Phenotypic Variation and Parasitic Fitness of Races of *Cochliobolus carbonum* on Corn in North Carolina

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ABSTRACT

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Two field populations of *Cochliobolus carbonum* on corn (*Zea mays*) in the piedmont of North Carolina, where races 2 and 3 coexist, were sampled repeatedly (four times during 51 days in the Wilkes County field and three times during 32 days in the Yadkin County field) during 1987. Isolates were tested for lesion type and several polymorphic traits, including mating type, fertility, and fungicide tolerance. The relative fitness of race 3, as determined from frequency changes, was significantly lower than the fitness of race 2 in each field. The fitness values of race 3, with an estimated generation time of 7 days, were 0.82 and 0.84 versus 1.0 for race 2. A third race, race 0, was also common in the Wilkes County field and had a relative fitness of 0.42. The mean frequency of race 3 was 29% in the Wilkes County field, 30% in the Yadkin County field, and 92% in a third field in the Appalachian Mountains in Tennessee that was sampled only once in 1987. These data show that the frequency of race 3 in the piedmont and mountains changed little from 1977 to 1987, and parasitic fitness alone cannot account for the distribution of race 3. The frequencies of seven polymorphic traits and the genetic diversity within races 2 and 3 remained stable over the 1987 sampling period, and race frequencies changed, suggesting the phenotypic traits are selectively neutral. As in previous years, trait frequencies differed significantly between races, indicating their genetic isolation. Within races, however, there was evidence that sexual reproduction may have occurred. In races 0 and 3, there were no indications of gametic phase disequilibrium typical of asexual populations. In race 2, there were no significant associations of pairs of traits, but some phenotypes occurred significantly more frequently than was expected based on the frequencies of their component traits. Also, there were significant differences in phenotype frequencies between mating types MAT-1 and MAT-2 in race 2. The two mating types differed more from a 1:1 ratio in race 2 than in races 0 and 3, suggesting that sex may be less important in race 2 than in races 0 and 3.

Additional keywords: *Bipolaris zeicola*, *Helminthosporium carbonum*, maize.

Cochliobolus carbonum R.R. Nelson (anamorph: *Bipolaris zeicola* (G.L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is a frequent foliar pathogen of corn (*Zea mays* L.) in North Carolina (16) and in many temperate corn-growing areas of the world (28). Pathogenic races 1, 2, and 3 have been described based on lesion type on corn. Race 0, which is nearly avirulent on corn, has been reported in North Carolina (33). Recently, Dodd and Hooker (4) reported a fifth race (designated race 4) that produces larger than usual lesions on some corn lines in the midwestern United States.

Race 1 produces the host-specific HC-toxin and induces large lesions on corn genotypes homozygous for the *hm1* gene, which conditions sensitivity to HC-toxin. Toxin-sensitive corn hybrids are no longer grown commercially, and race 1 has declined in frequency to less than 1% (13), probably because unnecessary toxin production confers a fitness disadvantage to race 1 (14). On corn lines and hybrids not sensitive to the toxin, the lesions caused by race 1 resemble those of race 2, small, round to oval necrotic spots. Race 2 is prevalent in most *C. carbonum* populations that have been sampled (13,16,17).

Race 3 induces long, linear lesions on most corn lines and hybrids and is more frequent than race 2 in the Appalachian Mountains from Georgia to Pennsylvania. It was first reported in 1973 (22) but has prevailed, at least in the mountains of Virginia, for more than 35 years (15). Race 3 caused concern when it extended its range from the mountains into eastern corn-producing regions of North Carolina during the 1970s (13). Repeated surveys (15,17), however, revealed that the increase of race 3 east of the mountains in North Carolina stopped for reasons that are unclear.

Races 2 and 3 are morphologically similar but genetically distinct. Although these races can be crossed readily in the laboratory, there is no evidence of hybridization between races in the field (15). The distinctive lesion type of race 3 is inherited polygenically (3), but no isolates with intermediate lesion types have been found in the field. Leonard and Leath (15) cited the significant differences in frequencies of genes for cycloheximide tolerance and ability to form pseudothecia in races 2 and 3 as additional evidence against sexual reproduction between these races in the field. Also, the apparently nonrandom associations of numerous traits in race 2 were taken as evidence against sexual reproduction within race 2 in the field (15). On the other hand, field populations of both races 2 and 3 have maintained the two mating types at relatively stable frequencies (between 30 and 70%). The persistence of both mating types in all populations sampled suggests either frequency-dependent selection, typical of sexual populations, or another type of stabilizing selection affecting mating-type alleles independently of the sexual cycle.

In previous surveys, race 2 was genetically more diverse than was race 3, and genetic diversity within populations in different fields of the same region varied greatly depending partially on the proportion of race 3 in the field (15). Calculated values of genetic distances between field populations of race 2 were not correlated with geographical distances between the fields, leading Leonard and Leath (15) to suggest that the traits studied were not selectively neutral.

In our study, we sampled *C. carbonum* populations sequentially during epidemics in fields in which both races 2 and 3 were known to occur to compare the relative fitness of races 2 and 3 in the field. We also sought to determine whether selection would lead to changes in trait frequencies and diversity within races and whether races 2 and 3 had remained genetically isolated in spite of their sympatry since earlier surveys.

MATERIALS AND METHODS

Sampling. Three fields were selected for sampling. Two were in the neighboring counties of Wilkes (elevation 285 m) and Yadkin (elevation 350 m) near the Blue Ridge escarpment of western North Carolina. These fields were in locations known to harbor both races 2 and 3 of *C. carbonum* (15,17). The third field was near Mountain City in eastern Tennessee (elevation 740 m), an area where race 3 predominates (17). There were four sampling dates (9 and 28 July and 10 and 28 August 1987). The Wilkes County field was sampled on all four dates, the Yadkin County field on the first three dates, and the Tennessee field only on 28 July.

Sampling was carried out systematically. A single leaf was taken per plant every 2 m within a row from three rows approximately 5–10 m apart. Each sample comprised 60 leaves. Leaves were picked without regard to symptoms. The oldest leaves that had not yet senesced were sampled to improve the probability of obtaining leaves infected by *C. carbonum* but not excessively colonized by saprophytes. The leaves were dried in a plant press and kept at room temperature (22–26 C), for up to 2 mo, until use.

