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Gametic phase disequilibria in populations of race 2 and race 3 of *Cochliobolus carbonum*

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Abstract

Over 3 years 15 samples of *C. carbonum* were collected from 11 corn fields in North Carolina and Tennessee. Among 514 isolates of race 2 and 319 isolates of race 3, five phenotypic characters (mating type, production of pseudothecia, production of asci and ascospores, tolerance to cycloheximide and carboxin, respectively) that are controlled by single genes at unlinked loci (*Mat, Psu, Asc, Cyh, Crb*) were examined. Gametic phase disequilibrium (GPD) was analyzed by three methods. First, observed and expected four-locus haplotype frequencies were compared in *G*-tests for goodness of fit (method 1) and second, four-locus disequilibrium was calculated using an index of association which is based on the variance of the number of loci at which two isolates in a given population differ (method 2). Third, observed and expected frequencies of allele pairs were compared in 2 × 2 contingency tables (method 3). Each test was performed on individual and pooled samples which also allowed assessment of heterogeneity among samples. In general this heterogeneity was low, i.e. the consistency of associations among samples was high. Four-locus disequilibrium was significant in race 2 with method 1 but not with method 2. In race 3 we found no disequilibrium with any of the two methods. Method 3 indicated that several allele pairs were significantly associated in race 2 but not race 3. Thus GPD was significant in race 2 but not in race 3. Mating type frequencies were close to 0.50 in race 2 and race 3. This and the non-significant index of association indicate that the sexual stage of *C. carbonum* is operating within each of the two race populations. It is suggested that selection favours different haplotypes in the asexual than in the sexual stage of the pathogen. Such disruptive selection may have maintained polymorphisms of genes related to sexual fertility and stabilized gene frequencies in *C. carbonum* over a period of 15 years (1972–1987).

Introduction

Cochliobolus carbonum Nelson (anamorph: *Bipolaris zeicola* (G.L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is a ubiquitous foliar pathogen of maize (*Zea mays* L.) in many regions of the world [Shurtleff, 1980; Welz and Geiger, 1994]. In western Europe the leaf spot disease caused by this fungus is not considered important in commercial corn production fields [Smith *et al.*, 1988; Krüger, 1989]. However, in hybrid seed production fields in southern Switzerland severe yield losses were observed in recent years [Winter, 1978; Winter and Menzi, 1991] indicating the inferior resistance of some inbred lines to *C. carbonum* and the importance of resistance breeding. These observations and the presumed occurrence of new pathotypes

in the USA [Dodd and Hooker, 1990] and former Yugoslavia [Lević and Penčić, 1993] warrant increased attention to *C. carbonum* in Europe.

C. carbonum is a typical representative of the facultative parasites that infect leaves and stems of many grasses [Nelson and Kline, 1971] necrotrophically and survive on crop residues between growing seasons. In the USA five pathogenic races of *C. carbonum* have been reported on corn. Race 0 is avirulent on corn and causes only flecks or minute lesions on inoculated leaves. It has only been found in North Carolina [Welz and Leonard, 1993]. Race 1 produces the HC-toxin inducing large lesions specifically on corn genotypes homozygous for the *hm1* gene. Present day corn hybrids are not sensitive to the toxin because they do not carry the susceptibility gene. Therefore the frequency of race 1 has dropped below 1 % in the US population of the fungus [Leonard, 1987; Welz and Leonard, 1993]. Race 2 induces round to oval, necrotic lesions on all corn genotypes whereas lesions by race 3 are larger and linearly shaped. Size and number of lesions by races 2 and 3 are controlled by polygenic resistance. Race 2 has been found most frequently in population surveys of *C. carbonum* [Leonard, 1978; Leonard *et al.*, 1988; Welz and Leonard, 1993]. Only in the Appalachian mountains from Georgia to Pennsylvania race 3 appears to be the prevalent pathotype [Leonard, 1978; Leonard *et al.*, 1988; Welz and Leonard, 1993]. Race 4 is characterized by large (5–10 mm) round lesions and less virulence on inbred line W64A [Dodd, 1993]. Lesion types associated with race 4 have been observed only recently in the midwestern US and in southern France [Dodd and Hooker, 1990; Dodd, 1993].

