Isozyme Variation Within and Among Pathogenic Races of *Cochliobolus carbonum* on Corn in North Carolina

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**Abstract**


We used starch gel electrophoresis to investigate isozyme variation among 194 isolates of races 0, 2, and 3 of *Cochliobolus carbonum* collected in 1987 in two corn fields in North Carolina as well as six isolates of race 1 from previous collections in different states of the United States. Of eight enzyme systems studied in detail, only one, phosphoglucose dehydrogenase, was monomorphic. Diaphorase, malate dehydrogenase, glutamate oxaloacetate transaminase, and esterase were highly polymorphic in race 0, whereas isozyme variation in races 2 and 3 was very limited. Race 0 accordingly had a significantly higher average gene diversity than did race 2 or race 3. Genetic similarity between races 2 and 3 was very high. Their allele frequencies differed clearly at only two putative esterase loci. Races 2 and 3 were only distantly related to race 0, as demonstrated by cluster analysis and principal coordinates analysis. There was no apparent genetic variation among five race 1 isolates from North Carolina and South Carolina. Their common electrophoretic type occurred also in race 2 but not in race 0 or race 3, suggesting a common ancestry with race 2. Only a small proportion of race 2 and race 3 isolates or race 2 and race 0 isolates shared common electrophoretic types, which suggests that there is little, if any, genetic exchange between these races.

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

*Cochliobolus carbonum* R. R. Nelson (anamorph, *Bipolaris zeicola* (G. L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is an ubiquitous foliar pathogen of corn (*Zea mays* L.) and other grass hosts in many temperate regions of the world (18,34). Several races in this fungus have been described on the basis of their lesion types on corn. The avirulent race 0, observed only in western North Carolina (43,44), causes flecks or minute necrotic lesions on corn. Race 1 produces a host-specific toxin and induces large lesions in susceptible corn genotypes but only small lesions on hybrids that are resistant to the toxin. Since the removal of susceptible corn lines from commercial production, race 1 rarely has been found in the United States (16). In our 1987 survey of two corn fields in North Carolina and one in Tennessee, we found no race 1 isolates among the 90 *C. carbonum* isolates that we tested on the susceptible inbred N31 (44). Race 2 induces small, round to oval lesions and is the most prevalent race in the United States, except in the Appalachian mountains where race 3 predominates (16,17,22,44). Race 3 induces long, linear lesions on corn and has been reported in Japan as a foliar pathogen of both corn (41) and rice (46). Recently, Dodd and Hooker (5) reported a fifth race that causes large, zonate lesions and early leaf death on certain corn genotypes but that is less virulent on inbred W64A.

Races 1, 2, and 3 have different genes for pathogenicity on corn. Race 0 has no known pathogenicity gene (45), but it may be pathogenic on wild grasses. Race 1 expresses a single gene for toxin production (33) and another independent gene or genes for lesion induction (26). The lesion type of race 2 is inherited as two major genes (45) that also may be shared by race 3. Crosses of race 2 × race 3 by Dalmacio et al (4) gave no indication of major gene segregation. Instead, evidence from these crosses and similar crosses by Leonard (14) indicated that the linear lesion type of race 3 is under polygenic control.

The races of *C. carbonum* in North Carolina appear to be genetically isolated because 1) gene frequencies of several phenotypic traits differ significantly between races (44), and 2) no isolates with lesion types intermediate between those of race 2 and race 3 have been observed in the field (16,17,22,44). However, little is known about the genetic relatedness and evolution of *C. carbonum* races. It was suggested that race 3 may be a biotype of race 2, but no evidence was presented (34). Using seven phenotypic markers, Welz and Leonard (44) found that some haplotypes (i.e., marker combinations) were in fact shared by races 2 and 3, while race 0 was relatively distinct from races 2 and 3. Jones and Dunkle (12) presented evidence from polymerase chain reaction amplifications with arbitrary and gene-specific primers that race 0 is genetically distinct from races 1, 2, 3, and 4 of *C. carbonum*.

Isozyme analysis has been a successful tool in studying the evolution of races or formae specialae in other fungal species (e.g., 1,3,7,13). In several of these studies, isozyme markers were particularly helpful, because no other genetic markers were available or because the only available markers, viz. virulence genes, were not selectively neutral. Similarly, the phenotypic traits in *C. carbonum*, such as mating type, fertility, fungicide tolerance, and colony morphology, may affect fitness (17) and, therefore, are not ideal markers for many population studies. Isozymes, on the other hand, have proved to be neutral with regard to fitness in nearly all organisms examined (20).