Isolation. Three to four segments, 3- to 4-cm long, were cut from each leaf and immersed for 30 s in 70% ethanol followed by 30 s in 0.5% NaOCl to kill surface contaminants. The leaf segments were rinsed 30 s or more in tap water and incubated on moist filter paper in petri plates at 22–24 C with 12 h of light per day from two cool-white fluorescent lamps positioned approximately 80 cm above the plates. After 2–4 days, the leaf segments were examined under a dissecting microscope for colonies of *C. carbonum*. Single conidia were picked from conidiophores with a sterile needle and transferred to petri plates of potato-dextrose agar (PDA, 10 g of dextrose/L). Isolates were established by subculturing from mycelium at the margin of a single colony per plate 3 days after inoculation. Isolates with slow growth on PDA were transferred to potato-lactose agar (PLA, 10 g of lactose/L), which supported mycelial growth and conidiation, particularly of race 0 isolates, much better than did PDA. Isolates were stored as conidial suspensions in 30% glycerol at −70 C. Usually one isolate per leaf was kept for further experiments. Two isolates per leaf were kept only when the incidence of *C. carbonum* in a sample was low and when the two isolates were obtained from different leaf segments to assure their independence.

Race identification. Isolates were classified as race 0, 2, or 3 on the basis of their lesion type on seedlings of Pioneer Brand 3369A (a hybrid resistant to race 1) or inbred line N31 (susceptible to race 1). Comparative tests showed that lesion types and race designations were consistent on these hosts for races 0, 2, and 3. Seedlings were grown for 3–4 wk in the greenhouse, until the four- to six-leaf stage, for inoculation. Inoculum was prepared by washing conidia from 7- to 10-day-old cultures, one petri plate per isolate. The resulting conidial suspensions (about 20 ml per isolate at $1–20 \times 10^3$ conidia/ml) were filtered through four layers of cheesecloth to remove mycelial fragments. An atomizer (DeVilbiss Co., Somerset, PA) attached to an air pump was used to spray the conidial suspensions onto the seedlings. Atomizers were rinsed with 70% ethanol and tap water between inoculations with different isolates. Inoculated seedlings were incubated at 100% relative humidity in the dark in a moist chamber overnight. Lesion types were recorded 7 days after inoculation. Tests of isolates classified as race 0 were repeated to confirm that their lack of lesion production was not due to a failure of the inoculation.

Sporulation on PDA. Cultures with clearly restricted growth and poor sporulation on PDA were classified as PDA−. Colonies of PDA− isolates grew to less than 20% of the diameter of wild-type race 2 or 3 isolates and developed very few conidia on PDA, whereas PDA+ isolates grew normally and sporulated abundantly.

Mating type and fertility. All isolates were paired with albino tester isolates of *C. carbonum* (12) of known compatibility (mating types MAT-1 [A] and MAT-2 [a]) and fertility as described by Leonard and Leath (15). Each mating test consisted of one petri plate for each of the two mating-type testers, with two matings on senescent corn leaf disks on modified Sachs agar (8) per plate. Compatible matings of albino testers with isolates possessing a single gene for inability to form pseudothecia (21) formed only white pseudothecia; matings with isolates with the allele for pseudothecial production formed black, wild-type pseudothecia next to albino pseudothecia. After 20–25 days, five to 10 black pseudothecia from each mating containing them were crushed in a drop of water under a dissecting microscope to assess the presence or absence of asci and ascospores. Matings in which field isolates failed to form black pseudothecia, asci, or ascospores were repeated to confirm the results.

Fungicide tolerance. Isolate tolerances of 2 μg of cycloheximide [3-2-(3,5-dimethyl-2-oxocyclo-hexyl)-2-hydroxyethyl glutarimide], 100 μg of carboxin (5,6-dihydro-2-methyl-1,4-oxanthiin-3-carboxanilide), and 100 μg of cadmium acetate per milliliter were determined. Cycloheximide (CyhR) (18) and cadmium (CadR) (13,18) tolerances in *C. carbonum* are controlled by single genes, whereas carboxin tolerance (CrbR) is controlled by a single gene plus modifier genes (13). Fungicides from stock solutions were added to melted PLA in bottles immediately before pouring the media. For each field isolate, a single 5-mm-diameter mycelial plug from the margin of a 5- to 7-day-old culture was transferred to plates of the three fungicide-amended PLA media and to a plate of unamended PLA as a control. After 5 days, mycelial diameters of cultures on amended and unamended PLA were compared. Isolates with a colony diameter on fungicide-amended medium at least 20% as large as that on unamended PLA were considered tolerant to the fungicide, as described previously (13,15).

Data analysis. The relative fitness of the three races of *C. carbonum* was estimated with a simple discrete generation model from population genetics first used in plant pathology by Leonard (11) and extended by Bronson (2). In this model, fitness is assumed to be constant. Let W_q be the fitness of race q and let $W_p = 1.0$ be the fitness of a reference race p. If the relative frequency of q changes over n generations from q_0 to q_n while p_0 goes to p_n, then

$$\frac{q_n}{p_n} = \frac{q_0}{p_0} (W_q)^n$$

Transformation of the equation yields

$$\ln \frac{q_n}{p_n} = \ln \frac{q_0}{p_0} + n(\ln W_q)$$

This is a linear regression equation of the form $y = a + bx$ with a slope of $\ln W_q$. Thus, W_q, the fitness of race q, is equal to the antilog of the slope of the regression of q/p on n. Based on previous experience (17), the generation time (i.e., latent period of the isolates in the field) was estimated to be from 7 to 10 days throughout the time period studied (9 July to 28 August 1987). The number of elapsed generations, n, was derived from the generation time and the number of days elapsed between samplings. W_q was calculated both for an assumed generation time of 7 days and also for 10 days. Using a generalized logistic regression procedure (PROC CATMOD; 27) suggested by Østergaard (24) that employs a maximum likelihood technique, intercept and slope of the regression line were estimated simultaneously for all races and directly from the absolute race frequencies in the samples. An analysis of variance was performed on the estimates to test the fit of the model to the data. The

advantage of this technique over the simple regression used by Leonard (11) and Welz et al (34) lies in the fact that absolute frequencies can be utilized directly, allowing a weighting of subsamples according to their size.

Standard errors (m) of relative frequencies (p in a sample of size n) were calculated (30) as

$$m = \sqrt{\frac{p(1-p)}{n}}$$

Phenotype codes based on the binary scheme of Habgood (7) were assigned to individual character combinations of isolates within races. The coding, which was performed by the VIRULA program (31), replaced the seven-digit character formula. For example, a phenotype expressing the characters MAT-2, Psu+ (ability to form pseudothecia), Asc− (inability to form ascospores), CyhR, CrbS, CadS, and PDA− (inability to sporulate on PDA) has a seven-digit formula of 0,1,0,1,0,0,0 and is coded $0+2^1+0+2^3+0+0+0=10$.

The Shannon index of diversity (6) was calculated from the frequency of phenotypes within races as

$$H' = \sum_{i}^{n} p_i \ln(p_i)$$

in which p_i is the frequency of the ith phenotype and n is the total number of phenotypes found. Standard errors of H' and a t-statistic to compare H' values were calculated as described by Poole (25).

