Analysis of *Cochliobolus carbonum* Races by PCR Amplification with Arbitrary and Gene-Specific Primers

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ABSTRACT

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The pathogenic races of *Cochliobolus carbonum* cause necrotic lesions of characteristic sizes and shapes on maize leaves. To distinguish the races at the molecular level, isolates of *C. carbonum* races as well as other related species were analyzed by PCR (polymerase chain reaction) amplification of genomic DNA using either arbitrary oligonucleotide primers or primers with homology to sequences within the *Tox2* locus, which is essential for production of a host-specific toxin. Amplification products from isolates of the four pathogenic races of *C. carbonum* were very similar to each other and to those from species thought to be closely related but were substantially different from nonpathogenic race 0 and from most other species. One of the arbitrary primers tested distinguished isolates of *C. carbonum* race 3 by the absence of two amplification products present in the other pathogenic races. The patterns of amplification products from races 2 and 4 were indistinguishable with the primers tested, suggesting that the recently described race 4 is not substantially different from race 2. Primers from the *Tox2* locus distinguished race 1 isolates from isolates of other races. Only isolates of race 1 contained a single amplification product of the expected length when these primers were used under stringent annealing conditions. The results indicate that PCR amplification with arbitrary primers or gene-specific primers is useful for differentiating the races of *C. carbonum* and for examining their origins.

Additional keywords: *Bipolaris zeicola*, HC-toxin, *Helminthosporium carbonum*, Helminthosporium leaf spot of maize, random amplified polymorphic DNA (RAPD).

The genus *Cochliobolus* (members in the anamorphic genera *Helminthosporium* or *Bipolaris*) is composed of a diverse group of fungi pathogenic on different graminaceous host species. Perhaps because of the interspecific fertility within the genus (16,26) and the marked differences in pathogenicity and virulence among isolates of a species, identification based on morphological and cultural characteristics or on disease symptoms is not straight forward. Assessment of genetic variability within and among these species would provide valuable information on the relatedness and origin of the different species.

The fungus *Cochliobolus carbonum* R.R. Nelson (anamorph, *Bipolaris zeicola* (G.L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) causes Helminthosporium leaf spot and ear rot of maize (*Zea mays* L.) (34). In 1941, two races were described and differentiated on the basis of the size and shape of leaf lesions on specific maize inbreds (33). Race 1 causes large oval lesions on susceptible inbred lines with recessive alleles at the *Hm* locus (15); race 2 causes small lesions on most inbreds, including *hmhm* genotypes (Fig. 1). The greater virulence of race 1 is the result of the production of HC-toxin (18,27), a host-specific cyclic tetrapeptide (21,37) that affects only genotypes of maize susceptible to the toxin-producing race. Synthesis of HC-toxin is controlled by the *Tox2* locus (20), which encodes at least two enzyme activities (HTS-1 and HTS-2) of a cyclic peptide synthetase (36,38).

In addition to races 1 and 2, *C. carbonum* is composed of other races that vary in their cultural, morphological, or pathogenic characteristics (12,13). Consequently, race identification based on disease reaction is equivocal, except when identifying race 1. This is in part because the disease phenotypes vary with the isolate/cultivar combinations (8) and because a consistent set of inbred lines has not been used to characterize the races (9,17). During the early 1970s, strains of *C. carbonum* that incited a phenotypically distinct lesion size and shape (Fig. 1) were designated race 3 (17). Race 3 isolates were pathogenic on numerous maize inbreds with no differential host-genotype response (7,8). Race 3 has persisted in North America and has been identified as the pathogen causing a disease of sweet corn in Japan (30,31). Isolates from Japan also infect rice and produce a toxin with selective activity against rice cultivars (41). Still other forms of *C. carbonum*, differing in conidial and cultural characteristics, were described during the early 1970s and were thought to be distinct from the other races (9,35). Recently, another race of *C. carbonum* was reported in the the U. S. corn belt (3). This race, designated pathotype 4, infects a number of inbreds commonly used in hybrid seed production and causes large lesions with concentric rings (Fig. 1). Plants die in the later stages of disease development and express symptoms that suggested to Dodd and Hooker (3) that a toxin is involved. Whether this pathotype differs from the previously described races and whether it represents a new race with unique specificity are unknown. In addition to pathogenic races, a nonpathogenic race of *C. carbonum* with different colony morphology was recently described by Welz and Leonard (39) and was designated race 0.

