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Virulence and Molecular Diversity in the *Cochliobolus sativus* Population Causing Barley Spot Blotch in China

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Abstract

Spot blotch, caused by the fungal pathogen *Cochliobolus sativus*, is a limiting factor for barley (*Hordeum vulgare*) production in northeast China, which causes significant grain yield losses and kernel quality degradation. It is critical to determine the virulence diversity of *C. sativus* populations for barley resistance breeding and the judicious grouping of available resistance varieties according to the predominant pathotypes in disease epidemic regions. With little information on the barley pathogen in China, this study selected 12 typical barley genotypes to differentiate the pathotypes of *C. sativus* isolates collected in China. Seventy-one isolates were grouped into 19 Chinese pathotypes based on infection responses. Seventeen isolates were classified as pathotype 3, which has only been identified in China, whereas most (52 of 71) were classified as pathotype 1. All of the tested isolates had low virulence on the North Dakota (ND) durable, resistant line ND B112. Using 22 selected amplified fragment-length polymorphism (AFLP) primer combinations, genetic polymorphism was used to analyze 68 isolates, which clustered into three distinct groups using the unweighted pair group method average with the genetic distance coefficient. No relationship was found between the virulence of isolates and their origins. Isolates of the same pathotype or those collected from the same location did not group into clusters based on the AFLP analysis.

Keywords: *Bipolaris sorokiniana*, differential genotype, genetic diversity, virulence variation

Barley (*Hordeum vulgare* L) is the fourth most important cereal crop following maize (*Zea mays* L), rice (*Oryza sativa* L), and wheat (*Triticum aestivum* L) in terms of planted acreage and grain yield in China. Barley acreage is mainly in semiarid areas with higher altitudes in northeast and northwest China and the Qinghai-Tibet Plateau as well as small areas in the Jiangsu and Zhejiang Provinces of east China. Barley is used mainly as animal feed, and the raw material is used for brewing beer. In the Qinghai-Tibet Plateau and some regions of Yunnan Province in the southwest region, hull-less barley (*H. vulgare* L var. *nudum*) is the only staple crop for local people. In northeast China, cultivation of six-rowed barley has a long history. Since the 1990s, frequent epidemics of spot blotch—caused by *Bipolaris sorokiniana* (Sacc) Shoemaker (teleomorph *Cochliobolus sativus* [S Ito & Kurib] Drechsler ex Dastur)—have occurred in the eastern regions of Inner Mongolia and Heilongjiang, the major brewing barley production regions. Spot blotch caused remarkable yield losses from 2011 to 2013 in Hailar, Inner Mongolia. The major reason for the disease epidemic was the introduction of a susceptible barley cultivar Kenpimai 7 from Heilongjiang in 2004 and the subsequent buildup of infected crop residues in the fields. Indeed, most of the seeds that were sown in the field were not treated with fungicides. In addition, the prevalence of barley common root rot and black point caused by *C. sativus* increases with cool, rainy conditions at the seedling stage and high temperature and relative humidity at flowering (Kumar et al. 2002). During the disease epidemics, susceptible barley cultivars had yield losses of 20 to 30% owing to spot blotch or up to 50% in some fields according to local farmer estimates. Spot blotch is regarded as the most devastating foliar disease of barley cultivation in northeast China.

Barley spot blotch is prevalent in Asia, North America, Latin America, the Middle East, and North Africa, and it causes up to 30% yield losses in susceptible barley cultivars (Clark 1979). In 2002, Canada reported an epidemic of spot blotch in barley (Tekauz et al. 2003). Pathogenic variability exists among *C. sativus* populations (Ghazvini and Tekauz 2007; Gurung et al. 2013; Knight et al. 2010; Zhong and Steffenson 2001). To develop cultivars resistant to dominant local populations of the pathogen, the virulence patterns of the pathogen need to be determined. Three pathotypes (0, 1, and 2) of *C. sativus* were identified on differential barley cultivars (North Dakota [ND] 5883, Bowman, and ND B112) from isolates mostly collected from North Dakota, U.S.A. (Valjavec-Gratian and Steffenson 1997b). New *C. sativus* pathotypes with virulence on ND B112 and Bowman—designated virulence groups 7.7.5.1, 7.7.5.4, 7.7.7.5, and 6.3.5.0—were identified in Manitoba, Canada (Ghazvini and Tekauz 2007). In Longdon, North Dakota, an isolate ND 4008—designated pathotype 7— was identified from a barley root sample, which exhibited high virulence on ND 5883, Bowman, and ND B112 (Gyawali 2010). However, ND B112 has remained the primary source of resistance to *C. sativus* for >50 years (Steffenson et al. 1996), and it maintains effectiveness for most *C. sativus* pathotypes worldwide. All of the six-rowed malting cultivars released in the Upper Midwest of the U.S.A. since 1964 carry ND B112 resistance and remain highly resistant to spot blotch (Roy et al. 2010). Differential barley genotypes have been analyzed for virulence variation among *C. sativus* populations in disease epidemic regions. In Australia, 12 differential barley cultivars (ND B112, Bowman, Klaxon/Tallon-34, Stirling, Gilbert, CI 1227, CI 6311, ND 5883, Klaxon/Tallon-45, Lindwall, Lofa Abed, and Skiff) were used to identify six pathotypes of *C. sativus* among 34 isolates (Meldrum et al. 2004). The virulence diversity of 127 *C. sativus* isolates from Canada and other countries was evaluated on 12 differential barley cultivars (AC Metcalfe, Bowman, CDC Bold, CDC Stratus,

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Conlon, ND 5883, ND B112, Newdale, Robust, Stander, TR 251, and TR 261), with eight pathotypes identified (Ghazvini and Tekauz 2007). Three virulence groups from *C. sativus* isolates collected in Uruguay were identified from the infection responses (IRs) of 20 Uruguayan barley genotypes (Gamba and Estramill 2002). A survey of IRs of 31 Syrian *C. sativus* isolates using 13 differential barley cultivars (AECS 83, AECS 76, AECS 71, Arabi Abiad, Furat-2, Arizona, Arrivate, CI-5791, Golf, Thibaut, Selina, WI 2291, and Smash) identified four pathotypes (Arabi and Jawhar 2003). Because different differential barley genotypes were used to analyze the virulence among *C. sativus* populations in the above countries, it is not possible to compare the pathotype designations according to the coded triplet nomenclature system. Fortunately, three genotypes—ND 5883, Bowman, and ND B112—were common components of the sets of differential barley genotypes used in Canada, the U.S.A., and Australia, providing limited information for cross-analysis of virulence variation among *C. sativus* populations from these epidemic regions.

The high level of virulence variability in *C. sativus* populations was evaluated directly from the IRs of differential barley genotypes. Under natural conditions, *C. sativus* virulence is vulnerable to selection pressure from disease-resistant host barley cultivars. Genetic variation in the pathogen is mainly responsible for its virulence diversity and various pathotypes. Also, heterokaryosis and parasexuality are considered major factors for genetic variability in *C. sativus* pathotypes (Glass et al. 2000; Tinline 1962). An investigation of the genetic variation among Australian isolates of *C. sativus* from different cereal tissues using the amplified fragment-length polymorphism (AFLP) identified that isolates collected from spot blotch infections generally clustered separately from those from common root rot infections (Knight et al. 2010). Molecular diversity in *C. sativus* isolates from North America and other countries has also been analyzed. The pathogen isolates had a high level of genetic variability, but molecular cluster analysis did not reveal a close correlation between pathogen pathotypes and AFLP groups (Ghazvini and Tekauz 2012; Zhong and Steffenson 2001). These results offer genetic clues for analyzing virulence variation and host specificity of the pathogen.

