Inherited and Environmentally Induced Differences in Mutation Frequencies Between Wild Strains of *Sordaria fimicola* From "Evolution Canyon"

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ABSTRACT

We have studied whether there is natural genetic variation for mutation frequencies, and whether any such variation is environment-related. Mutation frequencies differed significantly between wild strains of the fungus *Sordaria fimicola* isolated from a harsher or a milder microscale environment in "Evolution Canyon," Israel. Strains from the harsher, drier, south-facing slope had higher frequencies of new spontaneous mutations and of accumulated mutations than strains from the milder, lusher, north-facing slope. Collective total mutation frequencies over many loci for ascospore pigmentation were 2.3, 3.5 and 4.4% for three strains from the south-facing slope, and 0.9, 1.1, 1.2, 1.3 and 1.3% for five strains from the north-facing slope. Some of this between-slope difference was inherited through two generations of selfing, with average spontaneous mutation frequencies of 1.9% for south-facing slope strains and 0.8% for north-facing slope strains. The remainder was caused by different frequencies of mutations arising in the original environments. There was also significant heritable genetic variation in mutation frequencies within slopes. Similar between-slope differences were found for ascospore germination-resistance to acriflavine, with much higher frequencies in strains from the south-facing slope. Such inherited variation provides a basis for natural selection for optimum mutation rates in each environment.

FOR understanding adaptation, evolution and biodiversity, one must explore the causes of variation and the controls of processes causing genetic variation. We have studied whether there is natural genetic variation for mutation frequencies and whether any such variation is environment-related. We looked for variation in mutation frequencies between different wild strains of a fungus, *Sordaria fimicola*.

We isolated strains from different microscale environments in "Evolution Canyon," Israel, from different altitudes on different slopes, and from the valley bottom. The contrasting south- and north-facing slopes of Evolution Canyon, Lower Nahal Oren, Mount Carmel, Israel, are suitable for testing a range of points in genetic, evolutionary and ecological theory (Nevo 1995, 1997). The south-facing slope (SFS) bears African and Asian xeric tropical species, has higher solar radiation, and is warmer, drier, more fluctuating, and more heterogeneous than the lush "temperate European" north-facing slope (NFS), with only 200 to 500 meters between the slopes (Nevo 1995, 1997). The Canyon is 100 m deep, with a 35° dip on the 120-m-long SFS, and a 25° dip on the 180-m-long NFS (Nevo 1997).

Sordaria fimicola is an Ascomycete fungus that occurs on dung or plant remains. It is homothallic, self-fertile and haploid, with no asexual spores and no distinct gametes. The black sexual perithecia can result from a strain self-fertilizing, or from the meeting of different strains, if vegetatively compatible. Meiosis plus a mitosis produces asci containing eight haploid, binucleate, black ascospores. Ascus dehiscence projects groups of ascospores into the air over short distances. They are reproductive, dispersive and resting agents.

The working hypothesis was that the harsher, more changeable and more diverse SFS environment might favor processes increasing variation, including a higher mutation rate and more recombination through crossing-over and/or gene conversion. For an account of wider aspects of the whole Evolution Canyon project and background references on evolution, environmental stress, genetic diversity, biodiversity, and the roles of recombination, mutation and genetic drift in evolution and adaptation, see Nevo (1997).

Our aims were the following: to see whether there were significant differences between the nine wild-type strains in mutation frequencies; to see if any such variation was random between the strains or whether strains from the south-facing slope (SFS) had mutation frequencies generally different from those of strains from the north-facing slope (NFS); to determine whether any such between-slope differences were because of different rates of mutation arising in the different original environments and/or inherently different rates of spontaneous mutation that would persist in a uniform lab environment, and if mutation frequencies depended on

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whether strains had been isolated from dung or soil. We were particularly interested in whether any differences found were environment related and possibly adaptive. Mutation was studied from the frequency of ascospore pigmentation mutations, and from the frequency of ascospores able to germinate on particular concentrations of acriflavine.

MATERIALS AND METHODS

Isolation and strain details: Wild strains of the Ascomycete fungus *Sordaria fimicola* were isolated between August 1994 and February 1995 from three different levels, 60, 90 and 120 m above sea level on each slope, south facing and north facing, and from the valley bottom (strain B). The wild isolates were obtained vegetatively, not from ascospores, and were subcultured as little as possible, using large inocula to minimize genetic drift, before the determination of mutation frequencies. From the SFS, S1 was isolated from near the top, S2 from near the middle, and S3 from near the bottom. From the NFS, N5(i) and N5(ii) were isolated from the bottom, N6 from near the middle, and N7(i) and N7(ii) from near the top. S1, S2, and N7(i) were isolated from dung, S3, N5(i) and N6 from soil, but it is not known whether N5(ii), N7(ii) or B came from soil or dung.

S. fimicola strains were isolated from soil by the dilution plating method (Waxman 1916), using dilutions of 1:10, 1:100 and 1:1000 for soil:water. Isolation from dung was by the humid chamber method at 26°: after surface sterilization, dung was put in Petri dishes with sterile paper discs soaked in sterile water, and *S. fimicola* was identified by its sporulation pattern (Domasch *et al.* 1980). Perithecia from the dung were transferred to fresh nutrient agar, with cultivation of vegetative hyphae from the perithecia.

Growth, germination and crosses: Crosses were made at 17.5° by inoculation of one strain in the center (selfing), or two strains on opposite sides, of 9-cm Petri dishes of minimal medium (Olive 1956). Growth and ascospore germination were on cornmeal agar with sodium acetate (Kitani and Olive 1967), with 18° for germination. Dehisced asci for visual scoring of ascospore color mutation frequencies were collected on plates of 1.7% water agar with 0.7 g/liter methyl-p-hydroxybenzoate to inhibit spontaneous ascospore germination. Each repeat experiment consisted of three or four petri dishes (replicates), and, to avoid overcrowding of dehisced asci, several different collecting lids were placed sequentially on each dish for scoring. Samples of nonblack ascospores were isolated, germinated and allowed to self-fertilize, to test for phenocopies and spontaneous reversion. Some of the ascospores with mutant color had low germination frequencies, but phenocopies were rare. Acriflavine, an acridine dye, was used as acriflavine hydrochloride (Sigma, Poole, UK), which consists of acriflavine HCl and proflavine HCl. According to Pelczar *et al.* (1986), acriflavine exhibits selective inhibition against staphylococci and gonococci, but possesses little antifungal activity.

Black ascospores from selfed perithecia of the wild strains were germinated to obtain the Selfed Generation 1 strains, which were used in turn to get Selfed Generation 2 strains from their black ascospores. Because the fungus is homothallic, it is difficult to see if two wild-type strains are interfertile, as both produce asci with eight black ascospores in crosses or selfings. We therefore tested crossing ability by plating together a strain with black ascospores and one with a spore color mutant, looking for crossed perithecia with four black: four nonblack segregations.

