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Small fitness effects and weak genetic interactions between deleterious mutations in heterozygous loci of the yeast *Saccharomyces cerevisiae*

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Summary

Rare, random mutations were induced in budding yeast by ethyl methanesulfonate (EMS). Clones known to bear a single non-neutral mutation were used to obtain mutant heterozygotes and mutant homozygotes that were later compared with wild-type homozygotes. The average homozygous effect of mutation was an approximately 2% decrease in the growth rate. In heterozygotes, the harmful effect of these relatively mild mutations was reduced approximately fivefold. In a test of epistasis, two heterozygous mutant loci were paired at random. Fitness of the double mutants was best explained by multiplicative action of effects at single loci, with little evidence for epistasis and essentially excluding synergism. In other experiments, the same mutations in haploid and heterozygous diploid clones were compared. Regardless of the haploid phenotypes, mildly deleterious or lethal, fitness of the heterozygotes was decreased by less than half a per cent on average. In general, the results presented here suggest that most mutations tend to exhibit small and weakly interacting effects in heterozygous loci regardless of how harmful they are in haploids or homozygotes.

1. Introduction

Random mutations, unless neutral to the phenotype, are much more likely to impair biological functions than to improve them. This is often seen as a simple consequence of a long history of natural selection, which has already fixed practically all beneficial mutations (Crow & Simmons, 1983). Another common feature of mutations, especially the null ones, is their recessivity. The latter results usually from basic features of the dynamics of metabolic pathways (Wright, 1934; Kacser & Burns, 1981; Mayo & Bürger, 1997; Bourguet, 1999). Thus, mutations tend to be deleterious and recessive, but do they interact with each other in ways that either reinforce or weaken their individual effects (Kimura & Maruyama, 1966)? No straightforward arguments or obvious observations are known to answer this question. Quantification of the above-mentioned three factors – selection coefficients, degree of dominance, and direction and intensity of genetic interactions – is critical to understanding how natural selection works against the 'load of mutations' (Muller, 1950). Genetic variation of mutational origin is likely to be ubiquitous, even in populations well adapted to their environment. This is the reason why deleterious mutations have attracted much attention and have been invoked to explain the evolution of several major biological phenomena. One of these phenomena is diploidy.

The evolutionary advantage of diploidy is best evidenced by its widespread occurrence among both multicellular and unicellular eukaryotes (Bell, 1982). In diploid genomes, deleterious mutations can be 'masked' by non-mutated alleles. Masking ensures immediate benefits for individuals but has long-term costs for populations because mutations will persist for a longer time (Haldane, 1937). The balance in the relative length of haplo- and diplophase may be affected by several genetic and ecological factors, but the degree of recessiveness of the mutated alleles is probably one of the most important (Kondrashov & Crow, 1991; Perrot *et al.*, 1991; Jenkins & Kirkpatrick,

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Table 1. *Experimental crosses*

Clone	Locus[a]	
	i	*j*
Original heterozygous mutants[b]	$\alpha\, i^m/\mathbf{a}\, i^w$	$\alpha\, j^m/\mathbf{a}\, j^w$
Derived haploids	$\alpha\, i^m\ \alpha\, i^w\ \mathbf{a}\, i^m\ \mathbf{a}\, i^w$	$\alpha\, j^m\ \alpha\, j^w\ \mathbf{a}\, j^m\ \mathbf{a}\, j^w$
	Single-locus crosses in the dominance experiment leading to	
Wild-type homozygote	$\alpha\, i^w \times \mathbf{a}\, i^w$	$\alpha\, j^w \times \mathbf{a}\, j^w$
Heterozygote	$\alpha\, i^m \times \mathbf{a}\, i^w$	$\alpha\, j^m \times \mathbf{a}\, j^w$
Mutant homozygote	$\alpha\, i^m \times \mathbf{a}\, i^m$	$\alpha\, j^m \times \mathbf{a}\, j^m$
	Two-locus crosses in the epistasis experiment leading to	
Double wild-type	$\alpha\, i^w\, j^w \times \mathbf{a}\, i^w\, j^w$	
Single heterozygote	$\alpha\, i^m\, j^w \times \mathbf{a}\, i^w\, j^w$	
Single heterozygote	$\alpha\, i^w\, j^w \times \mathbf{a}\, i^w\, j^m$	
Double heterozygote	$\alpha\, i^m\, j^w \times \mathbf{a}\, i^w\, j^m$	

[a] Two example clones with mutated locus *i* or *j*.
[b] The wild-type and mutant alleles of *i* and *j* are abbreviated as *w* and *m*, respectively. The symbols α and **a** denote the two alleles of the mating type locus *MAT*.

1995; Otto & Goldstein, 1992). Other phenomena whose evolution is postulated to be explained by the existence of deleterious mutations are sexual reproduction and recombination. These may have evolved to strengthen selection against deleterious mutations, especially if the mutations were frequent and their negative effects reinforced by synergistic interaction (Feldman *et al*., 1980; Kondrashov, 1988; Barton, 1995; Otto & Lenormand, 2002). In asexual organisms, mutations arise and are selected against in each clone independently. When populations are small, mutations can be fixed even in the most fit clone, leading to a gradual ratchet-like decrease in both the maximum and average fitness (Muller, 1950). This process can develop into 'mutational meltdown' and, ultimately, lead to population extinction (Gabriel *et al*., 1993). The chances that the ratchet slows down or stops increase when mutational effects are substantial and tend to interact synergistically (Charlesworth *et al*., 1993; Kondrashov, 1994). Among other intensively studied issues related to deleterious mutations are inbreeding depression (Fu & Ritland, 1994; Dudash & Carr, 1998) and evolution of selfing (for a review see Charlesworth & Charlesworth, 1998).

In the present paper we present an experimental study of the effects of dominance and epistasis. Several statistical methods have been developed to quantify these factors (Lynch & Walsh, 1998). They are generally based on the analysis of variance components. Therefore they provide only the means of estimated parameters and are subject to biases if assumptions of the quantitative genetics models are not met. We applied a straightforward approach based on simple genetic crosses in which we could directly estimate fitness of individual mutations in haploid, homozygous and heterozygous loci.