Spot blotch of barley has broken out several times in northeast China when weather conditions during the growing season were suitable. However, there are no special spot blotch resistance breeding programs or reports on virulence variability in the *C. sativus* population in China. This study aimed to analyze the virulence variation and genetic diversity of *C. sativus* isolates collected from major malting barley areas in northeast and northwest China and determine the distribution of predominant pathotypes. A collection of barley varieties with known spot blotch resistance levels along with widely grown cultivars and core barley breeding parental lines was assessed for resistance to spot blotch to identify a set of differential barley genotypes. The characterization of virulence variation of *C. sativus* will benefit resistance breeding and the application of barley resistance sources.

Materials and Methods

Collection of fungal isolates. Seventy-one isolates of *C. sativus* were collected from 2012 to 2015, including 43 from Inner

Fig. 1. Geographic distribution of *Cochliobolus sativus* pathotypes identified from 71 isolates of barley spot blotch collected in China: Jinchang and Zhangye of Gansu Province in northwest China; Beijing and Langfang of Hebei Province and Hohhot of Inner Mongolia in north China; Hailar of Inner Mongolia; and Harbin, Jiamusi, Heihe, and Bei'an of Heilongjiang Province in northeast China. Numbers in parentheses denote the numbers of isolates collected from a location and the corresponding pathotype numbers.

Mongolia, 20 from Heilongjiang, four from Gansu, two from Beijing, and two from Hebei (Fig. 1 and Table 1). All of the isolates were obtained from diseased barley leaf tissue with typical spot blotch symptoms. Tissue sections cut from a putative spot blotch lesion were surface sterilized and incubated on a petri dish containing potato dextrose agar (PDA) for 2 to 3 days at 21°C. The growth medium of a culture around a leaf tissue specimen was transferred to a new PDA plate with sterilized 0.5 × 0.5-cm pieces of Whatman filter paper for 6 to 7 days until they were covered with mycelia. After morphological identification of pathogen conidia under a microscope, the filter paper pieces with mycelium and spores of an isolate were collected as an original stock and stored at –20°C after drying. The isolate stock was incubated for 6 to 7 days to induce fungal sporulation, and the conidia were then used to establish single-spore isolations and stored as described above. Surface sterilization and growing conditions for the conidial cultures were conducted following the protocols described by Fetch and Steffenson (1994), except that PDA medium was used instead of yeast peptone soluble starch agar.

Differential barley genotypes. The spot blotch resistance levels of a collection of 77 barley genotypes, mostly Chinese cultivars and varieties or parent lines with different disease resistance levels to *C. sativus* isolates collected from China, were evaluated at the seedling stage using 21 isolates randomly selected from Table 1. The triticale line H 1890 was used as a resistant control for spot blotch. Seventeen candidate genotypes were selected according to their resistance spectra and genetic origins (Supplementary Fig. S1 and Supplementary Table S1). Combined with three American differential genotypes (ND 5883, Bowman, and ND B112) from the University of Minnesota, the 20 candidate barley genotypes (Table 2) were further inoculated with 55 isolates. Six-rowed barley cultivar Mirco and a triticale line H 1890 were used as susceptible and resistant controls to spot blotch, respectively.

Preparation of host plants. Barley genotypes were seeded about 2 weeks before inoculation, with four lines grown as clumps of 8 to 12 seeds per 15-cm-diameter clay pot filled with a mixture of sieved soil, turf soil, and vermiculite in a 4:1:1 ratio. Nitrogen-based fertilizer (2.0 g per pot) was mixed into the soil in each pot just before planting. Plants were grown in a controlled cabinet at 21 ± 1/19 ± 1°C day/night, with a 16-h photoperiod under 6,000 lux.

Inoculum preparation. The inoculum was prepared by transferring one or two pieces of filter paper stock with conidia of *C. sativus* onto 9-cm petri plates containing PDA medium and incubating for 7 to 8 days for conidia production. To test the virulence of different fungal isolates, conidial suspensions (1×10^4 conidia per 1 ml) in 0.05% vol/vol Tween-20 were used for greenhouse inoculation. The individual inoculum was prepared as described by Fetch and Steffenson (1994).

Inoculation and disease assessment. Seedlings were inoculated with the conidial suspension at the two-leaf stage (12 to 14 days old) for resistance evaluation. The inoculation method is detailed in Fetch and Steffenson (1999). After inoculation, seedlings were incubated in dark chambers at 21 ± 1°C with 100% relative humidity for 24 h. Plants were then returned to the greenhouse under the same conditions as those for seedling cultivation; 10 to 12 days after inoculation, the second leaves of 8 to 12 inoculated seedlings of each candidate genotype were assessed for IRs using the 0 to 9 rating scale

Table 1. Isolates of *Cochliobolus sativus* from infected barley leaf tissue with typical spot blotch collected in China for virulence and genetic diversity analyses

Isolate	Geographic origin	Year collected	Host/tissue
Z 12004	Hailar, Inner Mongolia	2012	Barley/leaf
Z 12007	Hailar, Inner Mongolia	2012	Barley/leaf
Z 12010	Hailar, Inner Mongolia	2012	Barley/leaf
Z 12011	Hailar, Inner Mongolia	2012	Barley/leaf
Z 12014	Hailar, Inner Mongolia	2012	Barley/leaf
Z 12028	Harbin, Heilongjiang	2012	Barley/leaf
Z 13001	Harbin, Heilongjiang	2013	Barley/leaf
Z 13004	Harbin, Heilongjiang	2013	Barley/leaf
Z 13005	Harbin, Heilongjiang	2013	Barley/leaf
Z 13006	Harbin, Heilongjiang	2013	Barley/leaf
Z 13010	Harbin, Heilongjiang	2013	Barley/leaf
Z 13011	Harbin, Heilongjiang	2013	Barley/leaf
Z 13012	Harbin, Heilongjiang	2013	Barley/leaf
Z 13013	Harbin, Heilongjiang	2013	Barley/leaf
Z 13015	Bei'an, Heilongjiang	2013	Barley/leaf
Z 13016	Heihe, Heilongjiang	2013	Barley/leaf
Z 13017	Hailar, Inner Mongolia	2013	Barley/leaf
Z 13020	Hailar, Inner Mongolia	2013	Barley/leaf
Z 13022	Hailar, Inner Mongolia	2013	Barley/leaf
Z 13024	Hailar, Inner Mongolia	2013	Barley/leaf
Z 13027	Hailar, Inner Mongolia	2013	Barley/leaf
Z 13036	Hohhot, Inner Mongolia	2013	Barley/leaf
Z 14200	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14207	Hohhot, Inner Mongolia	2014	Barley/leaf
Z 14361	Hohhot, Inner Mongolia	2014	Barley/leaf
Z 14362	Hohhot, Inner Mongolia	2014	Barley/leaf
Z 14372	Beijing	2014	Barley/leaf
Z 14382	Beijing	2014	Barley/leaf
Z 14386	Langfang, Hebei	2014	Barley/leaf
Z 14474	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14476	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14479	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14480	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14481	Heihe, Heilongjiang	2014	Barley/leaf
Z 14482	Jiamusi, Heilongjiang	2014	Barley/leaf
Z 14483	Heihe, Heilongjiang	2014	Barley/leaf
Z 14484	Jiamusi, Heilongjiang	2014	Barley/leaf
Z 14485	Jiamusi, Heilongjiang	2014	Barley/leaf
Z 14486	Jiamusi, Heilongjiang	2014	Barley/leaf
Z 14487	Jiamusi, Heilongjiang	2014	Barley/leaf
Z 14489	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14490	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14491	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14492	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14494	Hailar, Inner Mongolia	2014	Barley/leaf
Z 14495	Jinchang, Gansu	2014	Barley/leaf
Z 14496	Zhangye, Gansu	2014	Barley/leaf
Z 14497	Langfang, Hebei	2014	Barley/leaf
Z 15418	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15419	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15420	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15515	Jinchang, Gansu	2015	Barley/leaf
Z 15521	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15525	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15530	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15534	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15538	Jinchang, Gansu	2015	Barley/leaf
Z 15568	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15571	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15580	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15600	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15657	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15658	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15660	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15661	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15663	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15670	Hailar, Inner Mongolia	2015	Barley/leaf
Z 15687	Harbin, Heilongjiang	2015	Barley/leaf
Z 15688	Harbin, Heilongjiang	2015	Barley/leaf
Z 15691	Hohhot, Inner Mongolia	2015	Barley/leaf
Z 15692	Hohhot, Inner Mongolia	2015	Barley/leaf

developed by Fetch and Steffenson (1994, 1999), with 0 to 5 being resistant and 6 to 9 being susceptible. The barley genotypes were inoculated in two separate experiments to determine their resistance, and SPSS 16.0 (SPSS Inc.; https://spss.en.softonic.com/) software was used to determine significant differences by one-way analysis of variance (ANOVA), taking the $P < 0.05$ level as significant according to Duncan's multiple range test between two replicates. The mean IR scores were calculated by averaging IR scores across two separate replicates.