Mutation: Mutation was studied collectively over a range of different loci for ascospore color, where genes for wild-type black can mutate to give white or other colors (Olive 1956; Kitani and Olive 1967). From the frequency of octads with 4 black (+): 4 nonblack, for example white (*w*) or gray ascospores, one gets an overall mutation frequency for many loci. Catalogue (1996) lists 16 such loci, but the present mapping data showed additional loci. Using the overall frequency gives an average response over all those loci, which is more representative than results from any one locus. As there was some ascus-to-ascus variation in wild-type ascospore pigment intensity, minor increases or decreases in the amount of black-gray pigment were ignored during the scoring of mutation frequencies. Mutations occurring late in ascus development could give 5:3, 6:2 or 7:1 ratios of +:*w*, but, as those ratios could also arise by gene conversion from what would have been a 4:4 ascus, those extremely rare classes were not used to estimate late mutations in the ascus.

When the original wild strains were tested in the lab, their mutations included those from spontaneous mutation under standard lab conditions and pre-existing mutations accumulated in hyphae in the wild on the SFS or the NFS after those hyphae or their forerunners arose from wild-type ascospores. Separating the frequencies of these two types of mutation was easy for ascospore color: new strains were isolated from germinated black, haploid, ascospores from selfed wild-strain perithecia. The new Selfed Generation 1 strains' inherited spontaneous mutation frequencies were then determined under standard lab conditions. By subtracting the spontaneous mutation frequencies in the lab (in the Selfed Generation 1 strains) from the mutation frequencies in the original wild strains, the frequencies of mutations accumulated in the wild could be estimated.

These mutation studies were repeated using a different criterion, the frequency of ascospores able to germinate on medium with 150 or 250 μg/ml acriflavine. A level of 50 μg/ml acriflavine was used by Perkins (1996) for selecting acriflavine-resistant *Neurospora crassa acr-2*^{R} strains from ascospore germination, so the present level was more stringent. None of these strains had previously been exposed to acriflavine.

The ability of ascospores to germinate on acriflavine could result from a mutation to acriflavine resistance or from segregation at polymorphic loci for acriflavine resistance, perhaps with different loci giving different levels of resistance, but segregation would only occur if genetically different strains were crossed, which was not done in these experiments. No segregation should take place in progeny of selfed homokaryotic haploid strains. The haploid progeny in Selfed Generations 1 and 2 cannot therefore show genetic segregation for germination properties, but can show spontaneous mutation to acriflavine resistance in germination. The genetics of acriflavine resistance has not previously been studied in *S. fimicola*, to our knowledge. In other coprophilous ascomycetes, there is usually more than one locus for this character (Catalogue 1996), so the present results may well include mutations at more than one locus, which would help to account for their high frequency, as does the presence of frameshift-inducing mutagen proflavin in the acriflavine, although the experiment does not allow time for segregation delay before expression of resistance.

Mapping: Some of the mutant ascospores were germinated, and the resulting colonies were crossed to map the mutations. Recombination frequencies were obtained from the numbers of parental ditype, nonparental ditype and tetratype asci from repulsion-phase dihybrid crosses, and centromere distances were obtained from half the percentage of asci with second division segregation in monohybrid crosses of mutant × wild-type.

RESULTS

There were clear differences in mutation frequencies for ascospore color mutations among the nine original wild-type strains (Table 1), with generally good agreement between repeats and replicates. There were consistently higher mutation frequencies in strains from the south-facing slope (2.27 to 4.41%) than in strains from the north-facing slope (0.89 to 1.27%). This highly significant between-slope difference was also found (Table 2) in Selfed Generation 1 and 2 strains, so it was partly heritable. A similar pattern of mutation differences between strains and between slopes was found for acriflavine resistance of ascospores (Table 3), with 4.5 to 5.9% for SFS strains and 0.45 to 0.76% for NFS strains in the 1996 data.

Controls on the homogeneity of repeats and replicates, the time of most mutations, storage time, and numbers of subcultures: For each strain, there was generally good agreement for mutation frequency for ascospore color mutations between the three repeats, usually made over a period of five months, showing that strain storage and chance variations in experimental conditions in the lab had little effect. Tests with χ^2 (Table 1) showed significant heterogeneity for the repeats of S1 and S3, but replicate dishes made at the same time and repeat experiments made months or even a year apart generally gave homogenous results for mutation frequency. Variation between repeats or replicates could arise because mutation can occur at any time during colony growth. A mutation, say to *white*, arising early in a culture would cause more 4+:4*w* asci than one arising late in that culture, but the asci with mutant ascospores were well distributed within and between plates, with no obvious clustering. This suggests that most mutations were independent events arising late in colony development, when there are far more nuclei present than there are earlier. Early mutations in Ascomycetes can cause noticeable clustering of mutant asci (see Figure 1; Lamb and Wickramaratne 1973), or unexpectedly high mutation frequencies, as in the acriflavine data here in Table 3, 1996 data, strain S1 in Selfed Generation 2. As this fungus is homothallic, an early mutation to *white*, say before perithecial formation, could cause perithecia with only 0+:8*w* octads, giving clusters of such asci on collecting lids, but such clusters were not observed.

As each ascus comes from two fusing nuclei, counting

TABLE 1

Total mutation frequencies for ascospore color loci in nine strains originally isolated from Evolution Canyon

		4+:4*w* octads[a]		
Wild strain	Total octads	Mean (%)	SE	Limits of three repeats (%)
South-facing slope strains				
S1	61,325	3.49	± 0.12	3.34–3.81
S2	74,743	2.27	± 0.06	2.14–2.39
S3	51,077	4.41	± 0.24	4.09–4.99
Bottom of valley				
B	7,522	2.14	± 0.08	2.00–2.32
North-facing slope strains				
N5(i)	68,750	1.25	± 0.03	1.19–1.31
N5(ii)	7,262	1.23	± 0.06	1.08–1.31
N6	66,077	1.27	± 0.00	1.21–1.30
N7(i)	59,969	0.89	± 0.05	0.79–1.00
N7(ii)	8,883	1.13	± 0.13	0.81–1.36
Slope totals, excluding valley bottom:				
South-facing slope strains	187,145	3.25	± 0.13	3.02–3.57
North-facing slope strains	210,941	1.13	± 0.03	1.09–1.21

Statistical analysis, using $2 \times n$ χ^2:[b] Homogeneity between results from south-facing and north-facing slope strains: d.f. = 1; total values, $\chi^2 = 2140$***; separate repeats: repeat 1, $\chi^2 = 812$***, repeat 2, $\chi^2 = 584$***; repeat 3, $\chi^2 = 728$***. Homogeneity between different strains from the same slope: $\chi^2 = 457$***, d.f. = 2, for three different south-facing slope strains; $\chi^2 = 51$***, d.f. = 4, for five different north-facing slope strains. Homogeneity between different strains from the same site on the same slope: N5(i) and N5(ii), $P > 0.05$; N7(i) and N7(ii), $\chi^2 = 13$***. Homogeneity between the three repeat experiments for each of the nine strains: d.f. = 2. All were homogeneous at $P = 0.05$, except for S1, $\chi^2 = 8.0$* and S3, $\chi^2 = 21$***. Homogeneity between strains isolated from soil or dung, within slopes: The SFS strain from soil, S3, had a higher mutation frequency ($\chi^2 = 202$***), than the two from dung, S1 and S2; two NFS strains from soil, N5(i) and N6, had higher mutation frequencies ($\chi^2 = 50$***) than one from dung, N7(i).

[a] 4+:4*w* here includes mutations to any nonblack color, not just to white.

