doi:10.1111/j.1420-9101.2005.00934.x

Comparing artificial and natural selection in rate of adaptation to genetic stress in *Aspergillus nidulans*

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Keywords:

artificial selection;
experimental evolution;
fungi;
genetic stress;
natural selection.

Abstract

In an experimental study of adaptation to negative pleiotropic effects of a major fungicide resistance mutation in the filamentous fungus *Aspergillus nidulans* we have investigated the relative effectiveness of artificial selection vs. natural selection on the rate of compensatory evolution. Using mycelial growth rate as a fitness measure, artificial selection involved the weekly transfer of the fastest growing sector onto a fresh plate. Natural selection was approximated by transferring random samples of all the spores produced by the mycelium. Fungicide resistant and fungicide sensitive haploid and diploid strains were used in an evolution experiment over 10 weekly transfers, which is equivalent to 1200 cell cycles. Two different environmental conditions were applied: a constant fungicide-free environment and a weekly alternation between presence and absence of fungicide. Results show that for all strains and conditions used the transfer of a random sample of all spores leads to more rapid adaptation than the transfer of the visually 'fittest' sector. The rates of compensatory evolution in the constant and the alternating environment did not differ. Moreover, haploid strains tend to have a higher rate of adaptation than isogenic diploid strains.

Introduction

Antibiotic resistance easily develops in microorganisms by mutation. Typically, such resistance mutations display deleterious pleiotropic effects, which become apparent in an antibiotic-free environment (Andersson & Levin, 1999; Reynolds, 2000). These negative side-effects can be viewed as genetically induced stress. Adaptation to such stresses occurs by subsequent compensatory evolution reducing resistance associated fitness costs. Compensatory evolution has been studied experimentally, initially using mainly bacterial systems (Andersson & Levin, 1999; Elena & Lenski, 1997). The process of fitness compensation has been shown to involve second site mutations rather than reversion to antibiotic sensitivity, resulting in resistant strains not only growing well in the presence of the antibiotic but also in its absence (Bouma & Lenski, 1988; Schrag & Perrot, 1996; Moore *et al.*, 2000; Reynolds, 2000).

We recently studied fungicide resistance development and subsequent compensatory evolution in the filamentous fungus *Aspergillus nidulans* (S. E. Schoustra, A. J. M. Debets, S. M. Slakhorst & R. F. Hoekstra, unpublished). In that study, one mutant resistant to the fungicide fludioxonil with associated fitness costs was selected for an experimental study of compensatory evolution over 3000 cell cycles where 40 parallel strains of this mutant evolved under three different environmental conditions. In that study we defined fitness as the mycelial growth rate (MGR). An adaptive mutation occurring in a nucleus in growing mycelium can be recognized as a sector in the mycelium with an enhanced growth rate (Fig. 1). Strains were cultured by weekly transferring a fraction of the mycelium most distant to the point of inoculation onto fresh medium. We hypothesized that this artificial

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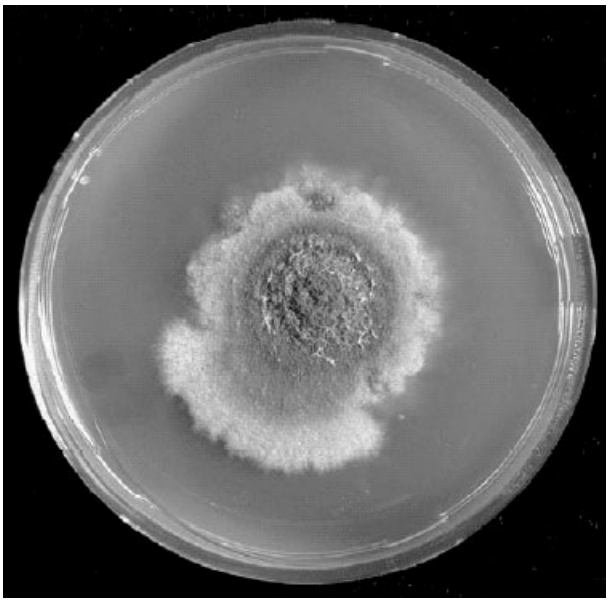