Pathotype designation. The designation of pathotypes was according to the coded triplet nomenclature system (Limpert and Müller 1994), and *C. sativus* isolates were described as per Valjavec-Gratian and Steffenson (1997b). Host differentials were ordered in subsets of three genotypes from low- to high-resistance levels. Binary values of each component of a subset were further converted to decimal values that were then summed to derive a single number for each subset.

Stability test of IRs to temperature. To test the stability of IRs of the 12 differential barley cultivars (ND 5883, Zaoshu 3, Kenpimai 7, Morex, Varunda, Bowman, Tradition, Mengpimai 3, Kenpimai 9, 10PJ-24, Kenpimai 11, and ND B112) to *C. sativus* under different temperature conditions during the disease latent period, the barley genotypes were inoculated with three isolates (Z 13027, Z 14486, and Z 15525) at the two-leaf stage. After incubation in dark chambers for 24 h at 21°C and 100% relative humidity, the plants were transferred to growth chambers set at 18, 21, and 25°C, respectively. Ten seedlings were scored for every genotype inoculated with an isolate. The experiment was replicated three times. Inoculation, disease assessment, and rating scales were the same as described above. The mean IR value of a variety at a latent temperature was calculated by averaging IR values after inoculating with an isolate. There were three independent biological replicates for each isolate used. SPSS 16.0 (SPSS Inc.; https://spss.en.softonic.com/) software was used to determine significant differences by one-way ANOVA, taking the $P < 0.05$ level as significant according to Duncan's multiple range test between infection types of a differential genotype at different latent temperatures after inoculation.

Cluster for virulence of isolate and resistance of barley genotypes. The IRs of the 12 barley genotypes to each of the *C. sativus* isolates tested are illustrated in a resistant/susceptible matrix (Table 3). The reaction of each isolate to a differential host component was described as zero for high virulence (IRs from 6 to 9 for susceptible responses) and one for low virulence (IRs from 0 to 5 for resistant responses) for cluster analysis. Hierarchical cluster analysis was performed using the SAHN program in the NTSYS-pc software (version 2.2; Exeter Software) to evaluate the relationships among isolates of *C. sativus* in each pathotype. To compare resistance pattern similarity to spot blotch among the candidate barley genotypes, a matrix of the IRs of 20 barley genotypes and a resistant triticale control to each of the 55 *C. sativus* isolates was constructed, and the hierarchical cluster analysis method was the same as that used for the *C. sativus* isolates analysis. Cluster analysis was performed using the one and zero values for resistant/susceptible responses of barley genotypes to each isolate.

Genomic DNA extraction and quantification. To extract fungal genomic DNA, each isolate was grown on PDA plates. When mycelia grew for 7 to 10 d, the petri dishes were covered with two layers of sterilized medical gauze and placed on a well-ventilated bench for 2 days at room temperature to dry the fungal mycelia. The mycelia were then gently scraped directly with a sterilized blade. Harvested mycelia were placed in a 2-ml centrifuge tube and stored at –80°C. The mycelia were ground to fine powder using a high-speed mixer mill (Model TL2020; DHS). Genomic DNA was isolated using a revised cetyltriethylammnonium bromide (CTAB) extraction procedure (Gawel and Jarret 1991). Briefly, 0.65 ml preheated DNA isolation buffer (100 mM Tris-HCl at pH 8.0, 20 mM EDTA, 2% wt/vol CTAB, 1.4 M NaCl, and 2% vol/vol mercaptoethanol) was added and incubated at 65°C for 30 min. An equal amount of chloroform and Tris-Phenol (1:1; vol/vol) was added, and the contents in the tube were gently stirred for 5 min before centrifuging at 12,000 rpm for 5 min at room temperature. The aqueous phase containing DNA was transferred to a new 2-ml tube. Finally, 1 ml of absolute ethyl alcohol was added, keeping the contents at 4°C for about 1 h before centrifuging at 12,000 rpm for 15 min to reveal the precipitate. The precipitate was rinsed with 0.5 ml of 70% ethanol and centrifuged at 12,000 rpm for 2 min. The DNA was then dissolved in 100 μl double distilled water with 10 mg/ml RNAse, incubated for 1 h at 37°C, and stored at 4°C until needed. The DNA samples were quantified using a NanoDrop (Model ND-1000; Thermo Scientific Inc.) and adjusted to 500 ng/μl for the AFLP analysis.

Table 2. Typical barley varieties and lines used for selecting differential genotypes to assess virulence variability of *Cochliobolus sativus* isolates from China

Genotype	Pedigree	Two or six rowed	Growth habit	Geographic origin
Ganpi 2	Medusa/Diamant//Frallf"S"	2	Spring	China
Mengpimai 1	Bowman/91 Dong 27//91G318	2	Spring	China
Mengpimai 3	Guopin11/Gienm	6	Spring	China
Kenpimai 7	TR212//Proctor/Prior A/3/Ant90-2	2	Spring	China
Kenpimai 9	Azare/Hazen//Robust/Azare/3/Azare/Hazen	6	Spring	China
Kenpimai 11	02SK046/He5232	2	Spring	China
10PJ-24	Hunxuan 2/Logan	2	Spring	China
09GW-07	–	2	Spring	China
Zaoshu 3	Selection from cultivar Kando Nijo 3	2	Spring	Japan
Mazurka	Hijlkema 1148/Heine 4808	2	Spring	The Netherlands
Varunda	Vada/Hijlkema 1148	2	Spring	The Netherlands
Mirco[a]	–	6	Winter	Italy
Golden Promise	Gamma-ray mutant of Maythorpe	2	Spring	England, United Kingdom
Tradition	B1603/3/Robust//M74-10/ND2670/4/Hazen/ 3/ND5570//Glen/Karl	6	Spring	U.S.A.
Legacy	6B86-3517/4/Cree/Bonanza//Manker/3/ 2*Robust	6	Spring	U.S.A.
Steptoe	WA3564/Unitan	6	Spring	U.S.A.
Morex	Cree/Bonanza	6	Spring	U.S.A.
ND 5883[b]	Clipper/702-10	2	Spring	U.S.A.
Bowman	Klages//Fergus/Nordic/3/ND1156/4/Hector	2	Spring	U.S.A.
ND B112[b]	CI 7117-77/Kindred	6	Spring	U.S.A.
Triticale[c]	–	–	Winter	China

[a] Barley cultivar Mirco was used as a susceptible control for spot blotch.
[b] ND, North Dakota.
[c] Triticale line H 1890 was used as a resistant control for spot blotch.