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Recently, Simcox et al. (35) reported isozyme polymorphisms in races 1, 2, and 3 of *C. carbonum* in a small collection of isolates from different geographical regions. As in the study by Simcox et al., one of our objectives was to find a set of useful isozyme markers for investigating genetic variation within and among races of *C. carbonum*. Our second objective was to use the isozyme markers to shed some light on the evolution of races of *C. carbonum*. For our study, we selected 194 isolates of races 0, 2, and 3 from our 1987 collection, which had already been characterized for seven other phenotypic traits (44), as well as six race 1 isolates from previous collections.

**MATERIALS AND METHODS**

**Isolates.** We tested 29 isolates of race 0, 90 of race 2, and 75 of race 3 that represent subsamples from a larger collection of *C. carbonum* isolates made in 1987 in two fields in North Carolina (44). We also tested six isolates of race 1, five of which were collected between 1965 and 1976 in two fields in North Carolina (four isolates) and one in South Carolina. The sixth race 1 isolate came from Michigan and was also collected before 1977. All isolates were stored as frozen conidial suspensions in 30% glycerol at –70°C.

**Isozyme analysis.** Isozymes were identified by starch gel electrophoresis. Samples were prepared by growing isolates on potato-lactose agar for about 4 days. When isolates produced a crop of conidia in amounts similar to that of a wild-type race 2 isolate (>10⁶ conidia per plate), a single plate yielded enough spores for electrophoresis. If sporulation was sparse, conidia from several cultures were combined. The conidia were harvested and suspended in 25 ml of a phosphate buffer (0.01 M, pH 7.0, from a 0.1 M stock solution of 61 ml of 0.2 M disodium hydrogen phosphate, 39 ml of 0.2 M sodium dihydrogen phosphate, and 100 ml of distilled water containing 2.5 ml of Tween 20 and 10 μl of ethanol). Flasks containing the conidial suspensions were placed on a revolving shaker run at three agitations per second for 5 min to wash off substances that might inhibit germination (24). The suspension was then centrifuged at 1,000 rpm for 4 min, the supernatant was discarded, and the conidia were resuspended in 25 ml of phosphate buffer. This suspension was shaken for 6 h to induce conidial germination and then centrifuged. The conidia were resuspended in a few drops of phosphate buffer and transferred to 2-ml reaction vials, which were stored at 80°C until further use. Usually fewer than 50% of the conidia germinated under the conditions described, but comparative tests indicated that their enzyme activity was higher than that of untreated conidia. Storage for several weeks did not reduce the enzyme activity of the samples.

For enzyme extraction, the frozen conidial suspensions were allowed to thaw and were then cooled over ice. A few grains of quartz sand were added, and the conidia were homogenized with a glass rod for a few seconds. Solutions were then centrifuged for 10 min (15,000 rpm, 4°C). The supernatant was absorbed onto paper chromatography wicks (7 X 3 mm), and the wicks were inserted into a sample slot 2 cm from the cathodic end of the starch gel (12% [w/v] starch, thickness 7 mm, solidified overnight). Gels were cooled on both sides (4°C).

After electrophoresis, each gel was cut into three slices. Three buffer systems were used (Table 1). Eight enzyme systems yielding high activity and well-separated bands were selected for routine work (Table 2): phosphogluconate dehydrogenase (PGD), NADH-diaphorase (DIA), α-esterase (EST), glucosephosphate isomerase (GPI, syn. PG1 = phosphoglucone isomerase), aconitase (ACO), phosphoglucomutase (PGM), malate dehydrogenase (MDH), and glutamate oxaloacetate transaminase (GOT, syn. AAT = aspartate aminotransferase). Enzymes were stained according to the protocols of Harris and Hopkins (11) and Solits et al. (37). EST and GOT were stained in solution baths, whereas the agar overlay technique was applied with the other enzymes.

In preliminary experiments, we also tested a number of additional enzyme systems that did not give satisfactory activity or band resolution (Table 2) with the three buffer systems used (Table 1). When the genetic basis of isozymes was clear from this or a related (45) study, enzyme alleles were coded according to the relative migration distance (*R*) from the origin near the cathode, with the most frequent allele representing, by definition, *R* = 100. When the genetic basis of isozyme banding patterns was not clear, each band was considered to be due to a different locus, and loci were designated tentatively with the prefix *B* for band followed by the migration distance (e.g., *Got-B62*).

**Data analysis.** All computations were done with the program package NTSYS-pc, Numerical Taxonomy and Multivariate Statistical Analysis System (31), unless stated otherwise.

The Dice index of similarity (36) was computed to qualitatively compare different electrophoretic types (ETs). The Dice index is computed as

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

in which *F* represents the proportion of alleles shared by two ETs (*n*<sub>ab</sub>) among the sum of alleles that ETs *a* and *b* express (*n*<sub>a</sub> and *n*<sub>b</sub>).