Pathological races are usually identified by the disease response

of selected genotypes of the host. Other approaches to identifying and characterizing races include tests for heterokaryon incompatibility, isozyme analysis, electrophoretic karyotyping, and genetic comparisons by restriction fragment length polymorphisms (RFLPs) and by polymerase chain reaction (PCR) fingerprinting. Of the DNA-based methods of examining genetic relationships among taxonomic groups, analyses based on PCR polymorphisms generated from arbitrary decamer primers (40) are gaining wide usage. Thus far, PCR-based methods of detecting and quantifying differences in DNA sequences have been applied to distinguish species or races of fungal pathogens (2,5) as well as to analyze isolates of extremely heterogeneous species, such as *Colletotrichum graminicola* (6).

The objectives of this study were to examine the genetic relatedness among the races of *C. carbonum* and among similar species and to determine the feasibility of distinguishing the races by their PCR-amplification products. To this end, we used single arbitrary primers to amplify DNA sequences and compared the amplification products as an indication of genetic relatedness. In addition, to investigate the possibility that races other than race 1 possess the genetic potential to produce toxic metabolites similar to the HC-toxin, we used primers from the *Tox2* locus to specifically amplify *Tox2* DNA.

MATERIALS AND METHODS

Source and culture of fungal strains. The fungi used in this study and their sources are listed in Table 1. Members of the genus *Cochliobolus* included in this study, in addition to *C. carbonum*, are *C. heterostrophus* (Drechs.) Drechs. (= *Bipolaris maydis* (Nisikado & Miyake) Shoemaker), *C. miyabeanus* (Ito & Kuribayashi) Drechs. ex Dastur (= *Bipolaris oryzae* (Breda de Haan) Shoemaker), *C. sativus* (Ito & Kuribayashi) Drechs. ex Dastur (= *Bipolaris sorokiniana* (Sacc.) Shoemaker), *C. victoriae* R.R. Nelson (= *Bipolaris victoriae* (F. Meehan & Murphy) Shoemaker), and *Bipolaris sacchari* (E.J. Butler) Shoemaker, which has no known teleomorphic stage. Also included in the study was *Setosphaeria turcica* (Luttrell) K.J. Leonard & E.G. Suggs (= *Exserohilum turcicum* (Pass.) K.J. Leonard & E.G. Suggs) (= *H. turcicum* (Pass.)).

Conidia from cultures or diseased leaf tissue were germinated on water agar, and single germlings were transferred to V8-juice agar (32) or to lactose casein hydrolysate medium (32) for *S. turcica*. Cultures were grown for 7–10 days at 26 C under fluorescent light with a 12-h photoperiod. Isolates were preserved as conidia from the single-spore cultures stored in 20% glycerol at −80 C. For mycelium production, conidia were used to inoculate liquid-modified Fries medium (23), and cultures were grown in Erlenmeyer flasks at 26 C on a rotary shaker for 24 h.

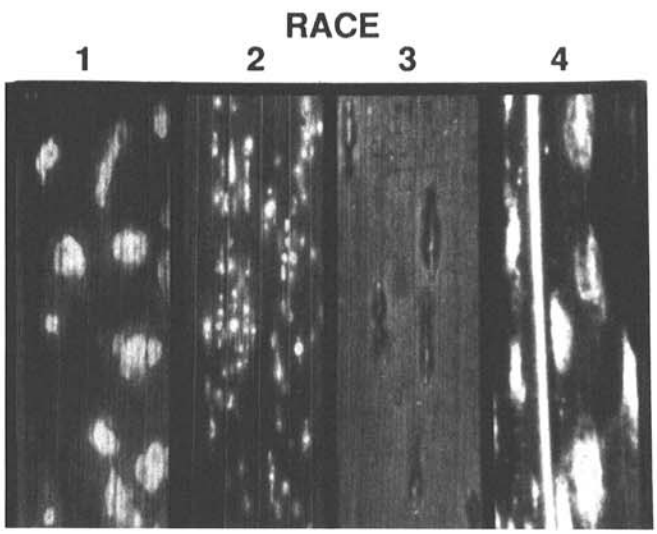

Fig 1. Disease phenotypes of the four pathogenic races of *Cochliobolus carbonum* on susceptible maize leaves.

