–0.667)		(0.626–0.647)
MA lines	105	0.561 \pm 0.021	(0.520–0.602)	(0.308–0.334)*	(0.344–0.369)*	(0.508–0.544)	(0.574–0.596)
	208–209	0.539 \pm 0.016	(0.508–0.570)	(0.095–0.106)*	(0.146–0.161)*	(0.401–0.444)*	(0.540–0.572)
	210	0.530 \pm 0.040	(0.452–0.608)	(0.093–0.105)*	(0.145–0.161)*	(0.400–0.443)*	(0.539–0.571)

¹ Standard errors of observed mean viabilities were calculated from the observed within- and among-generation environmental sampling variances evaluated at the corresponding generations. (For generation 210, the among-generation variance used was that from generations 208–209.)

² Confidence interval obtained as mean \pm 1.96 SE.

³ Selection within and between lines in the MA lines.

⁴ Confidence interval of simulations obtained from the 2.5 and 97.5 percentiles of the distribution of means from 200 replicates of each simulation.

* Significant difference ($P < 0.05$) between observed and simulated results.

at generation 255 under competitive conditions using balancer chromosomes (Chavarrías et al. 2001). The inbreeding depression predicted by the BM-Mukai model, however, was one order of magnitude larger than the observed one (0.37% with confidence limits 0.31% and 0.44%). The two models predicted a rather different genetic architecture for the control. The BM-Mukai model implied a large number of mutations segregating in the population with effects between 10^{-4} and 0.1, with the majority of them being at frequencies below 0.1, but a substantial number having larger frequencies. For the MD-Mukai model the number of segregating mutations in the control was very much smaller, with most of them at low frequencies.

The effect of selection on the MA lines was rather small for the BM-Mukai model (Fig. 1). The reason is that purging of mildly detrimental mutations, which are very common under this model (see below), is not efficient in full-sibling lines. For the MD-Mukai model the effect of selection was more substantial. The observed means for MA lines (triangles) were nonsignificantly different from the predictions of this model, and they completely disagreed with the predic-

TABLE 3. Simulation results for parameters in the control population at generation 210.

	BM-Mukai model	MD-Mukai model
Inbreeding depression ¹	0.37	0.04
No. of fixed mutations	0.85	0.00
No. of segregating mutations	682.66	11.02
No. of segregating mutations at frequencies (q):		
0.0 < q \leq 0.1	518.24	10.28
0.1 < q \leq 0.2	80.59	0.57
0.2 < q \leq 0.3	35.52	0.15
0.3 < q \leq 0.6	37.74	0.02
0.6 < q < 1.0	10.57	0.00
No. of segregating mutations with deleterious effects (s):		
0.0 < s \leq 10^{-6}	3.06	0.00
10^{-6} < s \leq 10^{-4}	32.66	0.00
10^{-4} < s \leq 10^{-2}	306.16	0.00
0.01 < s \leq 0.1	311.61	2.50
0.1 < s \leq 0.2	26.18	4.31
0.2 < s \leq 0.4	2.92	3.71
0.4 < s \leq 0.6	0.07	0.47
0.6 < s \leq 1.0	0.00	0.03

¹ Percent reduction in mean per 1% increase in inbreeding.

tions from the BM-Mukai one (Table 2), even though the initial viability assumed for this latter was the maximum value of one.

The empirical and simulated distributions of line mean viabilities at generation 210 are shown in Figure 2 (arcsine scale in radians, note that viability one corresponds to 1.57 radians). The observed distribution of mean viability for the control (Fig. 2b) did not depart from normality ($P < 0.10$ in a Shapiro-Wilks test). The simulated distribution for the MD-Mukai model (Fig. 2c and especially 2d accounting for selection) agreed quite well with the observed distribution (Fig. 2a). Those for the BM-Mukai model (Fig. 2e, f) were clearly inconsistent with the observations.

Table 4 shows the number of mutations with different effects fixed in the simulated MA lines. A comparison between the first column (no selection), second column (selection within lines but without line losses), and third column (selection within and between lines) allows us to see the effectiveness of selection in removing mutations of different effects. The table also includes the actual values of λ and $E(s)$ for each case (the rate and mean effect of mutations actually fixed in the surviving lines). It appears that about half the selection occurs within lines, whereas the other half is due to line extinction. In agreement with the conclusions based on viability decline (see Fig. 1), it is clear that selection has very small effect under the BM-Mukai model, but is much more effective under the MD-Mukai model. The data confirm that selection removes some of the mutations with effect larger than about 0.1, but is inefficient against mildly deleterious mutations.