Fig. 1 An *Aspergillus nidulans* colony (WG561) after 4 days of growth showing a sector with enhanced growth rate. The formation of a colony starts after germination of an asexually or sexually derived spore or from mycelium containing at least one nucleus. A dense network of hyphae develops by expansion of mitotically active apical cells that contain up to 40 nuclei (Kaminskyj & Hamer, 1998). Twenty-four hours after the formation of a hypha, the asexual cycle has completed with the formation of spores on conidiophores (Fig. 2) (Timberlake, 1990; Adams *et al.*, 1998). The sexual cycle commences around 3 days after termination of the asexual cycle. The production of sexual spores results in fruiting bodies (cleistothethia), each containing up to 100 000 spores (Adams *et al.*, 1998; Casselton & Zolan, 2002).

selection procedure would maximize the chance of an adaptive mutation to be selected. Our results showed that in all environmental conditions used the mutant strain was able to restore fitness. Genetic analyses of crosses between different evolved stains and between evolved and ancestral strains showed that a limited number of one, two or three major loci were involved in fitness compensation and revealed interaction between compensatory mutations.

In the present study we used fungicide resistant *A. nidulans* strains to investigate the relative effectiveness of two modes of selection on the rate of compensatory evolution. Artificial selection by transplanting the 'fittest' sector of a colony to fresh medium at certain time intervals was compared with natural selection, which was approximated by transfer of random samples of 0.1% of all the spores of the fungal mycelium. *Aspergillus nidulans* mycelium produces numerous asexual and sexual spores (up to 10^9 on the surface of a colony the size of a Petri Dish). Under natural conditions their dispersal and subsequent outgrowth is likely to be of much greater importance than mycelial extension, as most mycelial colonies tend to be short-lived due to rapid exhaustion of the substrate. However, we hypothesize that this random selection procedure decreases the chance of an adaptive mutation to be directly selected in an evolution experiment, hence that the direct selection of adaptive mutations by selection of the 'fittest' sector, as practised in our previous study (S. E. Schoustra, A. J. M. Debets, S. M. Slakhorst & R. F. Hoekstra, unpublished) overestimates the rate of compensatory evolution in nature.

The genetics of *A. nidulans* are well studied (Pontecorvo *et al.*, 1953; Clutterbuck, 1974; Timberlake, 1990; Adams *et al.*, 1998; Swart & Debets, 2004) and this fungus is a suitable model system to experimentally address evolutionary questions. It combines several of the characteristics of unicellular microorganisms used in experimental evolution (Elena & Lenski, 2003) with additional advantages of multicellularity in a somatic structure of growing mycelium containing nuclei in spatially defined positions. The MGR is an easily to assess and highly reproducible fitness measure (Pringle & Taylor, 2002; Bruggeman *et al.*, 2003). The number of cell cycles (successive nuclear mitotic divisions) in the growing front of the mycelium is up to 112 per week (Bergen & Morris, 1983; Kaminskyj & Hamer, 1998) and the parasexual and sexual cycle is of use for genetic analysis (Kafer, 1958; Clutterbuck, 1974). Samples taken at different time points can be stored in a nonevolving state at −80 °C and later used for comparative analysis. Vegetatively growing isogenic haploid and diploid strains can be used (Pontecorvo *et al.*, 1953; Kafer, 1958).