DNA amplification conditions. Fungal DNA was extracted from 1–3 g of mycelium as described by Panaccione et al (19). Preparations were enriched for nuclear DNA with two successive bisbenzimide-CsCl gradients (4,10), but methods without the gradient steps yielded DNA of sufficient purity for analysis. The DNA from all isolates of each race was analyzed to establish the reproducible and characteristic patterns of amplification products for each race, but the data presented are for a representative isolate of each race to illustrate the major differences and distinguishing features of the patterns. Two to four isolates of each race were initially screened for race-specific bands detected by each of 20 primers, as described below. Primers that yielded putative race-specific bands were used to analyze a minimum of nine different isolates of the same race, except for race 0, which was represented by only two isolates in our collection.

The DNA was amplified in a 25-μl reaction mixture containing 12.5 ng of template DNA, 0.4 μM primer, 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 75 μM each dATP, dCTP, dGTP, and dTTP (Perkin-Elmer/Cetus), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, either 1.5 or 3.5 mM $MgCl_2$,

TABLE 1. Isolates and origins of fungal species

Species (race)	Isolate designation	Origin
C. carbonum		
race 0	CC011	North Carolina
	CC021	North Carolina
race 1	CC111	Unknown
	CC121	Indiana
	CC131	Unknown
	CC141	Illinois
	CC151	Michigan
	CC161	North Carolina
	CC171	Michigan
	CC181	South Carolina
	CC191	Unknown
race 2	CC211	Unknown
	CC221	North Carolina
	CC231	Illinois
	CC241	Illinois
	CC251	Minnesota
	CC261	Missouri
	CC271	North Carolina
	CC281	Tennessee
	CC291	North Carolina
	CC2101	North Carolina
race 3	CC321	North Carolina
	CC331	Virginia
	CC341	Virginia
	CC352	Virginia
	CC361	Virginia
	CC372	New York
	CC381	Pennsylvania
	CC391	North Carolina
	CC3101	Ohio
	CC3111	North Carolina
	CC3121	North Carolina
race 4	CC411	Illinois
	CC421	Illinois
	CC431	Illinois
	CC441	Iowa
	CC451	Indiana
	CC461	Minnesota
	CC471	Unknown
	CC481	Illinois
	CC491	Illinois
	CC4101	Illinois
	CC4111	Illinois
C. heterostrophus	CH021	Iowa
S. turcica	HT151	North Carolina
C. victoriae	CV031	Iowa
C. miyabeanus	CM011	Unknown
C. sativus	CS011	Unknown
B. sacchari	BSO11	Unknown

4 μM EDTA, 0.006% Tween 20, and 0.006% Nonidet P40, and overlaid with mineral oil. Decamer primers (A01–A20) were obtained from Operon Technologies (Alameda, CA). To detect the presence of *HTS1* sequences, we used 17-mer primers (5′-TGTCAACTTCTGTATCT-3′ and 5′-GTTTGTTGACAGCTG-AT-3′) generously provided by D. G. Panaccione and J. D. Walton (Michigan State University, East Lansing).