Finally, Figure 3 shows the number of surviving lines obtained from the simulations and that actually observed at different times. At each model, the reproductive capacity R (the maximum number of attempts of a couple to produce progeny) was adjusted to simulate the observed number of line extinctions at generation 210. This was achieved by using $R = 45$ (BM-Mukai model) and $R = 11.5$ (MD-Mukai model). Therefore, the number of extinctions with the two models converged at this generation. Other values of R produced results clearly incompatible with the observations and are not shown. The BM-Mukai model predicted no extinction up to about generation 125, followed by an accelerated loss rate, the proportion of lines surviving by generation 255 being

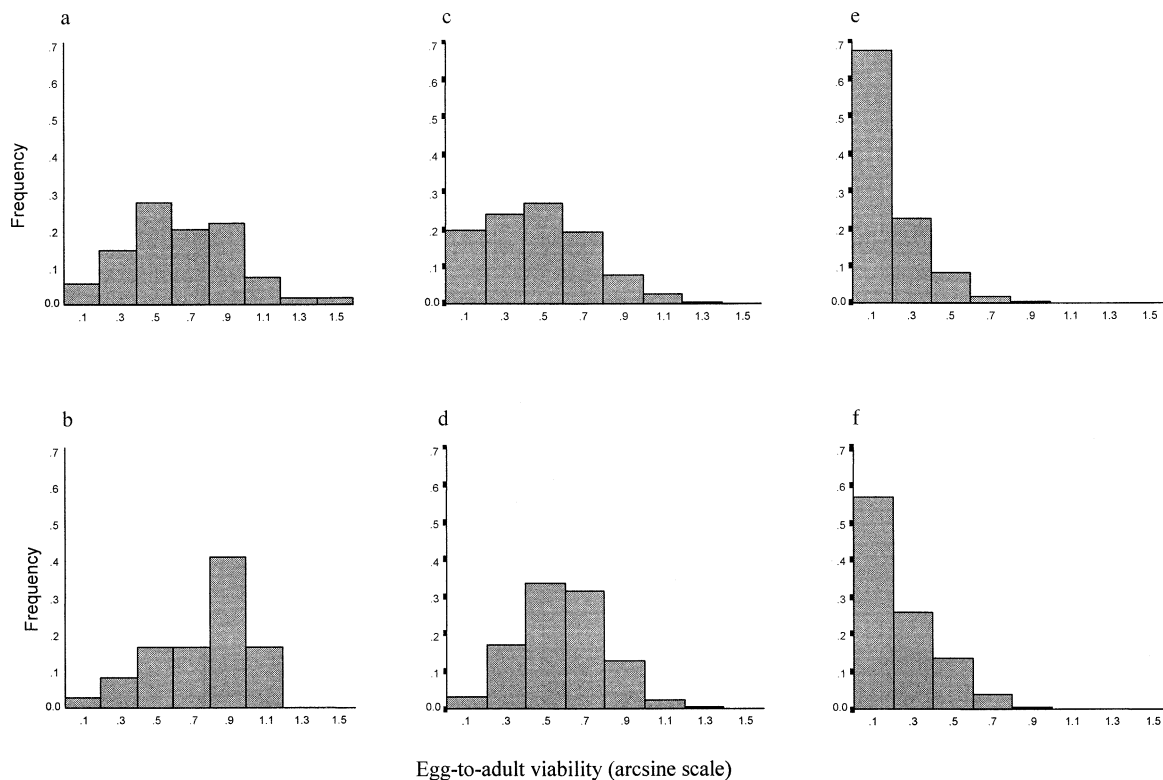


FIG. 2. Distribution of line means. (a) Observed in $n = 55$ MA lines. (b) Observed in $n = 37$ replicates of the control lines. Simulated MA lines: (c) MD-Mukai model with no selection ($n = 2000$); (d) MD-Mukai model with selection within and between lines ($n = 1129$); (e) BM-Mukai model with no selection ($n = 2000$); (f) BM-Mukai model with selection within and between lines ($n = 1090$).

TABLE 4. Simulation results for the number of mutations fixed for each of the mutational models in the surviving lines at generation 210. λ , $E(s)$: haploid mutation rates per generation and mean effect of mutations fixed in the surviving lines at generation 210.