We have studied the effect of artificial and natural selection in an evolution experiment over 10 weekly transfers (equivalent to 1200 cell cycles for the growing front of the colony) in different situations. Fungicide resistant and fungicide sensitive haploid and diploid strains were used growing in the absence of fungicide. Because of their cost of resistance, resistant strains are more distant from a fitness optimum than the fungicide sensitive strains. Therefore, mutations with large effect are more likely to be adaptive in resistant than in sensitive strains that are on or around a fitness optimum (Fisher, 1930). For the resistant strains, we expect the transfer of the mycelial sector directly pointing to an adaptive mutation to produce a faster rate of compensatory evolution than by the transfer of a random spore sample. For the sensitive strains, where adaptive mutations will have much smaller effects and therefore are likely to escape visual detection, we expect the transfer of random spore samples to be more successful.

We used isogenic haploid and diploid strains to assess the influence of ploidy on the rate of adaptation in the two modes of selection. *A priori* we do not know what outcome to expect comparing evolving haploid and diploid strains. There are physiological differences between haploid and diploid strains. Diploid strains only produce around 25% of the number of spores haploids produce (see Methods), however, diploid hyphae expand around 5% more rapidly

than haploid hyphae (S.E. Schoustra, unpublished observation). In diploid strains, the phenotypic effect of recessive adaptive mutations can be masked, but successful combinations of adaptive mutations may be formed after haploidization due to chromosome loss by repeated nondisjunction or mitotic recombination of a diploid (Pontecorvo & Kafer, 1958).

Compensatory evolution in fungicide resistant haploid and diploid strains was compared in two different environments: a constant fungicide-free environment and the alternating absence and presence of fungicide. In the alternating environment we expect the random spore sample propagation to lead to a higher rate of adaptation than the mycelial sector propagation, as the conditions after a transfer are different from the conditions where selection took place.

Methods

Strains

Aspergillus nidulans strains used were derived from the original Glasgow strains (Clutterbuck, 1974). Haploid and diploid strains both sensitive and resistant to the fungicide fludioxonil [4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril] were used. WG562 (*lys*B5) and WG562//145 (*lys*B5 // *w*A3, *pyro*A4) were sensitive. WG561 (*fld*A1, *lys*B5) and WG561//615 (*fld*A1, *lys*B5 // *w*A3, *fld*A1, *pyro*A4) were resistant to the fungicide fludioxonil and had associated fitness costs.

Media and culturing

Strains were cultured in Petri dishes with solid Minimal Medium (MM) or Complete Medium (CM). MM consists of 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 mg of $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$ and agar 15 g + 1000 mL H_2O (pH 5.8). For CM, 2.0 g neopeptone, 1.0 g vitamin assay casamino acids, 1.0 g yeast extract and 0.3 g ribonucleic acids for yeast was added to MM. After sterilization of the media, sucrose was added to a final concentration of 25 mmol L^{-1} to both media. CM was supplemented with a vitamin solution after sterilization (2 mL solution/1000 mL medium) containing thiamin 100 mg L^{-1}, riboflavin-Na 1.25 g L^{-1}, para-aminobenzoicacid 100 mg L^{-1}, nicotinamide 1.0 g L^{-1}, pyridoxin-HCl 500 mg L^{-1}, D-pantothenic acid 100 mg L^{-1} and biotin 2.0 mg L^{-1}. MM always was supplemented with lysine to a final concentration of 2.0 mmol L^{-1} in the medium.

Spore suspensions were made in saline (distilled water with NaCl 0.8 g L^{-1}) supplemented with Tween80 (0.01%) by washing off all spores from the surface of all mycelium on a plate with 5 mL saline-tween. Agar plates were inoculated in the centre either with an inoculation needle or with 5 μL of spore suspension. Plates were always incubated at 37 °C. Two different growth conditions were used: fludioxonil-free MM and MM with weekly alternating with or without fludioxonil (Novartis, Basel, Switzerland; 0.2 ppm). For the latter condition, only the resistant haploid and diploid strains were used. Ploidy levels of all initially diploid strains were assessed after the last transfer by growing them on CM with benomyl 1.7 ppm (Kafer, 1958). Maintenance of resistance was assessed by growing all initially resistant strains on MM with fludioxonil (0.2 ppm).

