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TEST OF INTERACTION BETWEEN GENETIC MARKERS THAT AFFECT FITNESS IN *ASPERGILLUS NIGER*

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Abstract.—In this paper we study whether and how a number of arbitrarily chosen marker mutations interact in their effect on fitness, which is relevant for our understanding of the evolution of sex. If epistasis is synergistic, the main function of sex may be to facilitate selection against deleterious mutations. We use strains of the filamentous fungus *Aspergillus niger* with variable combinations of marker mutations that have been obtained by isolating segregants from a diploid between a wild-type strain and a related strain carrying a marker mutation on each of its eight chromosomes. The marker mutations include five auxotrophic and two resistance mutations. As a measure of fitness the mycelium growth rate on supplemented medium has been used. The results suggest that the marker mutations have independent effects on fitness, and hence they do not support the deterministic mutation hypothesis of the evolution of sex. The apparent linear relationship between mutation number and log fitness is the result of interactions of opposite type (i.e., synergistic and antagonistic) that cancel each other's effect. However, due to an isolation bias caused by the fact that not all possible strains with many mutations could be isolated, the results may be relatively biased towards an antagonistic relationship between mutation number and log fitness.

Key words.—*Aspergillus*, evolution of sex, synergistic epistasis.

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Whether or not deleterious mutations interact in affecting fitness can have important consequences for the mutation load of a population (Kimura 1961; Kimura and Maruyama 1966), and for the advantage that sex and recombination can provide to reduce this load (Crow 1970; Kondrashov 1982). If the interaction between mutations is synergistic, that is, if the logarithm of fitness declines more rapidly than linearly with an increase in the number of mutations, sexual reproduction may be maintained because it reduces the mutation load (Kondrashov 1988; Charlesworth 1990). At present, the empirical evidence for synergistic epistasis is rather sparse (e.g., Hurst and Peck 1996). Furthermore, most experimental studies present indirect tests that produce ambiguous data (Kondrashov 1993).

The simplest way to study experimentally the relationship between the number of deleterious mutations and fitness, would be to measure the fitness of individuals with a varying, but known, number of deleterious alleles. However, direct information on mutation number of particular individuals is very difficult to obtain. Therefore, experimental studies on epistasis between deleterious mutations have been based on indirect estimates of the individual mutation load, by assuming a relationship between mutation number and some other experimental variable that can be known more accurately. Examples of such assumptions include a linear relationship between mutation number and the period of mutation accumulation (Mukai 1969), a linear relationship between mutation number affecting fitness and inbreeding coefficient (Willis 1993, and references therein), a rank-order relationship between mutation number and fitness (de Visser et al. 1996), and a symmetrical distribution of individual mutation number among the offspring from a cross (de Visser et al. 1997). Assumptions like these are likely to be a source of error and may, therefore, obscure the conclusions from such studies on the form of the relationship between mutation number and fitness.

In this paper, we report on direct measurements of fitness interactions between deleterious mutations. We constructed strains of the filamentous fungus *Aspergillus niger* by isolating haploid segregants from a diploid between a wild-type strain and a strain with a marker mutation on each of its eight chromosomes. Since both strains share a recent common ancestor, they are presumed to have a common genetic background and to vary only with respect to the marker mutations. The strain carrying the marker mutations was more or less arbitrarily chosen, the only selection criterion being that the markers could be accurately tested (e.g., that are not "leaky"). The markers involved are two resistances and five auxotrophies. As a component of fitness, we measured the mycelial growth rate of all strains on supplemented medium to reduce the severe fitness effects of the auxotrophic mutations. Consequently, a marker mutation that causes a fitness decrease can be interpreted as a deleterious mutation with small effect in the relevant part of the underlying metabolism. For instance, an auxotrophic mutation with decreased fitness on supplemented medium relative to the wild type can be interpreted as a deleterious mutation in the uptake system of the nutrient involved.