C. carbonum is a haploid ascomycete that is typically homokaryotic. It has a sexual stage that can be readily induced in the laboratory but has never been observed in the field. Long lasting polymorphisms for several traits have been reported in race 2 and race 3 populations. Between 1972 and 1987 there has been little change in gene frequencies of mating type (*MAT-1*), pseudothecium production (*Psu+*), ascus and ascospore formation (*Asc+*) and tolerance to cycloheximide (*CyhR*) and carboxin (*CrbR*) [Welz and Leonard, 1993]. At the same time gene frequencies were consistently different between races suggesting that race 2 and race 3 are genetically isolated [Leonard, 1978; Leonard and Leath, 1990; Welz and Leonard, 1993; Welz *et al.*, 1994]. Comparisons of observed and expected frequencies of character combinations and haplotypes revealed that the total population (comprising races 0, 2, 3) was in highly significant gametic phase disequilibrium (GPD) typical of asexual fungi [Leonard and Leath, 1990; Welz and Leonard, 1993]. In contrast, there was only weak GPD within races, particularly in race 3, suggesting that sexual recombination may be relevant within but not between races [Welz and Leonard, 1993].

When Leonard and Leath [1990] analyzed GPD, they pooled samples within races before calculating expected frequencies of allele associations. As gene frequencies were different among samples this may have led to an overestimation of GPD. Here we reexamine the strength, the stability and causes of GPD in previously described [Leonard and Leath, 1990; Welz and Leonard, 1993] and additional samples of *C. carbonum* in order to understand how genetic variation is maintained in parasitic fungi that have a presumably functioning sexual stage in their off-season survival phase, but reproduce strictly asexually during epidemics.

Materials and methods

Sampling. Fifteen samples of 50 or more leaves were collected from 11 commercial corn fields without regard to cultivar and disease symptoms in North Carolina (six counties) and Tennessee (one county) in 1985, 1986, and 1987. Samples collected in 1985 and 1987 were described by Leonard and Leath [1990] and Welz and Leonard [1993], respectively. The two samples collected in 1986 were from corn fields in Wilkes Co., North Carolina, and Johnson Co., Tennessee. From each leaf harbouring *C. carbonum*, one isolate was established. This resulted in a total of 833 isolates. The location of fields and sampling techniques were described in detail by Leonard and Leath [1990] and Welz and Leonard [1993].

Race identification. Individual isolates were inoculated onto hybrid cv. Pioneer Brand 3369A as conidial suspensions. On this genotype races

0, 2, and 3 can be distinguished by their lesion type. Race 1 cannot be identified on this HC-toxin resistant hybrid but a subsample which was tested on inbred line N31 (susceptible to race 1) indicated its rareness [Welz and Leonard, 1993]. Race 4 was never observed before 1989 [Dodd, 1993] and thus should not have affected results. The relatively few isolates of the avirulent race 0, obtained in 1987, are not considered in this paper. For further details concerning methodology see Welz and Leonard [1993]. The total sample comprised 514 isolates of race 2 and 319 isolates of race 3.

Mating type and fertility. Each field isolate was paired with fertile albino tester isolates [MAT-1 (A) and MAT-2 (a)] on corn leaf disks on modified Sachs agar as described by Leonard and Leath [1990]. Compatible matings with isolates carrying a single gene for pseudothecium production [Nelson, 1964], *Psu+*, formed both albino and black wild-type fruiting bodies while those with isolates lacking this gene formed only albino fruiting bodies. Three weeks after the matings were set up, five to 10 black pseudothecia were crushed in a drop of water under a dissecting microscope to assess the production of asci and ascospores. This character is controlled by another single gene [Dalmacio and Nelson, 1976], *Asc+*. Matings yielding no black pseudothecia, asci or ascospores were repeated to confirm the postulated genotype. Note that in this study, *Psu– Asc+* and *Psu– Asc–* types were not distinguishable with the *Psu+ Asc+* mating testers used.

