Phenotypic and Molecular Diversity of *Cochliobolus sativus* Populations from Wheat

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Abstract

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Spot blotch, caused by *Cochliobolus sativus*, is a devastating foliar disease of wheat in Nepal and in the Northern Great Plains of the United States. However, limited information on variation in virulence and genetic structure of *C. sativus* from wheat is available. In this study, pathogenic variation of 96 isolates of *C. sativus* from the Hill and Plain areas in Nepal ($n = 48$) and in the Central and Northern areas in North Dakota ($n = 48$) were evaluated on 12 differential wheat lines. DNA polymorphisms in all isolates were analyzed using eight selected amplified fragment length polymorphism primer combinations. Phenotypic data analysis showed the isolates varied greatly and were classified into 47 pathotypes. Cluster analysis indicated the isolates fell into

three distinct groups with low, intermediate, and high virulence. Population genetic analysis revealed significant linkage disequilibrium ($\bar{r}_d = 0.066$ to 0.292), indicating that sexual reproduction plays little or no role in evolution and disease epidemiology in wheat fields. Furthermore, the corrected standardized fixation index ($G'_{ST} = 0.05$ and 0.02) showed no evidence of genetic differentiation in *C. sativus* populations. Collectively, these results confirmed high pathogenic and molecular diversity in the *C. sativus* populations collected from wheat foliar infections and will be useful to assist in developing resistant cultivars to manage this disease.

Spot blotch, caused by *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur (anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker), is an important disease of wheat and barley worldwide (23,43,56). Duveiller et al. (12) reported that seed was the primary source of inoculum, for wheat in South Asia. However, the airborne conidia occurring from soil debris, crop residues, and wild grasses can also act as primary inoculum sources (32). Spot blotch is less problematic under dry and cool weather conditions, whereas warm humid conditions increase disease severity (12). The teleomorph of this fungus has been reported from Zambia under natural conditions (36). However, in general, the sexual stage is uncommon and contributes little to genetic variation in this fungus (56).

Knowledge of the pathogenic and genetic structure of the pathogen populations has implications for formulating disease management strategies and developing effective resistance breeding programs (37). The virulence of *C. sativus* populations from barley has been analyzed in Australia, Canada, and the United States (17,18,23,48,56). Pathotype 2 was reported to be common in North Dakota and had lower allelic diversity compared with pathotypes 0 and 1 (56). Recently, Knight et al. (23) identified 11 pathotypes of *C. sativus* from leaves and roots of both barley and wheat from Australia. *C. sativus* in wheat has been reported from North America, South America, South and Central Asia, Australia, Europe, and Africa (5,26,42,52). In particular, this pathogen is regarded as a major biotic stress, limiting production of wheat in the Northern

Great Plains (53) and barley in the Canadian Prairie Provinces in North America (17,18). In addition, *C. sativus* has been reported to be one of the most destructive pathogens in wheat in warm areas, particularly in the intensive rice-wheat cropping system (12), and has significant impact on wheat production in South Asia (22,42,44).

Analysis of virulence and population genetic structure of *C. sativus* can assist plant pathologists and breeders in deploying resistance genes in wheat breeding programs. However, little information on pathogenic variation and genetic structure of *C. sativus* from wheat in Nepal and North Dakota is available. Spot blotch is one of the most devastating diseases of wheat in Nepal and causes up to 30% yield loss (12). In addition, Nepal's environment is conducive for the occurrence of spot blotch epidemics and, thus, the International Maize and Wheat Improvement Center has chosen it as one of the primary spot blotch screening sites (21,39-41,43). Therefore, it is imperative to know about phenotypic and molecular variations in *C. sativus* in this region where wheat is planted in both hills (elevation of 800 to 4,000 m, with subtropical climate) and plains in Nepal (elevation of 75 to 1,000 m, with tropical climate). The climatic conditions and cultural practices for wheat cultivation in Nepal are quite distinct from North Dakota. Wheat cultivars grown in Nepal are mostly either introduced from other countries or released by the National Wheat Research Program (NWRP), Bhairahawa, Nepal. In contrast, North Dakota has a temperate climate and is one of the largest wheat producing states in the United States (47), where spot blotch has recently emerged as an important foliar disease (46). Wheat cultivars in North Dakota are exposed to great differences in weather conditions and length of host-free periods, and these cultivars have various sources of spot blotch resistance incorporated by wheat breeding programs (19). In addition, the spring wheat cultivars are planted in the spring and harvested during late summer or early fall whereas the winter wheat cultivars are sown in the fall, remain dormant during the winter, and are harvested during June or July. *C. sativus* populations infecting spring wheat survive a host-free period of extremely cold winter weather, whereas those on winter wheat survive in a host-free period of hot and dry weather during summer

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and early fall (19). We hypothesize that populations of *C. sativus* in the Hill and Plain areas in Nepal and in the Central and Northern areas of North Dakota might evolve high virulence and could be genetically diverse. The main objectives of this study were to determine virulence in *C. sativus* populations from wheat in Nepal and North Dakota on differential wheat lines and cultivars and examine their genetic variation using selected polymorphic amplified fragment length polymorphism (AFLP) primers. Given the lack of knowledge of pathogenic and genetic diversity of *C. sativus* from wheat, this work will provide descriptive data to fill in an important information gap.

Materials and Methods

Fungal isolates. The isolates from Nepal were collected from major wheat-producing areas in the hilly production areas of the Kathmandu, Lalitpur, Bhaktapur, Pokhara, Kavreplanchowk, Solukhumbu, Parbat, Baglung, and Dhankuta districts and in the Plain areas from the Sunsari, Morang, Dhanusha, Bara, Parsa, Chitwan, Rupandehi, Banke, Bardiya, and Kanchanpur districts during 2009 (Table 1). Wheat leaves showing typical spot blotch symptoms were collected randomly from wheat fields in Central and Northern areas of North Dakota during 2006 and 2007 (Table 1). Single-spore isolation of *C. sativus* from the Central and Northern areas of North Dakota was performed by S. Ali at North Dakota State University (NDSU), Fargo (Table 1). Single-spore isolation of *C. sativus* from the Hill and Plain areas of Nepal was done at the Division of Plant Pathology, Nepal Agriculture Research Council, Khumaltar, Nepal and brought to NDSU under United States Department of Agriculture Animal and Plant Health Inspection Service permit number P526P-09-02536.

For isolation, diseased leaf samples were cut into 2- to 3-mm segments, placed in a plate with 0.5% sodium hypochlorite solution for 30 s, and later rinsed twice with sterile distilled water. Leaf segments were dried on sterile Whatman filter paper in a laminar flow hood and later placed in moist filter paper and kept in an incubator for 24 to 48 h at 21°C. The leaves were then observed under the stereoscope and a single conidium was picked using a flame-sterilized steel needle. Each single conidium was transferred to a petri dish containing V8-potato dextrose agar (PDA) (150 ml of V8 juice, 10 g of Difco PDA, 3 g of CaCO₃, 10 g of Bacto agar, and 850 ml of distilled water) and incubated at 21°C for 8 to 10 days under continuous light condition. At least two subsequent single-spore transfers were made to ensure the purity of each isolate. Later, the single-spore isolates from each V8-PDA plate were cut into small blocks, air dried under a laminar flow hood for 24 h, and stored at -80°C. In total, 96 putative isolates of *C. sativus* from the Hill ($n = 24$) and Plain ($n = 24$) areas in Nepal and from the Central ($n = 24$) and Northern ($n = 24$) areas in North Dakota and were used in this study (Table 1).

Virulence analysis. Twelve differential wheat lines (Table 2) originating from Brazil, China, India, and Nepal (22,24,25,33, 34,41–43) with different levels of resistance to *C. sativus* were selected based on differential responses in previous studies (21,29). Ten of these wheat lines have exhibited resistance to *C. sativus* under field conditions and have been used as potential sources of resistance to *C. sativus* in wheat breeding programs in Asia (24,25,33,40–44). Two differential wheat lines (Sonaliika and BL 1473) were included as susceptible checks.

In total, 95 isolates of *C. sativus* (Central [$n = 24$] and Northern [$n = 24$] areas of North Dakota and Hill [$n = 24$] and Plain [$n = 23$] areas of Nepal) were evaluated for their virulence on the 12 differential wheat lines. Pathogen inoculation was conducted under controlled conditions in growth chambers (16). Each experiment was conducted using a randomized complete block design with three replications. Each experimental unit had three seeds of a cultivar planted in a plastic “conetainer” (3.8 cm in diameter and 20 cm long) filled with Fison sunshine Mix number 1 (Fison Horticulture). Each plant in the conetainer was considered a sampling unit. The conetainers were placed in RL98 trays (Stuewe and Sons, Inc.). For each cultivar, nine plants were used and tested for each

isolate. In each experiment, wheat lines were considered a fixed effect and isolate was considered a random effect. The experiment was repeated once.

