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Genetic and molecular characterization of mating type genes in *Cochliobolus sativus*

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Abstract: Genetic and molecular approaches were used to characterize the mating type (*MAT*) genes in *Cochliobolus sativus*. One hundred and four ascospore progeny derived from a cross of *C. sativus* isolates ND93-1 (*MAT*-1) × ND90Pr (*MAT*-2) were backcrossed with their parents to determine mating type, but only five progeny produced pseudothecia with asci and/or ascospores. When degenerate primers from the conserved high mobility group (HMG) protein domain encoded by the *MAT*-2 gene in *Cochliobolus* species were used in polymerase chain reaction (PCR) with genomic DNA of *C. sativus* as templates, an amplicon of predicted size was amplified only from *MAT*-2 isolates. The presence of a *MAT*-2 homolog in these *MAT*-2 isolates was confirmed by Southern hybridization with the HMG box as a probe. Additionally, the presence or absence of the HMG homolog in the progeny segregated in a 1:1 ratio, as expected for the single gene control of mating type. Using primers based on the conserved regions at the 5' and 3' flanks of the idiomorphs in the *MAT* genes of other *Cochliobolus* species, the full-length *MAT*-1 and *MAT*-2 idiomorphs were cloned by PCR from *C. sativus* isolates ND93-1 and ND90Pr, respectively. DNA sequence analysis indicated that these two idiomorphs are organized in a manner similar to their respective counterparts in other *Cochliobolus* species. DNA hybridization and PCR amplification analysis of 54 field isolates of *C. sativus* collected worldwide showed that both mating types exist in populations around the world. The low frequency of successful backcrosses of progeny to parents in the ND93-1 × ND90Pr cross, combined with the fact that many crosses between isolates of opposite mating type are unsuccessful, suggests that genetic factors other than *MAT* genes affect the fertility of the fungus.

Key Words: DNA binding protein, high mobility group, infertility

INTRODUCTION

In all Ascomycetes tested to date, sexual reproduction is controlled by mating type (*MAT*) genes. Knowledge of the structure and function of these genes is essential for understanding the evolution of the mating system and pathogenicity in plant pathogenic fungi (Turgeon 1998). Using a molecular genetic approach, *MAT* genes were recently cloned from several ascomycetous plant pathogens in the genus *Cochliobolus*, including *C. heterostrophus* (Turgeon et al 1993), *C. carbonum* (Christiansen et al 1997), and *C. victoriae* (Christiansen et al 1997). Analysis of the molecular organization of these *MAT* genes revealed that the two opposite mating type genes occupy the same locus but consist of completely unrelated sequences known as idiomorphs (Metzenberg and Glass 1990). *MAT*-1 idiomorphs of *Cochliobolus* species encode a DNA-binding protein (alpha domain), which corresponds to one of several proteins encoded by the *mt A* idiomorph of *Neurospora crassa* and *mat*- idiomorph of *Podospora anserina* (Turgeon et al 1993). *MAT*-2 idiomorphs encode a different DNA-binding protein (high mobility group [HMG]), which is also encoded by the *mt a-1* idiomorph of *N. crassa* and *mat+* idiomorph of *P. anserina* (Turgeon et al 1993). These alpha and HMG domains are highly conserved across all Ascomycetes studied to date. Although the similarity between opposite idiomorphs of mating type genes in *Cochliobolus* is low (33%), high sequence conservation was found in the flanking regions (Christiansen et al 1997; Turgeon 1998).

Cochliobolus sativus (Ito & Kurib.) Drechs. ex Sacc. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.] is a plant pathogen of worldwide importance causing spot blotch, common root rot, and black point of barley and wheat. The sexual stage of the fungus can be induced in the laboratory (Tinline 1951, Shoemaker 1955), but has not been found in nature (Tinline 1988). Like other heterothallic Ascomycetes, two mating types designated A and a, are required for successful matings (Tinline 1988). Early genetic studies indicated that a single locus controls

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TABLE I. *Cochliobolus sativus* isolates with known mating type used in this study

Isolate ^a	Geographic origin	Source	Mating type ^b
ND93-1	North Dakota	B. J. Steffenson	1
ND90Pr	North Dakota	B. J. Steffenson	2
D9	Canada	R. D. Tinline	2
D14	Canada	R. D. Tinline	2
D8	Canada	R. D. Tinline	1
A20	Canada	R. D. Tinline	2
A18	Canada	R. D. Tinline	1
K3	Canada	R. D. Tinline	2
J1-15	Canada	R. D. Tinline	1
R3-11	Canada	R. D. Tinline	1

^a Mating type of isolates ND93-1 and ND90Pr was determined by Valjavec-Gratian and Steffenson (1997b); mating type of the remaining isolates was determined by R. D. Tinline (Harding and Tinline 1983).