MATERIALS AND METHODS

Parental Strains

Strain N411 and N890 of *A. niger* were used as parental strains for the construction of the multiple-marker strains. The progenitor of both strains is a mutant with short conidiophores (*cspA1*, strain N402) of wild-type strain N400 (ATCC 9029). Strain N411 carries one marker mutation on chromosome 1, which causes olive instead of fawn-colored spores (*olvA1*) and that has been induced by a low dose of UV to limit multiple hits (Bos 1987). We used N411 instead

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of wild-type strain N402 (which produces black-colored spores) as one of the parental strains, because it facilitated the isolation of haploid segregants from the diploid mycelium, which forms black spores due to complementation. Strain N890 carries eight marker mutations, one on each of its eight chromosomes. It has been constructed by a combination of low-dose UV induction of strain N402 (one induction for each marker) and mitotic recombination by inducing segregation of spontaneously arising diploid conidiospores isolated from a heterokaryon (e.g., Debets et al. 1993). The construction history of strain N890 is given by Bos et al. (1993). The eight marker mutations of strain N890 are (on increasing chromosome number): a color mutation, *fwnA1* (fawn-colored conidiospores); five auxotrophic markers, *argH12* (arginine deficiency), *pyrA5* (pyrimidine deficiency), *leuA1* (leucine deficiency), *pheA1* (phenyl-alanine deficiency), and *lysD25* (lysine deficiency); and two resistances *oliC2*, (oligomycin resistance) and *crnB12* (chlorate resistance).

Media

The basic medium was a minimal medium (MM), containing 8.5 g sucrose; 6 g $NaNO_3$; 1.5 g KH_2PO_4 and K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g KCl; about 1 mg of the elements $FeSO_4$, $ZnSO_4$, $MnCl_2$, $CuSO_4$, and 15 g agar per liter; pH = 5.8. Isolation of the mutants and the fitness assays were performed on this medium, supplemented (MMsup) with 348 mg arginine, 244 mg uridine, 197 mg leucine, 165mg phenylalanine, 365 mg lysine, and 200 mg ureum per liter. For testing the genotype of haploid segregants, 200 mM $KClO_3$ and 20 mM ureum were added to MM to test for *crnB12*, and 1 mM oligomycin to test for *oliC2*.

Construction of Multiple Marker Strains

Although *A. niger* is an asexual fungus, segregants of the two parental strains can be obtained by forced haploidization of heterozygous diploids that arise spontaneously in a heterokaryon (Pontecorvo et al. 1953). In this way, from a diploid of strain N411 and N890, theoretically $2^8 = 256$ different haploid segregants can be obtained with all possible combinations of the parental markers. Methods for obtaining heterokaryons, isolating diploids, haploidizing diploids, and isolating haploid segregants have been described by Bos et al. (1988). Mitotic crossing over is known to occur in *A. niger* with a frequency lower than 4% per chromosome (Debets et al. 1993). Given the low doses of UV used for the generation of mutants, most crossing-over events will happen between identical chromosomes and thus will not result in genetic recombination. Therefore, it is unlikely that the segregants we isolated differ at more loci than those carrying the marker mutations.

About 2500 segregants were isolated, purified by transferring spores from single spore heads and tested for marker genotype on eight selective media: MMsup (control); MMsup in which serially arginine, lysine, leucine, phenyl-alanine, and uridine were left out; MMsup with $KClO_3$ and ureum; and MMsup with oligomycin. It appeared difficult to isolate segregants with many marker mutations, due to poor growth. We isolated 186 of all 256 possible multiple-marker strains (73%).

Fitness Assay

The mycelial growth rate on supplemented medium was measured as a component of the fitness of each strain. Since the rate of spore production, rather than the growth rate of the mycelium, is a fitness-related trait, we estimated the correlation between colony surface area and the total number of spores of that colony in a pilot experiment. The correlation between colony surface area and total spore number was measured for five multiple-marker strains that vary in marker number from one to eight. Each strain was grown on 10 plates that were placed at 5°C after varying periods of growth. All spores were harvested and suspended into 20 mL of saline and their total number was estimated from a sample (0.5 mL) with the aid of a Coulter counter (Coulter ZF6 system, Coulter Electronics, Ltd.). Within strains the correlation between colony surface area and spore number was highly significant in all cases (at least, $\rho = 0.95$, $n = 10$, $P < 0.001$). The overall correlation for the five strains, by lumping all data, was slightly lower but still highly significant ($\rho = 0.87$, $n = 43$, $P < 0.001$). Thus, the mycelial growth rate, expressed as the increase of mycelial surface area per unit time, seems a good predictor of the rate of spore production and hence fitness.