We calculated Nei's standard genetic distance for comparisons between races of *C. carbonum* (25). It is defined as

\[
d_{ab} = -\ln \left( \frac{\sum_{i=1}^{k} x_{ia} x_{ib}}{\left( \sum_{i=1}^{k} x_{ia}^2 \sum_{i=1}^{k} x_{ib}^2 \right)^{1/2}} \right)
\]

in which two taxonomic units, *a* and *b*, are compared over *k* loci, and *x*<sub>ia</sub> and *x*<sub>ib</sub> denote the relative frequency of the *i*th allele in units *a* and *b*.

The UPGMA (unweighted pair-group method with arithmetic averaging) clustering method (38) was used to group ETs on the basis of their qualitative similarity (Dice index) and to compare the relatedness of races by using the Nei's standard genetic distance measure, *d*. Results are presented as phenograms prepared by programs SAnH and TRiEg in NTSYS-pc.

We also used principal coordinates analysis (9,10) to corroborate the result from the cluster analysis of ETs. The same similarity matrix used for cluster analysis of ETs (Dice index) was transformed to scalar product form by program DCENTER in NTSYS-

**TABLE 1. Buffer composition and run parameters for starch gel electrophoresis of isozymes from isolates of Cochliobolus carbonum**

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>Gel buffer</th>
<th>Tray buffer</th>
<th>Parameters</th>
<th>Target enzyme*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-borate</td>
<td>0.015 M Tris, pH 7.7 with citric acid</td>
<td>0.3 M boric acid, pH 7.5 with 1 M NaOH</td>
<td>3 h, 60 mA</td>
<td>PGD, DIA, EST</td>
</tr>
<tr>
<td>Histidine-citrate</td>
<td>0.02 M l-histidine-HCl, pH 5.7 with 1 M NaOH</td>
<td>75 mM Na&lt;sub&gt;2&lt;/sub&gt;-citrate, pH 5.7 with citric acid</td>
<td>3.5 h, 100 mA</td>
<td>GPI, ACO</td>
</tr>
<tr>
<td>Tris</td>
<td>9 mM Tris, 0.3 mM citric acid, pH 7.0 with Tris</td>
<td>0.135 M Tris, 0.045 M citric acid, pH 7.0 with 1 M NaOH</td>
<td>3 h, 100 mA</td>
<td>PGM, MDH, GOT</td>
</tr>
</tbody>
</table>

*PGD = phosphogluconate dehydrogenase; DIA = NADH-diaphorase; EST = α-esterase; GPI = glucosephosphate isomerase; ACO = aconitase; PGM = phosphoglucomutase; MDH = malate dehydrogenase; and GOT = glutamate oxaloacetate transaminase.*

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pc, and eigenvalues and eigenvectors were computed by program EIGEN in NTSYS-pc. These vectors are the principal coordinate axes. The first three principal coordinates explained a great part of the similarity among ETs, so they were depicted graphically with Proc G3D in SAGROPE (32). With no missing values and with the Dice similarity index, results from a principal coordinates analysis are equal to those from a principal components analysis (31). The two techniques differ in that a principal components analysis is based on a covariance matrix of allele frequencies, whereas in a principal coordinates analysis, a genetic distance or similarity matrix of taxonomic units serves as the basis for the actual multivariate analysis. Consequently, computation of the principal coordinates analysis is more convenient when the number of allele frequencies is greater than the number of taxonomic units (9), a situation met frequently in restriction fragment length polymorphism and isozyme analyses.

Average gene diversity $\hat{H}$ was computed as

$$\hat{H} = \frac{\sum \hat{h}_j}{r},$$

in which $\hat{h}_j$ is the gene diversity at the $j$th locus and $r$ is the number of loci. An unbiased estimate of gene diversity in a selfing population, being equivalent to a haploid population, was given by Nei (25) as

$$\hat{h} = \frac{n(1-\Sigma x_i^2)}{n-1},$$

in which $n$ is the number of isolates and $x_i$ is the frequency of the $i$th allele at the locus considered. The sampling variance of $\hat{H}$ was obtained by

$$V(\hat{H}) = \frac{V(\hat{h})}{r},$$

in which $V(\hat{h})$, the variance of $\hat{h}$ is

$$V(\hat{h}) = \frac{\Sigma (\hat{h}_j - \hat{H})^2}{r-1}.$$  

The standard error of $\hat{H}$ is the square root of the total variance $V(\hat{H})$. $H$ values of different races were compared by a $t$ test for independent observations as outlined by Nei (25).

To compare the richness of variation in ETs among races, i.e., multilocus isozyme genotypes, the Shannon index of diversity (29) was calculated from the equation

$$H' = -\Sigma p_i \ln p_i,$$

in which $p_i$ is the proportion of the $i$th ET in a race. Standard errors and $t$ tests to compare $H'$ values of different samples were computed according to Poole (29).

