Virulence and Molecular Diversity in *Cochliobolus sativus*

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ABSTRACT

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Spot blotch, caused by the fungal pathogen *Cochliobolus sativus*, is an important disease of barley in many production areas of the world. To assess genetic diversity in this pathogen, a worldwide collection of *C. sativus* isolates was evaluated for virulence on barley and DNA polymorphism. Three pathotypes (0, 1, and 2) were identified among the 22 isolates tested in this study and the 36 isolates characterized previously on three barley differentials (ND5883, Bowman, and NDB112) that differ in their resistance to *C. sativus*. Pathotype 2, which exhibits high virulence on cv. Bowman, was only found in North Dakota, whereas the other two pathotypes occurred in many other regions of the world. Genetic diversity of the 58 *C. sativus* isolates, together with isolates of three related pathogenic *Cochliobolus* spp. (*C. heterostrophus*, *C. carbonum*, and *C. victoriae*) was analyzed using amplified fragment length polymorphism (AFLP) markers. A total of 577 polymorphic AFLP markers were recorded among the 70 isolates of the four *Cochliobolus* spp. using eight

primer combinations. Cluster analysis revealed distinct groups corresponding to the four different species, except in one case where race 0 of *C. carbonum* was placed in an outgroup that may belong to a different species. In *C. sativus*, 95 polymorphic AFLP markers were detected with the eight primer pairs used, and each isolate exhibited a unique AFLP pattern. Allelic diversity in the pathotype 2 group was lower (0.10) than in the pathotype 0 (0.23) and pathotype 1 (0.15) groups, indicating that pathotype 2 may have arisen more recently. Cluster analysis did not reveal a close correlation between pathotypes and AFLP groups, although two AFLP markers unique to pathotype 2 isolates were identified. This low correlation suggests that genetic exchange may have occurred through parasexual recombination in the fungal population. Some isolates collected from different regions of the world were clustered into the same AFLP group, suggesting that migration of the fungal pathogen around these regions has occurred.

Additional keywords: DNA markers, *Hordeum vulgare*.

Cochliobolus sativus (Ito & Kurib.) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* [Sacc. in Sorok.] Shoem.) is the causal organism of barley spot blotch, an important disease commonly occurring in areas with a warm, humid climate such as the Upper Midwest region of the United States and the Prairie Provinces of Canada (22). Under conditions favorable for the disease, yield losses from 16 to 33% can occur in susceptible barley cultivars (9,36). Through the deployment of resistant cultivars, spot blotch has been effectively controlled in six-rowed malting barley in the Upper Midwest since the mid-1960s (30,36). However, the disease is still an important problem on two-rowed barley cultivars because of their lower levels of resistance to *C. sativus* and the recent occurrence of a new pathotype (pathotype 2) in the region (12,33).

C. sativus isolates can be highly variable in pathogenicity and virulence. Christensen (7) reported physiologic forms of the fungus causing different degrees of infection on a variety of cereals and grasses. Dosdall (11) described *C. sativus* isolates that induced differential infection reactions on barley cv. Lion. Kline and Nelson (16) identified 13 pathogenicity types from 28 isolates based on their varying capacity to cause disease on selected species of *Poaceae*. In North Dakota, Fetch and Steffenson (12) identified two pathotypes of *C. sativus* that exhibited a differential virulence pattern on several barley genotypes. Further evaluation of 36 *C. sativus* isolates collected from North Dakota and other regions on three differential barley genotypes (ND5883, Bowman,

and NDB112) revealed three pathotypes (33). Pathotype 1 exhibits high virulence on ND5883, but low virulence on Bowman and NDB112. Pathotype 2 exhibits high virulence on Bowman, but low virulence on ND5883 and NDB112, and pathotype 0 exhibits low virulence on all three barley genotypes (33).

Virulence assessments can provide useful information regarding virulence variation in plant pathogenic fungi and the effectiveness of host resistance genes, which are very important for breeding programs. However, these assessments may only detect a limited number of virulence phenotypes because of the difficulty in identifying genetically characterized host differentials. Furthermore, virulence types are subject to strong selection by the host (17), thus limiting their application in genetic diversity studies (19,23). In contrast, molecular markers (e.g., allozymes, restriction fragment length polymorphism [RFLP], random amplified polymorphic DNA, and amplified fragment polymorphism [AFLP]) are abundant and selectively neutral, and therefore can provide more detailed information of the genetic diversity of pathogen populations (3,6). In this study, genetic variation among *C. sativus* isolates collected from North Dakota and other regions of the world was assessed based on virulence and AFLP markers.