Amplification was performed in either a Coy tempcycler (Coy, Grass Lake, MI) or in a Perkin-Elmer/Cetus thermal cycler using the fastest heating and cooling rates and programmed for 45 cycles of 94 C for 1 min, 37 C for 1 min, and 72 C for 2 min, followed by a final 7-min extension at 72 C. The data presented were obtained with the Coy tempcycler. The concentrations of reaction components and the thermocycling parameters were optimized, as recommended by Innis et al (11), to generate discrete amplification products and reproducible patterns. In some experiments with the *HTS1* primers, the annealing temperature was elevated to 50 C to increase the specificity of annealing. The amplification products were resolved by electrophoresis in a 1.2% agarose gel (Gibco BRL, Grand Island, NY) with 0.5× TBE buffer (0.045 M Tris-borate and 0.001 M EDTA) (24) for 17 h at 1.2 V/cm or 2 h at 5 V/cm and were stained with ethidium bromide.

RESULTS

Analysis of related species. Distinctive patterns of amplification products differentiated most of the seven species (Fig. 2). Primer A20 in the presence of 1.5 mM $MgCl_2$ and at an annealing temperature of 37 C readily distinguished *B. sacchari, C. heterostrophus, C. miyabeanus, C. sativus,* and *S. turcica.* However, the *C. victoriae* isolate analyzed was indistinguishable from the pathogenic races of *C. carbonum* using A20 (Fig. 2) and four other primers tested (A02, A04, A08, and A10) that also distinguished the other species (data not shown). The patterns of *B. sacchari* and *C. heterostrophus* were similar to each other but distinct from the other species. Interestingly, the pattern of *C. carbonum* race 0 was different from all the pathogenic races of *C. carbonum* and from all the other species.

Analysis of *C. carbonum* races. With each of the 20 decamer primers, amplification produced different banding patterns (data not shown). Depending on the primer, from one to 10 (average of six) amplification products were generated with lengths ranging from 500 to 3,000 bp. In some reactions, smaller and larger products were observed, but they were not consistently present. In addition, analysis of the amplification products from representative isolates of each race revealed band(s) that were unique to individual isolates and not necessarily characteristic of a given race, as noted for the race 3 isolates in Figure 3.

The 20 random primers were tested on all five races, and the banding patterns were similar for all the pathogenic races when using 19 of the primers (data not shown). However, with primer A03, race 3 isolates consistently lacked two amplification products, approximately 2,300 and 2,700 bp, that were amplified in isolates of all other pathogenic races (Fig. 3). Thus, A03 generated a pattern that was diagnostic for the race 3 isolates analyzed in this study.

Strains of races 2 and 4 were not differentiated by any of the initial 20 arbitrary primers we tested. Further analyses were carried out by pooling DNA preparations from 10 isolates of each race and analyzing each pool separately. Pooling the DNAs assured that differences among isolates of a race would not obscure the potential race-specific differences or complicate the detection of consistent differences between the two races. Analysis with 50 additional decamer primers failed to resolve these races on the basis of unique, race-specific amplification products (data not shown). This approach was effective, however, because analysis of pooled DNA preparations from the other races confirmed our observations that two products amplified with primer A03 in pathogenic races 1, 2, and 4 were absent from the collection of race 3 isolates. The process of screening primers for those that generated diagnostic patterns was facilitated greatly by pooling DNA preparations.

At low annealing temperatures, primers with sequence homology to the *HTS1* gene amplified two DNA fragments, a 950- to 1,000-bp product in all race 1 isolates and a 1,400-bp product in all the pathogenic races of *C. carbonum* (Fig. 4A). These products were not detected in the nonpathogenic race 0 (Fig. 4A) or in any of the other species (data not shown). A 967-bp product was predicted from sequence data (J. S. Scott-Craig,

