Evidence for Mutation and Migration as Sources of Genetic Variation in Populations of *Rhynchosporium secalis*

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ABSTRACT

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Four isolates of the barley scald disease pathogen, *Rhynchosporium secalis*, that differed both for pathogenicity to 14 barley differential cultivars and for isozyme genotype were coinoculated onto barley plants in the field and the greenhouse to test for mutation, migration, and parasexual recombination. Ten isozyme genotypes and 37 pathotypes were identified among 96 isolates recovered from 10 field plots. Most (62%) of the isolates recovered in the field experiment had an isozyme genotype like that of one of the four inoculated isolates. Among the remaining isolates were six different isozyme genotypes, two of which were probably introduced by migration; the other four could have arisen by mutation, migration, or parasexual recombination. Most of the new pathotypes probably arose by mutation or migration. Fifteen single-spore derivatives of the original isolates had changes for pathogenicity, which were almost certainly the result of mutation, to one or more of the differential cultivars, but there were no changes in isozyme genotype among single-spore derivatives. The four original isolates were also inoculated pairwise onto a susceptible barley cultivar in the greenhouse. No isozyme recombinants were found among 240 isolates recovered after two asexual generations; three greenhouse isolates were definitely migrants; one was a mixed culture of parental isozyme genotypes; and the remaining 236 were parental types. Among more than 1,000 isolates analyzed in this and two other studies, no three-banded (heterodimeric) isozyme phenotypes (indicating heterozygotes at intermediate stages in the parasexual cycle) were found. Taken together, these results indicate that parasexual recombination, if it occurs at all, probably plays at most a small role in generating genetic variability in *R. secalis* populations in nature. However, there is clear evidence that mutation and migration are very important, and the mutation rate for pathogenicity appears to be very much higher than that for isozymes.

Additional keywords: Deuteromycetes, leaf blotch.

There are at least four mechanisms that can increase genetic and/or genotypic diversity in populations of deuteromycete fungi: mutation, migration, an unobserved sexual stage, or parasexual recombination. Perhaps the most intriguing of these is the parasexual cycle. Briefly, in haploids, the parasexual cycle begins with the fusion of two compatible hyphae to form a heterokaryon (20). Occasionally, the nuclei fuse to form a diploid; and in a small proportion of diploids, the haploid condition is regenerated by the loss of duplicate homologous chromosomes. Mitotic recombination and the random assortment of chromosomes in the diploids lead to new haploid genotypes. Variations of the parasexual cycle can lead to higher rates of recombination in some fungi (1); in theory, the range of new genotypes generated by the parasexual cycle can be equal to that produced by sexual recombination.

In parasexual analysis, auxotrophic mutants that cannot survive separately on minimal media are usually used for the production of heterokaryons (4,12,16,23). Even under these artificial conditions, the fusion of nuclei in the heterokaryons, mitotic crossing over, and haploidization of diploid nuclei are each rare events (20). Although parasexual recombination has been demonstrated in a wide range of fungi in the laboratory (20), it remains speculative whether or not it occurs under natural conditions.

One fungus in which parasexual recombination has been postulated is *Rhynchosporium secalis* (Oudem.) J. J. Davis. This deuteromycete causes scald disease (also known as leaf blotch) on barley (*Hordeum vulgare* L.), rye, and a number of wild grass species (22). Scald occurs throughout the world and can cause economically important yield loss. Part of the difficulty in the genetic control of scald is that populations of *R. secalis* are extremely variable. For example, more than 350 pathogenic races (pathotypes) have been identified in California alone (3) on the basis of their ability to infect 14 barley cultivars with different resistance genes. This wide diversity of pathotypes could have been generated in part by parasexual recombination. Jackson and Webster (11) attempted to test this hypothesis by coinoculating five pathotypes on a susceptible barley cultivar in the greenhouse. After two asexual generations, only 17 among 100 reisolates had a pathotype like one of the original five pathotypes. Most of the remaining 83 isolates were different from one or more of the original pathotypes on one or two of the 14 barley differential cultivars, but some had undergone change on up to six of the differentials (11). Because the pathogenicity assay appeared to be clear and repeatable, the authors concluded that the changes must have been the result of some unknown mechanism(s) of recombination and segregation. Similarly, Newman and Owen (18) reported what they believed were asexual recombinants in one of 20 lesions resulting from coinoculation with isolates of *R. secalis* that differed for isozymes of esterase and β-glucosidase (β-GLU).

Unfortunately, the markers used in these experiments did not have the resolving power to distinguish mutants and migrants from parasexual recombinants. Most of the isolates recovered by Jackson and Webster (11) differed on only one or two of the differential cultivars; mutation may be a more likely explanation for these changes than recombination. Migration was also a possible source of new pathotypes, particularly those that had

changes on many of the differential cultivars compared with the original five isolates. A similar problem occurred with the isozyme markers used by Newman and Owen (18), where the putative recombinants had an esterase pattern like that of one parent but a β-GLU pattern like that of the second parent. The only change from the first parent involved the gain of a β-GLU band. However, the genetic interpretation of this polymorphism was not clear (17), and such a change could have arisen just as easily from mutation or migration as from parasexual recombination. Furthermore, the apparent rates of parasexual recombination reported in both of these studies were high compared with those in other fungi (20). Neither of these studies adequately tested the alternative hypotheses of mutation or migration and therefore provided no convincing evidence for parasexual recombination in *R. secalis*.