Table 3. The infection responses of 71 isolates of *Cochliobolus sativus* on differential barley genotypes

	Infection response[a]														
Isolate	ND 5883	Zaoshu 3	Kenpimai 7	Morex	Varunda	Bowman	Tradition	Mengpimai 3	Kenpimai 9	10PJ-24	Kenpimai 11	ND B112	Code[b]	Pathotype in ND[c]	Pathotype in China[d]
Z 14382	R	S	R	R	R	R	R	R	R	R	R	R	2.0.0.0	0	1
Z 14474	R	S	R	R	R	R	R	R	R	R	R	R	2.0.0.0	0	1
Z 14482	S	R	R	R	R	R	R	S	S	R	R	R	1.0.6.0	1	2
Z 12004	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 14372	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 14489	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 15515	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 15580	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 15568	S	S	R	R	R	R	R	R	R	R	R	R	3.0.0.0	1	3
Z 13011	S	S	R	S	R	R	R	R	R	R	R	R	3.1.0.0	1	4
Z 13013	S	S	R	S	R	R	R	R	R	R	R	R	3.1.0.0	1	4
Z 14485	S	S	R	S	R	R	R	R	R	R	R	R	3.1.0.0	1	4
Z 15687	S	S	R	S	R	R	R	R	R	R	R	R	3.1.0.0	1	4
Z 15688	S	S	R	S	R	R	R	R	R	R	R	R	3.1.0.0	1	4
Z 13005	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 14491	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 14492	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15419	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15420	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15657	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15600	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15661	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 15663	S	S	R	S	S	R	S	R	R	R	R	R	3.3.1.0	1	5
Z 12007	S	S	R	R	R	S	R	R	R	R	R	R	3.4.0.0	3	6
Z 13024	S	S	R	R	R	S	R	R	R	R	R	R	3.4.0.0	3	6
Z 15534	S	S	R	R	R	S	R	R	S	R	R	R	3.4.4.0	3	7
Z 12011	S	S	R	R	R	S	R	R	S	S	R	R	3.4.4.1	3	8
Z 14486	S	S	R	R	R	S	R	R	S	S	R	R	3.4.4.1	3	8
Z 15660	S	S	R	R	R	S	R	R	S	S	R	R	3.4.4.1	3	8
Z 13022	S	S	R	S	R	S	R	R	R	R	R	R	3.5.0.0	3	9
Z 14490	S	S	R	S	R	S	R	R	R	R	R	R	3.5.0.0	3	9
Z 15571	S	S	R	S	R	S	R	R	R	R	R	R	3.5.0.0	3	9
Z 13020	S	R	S	S	R	R	R	R	S	R	R	R	5.1.4.0	1	10
Z 14487	S	R	S	R	S	S	R	S	R	R	R	R	5.6.2.0	3	11
Z 14483	S	R	S	R	S	S	R	R	S	R	R	R	5.6.4.0	3	12
Z 13006	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 13012	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 14386	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 14495	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 14496	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 14497	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 15538	S	S	S	R	R	R	R	R	R	R	R	R	7.0.0.0	1	13
Z 13001	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 13010	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 13016	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 13017	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 14200	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 14480	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 14494	S	S	S	R	S	R	R	R	R	R	R	R	7.2.0.0	1	14
Z 12010	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 13015	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 13036	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 14476	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 15521	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 15418	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 15670	S	S	S	S	S	R	R	R	R	R	R	R	7.3.0.0	1	15
Z 14207	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 14361	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 14362	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 14484	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 15658	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 15691	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 15692	S	S	S	S	S	R	R	S	R	R	R	R	7.3.2.0	1	16
Z 12014	S	S	S	R	S	S	R	R	R	S	R	R	7.6.0.1	3	17
Z 13027	S	S	S	R	S	S	R	R	R	S	R	R	7.6.0.1	3	17
Z 14481	S	S	S	R	S	S	R	R	R	S	R	R	7.6.0.1	3	17
Z 15530	S	S	S	R	S	S	R	R	R	S	R	R	7.6.0.1	3	17
Z 12028	S	S	S	S	R	R	S	S	S	S	R	R	7.1.7.1	1	18
Z 13004	S	S	S	S	R	R	S	S	S	S	R	R	7.1.7.1	1	18
Z 14479	S	S	S	S	R	S	S	S	S	R	S	R	7.5.7.2	3	19
Z 15525	S	S	S	S	R	S	S	S	S	R	S	R	7.5.7.2	3	19

[a] All infection responses were observed on 8 to 12 seedlings of a differential genotype over two independent experiments based on the 0 to 9 scale of Fetch and Steffenson (1994, 1999). With this rating scale, infection response scores from 0 to 5 are resistant (R), and those from 6 to 9 are susceptible (S) (Valjavec-Gratian and Steffenson 1997a, b).

[b] Coded triplet nomenclature system of Limpert and Müller (1994).

[c] Pathotype in North Dakota (ND), U.S.A. was designated according to the infection responses on the differential genotypes of ND 5883, Bowman, and ND B112.

[d] Pathotype in China was based on the infection responses on the 12 differential lines selected in this study.

AFLP analysis. Genetic diversity among the *C. sativus* isolates was analyzed using the AFLP method. DNA amplification was conducted as described by Vos et al. (1995). Genomic DNA (500 ng/μl) of each isolate was digested with *Eco*R I and *Mse* I at 37°C for 3 h and deactivated at 80°C for 20 min; the restricted DNA fragments were then ligated to AFLP adaptors at 37°C for 10 h and deactivated at 80°C for 10 min. The final templates were diluted in a 1:10 ratio with sterile distilled water to be used in the preamplification. Preamplification reactions and polymerase chain reaction (PCR) conditions are described in Gurung et al. (2013). Primers complementary to the adapter sequences plus one selective base at the 3′ end were used. Each preamplification reaction contained the DNA template (6.25 ng/μl), 0.5 μmol/μl each *Eco*R I primer (E-A, 5′-GACTGCGTACCAATTCA-3′) and *Mse* I primer (M-C, 5′-GATGAGTCCTGAGTAAC-3′), 0.2 mM dNTPs, 1× buffer, and 1 unit of *Taq* DNA polymerase. The preamplifications were done using the PCR program of 95°C for 3 min followed by 95°C for 30 s, 56°C for 60 s, and 72°C for 60 s for 30 cycles and 72°C for 10 min for 1 cycle. The PCR products were kept at 4°C. The preamplification PCR products were diluted in a 1:10 ratio with sterile distilled water as templates for selective amplification. The *Eco*R I and *Mse* I primers with three selective bases were used for selective amplification at 95°C for 4 min followed by 95°C for 30 s, 65°C for 30 s (–7°C per cycle), and 72°C for 60 s for 12 cycles; 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 23 cycles; and 72°C for 10 min for 1 cycle, and they were stored at 4°C. Eight primers each of *Eco*R I and *Mse* I were used for the AFLP analysis (Table 4). Selective PCR amplification products were mixed with formamide loading dye, denatured at 95°C for 5 min, and then, separated in 6% denaturing polyacrylamide gels as described by Vos et al. (1995). The DNA silver staining system was used to detect the AFLP bands (Zhong and Steffenson 2000).

The reproducible DNA bands (50 to 600 bp in length) were scored manually with reference to a 20-bp DNA ladder (TaKaRa). The DNA fragments were scored manually in a binary system, where the presence of a band was coded as one and absence was coded as zero. Hierarchical cluster analysis of the matrix values used unweighted pair group method average (UPGMA) with the SAHN program in NTSYS-pc 2.0 (version 2.2; Exeter Software) and the Tree plot model to construct the AFLP cluster. To determine the robustness of the dendrogram, the presence or absence of data was resampled by replacement with 1,000 bootstrap replicates.