[b] Significant χ^2 values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2

Comparison of total mutation frequencies for ascospore color loci in wild strains with those from Selfed Generation 1 and Selfed Generation 2 strains

Wild strain		Selfed Generation 1			Selfed Generation 2		
Total octads	4+:4*w* octads (%)[a]	Strain	Total octads	4+:4*w* octads (%)	Strain	Total octads	4+:4*w* octads (%)
Strain S1 and its derivative from selfing							
61,325	3.49	S1(1;4.1)	18,179	1.52	S1(2;1.2)	33,634	1.51
		S1(1;5.3)[b]	19,908	1.88	S1(2;2.3)	40,815	1.47
		Total	38,087	1.71	Total	74,449	1.49
Strain S3 and its derivatives from selfing							
51,077	4.41	S3(1;1.2)[b]	19,356	2.07	S3(2;1.4)	29,595	2.03
		S3(1;2.4)	18,726	2.04	S3(2:2.4)	32,107	1.85
		Total	38,082	2.06	Total	61,702	1.94
Pooled south-facing slope strain values for each generation							
112,402	3.91		76,169	1.88		136,151	1.69
Strain B, from valley bottom							
7,522	2.14	B, Selfed	5,782	1.87	—	—	—
Strain N5(i) and its derivatives from selfing							
68,750	1.25	N5(i)(1;2.1)	24,454	0.91	N5(2;1.1)	23,317	0.71
		N5(i)(1:2.3)[b]	17,201	0.74	N5(2;3.2)	24,381	0.83
		Total	41,655	0.84	Total	47,698	0.77
Strain N5(ii) and its derivative from selfing							
7,262	1.23	N5(ii), Selfed	7,488	0.99	—	—	—
Strain N7(i) and its derivatives from selfing							
59,969	0.89	N7(i)(1;1.4)	18,434	0.65	N7(2;1.3)	35,775	0.47
		N7(i)(1;3.2)	20,326	0.67	N7(2;4.2)	13,825	0.69
		Total	38,769	0.66	Total	49,600	0.53
Strain N7(ii) and its derivative from selfing							
8,883	1.13	N7(ii), Selfed	6,148	0.62	—	—	—
Pooled north-facing slope strain values for each generation							
144,864	1.09		94,051	0.77		97,298	0.65

Statistical analysis, using $2 \times n$ χ^2:[c] Homogeneity between generations: d.f. = 1; comparing pooled wild-strain values with pooled Selfed Generation 1 values, the generations had highly significant differences; for SFS strains, $\chi^2 = 623.5$***; for NFS, $\chi^2 = 65.4$***. Comparing pooled Selfed Generation 1 strains with pooled Selfed Generation 2 strains, the generations had lesser differences, but they were significant; for SFS strains, $\chi^2 = 9.9$**; for NFS strains, $\chi^2 = 8.7$**. Homogeneity between strains from south- and north-facing slopes in Selfed Generation 1 and 2: d.f. = 1; The mutation frequencies were highly significantly different between slopes in both generations: Selfed Generation 1, $\chi^2 = 415$***; Selfed Generation 2, $\chi^2 = 496$***. Homogeneity between the two strains derived from selfing one parental strain: d.f. = 1; The two strains were homogeneous ($P > 0.05$) in all cases except for S1 Selfed Generation 1 strains ($\chi^2 = 7.4$**) and the N7(i) Selfed Generation 2 strains ($\chi^2 = 8.9$**). Homogeneity between different strains from the same site, Selfed Generation 1: N5(i) and N5(ii), $P > 0.05$; N7(i) and N7(ii), $P > 0.05$.

[a] 4+:4*w* here includes mutations to any nonblack color, not just to white.

[b] The Selfed Generation 1 strains used as parents of the Selfed Generation 2 strains; numbers in parentheses are the Selfed Generation number, followed, if known, but the number of the octad, a period and the number of the ascospore within the octad.

[c] Significant χ^2 values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

each ascus as a single event instead of a double event for mutation scoring underestimates the sample size (a 4+:4*w* octad comes from one mutant and one nonmutant nucleus), but this is somewhat counteracted by the fact that an early mutation in a culture can give rise to a number of different asci scored as mutant. The number of subcultures made before starting a cross had no effect on mutation frequency. The numbers of subcultures from isolation of the strain from the wild to making the crosses were as follows: four for S2, N5(i) and (ii) and N7(ii), and B; four to seven for different S1 crosses; eight for S3 and N7; and 16 or 17 for N6, for which fertility was initially low. These numbers bear no relation to the strains' mutation frequency differences (Table 1).

Differences between strains: The nine wild isolates showed clear differences in their frequencies of octads with 4 black:4 nonblack ascospores (Table 1), ranging from 0.89 to 4.41% and totalled over more than 16 loci. From the SFS, all three strains, S1, S2 and S3, were significantly different from each other ($P < 0.001$) in mutation frequency, with values of 3.5%, 2.3% and 4.4%, respectively. From the NFS, N7(i) had significantly less ($P < 0.05$) mutation, with 0.89%, than N5(i),

TABLE 3

Ascospore germination resistance to acriflavine in the original wild strains and Selfed Generations 1 and 2

1996 data			1997 data				
Acriflavine HCl: 150 μg/ml			150 μg/ml			250 μg/ml	
Strain	Total ascospores	Survival[a] (%)	Strain	Total ascospores	Survival (%)	Total ascospores	Survival (%)
Original strains, south-facing slope							
S1	1181	5.1		371	6.3	390	4.9
S2	603	5.9		419	8.9	397	2.5
S3	1436	4.5		395	6.0	393	3.6
Total	3220	5.0		1185	7.1	1180	3.9
Original strains, north-facing slope							
N5(i)	791	0.76		371	1.9	401	0.6
N6	823	0.62		427	1.3	390	0.6
N7(i)	1446	0.45		414	0.9	396	1.0
Total	3060	0.59		1212	1.4	1187	0.7
Selfed Generation 1 strains from selfing original strains, south-facing slope							
S1(1;4.1)[c]	1058	2.5	S1(1;4.1)	424	4.9	428	3.3
			S1(1;5.3)	406	8.7	405	3.6
S3(1;1.2)	1794	4.1	S3(1;1.2)	399	4.6	377	4.5
Total	2852	3.5		1229	6.1	1210	3.8
Selfed Generation 1 strains from selfing original strains, north-facing slope							
N5(i)(1;2.1)	1532	0.77	N5(i)(1;2.1)	426	1.5	409	0.0
N7(i)(1;3.2)	1133	0.38	N7(i)(1;3.2)	375	1.0	417	0.5
			N7(i)(1;1.4)	372	1.1	421	0.0
Total	2665	0.60		1183	1.2	1247	0.2
Selfed Generation 2 strains from selfing first generation, south facing slope							
S1(2;1.2)	1154	9.5[b]	S1(2;1.2)	637	8.1	605	7.0
S3(2;1.4)	987	2.7	S3(2;1.4)	621	6.4	588	4.6
			S3(2;2.4)	617	6.8	574	5.4
Total	2141	6.4		1875	7.1	1767	5.7
Selfed Generation 2 strains from selfing first generation, north-facing slope							
N5(i)(2;3.2)	717	0.53	N5(i)(2;3.2)	630	2.5	596	1.2
			N7(i)(2;3.1)	633	2.0	641	1.8
N7(i)(2;4.2)	816	0.51	N7(i)(2;4.2)	590	1.2	605	0.4
Total	1533	0.52		1853	1.9	1842	1.1