MATERIALS AND METHODS

Fungal isolates. Fifty-eight isolates of *C. sativus* were used in this study. Most isolates were from the United States (primarily North Dakota, but also Colorado, Minnesota, South Dakota, and Virginia), with additional isolates collected from Canada, Poland, Brazil, Uruguay, China, and Japan (Table 1). In addition, three isolates of *C. heterostrophus* (the southern corn leaf blight pathogen), six isolates of *C. carbonum* (the corn leaf spot pathogen), and three isolates of *C. victoriae* (the oat leaf blight pathogen)

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TABLE 1. *Cochliobolus* isolates used for virulence and molecular diversity analysis

Isolate	Year collected	Host origin	Geographic origin (location/state/country)	Source	Pathotype ^a
<i>C. sativus</i>					
ND93-1	1993	Kernel/barley	Walsh/ND/U.S.	B. J. Steffenson	0*
ND90Pr	1990	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	2*
ND85F	1985	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	1*
R002	1976	Root/wheat	Walsh/ND/U.S.	R. W. Stack	0*
ND89-1	1989	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	0*
ND89-8	1989	Leaf/barley	Emmons/ND/U.S.	B. J. Steffenson	0*
ND89-25	1989	Leaf/barley	Rolette/ND/U.S.	B. J. Steffenson	0*
ND89-33	1989	Leaf/barley	Mountrail/ND/U.S.	B. J. Steffenson	0*
ND89-37	1989	Leaf/barley	Dunn/ND/U.S.	B. J. Steffenson	0*
ND89-38	1989	Leaf/barley	Mercer/ND/U.S.	B. J. Steffenson	0*
ND89-40	1989	Leaf/barley	Sheridan/ND/U.S.	B. J. Steffenson	0*
ND91-Bowman	1991	Leaf/barley	Minot/ND/U.S.	B. J. Steffenson	2
SD91-7	1991	Leaf/barley	Roberts/SD/U.S.	B. J. Steffenson	0*
MN91-11	1991	Leaf/barley	Traverse/MN/U.S.	B. J. Steffenson	0*
MN92-5	1992	Leaf/barley	Polk/MN/U.S.	B. J. Steffenson	0
MN92-6	1992	Leaf/barley	Ramsey/MN/U.S.	B. J. Steffenson	0
ND92-1	1992	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	0*
ND92-2	1992	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	0*
ND92-8	1992	Leaf/barley	Stutsman/ND/U.S.	B. J. Steffenson	2*
ND92-10	1992	Leaf/barley	Logan/ND/U.S.	B. J. Steffenson	2*
ND92-11	1992	Leaf/barley	Emmons/ND/U.S.	B. J. Steffenson	2*
ND93-11	1993	Kernel/barley	Cavalier/ND/U.S.	B. J. Steffenson	0*
ND93-12	1993	Kernel/barley	Ramsey/ND/U.S.	B. J. Steffenson	2*
ND93-18	1993	Leaf/barley	Cass/ND/U.S.	B. J. Steffenson	0
ND95-17	1995	Leaf/barley	Towner/ND/U.S.	B. J. Steffenson	1*
ND95-31	1995	Leaf/barley	Hettinger/ND/U.S.	B. J. Steffenson	2*
KB001	1993	Kernel/barley	Cass/ND/U.S.	B. J. Steffenson	0*
KB002	1993	Kernel/barley	Cass/ND/U.S.	B. J. Steffenson	1*
KB005	1993	Kernel/barley	Cass/ND/U.S.	B. J. Steffenson	0*
KB008	1993	Kernel/barley	Cass/ND/U.S.	B. J. Steffenson	0*
KB361	1994	Kernel/barley	Larimer/CO/U.S.	B. J. Steffenson	0*
KB362	1994	Kernel/barley	Larimer/CO/U.S.	B. J. Steffenson	0*
KB363	1994	Kernel/barley	Larimer/CO/U.S.	B. J. Steffenson	0*
KB364	1994	Kernel/barley	Larimer/CO/U.S.	B. J. Steffenson	1*
JPN95-1	1995	Leaf/barley	Shikoku/Japan	B. J. Steffenson	1*
JPN95-2	1995	Leaf/barley	Shikoku/Japan	B. J. Steffenson	0*
CHN95-1	1995	Leaf/barley	Hongshan/Zhejiang/China	B. J. Steffenson	0*
CHN95-2	1995	Leaf/barley	Hongshan/Zhejiang/China	B. J. Steffenson	1*
ND97-Foster	1997	Leaf/barley	Towner/ND/U.S.	B. J. Steffenson	0
VA98-1	1998	Leaf/barley	Montgomery/VA/U.S.	B. J. Steffenson	1
A18	...	...	Canada	R. D. Tinline	0
A20	...	...	Canada	R. D. Tinline	0
Brier	...	...	Canada	R. D. Tinline	0
POL97-KD31	1997	Kernel/wheat	Poland	J. Chelkowski	1
POL97-KD32	1997	Kernel/wheat	Poland	J. Chelkowski	1
POL97-KD33	1997	Kernel/wheat	Poland	J. Chelkowski	1
POL97-KD34	1997	Kernel/wheat	Poland	J. Chelkowski	0
POL97-KD35	1997	Kernel/wheat	Poland	J. Chelkowski	0
POL97-KD36	1997	Kernel/wheat	Poland	J. Chelkowski	1
BRZ97-PF1	1997	Leaf/barley	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	1
BRZ97-PF2	1997	Leaf/barley	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	1
BRZ97-PF3	1997	Leaf/barley	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	1
NZ97-Pickering	1997	Leaf/barley	New Zealand	P. Pickering	1
URY98-Gamba18	1997	Leaf/barley	Uruguay	F. Gamba	0
URY98-Gamba30	1997	Leaf/barley	Uruguay	F. Gamba	1
URY98-Gamba51	1997	Leaf/barley	Uruguay	F. Gamba	1
URY98-Gamba54	1997	Leaf/barley	Uruguay	F. Gamba	1
URY98-Gamba55	1997	Leaf/barley	Uruguay	F. Gamba	1
<i>C. carbonum</i>					
CC021	...	Maize	NC/U.S.	L. D. Dunkle	Race 0**
SB111	...	Maize	...	J. D. Walton	Race 1**
SB114	...	Maize	...	J. D. Walton	Race 1**
CC221	...	Maize	NC/U.S.	L. D. Dunkle	Race 2**
CC323	...	Maize	NC/U.S.	L. D. Dunkle	Race 3**
CC411	...	Maize	IL/U.S.	L. D. Dunkle	Race 4**
<i>C. heterostrophus</i>					
IA1	...	Maize	IA/U.S.	C. R. Bronson	Race O***
B30.A3.R45	...	Maize	...	C. R. Bronson	Race T***
Hm540	...	Maize	NC/U.S.	C. R. Bronson	Race O***
<i>C. victoriae</i>					
#3	...	Oat	...	O. C. Yoder	...
#23	...	Oat	...	O. C. Yoder	...
HvW	...	Oat	...	O. C. Yoder	...