^b MAT-1 and MAT-2 correspond to the mating type designated as a and A, respectively, by Tinline (1951).

mating type in *C. sativus* (Tinline 1951, Tinline and Dickson 1958), however, the fertility of matings among field-collected isolates is highly variable (Kline and Nelson 1968, Harding and Tinline 1983). The objectives of this study were to study the genetics of *MAT* genes in a *C. sativus* cross, and to clone the mating type idiomorphs by PCR amplification using primer pairs corresponding to the conserved regions of the *MAT* genes in other *Cochliobolus* species.

MATERIALS AND METHODS

Fungal isolates and crosses.—Isolates used in this study included the *C. sativus* parental isolates ND90Pr and ND93-1, 104 progeny derived from their hybridization, 8 experimental isolates (TABLE I), and 54 field isolates collected around the world (TABLE II). Methods for producing the ND90Pr × ND93-1 cross and information on the parental isolates and resulting progeny population are described in Valjavec-Gratian and Steffenson (1997b). The mating type of isolate ND90Pr was determined to be MAT-2 (formerly A) by crossing tests with the *C. sativus* tester isolates A18 (MAT-1, formerly a) and A20 (MAT-2, formerly A), provided by K. L. Bailey, Agriculture & Agri-Food Canada Research Station, Saskatoon, Saskatchewan. The mating type of isolate ND93-1 was determined to be MAT-1, based on the fact that it is compatible with ND90Pr (Valjavec-Gratian and Steffenson 1997b). To determine the mating type of the progeny isolates, the two parents ND93-1 and ND90Pr were crossed with each of the progeny using a method modified from Tinline (1951) and described by Valjavec-Gratian and Steffenson (1997b). For each progeny isolate, at least two mating attempts were made to each parent. Selfings of 20 progeny isolates and matings between the original parental isolates ND90Pr and ND93-1 were included as con-

trols in the experiment. For each backcross or cross, 20 to 30 pseudothecia-like structures were crushed and checked for the presence of asci and ascospores.

DNA isolation, manipulation, and hybridization.—Plasmid DNA isolation and subcloning were according to Sambrook et al (1989). Genomic DNA was isolated from *C. sativus* isolates using the method described by Yoder (1988). For Southern hybridization, genomic DNA of *C. sativus* was digested with restriction enzymes according to the conditions recommended by the manufacturer (New England Biolabs, Beverly, Massachusetts). Digested DNA fragments (2 µg per lane) were separated on 0.9% agarose gels, treated with 0.25 N HCl for 10 min, and blotted to Hybond+ filters in 0.4 N NaOH for 12–18 h. Filters were pre-hybridized at 65 C in a pre-hybridization buffer (0.5 M NaPO₄, 7% SDS, and 1% BSA) for 2–3 hours before a DNA probe was added. Two clones were used as probes. One was the HMG box cloned into the TA cloning vector (Invitrogen, Carlsbad, California), and the other was a 0.25 kb *HindIII-EcoRI* fragment sub-cloned from the *MAT-1* gene of isolate ND93-1. These clones were labeled with α-[³²P]dCTP by the random hexamer labeling method (Feinberg and Vogelstein 1983). DNA-DNA hybridization was carried out at 65 C for at least 16 hours, and the filters were sequentially washed in 2× SSC/1% SDS at 65 C for 10 min, 1× SSC/0.5% SDS at 65 C for 10 min, and 0.5× SSC/0.25% SDS at 65 C for one hour. Filters were then exposed to Kodak XAR-5 film for 16–96 hr depending on the strength of the radioisotope signal. Labeled filters were stripped before reuse by washing in 0.5% SDS at 95 C for 1–2 min.

PCR amplification.—The degenerate primer pair 5'-AAGGCNCCNCGYCCNATGAAC-3' (ChHMG1) and 5'-CTNGGNGTGTAYTTGTAATTNGG-3' (ChHMG2), designed by Arie et al (1997), was used in PCR to amplify the HMG box homolog from isolate ND90Pr. Specific primers (CsPrimer1 and CsPrimer2) based on the *C. sativus* HMG box sequence were used to amplify the HMG box from other *C. sativus* isolates. Other primers (TABLE III) corresponding to the highly conserved regions at the 5' and 3' ends of the mating-type genes in several *Cochliobolus* species (*C. heterostrophus*, *C. carbonum*, and *C. victoriae*) were used to amplify the flanking regions of the HMG box and the entire *MAT* idiomorphs. Amplification was performed in a 50 µL PCR solution, which contained 1× buffer (10 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM MgCl₂, 200 µM dNTP, 1.0 unit of *Taq* DNA polymerase (Promega, Madison, Wisconsin), 36 ng of each primer, and 25 ng of template DNA. Denaturation at 95 C for 2 min was followed by 30 cycles of 94 C for 1 min, 55 C for 30 s, and 72 C for 1 min in a thermocycler (PTC-100™ Programmable Thermal Controller, MJ Research Inc., Watertown, Massachusetts). PCR products were separated in 1.4% agarose gels in 1× TAE buffer, stained with ethidium bromide, and photographed.