The purpose of this study was to test the hypothesis that parasexual recombination occurs in *R. secalis* as well as the alternative hypothesis that mutation and/or migration could be sources of genetic variation within populations. Isozyme and pathogenicity markers were used in both the field and the greenhouse. Isozymes are codominant and often heterodimeric; presence or absence of a heterodimer band can be used to distinguish heterokaryons from physical mixtures of cultures. Detection of heterodimer bands or of novel genotypes that were unlikely to have arisen by mutation or migration would provide strong evidence for parasexuality in *R. secalis*. A secondary goal was to quantify the rate of parasexual recombination, if detected. The possibility of an unobserved sexual cycle in *R. secalis* seemed very unlikely under our experimental conditions and so will not be considered further in this paper.

MATERIALS AND METHODS

Growth and characterization of isolates. Isolates of *R. secalis* were obtained from infected barley leaves by transferring single, germinated conidia or wefts of mycelium to potato-dextrose agar (PDA) plates (8). Isolates were subsequently maintained on PDA slants at 15 C in the dark, and transfers to fresh PDA slants were made approximately every 6 mo. Barley seedlings were inoculated with conidia grown on PDA plates (6). Pathogenicity testing was done with 14 barley differential cultivars (10). Five or six seedlings of each differential cultivar plus eight to 10 seedlings of the susceptible cultivar UC 566 (C.I. 15477) were grown in large metal flats and spray inoculated (at the two- to three-leaf stage) with 50 ml of a suspension containing approximately 2×10^5 conidia per milliliter. Inoculated seedings were placed in a mist chamber at 17 C for 2 days and then removed to a greenhouse bench until symptoms developed. Each inoculated seedling was scored for disease after 10–14 days on a scale of 0–4 (10), where 0 = no symptoms, 1 = very small lesions confined to leaf margins, 2 = small lesions not confined to leaf margins, 3 = large coalescing lesions, and 4 = total collapse of the leaf. Differentials with scores of 0–2 were considered resistant, while those with ratings of 3 or 4 were considered susceptible. Generally, all seedlings of each cultivar reacted uniformly, so disease scoring was straightforward. Only those inoculations that resulted in large lesions (scores of 3 or 4) on the susceptible control were scored. Inoculation results that were ambiguous or that could not be repeated were deleted from further analysis. Pathotypes were named by an octal nomenclature (7). These inoculation techniques have been shown to give clear, repeatable results (10). Starch gel electrophoresis to distinguish isozymes of glucose-6-phosphate isomerase (GPI), phosphoglucomutase (PGM), cytosol (leucine) aminopeptidase (LAP), catalase (CAT), and β-GLU was as described by Goodwin et al (8). From two to four isolates with known genotypes were run on each gel as controls.

Field experiment. The field plots were from the first year of a 3-yr experiment to determine the effect on disease of mixtures of barley lines inoculated with a variable pathogen population, as described by McDonald et al (15). Plots were planted with equiproportional (by number) mixtures of seed of barley lines that varied in resistance to four pathotypes of *R. secalis* (Table 1), races 40, 61, 72, and 74 of Jackson and Webster (10). These correspond to pathotypes 03475, 36317, 36777, and 37777, respectively, in the octal nomenclature (7). Host plots were inoculated with a mixture of conidia from all four pathotypes approximately 10 wk after planting (15). Inoculations were carried out in the evening with a backpack sprayer. Leaves containing scald lesions were sampled randomly from the plots 10 wk after inoculation, and the fungus was reisolated as indicated above. Disease levels varied widely among plots (15), and infected leaves were collected from plots with different host compositions to obtain a representative sample of the diversity present in the total experiment. These isolates were tested for pathogenicity on the 14 barley differential cultivars and for electrophoretic variation at the four isozyme loci *Gpi, Pgm, Lap,* and *Cat*. Not all of the isolates tested for isozyme genotype were also tested for pathogenicity and vice versa, so sample sizes for the different markers varied.

To test for mutation, single-spore isolations were made from the original parent cultures of the isolates of races 40, 61, and 74 by isolating single, germinated conidia (10). These isolates were then tested for pathogenicity and isozyme genotype as described above.

Greenhouse experiments. Seedlings of the scald-susceptible barley cultivar UC 566 were grown to the five- to six-leaf stage in a greenhouse in metal flats in UC soil mix C-2 (14). There were approximately 100 seedlings per flat. The four isolates used in the field experiment, races 40, 61, 72, and 74 (Table 1), were used for inoculations in all six pairwise combinations plus the four-way combination. Conidial concentrations were adjusted so that the final concentration of conidia of each isolate in the binary mixtures was $5–7.5 \times 10^5$ conidia per milliliter. There were 2.5×10^4 conidia per milliliter for each isolate in the four-way mixture. Four replications containing approximately 100 seedlings each (400 seedlings total) were inoculated with 400 ml (100 ml per flat) of each conidial mixture. To minimize contamination, different mixtures were inoculated at least 2 days apart, so only one mixture was in the inoculation chamber at a time. As an additional precaution, flats inoculated with different mixtures were grown on separate greenhouse benches and were surrounded by 3-ft-high wooden frames that were covered with plastic sheeting on all four sides to prevent splash dispersal of conidia during watering.