Replication and propagation

Per strain and condition, 12 replicates of the same strain were used. After incubation of 6 days, all plates were placed at 4 °C for 24 h after which strains were transferred to fresh medium using two ways of transfer. The first way was by transfer of a part of mycelium from the growing front most distant to the point of inoculation; in this way 10 000–50 000 nuclei are transferred. The number of nuclei transferred was measured by a plate count of a suspended inoculum in saline-tween [diploids: mean ($n = 4$) = 4.6×10^4, standard error of the mean – SEM = 4.3×10^3; haploids: mean ($n = 4$) = 1.6×10^4, SEM = 1.3×10^3].

The second way was by washing off all spores from the surface of the colony with 5.0 mL of saline-tween of which 5 μL was used to inoculate fresh medium, giving a fixed bottleneck size of 0.1%. Of the total 12 replicate populations per condition, six were transferred with mycelium of the growing front of the fittest sector of the colony, six by transferring a 0.1% sample of all spores. After 6 days of growth, a fungicide sensitive haploid colony (WG562) produces on average around 1.6×10^9 spores ($n = 12$; SEM = $2{,}4 \times 10^8$); a diploid colony (WG562//145) 5.2×10^8 spores ($n = 12$; SEM = 3.9×10^7). The evolution experiment consisted of 10 weekly transfers, which amounts to approximately 1200 cell cycles for the growing front of the colonies.

Fitness assays

We defined fitness as the MGR (Pringle & Taylor, 2002). We measured the MGR for all strains at every transfer by averaging the colony diameter as measured in two randomly chosen perpendicular directions. The MGR of the fungicide sensitive strain WG562 (weekly taken from the −80 °C stock, grown in sixfold as a nonevolving control) was used as a reference to calculate the relative fitness of the other strains. The slope of the fitness trajectory over the 10 transfers of a strain, computed from a linear regression analysis, defines its rate of adaptation in this evolution experiment.

Results

In an evolution experiment, artificial and natural selection have been compared using fungicide sensitive and

Table 1 Rates of adaptation given by the mean slope (×1000) of fitness trajectories in time of all ($n = 6$) individual replicate fungicide resistant or sensitive strains per environmental condition and mode of transfer. The value for the 95% Confidence interval (CI) of the mean is indicated. There were 12 replicate populations per growth medium per strain, six of which were transferred using the fittest sector and six using a random sample of all spores. Alternating growth medium is medium with alternating absence and presence of fungicide at each transfer. When calculating the slope of strains growing in alternating absence and presence of fungicide, the MGR measurements on medium with fungicide were omitted.

Strain	Environment	Transfer by 'fittest' sector	95% CI	Transfer by random spore sample	95% CI
Resistant diploid	Fungicide free	10.1	13.8	30.9	11.8*
Sensitive diploid	Fungicide free	3.2	0.6*	5.0	1.6*
Resistant haploid	Fungicide free	28.0	24.4*	52.4	14.6*
Sensitive haploid	Fungicide free	3.0	1.2*	9.0	2.2*
Resistant diploid	Alternating	3.6	12.0	21.4	12.7*
Resistant haploid	Alternating	8.2	11.2	43.4	14.0*
Average over all strains and media		9.4	14.2	27.0	17.2*
Average over all strains. media and mode of transfer		18.2	4.8*		

*Confidence interval does not comprise zero ($P < 0.05$), showing that the mean rate of adaptation was >0.

resistant haploid and diploid strains in two different environmental conditions. Figure 3 shows the mean trajectories of the relative fitness of the replicate strains grown under the same conditions and with the same mode of transfer. The rate of adaptation is given by the mean of the slopes of replicate strains of relative fitness against time for each condition. The mean slopes in time of all six replicates per treatment and ploidy are in Table 1. In individual adapting strains fitness increase shows a step-wise pattern, however, the mean fitness of all individual strains increase in a linear fashion because of differences in the time of appearance of beneficial mutations.