^a *, **, and *** indicate isolates previously characterized by Valjavec-Gratian and Steffenson (33), race information is from Jones and Dunkle (15), and race information is from Tzeng et al. (32), respectively.

were included for comparison with *C. sativus* (Table 1). Information on the origin, source, and pathotype (or race) of each isolate is listed in Table 1. All isolates were derived from single spores and stored on silica gel crystals in a refrigerator at 4°C (12) until needed.

Virulence assessment. The virulence phenotype of the *C. sativus* isolates was evaluated on three differential barley genotypes, ND5883, Bowman, and NDB112. The sowing and growing of the differential host lines, inoculum preparation, inoculation, infection response scoring, and pathotype nomenclature were as previously described (13,33).

DNA isolation. Genomic DNA was extracted from fungal isolates by a procedure modified from Yoder (37). Briefly, conidia harvested from 10- to 12-day-old cultures were inoculated into a 500-ml flask containing 100 ml of liquid complete medium (37) with a final concentration of approximately 10⁶ conidia per ml. After incubating at 28°C on a rotary shaker at 150 rpm for 24 h, the resulting mycelium was harvested by pouring the solution through four layers of cheesecloth and thoroughly washing with sterile distilled water. The mycelium was blotted dry on paper towels and ground in liquid nitrogen to a fine powder with a mortar and pestle. The ground mycelium (wet weight yield of 5 to 8 g) was placed into a 50-ml plastic centrifuge tube. Seven milliliters of DNA isolation buffer (50 mM Tris-HCl at pH 8.0, 150 mM EDTA, 1% sarkosyl, and 300 µg of proteinase K per ml) was added and stirred with a glass bar to homogenize the mixture. After incubating at 65°C for 30 min, an equal amount of chloroform was added, mixed by shaking on a rotary shaker for 30 min, and centrifuged at 5,000 rpm for 10 min at room temperature (22 to 25°C). The aqueous phase containing DNA was transferred to a new 50-ml centrifuge tube. DNA was precipitated by adding an equal volume of isopropanol. The precipitate was collected by centrifugation at 5,000 rpm for 5 min, rinsed with 70% ethanol, and air dried. The DNA pellet was then resuspended in 500 to 1,000 µl of 10 mM Tris-HCl and 0.1 mM EDTA or sterile distilled water. DNA concentration was measured with a fluorometer (TKO 100 Fluorometer, Hoefer Scientific Instruments, San Francisco), treated with 5 µl of RNase (10 mg/ml), and stored at 4°C until needed.