All lesions were harvested approximately 4 wk after inoculation and stored in the laboratory for an additional 10–12 wk to simulate

TABLE 1. Pathogenicity of the original four parent isolates of *Rhynchosporium secalis* and of 15 single-spore progeny from the isolates of races 40, 61, and 74

Parent isolate[a]	Pathogenicity[b]	Changes from original		Number recovered	Octal name
		Gains	Losses		
Race 40	000011100111101	...	...	...	03475
Race 61	011110011001111	...	...	...	36317
Race 72	011110111111111	...	...	...	36777
Race 74	011111111111111	...	...	...	37777
Race 40	000011101111101	1	0	4	03575
	000011110111101	1	0	1	03675
	000011111111101	2	0	4	03775
Race 61	000001011000101	1	6	1	01305
	100011011001101	2	4	1	43315
Race 74	000011111111111	0	3	3	03777
	001011111111111	0	2	1	13777

[a] The original reactions as reported by Jackson and Webster (10) are indicated in the first four rows. New pathotypes identified among single-spore derivatives from three of the original four isolates are shown in the bottom seven rows.

[b] According to the octal nomenclature proposed by Goodwin et al (7). Pathogenicity to the differential cultivars C.I. 5831, C.I. 2376, Kitchin (C.I. 1296), Osiris (C.I. 1622), Steudelli (C.I. 2266), Atlas (C.I. 4118), Turk (C.I. 5611-2), Trebi (C.I. 936), Modoc (C.I. 7566), Hudson (C.I. 8067), Brier (C.I. 7157), Wisconsin Winter × Glabron (C.I. 8162), Atlas 46 (C.I. 7323), La Mesita (C.I. 7565), and the susceptible cultivar UC 566 (C.I. 15477) are listed from left to right, where 1 = pathogenicity and 0 = nonpathogenicity.

the saprophytic stage of the fungus as it occurs in nature (11). Only those combinations that produced at least 50 lesions per flat (200 lesions total) were saved for the second set of inoculations. The infected leaves then were moistened with distilled water and suspended on plastic screens over water in plastic crispers at 15 C for 4 days to induce sporulation of the fungus. Conidia were harvested by macerating the sporulating tissue in a Waring blender in a small amount of water and straining it through two layers of cheesecloth. The resulting suspensions were inoculated onto another 400 seedlings of UC 566 as described above to allow for completion of the parasexual cycle. Lesions were harvested approximately 3 wk after the second inoculation, and *R. secalis* was isolated as indicated above. The isozyme genotypes of these isolates at the five loci, *Gpi, Pgm, Lap, Cat,* and *β-Glu,* were determined as described by Goodwin et al (8). Isozyme analysis provided repeatable, unambiguous identification of *R. secalis* genotypes (Fig. 1).

RESULTS

Variation in single-spore derivatives. Nine single-spore derivatives of the race 40 isolate had the same isozyme genotype as the original race 40 control. However, there was variation in pathogenicity among single-spore cultures of the isolates of races 40, 61, and 74 (the race 72 isolate was not tested). Single-spore derivatives from the race 40 isolate had all gained pathogenicity to cultivars Trebi and/or Modoc (Table 1) for a mean change of +1.4 differentials. The original race 40 mass transfer isolate had also gained pathogenicity to Modoc compared with its original assessment (10) more than 12 yr earlier. The two single-spore derivatives of the race 61 isolate showed both gains and losses of pathogenicity (Table 1), with a mean of 6.5 changes compared with the original description (10). Four single-spore derivatives from the race 74 isolate had lost pathogenicity to three (three isolates) or two (one isolate) of the differential cultivars (Table 1) for a mean of 2.8 changes. Overall, analysis of single-spore derivatives from the original three races yielded seven new pathotypes, with a mean of 2.5 changes from the original isolates. In contrast, no isozyme changes were observed in the single-spore derivatives or in any of the other cultures over a 2-yr period.

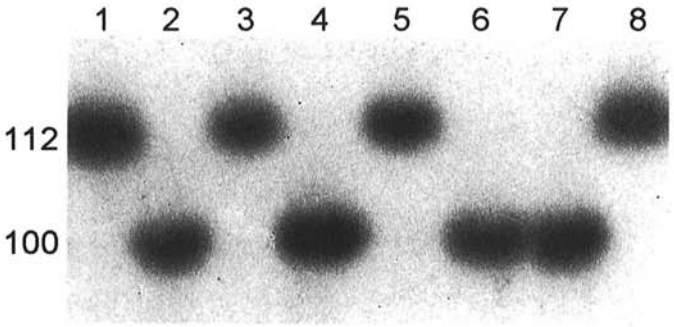

Fig. 1. Isozymes at the phosphoglucomutase (*Pgm*) locus of *Rhynchosporium secalis.* Lane numbers are indicated at the top. The isolates in lanes 1, 3, 5, and 8 have the *112* allele, while those in lanes 2, 4, 6, and 7 have the *100* allele. Isozymes provided clear, repeatable, unambiguous identification of *R. secalis* genotypes.