The medium for this assay (MMsup) was prepared in a single batch. Petri dishes (9-cm diameter) were filled with 20 mL of medium with the aid of a calibrated pump. Each of the 186 isolated strains was inoculated on two replica plates by pricking a platinum needle with spores in the middle of the plate. Plates were randomized and put in the dark at 26°C. The colony diameter was measured in two perpendicular directions after three days and again after 12 days. The colony surface area at both time points was estimated as a circle, and the difference divided by the 234 hours of growth.

Statistical Analyses

Due to infection or breakage of cultures, five individual estimates of the mycelial growth rate have been omitted from the analysis. Where the mean fitness of genotypes is used in the analysis, the value of the remaining of the two replicates was used for these genotypes.

In the analysis, the logarithm of the fitness relative to the wild type carrying the same spore-color marker was used. The form of the relationship between marker number and log fitness was tested by comparing the fit of a linear model and of a model that includes a quadratic term with the log mean fitness of each strain. The fit of both models was compared with a partial F-test (Kleinbaum and Kupper 1978, pp. 118–119). To exploit the potential power of our large dataset, we also used Tukey's jackknife procedure (Sokal and Rohlf 1981) to generate confidence limits for the coefficient of the linear (α) and quadratic (β) term in the respective models. This procedure yielded 363 (184 strains [all but the two wild-type strains] $\times$ two replicates—five missing estimates due to infection or breakage) estimates for both coefficients for the total dataset, and 62 (31 strains $\times$ two replicates) esti-

TABLE 1. Numbers of expected and isolated multiple-marker strains from parental strains N411 and N890, classified into categories with equal mutation number.

Marker number	Number of strains expected	Number of strains isolated
0	2	2 (100%)
1	14	13 (93%)
2	42	36 (86%)
3	70	51 (73%)
4	70	46 (66%)
5	42	31 (74%)
6	14	7 (50%)
7	2	0 (0%)
Total	256	186 (73%)

mates for the two "complete subsets" of 32 strains (see results).

Five-way analysis of variance was performed on the two complete subsets of strains to estimate the relative contribution of single markers and their interactions to the total variation in log fitness observed. For this purpose, a Model I ANOVA was applied assuming fixed effects of the markers (Sokal and Rohlf 1981). We could not accurately test the homogeneity of variances, because only two replicates were used. However, the absence of a correlation between mean and variance of the fitness estimates of the two replicates ($\rho = -0.101$, df = 181, $P > 0.05$) suggests that there is no strong dependence of the error variance on the mean value.

As a method to correct for multiple comparisons, we used the sequential Bonferroni technique, which has been explained by Rice (1988).

RESULTS

Number and Genotype of Strains Isolated

We isolated 186 of all 256 possible multiple-marker strains (73%). As can be seen in Table 1, the fraction of all strains that was isolated decreased with increasing marker number. This was probably caused by the small size and poor sporulation of strains with many markers, which made them very inconspicuous among other more fit haploid segregant sectors and the diploid parental mycelium. It is therefore likely that of the strains with many markers, we isolated only those with a relatively high fitness. Consequently, the dataset as a whole may be biased toward a convex (i.e., antagonistic) relationship between marker number and *log* fitness, and therefore does not represent a random sample of all possible haploid segregants of the diploid we used.

In Table 2, the number of strains isolated with a specific marker, and with a specific combination of two markers is given. A relatively low number of strains carried the *lys* marker (significantly less than 50%: $\chi^2 = 15.7$, df = 1, $P < 0.001$), while *crn* was found in more than 50% of the strains ($\chi^2 = 6.2$, df = 1, $P < 0.05$). Furthermore, the combination of *lys* and *crn* was found in more strains than expected from the frequency of the individual marker mutations ($\chi^2 = 4.7$, df = 1, $P < 0.05$).