To study the genetic relationships among phenotypes, a phylogenetic tree was constructed with the UPGMA method (unweighted pair group method with arithmetic mean) (20) from a data matrix of similarity values by the program NTSYS-pc (26). We used the Dice index of similarity

$$F = \frac{2n_{ab}}{n_a + n_b}$$

in which F represents the proportion of characters shared by two phenotypes (n_{ab}) among the sum of characters that phenotype a and b express (n_a and n_b).

To determine whether the phenotype frequency distributions were homogeneous between the two mating types of each race, a homogeneity test (10) was performed. In this test, two sample distributions are considered homogeneous if their observed character frequencies are homogeneous with their expected character frequencies ($E_{ij} \geq 1$).

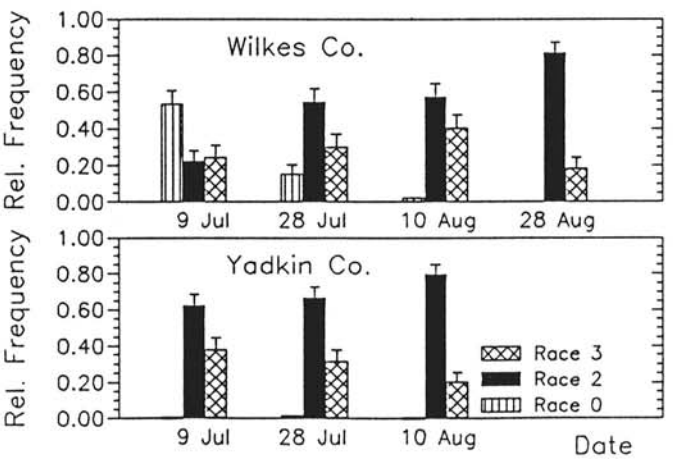

Fig. 1. Relative frequencies of races 0 (vertically hatched bars), 2 (filled bars), and 3 (cross hatched bars) of *Cochliobolus carbonum* in sequential samples of leaves from two corn fields in Wilkes and Yadkin counties, NC. Standard errors of frequencies are indicated.

Gametic phase disequilibrium of phenotypic traits was tested in two ways. First, expected phenotype frequencies were computed using the VIRULA program (31) and were compared to the observed phenotype frequencies in the sample by a chi-square test. Expected phenotype frequencies were computed as the product of individual character frequencies in the sample. For example, consider the two characters MAT-1 and Psu+. There are 2^2 possible phenotypes (MAT-1/Psu+; MAT-1/Psu−; MAT-2/Psu+; and MAT-2/Psu−). If frequencies of MAT-1 and Psu+ in a sample were 0.4 and 0.3, respectively, the expected phenotypes of the four possible phenotypes would be 0.12, 0.28, 0.18, and 0.42. Likewise, VIRULA computed the expected frequencies (E_{ij}) of all 128 ($=2^7$) phenotypes that are possible with seven dichotomous characters. As above, phenotype classes were combined so all $E_{ij} \geq 1$, to increase the accuracy of the chi-square test. As a second test of gametic phase disequilibrium or association of phenotypic characters, observed and expected frequencies of pairwise character combinations were computed by a G-test of independence as described by Alexander et al (1). This test was also implemented in the VIRULA program.

RESULTS

Frequency and fitness of races. The incidence of *C. carbonum* was high in each of the eight field samples. The lowest proportion of *C. carbonum*-infected leaves/total leaves was 40/60 (median 48/60). Among 90 isolates that were tested on inbred line N31, a genotype sensitive to the HC-toxin, no race 1 was detected. Races 2 and 3 occurred in each sample, and race 0, a newly described race (33) that causes only flecks or very small lesions on all corn genotypes tested, was found on 21 of 60 leaves in the first sample taken from the Wilkes County field. A striking feature of race 0 isolates was their poor growth on PDA. Race 0 colonies rarely grew beyond a diameter of 2 cm, had a dark-brown, leathery appearance, and rarely formed conidia. This caused difficulties early in the study, when PDA was the standard medium used. By screening various carbohydrate sources, we found that race 0 isolates grew without restriction and formed conidia abundantly on media containing lactose instead of dextrose (32). In the Yadkin County field, only a single race 0 isolate was found on 28 July (Fig. 1). Among 52 isolates from the Tennessee field, one was race 0, only three were race 2, and 48 were race 3.

The frequency of race 0 declined rapidly in the Wilkes County field to below the detection level on the last sampling date. Race 2 increased steadily in both the Wilkes and Yadkin fields (Fig. 1). Race 3 first increased gradually in the Wilkes County field at the expense of race 0 but later declined as it did from the first sampling date in the Yadkin County field (Fig. 1). The simple selection model closely fit the frequency data (Fig. 2), as indicated by the nonsignificant likelihood ratios ($P = 0.532$ for both 7-

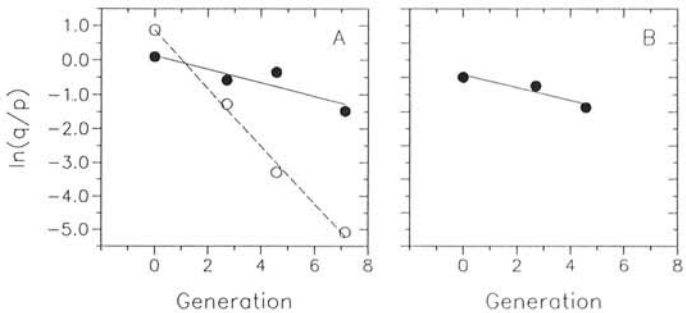

Fig. 2. Change in frequency of races 0 (open circles) and 3 (filled circles) of *Cochliobolus carbonum* relative to race 2 in **A,** a Wilkes County and **B,** a Yadkin County field in North Carolina. Data are expressed as log frequency of race 0 (or 3) divided by log frequency of race 2 and are plotted against the number of generations, based on an estimated generation time of 7 days. Race frequencies are presented in Figure 1. Regression lines were estimated by a generalized logistic regression model. Only one isolate of race 0 was found in the Yadkin County field.

and 10-day generation times in the Wilkes County field and $P = 0.266$ for both generation times in the Yadkin County field). The fitness of races 0 and 3 was significantly lower than that of race 2 in the Wilkes County field, where all three races occurred, and the fitness of race 3 was significantly lower than that of race 2 in the Yadkin County field, where only one isolate of race 0 was found (Table 1).