Statistical analysis, using $2 \times n$ χ^2:[c] Homogeneity between results from south- and north-facing slope strains, on pooled slope totals: d.f. = 1; all generations showed highly significant differences; original strains, 1996 data, $\chi^2 = 110$***; 1997 data, 150 μg/ml, $\chi^2 = 33$***; 250 μg/ml, $\chi^2 = 19$***. Selfed Generation 1 strains, 1996 data, $\chi^2 = 57$***; 1997 data, 150 μg/ml, $\chi^2 = 28$***; 250 μg/ml, $\chi^2 = 29$***. Selfed Generation 2 strains, 1996 data, $\chi^2 = 81$***; 1997 data, 150 μg/ml, $\chi^2 = 36$***; 250 μg/ml, $\chi^2 = 33$***. Homogeneity between different strains from the same slope: Most results showed homogeneity, with these exceptions: south-facing slope strains, 1996 data, Selfed Generation 1 strains, $\chi^2 = 5.5$*, d.f. = 1; Selfed Generation 2, $\chi^2 = 41$***, d.f. = 1. Homogeneity between different generations: d.f. = 1; some differences were significant, some were not; original strains with Selfed Generation 1 strains: south-facing slope, 1996, $\chi^2 = 8.2$**; 1997, 150 μg/ml, $\chi^2 = 0.92$; 250 μg/ml, $\chi^2 = 0.05$; north-facing slope, 1996, $\chi^2 = 0.02$; 1997, 150 μg/ml, $\chi^2 = 0.30$; 250 μg/ml, $\chi^2 = 3.0$. Selfed Generation 1 strains with Selfed Generation 2 strains, south-facing slope strains, 1996, $\chi^2 = 22$*** (but this includes the atypical S1 Selfed Generation 2 result); 1997, 150 μg/ml, $\chi^2 = 0.02$; 250 μg/ml, $\chi^2 = 1.5$; north-facing slopes, 1996, $\chi^2 = 0.11$; 1997, 150 μg/ml, $\chi^2 = 0.64$; 250 μg/ml, $\chi^2 = 5.8$*.

[a] Calculated as germination percentage on acriflavine/(control germination percentage without acriflavine/100). The control germination percentages, without acriflavine, ranged from 78 to 95%, with no systematic trends between slopes or generations.

[b] Unusually high, so a mutation presumably occurred very early in the origin of this strain, which gave heterogeneous results between replicates.

[c] Significant χ^2 values are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

N5(ii), N6 and N7(ii), which ranged from 1.13 to 1.27%, with no significant differences between the last four strains. There were therefore five groups of strains for mutation frequencies, with the valley bottom strain, B, not significantly different from the lowest value SFS strain, S2, although strain B was significantly different

from all NFS strains and from the other SFS strains. The significant differences among SFS strains and among NFS strains bore no clear relation to their positions (top, middle or bottom) on each slope, and different strains from the same site on the same slope were sometimes homogeneous [N5(i) and N5(ii)], and sometimes not [N7(i) and N7(ii)] (Tables 1 and 2).

Differences between slopes: Table 1 shows highly significant differences for color mutations between the three strains from the south-facing slope, with overall mutation frequencies of 2.27 to 4.41%, and the five from the north-facing slope, with only 0.89 to 1.27%. In the pooled values for slope totals, the SFS strains had significantly higher mutation frequencies than the NFS strains, with $P < 0.001$ (Table 1). The difference between slopes was consistent across all three repeat experiments, with repeats from the SFS varying from 2.14 to 4.99% and repeats from the NFS varying from 0.79 to 1.36%.

Were the differences a result of different induction rates in the two different wild environments (slopes), or a result of inherently different rates of spontaneous mutation in strains from the different slopes? The inherent frequency of spontaneous mutation under standard lab conditions was determined from Selfed Generation 1 colonies. This frequency will exclude any ascospore-pigmentation mutations that occurred in the wild environment, because the ascospores germinated to get the Selfed Generation 1 strains were black and haploid, with nearly all mutations being autonomous and expressed. By subtracting this spontaneous frequency from the wild strains' mutation frequency, one obtains the frequency of mutations that accumulated in the wild strains and that will reflect the mutation frequency occurring in their respective wild environments, SFS or NFS conditions.

The Selfed Generation 1 and 2 crosses were made with one subculture after their isolation from a germinated ascospore. Mutation frequencies in Selfed Generation 1 strains (Table 2) were significantly lower than those from their own parental wild isolates, for all seven cases except N5(ii), through the elimination of accumulated mutations from the wild by germinating only black, wild-type ascospores. For S1 and S3, mutation frequencies were roughly halved, from 3.49 and 4.41% in the wild strains to 1.52 to 2.07% in the Selfed Generation 1 strains. For N5(i) and (ii), the reduction was less, from about 1.24% in the wild strains to 0.84 and 0.99% in the Selfed Generation 1 strains. For N7(i) and (ii), the reduction was from 0.89 to 0.66% and from 1.13 to 0.62%, respectively.

The differences in mutation frequency between the SFS and NFS original wild strains were thus partly a result of differences in the inherited spontaneous mutation rate (shown by the Selfed Generation 1 values) and partly a result of differences in accumulated mutations. From pooled values in Table 2, Selfed Generation 1 SFS strains had a pooled mutation value of 1.88% and the wild SFS strains had a value of 3.91%, so the frequency of accumulated mutations from the wild was 2.03%. Similarly, the frequency of accumulated mutations from the wild for NFS strains was 1.09% − 0.77% = 0.32%. There were therefore highly significant differences between SFS and NFS strains in both the frequency of mutations accumulated in the wild and in the inherited frequency of spontaneous mutations.

The relative frequencies of accumulated and spontaneous mutations were also different for strains from the two slopes. For the original SFS strains, 48% (1.88 as a percentage of 3.91) of the ascospore color mutations were caused by the inherited spontaneous mutation frequency, and 52% were accumulated from a build-up of mutations in the wild before the strains were isolated to the lab. For NFS strains, 71% (0.77 as a percentage of 1.09) of mutations were newly spontaneous and 29% were accumulated.

The harsher SFS environment, with much stronger solar radiation and more variable temperatures, induced spore color mutations at a higher frequency (2.03%) than did the temperate NFS environment (0.32%), a highly significant difference ($P < 0.001$). The difference between mutation frequencies in the SFS and NFS strains persisted through Selfed Generation 1 to Selfed Generation 2, in which the pooled SFS total was 1.69% compared with 0.65% for NFS strains (Table 2). The small but significant reduction in mutation frequencies between Selfed Generations 1 and 2 was unexpected and did not occur for the acriflavine results (see below).

Strains isolated from dung or from soil: Within a slope, strains from soil had higher ($P < 0.001$) mutation frequencies than those from dung (Table 1), but the number of strains was small.