Inoculum of each isolate was prepared as described previously by Mahto et al. (30). The conidial suspension was adjusted to 3.5×10^3 conidia/ml and conidia were counted using a hemacytometer. One drop of Tween 20 per 100 ml of spore suspension was added as a surfactant. Two-week-old seedlings at Zadoks growth stage 13 (55) were spray inoculated with the spore suspension until run-off. Following inoculation, seedlings were moved into a mist chamber to provide continuous leaf wetness. After 24 h, seedlings were moved to a growth chamber at 21 and 18°C (day and night, respectively) with a 16-h photoperiod. Disease scores were assessed on second leaves between 9 and 10 days after inoculation using a 1-to-9 scale which was developed for scoring *C. sativus* infection response in barley (16). However, disease scores from 1 to 4 were considered resistant (R) because they had minute to small round or oblong necrotic lesions (<2 mm²) with or without restricted chlorotic margins. Similarly, disease reactions >4 were considered susceptible (S) because they had medium to large elliptical or ovate necrotic lesions (>2 mm²) with chlorotic margins and expanding diffuse chlorosis.

Virulence and cluster analysis. The term “virulence” is defined as the ability of an isolate to cause disease by overcoming a specific host *R* gene and “pathogenicity” as both the specific and non-specific disease-causing abilities of a pathogen (49). However, herein, we have used the term “virulence group” as the ability of a group of isolates to cause disease across a set of 12 differential wheat lines as previously mentioned by Ghazvini and Tekauz (17). Similarly, the term “pathotype” was used to describe levels of aggressiveness of isolates with a smaller set of differential genotypes for *C. sativus* (17,23), and *Pyrenophora teres* f. *teres* from barley (7,28). Nonparametric analysis (39) was performed using disease scores obtained from each cultivar–isolate combination from each experiment. The median values were estimated using the SAS program PROC Mixed (version 9.2; SAS Institute) (38). To test whether the median disease scores of both experiments were similar, the interactions between the wheat lines and the fungal isolates and between two experiments were calculated (1). To test the homogeneity of variances among the two experiments, Bartlett’s χ^2 values were estimated using SAS as described previously (1).

The reactions of 12 wheat differential lines to each of the 95 *C. sativus* isolates were shown by preparing a resistance/susceptibility (R/S) matrix (Table 3). The triplet coded nomenclature system, as proposed by Limpert and Muller (27), was also used to designate the virulence group of the 95 isolates of *C. sativus*. This system has been used to classify virulence group of *C. sativus* previously (17,18). Hierarchical cluster analysis was performed using the SIMINT program in the NTSYS-pc software (version 2.1; Exeter Software) in order to evaluate the intra- and inter-relationships among isolates of *C. sativus* in each pathotype. Cluster analysis was performed using the median disease rating score of differential wheat lines to each isolate (18,23).

Genomic DNA extraction and quantification. To extract genomic DNA, each isolate was grown on V8-PDA plates overlaid with cellophane sheets, and mycelia grown on the cellophane surface were scraped directly with a sterile scalpel and harvested from each plate after 15 days. Harvested mycelia were placed in a 2-ml centrifuge tube and freeze dried at -80°C for 48 h. The lyophilized mycelia were ground to fine powder using a high-speed mixer mill (Model MM301; Retsch Inc.). Genomic DNA of each isolate (total of 96 isolates; 48 isolates each from Nepal and North Dakota) was extracted using a DNeasy Plant Mini Kit (Qiagen Inc.) and was quantified using a Nano Drop (Model ND-1000; Thermo Scientific Inc.). Each DNA sample was adjusted to 30 ng/μl for AFLP analysis.

Sequence analysis. To confirm the identity of *C. sativus* isolates, we sequenced and compared internal transcribed spacer (ITS)4 and ITS5 and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) regions from the 96 isolates of *C. sativus*. ITS and *gpd* sequences were further compared with ITS and *gpd* sequences

Table 1. *Cochliobolus sativus* isolates collected from wheat in Hill and Plain areas in Nepal and Central and Northern areas in North Dakota (ND) which were used for pathogenic and molecular diversity studies

Serial number	Isolate designation	Date of collection	Cultivar or wheat type ^a	Origin	Region
1	CS1	2009	Spring wheat	Solukhumbu	Hill
2	CS2	2009	Spring wheat	Solukhumbu	Hill
3	CS3	2009	Spring wheat	Dhankuta	Hill
4	CS4	2009	Spring wheat	Dhankuta	Hill
5	CS5	2009	Spring wheat	Dhankuta	Hill
6	CS6	2009	Spring wheat	Kavrepalanchowk	Hill
7	CS7	2009	Spring wheat	Kavrepalanchowk	Hill
8	CS8	2009	Spring wheat	Kavrepalanchowk	Hill
9	CS9	2009	Spring wheat	Kavrepalanchowk	Hill
10	CS10	2009	Spring wheat	Kavrepalanchowk	Hill
11	CS11	2009	Spring wheat	Bhaktapur	Hill
12	CS12	2009	Spring wheat	Bhaktapur	Hill
13	CS13	2009	Spring wheat	Bhaktapur	Hill
14	CS14	2009	Spring wheat	Bhaktapur	Hill
15	CS15	2009	Spring wheat	Bhaktapur	Hill
16	CS16	2009	Spring wheat	Kathmandu	Hill
17	CS17	2009	Spring wheat	Kathmandu	Hill
18	CS18	2009	Spring wheat	Kathmandu	Hill
19	CS19	2009	Spring wheat	Kathmandu	Hill
20	CS20	2009	Spring wheat	Kathmandu	Hill
21	CS21	2009	Spring wheat	Kathmandu	Hill
22	CS22	2009	Spring wheat	Lalitpur	Hill
23	CS23	2009	Spring wheat	Baglung	Hill
24	CS24	2009	Spring wheat	Parbat	Hill
25	CS25	2009	Spring wheat	Morang	Plain
26	CS26	2009	Spring wheat	Morang	Plain
27	CS27	2009	Spring wheat	Morang	Plain
28	CS28	2009	Spring wheat	Morang	Plain
29	CS30	2009	Spring wheat	Sunsari	Plain
30	CS31	2009	Spring wheat	Sunsari	Plain
31	CS32	2009	Spring wheat	Sunsari	Plain
32	CS33	2009	Spring wheat	Janakpur	Plain
33	CS34	2009	Spring wheat	Janakpur	Plain
34	CS35	2009	Spring wheat	Janakpur	Plain
35	CS36	2009	Spring wheat	Bara	Plain
36	CS37	2009	Spring wheat	Bara	Plain
37	CS38	2009	Spring wheat	Bara	Plain
38	CS39	2009	Spring wheat	Parsa	Plain
39	CS40	2009	Spring wheat	Parsa	Plain
40	CS41	2009	Spring wheat	Parsa	Plain
41	CS42	2009	Spring wheat	Chitwan	Plain
42	CS43	2009	Spring wheat	Chitwan	Plain
43	CS44	2009	Spring wheat	Chitwan	Plain
44	CS45	2009	Spring wheat	Rupandehi	Plain
45	CS46	2009	Spring wheat	Rupandehi	Plain
46	CS47	2009	Spring wheat	Rupandehi	Plain
47	CS48	2009	Spring wheat	Banke	Plain
48	CS49	2009	Spring wheat	Banke	Plain
49	CS50	2007	Goodstreak (HRWW)	Bottineau, ND	Northern
50	CS51	2006	Spring wheat	Bottineau, ND	Northern
51	CS52	2007	Wesley (WW)	Lisbon, ND	Central
52	CS53	2006	Spring wheat	Griggs, ND	Northern
53	CS54	2006	Spring wheat	Griggs, ND	Northern
54	CS55	2006	Spring wheat	Griggs, ND	Northern
55	CS56	2006	Spring wheat	Nelson, ND	Northern
56	CS57	2006	Spring wheat	Towner, ND	Northern
57	CS58	2006	Spring wheat	Bottineau, ND	Northern
58	CS59	2007	Wesley (WW)	Lisbon, ND	Central
59	CS60	2006	Spring wheat	Bottineau, ND	Northern
60	CS61	2007	Spring wheat	Minot, ND	Northern
61	CS62	2007	SD 96240-3-1 (WW)	Lisbon, ND	Central
62	CS63	2007	Jerry (WW)	Prosper, ND	Central
63	CS64	2007	SD 96240-3-1 (WW)	Lisbon, ND	Central
64	CS65	2007	Millennium (WW)	Lisbon, ND	Central
65	CS66	2006	Spring wheat	Carrington, ND	Central
66	CS67	2007	SD 96240-3-1 (WW)	Lisbon, ND	Central
67	CS68	2006	Spring wheat	Carrington, ND	Central
68	CS69	2007	Goodstreak (HRWW)	Prosper, ND	Central
69	CS70	2007	Darrel (HRWW)	Lisbon, ND	Central
70	CS71	2006	Spring wheat	Griggs, ND	Northern
71	CS72	2006	Spring wheat	Griggs, ND	Northern
72	CS73	2007	Goodstreak (HRWW)	Prosper, ND	Central

(continued on next page)

^a HRWW= hard red winter wheat, HRSW= hard red spring wheat, SW = spring wheat, and WW = winter wheat.