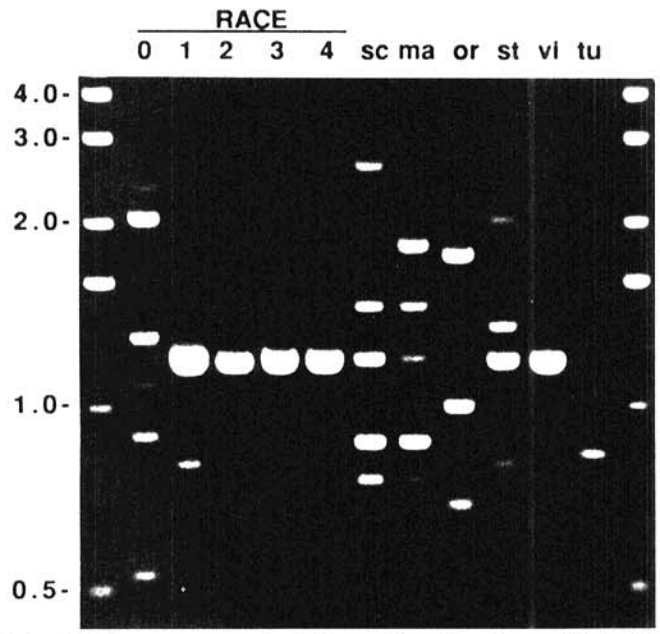

Fig 2. DNA from representative isolates of fungal species amplified with a single arbitrary primer (A20) in 1.5 mM $MgCl_2$ at an annealing temperature of 37 C. Amplified fragments were separated by electrophoresis, and gels were stained with ethidium bromide. Lanes contain, from left to right, *Cochliobolus carbonum* nonpathogenic race 0 (CC021), pathogenic races 1 (CC141), 2 (CC221), 3 (CC321), and 4 (CC411), respectively, and species *B. sacchari* (sc) (BS011), *C. heterostrophus* (ma) (CH021), *C. miyabeanus* (or) (CM011), *C. sativus* (st) (CS011), *C. victoriae* (vi) (CV031), and *S. turcica* (tu) (HT151). Values on the left indicate the position of markers in kilobase pairs, shown in the outside lanes by size standards.

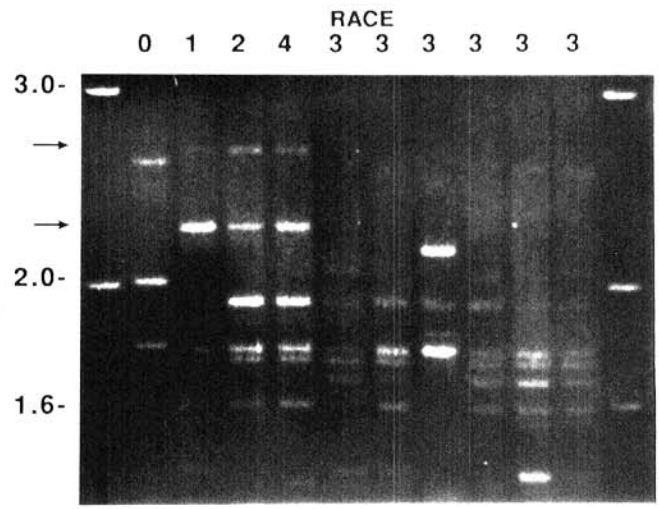

Fig 3. Differentiation of races 0 and 3 of *Cochliobolus carbonum.* DNA was amplified with a single arbitrary primer (A03) in 3.5 mM $MgCl_2$ at an annealing temperature of 37 C. Amplified fragments were separated by electrophoresis, and gels were stained with ethidium bromide. Lanes contain, from left to right, nonpathogenic race 0 (CC011) and pathogenic races 1 (CC121), 2 (CC211), 4 (CC411), and 3 (CC321, CC341, CC352, CC361, CC372, CC391), respectively. Values on the left indicate the position of markers in kilobase pairs, shown in the outside lanes by size standards.

D. G. Panaccione, J.-A. Pocard, and J. D. Walton, *unpublished data*). Higher temperatures during the primer/template annealing step were used in an attempt to eliminate the larger amplification product. At an annealing temperature of 50 C, the *HTS1* gene primers generated a single 950- to 1000-bp product only in the race 1 isolates (Fig. 4B), illustrating the diagnostic character of this fragment.