AFLP analysis. AFLP analysis was conducted according to a method modified from Vos et al. (34) and described by Zhong and Steffenson (40). Primers with one selective base at the 3' end were used in preselective amplification. For selective amplification, primers with two selective bases were used. The eight primer pairs used and the number of polymorphic AFLP markers generated by each are listed in Table 2. The DNA silver-staining system (Promega, Madison, WI) was used to detect the AFLP bands (40).

Data analysis. For each primer pair, AFLP bands of 80 to 800 bp in size were scored for all isolates of the four *Cochliobolus*

spp. DNA bands smaller than 80 bp or larger than 800 bp were often not repeatable and were therefore not scored. AFLP bands that were present in some isolates but absent in other isolates were considered polymorphic markers. The presence and absence of a polymorphic marker were coded with 1 and 0, respectively. Allelic diversity was assessed using Nei's allelic diversity index (*h*), which was calculated by the equation $h = 1 - \sum P_i^2$ (24), where *P_i* is the frequency of the *i*th allele. The mean allelic diversity over all loci was used to measure the genetic diversity for the whole population and also for each pathotype. A similarity matrix was constructed based on the Dice coefficient (10) using the SIMQUAL program in the NTSYS-pc software package (version 2.0; Exeter Software, Setauket, NY). Cluster analysis of the matrix values was done using the unweighted pair-group method with arithmetic averages using the SAHN program in NTSYS-pc 2.0. Separate dendrograms were constructed for the *C. sativus* isolates alone and together with the collective group of *C. heterostrophus*, *C. carbonum*, and *C. victoriae* isolates. Correlation between the virulence matrix and the AFLP matrix was calculated using the MXCOMP program. To estimate the strength of the grouping generated by cluster analysis, bootstrap analysis was conducted with 500 replications using the Winboot computer program (25).

RESULTS

Virulence assessment. Thirty-six of the *C. sativus* isolates were previously tested (33) for their virulence phenotype on the

Fig. 1. A partial polyacrylamide gel containing the silver-stained amplified fragment length polymorphism markers of isolates of four *Cochliobolus* spp. amplified with primer pair E-AG+E-CG. The name of each isolate/species is indicated at the top of the panel. *C. s* = *Cochliobolus sativus*, *C. c* = *C. carbonum*, *C. h* = *C. heterostrophus*, and *C. v* = *C. victoriae*.

TABLE 2. Primer combinations used for amplified fragment length polymorphism (AFLP) analyses of *Cochliobolus* spp.

Primer combination	Number of polymorphic AFLP markers	
	<i>C. sativus</i> ^a	<i>Cochliobolus</i> spp. ^b
E-AA+M-CA	13	85
E-AA+M-CC	8	59
E-AC+M-CA	6	64
E-AC+M-CC	13	62
E-AC+M-CT	10	66
E-AG+M-CA	15	88
E-AG+M-CG	7	66
E-AT+M-CT	23	86
Total	95	577

^a Includes 58 isolates of *C. sativus*.

^b Includes 58 isolates of *C. sativus*, six isolates of *C. carbonum*, three isolates of *C. heterostrophus*, and three isolates of *C. victoriae*.

barley genotypes ND5883, Bowman, and NDB112 (Table 1). These isolates were differentiated into three pathotypes (0, 1, and 2) according to the nomenclature of Limpert and Miller (21). The remaining 22 *C. sativus* isolates were evaluated in the present study and all were of the same three pathotypes of 0, 1, and 2 (Table 1). Only one pathotype 2 isolate (ND91-Bowman) was detected among these 22 isolates. In total, seven pathotype 2 isolates were identified, and all of them were collected from North Dakota. Pathotype 1 isolates were found in all regions of the world surveyed, whereas pathotype 0 isolates were found only in North Dakota, Minnesota, Canada, Poland, and Uruguay.