**RESULTS**

Of the eight enzymes that performed well, five were moderately to highly polymorphic, two were weakly polymorphic, and only one was monomorphic (Table 2). We did not investigate whether the inconsistent staining of acid phosphatase (ACP), leucine amino peptidase (LAP), and xanthine dehydrogenase (XDH) reflected inconsistent sample treatment or genetic polymorphism (i.e., functional and null alleles).

The genetic interpretation of PGD, PGM, ACO, and DIA banding patterns was straightforward; they were controlled by single loci with two to three alleles each (Table 3). The patterns of MDH, GOT, and EST indicated a rather complex genetic basis. In interpreting these patterns, we assumed that our isolates

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**TABLE 2. Activity, resolution of bands, and morphism of enzyme systems in Cochliobolus carbonum**

<table>
<thead>
<tr>
<th>Activity and resolution</th>
<th>Enzyme</th>
<th>Morphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A+ R+</td>
<td>ACO (aconitase, EC 4.2.1.3)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>DIA (NADH-diaphorase, EC 1.6.2.2)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>EST (a-esterase, EC 3.1.1.1)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>GPI (glucosephosphate isomerase, EC 5.3.1.1)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>GOT (glutamate oxaloacetate transaminase, EC 2.6.1.1)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>MDH (malate dehydrogenase, EC 1.1.1.37)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>PGD (phosphogluconate dehydrogenase, EC 1.1.1.44)</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>PGM (phosphoglucomutase, EC 2.7.5.1)</td>
<td>P+</td>
</tr>
<tr>
<td>A+ R+</td>
<td>ACP (acid phosphatase, EC 3.1.3.2)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>LAP (leucine amino peptidase, EC 3.4.11)</td>
<td>EC 1.2.1.37</td>
</tr>
<tr>
<td></td>
<td>XDH (xanthine dehydrogenase, EC 1.1.1.49)</td>
<td>P+</td>
</tr>
<tr>
<td>A+ R−</td>
<td>ADH (alcohol dehydrogenase, EC 1.1.1.1)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>GD (glucose-6-phosphate dehydrogenase, EC 1.1.1.49)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>SOD (superoxide dismutase, EC 1.15.1.1)</td>
<td>P</td>
</tr>
<tr>
<td>A− R+</td>
<td>ALP (aluminum phosphatase, EC 3.1.3.1)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>F-1,6-DP (fructose-1,6-diphosphatase, EC 3.1.3.1)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>FH (fumarate hydratase, EC 4.2.1.2)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>GDH (glucose dehydrogenase, EC 1.1.1.47)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>GPT (glutamate-pyruvate transaminase, EC 2.6.1.2)</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>SDH (shikimate dehydrogenase, EC 1.1.1.25)</td>
<td>P+</td>
</tr>
</tbody>
</table>

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**TABLE 3. Relative allele frequencies (%) among races of Cochliobolus carbonum**

<table>
<thead>
<tr>
<th>Putative isozyme allele</th>
<th>Pgd</th>
<th>Gpi</th>
<th>Pgm</th>
<th>Aco</th>
<th>Dia</th>
<th>Mdh1</th>
<th>Mdh2</th>
<th>Got-B</th>
<th>Est-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race n*</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>29</td>
<td>99</td>
<td>0</td>
<td>1</td>
<td>96</td>
<td>4</td>
<td>0</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>100</td>
<td>0</td>
<td>1</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Number of isolates.

* Alleles are designated by locus name (e.g., Mdh1 = malate dehydrogenase locus 1) and relative allozyme mobility.
were typical haploid monokaryons. This assumption is based on the following: Heterokaryon incompatibility severely limits the potential for heterokaryon formation by Cochliobolus spp. in nature (40, 47). Conidia produced by forced heterokaryons on artificial media are nearly always monokaryotic (40). Our isolates were derived from single conidia (44). The phenotypes of our isolates were stable.

In vertebrates, MDH usually has a dimeric structure (11), but in all isolates of races 1 and 2 and in all but 3 of 75 isolates of race 3, MDH appeared to be tetrameric. These isolates expressed a pattern of five regularly spaced MDH bands (Rf = 100, 83, 67, 50, and 33) with darker staining in the intermediate migration distances (Rf = 83, 67, and 50). This is typical of a tetrameric enzyme forming two homotetramers, Rf = 100 and 33, and three heterotetramers, Rf = 83, 67, and 50 (6). This pattern may be explained by alleles at two MDH loci, Mdhl 100 and Mdhd 33 (Table 3). The deviant two-banded MDH pattern of all race 0 isolates (Rf = 100 and 56, or Rf = 133 and 56) and the threecolored pattern of a single race 3 isolate (Rf = 111, 89, and 67) suggest that there may be three alleles at each MDH locus and that only the alloseymes of Mdhl 100 and Mdhd 33 can form tetramers. This would be unusual, but variation is known to occur in subunit assembly of oligomeric enzymes among different taxa (6).