The calculated fitness values depend, of course, on the estimated generation time. Considering that mean summer temperatures in western North Carolina are approximately 20–22 C (17), we think that 7 days is a better estimate than 10 days for generation time for *C. carbonum* on corn. The fitness values of race 3 in the two fields were similar (Table 1; Fig. 2)

TABLE 1. Relative fitness[a] of races 0, 2, and 3 of *Cochliobolus carbonum* in two North Carolina corn fields

	Fitness in Wilkes Co. field		Fitness in Yadkin Co. field	
Race	7 days/gen.	10 days/gen.	7 days/gen.	10 days/gen.
0	0.42*** [b]	0.29***	...[c]	...
2	1.00	1.00	1.00	1.00
3	0.82**	0.76*	0.84*	0.78*

[a] Relative fitness of races 0 and 3 relative to race 2 was estimated from a generalized logistic regression model assuming constant fitness and a mean generation time (days/generation) of either 7 or 10 days.
[b] Fitness values significantly less than 1.0 are indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).
[c] Race 0 was recovered only once from the Yadkin Co. field.

Distribution phenotypes among races. Within each race, several distinct phenotypes were found (Table 2). We identified 14 phenotypes among 33 isolates of race 0, of which 13 occurred only in race 0; 19 phenotypes were identified among 185 isolates of race 2; and 11 phenotypes were identified among 146 isolates of race 3. Several phenotypes, particularly the more frequent ones, were shared by races 2 and 3. The genetic distinctiveness of race 0 is mainly due to cadmium tolerance (CadR) and inability to sporulate on PDA (PDA−), which were expressed by almost all isolates of race 0 but by very few isolates of race 2 or 3 (Table 2). The relative genetic similarity of the three races is further illustrated by the phylogenetic tree for the 352 phenotypes that were found more than once. At a similarity value of 0.64, the phylogenetic tree has four branches (Fig. 3). The uppermost branch contains exclusively race 0 phenotypes. The second branch contains phenotypes 64 and 72, which share traits MAT-2, Psu−, Asc−, CrbS, CadS, and PDA+. The third branch contains phenotypes with Psu+, Asc+, CadS, and PDA+, and the fourth branch contains phenotypes with Asc−, CrbR, CadS, and PDA+. Of the 352 isolates represented in the tree, 254 (72%) fall into the fourth group.

Phenotype-frequency distributions varied between races (Fig. 4). Individual phenotypes of race 0 were found either one, two, four, or seven times in a pattern of decreasing numbers of phenotypes at increasing numbers of times found. Races 2 and 3 had a more skewed distribution, with five or six phenotypes found only once and single phenotypes found 10 or more times. Pheno-

TABLE 2. Binary codes[a] for phenotypes and their absolute frequencies[b] among isolates of races 0, 2, and 3 of *Cochliobolus carbonum* from four sampling times in two corn fields in North Carolina and one sample from a corn field in Tennessee

	No. of isolates of race			Presence or absence of character[c]						
Code	0	2	3	MAT-1	Psu+	Asc+	CyhR	CrbR	CadR	PDA+
10	1			0	1	0	1	0	0	0
24	1			0	0	0	1	1	0	0
31	2			1	1	1	1	1	0	0
40	4			0	0	0	1	0	1	0
41	4			1	0	0	1	0	1	0
42	2			0	1	0	1	0	1	0
43	2			1	1	0	1	0	1	0
46	2			0	1	1	1	0	1	0
57	1			1	0	0	1	1	1	0
58	1			0	1	0	1	1	1	0
59	7			1	1	0	1	1	1	0
62	1			0	1	1	1	1	1	0
63	4			1	1	1	1	1	1	0
64		2		0	0	0	0	0	0	1
71		2		1	1	1	0	0	0	1
72		8	1	0	0	0	1	0	0	1
73		1		1	0	0	1	0	0	1
74			1	0	1	0	1	0	0	1
75			1	1	1	0	1	0	0	1
78		4		0	1	1	1	0	0	1
79		1	1	1	1	1	1	0	0	1
80		30	1	0	0	0	0	1	0	1
81		8		1	0	0	0	1	0	1
86		3		0	1	1	0	1	0	1
87		7		1	1	1	0	1	0	1
88		48	49	0	0	0	1	1	0	1
89		24	71	1	0	0	1	1	0	1
90		1	7	0	1	0	1	1	0	1
91		6	9	1	1	0	1	1	0	1
94		27	3	0	1	1	1	1	0	1
95		10	2	1	1	1	1	1	0	1
104		1		0	0	0	1	0	1	1
107		1		1	1	0	1	0	1	1
120	1			0	0	0	1	1	1	1
122		1		0	1	0	1	1	1	1

[a] Binary codes computed according to the scheme of Habgood (7).
[b] Frequencies represent sums over all field samples ($n = 364$ isolates); isolates for which complete phenotype data were not determined are not included.
[c] 1 indicates the character is present; 0 indicates the alternative character (allele) is present. The characters are: MAT-1, mating type 1; Psu+, ability to form pseudothecia; Asc+, ability to form ascospores; Cyh, tolerance of cycloheximide; CrbR, tolerance of carboxin; CadR, tolerance of cadmium; and PDA+, ability to sporulate on potato-dextrose agar.

types of race 3 were either frequent (found 49 or 71 times) or rare (found less than 10 times), whereas race 2 phenotypes had a more continuous distribution of frequencies (Fig. 4). The lack of evenness in distribution of frequencies of race 3 phenotypes also can be seen in the plot of diversity-index values shown in Figure 5. At each sampling time, the Shannon index of diversity was significantly lower for race 3 than for race 2 in the fields in both Wilkes and Yadkin counties. It was only in the Tennessee field that race 3 appeared to be as diverse as race 2, but in that field, only three isolates of race 2 were found, compared with 49 of race 3.

Diversity did not change dramatically over time within any of the race populations (Fig. 5). Only in race 3 in the second sample from Yadkin County was there a significant decrease in the Shannon index ($t = 3.15$, $P < 0.01$). Three phenotypes (codes 72, 90, and 91) found once or twice in the first Yadkin County sample were missing from the second sample.

Frequencies of polymorphic traits. The frequencies of most of the polymorphic traits remained relatively constant over the sampling periods in Wilkes and Yadkin counties. The frequency of PDA+ in race 0 was somewhat higher in the second than in the first sample from the Wilkes County field (Fig. 5, PDA+). No PDA− isolates were found among race 2 or 3 isolates from any of the samples. The frequency of CadR remained relatively unchanged. Most race 0 but very few race 2 isolates and no race 3 isolates were CadR (Fig. 5, CadR). Nearly all race 3 isolates from all samples were CrbR, whereas a small but consistent proportion of the race 2 isolates were CrbS (Fig. 5, CrbR). Also, nearly all race 3 isolates were CyhR, but CyhS in race 2 fluctuated around 30% (Fig. 5, CyhR). All race 0 isolates were CyhR, but a high proportion were CrbS, especially in the second sample from the Wilkes County field.