Field experiment. In all, 96 isolates were recovered from 10 plots in the field experiment. Isozyme analysis of 69 of these isolates revealed 10 multilocus genotypes (Table 2), four of which were the same as those possessed by the inoculated isolates of races 40, 61, 72, and 74 (62% of the sample). The remaining 38% of the isolates had isozyme genotypes that were not like those of any of the original isolates. These genotypes must have arisen by mutation, migration, or parasexual recombination. Two of the new isozyme genotypes (variants 4 and 6, Table 2) had combinations of alleles that could not have arisen by recombination between any pair of the original four isolates. These two genotypes were also at least two mutational steps away from any of the original isolates. Because of the vanishingly small probability of two independent mutations occurring in the same individual, migration was the most likely source of these genotypes. The four remaining genotypes (variants 1, 2, 3, and 5) might have arisen by pairwise recombination between the original isolates. For example, recombination between isolates 40 and 74 could have given rise to the variant 2 genotype. These genotypes were also only one mutational step away from one of the original isolates (Table 2). Two of these genotypes (variants 2 and 5) had four-locus isozyme genotypes that were identical to isolates collected previously in California, as revealed by comparison with a database of isolates described by Goodwin et al (8), so they could have been introduced by migration. Therefore, it was not possible to eliminate any of the three potential sources for the genotypes of variants 1, 2, 3, and 5.

Pathogenicity analysis of 68 isolates revealed even more diversity. Isolates with the same isozyme genotype often had different pathotypes (Table 2). A total of 37 pathotypes was identified, 31 of which were found only once (Table 3). Only one of the 68 isolates tested had a pathotype like that of one of the original four isolates. This isolate had the same pathotype as race 74, but its isozyme genotype was like that of the race 72 isolate. It was isolated from a plot planted with a barley line that was resistant to all inoculated races except race 72 (Table 2). Thus, none of the isolates tested had the same pathogenicity and isozyme genotype as any of the four inoculated isolates. Although the original four pathotypes were very complex (infecting from seven to 13 of the 14 differential cultivars), most of the recovered isolates had very simple pathogenicities; each infected only a few of the differential cultivars in addition to the universally sus-

TABLE 2. Frequency of *Rhynchosporium secalis* isozyme genotypes and the number of pathotypes per isozyme genotype of isolates recovered from field plots inoculated with isolates of races 40, 61, 72, and 74

Genotype name	Isozyme genotype[a]				Number recovered	Percentage of sample	Number of pathotypes[b]	Mean number of changes from closest inoculated pathotype[c]
	Gpi	*Pgm*	*Lap*	*Cat*				
Race 40	*100*	*100*	*100*	*86*	5	0.07	4/5	4.4
Race 61	*87*	*112*	*100*	*100*	35	0.51	17/23	7.9
Race 72	*87*	*100*	*100*	*100*	1	0.01	1/1	1.0
Race 74	*100*	*100*	*112*	*100*	2	0.03	1/2	11.0
Variant 1	*100*	*100*	*100*	*100*	2	0.03	1/1	3.0
Variant 2	*100*	*100*	*112*	*86*	15	0.22	4/6	5.2
Variant 3	*100*	*112*	...[d]	*100*	1	0.01	1/1	1.0
Variant 4	*100*	*112*	*112*	*86*	4	0.06	...	...
Variant 5	*87*	*100*	*100*	*86*	2	0.03	1/1	7.0
Variant 6	*87*	*112*	*112*	*86*	2	0.03	2/2	6.5

[a] Allele designations at each locus were as described by Goodwin et al (8). These isolates were not tested for β-glucosidase activity.
[b] Number of pathotypes/number tested for pathogenicity. Not all isolates that were tested for isozymes were tested for pathogenicity.
[c] Includes both gains and losses of pathogenicity.
[d] Not determined.

ceptible cultivar UC 566. For each isozyme genotype, the mean number of pathogenicity changes from the closest of the inoculated isolates ranged from one to 11 (Table 2). Five of the six pathotypes that were identified more than once occurred in more than one plot (Table 4) and/or with multiple isozyme genotypes. Isolates with the same pathogenicity were usually genetically heterogeneous on the basis of isozyme analysis.

Multiple isozyme genotypes and pathotypes were recovered from most of the field plots sampled (Table 4). Isolates with an isozyme genotype like that of the race 61 isolate were recovered from six of the 10 plots sampled. The next most common genotype, variant 2, was isolated from four plots. Isolates with an isozyme genotype like that of the race 40 isolate were recovered from two plots, and the remaining genotypes were each isolated only from a single plot. There was no clear association between isozyme genotype and pathogenicity to any of the 14 differential cultivars.

Greenhouse experiment. To provide the greatest opportunity for observing parasexual recombination among the seven inoculated combinations (six binary, one four-way), only those that produced large, coalescing lesions with ample opportunity for hyphal contact were saved for the second set of inoculations. Four combinations (40 and 61, 40 and 72, 61 and 72, and the four-way mixture) were discarded because the lesions resulting from the first set of inoculations were too small and too few to provide many opportunities for hyphal anastomosis. Two of the remaining three combinations gave good infection in the second set of inoculations. Among 240 isolates examined from these two combinations, none were recombinants; the vast majority (more than 98%) were parental types (Table 5). Most (83%) of the 121 isolates recovered from the mixed inoculation of the isolates of races 61 and 74 were identical to the race 61 isolate (Table 5). One isolate had a zymogram that was a combination of the genotypes for the isolates of races 61 and 74 but without heterodimer bands at the three heterozygous loci (Table 5). Therefore, it was almost certainly a mixed culture of both parents and not a hybrid. No heterodimer bands were observed at any locus in any of the 309 isolates (240 from the greenhouse and 69 from the field experiment) examined for this study.