Results

Genotype selection. Twenty-one isolates were randomly selected to identify the IRs of 77 barley genotypes used in commercial production or breeding programs in China. After evaluation of spot blotch resistance in the barley accessions, 17 candidate cultivars (Mirco, Gold Promise, Ganpi 2, Mengpimai 1, Steptoe, Mazurka, Zaoshu 3, 09GW-07, Varunda, Morex, Kenpimai 7, Tradition, Mengpimai 3, Legacy, Kenpimai 9, Kenpimai 11, and 10PJ-24) were selected based on cluster analysis of their IRs to the pathogen isolates and genetic origins. Another three candidate barley genotypes—ND 5883, Bowman, and ND B112—were the first differential set of genotypes for barley spot blotch pathogen developed by Valjavec-Gratian and Steffenson (1997b) and widely used in the U.S.A., Canada, and Australia. Thus, the resulting 20 candidate genotypes used in this study genetically represent the disease resistance levels to isolates collected across China and other disease epidemic regions. These 20 barley genotypes and a triticale line H 1890 as a resistance control (Table 2) were further inoculated with 55 isolates of *C. sativus*. According to cluster analysis of the resistance spectra, the barley genotypes and triticale line H 1890 were classified into four clusters (Fig. 2). The first cluster contained eight highly susceptible barley cultivars or lines, including Zaoshu 3 and ND 5883. The second cluster contained four moderately susceptible cultivars, including Morex and Kenpimai 7. The third cluster consisted of three highly resistant barley genotypes, including ND B112, Kenpimai 11, and 10PJ-24, and the resistant control triticale, and the fourth cluster contained five moderately resistant genotypes, including Bowman and Kenpimai 9. In addition to the resistance spectrum of each differential candidate genotype, their use in barley production or breeding programs in China and pedigree relationships between candidate genotypes were considered in the selection of appropriate differential genotypes. The resulting 12 differential barley genotypes were ordered from low- to high-resistance levels as follows: ND 5883, Zaoshu 3, Kenpimai 7, Morex, Varunda, Bowman, Tradition, Mengpimai 3, Kenpimai 9, 10PJ-24, Kenpimai 11, and ND B112. The pathotypes identified within the differential set developed in this study revealed the virulence variation among the Chinese *C. sativus* population and provided limited information for comparing those in other regions, such as the U.S.A., Canada, and Australia. According to the coded

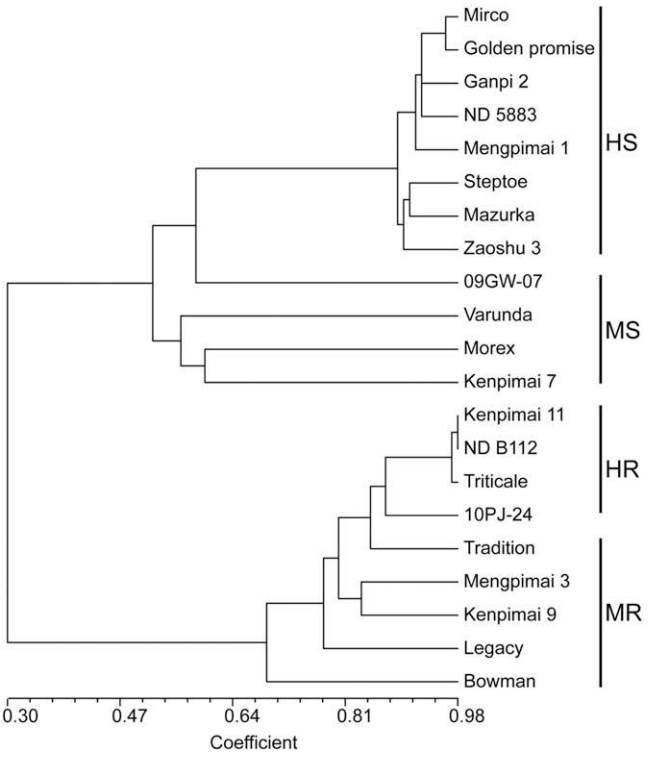

Fig. 2. Dendrogram based on the similarity of infection responses of 55 isolates of *Cochliobolus sativus* on 20 barley genotypes and a resistant triticale line control. HR, high resistance; HS, high susceptibility; MR, moderate resistance; MS, moderate susceptibility.

Table 4. The selective primer sequences of the amplified fragment-length polymorphism analysis

Primer number	Primer name	Sequence (5′ to 3′)	Primer number	Primer name	Sequence (5′ to 3′)
EI-1	E-AAA	GACTGCGTACCAATTCAAA	MI-1	M-CCA	GATGAGTCCTGAGTAACCA
EI-2	E-AAC	GACTGCGTACCAATTCAAC	MI-2	M-CCC	GATGAGTCCTGAGTAACCC
I-3	E-AAG	GACTGCGTACCAATTCAAG	MI-3	M-CCT	GATGAGTCCTGAGTAACCT
EI-4	E-AAT	GACTGCGTACCAATTCAAT	MI-4	M-CCG	GATGAGTCCTGAGTAACCG
EI-x-1	E-AA	GACTGCGTACCAATTCAA	MI-x-1	M-CA	GATGAGTCCTGAGTAACA
EI-x-2	E-AC	GACTGCGTACCAATTCAC	MI-x-2	M-CC	GATGAGTCCTGAGTAACC
EI-x-3	E-AG	GACTGCGTACCAATTCAG	MI-x-3	M-CT	GATGAGTCCTGAGTAACT

triplet nomenclature system of Limpert and Müller (1994), the 12 differential genotypes could be divided into four subsets, with each one consisting of three components. Of the 12 differential barley genotypes tested in this study, ND B112 was the only one resistant to all isolates originating from China at the seedling stage, being highly resistant to 65 of 71 isolates tested and moderately resistant to the other 6 isolates. ND 5883 was the most susceptible differential genotype, being moderately resistant to 2 of 71 isolates and highly susceptible to the other 69 isolates, followed by Zaoshu 3, which was susceptible to 67 of 71 isolates.

Environmental factors, especially temperature, often affect plant IRs to pathogens. To test the stability of IRs of the 12 differential genotypes under different latent temperatures, the genotypes were kept at 18, 21, and 25°C during the latent period after inoculation with three randomly selected isolates. In the one-way ANOVA *F* test, all of the *P* values for the differential genotypes inoculated with the three isolates were >0.05, with no significant differences observed in the IRs of the differential cultivars at different latent temperatures (Supplementary Fig. S2).

Virulence diversity. All isolates of *C. sativus* in this study produced tiny necrotic lesions or typical spot blotch symptoms on the 12 differential barley genotypes tested at the seedling stage, with variation in virulence levels between genotypes and none immune to spot blotch. Based on the IRs, the 71 isolates were grouped into 19