Clear differences existed between races 2 and 3 in characters controlling mating capacity and fertility (MAT, Psu, and Asc). In both fields, MAT-1 was more frequent in race 3 than in race 2 (Fig. 5, MAT-1), but fewer isolates of race 3 could form pseudothecia (Fig. 5, Psu+) or asci and ascospores (Fig. 5, Asc+). Isolates unable to form pseudothecia also were classified as incapable of forming asci or ascospores. Most (54 of 63) race 2 isolates that formed pseudothecia also formed asci and ascospores in those pseudothecia, whereas few (six of 24) race 3 isolates that formed pseudothecia also were capable of forming asci and ascospores. All of the *C. carbonum* isolates were male-fertile and induced compatible, female-fertile tester isolates to form fertile pseudothecia in mating tests.

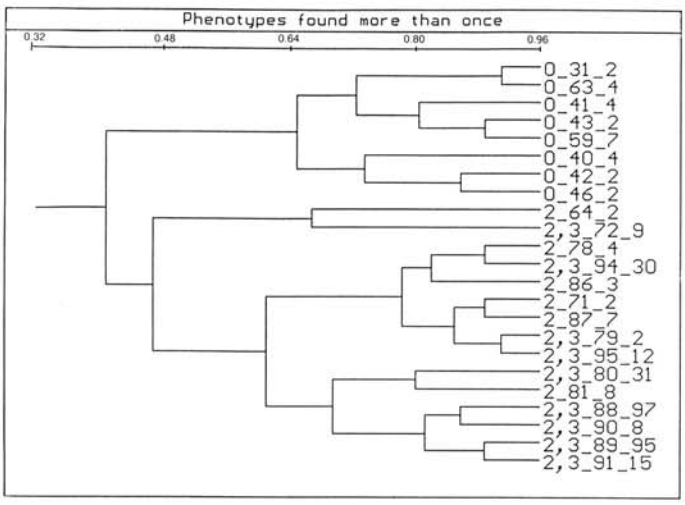

Fig. 3. Phenogram of relationships among phenotypes of *Cochliobolus carbonum* isolates from two corn fields in North Carolina and one in Tennessee. The phenogram is based on the Dice index of similarity and the unweighted pair group method with arithmetic mean. Numbers at the right indicate race_phenotype code_number of times found. The character combinations represented by each phenotype code are listed in Table 2.

The fertility classes were more nearly balanced in race 0 than in races 2 and 3. Among the 33 race 0 isolates collected, nine formed pseudothecia and ascospores, 13 formed pseudothecia but not ascospores, and 11 did not form pseudothecia. Thus, race 0 appeared to be intermediate between races 2 and 3 in fertility in mating tests.

Gametic phase disequilibria. The frequency of identified phenotypes was compared between the two mating types for races 0, 2, and 3 (Table 3). If each race represented a randomly mating subpopulation of *C. carbonum* in the fields that we sampled, phenotype-frequency distributions should be homogeneous between the MAT-1 and MAT-2 subgroups of each race. This, in fact, was observed with races 0 and 3 (Table 3), but phenotypes of race 2 occurred at different frequencies in the two mating types.

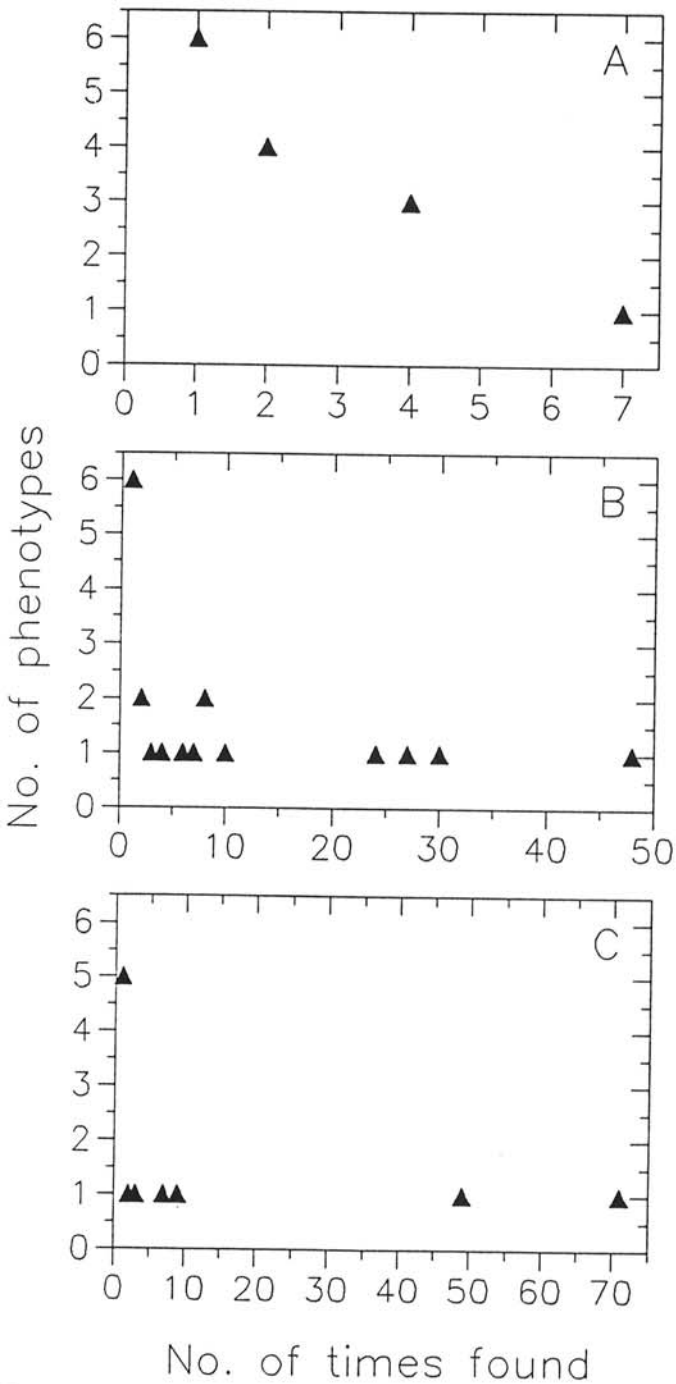

Fig. 4. Comparison of numbers of phenotypes in different frequency classes for races **A,** 0, **B,** 2, and **C,** 3 of *Cochliobolus carbonum* among isolates collected from two corn fields in North Carolina and one in Tennessee ($n = 364$)

Comparison of observed and expected phenotype frequencies (Table 4) suggests that the populations of races 0 and 3 are closer to gametic phase equilibrium than is the population of race 2. Observed phenotype frequencies did not deviate significantly from expected frequencies for race 0 or 3 in any of the field samples that yielded enough race 0 and 3 isolates for statistical analyses. With race 2, all three samples from the Yadkin County field and one of three samples from the Wilkes County field indicated heterogeneity (Table 4). When frequencies were pooled over samples within races or over races within samples, the respective populations appeared to be in even greater disequilibrium.