Table 1. (continued from preceding page)

Serial number	Isolate designation	Date of collection	Cultivar or wheat type ^a	Origin	Region
73	CS74	2006	Spring wheat	Ramsey, ND	Northern
74	CS75	2007	Wesley (WW)	Lisbon, ND	Central
75	CS76	2007	Alsen (SW)	Langdon, ND	Northern
76	CS77	2006	Spring wheat	Ramsey, ND	Northern
77	CS78	2006	Spring wheat	Nelson, ND	Northern
78	CS79	2007	Goodstreak (HRWW)	Prosper, ND	Central
79	CS80	2006	Spring wheat	Towner, ND	Northern
80	CS81	2007	SD 96240-3-1 (WW)	Lisbon, ND	Central
81	CS82	2006	Spring wheat	Carrington, ND	Central
82	CS83	2006	Spring wheat	Prosper, ND	Central
83	CS84	2007	Darrel (HRWW)	Lisbon, ND	Central
84	CS85	2006	Spring wheat	Nelson, ND	Northern
85	CS86	2006	Spring wheat	Nelson, ND	Northern
86	CS87	2006	Spring wheat	Rolette, ND	Northern
87	CS88	2006	Spring wheat	Rolette, ND	Northern
88	CS89	2006	Spring wheat	Rolette, ND	Northern
89	CS90	2007	Alice (HRWW)	Lisbon, ND	Central
90	CS91	2007	Darrel (HRWW)	Lisbon, ND	Central
91	CS92	2006	Spring wheat	Walsh, ND	Northern
92	CS93	2007	CDC Falcon (WW)	Lisbon, ND	Central
93	CS94	2007	Wesley (WW)	Lisbon, ND	Central
94	CS95	2006	Spring wheat	Prosper, ND	Central
95	CS96	2007	Howard (HRSW)	Langdon, ND	Northern
96	CS97	2007	Wesley (WW)	Lisbon, ND	Central

Table 2. Differential wheat lines used to assess virulence of *Cochliobolus sativus* isolates from wheat in Nepal and North Dakota

Genotype	Level ^a	Pedigree	CIMMYT CID ^b	Origin
BL 1473 ^c	S	Nepal 297/NL531	166468	Nepal/CIMMYT
Chirya 7 ^d	R	CS/Th. cu//Glen/3/Ald/Pvn/4/Ningmai No.4/Oleson//Ald/Yangmai No.4	66176	Nepal/CIMMYT
Chirya 3 ^{c,e}	R	CS/Th. cu//Vee/3/Ald/Pvn/4/Ningmai No.4/Oleson//Ald/Yangmai No.4	54384	CIMMYT
G 162 ^f	R	Guizhou large head no.7//Yangmai-1/Op	93926	China
Milan/Shanghai 7 ^c	R	VS73.600/MIRLO/3/BOW//YE/TRF/4/Shanghai7	20026	China
Ning 8201 ^e	R	Ningmai-4/Olesen/Alondra (Sib)/Yangmai-3	95659	China
Ning 8319 ^g	R	Ningmai-4/Oleson//Ald/Yangmai-3	3495	China
OCEPAR-7 ^h	R	Tzpp*2/An64//Inia66/3/Cno67/Jar//Kvz	7136	Brazil
PBW 343 ⁱ	R	Nd/VG9144//Kal/Bb/3/Yaco/4/Vee5	8890	India
Sonalika ^j	S	II53.388/AN//YT54/N10B/3/LR/4/B4946/Y53//3*Y50	6977	Nepal/CIMMYT
SW89.5422 ^g	R	Unknown	72403	China
Yangmai-6 ⁱ	R	Unknown	239288	China

^a Level of field resistance according to evaluation of lesion density (lesions cm²) and area under disease progress curve of seedling and adult plant resistance to spot blotch. S = susceptible and R = resistant.

^b International Maize and Wheat Improvement Center (CIMMYT) cross identification number (CID).

^c Neupane et al. (33).

^d Sharma et al. (42).

^e Kumar et al. (25).

^f Sharma et al. (43).

^g Sharma et al. (41).

^h Okuyama et al. (34).

ⁱ Joshi et al. (22).

^j Kumar et al. (24).

reported from closely related species, including *C. carbonum* (*gpd* = GenBank accession AF081382; ITS = AF158110), *C. victoriae* (*gpd* = AF158112; ITS = AF158109), *C. heterostrophus* (*gpd* = AF081380; ITS = AF071325), and *C. eleusines* (*gpd* = AF081405; ITS = AF081451) (*data not shown*). The ITS regions were amplified using the standard primers ITS4 and ITS5 (51) and the region coding for the nuclear gene *gpd* was amplified using the primer pair *gpd1/gpd2* (6) and, for both ITS and *gpd* sequence amplifications, polymerase chain reaction (PCR) cycles and conditions were similar to those described previously by Berbee et al. (6). For confirmation, 5 µl of the amplified PCR products were run in 2% (wt/vol) agarose gel (Sigma-Aldrich) in 0.5× Tris-borate-EDTA (TBE) buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). After confirming the PCR reaction, the remaining 20 µl of the amplified products were sent to the Molecular Cloning Laboratories DNA sequencing service (San Francisco, CA) for sequencing of ITS and *gpd* genomic regions in both sense and anti-sense directions. Sequences derived in this study were compared

with the ITS and *gpd* sequence with other closely related species sequence after downloading the sequences from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>). Both the ITS and *gpd* sequences were aligned using the software ClustalX and neighbor-joining trees, with 1,000 bootstraps built to obtain the phylogenetic trees.

AFLP analysis. AFLP analysis was performed using an AFLP core reagent kit (Invitrogen Life Technologies) and reactions were conducted as described previously (50). Genomic DNA (30 ng/µl) of each isolate was digested with *EcoRI* and *MseI* at 37°C for 3 h and, later, the restricted DNA fragments were ligated to AFLP adaptors at 20°C for 3 h. The digested and ligated templates were diluted in 1:2 ratios with sterile distilled water before being used in the preamplification. Primers complimentary to the adapter sequences plus one selective base at the 3' end were used for preamplifications. All the samples were preamplified in reactions containing the DNA template (30 ng/µl), 36 ng of each *EcoRI* primer (E-A) and *MseI* primer (M-C), 0.2 mM dNTPs, 1× buffer,

Table 3. Median disease values of 95 isolates of *Cochliobolus sativus* on 12 differential wheat lines and corresponding pathotypes and virulence types based on growth chamber studies^a