AFLP analysis. AFLP analysis was conducted on DNA prepared from all 70 isolates of the *Cochliobolus* spp. using eight primer pairs. For each primer pair, 35 to 60 DNA fragments of 80 to 800 bp in size were detected. The number of DNA fragments detected varied with the primer pair and the isolate used. Figure 1 shows a partial polyacrylamide gel containing silver-stained AFLP markers of isolates from the four *Cochliobolus* spp. that were amplified with primer pair E-AG+M-CG. The DNA banding patterns were distinct among the different species, but were very similar within a species. A notable exception to this pattern was with isolate CC021 (race 0), which showed a markedly different DNA banding pattern from other isolates of *C. carbonum* (Fig. 1). With the eight primer pairs combined, a total of 633 AFLP bands were recorded across all 70 *Cochliobolus* isolates. Among these AFLP bands, 577 were polymorphic (Table 2). Cluster analysis with the 577 polymorphic DNA markers showed that similarities among isolates within a species were high, ranging from 82 to 96% (Fig. 2). In contrast, the similarity between different *Cochliobolus* spp. was low to intermediate, ranging from 6 to 65%. The 70 isolates of *Cochliobolus* were classified into five groups at 80% similarity (Fig. 2). Except for CC021, isolates from each of the respective *Cochliobolus* spp. were clustered in the same group. The grouping was supported by the bootstrap analysis as bootstrap values ranged from 88 to 99. *C. carbonum* and *C. victoriae* had a 65% similarity, which was much higher than that found for all other pairs of species tested in this study.

With the 58 *C. sativus* isolates, a total of 346 AFLP bands were recorded for the eight primer pairs; however, only 95 (29.3%) of the AFLP bands were polymorphic among the isolates (Table 2). Fifty-eight different AFLP phenotypes were observed when the AFLP markers generated by all primer pairs were combined, indicating the genetic uniqueness of each *C. sativus* isolate. Nei's allelic diversity index (averaged over the 95 AFLP loci) was 0.22 for the entire population. The grouping of isolates according to pathotype revealed that pathotype 0 isolates had the highest allelic diversity (0.23) and pathotype 2 isolates the lowest (0.10). Pathotype 1 isolates had an allelic diversity value of 0.15. A dendrogram for the *C. sativus* isolates was generated based on the 95 AFLP markers (Fig. 3). At 65% similarity, the isolates were separated into five AFLP groups, but this grouping was not supported by the bootstrap analysis because the bootstrap value for each group branch was low (from 11 to 68). However, some isolates showed a high percentage of similarity to each other and formed a small cluster with a high bootstrap value (Fig. 3). For example, in group 2, isolates ND90Pr and ND91-Bowman, isolates ND89-38, JPN95-2, KB362, and POL97-KD34, isolates POL97-KD32 and POL97-36, and isolates POL97-31 and NZ97-Pickering, formed small clusters with high similarity and bootstrap value. This indicates that they are more closely related to each other genetically.

Two AFLP markers only present in pathotype 2 isolates were identified. One of the pathotype 2 specific markers is shown in Figure 4. No AFLP markers specific for pathotype 0 or pathotype 1 were found. A correlation analysis was made based on the similarity matrices for virulence and AFLP data. The correlation coefficient between the virulence phenotype and AFLP banding pattern of *C. sativus* isolates was 0.17.

DISCUSSION

Among the three identified pathotypes of *C. sativus*, pathotype 2 was found only in North Dakota, whereas the other two pathotypes occurred in almost every region where isolates were col-

Fig. 2. A dendrogram of isolates of *Cochliobolus* spp. revealed by cluster analysis with the unweighted pair group method with arithmetic averages. A total of 577 polymorphic amplified fragment length polymorphism markers were used in the analysis of 10 selected *C. sativus* isolates, three *C. heterostrophus* isolates, six *C. carbonum* isolates, and three *C. victoriae* isolates. The scale shown above the dendrogram is the genetic similarity coefficient calculated according to Dice (10). Numbers at the nodes of major clusters represent bootstrap values generated by 500 replications using the Winboot program (25).

lected (33; T. Fetch and B. Steffenson, *unpublished data*). It is interesting to speculate on the origin of pathotype 2 isolates in North Dakota. Spot blotch of barley has been kept in check for over 35 years by the wide-spread planting of durably resistant six-rowed malting cultivars (30,36). The initiation of a two-rowed feed barley improvement program in North Dakota in the late