TABLE 3. Pathotypes of *Rhynchosporium secalis* recovered from field plots inoculated with a mixture of conidia from isolates of races 40, 61, 72, and 74

Pathotype[a]	Pathogenicity[b]	Number recovered
00001	000000000000001	11
00401	000000100000001	2
01001	000001000000001	4
00005	000000000000101	1
00021	000000000010001	1
10003	001000000000011	1
01005	000001000000101	14
01101	000001001000001	1
00203	000000010000011	1
04003	000100000000011	1
10203	001000010000011	1
03005	000011000000101	1
01007	000001000000111	5
01015	000001000001101	1
01405	000001100000101	1
03007	000011000000111	2
41105	100001001000101	1
01445	000001100100101	1
01065	000001000110101	1
42225	100010010010101	1
01427	000001100010111	1
43017	100011000001111	1
03465	000011100110101	1
14323	001100011010011	1
11327	001001011010111	1
53107	101011001000111	1
53315	101011011001101	1
03675	000011110111101	1
57305	101111011000101	1
43707	100011111000111	1
53307	101011011000111	1
03775	000011111111101	1
53317	101011011001111	1
15737	001101111011111	1
15777	001101111111111	1
33777	011011111111111	1
37777	011111111111111	1

[a]Named according to the octal nomenclature of Goodwin et al (7).

[b]Pathogenicity to the differential cultivars C.I. 5831, C.I. 2376, Kitchin (C.I. 1296), Osiris (C.I. 1622), Steudelli (C.I. 2266), Atlas (C.I. 4118), Turk (C.I. 5611-2), Trebi (C.I. 936), Modoc (C.I. 7566), Hudson (C.I. 8067), Brier (C.I. 7157), Wisconsin Winter × Glabron (C.I. 8162), Atlas 46 (C.I. 7323), La Mesita (C.I. 7565), and the susceptible cultivar UC 566 (C.I. 15477) are listed from left to right, where 1 = pathogenicity and 0 = nonpathogenicity.

The race 74 isolate dominated the mixture when it was coinoculated with a race 72 isolate (Table 5); only one among the 119 isolates recovered was like the original race 72 isolate. Two additional genotypes were identified (Table 5). Because each had an allele at the *Pgm* locus (*112*) that was not present in either of the putative parent isolates, it seems unlikely they resulted from parasexual recombination. Both genotypes were also at least two mutations away from either of the parents, so it is extremely unlikely that they could have arisen by mutation. We consequently conclude that they were the results of migration. Two isolates had the same culture morphology and isozyme genotype as the race 61 isolate and thus were probably migrants from one of the other mixtures. The genotype of the remaining isolate was not like that of any of the inoculated isolates, so it was probably a migrant from outside the experiment.

The race 74 isolate was a component of both successful mixtures, and its fitness varied depending on the other isolate in the mixture. Fitnesses were estimated according to the method of Leonard (13) as described in Weltz and Leonard (24). The race 74 isolate had a competitive fitness of 0.37 compared with 1.0 for the race 61 isolate, while the race 72 isolate had a fitness of 0.09 compared with 1.0 for the race 74 isolate. Thus, the fitnesses of the isolates were ranked in the order $61 > 74 \gg 72$.

DISCUSSION

Mutation was probably a major source of pathogenicity changes in *R. secalis* populations. Single-spore derivatives of the original isolates had electrophoretic genotypes that were identical to those of the parent isolates but had changes in pathogenicity. These changes were almost certainly caused by mutation. These isolates had been in culture for more than 10 yr, and consequently, ample time had gone by for them to accumulate mutations even before the experiment began. In the field experiment, 37 different pathotypes were recovered, but there were only 10 isozyme genotypes, and most of the isolates (62%) had an isozyme genotype like that of one of the original four isolates. It appears very unlikely that the parasexual cycle could have rearranged the pathogenicities without altering the isozyme genotypes; consequently, many of these changes were also probably caused by mutation. Similar changes in pathogenicity have been reported by other investigators. Among 27 single-spore isolates of *R. secalis* made by Hansen and Magnus (9) from several parent isolates in Norway, 26% had pathogenicity changes from the original isolates. Jackson and Webster (11) reported gains in pathogenicity in one of their five control isolates after a single passage through the host, changes that were almost certainly caused by mutation. Differences in pathogenicity among *R. secalis* isolates from the same lesion in Australia (2) also may have been caused by mutation, although other mechanisms cannot be excluded in this case. There seems to be a much higher rate of mutation for pathogenicity than for isozymes in *R. secalis,* which suggests that imperfect fungal pathogens may have evolved mechanisms that increase the mutation rate for pathogenicity relative to other parts of the genome.

Migration also appears to be an important source of genetic variation in populations of asexually reproducing fungi. It is rather disturbing that migration occurred in the greenhouse experiment because great care was taken to prevent it. Inoculations were

carried out at least 2 days apart; only one combination was in the inoculation chamber at a time; and flats inoculated with different combinations were kept in separate enclosures on different greenhouse benches to prevent splash dispersal of conidia. Despite these precautions, 2.5% of the isolates recovered in the mixed inoculation of the isolates of races 72 and 74 were migrants. The two migrant genotypes are unlikely to have arisen from parasexual recombination because they had the *112* allele at the *Pgm* locus, which was not present in either of the parents. It is also unlikely that they were mutants because they would have needed mutations at two or more isozyme loci. One of the migrant genotypes was identical to the race 61 isolate in culture morphology and isozyme genotype and so was probably a contaminant from one of the other mixtures. The isozyme genotype of the other migrant was unlike that of any of the inoculated isolates, and so it almost certainly came from outside the experiment, either from other inoculation experiments or possibly as a seed contaminant. Osbourn et al (19) found that apparent changes in host adaptation in *Septoria nodorum* were also caused by migration. Evidently, efficient migration is common in fungi with splash-dispersed conidia.