We began with single deleterious mutations (one base random substitutions) caused by ethyl methanesulfonate (EMS) (Sega, 1984) obtained from a former study in which we examined a large number of diploid clones for the presence of a single allele generating visible phenotypic effects in the haploid products of meiosis (Wloch *et al*., 2001*b*). The other loci were generally isogenic and homozygous. If there were EMS-induced mutations other than the studied one, their phenotypic effects were not detectable under our assays. In the present experiments, we derived the wild-type and the mutated haploid clone from every heterozygote (except for the lethals). These haploids were then used in two types of experimental crosses (Table 1). In order to test for 'dominance', crosses were made between haploids derived from a single heterozygous mutant strain. A wild-type homozygote was obtained by crossing two wild-type haploids, a mutant homozygote by crossing two mutant haploids, and a (new) heterozygote by crossing a wild-type and a mutant haploid segregant. Because each trio was derived from a single original heterozygous mutant it was possible to compare the three single-locus genotypes on the same average genetic background. By this protocol, any cryptic mutations possibly residing on chromosomes or cytoplasmic elements would contribute equally to the average fitness of wild-type homozygotes, mutant homozygotes and heterozygotes.

In an experiment designed to test for 'epistasis', haploids derived from two different original heterozygous clones were mated (Table 1). In this case, two loci were involved and the diploids obtained were either wild-type homozygotes in both loci, heterozygous for mutations at one of the two loci, or heterozygous for mutations at both loci. Again, the genetic background was expected to be the same for

all four genotypes because it was derived in equal proportions from the same pair of original diploid clones. In yeast, mixing of genes in meiosis is efficient due to segregation of 16 chromosomes and recombination at about 100 cross-overs per meiosis (Roeder, 1995; Borts *et al*., 2000; Abdullah & Borts, 2001).

In addition to the study of dominance and epistasis in diploids, we also wanted to compare fitness effects of mutations in haploid and heterozygous diploid clones. This is similar to but not the same as a comparison between homozygous and heterozygous diploid mutant strains. Both in haploids and in homozygous mutant strains the masking effect of a wild-type allele is absent. However, phenotypes of, and challenges faced by, the two genetic phases differ in a number of significant aspects (Sherman, 1997; Galitski *et al*., 1999). For example, in normal circumstances, only haploids begin growth by germinating from a spore. The germinating haploids have to re-start metabolism, and this challenging situation is more likely to uncover genetic defects (Wendland, 2001). Finally and most importantly, comparisons between haploids and diploids were necessary to include haplo-lethal mutations in the study. Such lethal mutants could not have been used in crosses such as those described above (Table 1). Therefore, in order to estimate the effect of heterozygous lethal mutations, two samples of diploid clones were compared: one group was known to bear single heterozygous lethal alleles and the other was free of them.

2. Materials and methods

(i) *Strains*

The initial diploid strain was essentially homozygous at all loci except for the mating type locus which was *MAT***a**/*MAT*α. Homozygosity was obtained by a series of 10 sequential backcrosses to the same 'wild-type' strain (Wloch *et al*., 2001*b*). The only loci known not to be wild-type were three auxotrophic loci (*ura3*, *leu2* and *trp1*) and the *HO* (homothalism) gene. The latter was inactive, *ho*, which allows stable maintenance of the mating type of haploid clones.

A colony developed from a single cell was used to initiate a liquid culture and grown overnight. A 1 ml aliquot of this culture was treated with 2 μl of EMS for 1 h and then washed to terminate mutagenesis. A large sample of mutagenized culture was used to inoculate fresh medium in which eight generations of growth were completed. From this, replicate clones were obtained. Samples were diluted and spread as single cells on YPD (1% yeast extract, 2% peptone, 2% glucose) agar surface. The resulting colonies were replica-plated onto sporulation medium (1% potassium acetate, 0·05% glucose, 0·1% yeast extract, required supplements) where, after some additional growth, diploid cells underwent meiosis and developed into tetrads of haploid spores.

A single tetrad was chosen at random from each colony. The four spores were separated on a fresh YPD plate and grown into colonies. Most clones exhibited wild-type formation because the concentration of EMS was low. When a 2:2 segregation pattern for viability or small colony size was discovered within a tetrad, the parental diploid clone was considered to have a single mutation in a heterozygous locus. Such a pattern would also be produced if two clones within one tetrad happened to be altered due to purely phenotypic and not genetic reasons. Wloch *et al*. (2001*b*) provide extensive statistical arguments that such false 2:2 segregations were rare, such that over 90% of clones identified in this manner contained a single mutation. For the purpose of the present experiments, we repeated the tetrad analysis of the previously identified heterozygous diploids to confirm that all clones used in the following crosses did indeed contain a single mutation. The methods applied in this verification are described in the following section.

(ii) *Verification of mutants*

Samples of diploid clones that had been identified by Wloch *et al*. as containing single heterozygous mutations were kept at -70 °C. The present study was initiated by thawing these samples and spreading to single cells on YPD plates. A single colony of every clone was transferred onto sporulation medium. Three to nine tetrads per colony were dissected on a fresh YPD plate. After 48 h of incubation at 30 °C, the sizes of the resulting haploid colonies were assessed visually. The tetrads were grouped into five classes: (i) no visible mutation pattern, which usually meant that all four colonies were of normal wild-type size; (ii) two normal and two slightly smaller than normal size colonies; (iii) two normal and two medium-sized colonies; (iv) two normal and two small colonies; (v) two normal and two dead colonies. A mutation was considered as positively verified if the replicate tetrads of a given clone showed the expected 2:2 pattern, i.e. two of four colonies were smaller in a similar degree as seen by Wloch *et al*. (2001*b*).

A subset of clones was verified more thoroughly by measuring under $\times 40$ magnification the sizes of the haploid colonies obtained after tetrad dissection. This allowed quantitative comparison of the growth effects of the mutations obtained in the present study with those estimated by Wloch *et al*. (2001*b*).

The above assays, both qualitative and quantitative, were performed in order to verify the presence of single mutations and to identify the necessary haploid clones. From every diploid with a verified non-lethal mutation in a heterozygous locus, four haploids

were obtained: *MAT***a** and *MATα* of a 'wild-type' phenotype, and *MAT***a** and *MATα* of a 'mutant' one. These were subsequently used as described below. Precise measurements of the growth rate of all the resulting clones were obtained according to a uniform protocol described in section (vi), 'Fitness assays and comparisons'.