1970s led to the release of the first two-rowed barley cv. Bowman in 1984. Bowman was considered moderately resistant to spot blotch (14) when it was first released. Moreover, spot blotch infection had not been observed on cv. Bowman or its derivatives during the time they were being developed in North Dakota (J. D. Franckowiak, *personal communication*). In 1990, severe spot

Fig. 3. A dendrogram of 58 isolates of *Cochliobolus sativus* revealed by cluster analysis with the unweighted pair group method with arithmetic averages. A total of 95 polymorphic amplified fragment length polymorphism markers were used in the analysis. The scale shown above the dendrogram is the genetic similarity coefficient calculated according to Dice (10). Numbers at the nodes of major clusters represent bootstrap values generated by 500 replications using the Winboot program (25). The pathotype of the isolates and their geographic origin (countries) are indicated on the right side of the dendrogram.

blotch infection was first observed on cv. Bowman at an eastern North Dakota breeding nursery (12). Characterization of *C. sativus* isolates from cv. Bowman in this nursery revealed the presence of a new virulence type (i.e., pathotype 2 isolate ND90Pr) that was distinct from pathotype 1 isolates such as ND85F (12,33). ND85F is a standard pathotype 1 isolate that has been used in the routine screening of barley germ plasm at North Dakota State University since 1985, and the resistance of cv. Bowman to this pathotype is still effective today. The detection of pathotype 2 isolates coincided with an increase in the planting of cv. Bowman. This suggests that pathotype 2 isolates may have arisen *de novo* in North Dakota relatively recently. Newly evolved pathotypes often have lower genetic differentiation because they have had a shorter period of time to develop genetic variation. In this study, we found that pathotype 2 isolates had a lower allelic diversity than pathotype 0 and pathotype 1 isolates, thus supporting the hypothesis of a more recent origin for pathotype 2. The origin of

pathotype 2 isolates should be more rigorously investigated by evaluating additional isolates from different regions of the world. Cluster analysis based on the 95 AFLP markers did not separate the *C. sativus* isolates into groups that correspond to pathotypes, and in general, the correlation between virulence pattern and AFLP pattern was low. In asexual populations of plant pathogenic fungi, isolates of the same pathotype may have greater genetic similarity than isolates from different pathotypes due to their isolated clonal reproduction (4,20). In *Magnaporthe grisea*, a fungus that reproduces asexually in nature, Levy et al. (20) found that isolates genetically related on the basis of molecular markers were also closely related for virulence. This close correlation between molecular and virulence variation also was reported in other asexually reproducing populations such as *Fusarium oxysporum* f. sp. *vasinfectum* (1), *Puccinia graminis* f. sp. *tritici* (4,28), and *P. recondita* (18). In contrast, the degree of correlation between virulence and molecular markers is often low in populations that

Fig. 4. A partial polyacrylamide gel containing the silver-stained amplified fragment length polymorphism (AFLP) markers of isolates of *Cochliobolus sativus* amplified with primer pair E-AG+E-CA. The AFLP marker unique to pathotype 2 isolates is indicated with an arrow. The number above each lane represents the pathotype of the isolate.

reproduce sexually (5). The sexual stage of *C. sativus* has not been found in nature (31). The low degree of correlation found between virulence and AFLP markers in this study may be due to the occurrence of parasexual recombination, which has been demonstrated to occur in *C. sativus* in vitro (31). Further studies should be conducted to determine whether parasexual recombination occurs in *C. sativus* in the field.

Two AFLP markers unique to pathotype 2 isolates were identified in this study. Construction of a genetic map of *C. sativus* revealed that these two markers are closely linked to the locus, conferring high virulence on cv. Bowman in the pathotype 2 isolate ND90Pr (39). Thus, these two markers should be useful for identifying pathotype 2 isolates in *C. sativus* populations.

No close correlation was found between genetic similarity as measured by AFLP markers and geographic origin. Isolates from different regions and even distant countries had, in some cases, a high genetic similarity and were clustered into the same molecular group. For example, in group 2, four isolates collected from three different countries (Japan, Poland, and the United States) formed a cluster, and two isolates from Poland and New Zealand were clustered together (Fig. 3). This result suggests that these isolates were derived from the same founder source population and disseminated from one area to another in association with their hosts. This is highly plausible given that *C. sativus* has a wide host range (31) and is a seedborne pathogen (22).