Propagation by random spore samples resulted in more rapid adaptation than propagation by artificial selecting the 'fittest' sector. This was significant and independent of whether strains were fungicide sensitive or resistant, haploid or diploid, or whether environments were stable or changing (ANOVA, see Table 2).

Overall, there was a significant increase in relative fitness (MGR) after 10 transfers (the 95% confidence interval of the mean slope of fitness all fitness trajectories does not comprise zero, Table 1). However, this was not the case for all individual groups of replicate strains that were propagated with sectors, see Table 1. The fungicide resistant strains had a higher rate of adaptation than the fungicide sensitive strains (*t*-test, $t_{46} = 4.94$, $P < 0.001$).

Comparing haploid and diploid strains overall treatments and conditions showed that haploids had a higher rate of adaptation than diploids (ANOVA, $F = 9.48$, d.f. $= 1$, $P < 0.01$). However, comparison of haploid and diploid strains that had the same mode of transfer and growth conditions only shows statistically significant differences when strains are transferred with random spores samples, haploids having the highest rate of adaptation (*t*-tests, d.f. $= 10$, $P < 0.05$. After the tenth transfer, we determined the ploidy of all originally diploid strains. Out of the 36 initially diploid strains one strain that had been propagated by transfer of sectors and one that had been propagated by transfer of spores had become haploid. The two haploidized diploid strains had a higher rate of adaptation than the mean of all diploid strains, however, this difference was nonsignificant (*t*-test, $t_{34} = 1.34$, $P = 0.19$).

The difference observed in the rate of adaptation of resistant strains evolving in fungicide-free and alternating environments is nonsignificant (*t*-test, $t_{34} = 1.67$, $P = 0.10$). For each strain only the first five transfers to

Table 2 Three way analysis of variance (ANOVA) of the mean linear fitness increase of six replicate populations.

Source	Type III sum of squares	d.f.	Mean square	*F*	Significance
Alternating regime (ALT)	1.511E-03	1	1.511E-03	4.381	0.043
Ploidy	3.271E-03	1	3.271E-03	9.481	0.004
Inoculation method	7.236E-03	1	7.236E-03	20.974	0.000
ALT × PLOIDY	1.224E-04	1	1.224E-04	0.355	0.555
ALT × INOCULAT	4.540E-05	1	4.540E-05	0.132	0.719
PLOIDY × INOCULAT	3.333E-04	1	3.333E-04	0.966	0.332
ALT × PLOIDY × INOCULAT	1.456E-04	1	1.456E-04	0.422	0.520
Error	1.380E-02	40	3.450E-04		
Total	5.593E-02	48			

medium without fungicide were taken into account for this comparison. After 10 transfers, all initially resistant strains had retained their resistance to fludioxonil, indicating that compensation for fitness costs was by second-site mutations.

Discussion

In an evolution experiment over 10 weekly transfers, we have investigated the relative effectiveness of artificial selection vs. natural selection on the rate of adaptation to genetic stress caused by negative pleiotropic effects of a major resistance mutation in *A. nidulans* strains. Artificial selection involved the weekly transfer of the fastest growing sector onto a fresh plate (Fig. 1), whereas transferring random samples of all the spores produced by the mycelium approximated natural selection. Fungicide sensitive and resistant haploid and diploid strains were allowed to evolve in two different environments: a fungicide-free environment and an environment with a weekly alternation between presence and absence of fungicide.

The results of the evolution experiments show that in all conditions and with all strains used the transfer of random spore samples resulted in the most rapid adaptation. As a mycelial sector with enhanced growth rate directly points to a beneficial mutation of fairly large effect (Fig. 1), we hypothesized that for evolution starting under clearly sub-optimal conditions (resistant strains in a fungicide-free environment) artificial selection would result in a higher rate of adaptation than natural selection. However, our results clearly contradict this hypothesis.