TABLE 2. The isolated number of strains with a specific marker (in bold on diagonal), or combination of marker mutations.

	fwn	*olv*	*arg*	*pyr*	*leu*	*phe*	*lys*	*oli*	*crn*
fwn	**95**								
olv	—	**91**							
arg	47	43	**90**						
pyr	47	46	45	**93**					
leu	42	46	43	42	**88**				
phe	49	41	45	44	43	**90**			
lys	35	31	31	30	28	33	**66**		
oli	41	41	40	30	28	33	22	**82**	
crn	56	54	53	56	53	52	51	49	**110**

Individual Marker Effect

The effect of individual markers, measured relative to the wild-type strain with the same color marker are give in Table 3. Due to lack of power by having only two replicates, none of the individual selection coefficients are significantly different from zero with a *t*-test if corrected for multiple comparisons. However, the suggestion is raised that the individual marker effects depend on the background color marker. In the *fwn* background, the mean fitness effect of single markers is positive, while in the *olv* background the effect is deleterious. The reason for the differential effect of the background marker is unclear. When averaged over the two backgrounds, the mean fitness effect is deleterious, although not significantly ($t = 1.865$, df = 27, $P = 0.07302$). Of all markers *oli* seems to have the strongest deleterious effect on fitness, which is consistent with the fact that a relatively low number of strains carrying the *oli* marker could be isolated (see Table 2).

Relationship between Marker Number and Log Fitness

Figure 1 shows the natural logarithm of the mean relative fitness versus number of mutations for all 186 strains that could be isolated. A linear model describing the decline of *ln* fitness with number of mutations can explain most of the variation, if fitted to the *ln* mean fitness of all 186 strain ($r^2 = 0.591$, the linear coefficient, α, being -0.08944). If a quadratic term is added to the model, its coefficient is estimated to be positive ($\beta = 0.00634$), suggesting an antagonistic relationship between marker number and *ln* fitness, but the quadratic term does not improve the fit of the model significantly (partial *F*-test: $F_{1,184} = 1.602$, $P = 0.207$). Also, the jackknifed estimate of coefficient α estimated with a linear model is -0.07860, which is highly significant (SE = 0.00499, $t_{362} = 15.765$, $P < 0.0001$), while the jackknifed estimate of the quadratic term, β, is 0.00362, which is not significantly different from zero (SE = 0.00387, $t_{362} = 0.934$, $P = 0.175$). Therefore, the decrease of *ln* mean fitness is best described by a linear model.

However, as stated above, analysis of the whole dataset is likely to bias the relationship between marker number and *ln* fitness toward antagonism. In an attempt to overcome this bias, we extracted two sets of 32 strains from the 186 strains that comprise a complete subset of five markers, that is, a set of strains in which all possible combinations of the five markers involved are present. Thus, a complete subset of strains involving five markers includes the wild-type strain,

TABLE 3. Selection coefficients of individual markers, given separately for the two background color markers (*fwn* and *olv*). The values given separately for the two backgrounds are the mean of two replicates; no data are available for *lys* in the *olv* background and only one replicate for *leu* in the *fwn* background. Between brackets are standard errors. * $P < 0.05$, ** $P < 0.01$ (*t*-test).

Marker	*s* in *fwn* background	*s* in *olv* background	Mean *s*
arg	+0.1060 (0.0114)	−0.2272 (0.0681)	−0.0606 (0.1002)
pyr	+0.0189 (0.0142)	−0.3024 (0.0544)	−0.1417 (0.0955)
leu	+0.1056	−0.2432 (0.0243)	−0.1269 (0.1171)
phe	+0.2959 (0.0319)	−0.1737 (0.0553)	+0.0611 (0.1380)
lys	−0.0055 (0.0276)		
oli	−0.0445 (0.0177)	−0.4018 (0.0639)	−0.2232 (0.1066)
crn	+0.1935 (0.0634)	−0.3716 (0.0095)	−0.0891 (0.1652)
Mean	+0.0949* (0.0344)	−0.2866** (0.0287)	−0.0882 (0.0447)

the five strains with a single marker, the 10 strains with two, the 10 strains with three, the five strains with four, and the single strain with five markers. The only two complete subsets with five markers in the total set of 186 strains are a set including all possible combinations of the markers *arg*, *pyr*, *leu*, *oli*, and *crn*, further referred to as Complete Subset I (CSI), and a set including the markers *arg*, *pyr*, *leu*, *phe*, and *oli*, further referred to as Complete Subset II (CSII).