Isolate	BL 1473	Chirya-7	Chirya-3	G 162	MS-7	Ning 8201	Ning 8319	Ocepar-7	PBW 343	Sonalika	SW 89-5422	Yangmai-6	Code ^b	Group ^c	Pathotype ^d
Cs 51	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 82	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 78	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 4	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 93	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 16	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 2	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 12	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 65	R	R	R	R	R	R	R	R	R	R	R	R	0.0.0.0	L	1
Cs 90	R	R	R	R	R	R	R	R	R	S	R	R	0.0.0.1	I	2
Cs 64	R	R	R	R	R	R	R	R	R	S	R	R	0.0.0.1	I	2
Cs 21	R	R	R	R	R	R	R	S	R	S	R	R	0.0.2.1	I	3
Cs 36	R	R	R	R	R	R	R	S	R	S	S	R	0.0.2.3	I	4
Cs 1	R	R	R	R	R	R	R	R	S	S	R	R	0.0.4.1	I	5
Cs 30	R	R	R	R	R	R	R	R	S	S	R	R	0.0.4.1	I	5
Cs 92	R	R	R	R	R	R	R	R	S	S	R	R	0.0.4.1	I	5
Cs 59	R	R	R	S	R	R	R	R	R	S	R	R	0.1.0.1	I	6
Cs 9	R	R	R	S	R	R	R	R	R	S	R	R	0.1.0.1	I	6
Cs 96	R	R	R	S	R	R	R	R	S	S	R	R	0.1.4.1	I	7
Cs 73	S	R	R	R	R	R	R	R	R	R	R	R	1.0.0.0	I	8
Cs 87	R	R	R	S	R	R	R	S	S	S	R	R	0.1.6.1	I	9
Cs 88	R	R	R	R	S	R	R	S	S	S	R	R	0.2.6.1	I	10
Cs 26	S	R	R	R	R	R	R	R	R	S	R	S	1.0.0.5	I	11
Cs 62	S	R	R	R	R	R	R	R	S	S	R	S	1.0.4.5	I	12
Cs 63	S	R	R	R	R	R	R	S	S	S	R	R	1.0.6.1	I	13
Cs 52	S	R	R	R	R	R	R	S	S	S	R	R	1.0.6.1	I	13
Cs 6	S	R	R	S	R	R	R	S	R	S	R	R	1.1.2.1	I	14
Cs 8	S	R	R	S	R	R	R	S	R	S	R	R	1.1.2.1	I	14
Cs 7	S	R	R	S	R	R	R	S	R	S	S	R	1.1.2.3	I	15
Cs10	S	R	R	S	R	R	R	S	R	S	S	S	1.1.2.7	I	16
Cs 74	S	R	R	S	R	R	R	S	R	S	S	S	1.1.2.7	I	16
Cs 86	S	R	R	S	R	R	R	S	S	S	R	R	1.1.6.1	I	17
Cs 17	S	R	R	R	S	R	R	R	R	S	R	S	1.2.0.5	I	18
Cs 84	S	R	R	R	S	R	R	S	S	S	R	R	1.2.6.1	I	19
Cs 94	S	R	R	R	S	R	R	S	S	S	R	R	1.2.6.1	I	19
Cs 5	S	R	R	S	S	R	R	S	R	S	R	S	1.3.2.5	I	20
Cs 80	S	R	R	S	S	R	R	S	S	S	R	S	1.3.6.5	I	21
Cs 22	S	R	R	S	S	R	R	S	S	S	S	S	1.3.6.7	I	22
Cs 70	S	R	R	R	R	S	R	R	S	S	R	R	1.4.4.1	I	23
Cs 20	S	R	R	R	R	S	R	S	S	S	R	R	1.4.6.1	I	24
Cs 61	S	R	R	S	R	S	S	S	R	S	S	S	1.5.3.7	I	25
Cs 25	S	R	R	S	R	S	R	S	S	S	R	R	1.5.6.1	I	26
Cs 13	S	R	R	S	R	S	R	S	S	S	R	R	1.5.6.1	I	26
Cs 35	S	R	R	S	R	S	R	S	S	S	R	R	1.5.6.1	I	27
Cs 43	S	R	R	S	R	S	R	S	S	S	R	R	1.5.6.3	I	27
Cs 75	S	R	R	S	R	S	R	S	S	S	R	S	1.5.6.5	I	28
Cs 60	S	R	R	R	R	S	R	S	S	R	R	S	1.4.6.4	I	29
Cs 91	S	R	R	S	R	S	R	S	S	S	S	S	1.5.6.7	I	30
Cs 34	S	R	R	S	R	S	R	S	S	S	S	S	1.5.6.7	I	20
Cs 89	S	R	R	R	S	S	R	S	S	S	S	R	1.6.6.3	I	31
Cs 27	S	R	R	R	S	S	R	S	S	S	S	R	1.6.6.3	I	31
Cs 85	S	R	R	R	S	S	R	S	S	S	S	R	1.6.6.3	I	31
Cs 50	S	R	R	S	S	S	R	R	S	S	S	S	1.7.4.7	I	32
Cs 69	S	R	R	S	S	S	R	S	S	S	R	R	1.7.6.1	I	33
Cs 66	S	R	R	S	S	S	R	S	S	S	R	R	1.7.6.1	I	33
Cs 83	S	R	R	S	S	S	R	S	S	S	R	R	1.7.6.1	I	34
Cs 79	S	R	R	S	S	S	R	S	S	S	R	R	1.7.6.2	I	35
Cs 55	S	R	R	S	S	S	R	S	S	S	S	R	1.7.6.3	I	36
Cs 57	S	R	R	S	S	S	R	S	S	S	R	S	1.7.6.5	I	37
Cs 38	S	R	R	S	S	S	R	S	S	S	R	S	1.7.6.5	I	37
Cs 19	S	R	R	S	S	S	R	S	S	S	R	S	1.7.6.5	I	37
Cs 81	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs 43	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs 58	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38

(continued on next page)

^a One isolate (Cs 29) from Nepal (Plain) was not included in data analysis due to poor growth. R = resistant and S = susceptible.

^b Nomenclature code. Virulence of each isolate of *C. sativus* was evaluated on 12 differential wheat lines based the infection response (IR) and was assessed 9 to 10 days after inoculation using a 1-to-9 ordinal disease rating scale (16). The triplet coded nomenclature system as proposed by Limpert and Muller (27) was used to classify virulence of the 95 isolates of *C. sativus*. Nonparametric analysis (39) was performed using IR scores to obtain median disease values for each line-isolate combination as described in Materials and Methods.

^c Virulence group type: L = low, I = intermediate, and H = high.

^d Pathotype was identified according to virulence patterns on differential wheat lines and triplet coded nomenclature system as proposed by Limpert and Muller (27).

Table 3. (continued from preceding page)

Isolate	BL 1473	Chirya-7	Chirya- 3	G 162	MS-7	Ning 8201	Ning 8319	Ocepar-7	PBW 343	Sonalika	SW 89-5422	Yangmai-6	Code ^b	Group ^c	Pathotype ^d
Cs 15	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs 23	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs11	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs 72	S	R	R	S	S	S	R	S	S	S	S	S	1.7.6.7	I	38
Cs 56	R	S	R	R	R	S	R	S	S	S	R	R	2.4.6.1	I	39
Cs 18	S	R	S	R	R	S	R	S	S	S	S	S	5.4.6.7	I	40
Cs14	S	R	S	S	R	S	R	S	S	S	S	S	5.5.6.7	I	41
Cs 68	S	R	S	S	R	S	S	S	S	S	S	S	5.5.7.7	I	42
Cs 67	S	R	S	S	S	S	S	S	R	S	S	S	5.7.3.7	I	43
Cs 54	S	R	S	S	S	S	R	S	S	S	S	R	5.7.6.3	I	44
Cs 49	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 71	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 76	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 95	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 77	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 48	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 42	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 39	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 37	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 45	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 44	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 32	S	R	S	S	S	S	R	S	S	S	S	S	5.7.6.7	I	45
Cs 41	S	R	S	S	S	S	S	S	S	S	S	S	5.7.7.7	I	46
Cs 47	S	R	S	S	S	S	S	S	S	S	S	S	5.7.7.7	I	46
Cs 31	S	R	S	S	S	S	S	S	S	S	S	S	5.7.7.7	I	46
Cs 28	S	R	S	S	S	S	S	S	S	S	S	S	5.7.7.7	I	46
Cs 33	S	R	S	S	S	S	S	S	S	S	S	S	5.7.7.7	I	46
Cs 24	S	S	S	S	S	S	S	S	S	S	S	S	7.7.7.7	H	47
Cs 40	S	S	S	S	S	S	S	S	S	S	S	S	7.7.7.7	H	47
Cs 3	S	S	S	S	S	S	S	S	S	S	S	S	7.7.7.7	H	47
Cs 46	S	S	S	S	S	S	S	S	S	S	S	S	7.7.7.7	H	47

1.5 mM MgCl₂, and 1 unit of Taq DNA polymerase at 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s for 20 cycles. The *EcoRI* primers were labeled with the fluorescent dye IRD 700 (Li-COR Biosciences) and unlabeled *MseI* primers with two selective bases were used for selective amplification.

Eight *EcoRI* and *MseI* primer combinations (E-AA+M-CA, E-AA+M-CC, E-AC+M-CA, E-AC+M-CC, E-AC+M-CT, E-AG+M-CA, E-AG+M-CG, and E-AT+M-CT) revealed many polymorphisms in our preliminary as well as previous study (56). Therefore, these eight primer combinations were used in AFLP analysis (Table 4). Selective amplifications were done using the PCR conditions as described by Vos et al. (50). The selective amplified reactions were mixed with 6 µl of formamide loading dye and later denatured at 95°C for 5 min. Each PCR was run twice to determine variability between runs. The amplified products were separated on a LI-COR Global edition IR² System (Li-COR Biosciences) in 6% polyacrylamide gels within 1× TBE buffer (10.8 g of Tris base, 5.5 g of boric acid, 2 ml of 0.5 M EDTA, and 1,000 ml of sterile distilled water) as described previously (56). The polyacrylamide gel solution contained 20 ml of Rapid Gel-XL 6% liquid acrylamide (USB Corporation), 150 µl of 10% fresh ammonium persulfate, and 15 µl of tetramethylethylenediamine. The gel electrophoresis was run at 2,000 V with 40 mA of current and 50 W of power at 45°C temperature for 3 h with a Global IR² System (Li-COR Biosciences). Band sizes of 60 to 700 bp that were reproducible were scored manually with reference to a 50-bp ladder (Li-COR Biosciences). The DNA fragments were scored manually in a binary system, where presence of a band was coded as 1 and absence as 0. From the dissimilarity matrix, a dendrogram was obtained using the unweighted pair group mean analysis (UPGMA) with the SIMINT program in NTSYS-pc software (version 2.1; Exeter Software). To determine the robustness of the dendrogram, the presence or absence of data were resampled by replacement with 1,000 bootstrap replicates.