No isolate had more than one of the “fast” MDH bands at Rf = 100, 133, or 111. This is consistent with a model of an MDH locus, Mdhl, with three alleles. In crosses of two race 0 isolates (MDH bands 100 and 56) with two race 2 isolates (MDH bands 100, 83, 67, 50, and 33), there was a clear single gene segregation for MDH bands at Rf = 56 and 33 (45), suggesting that these bands are due to alleles at a single locus. Thus, there is additional evidence for at least two alleles at a second MDH locus, Mdhd. Both parents in each of the three crosses shared the Mdhl 100 allele, so no segregation was seen at the Mdhd locus (45).

Seven different well-resolved esterase bands were expressed among the races of C. carbonum (Table 4, Fig. 1), but the majority of isolates expressed only five (Table 3). Additional faint bands occurred at relative migration distances outside the range considered for analysis (Fig. 1). The genetic basis of the observed EST patterns was difficult to infer from these data alone, but crosses between isolates of race 0 and race 2 (45) suggested the presence of a single locus relating to each band, although not all of the seven EST bands segregated in a clear 1:1 fashion. The patterns we observed among natural isolates are consistent with this interpretation. The observed patterns are not consistent with interlocus oligomer formation nor with allelism of genes for the EST bands at Rf = 100, 97, 87, 80, and 73. However, they do not exclude the possibility that the gene for the band at Rf = 77 might be allelic with a gene for a band at Rf = 100, 92, or 73. For consistency and simplicity, we assigned each band a tentative (indicated by B for band) locus designation (Table 3).

Two GOT bands were expressed by most isolates (Table 3), but some had only a single band at Rf = 100. This band, GOT-B100, consistently stained more intensely when it occurred alone than when it occurred with band GOT-B62. The reason could be a duplication of the Got locus at Rf = 100 leading to stronger enzyme activity. Isolates with two (weaker) bands may indicate a mutation at one of the loci. The nonmutated wild-type allele at locus 1 could encode the “fast” band (Rf = 100) and the mutated allele at locus 2 the “slow” band (Rf = 62). Alternatively, one might suppose that the two bands are due to two independent loci, one monomorphic (Rf = 100) and the other polymorphic with one functional (Rf = 62) and one null allele. The latter model, however, does not take into account the consistent difference in staining intensity of the two bands. At any rate, there seem to be two Got loci, which we tentatively named Got-B100 and Got-B62.

GPI stains were difficult to score, because secondary bands frequently appeared on the gel about 10 min after the main band (Rf = 100) appeared. We followed the common practice of not regarding such bands as genetically distinct, although it appeared that some isolates produced these bands more intensely and reproducibly than others.

The isozyme data were used to compare the genetic relatedness of C. carbonum races and the level of genetic diversity within the races. Allele frequencies in races 1, 2, and 3 were highly correlated (Table 3), indicating a close genetic relation. Race 1 differed clearly from race 2 only in its much lower Got-B62 frequency (Table 3) (but the sample size of race 1 was small). The only major difference between race 2 and race 3 was their contrasting frequencies of Est-B97 and Est-B87 (Table 3, Fig. 1). Race 0, on the other hand, was distinctly different from the other three races in most allele frequencies (Table 3).

The high degree of similarity between race 1 and race 2 is most evident from the list of ETs (Table 5). Of the six race 1 isolates analyzed, five represented ET9, which was the second most frequent type in race 2. These five isolates all came from North Carolina (four isolates) or South Carolina (one isolate), whereas the unique ET10 in race 1 belonged to one isolate from Michigan.

 Races 2 and 3 shared similar phenotypes, but their shared phenotypes differed in frequency. ET12, the predominant type (93%) in race 3, ranked third at 4% frequency among race 2 isolates. ET11, the predominant type (82%) in race 2, ranked second at 5% among race 3 isolates (Table 5). Only one race 3 isolate had a type (ET16) not found among the race 2 isolates. In contrast, the only shared ET between race 0 and another race was ET6, which occurred once among race 0 isolates and once among race 2 isolates (Table 5).

### Table 4. Esterase phenotypes among field isolates of Cochliobolus carbonum

<table>
<thead>
<tr>
<th>Rf</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
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<tbody>
<tr>
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<tr>
<td>97</td>
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<td>73</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
<td>73</td>
<td>5</td>
<td>13</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*a Relative migration distance.

*b Additional esterase phenotypes were found among progeny of crosses between field isolates of C. carbonum (45).

*c Number of times found.

---

**Fig. 1.** α-Esterase banding patterns for one race 0, four race 2, and three race 3 isolates of Cochliobolus carbonum. Esterase bands with relative migration distance values of <73 or >100 were inconsistent and not included in the analysis.
TABLE 5. Frequencies of electrophoretic types among races of Cochliobolus carbonum

<table>
<thead>
<tr>
<th>Race</th>
<th>n°</th>
<th>ET</th>
<th>Pgd</th>
<th>Gpi</th>
<th>Pgm</th>
<th>Aco</th>
<th>Dia</th>
<th>Mdh1</th>
<th>Mdh2</th>
<th>Got-B</th>
<th>Est-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of isolates

* Electrophoretic type.