Mutational effects	No selection	Selection within lines	Selection within and between lines
BM-Mukai model			
$0.0 < s \leq 10^{-6}$	0.38	0.38	0.38
$10^{-6} < s \leq 10^{-4}$	3.63	3.75	3.77
$10^{-4} < s \leq 10^{-2}$	36.34	35.99	35.98
$0.01 < s \leq 0.1$	43.01	41.17	39.08
$0.1 < s \leq 0.2$	3.81	3.30	2.49
$0.2 < s \leq 0.4$	0.45	0.33	0.17
$0.4 < s \leq 0.6$	0.00	0.00	0.00
Total	87.63	84.93	81.87
λ	0.43	0.418	0.403
$E(s)$	0.026	0.025	0.022
MD-Mukai model			
$0.01 < s \leq 0.10$	0.44	0.42	0.38
$0.10 < s \leq 0.20$	0.87	0.70	0.53
$0.20 < s \leq 0.40$	0.78	0.50	0.19
$0.40 < s \leq 0.60$	0.09	0.04	0.01
$0.60 < s \leq 1.00$	0.01	0.00	0.00
Total	2.19	1.66	1.11
λ	0.011	0.008	0.005
$E(s)$	0.191	0.171	0.144

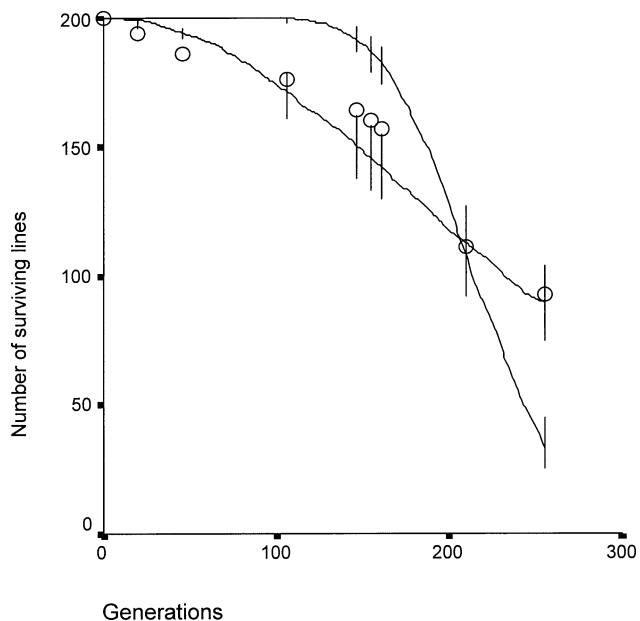


FIG. 3. Number of surviving lines. Upper line: BM-Mukai model. Lower line: MD-Mukai model. Circles: observed data from different experiments. Bars indicate 95% confidence limits of simulation results.

much smaller than the observed one. In contrast, the MD-Mukai model accounted for the loss of lines occurred in the early generations and agreed well with the observation at generation 255.

DISCUSSION

Empirical Estimates

We simultaneously evaluated egg-to-adult viability in a large control population and in 55 randomly chosen lines that had accumulated mutations for 210 generations. The rate of mutational viability decline observed ($\Delta M = 0.077\%$) was lower than that estimated after 105 MA generations without simultaneous evaluation of the control (0.156% from comparison to an adjusted control, 0.143% from MD methods). Our ΔM estimate was in close agreement with that obtained using MD ($\Delta M = 0.081\%$) from an independent but almost simultaneous evaluation of the same MA lines experiment (performed at generations 208–209 in Madrid on the whole set of 111 surviving lines). The rates of increase in variance estimated both in our evaluation and in the one of Madrid (0.189×10^{-3} , 0.179×10^{-3}) were similar to that estimated by generation 105 (0.199×10^{-3}). This mutational variance was in close agreement with those found in other studies (Mukai et al. 1972; Ohnishi 1977) if only quasi-normal lines are considered (see García-Dorado et al. 1999). This indicates that, although selection should have occurred against severely deleterious mutations, it would have not affected the accumulation of mildly deleterious ones.

The obtained BM (generation 210) and MD (generations 208–209) estimated parameters were in good agreement with each other, giving low deleterious mutation rates ($\lambda \approx 0.004$) and moderate mean deleterious effect ($E[s] \approx 0.2$). This is remarkable, because by generations 208–209 there was not an estimate of the control average that would allow to obtain a direct estimate of ΔM . The MD estimate was, therefore, unconstrained to any observed rate of viability decline. The validity of such unconstrained MD estimates had been previously documented at generations 104–105, for which the MD estimates conditional to the observed viability decline ($\lambda = 0.018$, $E[s] = 0.087$ for viability relative to a control average 0.65) were strikingly similar to those unconstrained by such decline ($\lambda = 0.016$, $E[s] = 0.089$; García-Dorado 1997).