When only the association of two characters at a time rather than the association of characters in complete phenotypes was analyzed, the race 2 population also showed some deviation from equilibrium when all samples were pooled (Table 5). The character combinations MAT-1/Psu+ and Psu+/CyhR were observed more frequently than expected from chance association of these pairs of traits. When these two character combinations were inspected separately for fields (Table 6), however, their frequencies did not differ significantly from equilibrium. The Psu+/CyhR combination in the Wilkes County field deviated from the expected frequency, but one cell of the expected frequency table contained only four entries (Table 6), at least five are needed for an adequate G-test (29). With races 0 and 3, only two character combinations could be analyzed (Table 5), and both were found in equilibrium.

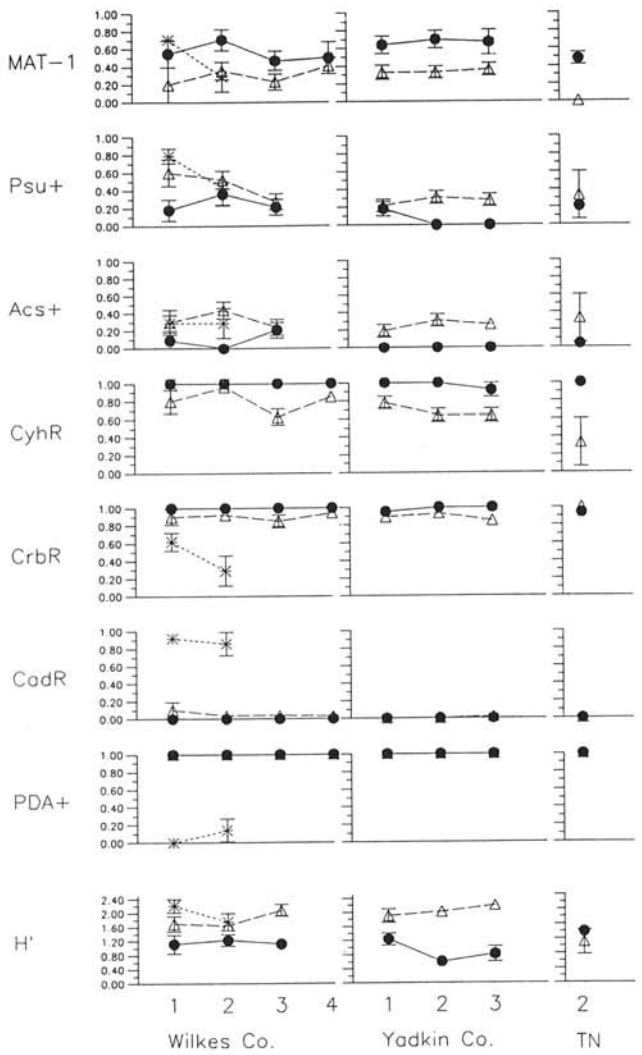

Fig. 5. Relative frequencies of seven phenotypic characters in races 0 (asterisks), 2 (triangles), and 3 (circles) among samples of *Cochliobolus carbonum* collected on four sampling dates in corn fields in Wilkes and Yadkin counties, NC and near Mountain City, TN. Samples 1, 2, 3, and 4 were collected on 9 and 28 July and 10 and 28 August 1987. Some data from Wilkes County sample 4 are missing. Phenotypic characters: MAT-1, mating type 1; Psu+, ability to form pseudothecia; Asc+, ability to form asci and ascospores; CyhR, tolerance of cycloheximide; CrbR, tolerance of carboxin; CadR, tolerance of cadmium; and PDA+, ability to sporulate on potato-dextrose agar. Genetic diversity in samples is indicated by H' (the Shannon index of diversity).

DISCUSSION

Race 3 was significantly less fit than race 2 in the fields in both Wilkes and Yadkin counties in which the populations of *C. carbonum* were sampled sequentially. This was surprising because race 3 induces larger lesions than does race 2. Nevertheless, the observed lower parasitic fitness of race 3 is consistent with the lack of increase of race 3 in the piedmont of North Carolina after 1977 (15). It is also consistent with the results of Welz et al (32), which showed that in vitro sporulation by race 3 on autoclaved senescent and green corn leaves was significantly lower than was sporulation by race 2. Thus, relative fitness in this pathogen may be correlated less strongly with lesion size than was previously thought (5). The reason for the apparent lack of correlation of fitness with lesion size may be that sporulation by *C. carbonum* is not confined to necrotic lesions. Instead, it occurs on senescing leaf tissue or on lower dead leaves of corn plants (17). Welz et al (32) found that sporulation by races 2 and 3 on senescent corn leaves in vitro was closely correlated with their parasitic fitness in corn fields in North Carolina.

Evidence that parasitic fitness of race 3 is lower than that of race 2 helps explain why the frequency of race 3 did not increase in the piedmont of North Carolina between 1977 and 1985 (15), but why race 3 invaded the piedmont between 1973 and 1976 and why race 2 has not displaced race 3 in the piedmont since then remains unclear. Parasitic fitness also does not explain why race 3 remains so much more abundant than race 2 in corn fields of the Appalachian Mountains. The sporulation study of Welz

TABLE 3. Pairwise frequency distributions of phenotypes of *Cochliobolus carbonum* races 0, 2, and 3 that are identical for six characters but that differ in mating type

Phenotype code[a]		No. of isolates/phenotype					
		Race 0		Race 2		Race 3	
MAT-1	MAT-2	MAT-1	MAT-2	MAT-1	MAT-2	MAT-1	MAT-2
11	10	0	1				
25	24	0	1				
31	30	2	0				
41	40	4	4				
43	42	2	2				
47	46	0	2				
57	56	1	0				
59	58	7	1				
63	62	4	1				
65	64			0	2		
71	70			2	0		
73	72			1	8	0	1
75	74					1	1
79	78			1	4	1	0
81	80			8	30	0	1
87	86			7	3		
89	88			24	48	71	49
91	90			6	1	9	7
95	94			10	27	3	2
105	104			0	1		
107	106			1	0		
121	120	0	1				
123	122			0	1		
N[b]		20	13	60	125	85	61
χ^2[c]		4.47		21.02		0.76	
df[c]		5		7		3	
P		ns[d]		<0.01		ns	

[a] Table 2 contains character combinations of phenotypes.
[b] Number of isolates.
[c] Degrees of freedom.
[d] No significant heterogeneity at $P \leq 0.05$.

et al (32) provides no evidence of differential adaptation to temperature, which might stabilize *C. carbonum* race frequencies in different parts of North Carolina. One explanation for the continued coexistence of races 2 and 3 could be that they have adapted to different niches during survival periods between corn crops. Such adaptation could make it unlikely that race 2 will ever displace race 3. For example, *C. heterostrophus* does not displace *C. carbonum* race 2 or 3, even though *C. heterostrophus* clearly has greater parasitic fitness on corn leaves than has race 2 or 3 of *C. carbonum* (16).