Cloning, sequencing, and analysis of PCR products.—PCR products were cloned into the vector pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The inserts were se-

TABLE II. Field isolates of *Cochliobolus sativus* assessed for mating type by DNA hybridization and PCR amplification

Isolate	Year collected	Geographic origin (location/state/country)	Source	Mating type ^a
ND85F	1985	Walsh/ND/USA	B. J. Steffenson	1
R002	1976	Walsh/ND/USA	R. W. Stack	1
ND89-1	1989	Cass/ND/USA	B. J. Steffenson	2
ND89-8	1989	Emmons/ND/USA	B. J. Steffenson	1
ND89-25	1989	Rolette/ND/USA	B. J. Steffenson	2
ND89-33	1989	Mountrail/ND/USA	B. J. Steffenson	2
ND89-37	1989	Dunn/ND/USA	B. J. Steffenson	1
ND89-38	1989	Mercer/ND/USA	B. J. Steffenson	2
ND89-40	1989	Sheridan/ND/USA	B. J. Steffenson	2
ND91-Bowman	1991	Minot/ND/USA	B. J. Steffenson	2
SD91-7	1991	Roberts/SD/USA	B. J. Steffenson	1
MN91-11	1991	Traverse/MN/USA	B. J. Steffenson	1
MN92-5	1992	Polk/MN/USA	B. J. Steffenson	2
MN92-6	1992	Ramsey/MN/USA	B. J. Steffenson	1
ND92-1	1992	Cass/ND/USA	B. J. Steffenson	1
ND92-2	1992	Cass/ND/USA	B. J. Steffenson	2
ND92-8	1992	Stutsman/ND/USA	B. J. Steffenson	1
ND92-10	1992	Logan/ND/USA	B. J. Steffenson	1
ND92-11	1992	Emmons/ND/USA	B. J. Steffenson	1
ND93-11	1993	Cavalier/ND/USA	B. J. Steffenson	1
ND93-12	1993	Ramsey/ND/USA	B. J. Steffenson	2
ND93-18	1993	Cass/ND/USA	B. J. Steffenson	1
ND95-17	1995	Towner/ND/USA	B. J. Steffenson	2
ND95-31	1995	Hettinger/ND/USA	B. J. Steffenson	1
KB001	1993	Cass/ND/USA	B. J. Steffenson	1
KB002	1993	Cass/ND/USA	B. J. Steffenson	1
KB005	1993	Cass/ND/USA	B. J. Steffenson	1
KB008	1993	Cass/ND/USA	B. J. Steffenson	1
KB361	1994	Larimer/CO/USA	B. J. Steffenson	1
KB362	1994	Larimer/CO/USA	B. J. Steffenson	1
KB363	1994	Larimer/CO/USA	B. J. Steffenson	2
KB364	1994	Larimer/CO/USA	B. J. Steffenson	1
JPN95-1	1995	^b —/Shikoku/Japan	B. J. Steffenson	2
JPN95-2	1995	—/Shikoku/Japan	B. J. Steffenson	2
CHN95-1	1995	Hongshan/Zhejiang/China	B. J. Steffenson	2
CHN95-2	1995	Hongshan/Zhejiang/China	B. J. Steffenson	1
ND97-Foster	1997	Towner/ND/USA	B. J. Steffenson	1
VA98-1	1998	Nottoway/VA/USA	B. J. Steffenson	1
Brier	—	—/—/Canada	R. D. Tinline	2
POL97-KD31	1997	—/—/Poland	J. Chelkowski	1
POL97-KD32	1997	—/—/Poland	J. Chelkowski	2
POL97-KD33	1997	—/—/Poland	J. Chelkowski	1
POL97-KD34	1997	—/—/Poland	J. Chelkowski	2
POL97-KD35	1997	—/—/Poland	J. Chelkowski	2
POL97-KD36	1997	—/—/Poland	J. Chelkowski	2
BRZ97-PF1	1997	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	2
BRZ97-PF2	1997	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	2
BRZ97-PF3	1997	Passo Fundo/Rio Grande do Sul/Brazil	G. Arias	2
NZ98-Pickering	1997	—/—/New Zealand	R. Pickering	1
URY98-Gamba18	1997	—/—/Uruguay	F. Gamba	1
URY98-Gamba30	1997	—/—/Uruguay	F. Gamba	1
URY98-Gamba51	1997	—/—/Uruguay	F. Gamba	2
URY98-Gamba54	1997	—/—/Uruguay	F. Gamba	2
URY98-Gamba55	1997	—/—/Uruguay	F. Gamba	2