(iii) *Crosses*

The experimental crosses carried out in this study are presented in Table 1. For a test of dominance, crosses involved two haploids of opposite mating types that were derived from one heterozygous diploid clone. The locus, which is putatively mutated, is denoted by *i*. The alleles at the locus are denoted by *w* (wild-type) or *m* (mutant). After the appropriate matings, three diploid clones were obtained: a wild-type homozygote, i^w/i^w, a mutant homozygote, i^m/i^m, and a heterozygote, i^w/i^m. Because both the haploids to be crossed and the resulting diploids had the same markers, complementation could not be used to select or screen for diploids. Therefore, two haploids were mixed, grown overnight on a YPD plate, and then spread to single cells on a new YPD plate. A number of resulting colonies were transferred onto sporulation medium and checked for sporulation, a marker of diploidy. Several matings, primarily those involving two mutants, did not yield diploids despite repeated efforts. This may mean that mating was impaired or that sporulation efficiency was very low. These unsuccessful crosses could bias our results only if mutations affecting mating or sporulation were associated with a specific effect on dominance or epistasis. There are no theoretical or experimental reasons to believe this to be true.

The study of epistasis was begun by random matching of two diploid clones, *i* and *j*, that were confirmed to have single heterozygous mutations. Each clone was used only once. A pair of haploid clones, wild-type and mutant, obtained from the *i* heterozygote were i^wj^w and i^mj^w, respectively. An analogous pair from the *j* heterozygous diploid were i^wj^w and i^wj^m (Table 1). In the subsequent matings, haploids with the different genotypes were mated to obtain a homozygous wild-type i^wj^w/i^wj^w, two single heterozygous mutants i^mj^w/i^wj^w and i^wj^m/i^wj^w, and a double heterozygous mutant, i^mj^w/i^wj^m.

(iv) *Lethal mutations*

A number of clones from the collection of Wloch *et al.* (2001*b*) that were previously identified as heterozygous for a single lethal allele were verified using the protocol described above. As a control, a similar number of diploid clones that yielded four normal-looking haploids were chosen. These clones were also subject to verification to ensure that they were indeed free of mutations. Both the heterozygous lethals and the control wild-types came from the same EMS-treated culture. Therefore, any very small, and thus invisible, mutational effects potentially present should have been, on average, the same in both groups.

(v) *Haploids versus heterozygotes*

Comparisons between haploid and heterozygous diploid clones were made by using data obtained from two studies. The data on heterozygous effects were collected from the present estimates gathered in both the dominance and epistasis studies. The estimates of haploid fitness, for both lethal and non-lethal mutations, came from our earlier experiments (Wloch *et al.*, 2001*b*). We chose the former haploid fitness estimates over the ones performed in the present study because the latter were most often qualitative estimates obtained simply to confirm that the expected phenotype was present.

(vi) *Fitness assays and comparisons*

Fitness of every clone was estimated as an average growth rate of a colony initiated by a single cell and incubated for 48 h at 30 °C. In our previous study, such an approach was natural because the tetrads were dissected to single haploid spores and placed at regular intervals on fresh YPD plates (Wloch *et al.*, 2001*b*). In this experiment, the aim was to measure growth rate of diploids in conditions as similar as possible to that used for haploids. Single vegetative diploid cells were moved with the aid of a micromanipulator and placed in fixed order on YPD agar in 90 mm Petri dishes. To make all positions uniform, the cells were laid at the same distance from the center of a plate on 12 evenly spaced points (Fig. 1). Samples of clones were put in the middle of a plate before being dispersed. The centre of the plate was subsequently removed so that the patches growing from the superfluous cells could not interfere with the colonies being assayed. The plates were photographed after 48 h of incubation using a digital camera. The area of every colony was then estimated using an image processing program. The estimate of the area was used to calculate colony volume. The shape of a colony was assumed to be hemispherical. While this is a simplification, the proportional volumes of colonies can be assessed correctly from the areas of their bases, as long as they are isomorphic. Although a few colonies were not isomorphic, we did not observe any large deviations, such as very flat or exceedingly steep-sided colonies, which might invalidate this assumption. The calculated volume was then divided by $1{\cdot}7 \times 10^{-7}\,\mu$l, the approximate volume occupied by a typical diploid cell within a colony (Sherman, 1991). From the