AFLP data analysis. The data analyses were performed using repeatable and polymorphic amplified bands. Different fragment

Table 4. Number of polymorphic amplified fragment length polymorphism bands generated by eight primer combinations in 96 isolates of *Cochliobolus sativus* from Nepal and North Dakota

Primer combinations	Total bands	Polymorphic bands (%)
<i>EcoRI</i> -AA/ <i>MseI</i> -CA	39	26
<i>EcoRI</i> -AA/ <i>MseI</i> -CC	35	31
<i>EcoRI</i> -AC/ <i>MseI</i> -CA	27	43
<i>EcoRI</i> -AC/ <i>MseI</i> -CC	28	89
<i>EcoRI</i> -AC/ <i>MseI</i> -CT	38	34
<i>EcoRI</i> -AG/ <i>MseI</i> -CA	38	82
<i>EcoRI</i> -AG/ <i>MseI</i> -CG	36	27
<i>EcoRI</i> -AT/ <i>MseI</i> -CT	41	54
Total	272	...

sizes of each AFLP marker were considered different alleles. Different allele sizes generated by each of the AFLP markers were recorded and entered in the GenAlex 6 format.

Population structure analysis. The affiliation of individual *C. sativus* isolates from sampling groups to specific genetic clusters and the possibility of admixture were tested using the software program STRUCTURE v.2.3.2 (35). STRUCTURE v.2.3.2 was run for Nepal (Hill and Plain areas) and North Dakota (Central and Northern areas) populations separately. Repeated gene flow might cause lack of significant population differentiation between sampling groups. For each number of clusters *K* (*K* = 1 through 10), defined as subpopulation, 10 runs were performed with a burn-in period of 10,000 iterations used for each run, and using 100,000 iterations in Monte Carlo Markov chain replications for the analysis. The number of subpopulations between 1 and 10 was analyzed for a total of five replicates using an admixture model. Further, an ad hoc statistic ΔK was used to confirm the exact number of subpopulation (*K*) using Structure Harvester software (v0.6.5). Here, the ΔK is solely based on the rate of change in log probability of data between its successive *K* values (13).

Genetic diversity analysis. Alleles generated from all loci were compiled to construct multilocus haplotypes (MLHTs). Isolates

with the same MLHT were considered clones. Clone-corrected data were used for all analyses. Estimation of total number of alleles, gene diversity (H), genetic distance (D), and Nei's unbiased genetic identity (I) were done using POPGENE version 1.32 (54). Genetic diversity in the total population (H_T) and within the geographic population (H_S) were estimated using Nei's unbiased gene diversity. Pairwise measures of population differentiation between *C. sativus* populations from the Central and Northern areas of North Dakota and between populations from the Hill and Plain areas of Nepal were done separately using a corrected standardized fixation index (i.e., G''_{ST}) using GenoDive 2.0 beta version software (20,31).

Linkage disequilibrium analysis. Multilocus linkage disequilibrium (LD) was assessed using the index of association ($\bar{r}_d$). The $\bar{r}_d$ is an alternative measure of index of association and is less sensitive to number of loci when compared with I_A . The $\bar{r}_d$ varies from 0 (random association of allele at different loci) to 1 (maximum possible LD among loci). The significance of $\bar{r}_d$ was tested by randomization (1,000 times) procedures by comparing the observed value of $\bar{r}_d$ with that expected under the null hypothesis of

complete panmixis (2). The $\bar{r}_d$ values were estimated for the Hill and Plain areas (Nepal) and for the Central and Northern areas (North Dakota) separately with MULTILOCUS program version 1.3 (2).

Analysis of molecular variance. Analysis of molecular variance (AMOVA) was used to partition genetic variation within and among populations for detecting population differences (14). Genetic variation was partitioned within and among populations from Central and Northern areas of North Dakota and within and among populations from Hill and Plain areas of Nepal using the Arlequin 3.1 software (14,15). Variance components and F_{ST} values were tested by 1,023 permutations of haplotypes between *C. sativus* populations to determine level of significance at $P \leq 0.001$ (14).

Results

Virulence analysis. Homogeneity tests performed using Bartlett's χ^2 indicated that the variance in the data sets was homogeneous ($\chi^2 = 2.98$, $P = 0.19$). Similarly, analysis of variance of the nonparametric data showed that the main effects of wheat differential lines ($P < 0.001$), isolates ($P < 0.001$), and interactions be-

Fig. 1. Dendrogram based on the similarity of the median disease scores of 95 isolates of *Cochliobolus sativus* on 12 differential wheat lines. Cluster analysis was based on unweighted pair group method using arithmetic averages. Pathotypes were designated according to the coded triplet nomenclature system.

tween wheat lines and isolates were significant ($P < 0.001$) (data not shown).

All isolates of *C. sativus* produced minute necrotic lesions with no or only slight chlorotic halos to large necrotic lesions surrounded by distinct yellow halos (typical spot blotch symptoms) on 12 differential wheat lines tested. On the basis of hierarchical cluster analysis, the 95 isolates were grouped into 47 pathotypes (Table 3) and were classified into three clusters (Fig. 1). The first cluster had low virulence and contained nine isolates. The second cluster had intermediate virulence isolates and consisted of 82 isolates. The third cluster had four isolates with high virulence on all of the 12 wheat differential lines tested (Fig. 1). No relationship was observed between the virulence group and country of origin (Fig. 1). Nine of the isolates from pathotype 1 produced small round or oblong necrotic lesions ($<2 \text{ mm}^2$) with or without restricted chlorotic margins on susceptible wheat line Sonalika (Table 3). The majority of isolates (82 isolates from pathotype 2 through pathotype 46) were classified as having intermediate virulence based on their ability to cause susceptible reactions on two susceptible wheat lines and on three to eight resistant wheat lines used in this study. The remaining four isolates from pathotype 47 were classi-

fied as highly virulent based on their ability to cause susceptible reactions in all of the resistant wheat lines used in this study. Pathotypes 45, 1, and 38 had the highest number of isolates consisting of 12, 9, and 7 isolates in each group, respectively (Table 3). The 30 randomly selected *C. sativus* isolates from Nepal and North Dakota produced minute necrotic lesions similar to those of resistant reactions on barley differential lines Bowman, NDB112, and ND5883. Based on infection response (IR) on the three barley differential lines and the triplet virulent group (i.e., pathotypes 0) (Supplementary Table S1), even the highly virulent isolate CS 46 was unable to cause susceptible reactions on the three differential barley lines. Thus, the disease reactions or scores of wheat *C. sativus* isolates on these barley differential lines were not included in our analysis. Of the 12 differential wheat lines or cultivars tested, Chirya 7 exhibited resistance to 91 of 95 isolates tested while Sonalika was the most susceptible differential host, being susceptible to 84 of 95 isolates. Other differential cultivars were resistant to 22 to 84 isolates.

Sequence analysis of ITS and *gpd* regions. The ITS (GenBank accession JX145649) and *gpd* (JX145648) sequences of the 96 *C. sativus* isolates were identical. Moreover, the *gpd* sequences from

Fig. 2. Estimates of genetic similarity among *Cochliobolus sativus* isolates collected from the Hill and Plain areas in Nepal and from the Central and Northern areas of North Dakota based on amplified fragment length polymorphisms. Bootstrap values (1,000 replicates) for branch nodes ranged from 10 to 100%.

C. sativus had 96 to 97% maximum identity and the ITS sequences had maximum sequence identity from 98 to 99% from those of the reference isolates of *C. carbonum*, *C. victoriae*, *C. heterostrophus*, and *C. miyabeanus* (data not shown).

AFLP data analysis. DNA polymorphisms between 60 to 700 bp allowed 108 polymorphic bands from eight AFLP primer combinations across 96 *C. sativus* isolates to be scored and used for data analysis. On average, 34 bands were scored per primer combination. On average, 48% polymorphic bands were observed for each primer combination. Two AFLP primer combinations (*Eco*RI-AC/*Mse*I-CC and *Eco*RI-AG/*Mse*I-CA) were highly polymorphic for *C. sativus* (Table 4). The dendrogram generated from combined AFLP data from eight primer pair sets revealed that the 96 isolates clustered as two distinct groups using UPGMA with the genetic distance coefficient (Fig. 2). The two isolates from North Dakota (Northern) clustered in one group and all isolates from Nepal (Hill and Plain areas) and North Dakota (Central and Northern areas) clustered together in another group. The isolates from same pathotype and geographic origins were unable to be grouped into the same cluster.