DISCUSSION

In this study, we examined PCR polymorphisms to evaluate genetic relatedness and diversity in an attempt to identify characteristics of *C. carbonum* races. Typically, pathological races are differentiated by quantitative or qualitative differences in host symptom expression. In some species, races are sufficiently different from each other to designate them as separate subspecies; in other species, a single gene difference may separate one race from another (1). If the amount of genetic variability within a race is very large relative to the amount of variability between races, then the differences that distinguish the races may be obscured. Thus, the success of any method of identifying pathogenic races as distinct entities requires sufficient heterogeneity between races combined with sufficient homogeneity within a race. Our results indicated that PCR amplification with arbitrary or sequence-specific primers can reveal race-specific and diagnostic features.

We found that *C. carbonum* races 0 and 3 represent two populations that could be distinguished from each other and from races 1, 2, and 4. All the pathogenic races were substantially different from nonpathogenic race 0. The two race 0 isolates we analyzed produced DNA patterns different from each other (data not shown). If the patterns are an indication of genetic relatedness, then race 0 is not as closely related to the other *C. carbonum* races as some of the other species appear to be. Such distinctive banding patterns may indicate a difference at the species level, as has been suggested for *Leptosphaeria maculans* (5,29). However, the isolates of race 0 are clearly members of *C. carbonum*; they produce conidia characteristic of the species and are compatible with tester strains in sexual crosses with other races (39).

Other studies also have found that race 3 may be a genetically distinct population. Leonard and Leath (13) compared frequencies of various traits in races 2 and 3 and concluded that these populations are distinct as a result of minimal gene flow between them. After isozyme analyses of three pathogenic races of *C. carbonum*, Simcox et al (28) concluded that races 1 and 3 are more similar to each other than to race 2, and the population of race 2 is more variable than the other races. They found isozyme polymorphisms for five enzymes and defined electrophoretic phenotypes that were diagnostic for each of the three races. Interestingly, race 1 was characterized by a single phenotype, race 2 by seven, and race 3 by three. In our analyses, we made no attempt to subdivide races based on the amplification patterns. Rather, we sought to define each race by a consistent and unique pattern superimposed on isolate variability.

Because the amplification patterns of some isolates contained unique bands, it was critical to include several isolates and several primers in the analyses before defining a given pattern as a diagnostic criterion for race identification. For example, analysis of the race 1 isolate shown in Figures 2 and 3 demonstrates the occurrence of isolate-specific amplification products. This isolate either lacks a product that is present in the other pathogenic races (Fig. 3) or contains a product that is absent from the other races (Fig. 2). However, these products were not consistent characteristics of all isolates of race 1 analyzed with those primers (data not shown) and, thus, were not a diagnostic feature of race 1. Races 2 and 4 were not differentiated by screening with a total of 70 decamer primers, suggesting that these races are apparently closely related to each other.

The number of primers that must be screened to distinguish the races reflects the extent of genetic variability among races. We found no consistent, race-specific differences among races 1, 2, and 4 with any of 20 decamer primers and consistently found race 3-specific differences with only one of the 20 primers. Similar analysis of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 showed distinct banding patterns detected by 13 of 14 primers (2). Unlike *C. carbonum*, the races of *F. s. cucurbitae* have reached total reproductive isolation (14) and thus, have become genetically distinct populations. Taken together, these studies indicate that the more races have diverged, the easier it is to identify primers that will generate race-specific amplification products.

We examined other fungi that produce phytotoxic compounds for the presence of HC-toxin-encoding gene sequences. With primers that anneal to sequences in the coding region of *HTS1*, all 10 of the race 1 isolates, but none of the other isolates or the isolates of other species, produced a fragment corresponding to the length predicted from sequence data. This result was expected because race 1 of *C. carbonum* is the only race known to produce HC-toxin. In separate experiments with the *HTS1* primers and with two additional primers with homology to sequences in the *Tox2* locus, we analyzed unrelated fungal species