Fungicide tolerance. Five to seven-day-old isolates were subcultured on PLA amended with 2 µg of cycloheximide [3–2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl glutarimide] and 100 µg of carboxin (5,6-dihydro-2-methyl-1,4-oxanthiin-3-carboxanilide) per milliliter, respectively. After 5 days the colony growth was compared to controls without fungicide. If the radial growth was ≥ 20% of the control, the isolates were rated tolerant of the fungicide. For more details see Leonard and Leath [1990]. Tolerance to cycloheximide [MacKenzie *et al.*, 1971] and carboxin [Leonard, 1978] is controlled by single genes, *CyhR* and *CrbR*, respectively.

Data analysis. Gametic phase disequilibrium (GPD) was only analyzed within races because race 2 and race 3 apparently are genetically isolated. Three methods were used. First, expected four-locus haplotype frequencies were calculated from the relative frequencies of *MAT-1*, *Psu+*, *CyhR*, and *CrbR* in each sample. The *Asc* locus was not considered in the multilocus analyses because its allelic state was unknown in *Psu–* isolates. Observed and expected haplotype frequencies were summed over samples and compared by a *G*-test for goodness of fit [Sokal and Rohlf, 1981]. To increase accuracy, haplotype classes with expected numbers < 3.0 were lumped with adjacent classes as suggested by Sokal and Rohlf [1981, p. 714]. The test statistic

$$G = 2 \sum m_O \ln(m_O/m_E), \quad (1)$$

where m_O is the observed and m_E is the expected number of isolates in each class. Replicated goodness of fit tests were also performed [Sokal and Rohlf, 1981, p. 722]: The goodness of fit was tested for each individual sample and *G*-values were summed to obtain a total *G*. By subtracting the pooled *G*, obtained for the pooled sample, from the total *G*, the *G* for heterogeneity was obtained, indicating whether the samples were as if collected from one population.

As a test of random mating we computed the index of multilocus association, I_A, suggested by Brown *et al.* [1980]. In this test the variance V_O of the distance K between two isolates – that is the number of loci at which they differ – in a sample of n isolates is calculated over n(n – 1)/2 isolate pairs – and compared to the expected variance of K, V_E. Then

$$I_A = V_O / V_E - 1. \quad (2)$$

The error variance of V_E was also computed according to Brown *et al.* [1980, equ. 22 minding the typing error] from which an upper 95% confidence limit of V_O was derived.

Pairwise associations of alleles were analyzed using 2 × 2 contingency tables, listing the four possible haploid genotypes that are obtained in this case, and the *G*-test of independence with Williams' correction [Sokal and Rohlf, 1981]. As with the four-locus haplotypes, expected fre-

quencies of allele combinations were calculated for each of the 15 samples individually, summing up subsequently, and using the equation for G given above. Replicated G-tests were also conducted to find out if the pooling of samples was appropriate.

Results

In each sample we found race 2 and race 3, i.e. both races coexisted in each of the 11 fields. Lesion types intermediate between race 2 and race 3 were not observed indicating genetic isolation [Leonard and Leath, 1990; Welz and Leonard, 1993]. Mating type allele frequencies were close to 0.50 in race 2 and race 3 (Table 1). The gene for pseudothecium production, *Psu+*, was significantly more frequent in race 2 but the gene for production of asci and ascospores, *Asc+*, was equally frequent in the two races. Tolerance to cycloheximide and carboxin was common in both races 2 and 3. However, the frequency of *CyhR* was significantly lower in race 2 than in race 3 (Table 1).