It has been argued that 10 MA generations is more efficient than a longer period to estimate deleterious mutation rates and effects (Deng et al. 1999). However, this conclusion relies on simulation results obtained for $\lambda = 0.1$. In this case, the expected number of deleterious mutations accumulated per line after 10 generations is one, which represents an optimal situation to infer the properties of individual deleterious mutations. For smaller λ (such as the estimate reported in this paper, $\lambda = 0.004$), longer MA periods (about 250 MA generations) are required to attain this optimal situation.

It should be noted that our estimates (and those of Mukai) refer to mutations detectable in laboratory experiments. The analysis of molecular evolution allows estimating the rate of point constrained mutations, which includes tiny deleterious effects that can pass undetected in MA experiments ($s < 5 \times 10^{-4}$; see Davies et al. 1999). For *Drosophila*, the small

estimated point constrained mutation rate (0.035 per gamete; Keightley and Eyre-Walker 2000) seems to leave no margin for important rates of deleterious mutation undetected in MA laboratory experiments. However, the particularly large transposition rate of this species, which produces up to 0.1 insertions per gamete and generation, could account for additional deleterious mutation and for important rates of recessive lethal mutations. Thus, the overall *Drosophila* rate of constrained mutations could be up to 0.14 per gamete, a value still inconsistent with the large Mukai's estimates for viability. Even if the rate of point constrained mutation could have been underestimated due to natural selection against synonymous mutations not due to codon bias (Kondrashov 2001), the shortcoming of these estimates is that they provide almost no information on the magnitude of the deleterious effects. Therefore, their evolutionary and practical consequences cannot be predicted. For example, for deleterious effects of the order of 10^{-4} or less, even more than one constrained mutation per zygote and generation can cause very small rates of genomic degradation.

Simulation Results: Effects of Selection on Mutational Parameter Estimates

The above results confirm that the low rates of deleterious mutation with relatively large mean effect estimated at generation 105 were not due to the lack of simultaneous control evaluation. However, the validity of those estimates could be challenged by the possibility that the control viability had declined due to mutation accumulation or that the decline viability of the MA lines had been substantially slowed down by selection. Therefore, we explored the magnitude of this phenomenon using simulation. To obtain simulation results that are independent of our data, we used two models obtained from the data of Mukai et al. (1972). The first one (BM-Mukai) is based on the original BM analysis, and the second (MD-Mukai) is based on MD reanalysis implying that a part of the fitness reduction observed by Mukai was nonmutational. The simulation results, as discussed below, show that neither mutation accumulation in the control nor natural selection in the MA lines can account for the experimental results under the classical BM-Mukai model.

To begin with, preliminary simulation runs using the BM-Mukai model showed that an initial egg-to-adult viability equal to one had to be assumed to reproduce the control mean experimentally observed by generation 210 and to obtain a mean viability for the MA lines that is not too close to zero (see Fig. 1). This is, on its own, a strong argument against the model. Mean egg-to-adult viability is obviously smaller than one even for segregating populations and should have been smaller for our original isogenic line. The mean egg-to-adult viability from five experiments of *D. melanogaster* was about 0.7–0.8 for outbred individuals (reviewed by Wang et al. 1998), and the mean inbreeding depression for the same trait was about 0.5–0.8% per 1% increase in inbreeding. Furthermore, the BM-Mukai model predicts that many mild deleterious mutations are segregating in natural populations. These would have been hardly purged during the construction of the original isogenic line used to start the control and MA lines. Thus, an initial mean viability lower than 0.7–0.8

would be expected under the BM-Mukai model. In addition, the rate of inbreeding depression obtained from the simulated control population under the BM-Mukai model was one order of magnitude larger than an empirical estimate obtained for competitive viability after a similar period (Chavarrías et al. 2001).

Within- and between-line selection on viability was simulated such that the total number of lines lost by generation 210 were as observed in the experiment. Although this is likely to represent a selective pressure larger than the experimental one and despite the unrealistic assumption of an initial mean viability equal to one, the BM-Mukai model predicted a final viability in the MA lines far below the observed one (Fig. 1). Thus, after 210 generations, the simulated distribution of the lines mean viability was markedly different from the observed one. It should be noted that between-loci fitness was simulated to be multiplicative. Under the BM-Mukai model, between-loci additive fitness would have produced negative viability means. Finally, this model predicts no initial line loss and a late acceleration of the rate of line extinction that does not fit to the observed pattern (Fig. 3).