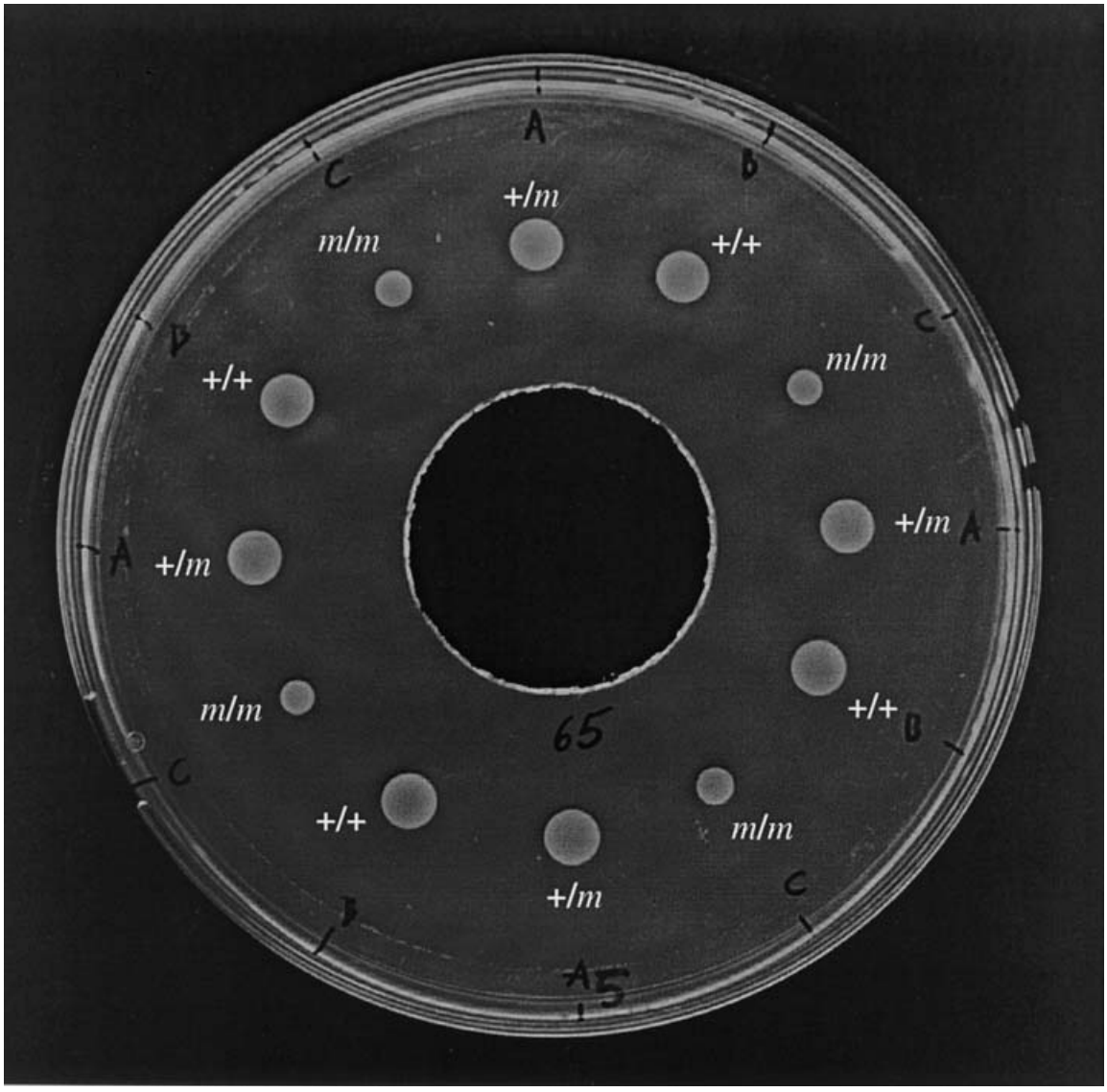

Fig. 1. An example of a plate with diploid clones positioned at 12 points. The plate contains clones resulting from a single mutation-cross (see Table 1 for details). An array of three clones – wild-type homozygote (+/+), mutant homozygote (*m*/*m*) and mutant heterozygote (+/*m*) – is repeated four times clockwise around the plate. (The letters A, B, C visible in the photograph were codes, randomly assigned to each plate. The measurements were done by a person who knew the codes but not the identity of the strains.)

number of cells, N, the number of divisions was calculated as $\log_2 N$ and then divided by 48 h. This yielded an estimate of the average rate of growth expressed as the number of doublings per hour.

A single plate contained clones resulting from one set of crosses. In the study of the dominance of non-lethal mutations, an array of cells of the three genotypes (i^w/i^w, i^m/i^m, i^w/i^m) was repeated four times around a circle. This resulted in 12 colonies, unless some of the cells were inviable (Fig. 1). Plates with many missing colonies were redone. The average growth rate of the four (sometimes three, rarely two) wild-type homozygous (i^w/i^w) colonies was calculated. Similar calculations were performed separately for the homozygous and heterozygous strains. The relative fitness, w, of wild-types was set to 1. The relative fitness of i^m/i^m and of i^w/i^m was calculated as the ratio of average growth rate of a given clone to the average growth rate of i^w/i^w from the same plate. In fact, growth rate is only one component of fitness, although an important one. Through the rest of the text the term 'fitness' will mean relative fitness or, strictly speaking, relative growth rate. In general, a single plate constituted an experimental block, in which the three clones were the compared groups, and the four cells were replicates.

This experimental design was applied to the two other experiments. As described above, dominance of the lethal mutations was studied by comparing heterozygotes, i^w/i^m, with diploids without any visible

mutations, i^w/i^w. The average growth rate of the i^wi^w colonies was set to 1 and used to calculate the relative fitness of heterozygotes. In the third experiment, the study of epistasis, an array of four clones – a wild-type i^wj^w/i^wj^w, two single mutants i^mj^w/i^wj^w, i^wj^m/i^wj^w, and a double mutant i^mj^w/i^wj^m – was repeated three times per plate. Two such plates were assayed per each pair of i and j. For the test of epistasis, two higher-order averages were calculated for a single plate. One was the 'disassociation' mean, i.e. an average relative fitness of two single heterozygotes, i^mj^w/i^wj^w and i^wj^m/i^wj^w, that can be expressed as $[(1-s_i)+(1-s_j)]/2=1-(s_i+s_j)/2$. The other was the 'association' mean, i.e. an average relative fitness of a wild-type, i^wj^w/i^wj^w, and a double heterozygote, i^mj^w/i^wj^m. The association mean can be expressed as $[1+(1-s_i-s_j+s_{i\times j})]/2$. It becomes equal to the disassociation mean, $1-(s_i+s_j)/2$, if there is no effect of interaction, $s_{i\times j}=0$. The data were analysed with a two-way ANOVA where the relationship of the mutations (associated or disassociated) and the cross (a pair of i and j) were the major effects and the two plates were replicates.

3. Results

(i) *Verification of haploid phenotypes*

Fitness effects of single mutations were quantified in replicate assays for 74 of the clones collected by Wloch *et al.* (2001*b*). These were compared with the original estimates using the same measurement and growth rate calculation methods as previously. The mean haploid relative fitness was 0·512 in the original assay and 0·513 in the present one. The difference was statistically insignificant ($t=0{\cdot}019$, $df=146$, $P=0{\cdot}985$). Individual estimates were also little changed between the two assays (Fig. 2). Correlation between the two measurements was high ($r=0{\cdot}825$, $n=74$, $t=12{\cdot}462$, $P<0{\cdot}001$), especially considering that the second assay was done a year later, after another round of thawing, growth, sporulation and digestion of asci. The few strong deviations might result not from an experimental error or phenotypic variation but from true genetic differences. The latter could happen when a colony that was thought to contain one clone with a single mutation was in fact founded by two or more genetically different cells. Thus it is possible that the original assays done by Wloch *et al.* (2001*b*) and the present assays involved different clones from such a mixed colony. Another possibility is that spontaneous mutations occurring during colony growth or freezing are a source of genetic polymorphism. These should be rare, however, because the present assays involved just a few dozens of generations and the spontaneous mutation rate per generation was estimated at $1{\cdot}1\times10^{-3}$ (Wloch *et al.*, 2001*b*).

The rest of the clones used in the present experiments were not subject to precise growth rate measurements but assigned to one of four mutant phenotypes (lethal, severe, moderate, slightly deleterious). In over 90% of comparisons it was found that the re-assayed phenotype was quantitatively similar to that originally observed. Only the positively verified clones were used in the experiments reported below.