A possible explanation for the more rapid adaptation when transferring random samples of all spores lies in the number of mitoses needed to produce a spore from a nucleus in the mycelium. After their development, some mycelial subapical cells differentiate into foot cells (Fig. 2), from which the condiophore with chains of asexual spores are formed. Up to 100 mitoses take place between the formation of mycelium and the formation of the asexual spores (Pontecorvo *et al.*, 1953; Clutterbuck, 1969). Clearly, these additional nuclear divisions during spore formation allow more genetic variation to be generated by mutation than in mycelial hyphae only. Apparently, the generated random variation includes many adaptive mutations that can be efficiently selected during spore germination. Moreover, the fittest sector approach only samples nuclei that manage to form a sector, i.e. are located in the growing front of the mycelium at the moment they receive a favourable mutation. The more central mycelium of the colony is not involved in the expansion of the colony, but serves mainly metabolic functions. Advantageous mutations occurring in nuclei in the central part of the colony will not end up in a recognizable fast growing sector. However, they do have a chance to get incorporated in spores, as spore formation occurs on the whole surface of the colony, both in the central part and in the expanding growth front. It is therefore quite possible that in the random spore sample transfer procedure the net is cast much wider for catching a favourable mutation than in the fittest sector approach, in which not necessarily the genotype with the highest fitness will be selected.

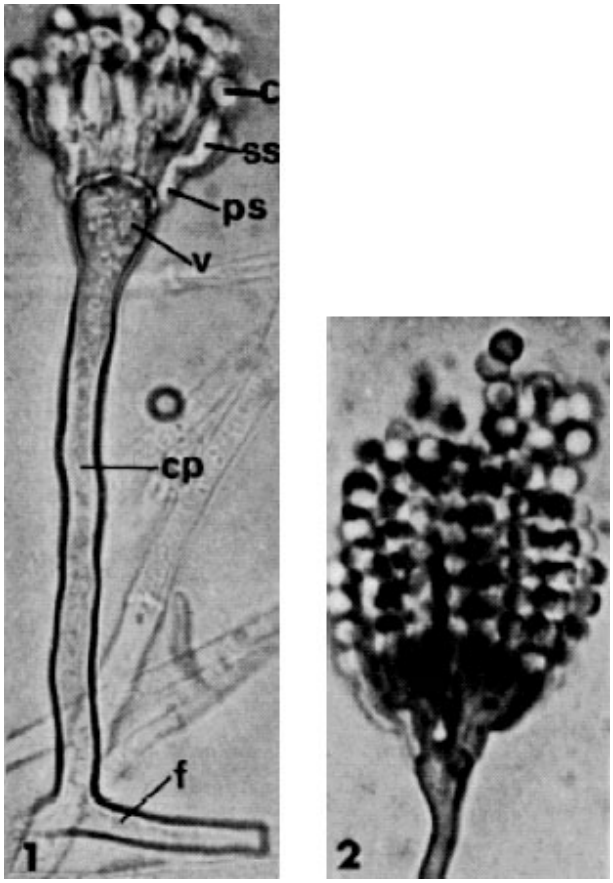

Fig. 2 Condiophore carrying asexual spores. Sixteen hours after formation of mycelium, subapical cells in hyphea differentiate into foot cells which give rise to a stalk with a vesicle and serigmata that carry chains of asexual spores. The left figure gives an overview of the total apparatus; c: conidium; ss, secondary serigmata; ps, primary serigmata; v, vesicle, cp, conidiophore stalk; f, mycelial foot cell. The right figure shows a fully-grown conidiophore with up to 10 000 asexual spores. These pictures were taken from (Clutterbuck, 1969).

In two previous studies of compensatory evolution in *A. nidulans* strains, the adaptive mutations found were recessive, in line with the general observation (e.g. Mukai, 1964) that most mutations are (partly) recessive. The chances of establishing an isogenic sector in mycelium consisting of multi-nucleate cells will be lower for a recessive mutation than for a dominant mutation. Therefore, maintenance of genetic variation in single-nucleate spores could have a large impact on the rate of adaptation.