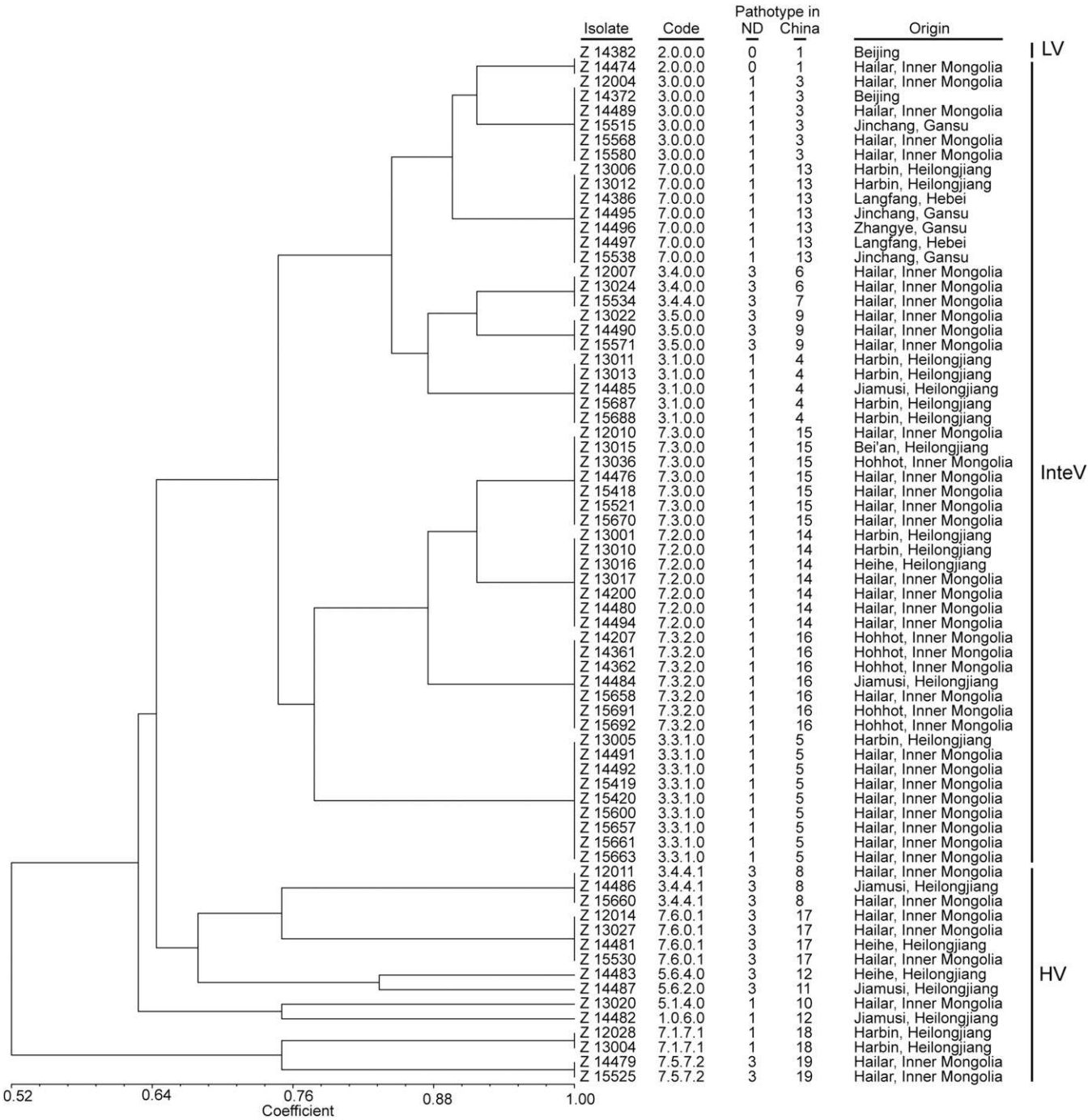

Fig. 3. Virulence cluster analysis on the similarity of infection responses (IRs) of 71 isolates of *Cochliobolus sativus* on 12 differential barley genotypes. Cluster analysis was based on the unweighted pair group method using a dissimilarity matrix of one for resistant (R) and zero for susceptible (S) infection response of an isolate on differential barley genotypes. Low IRs (0 to 3) and intermediate IRs (4 and 5) of host–parasite incompatibility are described as R, and high IRs (6 to 9) are described as S. HV, high virulence; InteV, intermediate virulence; LV, low virulence; ND, North Dakota.

pathotypes (Table 3) and classified into three clusters (Fig. 3). In the low-virulence cluster, only two isolates of pathotype 1 were included, which were highly virulent to Zaoshu 3. The intermediate-virulence cluster consisted of 54 isolates, all of which had low virulence to Kenpimai 9, 10PJ-24, Kenpimai 11, and ND B112, and Z 15534 had high virulence to Kenpimai 9. In the high-virulence cluster, 14 of 15 isolates were highly virulent to Kenpimai 9 and/or 10PJ-24, and 2 (Z 14479 and Z 15525) were highly virulent to resistant differential Kenpimai 11 (Fig. 3).

It has been reported that all *C. sativus* isolates from wheat roots were the least virulent (pathotype 0.0.0.0) on 12 differential barley genotypes (Knight et al. 2010). To confirm the virulence variation of *C. sativus* isolates from barley leaves on wheat plants, 28 isolates were randomly selected for testing on a set of Chinese differential wheat cultivars (Tiechun 1, Ningmai 15, Jimai 22, Longmai 15, Zhoumai 18, Lumai 14, Suwon 11, Ning 82109, Aikang 58, Shaanyou 225, Yangmai 6, and *Triticum spelta album*) at the two-leaf stage. Of these, 22 isolates were the least virulent to all differential wheat genotypes (pathotype 0.0.0.0); the other six, with pathotypes that were 3.1.0.0, 0.3.1.0, 0.1.2.0, 7.3.0.0, 0.2.0.0, and 3.5.1.0, had intermediate virulence on highly or moderately susceptible differentials (Supplementary Table S2), indicating that the virulence variation of *C. sativus* isolates from barley-growing areas in this study was derived primarily from resistance selection by host barley.

The frequency of isolates for each pathotype differed greatly. In the intermediate-virulence group, pathotype 5 (coded triplet 3.3.1.0) had the highest isolate number (nine)—of which eight were collected from Harbin in Heilongjiang and one was collected from Hailar in Inner Mongolia—followed by pathotypes 13, 14, 15, 16, 3, and 4, which had seven, seven, seven, seven, six, and five isolates in each subgroup, respectively (Table 3). In this study, the pathogen population from Heilongjiang had the highest virulence diversity, because 20 isolates from this region were grouped into 12 of 19 pathotypes. Similarly, 37 isolates collected from Hailar in Inner Mongolia were grouped into 13 of 19 pathotypes (Fig. 1 and Table 5). Interestingly, all members of the high-virulence group originated from Hailar in Inner Mongolia and Harbin, Jiamusi, and Heihe in Heilongjiang, where barley has been cultivated as a major cereal crop for several decades. No relationship was evident between virulence cluster and the origin of the cluster members in this study (Fig. 3 and Table 3). Compared with the virulence characterization results of the American differential genotypes (ND 5883, Bowman, and ND B112), two isolates of Chinese pathotype 1 can be equated to pathotype 0, and 17 isolates of pathotypes 6, 7, 8, 9, 11, 12, 17, and 19, being highly virulent to both ND 5883 and Bowman in this study, belonged to pathotype 3, whereas the other 52 isolates were grouped into pathotype 1. No isolates were grouped into pathotype 2, and all of the isolates were low virulence to differential ND B112 (Table 3).

Table 5. Geographical pathotype distribution of isolates collected from different origins

Origin	Pathotype	Isolate number	Origin	Pathotype	Isolate number
Beijing	1	1	Hailar, Inner Mongolia	17	3
	3	1		10	1
Langfang, Hebei	13	2		19	2
Jinchang, Gansu	3	1	Harbin, Heilongjiang	4	4
	13	2		13	2
Zhangye, Gansu	13	1		14	2
Hohhot, Inner Mongolia	15	1		5	1
	16	5		18	2
Hailar, Inner Mongolia	1	1	Jiamusi, Heilongjiang	4	1
	3	4		16	1
	5	8		8	1
	6	2		11	1
	7	1		2	1
	8	2	Heihe, Heilongjiang	12	1
	9	3		14	1
	14	4		17	1
	15	5	Bei'an, Heilongjiang	15	1
	16	1			

AFLP data analysis. For genetic diversity analysis using the AFLP method, 22 selective primer pairs were selected. The DNA polymorphic bands were scored, and 226 isolate-specific bands and 462 nonisolate-specific bands were produced for data analysis of 68 isolates of barley leaf spot blotch (Table 1). The DNA band number ranged from 19 to 42 in each primer combination. On average, 31 bands were produced for each combination, including 10 isolate-specific bands (Table 6). According to the dendrogram generated from the AFLP data from 22 primer pairs, the 68 isolates were clustered into three distinct groups using UPGMA with the genetic distance coefficient (Fig. 4). In group I, 28 of 48 isolates were collected from Hailar, with the remaining coming from Hohhot, Harbin, Jiamusi, Heihe, Jinchang, and Zhangye. In group II, two isolates from Beijing and two isolates from Langfang were contained. In group III, 8 of 16 isolates originated from Hailar, and three were from Hohhot. The remaining five isolates originated from Harbin, Bei'an, and Jiamusi. Most isolates of the same pathotype or origin were not grouped into the same cluster (Fig. 4).