Migration is also the probable source of the new electrophoretic genotypes in the field experiment. Two of the new genotypes could not have been generated by pairwise parasexual recombination or by single mutations, so were almost certainly migrants. They could have arisen by parasexual recombination involving three of the parents, but the lack of evidence for intermediates in the parasexual cycle or for parasexual recombination in general suggests this was extremely unlikely. It was not possible to exclude any of the potential sources of variation for the remaining four genotypes; they could have arisen from mutation, migration, or parasexual recombination. One of these new genotypes (variant 2) evidently had high fitness because it was recovered more frequently than three of the four inoculated isolates (Tables 2 and 4). Although parasexual recombination could not be eliminated as a possibility in every case, mutation and migration appear to be sufficient to explain all of the variation in both pathogenicity and isozyme markers seen in both the field and the greenhouse experiments.

No unequivocal evidence for parasexual recombination was

TABLE 4. Isozyme genotypes and pathotypes of *Rhynchosporium secalis* isolates recovered from field plots that were inoculated with isolates of races 40, 61, 72, and 74

Plot	Composition[a]	Isozyme genotypes[b]	Pathotypes[c]
19	RRSR	Race 72 (1)[d]	37777 (1)
22	SSSS RRSR	Race 61 (2), variant 6 (2)	00001 (2), 10003 (1), 10203 (1)
23	RRSR	Variant 2 (3), variant 5 (2)	00001 (1), 00401 (2)
24	SSSS	Race 61 (5), variant 2 (3)	00001 (2), 01001 (1), 00005 (1), 00021 (1), 01005 (2), 03005 (1), 03007 (1), 43017 (1)
36	SSSS RRSS SSSR	Race 61 (2)	01005 (1), 42225 (1)
39	RSSS SRSS SSSR	Race 40 (4), race 61 (20), race 74 (2), variant 2 (7), variant 3 (1), variant 4 (4)	00001 (6), 01001 (3), 01005 (9), 01101 (1), 00203 (1), 04003 (1), 01007 (5), 03007 (1), 41105 (1), 03465 (1), 14323 (1), 53315 (1), 03675 (1), 03775 (1), 15777 (1)
40	SSSS RRSR	Race 40 (1), race 61 (5), variant 1 (2), variant 2 (2)	01005 (2), 01015 (1), 01405 (1), 01445 (1), 01427 (1), 11327 (1), 57305 (1), 43707 (1), 53317 (1), 15737 (1), 33777 (1)
48	SSSS	Race 61 (1)	53307 (1)
123	SSSS	...[e]	53107 (1)
158	RSSS SRSS SSSR	...	01065 (1)

[a] Plots were planted with equiproportional mixtures of seed from one or more barley lines as described by McDonald et al (15). For example, there were two barley lines in plot 22, one line sensitive to each of races 40, 61, 72, and 74 (SSSS) and the other resistant to races 40 and 61, sensitive to race 72, and resistant to race 74 (RRSR).
[b] Isozyme genotypes correspond to those in Table 2.
[c] Pathotypes were named according to the octal nomenclature of Goodwin et al (7). In the octal nomenclature, race 40 is pathotype 03475, race 61 is pathotype 36317, race 72 is pathotype 36777, and race 74 is pathotype 37777.
[d] Number in parentheses is the number of times each genotype or pathotype was identified. Some isolates that were tested for isozymes were not tested for pathogenicity and vice versa, so the sample sizes for pathogenicity and isozymes are often unequal.
[e] Not determined.

TABLE 5. The results of pairwise mixed inoculations of *Rhynchosporium secalis* onto the highly susceptible barley cultivar UC 566

Isolates inoculated	Genotypes recovered	Isozyme genotype[a]					Number recovered	Percentage of sample
		Gpi	*Pgm*	*Lap*	*Cat*	*β-Glu*		
61 and 74	Race 61	*87*	*112*	*100*	*100*	*100*	100	82.7
	Race 74	*100*	*100*	*112*	*100*	*100*	20	16.5
	Mixture	*87/100*	*112/100*	*100/112*	*100*	*100*	1	0.8
72 and 74	Race 72	*87*	*100*	*100*	*100*	*105*	1	0.8
	Race 74	*100*	*100*	*112*	*100*	*100*	115	96.6
	Migrant (race 61)	*87*	*112*	*100*	*100*	*100*	2	1.7
	Migrant	*87*	*112*	*112*	*100*	*100*	1	0.8

[a] Allele designations at each locus are as described by Goodwin et al (8).

found in either of these experiments. Among the 240 isolates recovered in the greenhouse experiment, none were recombinants. This experiment should have been sufficient in scope to detect parasexual recombination if it occurred at high enough frequencies to account for the results of other investigators (11,18). One potential explanation for the lack of parasexual recombination is that the particular isolates chosen as parents were incompatible. It is also possible that because one parent in each combination had low fitness (particularly the race 72 isolate), opportunities for hyphal fusion were limited. However, neither of these explanations seems likely. Both Jackson and Webster (11) and Newman and Owen (18) obtained presumptive recombinants between randomly chosen isolates, and they reported no evidence for incompatibility. There also appeared to be large fitness differences among the isolates used in the previous studies (11,18) that evidently had no effect on the outcomes.