As relative fitness is calculated as the MGR of a strain relative to the MGR of the fungicide sensitive haploid strain (WG562) used as a control, several strains do not have a relative fitness of 1 at the start of the evolution experiment. When isogenic, diploids have a higher MGR than haploids and thus a different relative MGR at the start of the evolution experiment. For resistant strains, the MGR on medium with fungicide is higher than the MGR of fungicide sensitive reference strain growing in the absence of fungicide. This makes their relative fitness >1 (cf. Fig. 3). When calculating the rate of adaptation represented by the slope of the lines in Fig. 3, the weeks during which fungicide was present were omitted.

On average not only resistant strains but also sensitive strains gained in fitness, demonstrating that the sensitive strain was not optimally adapted to the experimental conditions we used. However, the resistant strains had a higher rate of adaptation than the sensitive strains, which can be explained by their lower initial fitness due to the cost of the resistance they carried. Because the resistant

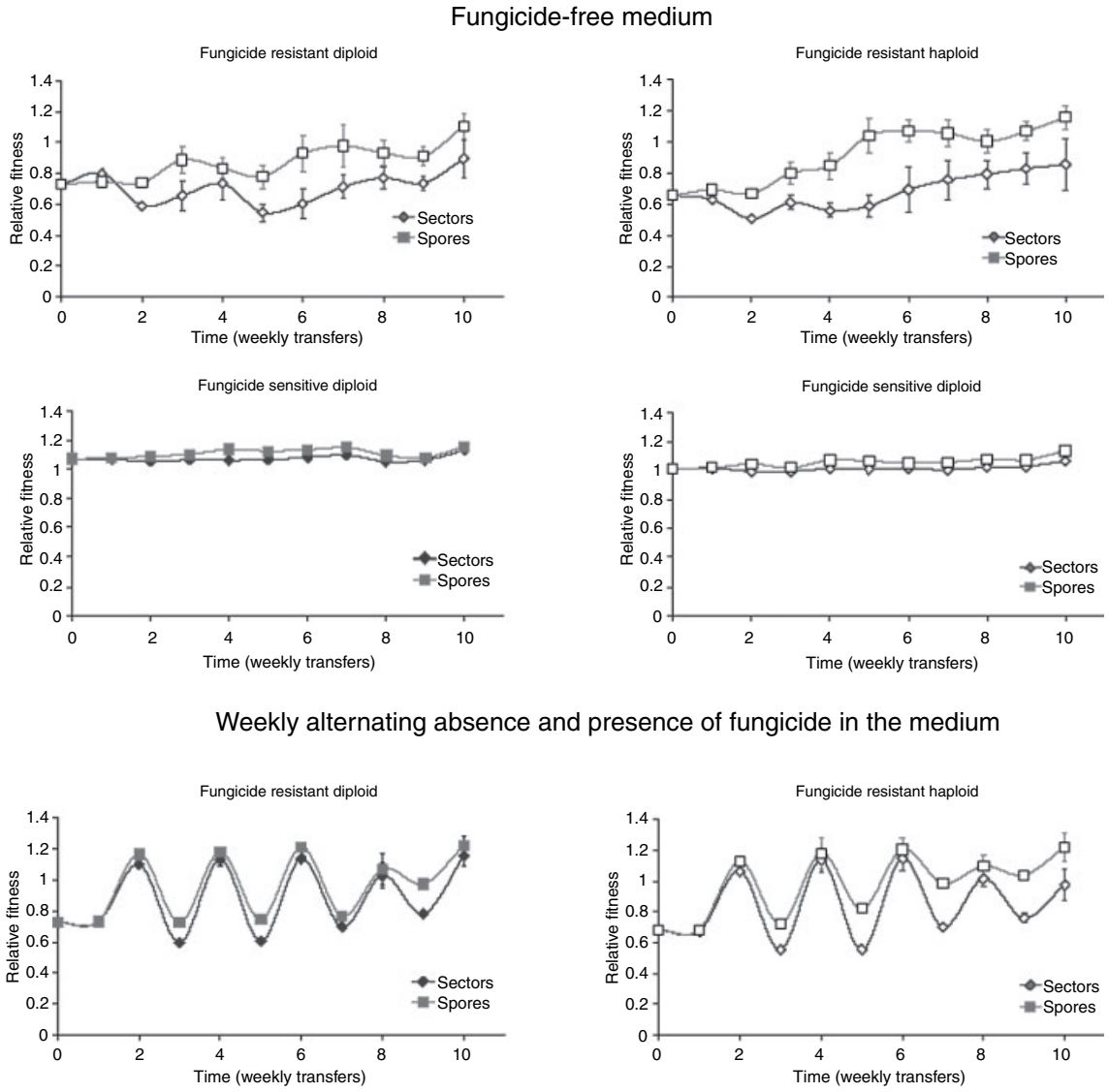

Fig. 3 Fitness trajectories for the evolution experiment over 10 transfers (equivalent to 1200 cell cycles). Two environmental conditions were used as indicated by the heading of a group of graphs. The titles of individual graphs show the strains used. Twelve replicates of each strain were used in every condition, six of which were propagated by transferring the 'fittest' sector and six by transferring a random sample of all spores (0.1%). Weekly the mycelial growth rate of all strains was measured and made relative to a fungicide sensitive reference strain (WG562) growing parallel in the absence of fungicide. Each line in each graph represents the mean of six replicate independently evolving strains that had the same treatment. Error bars indicate the 95% confidence interval for the mean. In several cases, the error bars are smaller than the symbols.

strains started at a lower relative fitness a larger fraction of all mutations is potentially adaptive than for fungicide sensitive strains that are closer to a fitness optimum (Fisher, 1930; Moore *et al.*, 2000).