* Alleles are designated by locus name (e.g., Mdh1 = malate dehydrogenase locus 1) and relative allozyme mobility.

---

**Dice Similarity Index**

0.48 0.64 0.80 0.96 1.12

---

**Fig. 2. Phenogram of similarity among 16 electrophoretic types (ETs) of races 0, 1, 2, and 3 of Cochliobolus carbonum. Some ETs occur in more than one race. The Dice index of similarity is based on the proportion of shared bands between ETs without regard to frequency of the ETs in the population. The phenogram was constructed by the unweighted pair-group method with arithmetic averaging.**

Further insight into the relatedness of ETs is provided by the cluster analysis of the Dice indices of similarity among ETs (Fig. 2). The majority of ETs among races 1, 2, and 3 were very closely related and fell into a single cluster. Exceptions to this were ET16 of race 3, differing in a number of isozyme alleles from the rest of race 3 isolates (Table 5), and ET6, being shared by race 0 and race 2.

Results of the principal coordinates analysis regarding the similarity of ETs corroborated this picture (Fig. 3). The typical ETs of races 1, 2, and 3 were well separated from those of race 0. Figure 3 presents the first three principal coordinates that contributed 36%, 20%, and 15% to the genetic similarity, respectively. ET16 is an outlier, but because of the minor importance of coordinate 3, it appears to be more similar to ET2, ET4, and ET5 of race 0 than to any other ET of races 1, 2, or 3 (Fig. 3).

The phenogram of the four C. carbonum races as constructed with Nei's standard distance measure (Fig. 4) is a condensed representation of their apparent genetic relatedness on the basis of race 3 isolates (Table 5), and ET6, being shared by race 0 and race 2.
of isozyme data. Races 1 and 2 appear to be most closely related in a cluster that includes race 3. The distance of this cluster from race 0 is comparatively large.

A general impression of the level of genetic diversity within the four races can be gained by inspecting their allele frequencies. Race 0 had more alleles at intermediate frequencies than were found in the other races, indicating a higher level of diversity. On the basis of the more stringent definition of polymorphism (less frequent allele[s] ≥ 0.05), nine of 16 putative isozyme loci were polymorphic in race 0, in contrast to only three polymorphic loci in race 1, three in race 2, and two in race 3 (Tables 3 and 6).

Nei's unbiased estimate of average gene diversity ($H$) also shows that race 0 was significantly more diverse than either race 2 or race 3 (Table 6). The test as suggested by Nei (25) demonstrated a significant difference between $H$ of race 0 and race 2 ($t = 3.368$, $P < 0.05$) but not between race 2 and race 3 ($t = 0.559$, $P > 0.05$). The same ranking in diversity was obtained by the Shannon index of diversity ($H'$) calculated from the proportions of ETs found in each race (Table 6). Not only was the difference between the $H'$ values of race 0 and race 2 highly significant ($t = 6.443$, $P < 0.001$), but the difference between race 2 and race 3 was significant as well ($t = 2.881$, $P < 0.01$).

DISCUSSION

Of the 20 enzyme systems that we evaluated, eight showed high activity and gave consistent band resolution under our conditions. Seven of these eight (all but PGD) were polymorphic at the 1% level. This high ratio of polymorphic enzymes may not be representative of the genome of *C. carinum*, because (1) we preselected enzyme systems known to be polymorphic in other pathogenic fungi (1,2,19,21), and (2) we refined the protocols for detecting those enzymes that showed signs of polymorphism in preliminary experiments. These same qualifications, however, probably apply to most organisms studied electrophoretically (25) and, thus, do not diminish the validity of our intraspecific comparisons.

Simcox et al. (35) tested 20 isolates of *C. carinum* for 36 enzyme systems and found polymorphisms for only five of the 19 that gave uniform staining and consistent resolution of bands. Three of the polymorphic systems identified by Simcox et al. (i.e., GOT, EST, and GPI) were also polymorphic among our isolates. LAP, which gave inconsistent activity in our study, was polymorphic among the race 1 and race 3 isolates of Simcox et al. (35), but none of their race 2 isolates showed LAP activity. Mannitol dehydrogenase (MADH) was polymorphic in their study but was not tested in ours. ACO, MDH, and PGM were polymorphic in our study but monomorphic among the isolates tested by Simcox et al. (35). DIA also was polymorphic in our study but stained inconsistently for Simcox et al. (35).