Acriflavine resistance: The 1996 data are considered first. As shown in Table 3, the three original strains from the SFS had much higher ($P < 0.001$) survival frequencies on 150 μg/ml acriflavine, 4.5 to 5.9%, than did the three NFS strains, 0.45 to 0.76%, with even bigger proportional differences than for ascospore color mutations. There was good agreement between two repeat experiments and between the three plates for each treatment in each experiment, except for one S3 plate that had far more spores (1275) than any other plate, with an atypically low germination frequency from overcrowding; its results have been excluded.

Going from the original wild strains to the Selfed Generation 1 strains in the 1996 data, the survival frequency reduced from 5.1 to 2.5% for S1, from 4.5 to 4.1% for S3, and from 0.45 to 0.38% for N7(i), but for N5(i) it remained unchanged at 0.77%. The average reduction in frequency was therefore greater for SFS strains than for NFS strains, as for ascospore color. The reduction between original strains and SG1 strains was significant for the SFS strains, pooled results, $P < 0.01$,

but not for NFS strains. Because of the unusual result for S1 in the Selfed Generation 2 strains, one cannot really compare results of Selfed Generations 1 and 2 for SFS strains, but there was no significant change between these two generations for the NFS strains.

If one uses pooled slope values from Table 3, 70% (3.5 as a percentage of 5.0) of the mutations in the original SFS strains were caused by the inherited spontaneous mutation frequency and 30% were accumulated from a build-up of mutations in the wild. For NFS strains, virtually all mutations for acriflavine resistance were newly spontaneous and virtually none was accumulated, because the survival percentages were almost the same from wild strains and from Selfed Generation 1 strains. When the results are looked at in terms of which strains originally came from dung and which from soil, there were no appreciable differences between them for acriflavine resistance frequencies.

The 1997 data come from crossing the same strains as for the 1996 data, but with a further subculture of each strain. For both concentrations of acriflavine, 150 and 250 μg/ml in Table 3, survival percentage was about five times higher for SFS strains than for NFS strains, in the original strains and in Selfed Generations 1 and 2, with all differences significant at $P < 0.001$. The reduction for SFS original strains going to Selfed Generation 1 was less than in the 1996 data. For the NFS strains, that reduction was small for 150 μg/ml, but was proportionately larger for 250 μg/ml. Going from Selfed Generation 1 to Selfed Generation 2, there were some increases in survival percentage, but they were not always significant. The 1996 and 1997 data were sometimes homogenous, sometimes heterogeneous between years, but the same general trends were apparent, especially the big between-slope difference in survival.

Mapping results: Six mutations from the SFS strains and five from the NFS have been partly mapped, with sample sizes of about 800 asci for mutation-to-mutation distances, and of about 25,000 asci for centromere distances. Most mutations are in a cluster of 10 cM length (SFS strains) or 8 cM (NFS strains), quite far from the centromere, with one mutation from each slope some way from the main cluster (Figure 1, separate results). In crosses between the SFS and NFS strains, these clusters coincide on the map (Figure 1, combined results). All 11 mutations tested are therefore in the same linkage group, unlike those in Catalogue (1996). The centromere distance data (not shown) agree well with the order and distances between mutations from the mutant × mutant data.

Fertility of different types of cross: Almost all SFS × SFS and NFS × NFS color mutant × wild-type crosses were fertile. The majority of SFS × NFS crosses were fertile, but not all. Out of 11 SFS white mutations, all crossed with SFS strains but two did not cross with any NFS strains. Of six NFS mutants, all crossed with other NFS strains; three white, one gray and one light gray crossed with SFS strains, but one gray did not. In SFS × NFS crosses of two ascospore color mutants, 6 out of 30 crosses failed. Fertility barriers to gene flow within slopes were thus almost nonexistent, but occurred between a minority of strains from different slopes.

DISCUSSION

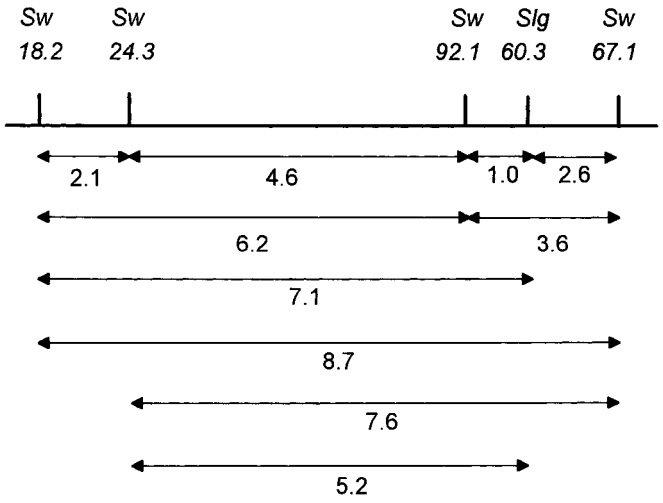
Mutation frequency differences between strains, slopes, and sites: Tables 1, 2, and 3 show highly significant differences between strains, between slopes, and between strains within slopes, with larger between-slope than within-slope differences. This was true for ascospore color mutations and for acriflavine resistance of ascospores.

These results show that there was natural variation in mutation frequencies. The fact that the variation was so consistent for ascospore color averaged over many loci, and for ascospore germination resistance to acriflavine, with high mutation frequencies for all three south-facing slope strains, and low mutation frequencies for all five north-facing slope strains, is completely consistent with the variation being environment related.

Spontaneous and induced mutation frequencies, and differently mutagenic environments on the two slopes: The differences between the nine wild strains could reflect different inherent frequencies of spontaneous mutation, and/or differences in accumulated mutations from the wild, including environmentally induced differences in mutation frequency, and/or differences in length of time or growth between a strain's origin from an ascospore and its vegetative isolation in this study. For inherited genetic variation in spontaneous mutation frequencies, it is therefore best to compare the Selfed Generation 1 strains with each other, and the Selfed Generation 2 strains with each other: equivalent times and amounts of growth were used for each strain in each Selfed Generation. In Table 2 for Selfed Generation 1, strains S1, S3, N5(i) and N7(i) had ascospore color mutation frequencies, respectively, of 1.71, 2.06, 0.84, and 0.66%, all of which are significantly different from each other at $P < 0.01$. In Selfed Generation 2, the values were 1.49, 1.94, 0.77 and 0.53%, again all significantly different at $P < 0.01$. There is therefore a high degree of inherited genetic variation between and within slopes, on which selection could act to optimize mutation rates for particular microscale environments.

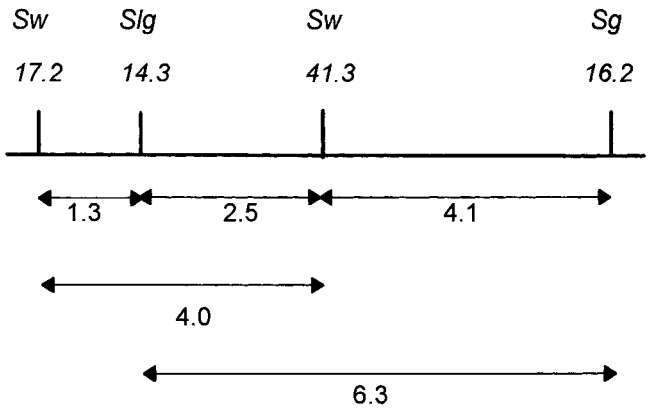
Strains from the harsher environment had the higher inherited spontaneous mutation frequencies as well as the higher accumulated mutation frequencies. All four SFS Selfed Generation 1 strains had higher inherited spontaneous mutation frequencies (1.5 to 2.07%) than the six corresponding NFS strains (0.62 to 0.99%) for ascospore color. The inherited spontaneous mutation frequencies for the SFS and NFS strains averaged 1.88 and 0.77%, respectively, a 2.4-fold difference. The average frequencies of accumulated mutations in the wild

South-facing-slope strains



The centromere is 23.9 cM to the left of *Sw18.2*; *Sg4.3* is 30.6 cM to the right of *Sw18.2*.