Population structure analysis. The value of $\log \text{Pr}(X/K)$ (i.e., the log likelihood of the model given that K clusters are present in the data) was significant for $K=1$ versus $K=2$ and revealed a single population for the Central and Northern populations from North Dakota and for the Hill and Plain populations from Nepal, respectively (Fig. 3A and B).

Genetic diversity analysis. Gene diversity analysis showed that the *C. sativus* populations had 108 alleles in the entire population. All of the isolates were of distinct haplotypes (Table 5). Gene diversity (H) values for Hill and Plain populations from Nepal were 0.10 and 0.08, respectively, whereas H values for Central and Northern populations from North Dakota were 0.11 and 0.16, respectively (Table 5).

LD analysis. Based on the multilocus LD statistic ($\bar{r}_d$) the null hypothesis of random recombination across loci was rejected for Central, Northern, Hill, and Plain populations from North Dakota and Nepal ($\bar{r}_d = 0.066$ to 0.292 , $P > 0.001$; Table 5), suggesting that all MLHTs resulted from asexual reproduction or parasexual recombination. Pairwise comparison of Nei's unbiased genetic identity (I) and genetic distance between the Central and Northern populations from North Dakota were 0.99 and 0.03, respectively.

Similarly, pairwise comparison of Nei's unbiased genetic identity (I) and genetic distance between the Hill and Plain populations from Nepal were 0.98 and 0.01, respectively. The G''_{ST} , which is a corrected standardized fixation index that is calculated as an independent measure of differentiation between populations, was 0.02 between Central and Northern populations from North Dakota and 0.05 between Hill and Plain populations from Nepal.

AMOVA. AMOVA revealed low genetic variation (4%) among Central and Northern populations whereas greater than 96% of the genetic variation was found within Central and Northern populations (Table 6). Similarly, low genetic variation (7%) also was observed among Hill and Plain populations from Nepal, whereas greater than 93% of the genetic variation was found within Hill and Plain populations. Individuals within Central and Northern populations and within Hill and Plain populations were statistically significant at $P < 0.001$ (Table 6).

Discussion

Virulence analysis of *C. sativus* on differential wheat lines and population genetic analysis using AFLP markers demonstrated that this pathogen is highly pathogenic and genetically diverse in Nepal and North Dakota. To our knowledge, this is the first study to determine virulence and molecular diversity of *C. sativus* populations from wheat foliar infections.

Valjavec-Gratian and Steffenson (48) were the first to develop the pathotype system for *C. sativus* using three barley differential lines (Bowman, NDB112, and ND5883) and using the coded triplet nomenclature system. They reported pathotypes 0, 1, and 2 for *C. sativus* isolated from barley leaves from North Dakota. Since then, the virulence spectra of *C. sativus* from barley have been analyzed in Australia, Canada, and the United States using the coded triplet nomenclature (17,18,23,48,56). Ghazvini and Tekauz (17) used 12 differential barley lines and identified eight virulence groups from 127 *C. sativus* isolates collected from barley in Canada. In another study, Arabi and Jawhar (4) found virulence of *C. sativus* from barley in Syria to be highly variable on barley lines possessing different levels of resistance. A study conducted in Australia identified 11 pathotypes among the 31 *C. sativus* isolates collected from barley and wheat, using 12 differential barley lines (23). All of the previous studies have used differential barley lines and the coded triplet nomenclature system to identify pathotypes of *C. sativus*.

Fig. 3. Affiliation of individual genotypes of *Cochliobolus sativus* as assessed using 108 polymorphic amplified fragment length polymorphism loci measured in Structure ver. 2.33 (35). A, Populations from the Hill and Plain areas of Nepal were determined to be $K = 1$ and B, populations from the Northern and Central areas of North Dakota were also determined to be $K = 1$.

(4,17,23,48). In this study, we found that *C. sativus* isolates from wheat can only induce minute necrotic lesions, similar to resistant reactions, on the three previously identified barley differential lines Bowman, NDB112, and ND5883. On the basis of IR on barley differential lines, all 30 randomly selected *C. sativus* isolates were grouped into pathotype 0 and were the least virulent pathotype. In contrast, these same isolates showed differential responses on the differential wheat lines tested and could be grouped into low- to high-virulence pathotypes. A similar result was reported by Knight et al. (23), who found that all of the *C. sativus* isolates from wheat roots were the least virulent (pathotype 0.0.0.0) on 12 differential barley lines. They further reported that the *C. sativus* population from wheat was clearly differentiated from the *C. sativus* population from barley on the basis of the AFLP marker system (23). Similarly, Conner and Atkinson (8) also found that *C. sativus* from wheat roots was less virulent on barley roots and vice versa. Our finding that the isolates from wheat were less virulent on barley differential lines indicates that there is the potential for host specificity (23). Thus, there is a need for differential wheat lines which can clearly define the virulence pattern of *C. sativus* isolated from wheat. We selected 12 wheat cultivars or lines based on their differential responses to *C. sativus* isolates in previous studies (24,25,30,33,40–44). We assume that this could be the first step for identifying such differential wheat lines to determine virulence in *C. sativus*.

All 95 isolates of *C. sativus* were classified into low, intermediate, and high virulence. The majority (86%) of isolates had intermediate virulence. The findings of intermediate virulence of wheat–*C. sativus* interactions suggest that the resistance in the host to the pathogen may be complex and governed by minor genes. Alternatively, there could be the possibility of differences in the virulence factors in this fungus. Kumar et al. (24,25) reported several major and minor resistance loci in some resistant wheat lines. Identification and characterization of necrotrophic effectors in this fungus need to be investigated.

Table 5. Genetic diversity of *Cochliobolus sativus* populations from Hill and Plain areas in Nepal, and Central and Northern areas in North Dakota

Population	Sample size ^a	Clonal fraction ^b	<i>H</i> ^c	$\bar{r}_d$ ^d
Nepal				
Hill	24	0.00	0.10	0.075**
Plain	24	0.00	0.08	0.066**
Total	48	0.00	0.16	...
North Dakota				
Central	24	0.00	0.11	0.082**
Northern	24	0.00	0.16	0.292**
Total	48	0.00	0.14	...

^a Clone-corrected sample size.

^b Estimated as $1 - [(\text{number of different genotypes})/(\text{total number of isolates})]$ (19).

^c *H* = gene diversity (POPGENE software; 54) within populations was calculated from clone-corrected data.

^d Standardized index of association ($\bar{r}_d$) statistic was estimated for each population (2) from clone-corrected data. Significance of $\bar{r}_d$ was tested with 1,000 randomizations of the data by comparing the observed value with that expected under the null hypothesis of $\bar{r}_d = 0$. The null hypothesis of multilocus linkage equilibrium was rejected if $P \leq 0.01$ or 0.05; ** = significant at $P \leq 0.01$ level.

The presence of highly virulent isolates of *C. sativus* in Nepal and North Dakota has raised a serious question regarding the effectiveness of “durable” resistance to *C. sativus* in these regions. The ability of a given population of *C. sativus* to quickly overcome the resistance presently deployed in the field is evident from this study. This study showed that there is immense need of resistance sources that provide better resistance than is currently available in commercial cultivars. Similarly, higher overall disease severity caused by highly virulent isolates from Nepal suggests that the aggressiveness of *C. sativus* population is shifting toward more aggressive types. Several wheat lines have been tested for resistance to *C. sativus* across multi-environments in South Asia (40–44). Most of these resistant lines originated from Brazil, China, and Zambia (11). A few resistant lines have also been derived from crosses with *Aegilops tauschii* and tetraploid wheat (42). Most of the lines tested across multiple locations have not shown stable or consistent resistance to *C. sativus*; however, three of the wheat genotypes (SW 89-5422, Yangmai-6, and Ning 8201) were reported to have stable spot blotch resistance across 20 environments in Bangladesh, India, and Nepal (41). In another study, Milan Shanghai 7 exhibited a high level of disease resistance and grain yield across 12 different sites in Bangladesh, India, and Nepal (40). The majority of the resistant lines used in this study had shown some level of resistance to *C. sativus* in the field. However, none of the wheat differential lines were completely resistant to the isolates tested. It is possible that some of these resistant wheat lines may have the genes responsible for resistance to *C. sativus* at the adult plant stage and that the gene might remain ineffective at the seedling stage. Interestingly, we found 27% (26 of 95 isolates) of the isolates able to break down the resistance in 10 resistant wheat lines. We were able to detect a broad spectrum of *C. sativus* pathogenicity in this study. Therefore, the virulence groups which were able to cause disease in all of the tested wheat differential lines would be the best isolates for screening wheat germplasm to identify additional sources of resistance to spot blotch in wheat.