There was also no evidence for intermediates in the parasexual process. A major advantage of isozymes is that many enzymes are dimeric, and heterozygotes for dimeric enzymes produce three-banded phenotypes, i.e., two homodimer bands plus the heterodimer. The heterodimer band establishes that two alleles are being expressed in the same cell. Because heterokaryons and diploids will have a heterodimer band, they can be distinguished easily from the parents and from physical mixtures of cultures. Heterokaryons and diploids are essential intermediates in the parasexual cycle, and they are often stable (20). Therefore, if parasexual recombination occurred frequently in *R. secalis,* heterozygous intermediates should be common and easily detectable. At least four of the five enzymes in this study (GPI, PGM, LAP, and CAT) are known to be dimeric, at least in some organisms. However, among over 1,000 isolates tested from diverse geographical origins, 309 in this study, 150 from around the world (8), and 566 additional isolates from Idaho, Oregon, and California (5), none had a three-banded phenotype at any locus. Occasional isolates had two-banded phenotypes, but these always segregated into single-banded parental types in single-spore progeny (5,8). Therefore, if parasexual recombination occurs at all in *R. secalis,* it is apparently rare and increases genetic variation only slowly in natural populations.

On the basis of these results, it may be possible to reinterpret previous reports of changes in controlled populations of *R. secalis.* Jackson and Webster (11) coinoculated five pathotypes of *R. secalis* onto a susceptible barley cultivar. After two disease cycles, only 17% of the reisolates had the same pathogenicity as one of the original isolates. Most of the new pathotypes had only one or two pathogenicity changes from one of the five inoculated isolates (11). This is consistent with expectations for changes caused by mutation. The same parent isolates were more stable when inoculated singly. However, there were changes in one of the five single inoculations (11). Furthermore, the controls were passed through the host only once, whereas the mixed inoculation was passed through leaves twice. It seems likely that more changes would have occurred in the control experiments had there been additional repetitions of host passages. Finally, Jackson and Webster (11) reported no particular efforts to prevent contamination. Migration is the most likely source for the pathotypes that had many changes from the original five isolates. A major problem with earlier experiments was that the pathogenicity markers used did not have the resolving power to distinguish mutants from migrants from parasexual recombinants. We conclude, therefore, that mutation and migration, rather than recombination, are the most likely explanations for virtually all of the pathogenicity variants detected in earlier experiments.

A similar reinterpretation can be made for the results of Newman and Owen (18), who reported what they believed were asexual recombinants of *R. secalis* with isozymes of esterase and β-GLU. The putative recombinants were identical to one of the parents, except for an extra band for β-GLU that they believed could have come from the other parent. However, the isozyme markers used in that study were too few to distinguish among the three potential sources of variation. Present results suggest that the observed changes were more likely to have been caused by mutation or migration rather than by parasexual recombination (although migration seems unlikely in this case). Another problem is that the isozymes scored by Newman and Owen (18) had multiple-banded phenotypes that were difficult to interpret (17). Goodwin et al (8) reported that esterase isozymes are highly variable in *R. secalis* and not repeatable, even with subcultures of the same isolate. On starch gels, there were five zones of β-GLU activity (5), four of which were faint and highly variable. The fifth band was always present and had a true polymorphism with two alleles (8). The β-GLU polymorphism scored by Newman and Owen (18) was evidently associated with two of the faint bands. It is not clear whether these represent real genetic differences or result from other factors. Therefore, the results of Newman and Owen (18) probably do not provide evidence for parasexual recombination in *R. secalis.*

Another potential explanation for these results is that there may be variations in the parasexual cycle that differ from classical parasexuality as described for *Aspergillus nidulans* (20). For example, in *Verticillium* (21), *Fusarium,* (16) and *Magnaporthe* (4), anastomosis between hyphae of auxotrophic mutants permitted prototrophic growth on minimal medium, but isolations from single hyphal tips and single conidia yielded only nonrecombinant parental types. Evidently, "single-cell" heterokaryons occurred proximal to the colony margin that fed the growing tips of the mycelia (21). The single-cell heterokaryons did not grow to produce stable heterokaryotic mycelia. Karyogamy occurred very rarely, and diploid colonies were recovered only by plating large numbers of conidia onto selective media (4,16,21).

In *Trichoderma,* colonies that differed from the parents in morphology and nutritional requirements were obtained after protoplast fusion (23). However, there was no recombination for isozyme markers, and as was the case with *R. secalis,* heterodimer bands were not observed at any of the isozyme loci (23). These authors concluded that there could be alternatives to the classical parasexual cycle. One possibility they proposed was that one nucleus in the heterokaryons might be degraded and small pieces of DNA incorporated into the other nucleus (23). Another possibility is that nonparental types arise from random assortment between nuclei and cytoplasmic inclusions (e.g., mitochondria and mycoviruses) during vegetative growth (23). However, many of the changes observed by Stasz and Harmon (23) also could have been caused by mutation. Any or all of these mechanisms could occur in *R. secalis.* Clearly, much remains to be learned about the mechanisms by which new genetic variation arises in deuteromycete fungi.