Comparison of AFLP banding patterns of the four *Cochliobolus* spp. showed greater differences between species than among isolates within a species (Fig. 1). The AFLP banding patterns of *C. carbonum* (excluding CC021) and *C. victoriae* were more similar than for any other pair of species, suggesting that these two species are closely related and may be derived from the same progenitor species. Two lines of evidence support this contention. First, crosses between some isolates of *C. carbonum* and *C. victoriae* were highly fertile, and some resulting progeny were able to infect both corn and oat because they inherited genes for the two toxins (HC-toxin and Victorin) from the parents (26,29). Second, the two species have almost identical DNA sequences in the ribosomal DNA internal transcribed spacer region (2), the glyceraldehyde phosphate dehydrogenase gene (2), and the mating type genes (8). The evolution of *C. carbonum* and *C. victoriae* into two different pathogens may have occurred after they obtained different genes for producing host selective toxins, probably through horizontal gene transfer (27,38).

In *C. carbonum*, five races have been identified (15). One of the races had the morphological characteristics of *C. carbonum*, but was not pathogenic on corn and thus was designated as race 0 (35). Other races are pathogenic on corn but show different symptoms (i.e., lesion size and shape) (15). The race 0 isolate (CC021) used in this study showed a markedly different AFLP banding pattern from other races of *C. carbonum*. The difference between CC021 and other races within *C. carbonum* was as great as that found among all species of *Cochliobolus* analyzed. This suggests that isolate CC021 may belong to a different species instead of a race of *C. carbonum*. Indeed, Jones and Dunkle (15) analyzed *C. carbonum* races by polymerase chain reaction amplification with arbitrary and gene-specific primers and showed that amplification products from isolates of the four pathogenic races were substantially different from the nonpathogenic race 0 isolates. The differences in DNA banding patterns found by Jones and Dunkle (15) reached the species level. An examination of additional race 0 isolates of *C. carbonum* should be made to further elucidate their taxonomic status.

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LITERATURE CITED

- Asigbetse, K. B., Fernandez, D., Dubois, M. P., and Geiger, J.-P. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA analysis. *Phytopathology* 84:622-626.
- Berbee, M. L., Pirseyedi, M., and Hubbard, S. 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* 91:964-977.
- Brown, J. K. W. 1996. The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytol.* 133:183-195.
- Burdon, J. J., and Roelfs, A. P. 1985. Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. *Phytopathology* 75:907-913.
- Burdon, J. J., and Roelfs, A. P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* 75:1068-1073.
- Chen, X. M., Line, R. F., and Leung, H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. *Phytopathology* 83:1489-1497.
- Christensen, J. J. 1922. Studies on the parasitism of *Helminthosporium sativum*. *Univ. Minn. Agric. Exp. Stn. Tech. Bull.* 11.
- Christiansen, S. K., Wirsal, S., Yun, S. H., Yoder, O. C., and Turgeon, B. G. 1997. The two *Cochliobolus* mating type genes are conserved among species but one of them is missing in *C. victoriae*. *Mycol. Res.* 102:919-921.
- Clark, R. V. 1979. Yield losses in barley cultivars caused by spot blotch. *Can. J. Plant Pathol.* 1:113-117.
- Dice, L. R. 1945. Measures of the amount of ecological association between species. *Ecology* 26:297-302.
- Dosdall, L. 1923. Factors influencing the pathogenicity of *Helminthosporium sativum*. *Univ. Minn. Agric. Exp. Stn. Tech. Bull.* 17.
- Fetch, T. G., Jr., and Steffenson, B. J. 1994. Identification of *Cochliobolus sativus* isolates expressing differential virulence on two-row barley genotypes from North Dakota. *Can. J. Plant Pathol.* 16:202-206.
- Fetch, T. G., Jr., and Steffenson, B. J. 1999. Rating scales for assessing infection responses of barley infected with *Cochliobolus sativus*. *Plant Dis.* 83:213-217.
- Franckowiak, J. D., Foster, A. E., Pederson, V. D., and Pyler, R. E. 1985. Registration of 'Bowman' barley. *Crop Sci.* 25:883.
- Jones, M. J., and Dunkle, L. D. 1993. Analysis of *Cochliobolus carbonum* races by PCR amplification with arbitrary and gene-specific primers. *Phytopathology* 83:366-370.
- Kline, D. M., and Nelson, R. R. 1963. Pathogenicity of isolates of *Cochliobolus sativus* from cultivated and wild gramineous hosts from the Western Hemisphere to species of the Gramineae. *Plant Dis. Rep.* 47:890-894.
- Kolmer, J. A. 1993. Selection in a heterogeneous population of *Puccinia recondita* f. sp. *tritici*. *Phytopathology* 83:909-914.
- Kolmer, J. A., Liu, J. Q., and Sies, M. 1995. Virulence and molecular polymorphism in *Puccinia recondita* f. sp. *tritici* in Canada. *Phytopathology* 85:276-285.
- Leung, H., Nelson, R. J., and Leach, J. E. 1993. Population structure of plant pathogenic fungi and bacteria. *Adv. Plant Pathol.* 10:157-205.
- Levy, M., Correa-Victoria, F. J., Zeigler, R. S., Xu, S., and Hamer, J. E. 1993. Genetic diversity of the rice blast fungus in a disease nursery in Columbia. *Phytopathology* 83:1427-1433.
- Limpert, E., and Miller, K. 1994. Designation of pathotypes of plant pathogens. *J. Phytopathol.* 140:346-358.
- Mathre, D. E. 1997. *Compendium of Barley Diseases*. 2nd ed. The American Phytopathological Society, St. Paul, MN.
- McDonald, B. A., and McDermott, J. M. 1993. Population genetics of plant pathogenic fungi. *Bioscience* 43:311-319.
- Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA* 70:3321-3323.
- Nelson, R. J., Baraoidan, M. R., Vera Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. *Appl. Environ. Microbiol.* 60:3275-3283.
- Nelson, R. R., and Kline, D. M. 1963. Gene systems for pathogenicity and pathogenic potential. I. Interspecific hybrids of *Cochliobolus carbonum* and *Cochliobolus victoriae*. *Phytopathology* 53:101-105.
- Panaccione, D. G., Scott-Craig, J. S., Pocard, J. A., and Walton, J. D. 1992. A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. *Proc. Natl. Acad. Sci. USA* 89:6590-6594.
- Roelfs, A. P., and Groth, J. V. 1980. A comparison of virulence phenotypes in wheat stem rust populations reproducing sexually and asexually. *Phytopathology* 70:855-862.