C. sativus has a high degree of morphological and phenotypic variation (4,17,23,46,48); however, little is known about the genetic variation in this pathogen (10). A high level of intraspecific genetic variability in *C. sativus* from Brazil and India has been reported using a random amplified polymorphic DNA (RAPD) marker (3,9). Baturo et al. (5) found low intraspecific differentiation in *C. sativus* populations collected over 15 years in different regions of Brazil using the same RAPD marker system. The present study provides insights into the genetic structure of *C. sativus* populations from two distinct geographic regions where different wheat genotypes are grown. The molecular diversity for both North Dakota (Northern and Central) and Nepal (Hill and Plain) was high, with 96 putative clones identified from 96 genotyped isolates. The genetic variations detected within individual isolates of *C. sativus* from North Dakota and Nepal populations were high (96 and 93%, respectively). In contrast, the genetic variation between Central and Northern populations from North Dakota was 4% and between Hill and Plain populations from Nepal was 7%, and they were also significant ($P < 0.001$).

Svobodova et al. (45) had shown a possible population differentiation among *C. sativus* populations collected from different regions of the Czech Republic using the AFLP marker system.

Table 6. Analysis of molecular variance (AMOVA) testing for genetic differentiation between Hill and Plain populations of *Cochliobolus sativus* from Nepal and Central and Northern populations of *C. sativus* from North Dakota, United States

AMOVA ^a	df	Variation (%)	<i>F</i> _{ST} ^b	<i>P</i>
Nepal				
Among Hill versus Plain populations	1	7	...	...
Within Hill versus Plain populations	46	93	0.072	0.000
North Dakota				
Among Central versus Northern populations	1	4	...	...
Within Central versus Northern populations	46	96	0.019	0.000

^a Genetic variation was grouped among Hill and Plain populations from Nepal and among Central and Northern populations from North Dakota.

^b *F*_{ST} = fixation index and the significance was determined by 1023 random permutations. Clone-corrected data were used for AMOVA. $P = 0.001$.

Similarly, Ruckstuhl (37) found population differentiation among South Asia, Mexico, and Bolivia *C. sativus* populations using RAPD markers. However, the result from this study contradicted the previous studies (37,45) because we did not find any significant population differentiation between North Dakota and Nepalese populations of *C. sativus*. Our results indicate that *C. sativus* populations from North Dakota (i.e., Northern versus Central) have very low population differentiation ($G'_{ST} = 0.02$). Similarly, *C. sativus* populations from Nepal (i.e., Hill versus Plain) also have low population differentiation ($G'_{ST} = 0.05$), and this result was also supported by Structure analysis. Thus, no evidence was found for population differentiation and structuring between *C. sativus* populations from North Dakota and Nepal. Most of the wheat planted in the Northern and Central areas in North Dakota are spring wheat habit and most wheat growers have a common seed source. Similarly, most of the wheat cultivars grown in Plain areas of Nepal are also grown in Hill areas. Because *C. sativus* is a seedborne pathogen (30), we hypothesize that migration of this fungus could have occurred from infected seed at the time of germplasm exchange between Northern and Central areas in North Dakota and, similarly, between Hill and Plain areas in Nepal. This information is useful for wheat breeders to deploy resistances in breeding programs in Nepal and North Dakota.

Our results showed significant ($P > 0.001$) LD for the Northern and Central populations in North Dakota and the Hill and Plain populations in Nepal. This suggests that sexual reproduction may play a less important role in disease epidemiology of this fungus. Our finding is in agreement with the previous study (45), which reported that recombination has a minor impact on the genetic structure of *C. sativus* populations. This is also true because the perfect stage of this fungus has been rarely observed in the field, and it has been reported only once from the field of Zambia (36). Svobodova et al. (45) observed significant LD in populations of *C. sativus* from Czech Republic, and our result was consistent with this previous study. The detection of significant LD values in *C. sativus* populations in North Dakota and Nepal is likely due to selection, migration of individuals or admixture of populations, and asexual reproduction.

The fungus reproducing asexually should result in high clonal populations and low genotypic diversity. Zhong and Steffenson (56) reported moderate allelic diversity in *C. sativus* populations from barley in North Dakota. Because the sexual phase of the life cycle of this fungus has not been found in the field, there should be other possible mechanisms for moderate to high genetic as well as pathogenic diversity detected in *C. sativus*. Some of the possible genetic mechanisms could be parasexual recombination (56), or translocations, chromosome deletions, and duplications as previously mentioned by Zhong and Steffenson (54).

In summary, this study demonstrated moderate to high pathogenic and genetic diversity of *C. sativus* populations collected from wheat in Nepal and North Dakota. The *C. sativus* isolates were classified into low, intermediate, and high virulence, and 47 distinct pathotypes were detected based on wheat cultivars or lines possessing different levels of resistance. Significant interactions between wheat differential lines and fungal isolates, and moderate population differentiation of *C. sativus*, suggest that available single genes for resistance to *C. sativus* are likely ineffective in diverse geographic areas. These virulent isolates of *C. sativus* identified in this study may overcome most of the resistant wheat genotypes that are commonly used in the current wheat breeding programs. There is immense need of wheat cultivars that have durable resistance to this fungus. To effectively manage this disease, wheat breeding programs should be initiated to pyramid different resistance genes in adapted wheat cultivars. In addition, identification of new sources of resistance to these virulent isolates of *C. sativus* from Nepal and North Dakota is warranted.

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Literature Cited

- Adhikari, T. B., Hansen, J. M., Gurung, S., and Bonman, J. M. 2011. Identification of new sources of resistance in winter wheat to multiple strains of *Xanthomonas translucens* pv. *undulosa*. *Plant Dis.* 95:582-588.
- Agapow, P. M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* 1:101-102.
- Aggarwal, R., Singh, V. B., Gurjar, M. S., Gupta, S., and Srinivas, P. 2009. Intraspecific variations in Indian isolates of *Bipolaris sorokiniana* infecting wheat based on morphological, pathogenic and molecular characters. *Indian Phytopathol.* 62:449-460.
- Arabi, M. I. E., and Jawhar, M. 2002. Virulence spectrum to barley (*Hordeum vulgare* L.) in some isolates of *Cochliobolus sativus* from Syria. *J. Plant Pathol.* 84:35-39.
- Baturo, A., Mehta, Y. R., and Sadowski, Cz. K. 2004. Identification of genetic variability in *Bipolaris sorokiniana* isolates from wheat in Brazil. *Summa Phytopathol.* 30:470-474.
- 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.
- Bouajila, A., Zoghalmi, N., Ahmed, M. A., Baum, M., Ghorbel, A., and Nazari, K. 2012. Pathogenicity spectra and screening for resistance in barley against Tunisian *Pyrenophora teres* f. *teres*. *Plant Dis.* 96:1569-1575.
- Conner, R. L., and Atkinson, T. G. 1989. Influence of continuous cropping on severity of common root rot in wheat and barley. *Can. J. Plant Pathol.* 11:127-132.
- de Oliveira, A. M. R., Matsumura, A. T. S., Prestes, A. M., and Van der Sand, S. T. 2002. Intraspecific variability of *Bipolaris sorokiniana* isolates determined by random amplified polymorphic DNA (RAPD). *Gen. Mol. Res.* 4:350-358.
- Dubin, H. J., and Rajaram, S. 1996. Breeding disease-resistant wheats for tropical highlands and lowlands. *Annu. Rev. Phytopathol.* 34:503-526.
- Duveiller, E. 2004. Controlling foliar blights of wheat in the rice-wheat systems of Asia. *Plant Dis.* 88:552-556.
- Duveiller, E., Kandel, Y. R., Sharma, R. C., and Shrestha, S. M. 2005. Epidemiology of foliar blights (spot blotch and tan spot) of wheat in the plains bordering the Himalayas. *Phytopathology* 95:248-256.
- Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. *Mol. Ecol.* 14:2611-2620.
- Excoffier, L., Laval, G., and Schneider, S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol. Bioinf.* 1:47-50.
- Excoffier, L., Smouse, P. E., and Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.
- Fetch, T. C., and Steffenson, B. J. 1999. Rating scales for assessing infection responses of barley infected with *Cochliobolus sativus*. *Plant Dis.* 83:213-217.
- Ghazvini, H., and Tekauz, A. 2007. Virulence diversity in the population of *Bipolaris sorokiniana*. *Plant Dis.* 91:814-821.
- Ghazvini, H., and Tekauz, A. 2008. Host-pathogen interactions among barley genotypes and *Bipolaris sorokiniana* isolates. *Plant Dis.* 92:225-233.
- Gurung, S., Goodwin, S. B., Kabbage, M., Bockus, W. W., and Adhikari, T. B. 2011. Genetic differentiation at microsatellite loci among populations of *Mycosphaerella graminicola* from California, Indiana, Kansas, and North Dakota. *Phytopathology* 101:1251-1259.
- Hedrick, P. W. 2005. A standardized genetic differentiation measure. *Evolution* 59:1633-1638.
- Ibeagha, A. E., Huckelhoven, R., Schafer, P., Singh, D. P., and Kogel, K.-H. 2005. Model wheat genotypes as tools to uncover effective defense mechanisms against the hemibiotrophic fungus *Bipolaris sorokiniana*. *Phytopathology* 95:528-532.
- Joshi, A. K., Ortiz-Ferrara, G., Crossa, J., Singh, G., Sharma, R. C., Chand, R., and Parsad, R. 2007. Combining superior agronomic performance and terminal heat tolerance with resistance to spot blotch (*Bipolaris sorokiniana*) of wheat in the warm humid Gangetic Plains of South Asia. *Field Crops Res.* 103:53-61.
- Knight, N. L., Platz, G. J., Lehmensiek, A., and Sutherland, M. W. 2010. An investigation of genetic variation among Australian isolates of *Bipolaris sorokiniana* from different cereal tissues and comparison of their abilities to cause spot blotch on barley. *Australas. Plant Pathol.* 39:207-216.
- Kumar, U., Joshi, A. K., Kumar, S., Chand, R., and Roder, M. S. 2009. Mapping of resistance to spot blotch caused by *Bipolaris sorokiniana* in spring wheat. *Theor. Appl. Genet.* 118:783-793.
- Kumar, U., Joshi, A. K., Kumar, S., Chand, R., and Roder, M. S. 2010. Quantitative trait loci for resistance to spot blotch caused by *Bipolaris sorokiniana*.