North-facing-slope strains



The centromere is 23.1 cM to the left of *Sw17.2*; *Sg21.3* is 22.7 cM to the right of *Sg16.2*.

Combined values from south- and north-facing-slope strains

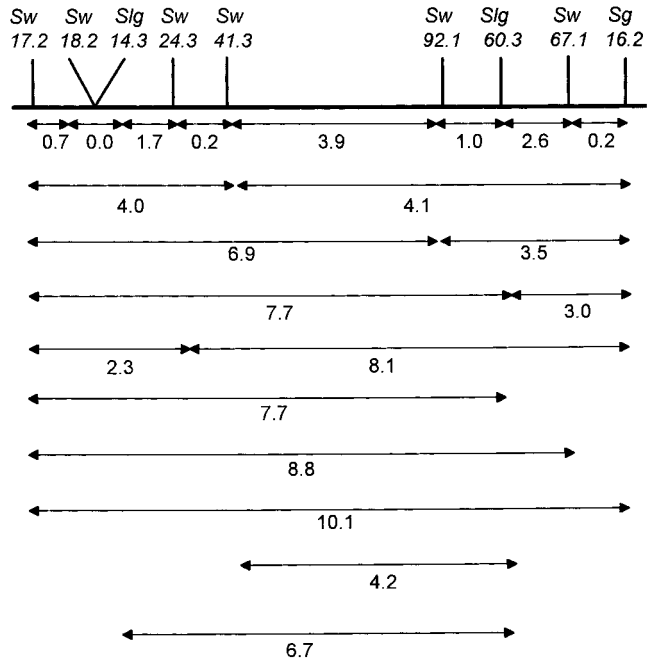


Figure 1.—Maps of the ascospore color mutations, not to scale. They are based on a series of repulsion-phase dihybrid crosses, sampling about 800 asci per cross for frequencies of parental ditypes, nonparental ditypes and tetratypes. Because of differences in recombination frequencies between SFS and NFS strains, separate maps are given for the two types of strain. In the combined map, only distances from SFS × NFS crosses are given. The two mutations not shown, *Sg21.3* and *Sg4.3*, are closely linked to some of the mutations shown but are only loosely linked to others, not fitting reliably on the combined map. In mutation names, *S* indicates Saleem, *w* indicates white ascospores, *g* indicates gray and *lg* indicates light gray. The first part of the number refers to the ascus number and the second part to the ascospore number.

for the SFS and NFS strains were 2.03 and 0.32%, respectively, a 6.3-fold difference. The difference in the frequency of accumulated mutations therefore cannot be only because of the difference in spontaneous mutation frequencies: the SFS environment is therefore more mutagenic than the NFS environment. Mutation-inducing factors in the wild include ultraviolet light from solar radiation and extremes of temperature, with much more radiation and greater temperature extremes on the SFS than on the NFS. Different rates of mutation induction might therefore be expected from different environmental conditions on the SFS and NFS, in the direction found. However, the inherited difference in spontaneous mutation frequencies between SFS and NFS strains under standard lab conditions must have internal inherited genetic causes, such as different effi-

ciencies of DNA repair or of proofreading systems at replication.

The unexpected small reductions in mutation frequencies between Selfed Generations 1 and 2 for ascospore color did not occur for acriflavine resistance, so it may not be a general effect. There were even some increases in mutation frequency for acriflavine resistance between Selfed Generations 1 and 2 within each slope, for example, 1997 data, 250 μg/ml, NFS ($P < 0.05$), but most were not significant.

Stability of mutation frequencies and of mutations: Although mutations arising at different times during colony growth could account for some heterogeneity between repeats or replicates, the fact that most replicates and repeats were homogenous for mutation frequencies suggests that this phenomenon had little effect on within-slope or between-slope results, especially as between-slope differences were consistent across all three repeats.

In this colonial fungus, there are many millions of nuclei in one Petri dish culture, and thousands even in a fragment of one hypha. A vegetative subculture will be started by very large numbers of nuclei, so genetic drift should be minimal and the number of subcultures before making crosses should not be crucial; this was borne out by the data, where results for N6, with 16 subcultures, were very similar for those of N5(i) and (ii), with only four subculturings since isolation from the wild. We have not found any marked changes in mutation frequencies from prolonged storage (more than one year) of strains at 4°, nor from vegetative subculturing, either for wild strains (which can be heterokaryotic) or for strains reisolated from ascospores, which are homokaryotic initially. A sexual ascospore will start a new isolate from a single haploid nucleus; the two nuclei in the spore come from mitosis of a single meiotic product.

During growth in monoculture on defined medium in the lab at 18° or during storage at 4 to 6°, the strains are not exposed to the natural mutagens of UV or extreme temperatures, nor to antibiotics from other fungi or bacteria, nor to any mutagenic chemicals in the natural environment. Induced mutations from such sources should therefore be absent from Selfed Generation 1 and 2 strains and should not arise during lab subculturing of wild or derived strains.

Kitani and Olive (1969) stated that they found no spontaneous mutants for the *g* (*gray* ascospore) locus in lab cultures of their 19 American and Canadian strains of *S. fimicola*. That seems strange after 10 or more years of work, as color mutants were induced by UV and it is generally accepted that mutations that can be induced can also occur spontaneously, although with lower frequencies. Of the *S. fimicola* ascospore color mutations of known origin listed in Catalogue 1996, only five were spontaneous, with 44 induced, so the Evolution Canyon strains probably have higher mutation frequencies than those from elsewhere. The induced mutations of Olive (1959) did not revert spontaneously or with UV, which would be unexpected unless they were all large deletions.

We do not know whether our strains differ from Olive's or from each other in mutagenic transposons or insertion sequences or in genetic mutator effects, and comparative studies would be useful. If such effects existed in our strains, then the color mutants that we isolated should often carry such mutagenic factors, when one might expect a high rate of reversion from color mutants to black ascospores. None of our color mutants showed a high rate of reversion: selfed crosses of such mutants showed no revertants in samples of over 20,000 asci from each of 11 color mutants.

Factors affecting the frequency of accumulated mutations, including selection: The frequency of accumulated mutations in hyphae from the wild depends on environmental conditions, the colony's age and size, and whether there is selection in hyphae for or against mutations for ascospore color genes. In the harsher SFS environment, vegetative strains are likely to die sooner than in the milder NFS, with re-establishment from ascospores when conditions are favorable. One would thus expect the SFS strains to be on average "younger," more recently from ascospores, than those on the NFS, so SFS strains would on average have had less time in which to accumulate mutations than would NFS strains. Therefore different times for accumulation of mutations do not explain the observed higher frequency of accumulated mutations in the SFS strains than in the NFS strains, whereas different levels of mutagenicity do explain it.