Figure 2 gives *ln* fitness of the two complete subsets of strains as a function of the number of markers. For both sets a linear model describing the decline of *ln* mean fitness of each strain involved can explain most of the variation in ln fitness (r^2 = 0.888 and 0.881, and α = -0.19157 and −0.18052 for CSI and CSII, respectively). The quadratic term, if added to the model, is estimated to have a positive coefficient for both subsets (β = 0.01742 in CSI, and β = 0.00845 in CSII), suggesting antagonism, but again adding a quadratic term does not improve the fit of the model significantly (CSI: $F_{1,30}$ = 1.862, P = 0.183; CSII: $F_{1,30}$ = 0.4390, P = 0.513). Similarly, the jackknifed estimates of coefficient α of a linear model were highly significant (CSI: α = −0.18045, SE = 0.01025, t_{61} = 17.613, $P < 0.0001$; CSII: α = −0.18205, SE = 0.00644, t_{61} = 28.274, $P < 0.0001$), while the jackknifed estimates of β are not significantly different from zero, if a sequential Bonferroni correction is applied for multiple comparisons (CSI: β = 0.01440, SE = 0.00765, t_{61} = 1.882, P = 0.0323; CSII: β = 0.00081, SE = 0.00452, t_{61} = 0.179, P = 0.429). Thus, an attempt to overcome the presumed isolation bias present in the total set of strains by restricting the analysis to two complete subsets of strains with five markers could also not reveal any significant marker interaction.

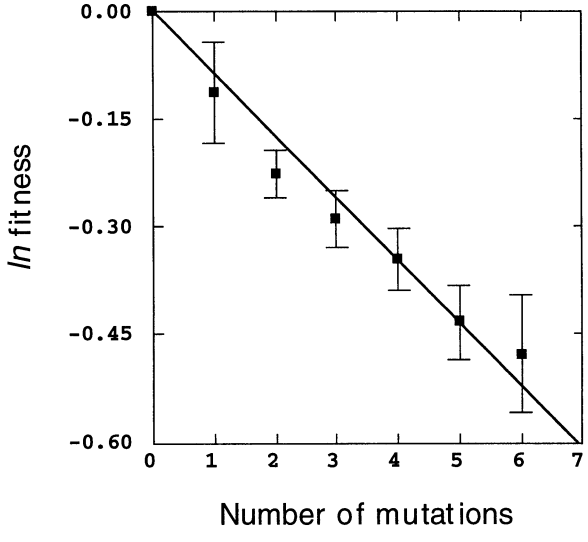

FIG. 1. The observed relationship between the number of marker mutations and the natural logarithm of fitness for all 186 strains that were isolated from a diploid of strain N411 and N890. Each value is the mean of a varying number of strains (see Table 1); error bars represent the standard error of the mean. The straight line shows the best fitting linear model (with coefficient −0.08944), which cannot statistically be rejected by a model including a quadratic term (see text).

Analysis of Variance in Complete Subsets of Strains

The absence of a prevalent type of marker interaction may either mean that none of the marker combinations show interaction, or that interactions of opposite type, that is, synergistic and antagonistic, (partly) cancel each other's effect. To investigate whether marker interactions occur at all, a five-way ANOVA was performed on the *ln* fitness of each of the two complete subsets of strains with five markers. As can be seen in Table 4, most variation is attributable to genetic differences between the 32 strains involved in the two subsets, that is, 93.7% in CSI and 93.4% in CSII, while most of the variation explained by genetic factors is due to the effect of single markers. However, the effect of the different orders of marker interaction are still highly significant in CSI as well. In CSII, the total marker interaction is marginally significant, which does not hold after a sequential Bonferroni correction for multiple comparisons. Thus, the *oli* marker appears to have a rather large impact on the overall occurrence of interactions. In the light of the absence of a significant type of epistasis, the significance of marker interactions found in the ANOVA suggests that interactions of opposite type occur that cancel each other's effect.