(ii) *Dominance of non-lethal mutations*

The relation between homozygous and heterozygous fitness is shown in Fig. 3. The homozygous selection coefficient, s, of a given mutation was calculated as the difference in relative fitness between the wild-type, i^w/i^w, and the homozygous mutant, i^m/i^m. An analogous difference between the wild-type and the heterozygote, i^m/i^w, was taken as an estimate of hs, where h stands for the coefficient of dominance. The mean hs was significantly lower than the mean s in a paired comparison ($\overline{hs}=0{\cdot}0042$, $\overline{s}=0{\cdot}0213$, $t=-5{\cdot}182$, $df=81$, $P<0{\cdot}001$). The mean coefficient of dominance calculated as the ratio $\overline{hs}/\overline{s}$ was 0·197.

The average standard errors of individual estimates of s and hs were 0·0051 and 0·0044, respectively. Therefore the negative values of some individual estimates of these two parameters probably indicate the measurement errors and not genetic effects resulting in an increased relative fitness (Fig. 3). A similar interpretation is likely to be true for the apparent fitness increases in the study of epistasis (Fig. 4) and in the comparison between haploids and heterozygotes (Fig. 5).

(iii) *Epistasis of non-lethal mutations*

This experiment tested the fitness effect of combining heterozygous mutations at two loci, i^m/i^w and j^m/j^w. The effects of the mutations on growth rate would be additive if the average fitness of two clones each bearing one such locus (disassociation) were equal to the average of two other clones, one with none and the other with both mutations (association). Since the calculations of growth rates are based on a logarithmic function, additivity of the growth rates is equivalent to multiplicity of non-logarithmic measures of fitness. For this reason, we judge single-locus effects multiplicative based on their additivity in terms of statistics. A result where the association average was lower than the disassociation average would mean synergism. Antagonism would be indicated by the opposite relationship. The results of an ANOVA test are presented in Table 2. Table 2 shows that one of the two major effects, the particular mutations, explained as much as 78% of genetic variation, while the other, the type of association, did not contribute at all. Interaction between these two factors was

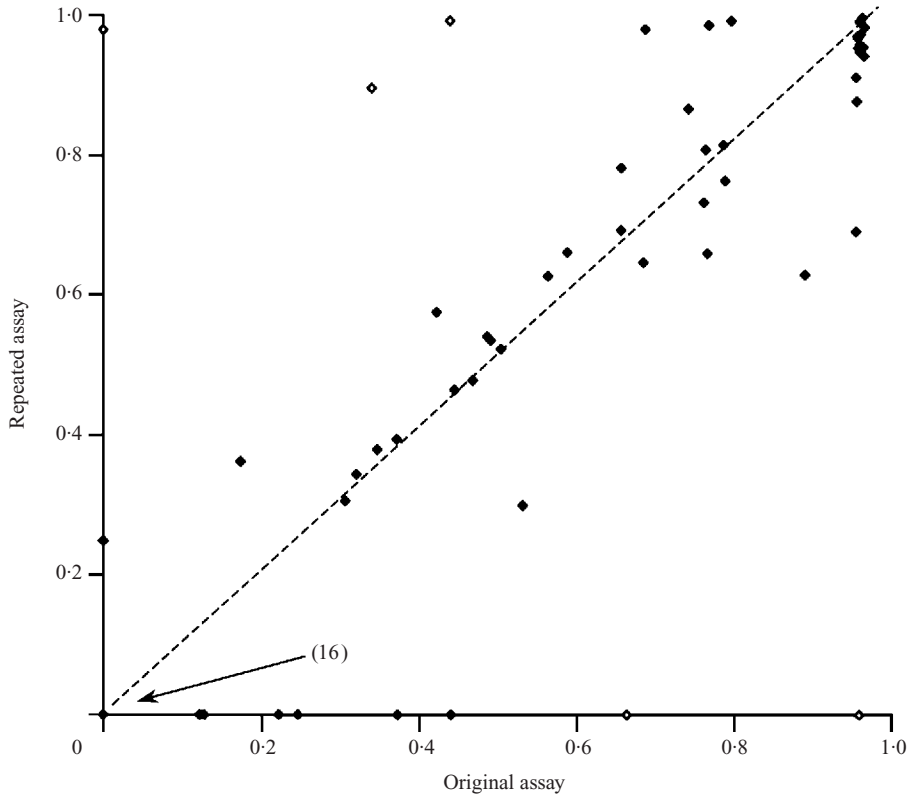

Fig. 2. Reproducibility of fitness assays. Values on the *x*-axis show relative fitnesses for a sample of the mutations found by Wloch *et al*. (2001*b*). The *y*-axis values show the results of assays repeated with the same clones in the present study. The five open diamonds mark the most aberrant cases; they were included in the correlation analysis reported in the main text.

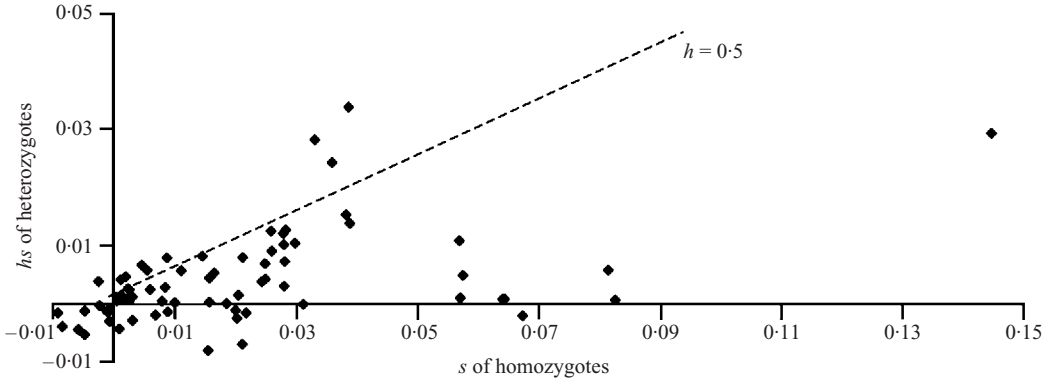

Fig. 3. Dominance experiment. The selection coefficient *s* of mutant homozygotes is related to the product of dominance and selection coefficients *hs* of mutant heterozygotes. The dashed line shows the region of co-dominance where the fitness effects of heterozygotes would be twofold smaller than those of homozygotes.

statistically significant and explained 22% of the genetic variation. This implies that some loci tended to interact synergistically and some antagonistically but that there was no average departure from multiplicative action of effects. The disassociation and association means would be distributed along a diagonal line $y = x$ if their deviation from the predicted values were random (Fig. 4). The actual principal major axis was $y = 0{\cdot}291 + 0{\cdot}709x$. The upper and lower limits for 95% confidence limits of the slope were 1·024 and 0·465, respectively (Sokal & Rohlf, 1995). Thus, the upper bound barely overlaps with the slope of the diagonal, leaving the null hypothesis viable.