29. Scheffer, R. P., Nelson, R. R., and Ullstrup, A. J. 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. *Phytopathology* 57:1288-1291.
30. Steffenson, B. J., Hayes, P. M., and Kleinhofs, A. 1996. Genetics of seedling and adult plant resistance to net blotch (*Pyrenophora teres* f. *teres*) and spot blotch (*Cochliobolus sativus*) in barley. *Theor. Appl. Genet.* 92:552-558.
31. Tinline, R. D. 1988. *Cochliobolus sativus*, a pathogen of wide host range. Pages 113-122 in: *Advances in Plant Pathology* Vol. 6. D. S. Ingram and P. H. Williams, eds. Academic Press, London.
32. Tzeng, T. H., Lyngholm, L. K., Ford, C. F., and Bronson, C. R. 1992. A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. *Genetics* 130:81-96.
33. Valjavec-Gratian, M., and Steffenson, B. J. 1997. Pathotypes of *Cochliobolus sativus* on barley. *Plant Dis.* 81:1275-1278.
34. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Freijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new concept for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
35. Welz, G., and Leonard, K. J. 1988. Genetic variation in field populations of races 0, 2, and 3 of *Bipolaris zeicola* in 1987. (Abstr.) *Phytopathology* 78:1574.
36. Wilcoxson, R. D., Rasmusson, D. C., and Miles, M. R. 1990. Development of barley resistant to spot blotch and genetics of resistance. *Plant Dis.* 74:207-210.
37. Yoder, O. C. 1988. *Cochliobolus heterostrophus*, cause of southern corn leaf blight. Pages 93-112 in: *Advances in Plant Pathology* Vol. 6. D. S. Ingram and P. H. Williams, eds. Academic Press, London.
38. Yoder, O. C., Macko, V., Wolpert, T. J., and Turgeon, B. G. 1997. *Cochliobolus* spp. and their host-specific toxins. Pages 145-166 in: *The Mycota: Plant Relationship Part V*. A. G. Carroll and P. Tudzynski, eds. Springer-Verlag, Berlin.
39. Zhong, S. 2000. Molecular genetics of *Cochliobolus sativus*. Ph.D. diss. North Dakota State University, Fargo.
40. Zhong, S., and Steffenson, B. J. 2000. A simple and sensitive silver-staining method for detecting AFLP markers in fungi. *Fungal Genet. Newsl.* 47:101-102.