Those ascospore genes that are only expressed during sexual reproduction should be selectively neutral in hyphae. Some ascospore color genes, however, have pleiotropic effects vegetatively. Thus Olive (1959) found that his *g* (gray ascospore) mutant had lighter hyphal pigmentation than wild type, and some ascospore color mutants in the present study also had paler hyphae than wild type. We found that the hyphal pigmentation was much darker when strains were grown in the dark than when grown in the light, the opposite of what one expects if dark hyphal pigments were giving protection from mutagenic effects of light. There could be a reversed selection on the basis of heat absorption, with dark pigments absorbing more heat from light than light pigments. If most mutations to lighter ascospore pigmentation exist as a very minor proportion of nuclei in heterokaryons with wild-type nuclei, they are unlikely to have much effect on the colony phenotype even if they have different amounts of hyphal pigment when homokaryotic, so they will usually be selectively neutral, like those with no vegetative effects. Acriflavine resistance, however, could be selectively disadvantageous in hyphae not exposed to the drug, which could explain

the very low frequency of accumulated acriflavine mutations in strains from the less mutagenic NFS.

In Evolution Canyon, strains of coprophilous fungi growing on the NFS are usually shaded by shrubs and other plants, but those on the exposed SFS are usually unshaded (Nevo 1995, 1997). We have no information on whether hyphae have different amounts of pigment in the two environments. Hyphae of SFS strains will often get direct sun exposure when on the surface of dung or soil, but be shaded when deeper. Dehisced ascospores will usually be exposed on surfaces. Lamb *et al.* (1992) showed that the black pigment of wild-type Sordaria ascospores gave much better UV protection than lesser amounts of pigment in mutant spores. For example, a UV dose of 96,000 ergs/cm^2 had no effect on the germination of wild-type ascospores, but gave only 49% germination for gray (g^-) and only 11% for hyaline (clear) (h^-) ascospores. Some, but not all, ascospore color mutations have much poorer germination than wild-type, so they would be selected against during sexual reproduction.

Whether selection for or against ascospore color mutations occurs vegetatively is important when we consider the frequencies of such mutations. Those that are not expressed vegetatively should be selectively neutral, and having many thousands of nuclei per hypha should mean that they do not fluctuate much from genetic drift in hyphae. Those that have some vegetative effect but which are heterokaryon-recessive to the much larger number of wild-type nuclei in the hyphae will also undergo little selection vegetatively. Only if they become frequent in relation to wild-type nuclei could selection have much effect on them. The only situation where one would expect strong selection is if some mutations are heterokaryon-dominant even over much larger numbers of wild-type nuclei in hyphae, and if they affect hyphal survival or growth rate. A. Farouk (personal communication to B. C. Lamb) found that the different original wild-type strains grew under lab conditions at approximately the same rates irrespective of their slope of origin, but some ascospore color mutants grew more slowly.

Theoretical aspects: Because most mutations are harmful, the increased production of beneficial mutations must outweigh the increased production of harmful mutations when higher mutation frequencies are selected in a particular environment, such as the south-facing slope. Kimura (1967) pointed out that on average a higher mutation rate is deleterious for short-term effects, so that a modifier that enhances the mutation rates of other genes will be selected against through intragroup selection, but if the mutation rate is too low, the species will not be able to cope with environmental changes. He suggested that there is an optimum mutation rate for the survival of a species, depending on how rapidly the environment changes, and the SFS is more changeable than the NFS. Kimura (1967) proposed that the mutation rate is adjusted in evolution in such a way that the sum of the mutation and substitutional load is minimized.

Mutation-rate evolution is vital for evolutionary theory (Korol *et al.* 1994). Under panmixis, zero mutation is the evolutionary stable state, though biologically impossible, but in a frequently changing environment, selection may favor the spread of modifiers causing nonzero mutation (Gillespie 1981; Ishii *et al.* 1989). Holsinger and Feldman (1983) showed that a nonzero optimum mutation rate was possible in systems with complete or partial selfing in a constant environment. For reviews of stress, habitats and evolutionary rates, see Parsons (1994) and Hoffmann and Parsons (1991). For a game-theory approach to mutation rates and for the importance of coevolutionary pressures, see Maley (1997). The spore color mutations in the present study are not "adaptive mutations" in the sense of Hall (1997), in that they are not specific to the challenge of selection, with only advantageous mutations arising.

Recent experimental (Sniegowski *et al.* 1997) and theoretical (Taddei *et al.* 1997) studies of mutation rates in asexually reproducing clonal populations—such as in *Escherichia coli*—adapting to new environments, have shown that mutator genes can accelerate adaptation even if the mutator gene remains at low frequency. Moxon and Thaler (1997) summarized ways in which an organism might control mutation rates by means of genes affecting DNA metabolism and processing and circumstances in which altering mutation rates might be advantageous.

S. fimicola usually has selfed perithecia; it has no gametes, but fusion of vegetative hyphae can lead to mixing of different nuclei in the multinucleate hyphae, and crossed perithecia can then result (Olive 1956). It differs from the systems modelled in the studies quoted above in that it is vegetatively haploid and multinucleate, with the diploid nucleus undergoing meiosis soon after formation in the perithecium, with no diploid mitosis and no diploid hyphae. This means that recessive or incompletely recessive alleles produced by mutation, and recessive or incompletely recessive mutations in loci controlling mutation frequency, are not hidden or partly hidden by dominance as they would be in a diploid organism with uninucleate cells. Their expression, however, would be affected by alleles dominant in multinucleate haploid hyphae, although chance or selective changes in nuclear ratio during hyphal growth and branching could give hyphal sections in which the mutant nuclei were the only or the major type, allowing them to be expressed and therefore be subjected to selection.

For a colonial fungus that can persist as hyphal fragments in soil or as dormant ascospores, one cannot really specify population sizes. One cannot say how many individuals there are on a piece of dung, as colonies derived from separate ascospores may fuse into one or

more large mycelium. On dung one gets a succession of different fungal species, with hyphae of different species coexisting even when no fruit bodies of the species are visible, and Sordaria can live saprophytically as well as on dung, often with no visible fruit bodies. Sexual reproduction occurs largely on dung, at the appropriate time in the fungal succession—see Webster (1980) for a biological summary. With Sordaria having such a different biology from many diploid organisms, such as Drosophila, which have discrete individuals that only reproduce sexually and among whom dominance is more important and population size better defined, some of the classic theoretical analyses of mutation and selection may not be completely applicable to homothallic, colonial, haploid, fungi like Sordaria.

The higher mutation rate to ascospore color mutants in the more light-exposed SFS strains will decrease fitness when producing less-pigmented mutations but could perhaps increase fitness if darker spores were produced. Octads of ascospores with four spores of the usual black-gray wild-type color and four spores darker than that were occasionally found. Because there was an almost continuous variation between normal wild-type spores and darker variants, the darker forms were not scored as separate color mutants, thereby slightly underestimating the mutation frequencies. The color intensity in wild-type spores looked the same in SFS and NFS strains under standard lab conditions, with some variation within a perithecium between different asci, which could be at different stages of maturity.