^a Mating types were determined by DNA hybridization with MAT-1 and MAT-2 specific probes and PCR amplification with primer pair ChPrimer1+ChPrimer2.

^b Unknown or not applicable.

TABLE III. Primers used in PCR for the cloning of mating type genes in *Cochliobolus sativus*

Num- ber	Name	Nucleotide sequence	Corresponding genomic location	Reference
1	ChHMG1	5'-AAGGCNCCNGYCCNATGAAC-3'	bp925-945 at the 5' end of the HMG box in the <i>C. heterostrophus</i> MAT-2 sequence	Arie et al (1997)
2	ChHMG2	5'-CTNGGNGTGTAATTGTAATTNGG-3'	bp1178-1200 at the 3' end of the HMG box in the <i>C. heterostrophus</i> MAT-2 sequence	Arie et al (1997)
3	CsPrimer1	5'-AAGGCTCCCCGCCGATGAAC-3'	5' end of <i>C. sativus</i> HMG box; corresponding to ChHMG1	This study
4	CsPrimer1-1	5'-GATGCAATGCACAAAGCAT-3'	5' end of <i>C. sativus</i> HMG box; nested with respect to ChHMG1	This study
5	CsPrimer2	5'-CTCGGCGTGTAATTGTAATTGGG-3'	3' end of <i>C. sativus</i> HMG box; corresponding to ChHMG2	This study
6	CvMAT2-5'fk	5'-GTGGTTGTGGCACCGGTAGC-3'	bp20-40 at the 5' end of the <i>C. victoriae</i> MAT-2 sequence	Christiansen et al (1997)
7	CvMAT2-3'fk-1	5'-CAACCATGTTTCGCATCTGCTG-3'	bp2548-2568 at the 3' end of the <i>C. victoriae</i> MAT-2 sequence	Christiansen et al (1997)
8	CvMAT2-3'fk-2	5'-GGAGCTGAATTGGCAGGTGC-3'	bp2578-2597 at the 3' end of the <i>C. victoriae</i> MAT-2 sequence	Christiansen et al (1997)

FIG. 1. Amplification of the HMG box in *Cochliobolus sativus* isolates. Primer pair ChHMG1 and ChHMG2 was used in PCR with genomic DNA of isolates ND93-1 and ND90Pr. Primer pair CsPrimer1 and CsPrimer2 was used in PCR with genomic DNA of the other isolates. PCR products were separated in a 1.4% agarose gel and stained with ethidium bromide.

quenced using the SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre, Madison, Wisconsin) with a LI-COR® Sequencer (LI-COR Inc., Lincoln, Nebraska). When the inserts were larger than 800 bp in size, sub-cloning was performed before sequencing. Double strand sequencing was performed for all clones. The computer software program MacVector 6.0 (Oxford Molecular, London) was used to assemble, align, and analyze the sequences. Sequence data were subjected to similarity searches against the GenBank databases using the BLAST search programs of Altschul et al (1997). The DNA sequences of the two mating type idiomorphs of *C. sativus* have been deposited in GenBank databases with the accession numbers AF275373 and AF275374 for *MAT-1* and *MAT-2*, respectively.

RESULTS

Testing of mating types of progeny by backcrossing.—One hundred and four ascospore progeny isolates derived from cross ND93-1 × ND90Pr (Valjavec-Gratian and Steffenson 1997b) were tested for mating compatibility by backcrossing to each of the respective parents. Only five backcross pairings produced pseudothecia containing asci, and all were with parental isolate ND90Pr. No fertile pseudothecia were found in backcrosses with isolate ND93-1. Even in matings where asci were observed, the frequency of pseudothecia with asci was low, ranging from 7% to 10%. Asci with ascospores were observed in only two of the matings, and the number of ascospores in each ascus ranged from one to two. Pseudothecia-like structures, which were called protothecia by Shoemaker (1955), were observed in 34% of the matings with isolate ND93-1 and 65% of the matings with isolate ND90Pr where no asci and/or ascospores were observed. Protothecia also appeared in the fer-

tile crosses and in the selfings (when a single isolate was plated). The protothecia superficially resembled mature pseudothecia, but lacked differentiation inside the centrum in addition to the absence of asci and/or ascospores. Eighteen percent of the progeny isolates did not produce any fruiting structures in the matings with either of the parents. In control crosses between isolates ND93-1 and ND90Pr, asci were observed in 35% of the pseudothecia evaluated, and only 23% of the asci contained ascospores, with the number ranging from one to five per ascus.