(iv) *Lethals, and haploids versus diploids*

The relationship between growth effects of the same mutation residing in a haploid or heterozygous diploid locus is shown graphically in Fig. 5. In the case of

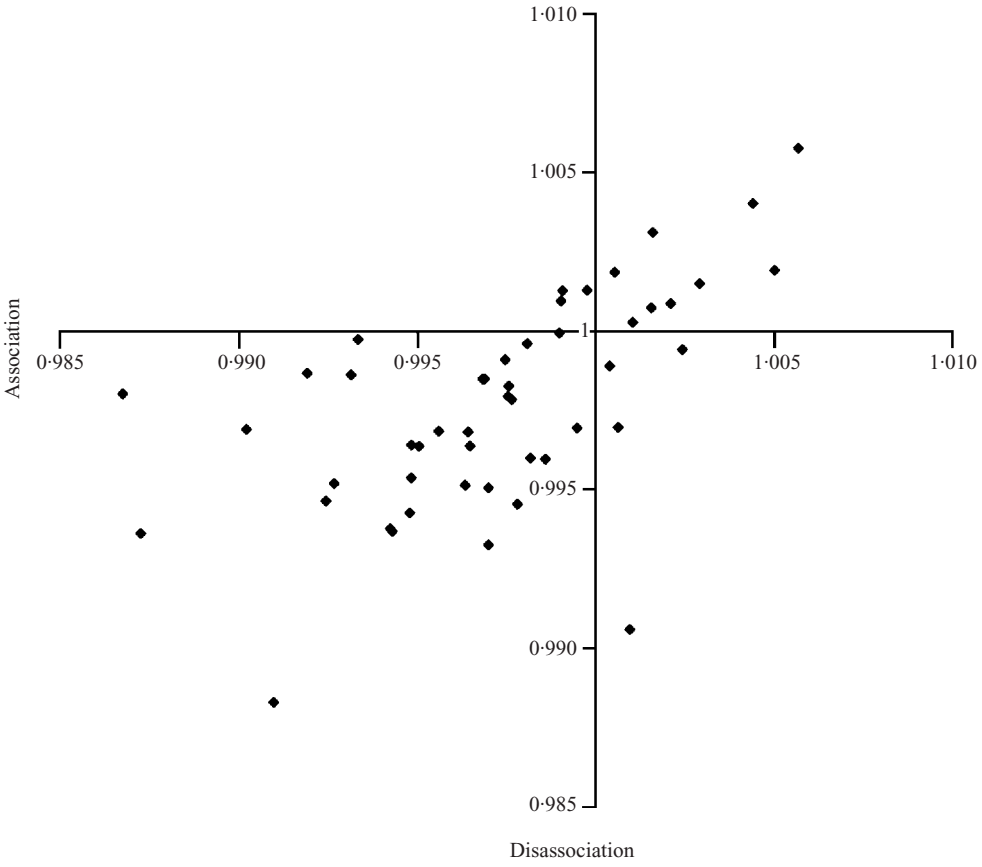

Fig. 4. Epistasis experiment. An 'association' estimate was calculated as the mean relative fitness of a wild-type and a double heterozygous mutant obtained from a given cross ($i \times j$). 'Disassociation' is the mean fitness of the two single heterozygotes resulting from the same cross.

lethal mutations, the haploid selection coefficient, s, was set to 1. The heterozygous selection coefficient of the lethal mutation, hs, was estimated by subtracting the relative fitness of a mutant heterozygote from that of a wild-type homozygote (the latter also set to 1). $\overline{hs}$ for the lethal mutations (and thus also their $\overline{h}$) was very low, at 0·0034.

The heterozygous selection coefficient was calculated separately, but in the same way, for the non-lethal mutations. It was found to be $\overline{hs} = 0{\cdot}0048$. (The figure of 0·0048 is somewhat different from the 0·0042 reported in section (ii), 'Dominance of non-lethal mutations', because the present estimate is based on the heterozygous fitness estimates collected from both the dominance and epistasis studies. See also Section 2, Materials and methods.) The heterozygous effects of the lethal and non-lethal mutations, 0·0048 and 0·0034, are statistically indistinguishable ($t = 0{\cdot}665$, $df = 169$, $P = 0{\cdot}507$). This suggests that the lethal mutations were about 8 times more successfully 'masked' than the non-lethal ones. The last conclusion can be made relating the average haploid selection coefficient of the lethal mutations, $\overline{s} = 1$, and that of the non-lethal mutations used in this comparison, $\overline{s} = 0{\cdot}1214$.

4. Discussion

(i) *Dominance*

Most of the mutations studied in the present experiments were strongly recessive. The average selection coefficient against single heterozygous deleterious, and even lethal, mutations was lower than half a per cent. Two features of the experimental system made it possible to study such small effects. One was the opportunity to carry out crosses in a way ensuring that the genetic background was homogenized and did not interfere with the small effects studied. The other was the way in which growth rate was measured. Single cells were used to initiate growing colonies. Therefore even small differences in the rate of growth translated to (roughly) exponentially growing differences in colony sizes, making the latter measurable.

(ii) *Dominance of non-lethal mutations: an overview*

The coefficient of dominance, h, was about 0·2 or 0·3 depending on the method of calculation used. Thus, the masking effect of a wild-type allele does not appear to be very strong when weakly deleterious mutations are considered.