Interaction between Pairs of Markers

To further investigate the occurrence of opposite interactions, the sign of the interaction between pairs of markers was tested. This was done by comparing the observed fitness of the genotype carrying both markers with the expected fitness based on the fitness of the two genotypes bearing the single marker, assuming independent effects (i.e., additivity on the log-scale). Table 5 gives the results of this analysis for those marker combinations that show a significant neg-

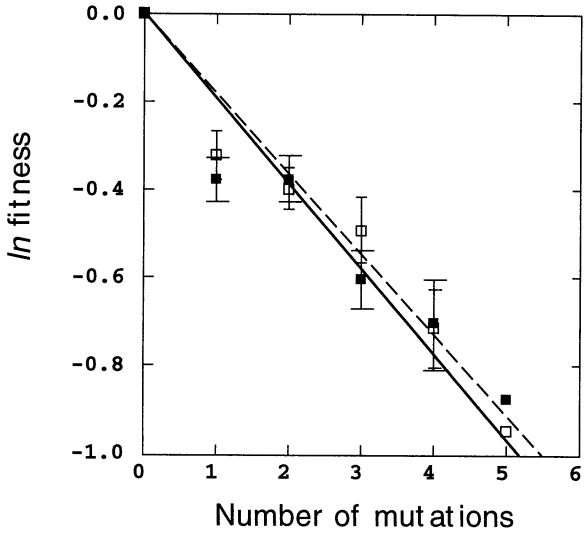

FIG. 2. The observed relationship between the number of mutations and the natural logarithm of fitness for two complete subsets of strains (see text) that represent all possible marker combinations of five markers. Solid squares and solid line represent all possible marker combinations of the markers *arg*, *pyr*, *leu*, *oli*, and *crn* (CSI); open squares and dashed line are for the complete subset *arg*, *pyr*, *leu*, *crn*, and *phe* (CSII). Each value is the mean of a varying number of strains: five for the single mutations, 10 for the double and triple mutations, five for the quadruple mutations, and one for the quintuple mutations; error bars represent the standard error of the mean. The solid and dashed lines represent the best fitting linear models (with coefficient $\alpha = -0.19157$ for CSI and $\alpha = -0.18052$ for CSII). A model including a quadratic term cannot significantly improve the fit (see text).

ative regression of *ln* fitness with marker number. Of the 28 combinations of two markers for which a complete dataset was available (i.e., both replicate fitness estimates for wild-type, the two single-marker, and the one double-marker strain), 18 show a decline in *ln* fitness with marker number, which is significant for only 14 combinations. As expected, both synergistic and antagonistic interactions occur, although the majority shows a tendency toward antagonism, while only one antagonistic (*oli* and *crn* in *olv* background) combination is significant after correcting for multiple comparisons. Also, the data suggest that the color marker affects the sign of interaction: although most do not show a significant decrease of *ln* fitness with marker number, the marker combinations in the *fwn* background all had a lower than expected *ln* fitness of the double mutant, suggesting synergism, while all combinations in the *olv* background interacted antagonistically. The reason for this differential effect of the color marker is unknown.

TABLE 5. The analysis of marker interaction between pairs of markers. Only marker combinations that show a deleterious effect, that is, that show a significant decrease of *ln* fitness when linearly regressed to marker number are given. The background color marker is given between brackets. $lnW_o - lnW_e$ = difference between observed and expected (i.e., multiplicative) *ln* fitness of genotype bearing both markers; negative values indicate synergism, positive values antagonism. $F_{1,4} = F$ corresponding to interaction term of two-way ANOVA. * = significant after sequential Bonferroni correction for multiple comparisons, using $\alpha = 0.05$.