Discussion

Barley spot blotch caused by *C. sativus* is a devastating leaf disease that has become prevalent in northeast China in the last three decades as the weather conditions have become more suitable for severe outbreaks in June and July when barley development is at the heading and anthesis stages. *C. sativus* can cause common root rot and leaf spot on barley seedlings in rainy spring weather and produce plenty of inoculum for spot blotch at later stages of growth. In this study, the first of its kind for Chinese *C. sativus* populations, we identified highly virulent variability and genetic diversity in the *C. sativus* isolates collected from diseased barley leaf tissues.

As a hemibiotrophic pathogen, *C. sativus* can exert high-virulence variability on host barley genotypes, which has been reported in

Table 6. Amplification results of the 68 isolates of *Cochliobolus sativus* with selective amplified fragment-length polymorphism primer pairs

Primer combination	Isolate-specific band	Nonisolate-specific band	Total band
EI-x-1 + MI-x-2	10	29	39
EI-x-1 + MI-x-3	9	16	25
EI-x-1 + MI-x-4	10	18	28
EI-x-2 + MI-x-1	10	22	32
EI-x-2 + MI-x-2	6	20	26
EI-x-2 + MI-x-3	15	26	41
EI-x-3 + MI-x-1	7	26	33
EI-x-3 + MI-x-2	11	17	28
EI-x-3 + MI-x-3	9	24	33
EI-x-3 + MI-x-4	9	24	33
EI-x-4 + MI-x-1	12	28	40
EI-x-4 + MI-x-2	12	23	35
EI-x-4 + MI-x-3	9	23	32
EI-x-4 + MI-x-4	12	25	37
EI-1 + MI-1	12	20	32
EI-1 + MI-2	12	11	23
EI-3 + MI-1	13	22	35
EI-3 + MI-4	13	19	32
EI-4 + MI-3	10	25	35
EI-2 + MI-1	9	14	23
EI-2 + MI-2	8	11	19
EI-2 + MI-3	8	19	27
Total band	226	462	688

several sets of differential barley genotypes worldwide. The first report on *C. sativus* pathotypes was from North Dakota, U.S.A. with three differential barley genotypes (ND 5883, Bowman, and ND B112) (Valjavec-Gratian and Steffenson 1997b), which used the coded triplet nomenclature system of Limpert and Müller (1994). Four pathotypes (0, 1, 2, and 7) of *C. sativus* isolates from barley leaves and roots have been identified in North Dakota. In Australia, 11 pathotypes were found among 30 *C. sativus* isolates collected from host barley and wheat, and 1 was from host prairie grass from the IRs of 12 differential barley cultivars (ND 5883, Bowman, ND B112, Stirling, Gilbert, Lindwall, Skiff, Delta, VB9524, CI 1227, CI 6311, and Sloop) (Knight et al. 2010). In another study, 12 differential wheat genotypes were selected to analyze the pathotypes among 96 *C. sativus* isolates collected from infected wheat leaves in North Dakota and Nepal; the isolates were classified into 47 pathotypes (Gurung et al. 2013). *C. sativus* isolates that cause common root rot on wheat were the least virulent (pathotype 0.0.0.0) when tested on 12 differential barley genotypes (Knight et al. 2010). Similarly, 30 randomly selected wheat isolates were grouped into pathotype 0 from the IRs of three barley genotypes (ND 5883, Bowman, and ND B112), and even the highly virulent isolate of wheat was unable to cause susceptible reactions on the differential barley genotypes (Gurung et al. 2013), indicating the potential for host specificity of *C. sativus* (Knight et al. 2010). In our study, we used *C. sativus* isolates collected from barley leaf spot blotch tissues in fields where no wheat had been grown for decades to confirm that the pathogen virulence variation was derived only by host barley genotypes. Twenty-eight of the barley spot blotch isolates showed the least virulence on differential wheat genotypes.

China has a long history of barley cultivation. Indeed, a new barley species (*Hordeum innermongolicum*), found in Inner Mongolia, is considered the oldest sect in the genus *Hordeum* such that Inner

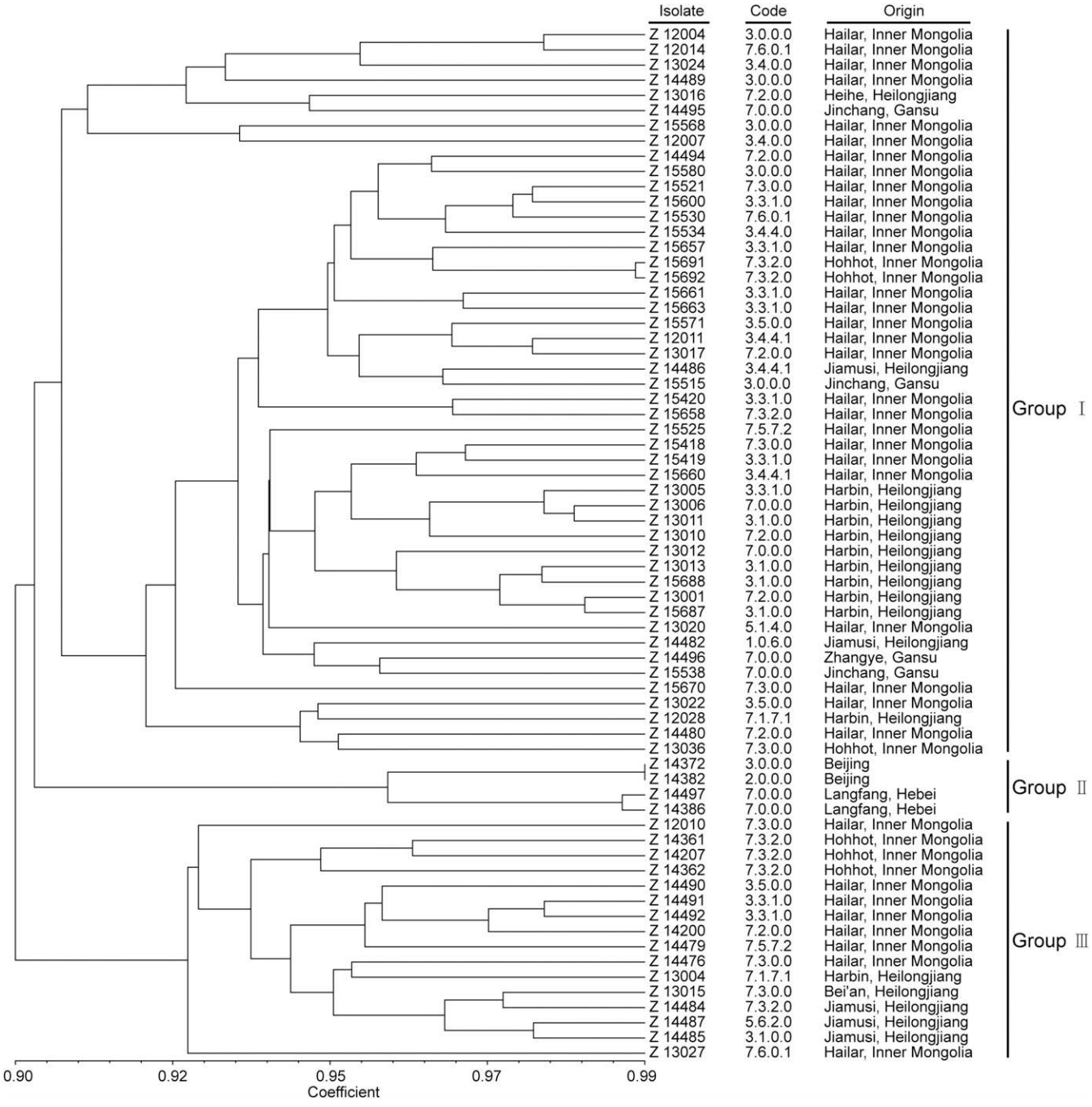

Fig. 4. Genetic diversity analysis of 68 isolates of *Cochliobolus sativus* collected in China using the amplified fragment-length polymorphism method.