Amplification of the HMG box from C. sativus.—Three PCR products were amplified from isolate ND90Pr, and two were amplified from isolate ND93-1 with primers ChHMG1 and ChHMG2 (FIG. 1). A DNA fragment of approximately 270 bp in size was only present in isolate ND90Pr. Cloning and sequencing of this DNA fragment revealed a 91% identity to the *C. heterostrophus* HMG box (Turgeon et al 1993) and a 99% identity to the HMG box homolog amplified from *C. sativus* isolate A20 (MAT-2) (Arie et al 1997).

Primers CsPrimer1 and CsPrimer2 (TABLE III) were designed based on the sequence of the PCR product (HMG box) specific for isolate ND90Pr. These primers were used in PCR amplification with genomic DNA of other *C. sativus* isolates whose mating types were previously characterized (TABLE I). A PCR product of the expected size (270 bp) was amplified only from DNA of MAT-2 isolates (FIG. 1). Probing of Southern blots with the cloned HMG box revealed a hybridization band present only in MAT-2 isolates (FIG. 2).

The primer pair CsPrimer1+2 was also used for PCR amplification with genomic DNAs of the asco-

FIG. 2. Hybridization of the HMG box to genomic DNAs of *Cochliobolus sativus* isolates of known mating type. Genomic DNAs from *C. sativus* isolates were digested with *EcoRV*, separated in a 0.9% agarose gel, blotted, and probed with the HMG box as a probe. Note that a hybridization signal was only present in MAT-2 isolates. Isolates ND90Pr, D9, D14, and K3 exhibited a hybridization band of about 14.8 kb, whereas isolate A20 had a polymorphic band of about 4.5 kb.

spore progeny serving as templates. A PCR product of the expected size (270 bp) was produced in 53 of the 104 progeny isolates. The other 51 progeny isolates, including the five that were compatible with isolate ND90Pr in the backcrossing experiments (see above), did not produce this DNA fragment in the PCR amplification. Southern hybridization of the HMG box to genomic DNAs from the progeny showed that a hybridization band was only present in the progeny isolates that produced the HMG amplicon (FIG. 3). The segregation of progeny for the presence or absence of the HMG box was not significantly different from a 1:1 ratio ($\chi^2 = 0.03$, $P = 0.88$) as expected for the segregation of a single locus in a haploid organism.

Cloning of *C. sativus* MAT-2 and MAT-1 idiomorphs.—Comparison of the *MAT* genes cloned from other *Cochliobolus* species (e.g., *C. heterostrophus*, *C. carbon-*

um, and *C. victoriae*) (Turgeon et al 1993, Christiansen et al 1997) revealed highly conserved regions at both the 5' and 3' flanks of the idiomorphs. Thus, primers complementary to these conserved regions were designed and used in combination with CsPrimer1-1 and CsPrimer2 for PCR amplification of the *MAT*-2 idiomorph from *C. sativus* (FIG. 4). A PCR product of approximately 1.3 kb in size was amplified from genomic DNA of isolate ND90Pr using primers CvMAT2-5'fk and CsPrimer2 (FIG. 5, lane 1). Cloning and sequencing of this product showed that it contained the HMG box and the 5' flanking region. In the PCR amplification with primers CsPrimer1-1 and CvMAT2-3'fk-2 and DNA of isolate ND90Pr, a fragment of approximately 1.3 kb was amplified, which contained the HMG box and the 3' flanking region (FIG. 5, lane 3). Alignment of DNA sequences of the two PCR products produced a DNA sequence of 2547 bp, which combined the HMG box and its 5' and 3' flanking regions. The entire DNA sequence was amplified when primers CvMAT2-5'fk and CvMAT2-3'fk-2 and genomic DNA of isolate ND90Pr were used for the PCR amplification (FIG. 5, lane 4). PCR with genomic DNA of isolate ND90Pr and primers CsPrimer1-1 and CvMAT2-3'fk-1 generated a product containing the HMG box but a shorter 3' flanking region (FIG. 5, lane 2).