Race 1 was not detected among 90 isolates tested on toxin-sensitive corn plants, which supports Leonard's (14) conclusion that in the absence of susceptible leaf tissue, unnecessary toxin production confers a selective disadvantage to race 1. The poor parasitic fitness of race 0 in corn fields seems to be due to its lack of two virulence genes necessary to induce necrotic lesions on corn, as was indicated by segregation from crosses of race 0 × race 2 isolates (H. G. Welz and K. J. Leonard, *unpublished data*). The history of race 0 is unclear. Race 0 may have occurred undetected in the piedmont before 1987, because in previous

TABLE 4. Gametic phase disequilibrium of phenotypic characters[a] in samples of *Cochliobolus carbonum* populations from two corn fields in North Carolina and one field in Tennessee

C. carbonum races	Wilkes Co., NC				Yadkin Co., NC				Tenn.
	9 July	28 July	10 August	Pooled	9 July	28 July	10 August	Pooled	28 July
0	ns[b]	ns	...[c]	<0.025	...	...	...	...	...
2	ns	<0.01	ns	<0.001	<0.05	<0.001	<0.001	<0.001	...
3	ns	ns	ns	ns	ns	ns	ns	ns	ns
Pooled	<0.001	<0.001	<0.01	<0.001	ns	<0.001	<0.001	<0.001	ns

[a] Phenotypes were defined by the seven characters described in Table 2; expected frequencies of phenotypes were calculated as the product of the frequencies of individual characters in each sample.
[b] Chi-square not significant at $P < 0.05$.
[c] Sample too small for statistical analysis.

TABLE 5. Association of phenotypic characters in isolates of races 0, 2, and 3 of *Cochliobolus carbonum* collected in two corn fields in North Carolina and one field in Tennessee in 1987

Race	Character combination[a]	No. of isolates in a class (no. observed/no. expected)[b]				Assoc.[c]	G-value[d]
		11	10	01	00		
0	MAT-1/Psu+	15/13	5/7	7/9	6/4	ns	1.49
2		27/20	33/40	36/43	89/82	+	4.60
3		13/14	71/70	11/10	51/52	ns	0.13
2	MAT-1/Asc+	20/18	40/42	34/36	91/89	ns	0.72
2	MAT-1/CyhR	43/43	17/17	90/90	35/35	ns	<0.01
2	MAT-1/CrbR	55/54	5/6	110/111	15/14	ns	0.57
2	Psu+/CyhR	51/45	12/18	82/88	40/34	+	4.00
2	Psu+/CrbR	55/56	8/7	110/109	12/13	ns	0.34
2	Asc+/CyhR	42/39	12/15	91/94	40/37	ns	1.33
2	Asc+/CrbR	47/48	7/6	118/117	13/14	ns	0.34

[a] Characters are MAT-1, mating type 1; Psu+, the ability to form pseudothecia; Asc+, the ability to form asci and ascospores; CyhR, tolerance of cycloheximide; and CrbR, tolerance of carboxin.
[b] Class designations represent the presence or absence of the two indicated characters (e.g., class 10 indicates that for the first character combination, MAT-1 is present [instead of MAT-2] and Psu+ is absent [replaced by Psu−]).
[c] ns = no significant association; + = a positive association of the indicated traits (i.e., observed frequencies greater than expected for classes 11 and 00).
[d] For G-values greater than 3.48, $P < 0.05$.

TABLE 6. Character combinations in race 2 of *Cochliobolus carbonum* that show gametic phase disequilibrium in samples pooled over sampling time from collections in two corn fields in North Carolina and one field in Tennessee

Character combination[a]	Field	No. of isolates in a class (no. observed/no. expected)[b]				Assoc.[c]	G-value[d]
		11	10	01	00		
MAT-1/	Wilkes Co.	10/7	7/10	15/18	25/22	ns	2.13
Psu+	Yadkin Co.	17/13	26/30	20/24	62/58	ns	2.99
	Tenn.	0/	0/	1/	2/		
	Wilkes Co.	...[e]	...	...	...	+[f]	6.19
Psu+/CyhR	Yadkin Co.	24/21	1/4	23/26	9/6	ns	0.60
	Tenn.	27/25	10/12	58/60	30/28		
		0/	1/	1/	1/		
		...[e]	...	...	...		

[a] Characters are MAT-1, mating type 1; Psu+, the ability to form pseudothecia; and CyhR, tolerance of cycloheximide.
[b] Class designations represent the presence or absence of the two indicated characters (e.g., class 10 indicates that for the first character combination, MAT-1 is present [instead of MAT-2] and Psu+ is absent [replaced by Psu−]).
[c] ns = no significant association; + = a positive association of the indicated traits (i.e, observed frequencies greater than expected for classes 11 and 00).
[d] For G-values greater than 3.48, $P < 0.05$.
[e] Sample too small to calculate expected frequencies.
[f] Indicated positive association is unreliable because the smallest number of isolates per cell is < 5.

surveys, samples were collected during late July or during August when race 0 populations in corn would have declined (13,15,17). Considering its lack of virulence to corn, race 0 may have spread to corn from some unknown weed host in the fields. Variation in pathogenicity to different grass species is widespread among *C. carbonum* isolates (9,23). Superior parasitic fitness of race 0 on another grass species would maintain the race 0 population in spite of its lack of parasitic fitness on corn.

Races 0, 2, and 3 were distinct not only in their lesion types on corn, but also in other phenotypic traits, such as fungicide tolerance and sporulation on PDA (Fig. 5) . Generally, most trait frequencies deviated little from values determined in previous years. CadR was rare among race 2 isolates (2%), absent in race 3 (0%), but frequent in race 0 (86%) in this survey. Leonard (13) reported 2 and 0% CadR during 1972–1975 in races 2 and 3, respectively. The frequency of CyhR in race 2 was 72% in 1987 compared to 67–79% during 1972–1985; in race 3, CyhR was 99% in 1987 compared to 91–100% during 1972–1985 (15). Also, the significantly greater genetic diversity observed in race 2 relative to race 3 in an earlier survey (15) was found again in our 1987 survey. The Shannon index of diversity for race 2 samples was 2.35 in 1985 (15) and 2.37 in 1987; for race 3, the Shannon index values were 1.67 in 1985 (15) and 1.34 in 1987.