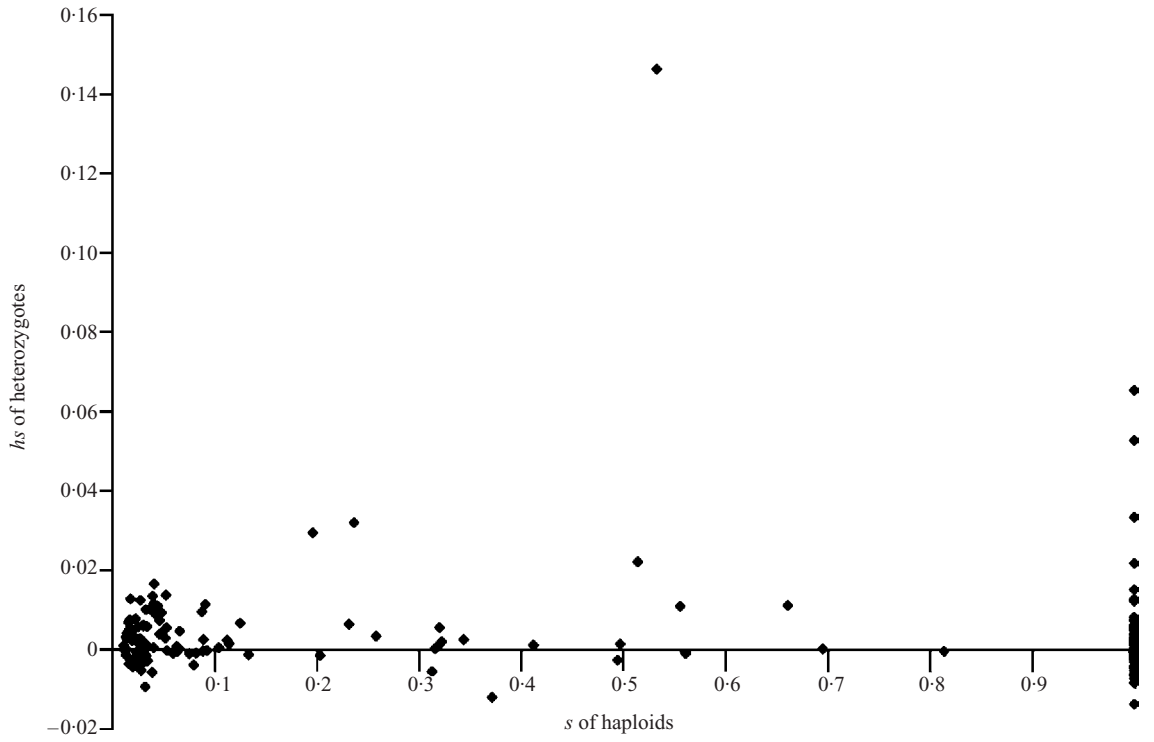

Fig. 5. Haploids versus heterozygotes. Fitness effects are expressed as *s* for haploids and *hs* for heterozygous diploids. The lethal haploid phenotypes are grouped at the right side of the graph because their *s* was set to 1.

Estimates of the dominance coefficients available for other organisms also suggest that small mutational effects are only moderately masked by wild-type alleles. The classic estimates of Mukai (1964) and Mukai *et al*. (1972) suggested that in *D. melanogaster* small viability effects caused by new mutations were only slightly recessive, $h = 0{\cdot}4$. This somewhat unexpectedly high dominance was confirmed in independent experiments by Ohnishi (1977*a*, *b*) although those results have recently been questioned. When Garcia-Dorado & Caballero (2000) reanalysed these two data-sets they argued that the estimate of *h* was about twofold lower. However, the original observation of the relatively high dominance of new mutations is supported by other work (Chavarrías *et al*., 2001). The value of *h* tended to be generally lower and more variable, however, when traits other than viability were studied in *Drosophila* (Fernández & Lopez-Fanjul, 1996; Houle *et al*., 1997). The values discussed above were obtained for spontaneous mutants. EMS mutagenesis yielded deleterious alleles with dominance coefficients of 0·1–0·3 (Ohnishi, 1977*a*, *b*). Among other invertebrates in which the impact of spontaneous mutations on fitness was studied are cladocerans and round-worms. In *Caenorhabditis*, the average dominance coefficient of mutations deleterious to fecundity was probably between 0·1 and 0·5 (Vassilieva *et al*., 2000). The estimates cited above were obtained in experiments where methods other than crosses of single mutations were applied. The mutations were usually accumulated in long-term experiments or sampled from extant populations. This was often followed by rounds of inbreeding. The exact number of mutations involved was generally not known. Nevertheless, both the reviewed studies and our data consistently show that alleles with small fitness effects retain approximately a quarter of their impact on fitness in heterozygotes.

Dominance of deleterious mutations in yeast has previously been estimated. Korona (1999*b*) obtained haploid strains that contained dozens of mutations accumulated during hundreds of generations of growth with an impaired DNA mismatch repair system. Although a mutational load was evidenced by a considerable drop in fitness, the strains were also known to have compensatory mutations that ameliorated some of the harmful effects. Such haploid clones were used in crosses with wild-type clones and the coefficient of dominance was found to be $h = 0{\cdot}08$. This value is lower than the present one, but it is less reliable because of the fact that mutations were numerous and not always deleterious. Mable & Otto (2001) compared haploid and diploid yeast cultures treated with EMS. The relatively large populations employed in that study could result in a loss of the more harmful mutations. The estimate of $h = 0{\cdot}30$ that they calculated is close to that obtained in the present study for mutations with small effects. Other studies employing yeast strains with numerous deleterious mutations suggest that the estimates of both *s* and *hs*

Table 2. *Relative fitness in the experiment measuring epistasis of non-lethal mutations* (*model II ANOVA*)

Source of variation	*df*	$MS \times 1000$	*F*	*P*	Component	Explained
Individual cross ($i \times j$)	47	4·693	3·907	$<0{\cdot}001$	0·873	78 %
Association vs disassociation[a]	1	1·001	0·833	0·366	−0·002	0
Interaction	47	1·201	1·706	0·014	0·248	22 %
Error	95	0·704			0·704	

[a] 'Association' is the average relative fitness of a wild-type ($i^w j^w / i^w j^w$) and a double heterozygous mutant ($i^m j^w / i^w j^m$) obtained from a given cross ($i \times j$). 'Disassociation' is the analogous average for the two single heterozygotes ($i^m j^w / i^w j^w$ and $i^w j^w / i^w j^m$) resulting from the same cross.

are dependent on environment. Harsh conditions tend to magnify the negative effects of mutations in both haploids and heterozygous diploids (Korona, 1999*a*; Szafraniec *et al*., 2001).