Comparing observed and expected four-locus haplotype frequencies in the individual samples resulted in G-values that were significant (P = 0.05) in two out of 12 samples in race 2, and in zero out of 11 samples in race 3 (some of the 15 samples contained too few isolates of either race 2 or race 3 and were thus excluded). Therefore replicated goodness of fit tests performed on the four-locus haplotype frequencies yielded non-significant G-values for heterogeneity among samples for race 2 and race 3 (Table 2), indicating that although samples were from different locations and years they were as homogeneous with regard to four-locus GPD as if collected from one population. In race 2, the total G was not significant whereas the pooled G was significant at the 0.1% level. This means that the gametic disequilibrium was too weak to be significant in the relatively small individual samples of race 2 whereas in the pooled sample it became significant due to the much larger sample size. Thus in

Table 1. Means and standard deviations of gene frequencies in race 2 (13 samples, 514 isolates) and race 3 (11 samples, 319 isolates) of *Cochliobolus carbonum*

Race	Gene[x]					Mean
	MAT-1	*Psu+*	*Asc+*[y]	*CyhR*	*CrbR*	Sample size
2	0.46 ± 0.19	0.53 ± 0.15	0.68 ± 0.19	0.72 ± 0.12	0.95 ± 0.05	39.5 ± 28.9
3	0.53 ± 0.11	0.18 ± 0.12	0.67 ± 0.36	0.95 ± 0.06	0.97 ± 0.04	29.0 ± 20.2
	ns[z]	*	ns	*	ns	

[x] Indicating genes for mating type 1, ability to produce pseudothecia, ability to produce asci and ascospores, tolerance to cycloheximide, and tolerance to carboxin, respectively.
[y] Frequencies estimated only within *Psu+* isolates in samples with ≥ 9 *Psu+* isolates (i.e. 12 samples in race 2 and seven samples in race 3, respectively) to avoid biasing of the mean.
[z] Means not significantly different (ns) or significantly different in t-test at $P < 0.05$ (*).

Table 2. Replicated G-tests for goodness of fit of observed and expected four-locus haplotype frequencies of races 2 (12 samples) and race 3 (11 samples) of *Cochliobolus carbonum*

	Race 2			Race 3		
	df[x]	G[y]	P	df	G	P
Total G	55	66.407	ns	19	9.232	ns
Pooled G	12	42.609	0.001	5	3.328	ns
Heterogeneity G	43	23.798	ns	14	5.904	ns

[x] Degrees of freedom. In each sample, classes with expected haplotype numbers < 3.0 were lumped with adjacent classes.
[y] Calculated without Williams' correction (for additivity).

both races pooling of samples was justified and increased the statistical power of the experiment.

Out of the 16 (= 2^4) detectable haplotypes (ignoring the *Asc* locus), 15 and 8 were likely to occur at least once among the 514 race 2 isolates or among the 319 race 3 isolates, respectively (Table 3, Exp > 1.0). However, only haplotypes with an expected number of isolates of ≥ 3.0 had a probability of P ≥ 95% not to be missed in a sample of 514 or 319 isolates. [The probability of missing a haplotype with true frequency q = 0.00584 = 3/514, in a sample of 514 isolates is $(1 - 0.00584)^{514} = 0.049$. Likewise, $(1 - 3/319)^{319} = 0.049$. If Exp = 1.0, i.e. q = 1/514 or 1/319, then P = 0.37]. Thus, among the haplotypes expected at a probability level of 95%, none was missed (Table 3). However, haplotype frequency distributions were quantitatively and qualitatively different among race 2 and race 3. The diversity, i.e. richness and evenness of distribution, of haplotypes was significantly (P < 0.001) greater in race 2 (Shannon index $H' = 1.94$) than race 3 ($H' = 1.27$). The second most frequent haplotype in race 2 (2–RR, i.e. *MAT-2 Psu– CyhR CrbR*) was also second most frequent in race 3. Haplotype 2++RR, which was most frequent in race 2 was much less frequent in race 3. Haplotype 1–RR was most frequent in race 3 but ranked only fifth in race 2 (Table 3). The greater genetic diversity in race 2 than race 3 was confirmed by the parameter D, the mean genetic distance per locus between isolates (Table 4), although we were unable to statistically test the significance of the difference in D.