When primers CvMAT2-5'fk and CvMAT2-3'fk-2 were used to amplify DNA from isolate ND93-1 (MAT-1), a fragment of approximately 2.7 kb was generated (FIG. 5, lane 5). Cloning and sequencing of this PCR product revealed that it is highly similar and presumably homologous to the *MAT*-1 idiomorph cloned from *C. heterostrophus* and *C. carbonum* (Turgeon et al 1993, Christiansen et al 1997) (see below).

Sequence analysis.—DNA sequences of the *MAT*-1 (2681 bp) (AF275373) and *MAT*-2 (2547 bp) (AF275374) idiomorphs cloned from the two *C. sativus* isolates ND93-1 and ND90Pr, respectively, were analyzed and compared with those of *C. heterostrophus* (Turgeon et al 1993, Wirsal et al 1998). As in *C. heterostrophus*, the overall nucleotide identity be-

FIG. 3. Hybridization of HMG box to genomic DNAs of a subset of *Cochliobolus sativus* progeny isolates derived from the ND93-1 $\times$ ND90Pr cross. Genomic DNAs from the progeny isolates were digested with *EcoRV*, separated in a 0.9% agarose gel, blotted and probed with the HMG box as a probe. Parental isolates ND93-1 (P1) and ND90Pr (P2) are MAT-1 and MAT-2, respectively. Progeny isolates 1, 6, 7, 8, 10, 11, 13, 14, 17, 18, 19, and 20 are MAT-1. Progeny isolates 2, 3, 4, 5, 9, 12, 15, and 16 are MAT-2.

FIG. 4. Diagram showing PCR cloning of the *MAT-2* gene in *Cochliobolus sativus*. The arrows represent primers and the direction for amplification. The number above each arrow corresponds to the primer number listed in TABLE III. A). Amplification of the HMG box using primers 3 and 5. B). Amplification of the HMG box and its 5' flanking region with primers 5 and 6. C). Amplification of the HMG box and its 3' flanking region with primers 4 and 7 or 4 and 8, respectively. D). Recovery of the entire *MAT-2* idiomorph with primers 6 and 8.

tween the idiomorphs of the two opposite mating type isolates in *C. sativus* was low (<40%), whereas the 5' and 3' flanking regions were 96% and 97% similar, respectively (TABLE IV). Sequence comparison of the *MAT* genes from *C. sativus* and *C. heterostrophus* revealed a 90% nucleotide identity for both *MAT-1* and *MAT-2* (TABLE IV).

FIG. 5. PCR products amplified with different primer pairs from genomic DNA of *Cochliobolus sativus* isolates ND90Pr and ND93-1. Lane 1) DNA fragment amplified from ND90Pr with primers CvMAT2-5'fk+CsPrimer2, 2) DNA fragment amplified from ND90Pr with primers CsPrimer1-1+CvMAT2-3'fk-1, 3) DNA fragment amplified from ND90Pr with primers CsPrimer1-1+CvMAT2-3'fk-2, 4) DNA fragment amplified from ND90Pr with primers CvMAT2-5'fk+CvMAT2-3'fk-2, 5) DNA fragment amplified from ND93-1 with primers CvMAT2-5'fk+CvMAT2-3'fk-2. M is DNA size marker (Hind III-digested λ DNA).

Several open reading frames (ORFs) were found in the *MAT* idiomorphs of *C. sativus*. The *C. sativus* *MAT-1*-specific ORF is interrupted by an intron, which was found at the same position as the intron in the *C. heterostrophus* *MAT-1* (Turgeon et al 1993). A putative intron also was found in the *C. sativus* *MAT-2*-specific ORF, similar to the *C. heterostrophus* *MAT-2* (Turgeon et al 1993). Removal of the introns generated continuous ORFs encoding 378 amino acids for *MAT-1* and 337 amino acids for *MAT-2* that were nearly the same length as their respective counterparts in *C. heterostrophus*. Similarity searches against the GenBank databases indicated that the translated proteins contained DNA binding domains (an alpha domain for *MAT-1* and a HMG motif for *MAT-2*) that have been found in the proteins encoded by *MAT* genes from several other filamentous fungi including *Cochliobolus* species (for review see Turgeon 1998). The alpha domain and HMG motif in *C. sativus* showed three and four amino acid differ-

TABLE IV. Nucleotide identity (%) between *MAT* genes of *Cochliobolus sativus* and *C. heterostrophus*

<i>MAT</i> genes compared ^a	5' flank (739 bp) ^b	3' flank (658 bp) ^c	Idiomorph
<i>ChMAT-1/ChMAT-2</i>	99.5	99.0	<40.0
<i>CsMAT-1/CsMAT-2</i>	96.0	97.0	<40.0
<i>ChMAT-1/CsMAT-1</i>	83.5	85.0	90.0
<i>ChMAT-2/CsMAT-2</i>	83.0	85.0	90.0

^a Ch = *C. heterostrophus*; Cs = *C. sativus*.