(iii) *Epistasis*

The mean fitness of two clones each carrying a single mutation (disassociation) was the same as the mean fitness of a double wild-type and a double mutant clone (association). This indicates an absence of genetic interaction. The mutations were in heterozygous condition and, consequently, the differences in fitness were slight. The most and the least fit clones differed by less than 2 % of their relative fitness (Fig. 4). This is an important feature of the present experiments because such small effects would be especially difficult to study with organisms other than yeast. Most of the genetic variation was attributable to differences between crosses, i.e. fitness was determined by a pair of particular mutations irrespective of whether they were in association or disassociation. These two findings mean that the growth rate effects are additive. As already mentioned in Section 3, this implies multiplicative action of deleterious mutational effects because additivity of growth rates means multiplicity of non-logarithmic measures of fitness, such as the number of progeny. Only about one-fifth of the variation was attributable to the genetic interaction (Table 2). This interaction was generally random, that is, estimates were distributed along a diagonal (Fig. 4). Epistasis of neither the 'diminishing returns' nor 'increasing returns' type was visible. This result was obtained with a very narrow range of relative fitness and a minimum number of interacting loci. Perhaps interaction between many loci or loci with large effects would be different. However, in a large equilibrium population the differences in fitnesses would also be small. The present data offer insight into the genetic interactions in individuals close to a 'wild-type' phenotype. They suggest that the phenotypes of these genotypes are not greatly influenced by the antagonistic or (with even higher certainty) the synergistic interaction of deleterious mutations.

Previous studies of the epistatic interactions of deleterious mutations have most often been done with haploids or homozygous diploids. Mukai (1969) found synergism of spontaneous deleterious mutations in fruit flies, although the validity of his data has been debated on several occasions (Shabalina *et al*., 1997; Fry *et al*., 1999; Keightley & Eyre-Walker, 1999; Fry, 2001). The authors of other studies, involving *Chlamydomonas* (de Visser *et al*., 1997*a*), higher plants (de Visser & Hoekstra, 1998), RNA viruses (Elena, 1999), nematodes (Peters & Keightley, 2000) and haploid yeast (Wloch *et al*., 2001*a*) also detected some signs of synergism although they all noted that their results were equivocal. As an alternative to the studies of random mutations, Elena & Lenski (1997) inserted genetic markers into defined positions of the *E. coli* genome. Bohannan *et al*. (1999) studied interactions between mutations conferring resistance of *E. coli* clones to bacteriophages. In yet another study, de Visser *et al*. (1997*b*) focused on existing chromosomal markers in *Aspergillus*. In these studies, there were interactions in both directions but no overall trend to synergism was seen. In a recent experiment, Yang *et al*. (2001) mutagenized laboratory populations of fruit flies with EMS and did not detect epistasis among heterozygous loci. Our results, also obtained with heterozygous loci, further weaken the evidence for synergism as a common and prevailing type of interaction among deleterious mutations.

(iv) *Lethals, and haploids versus diploids*

The fitness effects of mutations were much more visible in haploids than in diploids. This might be expected when haploids are compared with heterozygotes, but not necessarily expected when haploids are compared with homozygous mutant diploids. For example, the *x*-axis of the graph in Fig. 3 represents a sample of homozygous mutants. The mean *s* of the non-lethal mutations in homozygous diploids, 0·0213,

is considerably and statistically significantly smaller than the mean s of the very same mutations in haploids, 0·080: $t=3{\cdot}501$, $df=72$, $P<0{\cdot}001$. (The value reported here, $s=0{\cdot}080$, is smaller than the 0·1214 provided in the last line of Section 3 because in the present case only the haploid mutants that were used to form mutant homozygotes are considered. Some of the haploids containing severe mutations could not mate with an isogenic haploid of opposite mating type although they could mate with a wild-type and yield a heterozygote. See also section (iii) 'Crosses' in Section 2.) It might be premature to conclude that deleterious mutations are simply about 4 times less harmful in diploids than in haploids. The haploid clones were initiated from spores and therefore their fitness was affected both by the duration of germination and by the rate of subsequent growth. It usually takes about 4 h to complete germination but this time can vary substantially. It is likely that selective disadvantage of the haploid clones was sometimes caused by metabolic defects that interfered with germination but not, or much less, with vegetative growth. Therefore, the haploids and diploids would differ less in their selection coefficients if the vegetative growth dominated in both genetic phases and germination were a rare event.

Fitness of many heterozygous mutants was practically unaffected (Figs 3–5). This finding can partly explain the result obtained by Zeyl *et al.* (2001), whose mutator lines of diploid yeast accumulated mutations for 3000 generations and yet had a decrease in competitive ability that was undetectable in most of the lines. It also shows the important role of sporulation and mating within an ascus, which is more frequent than out-crossing in wild *S. cerevisiae* (Mortimer *et al.*, 1994). Intra-ascal mating results in homozygosity of mutations that would be essentially immune to natural selection in heterozygotes. Although the similarity of heterozygous fitness of the lethal and non-lethal mutations was striking in the present experiment, this phenomenon is probably not constrained to yeast. It has already been reported for *Drosophila* (reviewed by Mukai *et al.*, 1972; Crow & Simmons, 1983) and suggested for *Daphnia* (Lynch *et al.*, 1998).

A proper study of deleterious mutations requires that they are sheltered from selection before their selective value is estimated. Otherwise, the studied spectrum of mutations is different from that actually occurring. In this experimental system, mutagenesis was followed by eight generations of growth in a mass culture when mutations with large heterozygous effects would be out-competed. However, there were actually only approximately five generations of selection because the peak of mutant phenotypes occurs at the third generation after EMS treatment (Klein *et al.*, 1989, 1990). Mutations with heterozygous effects on the order of 0·10 or greater would be reduced in frequency by about a half after five generations of growth. There was only one such mutation among the 174 tested (Fig. 5). Of course, there could have been dominant, or partially dominant, lethal or seriously harmful mutations that would escape our assays. Such mutations would pose a problem to every experiment, however, and it is practically impossible to determine whether they in fact happen at an appreciable rate. A clear finding of the present study is that new mutations are readily detectable in haploids and very well masked in heterozygous diploids. It adds strength to our recent estimates of the mutation rate and distribution of fitness effects in budding yeast (Wloch *et al.*, 2001*b*).

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