Marker combination	Linear regression coefficient α	P	Interaction $lnW_o - lnW_e$	$F_{1,4}$	P
(*fwn*) *pyr, oli*	−0.130	0.00624	−0.351	24.797	0.00760
(*olv*) *arg, pyr*	−0.201	0.00102	+0.333	6.843	0.05905
(*olv*) *arg, leu*	−0.195	0.00016	+0.224	3.859	0.12093
(*olv*) *arg, phe*	−0.129	0.00630	+0.294	5.453	0.07981
(*olv*) *arg, oli*	−0.302	0.00023	+0.265	2.977	0.15953
(*olv*) *arg, crn*	−0.211	0.00331	+0.453	21.140	0.01005
(*olv*) *pyr, leu*	−0.242	0.00005	+0.234	5.614	0.07687
(*olv*) *pyr, phe*	−0.199	0.00053	+0.235	4.307	0.10657
(*olv*) *pyr, oli*	−0.370	0.00001	+0.212	2.310	0.20318
(*olv*) *pyr, crn*	−0.247	0.00126	+0.497	32.128	0.00478
(*olv*) *leu, phe*	−0.183	0.00006	+0.158	3.586	0.13120
(*olv*) *leu, oli*	−0.301	0.00017	+0.294	5.931	0.07157
(*olv*) *leu, crn*	−0.256	0.00026	+0.346	39.199*	0.00332
(*olv*) *phe, oli*	−0.280	0.00050	+0.227	2.772	0.17126

DISCUSSION

In this paper, we have studied whether and how mutations interact to affect fitness, which may have important consequences for understanding the maintenance of sexual reproduction (Kondrashov 1988). Our approach has been to measure a component of the fitness, that is, the mycelial growth rate, of strains of the filamentous fungus *A. niger* that carry a varying number of marker mutations from an arbitrarily chosen set of markers. Since the marker set included auxotrophic mutations, fitness of all strains was measured on a supplemented medium. The results suggested independent effects of the marker mutations involved, and therefore do not support the requirement of the deterministic mutation

TABLE 4. ANOVA results of the two complete subsets of strains with five markers. CSI: *arg, pyr, leu, oli,* and *crn*; CSII: *arg, pyr, leu, crn,* and *phe*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Source	df	CSI		CSII	
		MS	Explained variance	MS	Explained variance
Single markers	5	0.52896****	66.6%	0.65678****	83.0%
Interactions	26	0.04136****	27.1%	0.01588*	10.4%
Two markers	10	0.03860***		0.01164 (ns)	
Three markers	10	0.03574***		0.01924*	
Four markers	5	0.03879**		0.02067*	
Five markers	1	0.13786***		0.00064 (ns)	
Error	32	0.00784	6.3%	0.00811	6.6%

hypothesis (Crow 1970; Kondrashov 1982; Charlesworth 1990) that deleterious mutations interact synergistically.

The model system we used has at least three merits relative to existing studies on the nature of epistasis. First, the genotype of the mutants is directly known from testing on selective media. Thus, the error stemming from misinterpreting individual mutation numbers is negligible. Other studies on the nature of epistasis have always relied on certain assumptions to infer the number of deleterious mutations carried by individuals, which may obscure the conclusions on the relationship between mutation number and fitness (Mukai 1969; Willis 1993; de Visser et al. 1996, 1997). Second, the high fraction of the total fitness variance that could be explained by genetic differences between mutants (more than 93%) suggests that the combination of the marker mutations and the fitness assay we used provides a rather high degree of resolution. Third, exact knowledge of the genotypes of the mutants that were used allowed marker interaction to be studied by the powerful statistical tool of the analysis of variance. As a consequence, we could estimate the impact of marker interactions irrespective of their sign.

The results warrant at least two conclusions. First, the results show a linear relationship between the number of deleterious mutations and the logarithm of the mycelial growth rate, suggesting independent mutation effects. This conclusion is, however, limited by the fact that our isolation method favored antagonistic combinations of deleterious mutations, particularly when many mutations are involved. Consistent with the significance of an isolation bias in our data are: (1) the decrease of the number of strains that could be isolated with an increasing number of marker mutations; (2) the fact that the only two markers with a negative fitness effect in the *fwn* background, *oli* and *lys*, are also the markers that were least frequently found in the strains that were isolated; and (3) the observation that the relatively rare *oli* marker also seems to be more involved in synergistic interactions (see Table 5). Our attempt to overcome this isolation bias by studying a particular combination of five markers for which all 32 possible subcombinations were available possibly suffers from the same drawback, since a complete subset also has a higher probability to be isolated if the fitnesses of its strains with many markers are not too low, which is the case if they interact antagonistically. Therefore, it is unclear how generally valid our conclusion is that marker mutations have independent effects on fitness. However, in a number of studies on the mycelial growth rate in a variety of fungal species it was found that the alleles that segregated in the crosses had mainly additive effect if the parents involved were not of too divergent origin (Simchen 1966; Caten 1979 and references therein). Since these studies used a linear measure of the mycelial growth rate, their finding of additivity on the linear growth rate is consistent with our finding of additive marker effects on the natural logarithm of the mycelial surface area growth rate. The observation that in the unicellular alga *Chlamydomonas moewussii* naturally accumulated deleterious mutations show independent effects on the maximum growth rate (de Visser et al. 1997) seems also to be consistent with our conclusion for the mycelial growth rate.