The multilocus association test according to Brown *et al.* [1980] was performed on each individual sample as well as on the pooled samples. In race 2 and race 3, I_A was never significantly different from zero (Table 4, only results for pooled samples presented). This is in line with the results of the replicated goodness of fit tests of observed and expected four-locus haplotype frequency distributions. As a less rigorous test of random mating in populations with potentially mixed asexual and sexual reproduction $I_A$may be calculated for reduced data sets in which the occurrence but not the relative frequencies of haplotypes is considered. Reduced data sets of this type were analyzed by Maynard Smith *et al.*

Table 3. Observed and expected numbers of four-locus haplotypes in pooled samples of race 2 and race 3 of *Cochliobolus carbonum*. Significant heterogeneity among the frequency distributions was observed in race 2 but not in race 3 (see pooled *G*-values in Table 2)

Haplotype[x]	Race 2		Race 3	
	Obs	Exp	Obs	Exp
2–SS	4 ┐	2.8 ┐	0 ┐	*[z] ┐
1–SS	0 ├ 6	1.3 ├ 6.4[y]	0	*
2+SS	0	1.7	0	*
1+SS	2 ┘	0.6 ┘	0	*
2–RS	10	7.5	3 ├ 7	2.7 ├ 6.2
1–RS	3	3.8	1	2.7
2+RS	7	4.7	1	0.5
1+RS	3	3.1	2 ┘	0.3 ┘
2–SR	57	41.5	4 ┐	2.3 ┐
1–SR	34	30.6	1 ├ 8	3.6 ├ 7.1
2+SR	15	39.3	1	0.5
1+SR	43	34.3	2 ┘	0.7 ┘
2–RR	99	105.6	110	110.5
1–RR	56	67.4	153	149.8
2+RR	108	94.5	18	20.0
1+RR	73	70.8	23	24.0
Total	514	509.5	319	317.6

[x] Indicating alleles at *Mat, Psu, Cyh, Crb* loci.
[y] When expected haplotype numbers were < 3.0 they were lumped with adjacent classes (observed numbers likewise, to match).
[z] Expected number < 0.1.

Table 4. Measures of multilocus association in *Cochliobolus carbonum*

Race	D	I_A	V_{Obs}	V_{L-95}
2	0.378	0.017 ns	0.853	0.931
3	0.208	0.066 ns	0.560	0.602

D, mean genetic distance per locus between isolates; I_A, index of association; V_{Obs}, observed variance of the number of loci at which two isolates differ; V_{L-95}, upper 95% confidence interval of V_{Obs}; ns = not significant.

[1993], as examples of parasitic organisms in which there is genetic diversity resulting from recombination, but certain haplotypes have increased rapidly by asexual reproduction during epidemic outbreaks caused by those haplotypes. Reducing the data sets so that each haplotype is represented only once may remove GPD that was generated by the epidemic asexual reproduction and uncover the underlying random assortment of genes that preceeded the epidemic outbreaks. As expected, reducing the *C. carbonum* race 2 and race 3 data sets to consider each haplotype as a single individual resulted in an even greater probability of a non-significant I_A-value (data not presented).

Replicated goodness of fit tests with 2 × 2 contingency tables of allele pairs were only meaningful in a few cases because the requirement of a minimum expected cell frequency of 3.0 was not met with many individual samples. Where these tests could be made, sample heterogeneity was observed in three out of four cases (Table 5). However, when associations were significant in individual samples their sign was never different. The significance of heterogeneity occurred because in some samples associations were significant (Table 5; race 2, *Psu+ CyhR* in sample

Table 5. Replicated *G*-tests for goodness of fit of observed and expected allele pairs among samples of races 2 and race 3 of *Cochliobolus carbonum*