- kiniana* in wheat (*T. aestivum* L.) lines 'Ning 8201' and 'Chirya 3'. Mol. Breeding 26:477-491.
26. Ledingham, R. J., Atkinson, T. G., Horricks, J. S., Mills, J. T., Piening, L. J., and Tinline, R. D. 1973. Wheat losses due to common root rot in the prairies of Canada, 1969-71. Can. Plant Dis. Surv. 53:113-122.
 27. Limpert, E., and Muller, K. 1994. Designation of pathotypes of plant pathogens. J. Phytopathol. 140:346-358.
 28. Liu, Z. H., Zhong, S., Stasko, A. K., Edwards, M. C., and Friesen, T. L. 2012. Virulence profile and genetic structure of a North Dakota population of *Pyrenophora teres* f. *teres*, the causal agent of net form net blotch of barley. Phytopathology 102:539-546.
 29. Mahto, B. N., Gurung, S., and Adhikari, T. B. 2010. Morphological and pathogenic variability of *Cochliobolus sativus* from Nepal. (Abstr.) Phytopathology 100:S76.
 30. Mahto, B. N., Gurung, S., and Adhikari, T. B. 2011. Assessing genetic resistance to spot blotch, *Stagonospora nodorum* blotch and tan spot in wheat from Nepal. Eur. J. Plant Pathol. 131:249-260.
 31. Meirmans, P. G., and Hedrick, P. W. 2011. Assessing population structure: *Fst* and related measures. Mol. Ecol. Resour. 11:5-18.
 32. Neupane, A. C., Sharma, R. C., Duveiller, E., and Shrestha, S. M. 2010. Sources of *Cochliobolus sativus* inoculum causing spot blotch under warm wheat growing conditions in South Asia. Cereal Res. Commun. 38:541-549.
 33. Neupane, R. B., Sharma, R. C., Duveiller, E., Ortiz-Ferrara, G., Ojha, B. R., Rosyara, U. R., Bhandari, D., and Bhatta, M. R. 2009. Major gene controls of field resistance to spot blotch in wheat genotypes 'Milan/Shanghai #7' and 'Chirya 3.' Plant Dis. 91:692-697.
 34. Okuyama, L. A., Federizzi, L. C., and Neto, J. F. B. 2005. Grain yield stability of wheat genotypes under irrigated and non-irrigated conditions. Brazilian Arch. Biol. Technol. 48:697-704.
 35. Pritchard, J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.
 36. Raemaekers, R. H. 1991. Revised Checklist of Plant Diseases in Zambia. Belgian Development Cooperation Project, Chilanga, Zambia.
 37. Ruckstuhl, M. 1998. *Bipolaris sorokiniana*: studies on its epidemiology and population structure in the rice-wheat cropping pattern of Nepal. Pages 88-106 in: Helminthosporium Blights of Wheat: Spot Blotch and Tan Spot. E. Duveiller, H. J. Dubin, J. Reeves, and A. McNab, eds. International Maize and Wheat Improvement Center, CIMMYT, El Batan, Mexico, DF.
 38. SAS Institute Inc. 2010. SAS OnlineDoc, Version 9.2, SAS Institute, Cary, NC.
 39. Shah, D. A., and Madden, L. V. 2004. Nonparametric analysis of ordinal data in designed factorial experiments. Phytopathology 94:33-43.
 40. Sharma, R. C., and Duveiller, E. 2007. Advancement toward new spot blotch resistant wheats in South Asia. Crop Sci. 47:961-968.
 41. Sharma, R. C., Duveiller, E., Ahmed, F., Arun, B., Bhandari, D., Bhatta, M. R., Chand, R., Chaurasiya, P. C. P., Gharti, D. B., Hossain, M. H., Joshi, A. K., Mahto, B. N., Malaker, P. K., Reza, M. A., Rahman, M., Samad, M. A., Shaheed, M. A., Siddique, A. B., Singh, A. K., Singh, K. P., Singh, R. N., and Singh, S. P. 2004. Helminthosporium leaf blight resistance and agronomic performance of wheat genotypes across warm regions of South Asia. Plant Breed. 123:520-524.
 42. Sharma, R. C., Duveiller, E., and Ortiz-Ferrara, G. 2007. Progress and challenge towards reducing wheat spot blotch threat in the Eastern Gangetic Plains of South Asia: is climate change already taking its toll? Field Crops Res. 103:109-118.
 43. Sharma, R. C., Pandey-Chhetri, B., and Duveiller, E. 2006. Heritability estimates of spot blotch resistance and its association with other traits in spring wheat crosses. Euphytica 147:317-327.
 44. Siddique, A. B., Hossain, M. H., Duveiller, E., and Sharma, R. C. 2006. Progress in wheat resistance to spot blotch in Bangladesh. J. Phytopathol. 154:16-22.
 45. Svobodova, L. L., Minarikova, V., Kucera, L., and Pereyra, S. A. 2011. Structure of the *Cochliobolus sativus* population variability. Plant Pathol. Online publication. doi:10.1111/j.1365-3059.2011.02547.x
 46. Tamang, P., and Stein, J. 2011. Collection of *Cochliobolus sativus* isolates and assessment of virulence pattern. (Abstr.) Phytopathology. 101:S2.8.
 47. USDA. 2009. Agricultural Statistics. United States Department of Agriculture National Agricultural Statistics Service, United States Government Printing Office, Washington, DC.
 48. Valjavec-Gratian, M., and Steffenson, B. J. 1997. Pathotypes of *Cochliobolus sativus* on barley in North Dakota. Plant Dis. 81:1275-1278.
 49. VanderPlank J. E. 1968. Disease Resistance in Plants. Academic, New York. (2nd ed. 1984. Academic, Orlando, FL.)
 50. Vos, P., Hogers, R., Bleeker, M., Reijmans, 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.
 51. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to the Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
 52. Wildermuth, G. B. 1986. Geographic distribution of common root rot and *Bipolaris sorokiniana* in Queensland wheat soils. Aust. J. Exp. Agric. 26:601-606.
 53. Windels, C. E., Lamb, J. A., and Cymaluk, T. E. 1992. Common root rot and yield responses in spring wheat from chloride application to soil in northwestern Minnesota. Plant Dis. 76:908-911.
 54. Yeh, F. C., Yang, R. C., Boyle, T. B. J., Ye, Z. H., and Mao, J. X. 1997. POPGENE, the User Friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada.
 55. Zadoks, J. C., Chang, T. T., and Konzak, C.F. 1974. A decimal code for the growth stages of cereals. Weed Res. 14:415-21.
 56. Zhong, S., and Steffenson, B. J. 2001. Virulence and molecular diversity of *Cochliobolus sativus*. Phytopathology 91:469-476.