Second, the apparent pattern of multiplicative mutation effects on fitness is not the result of a general absence of interactions, but rather of interactions of both type, synergistic and antagonistic, occurring simultaneously and (partly) cancelling each other's effect. Many models in population genetics assume independent action of the genes involved. Our results apparently do not support this assumption for a small number of genes, but the results also suggest that the assumption seems reasonable if the effect of many genes is considered. The mutual masking of interactions between pairs of mutations is not understood, but it seems a quality of the underlying metabolism.

The generality of our conclusions depends on the generality of the metabolic mechanisms that are affected by the marker mutations. Only if these mechanisms are general, recurring slightly deleterious mutations will experience a similar type of epistasis. Of the marker mutations we used, *oli* is the only marker whose negative fitness effect can be directly understood at the metabolic level: *oli* mutants suffer a defective energy transduction due to a malfunctioning subunit of the enzyme ATPase (Edwards and Unger 1980). A deleterious effect of each of the five auxotrophic mutations (*arg*, *pyr*, *leu*, *lys*, and *phe*) on supplemented medium might be interpreted as a deleterious mutation in the uptake system of one of the substrates involved in its synthesis. In *Escherichia coli*, transport enzymes of nutrients that are regularly growth limiting are thought to be the major targets of selection (Dykhuizen and Dean 1990; Travisano et al. 1995). Since most active uptake systems are organized similarly, interactions between the five auxotrophic mutations may be relevant for the fitness of *Aspergillus* under growth limiting conditions. The *crn* marker of *A. niger* that we used is probably similar to the *crnA* marker in *A. nidulans* (Brownlee and Arst 1983), which causes a nitrate permease with a lower affinity for chlorate, and in young mycelium also for nitrate. Therefore, *crn* can be considered as a "leaky" auxotrophic mutation. Its effect on fitness was not strong (which is consistent with its high occurrence among the strains we isolated), which is possibly due to the compensatory uptake of ureum. The seven marker mutations used in this study thus are involved in different metabolic pathways, since even the amino acids involved in the auxotrophies do at least not share their immediate precursors. Consequently, our conclusions seem relevant only for interaction between deleterious mutations occurring in different metabolic routes.

Szathmáry (1993) studied the theoretical nature of epistasis between a pair of deleterious mutations that either affect the same or two different enzymes in a single metabolic pathway. He predicted synergism if two mutations affected the activity of the same enzyme and antagonism if two enzymes were affected and selection was favoring a maximum flux through the (growth limiting) pathway. The latter situation seems relevant for the mycelial growth rate as well, but since the mutations we studied relate to different metabolic routes, their interaction obviously cannot be explained by Szathmáry's model. Predictions from metabolic control theory on the interaction between mutations involved in a network of different metabolic pathways are needed for that purpose.

Whether the lack of support for synergistic epistasis in *A. niger* generated by the present study should disqualify the deterministic mutation hypothesis as a major explanation for the evolution and maintenance of sex is unclear. Other studies

have produced empirical support for the existence of synergistic epistasis between deleterious alleles affecting certain fitness components in a variety of organisms (Mukai 1969; Malmberg 1977; Willis 1993; de Visser et al. 1996, 1997). An interesting direct test would be to repeat the experiment described in this study with the related sexual fungus *A. nidulans*. Clearly, more data on the nature of epistasis for different organisms and, preferably, with respect to total fitness instead of fitness components are needed.

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