Sample	Race 2						Race 3		
	No. of isolates	*MAT-1 Psu+*		*MAT-1 CyhR*		*Psu+ CyhR*	No. of isolates	*MAT-1 Psu+*	
		G-value	Assoc.[w]	G-value	Assoc.	G-value Assoc.		G-value	Assoc.
1	34	0.216		..[x]		..	8	..	
2	26	0.351		1.293		0.541	20	..	
3	32	0.356		3.536		1.662	1	..	
4	37	0.374		8.780*	⊖	..	14	..	
5	21	5.074*	⊕	..		..	2	..	
6	44	0.046		0.0942		0.273	7	..	
7	26	..		..		..	13	..	
8	36	0.206		4.546*	⊖	9.011** ⊕	1	..	
9	6	..		..		..	0	..	
10	49	1.491		0.312		15.420*** ⊕	12	..	
11	21	1.041		..		..	38	..	
12	3	..		..		..	61	..	
13	57	2.197		..		6.521* ⊕	44	1.482	
14	125	3.039		0.249		0.608	54	..	
15	3	..		..		..	48	2.514	
Total G[y]		14.390		19.443**	⊖	34.036*** ⊕		3.996	
Pooled G[z]		11.137***	⊕	4.482*	⊖	12.132*** ⊕		0.082	
Heterogeneity G		3.253		14.961*		21.904***		3.914*	

[w] Association; positive or negative associations indicated. Asterisks indicate significance at P = 0.05, 0.01, or 0.001, respectively.

[x] Not tested because smallest expected cell frequency in 2 × 2 table < 3.0.

[y] Here, total G has k degrees of freedom (d.f.) where k = number of samples considered, pooled G has 1 d.f., and heterogeneity G has k-2 d.f.

[z] Calculated from marginal observed frequencies.

10) whereas in other samples (e.g. *Psu+ CyhR* in sample 14) they were not. In another case (Table 5; race 3, *MAT-1, Psu+*) there were only minute differences among samples 13 and 15 which cancelled in the pooled sample but resulted in a significant G for heterogeneity. Thus, because sample heterogeneity was limited and knowing that tests of independence in 2×2 tables are very sensitive to sample size we conducted another G-test of independence using the larger, pooled samples. This time we included each individual sample in which the two characters tested for independence were polymorphic, calculated expected frequencies for each individual sample, and entered the sum of those into the equation for G [1] which is not based on marginal observed frequencies, and used the Williams correction for G (to approximate the chi-square distribution) (Table 6). As it was not required that the minimum expected cell frequency in individual samples be ≥ 3.0, more locus pairs could be investigated with this technique. More locus pairs could also be tested in race 2 than in race 3 because gene frequencies, particularly frequencies of *Psu+* and *CyhR*, were more intermediate in race 2 (Table 1) and because of the larger sample size of race 2. Only the independence of *MAT-1 Psu+* and *MAT-1 Asc+* could be tested with races 2 and 3 (Table 6). In race 2 the two allele pairs were significantly associated (with different sign) whereas in race 3 there was no significant difference between observed and expected frequencies. Other allele combinations that were significantly associated in race 2 were *MAT-1 CyhR*, *Psu+ CyhR* and *Asc+ CyhR*. The G-value for *MAT-1 Psu+* was considerably smaller in race 2 when calculated with the composite expected frequencies (Table 6) than with the marginal observed frequencies (Table 5, pooled G). This indicates the greater conservatism of the former way of testing independence in a pooled sample of isolates.

Discussion

The two different techniques we used to analyze multilocus association in *C. carbonum* did not give exactly the same results. The index of association [Brown *et al.*, 1980] was not significant in race 2 nor race 3 suggesting that both populations are in equilibrium like randomly mating populations. G-tests for goodness of fit, however, pointed at significant multilocus association in race 2 but not race 3. The latter technique may thus be more

Table 6. Pairwise associations of alleles in pooled samples of *Cochliobolus carbonum* isolates from North Carolina and Tennessee