The greater number of polymorphic enzyme systems found in our study than in that of Simcox et al. (35) can be attributed to our larger sample size (194 isolates) compared with that of Simcox et al. (20 isolates). Even without the 29 isolates of the genetically diverse race 0, which was not tested by Simcox et al. (35), our 90 race 2 and 75 race 3 isolates from two fields in the piedmont of North Carolina showed combined polymorphism for seven enzyme systems.

Nei (25) suggested that the proportion of polymorphic loci ($P$) is not an accurate measure of genetic diversity, but we found general agreement between the three diversity measures, $P$, $H$ (average gene diversity), and $H'$ (Shannon index). The diversity of race 0 was dramatically greater than that of races 2 and 3, which exhibited only a small difference between their genetic diversities. The difference between race 2 and race 3 was not significant for $H$, which is based on allele frequencies, but was significant for $H'$, which is based on multilocus genotype frequencies. Thus, the Shannon index may be more sensitive than Nei’s average gene diversity index as a measure of genetic diversity in fungi such as *C. carinum*, in which reproduction is predominantly asexual. We also found a significantly greater Shannon index for race 2 than for race 3 in a previous study of genetic diversity in phenotypic markers such as mating capacity, fungicide tolerance, and colony morphology in the same populations of *C. carinum* from Wilkes and Yadkin counties in North Carolina (44). With regard to these phenotypic markers, as with isozymes, race 0 was significantly more diverse than either race 2 or race 3.

The small sample of race 1 isolates tested in this study came from earlier collections made in the 1960s and 1970s (15,16), because we found no race 1 isolates in the 1987 survey (44). It is remarkable that all five race 1 isolates in this sample from North Carolina and South Carolina had identical isozyme patterns even though they were collected from different fields and in different years. The fact that all five also were of the same mating type (MAT-2 = mating type a) contrasts with frequencies of mating types in races 0, 2, and 3 near the presumed equilibrium of 50% (44). These data suggest that the small race 1 population in the Carolinas may be a clone. This could be an effect of random genetic drift, since the frequency of race 1 dropped below 1% in the *C. carinum* population of North Carolina (16,44).

The sixth race 1 isolate came from Michigan and was collected before 1977. It also is mating type MAT-2 but has a unique isozyme profile. More isolates would be needed to determine whether this

![Fig. 4. Phenogram of Nei's standard genetic distance (25) between races 0, 1, 2, and 3 of *Cochliobolus carinum* on the basis of isozyme allele frequencies. The phenogram was constructed by the unweighted pair-group method with arithmetic averaging.](image)

<table>
<thead>
<tr>
<th>Race</th>
<th>$H^+$</th>
<th>$P$</th>
<th>$H'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.195 ± 0.047</td>
<td>0.563</td>
<td>1.592 ± 0.041</td>
</tr>
<tr>
<td>2</td>
<td>0.032 ± 0.011</td>
<td>0.188</td>
<td>0.730 ± 0.127</td>
</tr>
<tr>
<td>3</td>
<td>0.024 ± 0.011</td>
<td>0.125</td>
<td>0.277 ± 0.093</td>
</tr>
</tbody>
</table>

*Race 1 was not included because it was completely uniform in North Carolina.*

$H^+$ ± standard error was computed from allele frequencies at 16 putative isozyme loci according to Nei (25). A locus is defined as polymorphic when the most frequent allele is present in ≤ 95% of the individuals.

$H'$ ± standard error was computed from proportions of electrophoretic types according to Poole (29).
difference resulted from genetic divergence between the south-
easter and midwestern populations of race 1 in the United States. Our data and those of Simcox et al (35) do not allow a full comparison of isozyme patterns between their race 1 isolates from Urbana, Illinois, and our isolate from Michigan. The partial compa-
rison that is possible does not rule out the possibility that these 
race 1 isolates are similar, although some bands detected in our 
study were not found in theirs and vice versa.

The common electrophoretic type (ET9) of the race 1 isolates 
from North Carolina and South Carolina was shared by a significant 
part of the race 2 population (8%), suggesting a common ancestry 
of these races or genetic recombination between them. Race 1 and race 3 are known to differ not only in toxin production 
but also in polynenes for lesion type on corn lines insensitive 
to race 1 toxin. Therefore, it appears that race 1 is more closely 
related to race 2 than to race 3. The DNA region that encodes 
HC-toxin synthetase necessary for toxin production in race 1 
has no apparent homology with DNA in either race 2 or race 3, 
but border sequences on either side of this region are shared 
by race 1 and race 2, (28). The origin of the gene or gene complex 
for toxin production in race 1 is not known.

Leonard (16) and Leonard and Leath (17) concluded that race 
3 may have evolved more recently than race 2 on the basis of 
the following evidence: 1) race 3 shows less genetic variation 
than race 2; 2) race 3 was discovered more recently (27); and 3) race 
3 has a narrower known geographic range than race 2. Our 
observation that all but one isolate of race 3 exhibited ETs found 
also in race 2 suggests that race 3 may have arisen from race 
2. If race 3 arose from race 2, the completely different frequencies 
of two esterase alleles in race 2 and race 3 illustrate that the 
two race populations must have diverged genetically since the 
origin of race 3.