In contrast, the evolution of control and MA lines means simulated using the MD-Mukai model were compatible with the general observations from the experiment. Beginning with the control, the absence of appreciable empirical viability decline was in agreement with simulation results showing that only few deleterious mutations segregate in the control at low frequency (Fig. 1, Table 3). Caballero and Keightley (1998) reached a similar conclusion analyzing data from unselected control populations of *Drosophila* and mice initially devoid of variation. Furthermore, the inbreeding depression in the simulated control was comparable to the corresponding estimate for competitive viability (0.063%) found by Chavarrías et al. (2001).

The simulated distribution of the MA lines mean viability after 210 generations using the MD-Mukai model was reasonably similar to the observed one, the similitude improving when the simulation included selection (Fig. 2). Figure 1 shows that natural selection can appreciably slow the viability decline, which was largely due to moderately or severely deleterious mutation (Table 4). However, the predicted viability decline was always much smaller than under the BM-Mukai model. The available experimental estimates of the MA lines mean viability laid between the MD-Mukai simulation results obtained with and without selection (see Fig. 1). This suggests that the effect of natural selection in our lines was smaller than the simulated one. It should be noted that our simulation of within- and between-line selection assumes that lines are lost just because of selection on viability under limited reproductive capacity. This mechanism accounts for some random extinction. Thus, due to the nonzero initial mortality under the MD-Mukai model, lines can go extinct before they accumulate any deleterious alleles. However, this simulation ignores that some extinction can be due to accidents or to selection on other fitness components. Thus, this approach gives conservatively large selection effects.

The mean viability decline for the simulated MA lines for the MD-Mukai model in the absence of selection was almost perfectly linear. This suggests that, under this model, mutation accumulation is slow enough so that the assumption

of between-loci additive fitness (usual in both BM and MD analysis of MA data) can be safely used in long-term experiments. However, assuming within- and between-line selection, the simulated rate of viability decline between generations 105 and 210 was 37% of that observed during the first 105 generations. This can partly explain the rate of fitness decline empirically estimated at generation 210 being smaller than the one estimated at generation 105. Figure 1 shows that, with between-loci multiplicative viability, MA results up to generation 100 are expected to allow a better discrimination between models than later results. This effect would disappear using log-transformed data, which is appropriate for many viability and fitness measures. As explained in the Materials and Methods section, our data are better analyzed in the arcsine scale due to the nature of the variable and to the small viability decline observed. Thus, in future MA experiments middle-term assays are encouraged for this trait. In any case, an initial assay (not available for our data) would be very useful to discriminate between models.

Finally, the pattern of line extinction simulated using the MD-Mukai model was in better agreement with the experimental one, with a small but significant initial extinction rate and about half of the lines surviving by the end of the experiment. However, this observed pattern does not allow estimating the strength of natural selection. In fact, a model in which extinction is assumed to be accidental and to occur at a constant rate can satisfactorily predict the experimental observations (Chavarrías et al. 2001).

In summary, the mutational properties of our lines conform with a low rate of mildly deleterious mutations. MD estimates can be considered to be more likely unbiased than BM ones. However, the interpretation of these estimates (both the BM and the MD) should be cautious because it has been shown that the effects of selection may have been relevant. A possibility would be searching for mutational parameters that allow simulating the experimental results, but this is hampered by our limited information on the strength of selection that actually occurred. Even so, we could compare the MD estimates of λ and $E(s)$ obtained at generations 208–209 (Table 1) with those corresponding to mutations actually fixed in simulated lines (Table 4). Considering that the models used in the simulation were independent from our data, the coincidence found with values simulated using the MD-Mukai model under conservatively strong selection is extreme. Such coincident values should, to some extent, have occurred by chance. In fact, MD estimates obtained from our MA lines at generations 104–106 ($\lambda = 0.016$ and $E[s] = 0.09$) were different from those used in the MD-Mukai model. In any case the general conclusion is that the rate and effects of deleterious mutations in our MA lines are close to those previously estimated from the same MA lines (generations 104–106) or from Mukai's et al. (1972) data using the MD approach (García-Dorado et al. 1998), that is, $\lambda \approx 0.01$ or 0.02 , $E(s) \approx 10\%$ or 20% .

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