The comparison of rates of adaptation for haploids and diploids shows that haploid strains on average tend to have a higher rate of adaptation. This is in accordance with a similar study with haploid and diploid *Saccharomyces cerevisiae* strains (Zeyl *et al.*, 2003). Out of the total of 36 diploid strains, there were two diploid strains that reverted to haploidy and had a higher rate of adaptation than the all time haploids and diploids, although these differences were nonsignificant. However, it may point to a potential evolutionary advantage for a vegetative diploid phase in otherwise haploid filamentous fungi. In nature, most isolates of *A. nidulans* are haploid, with 0.01–0.1% diploids (Upshall, 1981).

For the resistant strains, adaptation takes place at an equal rate in a stable fungicide-free environment and an environment with alternating absence and presence of fungicide. Apparently, mutations that are adaptive in the environment without fungicide do not have (severe) negative side effects in the presence of fungicide. Whereas mutations causing fungicide resistance have large trade offs in the absence of fungicide, its compensatory mutations do not, showing that the topography of the adaptive landscapes in these two cases is different (Wright, 1982; Elena & Sanjuan, 2003). There are practical implications of this finding for agriculture where fungicides are widely applied to control plant pathogens. Fludioxonil is widely used in agriculture to control emerging fungal infections on grape vines in particular (Cabras *et al.*, 1997; Verdisson *et al.*, 2001). Our results suggest that discontinuous and irregular use of fungicide does not reduce the risk of spread of resistance.

In summary, in experiments of adaptive evolution with *A. nidulans* strains ameliorating costs of fungicide resistance, artificial selection by selecting the fastest growing part of the mycelium does not overestimate the rate of adaptation in more natural circumstances where total spore production of the fungal colony is taken into account. On the contrary, selecting only on the basis of mycelial expansion may lead to an underestimation of the rate of adaptation.

Acknowledgments

We wish to thank Marc Maas, Klaas Swart and an anonymous reviewer for help, suggestions and comments. The Netherlands Organization for Scientific Research (NWO-ALW) funded this project.

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Received 22 February 2005; accepted 27 February 2005