Race	Allele combination[w]	No. of isolates in class[x](no. observed / no. expected)				Assoc.[y]	G-value[z]
		11	10	01	00		
2	*MAT-1 Psu+*	121 / 109.3	93 / 104.7	130 / 141.7	170 / 158.3	+	4.36*
2	*MAT-1 Asc+*	77 / 91.6	44 / 29.4	118 / 103.4	12 / 26.6	–	20.51***
2	*MAT-1 CyhR*	126 / 136.6	79 / 68.4	212 / 201.4	76 / 86.6	–	4.29*
2	*MAT-1 CrbR*	143 / 140.2	8 / 10.8	216 / 218.8	21 / 18.2	ns	1.24
2	*Psu+ CyhR*	182 / 164.9	61 / 78.1	157 / 174.1	96 / 78.9	+	10.92***
2	*Psu+ CrbR*	168 / 168.1	12 / 12.0	191 / 191.0	17 / 17.1	ns	0
2	*Asc+ CyhR*	152 / 141.7	34 / 44.3	27 / 37.3	26 / 15.7	+	11.99***
2	*CyhR CrbR*	247 / 249.7	23 / 20.6	122 / 119.6	6 / 8.4	ns	0.46
3	*MAT-1 Psu+*	27 / 25.5	151 / 152.5	20 / 21.5	114 / 112.5	ns	0.18
3	*MAT-1 Asc+*	5 / 6.5	14 / 12.5	7 / 5.5	7 / 8.5	ns	1.15

[w] Allele functions are *MAT-1*, mating type 1; *Psu+*, ability to produce pseudothecia; *Asc+*, ability to produce asci and ascospores; *CyhR*, tolerance of cycloheximide; *CrbR*, tolerance of carboxin. Associations involving the *Asc* locus tested only within *Psu+* isolates.

[x] Class designations represent presence of indicated or opposite allele (e.g., for *MAT-1 Psu+*, class 00 represents *MAT-2 Psu–* phenotypes). Expected frequencies were calculated separately for each polymorphic (i.e., for the two characters considered) sample and then summed up. Equation [1] used to compute G (see Materials and methods section).

[y] ns = no significant association; + = positive association (i.e., observed frequencies greater than expected for classes 11 and 00.

[z] Calculated with Williams' correction; for $G > 3.84$, $P < 0.05$.

sensitive in detecting multilocus gametic disequilibrium. Maynard Smith *et al.* [1993] also used both techniques to demonstrate random association between loci in *Neisseria gonorrhoeae*. However, the non-significance of their χ^2-value for the goodness of fit of observed and expected electrophoretic type (ET) frequencies was not very meaningful because more than 70% of their isolates (160/227) were pooled in one large class.

By testing two-loci associations with 2 × 2 contingency tables we also detected gametic disequilibrium in race 2 of *C. carbonum*. This technique is very sensitive to sample size and so the *G*-values for *MAT-1 Psu+* and *MAT-1 CyhR* in the large pooled race 2 sample, which were just significant, must be interpreted as only weak associations. Yet this technique helps to understand which individual loci may cause multilocus disequilibrium and has been used most frequently in plant pathology [Alexander *et al.*, 1984; Welz, 1988; Hovmøller and Østergaard, 1991; Kolmer, 1992].

It is interesting that the replicated multilocus tests on individual and pooled samples, respectively, did not detect sample heterogeneity whereas the tests of independence of individual loci did. This emphasizes the suggested greater sensitivity of paired loci tests in contrast to multilocus tests. In the population genetics literature the usefulness of different measures of gametic disequilibrium has been debated. The disadvantage of all measures is that they are not completely independent of allele frequencies [Hedrick, 1987; Lewontin, 1988]. Therefore we have refrained from presenting the values of measures other than I_A and concentrated on significance tests.