The original goal of the field experiment was to follow the changes in frequency of the four pathotypes in response to host mixtures containing different combinations of resistance. However, this goal was not attainable because of apparently high mutation rates for pathogenicity and because it was not possible to control for migration. Only one isolate had a pathotype like that of one of the original four races (race 74); subsequent analysis revealed that this isolate had an isozyme genotype like that of the race 72 isolate. Therefore, none of the isolates recovered from the field experiment was identical in pathotype and isozyme genotype to any of the original four isolates. There were also large fitness differences among the isolates of races 61, 72, and 74 on a susceptible host in the greenhouse that correlated with the rate of recovery of each genotype from the field experiment. These intrinsic differences in parasitic fitness might obscure some differences caused by host genotype in the field. Therefore, pathogen populations in inoculated field experiments must be monitored carefully if the experiment is designed to test particular isolates. For example, the lack of a mixture effect in the majority of mixtures reported by McDonald et al (15) could have been caused by a change in the pathogen population; host lines chosen for resistance to particular isolates might have been ineffective against the genotypes that were actually present and caused disease. Because of mutation and migration in deuteromycete fungi, experiments that report changes in pathogen populations that are not verified with unambiguous genetic markers must be interpreted cautiously.

LITERATURE CITED

1. Bonatelli, R., Jr., and Azevedo, J. L. 1992. A system for increasing variability in filamentous fungi? Fungal Genet. Newsl. 39:90-91.
2. Brown, J. S. 1985. Pathogenic variation among isolates of *Rhynchosporium secalis* from cultivated barley growing in Victoria, Australia. Euphytica 34:129-133.
3. Crandall, B. A. 1987. Differentiation and distribution of *Rhynchosporium secalis* on composite cross populations and pureline cultivars in California. Ph.D. diss. University of California, Davis.
4. Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics 114:1111-1129.
5. Goodwin, S. B. 1988. Analysis of gene-for-gene interactions, and variability for isozyme and pathogenicity markers in *Rhynchosporium secalis*. Ph.D. diss. University of California, Davis.
6. Goodwin, S. B., Allard, R. W., Hardy, S. A., and Webster, R. K. 1992. Hierarchical structure of pathogenic variation among *Rhynchosporium secalis* populations in Idaho and Oregon. Can. J. Bot. 70:810-817.
7. Goodwin, S. B., Allard, R. W., and Webster, R. K. 1990. A nomenclature for *Rhynchosporium secalis* pathotypes. Phytopathology 80:1330-1336.
8. Goodwin, S. B., Saghai Maroof, M. A., Allard, R. W., and Webster, R. K. 1993. Isozyme variation within and among populations of *Rhynchosporium secalis* in Europe, Australia and the United States. Mycol. Res. 97:49-58.
9. Hansen, L. R., and Magnus, H. A. 1973. Virulence spectrum of *Rhynchosporium secalis* in Norway and sources of resistance in barley. Phytopathol. Z. 76:303-313.
10. Jackson, L. F., and Webster, R. K. 1976. Race differentiation, distribution, and frequency of *Rhynchosporium secalis* in California. Phytopathology 66:719-725.
11. Jackson, L. F., and Webster, R. K. 1976. The dynamics of a controlled population of *Rhynchosporium secalis,* changes in race composition and frequencies. Phytopathology 66:726-728.
12. Jinks, J. L. 1952. Heterocaryosis in wild *Penicillium*. Heredity 6:77-87.
13. Leonard, K. J. 1969. Selection in heterogeneous populations of *Puccinia graminis* f. sp. *avenae*. Phytopathology 59:1851-1857.
14. Matkin, O. A., and Chandler, P. A. 1957. The U.C.-type soil mixes. Pages 68-85 in: The U.C. System for Producing Healthy Container-Grown Plants. K. F. Baker, ed. Calif. Agric. Exp. Stn. Man. 23.
15. McDonald, B. A., Allard, R. W., and Webster, R. K. 1988. Responses of two-, three-, and four-component barley mixtures to a variable pathogen population. Crop Sci. 28:447-452.
16. Molnár, A., Sulyok, L., and Hornok, L. 1990. Parasexual recombination between vegetatively incompatible strains of *Fusarium oxysporum*. Mycol. Res. 94:393-398.
17. Newman, P. L. 1985. Variation amongst isozymes of *Rhynchosporium secalis*. Plant Pathol. 34:329-337.
18. Newman, P. L., and Owen, H. 1985. Evidence of asexual recombination in *Rhynchosporium secalis*. Plant Pathol. 34:338-340.
19. Osbourn, A. E., Scott, P. R., and Caten, C. E. 1986. The effects of host passaging on the adaptation of *Septoria nodorum* to wheat or barley. Plant Pathol. 35:135-145.
20. Pontecorvo, G. 1956. The parasexual cycle in fungi. Annu. Rev. Microbiol. 10:393-400.
21. Puhalla, J. E., and Mayfield, J. E. 1974. The mechanism of heterokaryotic growth in *Verticillium dahliae*. Genetics 76:411-422.
22. Sprague, R. 1950. Diseases of Cereals and Grasses in North America. Ronald Press, New York.
23. Stasz, T. E., and Harmon, G. E. 1990. Nonparental progeny resulting from protoplast fusion in *Trichoderma* in the absence of parasexuality. Exp. Mycol. 14:145-159.
24. Weltz, H. G., and Leonard, K. J. 1993. Phenotypic variation and parasitic fitness of races of *Cochliobolus carbonum* on corn in North Carolina. Phytopathology 83:593-601.