^b DNA sequence immediately at 5' end of the idiomorph.

^c DNA sequence immediately at 3' end of the idiomorph.

ences, respectively, when compared with their counterparts in *C. heterostrophus* (FIGS. 6, 7).

Molecular characterization of mating types in field isolates of C. sativus.—The *MAT-2*-specific HMG box cloned from isolate ND90Pr was used as a probe to hybridize with genomic DNA prepared from field isolates of *C. sativus* collected from different regions of the world (TABLE II). Among the 54 *Cochliobolus sativus* isolates surveyed, 28 showed a hybridization band, which indicated the presence of the *MAT-2* homolog. Southern hybridization of the HMG box with genomic DNA of a subset of the *C. sativus* isolates is shown in FIG. 8a. When the primer pair CsPrimer 1+2 was used for PCR amplification, the HMG box was only present in these 28 isolates (data not shown), corroborating further the *MAT-2* identity of these isolates. When a sub-clone containing a *MAT-1* specific fragment from isolate ND93-1 was used as a probe, a hybridization band occurred only in the remaining 26 isolates (FIG. 8b), indicating that they have a *MAT-1* homolog.

DISCUSSION

Our mating type testing experiments showed that most of the *C. sativus* progeny failed to produce fertile pseudothecia when backcrossed to the parents. This indicates that, in addition to mating type genes, other genetic factors may be involved in the development of the perfect stage in this fungus. In another genetic study, Hosford et al (1975) found that most progeny from a *C. sativus* cross did not produce fertile pseudothecia when crossed with either parent or with each other. They suggested that three genes controlled the events leading to the formation of pseudothecia, asci, and ascospores. Our results agree with those of Hosford et al (1975), since we found that only five of the 51 *MAT-1* progeny produced pseudothecia with asci or/and ascospores. This segregation fits a 1:7 ratio ($\chi^2 = 0.34$, $P = 0.56$), indicating that three genes might be involved in sexual fertility. No asci were observed among matings with the 53 *MAT-2* progeny. This may be due to the extremely low fertility of these progeny because even when some pseudothecia are produced, the low number of asci formed might go undetected when only 20 to 30 pseudothecia-like structures are checked in each cross as was done in this study.

For genetic studies of important characters in fungal pathogens, it is very useful to have compatible isolates that can be crossed with other isolates of interest. However, for many fungi, the perfect stage does not exist or cannot be induced in culture making genetic studies through crossing impossible. Even

for fungal pathogens whose perfect stage can be induced in culture, some critical isolates of interest may not be compatible with each other. Such is the case for *C. sativus*. In previous studies, three pathotypes of *C. sativus* inducing differential spot blotch infection responses on three barley genotypes (NDB112, ND5883, and Bowman), were identified (Fetch and Steffenson 1994, Valjavec-Gratian and Steffenson 1997a). Pathotype 1 (represented by isolate ND85F) is highly virulent on barley genotype ND5883, but expresses low virulence on barley genotypes Bowman and NDB112. Pathotype 2 (represented by isolate ND90Pr) is highly virulent on Bowman, but expresses low virulence on ND5883 and NDB112, and pathotype 0 (represented by isolate ND93-1) expresses low virulence on all three barley genotypes (Valjavec-Gratian and Steffenson 1997a). To study the genetics of differential virulence expressed by *C. sativus* isolates ND85F and ND90Pr on barley genotypes Bowman and ND5883, matings were attempted, but all were unsuccessful (Valjavec-Gratian and Steffenson 1997b). Many other mating attempts were made between pathotype 1 and pathotype 2 isolates, but again without success (Valjavec-Gratian and Steffenson 1997b, Valjavec-Gratian and Steffenson unpubl). Cloning and sequencing of a *MAT-1* homologue in isolate ND85F indicates that it is 97% identical to *MAT-1* in ND93-1, the isolate that was successfully hybridized to isolate ND90Pr. A key difference in *MAT-1* from isolate ND85F is a single base-pair deletion occurring in the region that encodes the alpha domain (S. Zhong and B. Steffenson unpubl). This nucleotide deletion leads to a frame-shift in the *MAT-1* specific ORF and premature termination of the protein. It is not known whether this difference in the *MAT-1* idiomorph is a major factor contributing to the infertility of matings involving ND85F. Transformation of isolate ND85F with a functioning *MAT* gene from *C. heterostrophus* or *C. sativus*, together with tests of fertility of such transformants with various isolates, would clarify this question. Moreover, transformation of a *MAT* deletion mutant of *C. heterostrophus* or *C. sativus* with the *MAT-1* idiomorph from ND85F would provide information regarding the function of the *MAT* gene in isolate ND85F. A similar strategy was used to test the function of several *MAT* genes cloned from *C. carbonum*, *C. victoriae*, and *Bipolaris sacchari* (Sharon et al 1996, Christiansen et al 1997).