Gametic phase disequilibrium (GPD) may arise from random genetic drift, migration or selection. It is broken down by recombination when the genes involved are unlinked. In this study, alleles at five pairs of loci were shown to be associated in race 2 but not in race 3: *Mat*, *Psu*; *Mat*, *Asc*; *Mat*, *Cyh*; *Psu*, *Cyh*; and *Asc*, *Cyh* (Table 6). Previous studies showed that four of these five pairs are unlinked. *Mat* is not linked to *Psu* [Nelson, 1964], *Asc* [Dalmacio and Nelson, 1976], or *Cyh* [Leonard, 1978; MacKenzie *et al.*, 1971; Welz and Leonard, 1994] and *Psu* is not linked to *Cyh* [Welz, unpublished]. No linkage information is available on the pair *Asc*, *Cyh*. Altogether it is most reasonable to assume that selection created and maintained the disequilibria observed, drift can be ruled out because of the consistency of GPD among races and samples (i.e. subpopulations). Migration among subpopulations of *C. carbonum* is presumably weak because gene frequencies even among geographically close subpopulations are significantly different [Leonard and Leath, 1990].

The frequencies of the genes involved in paired-loci associations have been very stable over 15 years (i.e., 1972–1987) [Leonard, 1978; Leonard and Leath, 1990; Welz and Leonard, 1993] and the individual alleles are thus probably neutral on long-term fitness. If selection affects haplotype frequencies, how can individual gene frequencies remain stable? Gene or haplotype frequency changes caused by selection during the epidemic (asexual) phase may be balanced by sexual selection. If asexual selection during the epidemic favours, for unknown reasons, *MAT-2 Asc+* haplotypes in race 2 the passage through the sexual stage would equalize *Mat* and *Asc* allele frequencies. This would occur because in the sexual stage opposite mating type isolates are required: *MAT-1* isolates being associated with the *Asc–* allele in race 2 would mate with *MAT-2* isolates associated with the *Asc+* allele. The effectiveness of any form of sexual selection should be correlated with the proportion of the *C. carbonum* population that undergoes the sexual cycle.

In contrast to obligate biotrophic pathogens like *Erysiphe graminis* where cleistothecia have an important role for oversummering in semi-arid areas [Koltin and Kenneth, 1970] it is unlikely that pseudothecia are essential or even important for off-season survival of *C. carbonum*. In this species the sole function of the sexual cycle appears to be the creation of genetic variability. In this respect fungi like *C. carbonum* are similar to "cyclical parthenogens" [Vrijenhoek, 1990] in the plant or animal kingdom. Cyclical asexuality has clearly an advantage for colonizing species such as *C. carbonum*. They do not suffer from the "twofold disadvantage" [Maynard Smith, 1978] of sexual reproduction (i.e., males produce no direct offspring) and at the same time have a high potential to create variability [Vrijenhoek, 1990]. Populations of cyclical parthenogens are often

found close to gametic phase equilibrium (GPE) [Vrijenhoek, 1990]. In the *C. carbonum* population studied here, several allele pairs were in GPE and others were found in weak GPD. Four-locus disequilibrium was also weak or non-significant. This strongly suggests that sexual recombination does occur in *C. carbonum* because in purely asexual populations as of *Puccinia graminis* f. sp. *tritici* (wheat stem rust fungus) or *P. recondita* (wheat leaf rust fungus) in the Great Plains of North America, GPD are typically extreme [Alexander *et al.*, 1984; Kolmer, 1992]. The fact that mating type frequencies in both race 2 and race 3 were close to 0.50, as in typical sexual species, is further evidence of sex in *C. carbonum*.

One problem remains. It might be argued that when sexual reproduction was important in *C. carbonum*, the genes related to fertility (*Psu+*, *Asc+*) should be more frequent than they were, particularly *Pšu+* in race 3. The reason may be the facultative nature of sex in this fungus. Because the sexual stage is not essential for survival, one part of the population will always survive asexually. If sexual fertility was associated with a fitness cost limiting the asexual reproduction rate on corn tissue, carriers of fertility genes may be at a selective disadvantage in this subpopulation [cf. Maynard Smith, 1978]. Thus a system of selection pressures may operate to maintain the polymorphism of fertility genes in *C. carbonum*.

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