The absence of hybridization between races 2 and 3 in the 
field (14,16,17,22) may be a consequence of, rather than the reason 
for, their divergent evolution. However, the lack of hybridization 
within these races. Welz and Leonard (44) found no significant genetic phase disequilibrium among pheno-
typic markers within race 2 or race 3. Some linkage disequilibrium 
might be expected in populations that completely lack sexual
reproduction. The isozyme data are not very suitable for analysis 
of linkage disequilibria, because the isozyme allele frequencies 
in races 2 and 3 were mostly 0-5% or 95-100%. However, the 
presence of both mating types in each electrophoretic type 
observed more than twice in each race (H. G. Welz, unpublished) 
indicates that there was genetic variation within electrophoretic 
types of races 2 and 3.

Although Simcox et al (35) found isozyme analysis to be 
extremely useful in identifying specific races of C. carbonum, 
we found that a significant number of isolates could be misclassi-
fied if only isozyme data were used. For example, if we regarded 
ET11 as a race 2 phenotype, then ET12 as a race 3 phenotype, 
we would have misclassified 5% of our race 3 isolates as race 
2 and 4% of our race 2 isolates as race 3. This difference between 
our results and those of Simcox et al (35) may be attributed 
to differences in the sources of isolates. Seven of the nine race 
2 isolates that Simcox et al (35) tested were from the Midwest, 
whereas eight of their nine race 3 isolates were from the southeast 
or the northeast areas of the United States. All 90 of our race 
2 isolates and all 75 of our race 3 isolates came from the same 
other two fields in North Carolina.

The avirulent race 0 of C. carbonum is phenotypically and 
genetically quite distinct from the other races in this fungus. 
This result had already emerged from the study of phenotypic 
markers (44) and was confirmed by our comparison of isozyme 
patterns as well as by Jones and Dunkle's (12) comparison of 
polymerase chain reaction amplification products from races 0, 
1, 2, 3, and 4. Only one of our eight electrophoretic types of 
race 0, ET6, occurred also in another race, viz, race 2. It is 
interesting that the single race 0 isolate with ET6 had another typical 
race 0 alleles (Pda— for inability to sporulate on potato-dextrose 
agar and CadR for cadmium tolerance), whereas the single race 2 isolate with ET6 had the Pda+ , CadR genotype (H. G. Welz, 
unpublished). Since cadmium tolerance is very rare (2%) in race 
2 (16,44), this isolate might have resulted from hybridization 
between race 0 and race 2. It is also interesting that both ET6 
isolates came from the same field.

The genetic diversity in race 0 was significantly greater than 
that in races 1, 2, or 3. This may indicate that the race 0 population 
in North Carolina is older than the race 1, 2, and 3 populations 
or that race 0 is subjected to less intense selection than are the 
other three races. The greater dependence of races 1, 2, and 3 
on agricultural production of corn could cause greater fluctuations 
in their population sizes than occurs in race 0 populations. In 
Magnaporthe grisea, the rice blast fungus, Leung and Williams 
(19) found much greater variation among grass isolates than 
among isolates from rice. They interpreted this as niche-dependent 
variation being correlated with variation in the host population.

We do not know the host range of race 0 of C. carbonum, but 
we have evidence of its low parasitic fitness on corn (42,44). Race 
0 may be more of a saprophyte than a parasite and may be broadly 
adapted to survive under varying conditions of the soil or crop 
debris, or race 0 may persist as a pathogen of some weedy grass 
species in roadsides and corn fields.

Laboratory experiments with Drosophila have shown that allo-
yzme heterozygosity was increased with environmental hetero-
genosity (23,30). Gillespie (8) showed in a model that polymorphism 
could be maintained mainly by spatial heterogeneity of the habitat. 
Spithi (39) extended Gillespie's theory to a model of "shifting 
multiple niches," in which the distribution of niches varies from 
one population to the next, causing different alleles and different 
levels of polymorphism to be favored in different populations. 
However, experimental evidence to support this theory is still 
lacking, mainly because selective effects of different allozyme 
alleles rarely could be proven (20). Furthermore, Lewontin (20) 
pointed out that, because of the typically large standard errors 
for average heterozygosity among allozyme loci, more than 100 
loci must be analyzed in a species for reliable estimates of the 
correlations between ecological parameters and the level of hetero-
zygosity. With molecular markers, it should be possible to reach 
that level of statistical power in pathogenic fungi. Jones and 
Dunkle's (12) recent study of polymerase chain reaction amplifi-
cation products from races 0, 1, 2, 3, and 4 of C. carbonum 
provides a good start in this direction.

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