It seems likely that the differences between NFS and SFS strains in mutation frequencies are part of a general control of mutation frequencies, not a specific control for ascospore mutations as such, especially as the acriflavine-resistance results showed the same trends, in the same direction over all three generations tested, as the ascospore color loci. Acriflavine resistance would be irrelevant in the wild.

One might predict that the strains exposed to greater UV levels might evolve more efficient repair systems, unless a higher mutation rate generally in that environment was advantageous. The SFS strains, when removed from that environment and reisolated from ascospores, actually showed higher spontaneous mutation frequencies than the NFS strains, which would fit with a higher mutation frequency being beneficial in the harsher environment.

Strains from dung or from soil: For ascospore color mutations, the mutation frequencies were higher in strains from soil than from dung. Such a difference is expected for accumulated mutations if hyphae from dung came more recently from ascospores (germinating in the dung after passage through a cow's alimentary canal) than did hyphae from soil. In the Selfed Generation data in Table 2, however, the higher mutation frequencies within slopes for strains from soil (S3, N5(i)) than for strains from dung (S1, N7(i)) persisted from the wild strains into Selfed Generations 1 and 2, showing that the differences were in inherited spontaneous mutation rates, not just in accumulated mutations.

The mutation frequency difference for strains isolated from dung compared with strains from soil did not occur for acriflavine resistance during ascospore germination in the same strains, so it may not be a general effect, especially as rather few strains were involved in the color mutations test.

Gene flow between slopes: There are no asexual reproductive propagules for dispersal in *S. fimicola*, and the ascospores dehisce over distances of <10 cm (Webster 1980). Some gene flow between SFS and NFS populations could occur for this coprophilous fungus, because browsing goats and cattle can move between the slopes. Movement of ascospores or hyphal fragments in rainwater is unlikely between the two slopes, as gravity would only give transport from the upper levels to the lower levels of the same slope. Any selection pressure for higher mutation rates on the SFS must be strong enough to overcome equalizing effects from any gene flow between the slopes. Gene flow between and within populations would be restricted by genes limiting fusion of different isolates (Carlile and Watkinson 1994); such genes are known in *S. fimicola* (Olive 1956). L. S. Olive found that different strains were often not cross-fertile. We found that some SFS strains would not cross with any NFS strains, and vice versa, and that some strains from one slope crossed with some but not all strains from the opposite slope, so there are some barriers to gene flow, but most SFS strains crossed well with NFS strains.

Substantial gene flow by migration between the slopes would lead one to expect that strains from the bottoms of the two slopes would be more alike than ones at the tops of the slopes, but that was not the case (Table 1). The mutation value for ascospore color of strain S3 from the bottom of the SFS (4.41%) was most different from the NFS values (0.89 to 1.27%). If substantial gene flow had occurred between the slopes, then mutation frequencies would be more evenly distributed between the slopes. If any of the strains sampled had been a heterokaryon from vegetative fusion of NFS and SFS strains, then it could have had an intermediate level of mutation frequencies, and Selfed Generation strains derived from it could show large mutation frequency differences between colonies derived from different ascospores. The differences in Table 2 between different Selfed Generation 1 strains from the same wild strain were usually small, whether they came from the same ascus, for example, N5(1;2.1) and N5(1;2.3), or from different asci, for example, S3(1;1.2) and S3(1;2.4), where the second number within the brackets indicates the ascus number and the third is the ascospore number within that ascus. None of the eight SFS Selfed Generation 1 or 2 strains, or of the ten NFS Selfed Generation 1 or 2 strains, had mutation frequencies deviating much

from typical values for that slope and generation, so there was no evidence of mixed-origin strains.

Mapping and recombination: The mapping data (Figure 1), with few recombination distances <1 cM, showed that a number of different ascospore color loci were involved in the mutation studies. All 11 mutants tested proved to be in the same linkage group, which was unexpected as spore color loci occur in several different linkage groups (Catalogue 1996). Any loci for which color mutants give poor ascospore germination would be scored in the mutation work but would be underrepresented in mapping data, as isolation of strains for mapping requires ascospore germination. The mapping results, with all 11 color mutations being linked, suggest that there are more than 16 loci for ascospore color. Although 4.41% seems very high for a mutation frequency, it is a total, not an average, over more than 16 spore-color loci. One 4+:4 *mutant* octad represents the fusion of one mutant and one nonmutant nucleus from the hyphae, so one must divide by more than 32 if comparing the results with conventional analyses at single loci. Although it would be useful to obtain mutation frequencies separately for the 16 or more different ascospore loci in this fungus, that is difficult because mutants at different loci may be very similar in color, and different mutations at a single locus may also differ, as with the *gray* locus having both hyaline and gray mutants (Catalogue 1996). Normal *cis*/*trans* allelism tests are not possible for mutations only affecting haploid ascospores. Most mutant spores were white, with some light gray or gray; a few were brown and a very few blue or green, with no yellow spores.

Derzhavets *et al.* (1996) found about four times as much (0.29%) male recombination in *Drosophila melanogaster* flies from the SFS than in ones from the NFS (0.07%) from Evolution Canyon, even after many generations in lab culture. That suggests that selection for recombination properties was sufficiently strong to overcome equalizing effects of migration even for such mobile organisms as fruit flies.

That the SFS strains are genetically different from the NFS strains in processes affecting genetic variation, and that migration does not overcome selection, was shown by the ascospore color mutation and acriflavine results and is also supported by the recombination results (M. Saleem and B. C. Lamb, unpublished results). The SFS strains, from the more stressful environment, all had higher frequencies than the NFS strains for crossing-over and gene conversion, in accordance with the working hypothesis.

Overall findings: There was natural genetic variation for mutation frequencies between strains, both for inherited spontaneous mutation frequencies and for accumulated mutation frequencies. The results showed different mutation frequencies between different strains from the wild, where the time between a colony's formation and its isolation could not be controlled, and also showed highly significant differences in mutation frequency between different Selfed Generations 1 and 2 strains, where the time, number of subcultures and total amounts of growth were carefully controlled and equal.

For ascospore color mutations, there were clear-cut, nonoverlapping differences between slopes for three SFS strains and five NFS strains in the original wild cultures, and for eight Selfed Generations 1 and 2 SFS strains and 10 Selfed Generations 1 and 2 NFS strains. These differences between strains and slopes were also found for acriflavine resistance, again with the SFS strains having much more mutation than the NFS strains. The difference between slopes was in the direction one would predict, so the evidence for environmentally related differences in mutation frequency is strong.

The south-facing slope conditions were more mutagenic than those on the north-facing slope. The highly significant and consistent differences in inherited spontaneous mutation frequencies for ascospore color and acriflavine resistance between strains from the south- and north-facing slopes may have a selective value in adapting the strains to their particular microscale environment, with more mutation in the harsher, more variable environment. The results displayed interslope mutation variation, probably in relation to environmental stress. They agree well with other findings from Evolution Canyon, especially those of Nevo (1995, 1997) on genetic diversity in diverse unrelated taxa, from multilocus allozymes and DNA diversities, and with those of Derzhavets *et al.* (1996) on recombination frequency in male *D. melanogaster*. In general, the evolutionary forces causing and maintaining genetic diversity are more pronounced in Evolution Canyon on the ecologically more heterogeneous and stressful south-facing slope. Environmental stress appears to be a major force driving evolution, with the more diverse and patchy habitat on the SFS also being relevant.

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