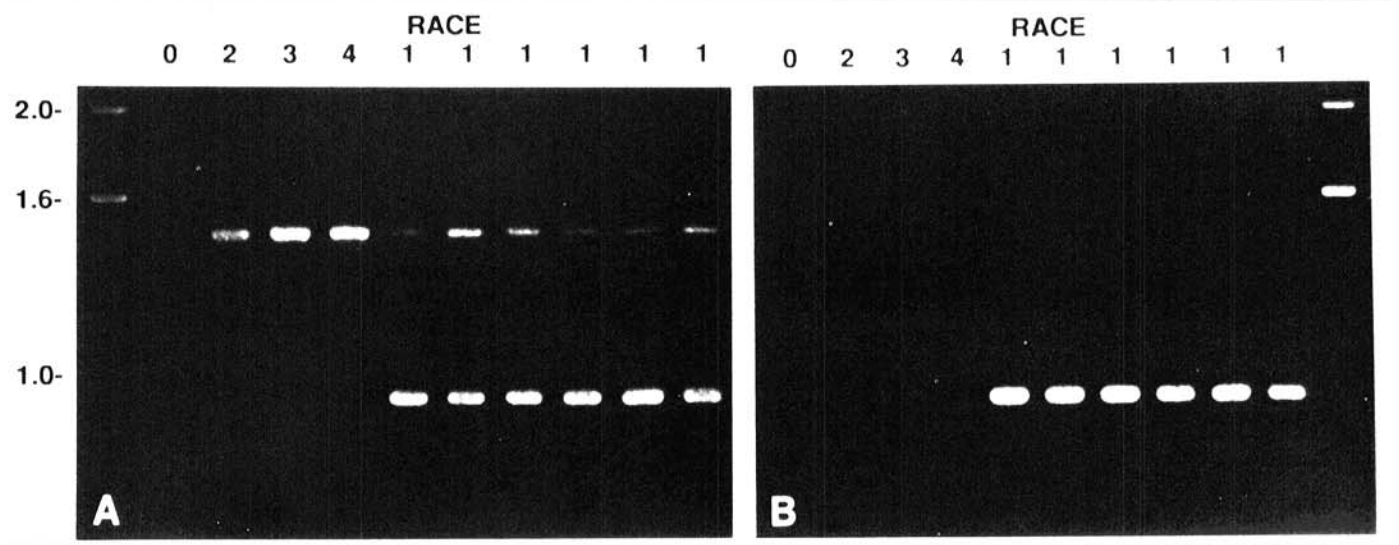

Fig 4. Distinction of races 0 and 1 of *Cochliobolus carbonum*. DNA from the isolates was amplified with a pair of primers from the *HTS1* region of the *Tox2* locus. **A,** 1.5 mM $MgCl_2$ at an annealing temperature of 37 C and **B,** 1.5 mM $MgCl_2$ at an annealing temperature of 50 C. Amplified fragments were separated by electrophoresis, and gels were stained with ethidium bromide. Lanes contain, from left to right, nonpathogenic race 0 (CC021) and pathogenic races 2 (CC221), 3 (CC321), 4 (CC411), and 1 (CC111, CC141, CC151, CC161, CC171, CC191), respectively. Values on the left indicate the position of markers in kilobase pairs, shown in the outside lanes by size standards.

that produce similar cyclic tetrapeptides containing the unusual amino acid amino-oxo-epoxydecanoic acid (AOE) (*Diheterospora chlamydosporia, Cylindrocladium macrosporum,* and *Petriella guttulata*) and found no evidence of amplified products of the expected size (M. J. Jones and L. D. Dunkle, *unpublished data*).

Our studies demonstrate the utility of PCR amplification with arbitrary primers in identifying and classifying the *Cochliobolus* species and in examining their relationships. It has been suggested that gradations in pathogenicity have evolved from a host-nonspecific progenitor (e.g., *C. sativus*), giving rise to more virulent, host-specific species (e.g., *C. carbonum, C. victoriae,* and *C. heterostrophus*) (22,25). We found distinct similarities or differences among different subgroups of *Cochliobolus* species. The similarity of the patterns of *C. sativus, C. victoriae,* and *C. carbonum* supports the hypothesis, based on similarities of secondary metabolites (22) and the extent of interfertility in sexual crosses (16,25), that they arose from a common progenitor. With further analysis, we may be able to determine genetic relatedness and eventually understand the dynamics of the host-specific interactions of these pathogens.

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