The apparent long-term stability of gene frequencies relates to the short-term stability displayed by the race populations in the fields in Wilkes and Yadkin counties (Fig. 5). Leonard and Leath (15) concluded that the phenotypic traits analyzed were not selectively neutral, assuming that stabilizing selection made gene frequencies fluctuate around equilibria. This may not be true. Leonard and Leath (15) presented evidence that gene frequencies could differ greatly between neighboring fields, suggesting little or no migration between fields. However, at some time, migrants must have founded local populations. If the number of migrants was low, which seems reasonable, genetic drift would have had a major impact (i.e., founder effect) on the structure of new subpopulations. In established populations, the significance of drift would be minimal, because the high-survival ability of *C. carbonum* (16) probably would keep population density reasonably high. This should allow frequencies of selectively neutral genes to maintain stable frequencies within each population although their frequencies differ between populations in different fields. Thus, gene frequencies could differ among fields, and the aggregate frequencies over many fields could remain stable.

It is interesting that in our sequential samples the frequencies of races changed significantly over time but gene frequencies and diversity within races remained largely unchanged. This also supports the conclusion that the phenotypic traits analyzed in our survey did not affect fitness.

Leonard and Leath (15) stated "The continuing presence of races 2 and 3 as genetically distinct populations in the same fields supports the conclusion that sexual reproduction by *C. carbonum* is rare or absent in these fields." They based their conclusion on the evidence of a lack of intermediate lesion types and on the significantly different frequencies of some traits (e.g., Psu+ and CyhR) in races 2 and 3. This evidence, however, is directly related only to sex between races 2 and 3. Leonard and Leath's (15) only evidence against sex within races was the lack of any observation of *C. carbonum* pseudothecia in the field and the suspected nonrandom association of genes in race 2 samples. In their analysis, Leonard and Leath (15) pooled data from the 10 fields they sampled. This, however, was not justified, because gene frequencies differed significantly between fields. Pooling population samples in which gene frequencies differ can yield apparently nonrandom gene associations even when the pooled subsamples are in perfect equilibrium (H. G. Welz, *unpublished data*). This effect is more pronounced with greater differences between subsamples. Therefore, Leonard and Leath's (15) data on gene associations provide no support for the conclusion that sexual reproduction does not occur within races of *C. carbonum*.

Our data support the conclusion that hybridization between races 2 and 3 is unlikely to occur in the field. None of our isolates induced intermediate lesion types. Furthermore, frequencies as well as diversity indices differed significantly between the two races. Similar evidence indicates an absence of hybridization between race 0 and race 2 or 3. For example, no isolates that produced lesions typical of race 2 or 3 were unable to sporulate on PDA, a trait shared by all but one of our race 0 isolates. On the other hand, our data do not rule out the possibility that sex within races, particularly race 3, may occur in the field.

Three lines of evidence suggest that genetic recombination may occur in race 3. First, the observed phenotype-frequency distributions in race 3 were homogeneous among both mating types (Table 3). Second, the observed phenotype-frequency distribution was homogeneous with the expected distribution calculated from the frequencies of individual traits within race 3 (Table 4). Third, pairwise comparisons showed no gametic phase disequilibrium of MAT-1 and Psu+ in race 3 (Table 5). All available samples were pooled for this gametic phase disequilibrium analysis because the gene frequencies in race 3 did not differ significantly between samples.

The evidence is weaker for genetic recombination in races 0 and 2. For race 0, there was no indication of gametic phase disequilibrium, but fewer isolates of race 0 than of race 3 were tested. Analysis of the frequency distribution of complete phenotypes in race 2 showed an apparent gametic phase disequilibrium, as would be expected from asexual reproduction, but the analysis of paired character combinations (Tables 5 and 6) in 2×2 contingency tables yielded little evidence of nonrandom gene associations. Thus, complete phenotype analysis may be a more sensitive test of gametic phase disequilibrium than analysis of individual character combinations. Additional evidence that sexual reproduction may be more frequent in races 3 and 0 than in race 2 is observed in the frequencies of MAT-1, which were closer to 50% in races 3 (58%) and 0 (61%) than in race 2 (32%). Using combined data from 10 fields in North Carolina, Leonard and Leath (15) found that MAT-1 occurred at nearly equal frequencies in races 2 (47%) and 3 (50%), but the standard deviation was greater in race 2 (17%) than in race 3 (12%).

If local race populations were founded by few individuals and reproduction of the new populations was purely asexual, the phenotype-frequency distributions would not be expected to be homogeneous with the expected distribution calculated from individual trait frequencies. Instead, the population structure should be more "clonal," with a few phenotypes dominating the populations. Therefore, the hypothesis that founder effects occurred is consistent with the observation of gametic phase equilibrium or near-equilibrium only if genetic recombination had rearranged the genetic structure of the race populations after their initiation. On the other hand, we do not have strong evidence of founder effects in the race 3 populations of the two fields in Wilkes and Yadkin counties. Gene frequencies did not differ significantly between the race 3 populations in those fields.

If some sexual reproduction does occur within one or more of the *C. carbonum* races, why is there no evidence of interracial hybrids in the field? It should be emphasized that we have not detected any genetic barriers against hybridization between *C. carbonum* races in the laboratory. Among the isolation mechanisms mentioned in the literature of evolutionary genetics as barriers to gene flow between populations in nature (19), seasonal isolation or lack of hybrid fitness seem the most likely to apply to *C. carbonum*. Seasonal isolation can occur when different stimuli or different levels of the same stimulus (e.g., day length, phytohormone level, etc.) induce the formation of sexual structures in different populations. Induction of the sexual stage may be artificially synchronized in the laboratory, and seasonal isolation may not be apparent in laboratory crosses. Observations of race $2 \times$ race 3 hybrids provide no evidence that these races lack fitness (3), but crosses of race $2 \times$ race 0 yielded progeny with lower sporulation ability than the mean of parental abilities (H. G. Welz and K. J. Leonard, *unpublished data*). The hybrids, however, did tend to sporulate as well or better than did the race 0 parent.

The results of our sequential sampling of field populations of *C. carbonum* in North Carolina show that race 3 is not a highly

aggressive pathogen of corn in the piedmont, even though it induces larger lesions than does race 2. The survey strengthened the previous conclusion that gene flow is rare or nonexistent between races 2 and 3 or between these races and the newly described race 0. However, the possible contribution of sexual reproduction to genetic variation within each of these races is still unclear. Evidence from the analysis of gametic phase disequilibrium suggests that sexual reproduction is most likely to occur in race 3. This evidence, however, is strangely inconsistent with the fact that genes required for the production of pseudothecia, asci, and ascospores occur less frequently among race 3 isolates than among those of either race 2 or 0. Obviously, there is still much to be learned about the extent, distribution, and mechanisms of genetic variation in plant pathogenic fungi that seem to have functional sexual stages but reproduce in nature primarily by asexual spores.

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