Mating type genes have been cloned from different types of fungi, including yeasts (Hicks et al 1979, Kelly et al 1988), filamentous ascomycetes (Glass et al 1988, Picard et al 1991, Turgeon et al 1993, Sharon et al 1996, Christiansen et al 1997) and basidiomycetes (Giasson et al 1989, Kronstad and Leong 1989,

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FIG. 6. Alignment of amino acids of the *MAT-1* specific ORFs between *Cochliobolus sativus* (Cs) and *C. heterostrophus* (Ch). Exact matches are shaded, whereas conserved substitutions are boxed but not shaded. The DNA binding domain (alpha box) (from amino acid 60 to 118) is flanked by two vertical lines (|). Note that there are three amino acid differences for the alpha box between the two species.

Mutasa et al 1990). Although no substantial DNA sequence similarity was found among the *MAT* genes from these different types of fungi, amino acid similarities occur in the DNA binding motifs encoded by

the *MAT* genes. Taking advantage of the highly conserved motifs, Arie et al (1997) developed degenerate primers and amplified DNA binding motif-specific PCR products from most genera of the loculoas-

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FIG. 7. Alignment of amino acids of the MAT-2 specific ORFs between *Cochliobolus sativus* (Cs) and *C. heterostrophus* (Ch). Exact matches are shaded, whereas conserved substitutions are boxed but not shaded. The DNA binding domain (HMG domain) (from amino acid 131 to 209) is flanked by two vertical lines (|). Note that there are four amino acid differences for the HMG domain between the two species.

comycetes and pyrenomycetes studied. These specific PCR products could be used as starting points for the cloning of mating type genes in many Ascomycetes by heterologous hybridization or various PCR approaches as demonstrated previously (Yun et al 1999) and in the present study. This same strategy should be applicable for the cloning of MAT genes from other *Cochliobolus* species and their asexual anamorphs or relatives (e.g., *Bipolaris* and *Curvularia*).

Mating types of fungal isolates are usually assessed through pairing with tester isolates of known mating type. This procedure is laborious and time consuming. With the successful cloning of MAT genes from various fungi, it is now possible to characterize the mating type of isolates using a molecular assay. This technique is especially useful in fungal species where low fertility or infertility is common, as with *C. sativus* (Kline and Nelson 1968, Harding and Tinline 1983,

FIG. 8a, b. Hybridization of HMG box and a 0.25 kb *Hind*III-*Eco*RI *MAT-1* clone (containing bp1100–1349 of the *MAT-1* idiomorph) to genomic DNAs of field-collected isolates of *Cochliobolus sativus*. Genomic DNAs from field isolates of *C. sativus* were digested with *Eco*RV, separated in a 0.9% agarose gel, blotted, and probed with the HMG box as a probe (a). After autoradiography, the filter was stripped and reprobbed with the 0.25 kb *Hind*III-*Eco*RI *MAT-1* clone (b). A subset of *C. sativus* isolates is shown here. Note that MAT-2 isolates show a hybridization band (about 14.5 kb) with the HMG box, and MAT-1 isolates show a hybridization band (about 14.8 kb) with the 0.25 kb *Hind*III-*Eco*RI clone of the *MAT-1* idiomorph.

Valjavec-Gratian and Steffenson 1997b, Zhong and Steffenson unpubl). Based on DNA hybridization and PCR amplification, we have determined the mating type of many *C. sativus* isolates. Within the group of 54 field isolates surveyed, the two mating types occurred with nearly the same frequency (48% for MAT-1 and 52% for MAT-2). Moreover, both mating types occurred in different geographic regions (TABLE II). This result is in agreement with the observation of Harding and Tinline (1983), who identified both mating types in *C. sativus* populations in Saskatchewan, Canada.

Even though both mating types of *C. sativus* have been identified in the same field or same population, the perfect stage of the fungus has not been found in nature (Tinline 1988). Several factors may prevent the development of the sexual stage in nature. As mentioned previously, many isolates are infertile even when paired with isolates of opposite mating type in culture, suggesting that other genetic factors for fertility are defective or not functioning. Additionally, critical environmental factors may be required for the induction of sexual structures (Tinline 1951, Shoemaker 1955). Further research on *MAT* genes and other genetic factors affecting fertility will increase our understanding of the mating mechanisms in the fungus.

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