Mongolia is likely one of the original centers of the genus *Hordeum* in China (Xu 1993). According to pathogen–plant interactions and the coevolution principle, the virulence variation of a pathogen naturally changes with plant resistance and evolves suitably to environmental conditions. For example, *C. sativus* has been an accidental pathogen on barley in northeast China and the eastern region of Inner Mongolia, but it has become a major biotic stress factor. However, no systemic reports on the pathotypes of Chinese *C. sativus* populations were available. Therefore, it was crucial to characterize the virulence patterns of *C. sativus* isolates collected from diseased barley leaf tissues in China. In this study, 12 barley genotypes were selected, including six key Chinese cultivars and six introduced cultivars or lines. The cultivars Kenpimai 11 and 10PJ-24, with slightly less resistance than ND B112, are promising brewing barley cultivars and remain highly resistant to most *C. sativus* isolates. Moderately resistant cultivars—Kenpimai 9, Mengpimai 3, Bowman, and Tradition—are extensively cultivated in northeast China. Bowman was introduced into China in the early 1990s and planted in eastern Inner Mongolia; it remains resistant to most isolates (53 of 71) but became susceptible in eastern North Dakota in 1990 (Fetch and Steffenson 1994). Tradition was introduced into China in 2003 and used as a major cultivar since 2008; it is resistant to 58 of 71 isolates. Kenpimai 7, Morex, and Varunda are moderately susceptible genotypes. Morex was introduced in the 1980s and used as a parent in breeding. Varunda, carrying resistance genes *rpsVa* and *rpsVa2* to stripe rust caused by *Puccinia striiformis* f. sp. *hordei* (Chen and Penman 2005), is an important parent in barley resistance breeding programs. Kenpimai 7 was released in 1996 and has been planted as a major cultivar for >20 years in northeast China. Zaoshu 3 was selected from a Japanese cultivar Kando Nijo 3 and is highly susceptible to *C. sativa*. It has been used as an important cultivar and core parent line in Chinese barley breeding for several decades. Fourteen of 155 Chinese cultivars developed in the last three decades are genetically related to Zaoshu 3 (Guo et al. 2016). Because most of the selected set of 12 differential genotypes were major cultivars or parent lines with a long history in China, they have interacted naturally with Chinese pathogen isolates and continuously exerted selection pressure on their pathogenicity. As a result, pathotypes among the Chinese *C. sativa* population could be identified more precisely in this set of differential barley genotypes.

Outside of the U.S.A., ND 5883, Bowman, and ND B112 have been used for pathotype identification in *C. sativus* populations in Canada and Australia (Ghazvini and Tekauz 2007; Knight et al. 2010; Meldrum et al. 2004). Advantages of the coded triplet nomenclature system by Limpert and Müller (1994) are obvious for designating pathotypes of plant pathogens. The system was adopted in previous studies on the pathotypes of *C. sativus* on barley and wheat (Ghazvini and Tekauz 2007; Gurung et al. 2013; Knight et al. 2010; Meldrum et al. 2004; Valjavec-Gratian and Steffenson 1997b). To compare the virulence variation of Chinese *C. sativus* populations with those from other regions of the world, we used ND 5883, Bowman, and ND B112 as differential components and adopted the coded triplet nomenclature for pathotype designation in this study.

The *C. sativus* isolates examined in this study were grouped into 19 pathotypes and three virulence clusters. Most isolates (54 of 71) were grouped into the intermediate-virulence cluster, with 15 in the high-virulence group and two in the low-virulence cluster (Fig. 3). All of the tested isolates had low virulence to ND B112. When comparing the *C. sativus* pathotypes detected with ND 5883, Bowman, and ND B112 in North America and Australia, we found that most isolates (52 of 71 and 17 of 71, respectively) were grouped into pathotypes 1 and 3, respectively, with none as pathotype 2 or 7. In Manitoba, Canada and North Dakota, *C. sativus* isolates exhibiting virulence on ND 5883, Bowman, and ND B112 were identified and designated pathotype 7 (Ghazvini and Tekauz 2007; Gyawali 2010). In Australia, the barley isolates of *C. sativus* designated pathotype 0 and 1 were identified (Knight et al. 2010; Meldrum et al. 2004). In the U.S.A. and Canada, four (0, 1, 2, and 7) and five (0, 1, 2, 6, and 7) pathotypes, respectively, have been characterized (Ghazvini and Tekauz 2007; Gyawali 2010; Zhong and Steffenson 2001), whereas three pathotypes (0, 1, and 3) were identified among the 71 *C. sativus* isolates collected from China. As such, pathotype 3 of *C. sativus* is specific to China, and pathotypes 2 and 7 are specific to North America.

Bowman has been planted in eastern Inner Mongolia for several years. It has also been used as parent material in breeding programs in the development of barley varieties, including Mengpimai 1 (Bowman/91W27//91G318), cultivar Mengpimai 2 (2001-146/Bowman), cultivar Mengpimai 4 (2001-146/Bowman), and cultivar Kenjianpimai 3 (TR212/Amagi Nijo//Bowman/Haruna Nijo), which have been planted in northeast China since the 1990s. These derivatives of Bowman have lost their resistance to most local isolates of *C. sativus*. Another important reason for the outbreak of barley spot blotch is that a major cultivar, Kenpimai 7 (TR 212/Clipper//Ant 90-2), became moderately susceptible in 2004. Cultivar Clipper is the common parent for Kenpimai 7 and ND 5883 (Clipper/702-10). Cultivar TR 212, Clipper, and cultivar Ant 90-2 were susceptible to most isolates at the seedling and adult stages. The application of Bowman's derivatives and Kenpimai 7 for so long may explain why pathotype 1 (52 of 71 isolates) is overwhelmingly predominant in the Chinese *C. sativus* population. Fortunately, pathotype 7 (highly virulent isolates to differential ND B112) was not found among the Chinese *C. sativus* isolates. Although *C. sativus* is a hemitropic disease agent, there is no indication that the gene-for-gene interaction model is the principal system operating in the *H. vulgare–C. sativus* pathosystem, but it plays a small role in some interactions (Ghazvini and Tekauz 2008; Valjavec-Gratian and Steffenson 1997a). Virulence variation of *C. sativus* is a long-term problem for barley resistance breeding against leaf spot blotch. *C. sativus* virulence variation was selected mainly by disease resistance of host plant. For example, the predominant pathotype 2 may be a specific virulence selection result from Bowman, which has been used as a major feed variety in North Dakota and other regions in the world since its release in 1984. Pathotype 3 is possibly a selection result from the cultivation of both derivatives of Bowman and Kenpimai 7 for a long time in northeast China.

High levels of genetic variability have been identified in *C. sativus* populations throughout the world (Ghazvini and Tekauz 2012; Gurung et al. 2013; Zhong and Steffenson 2001). However, this variability differs from other species of the genus *Cochliobolus* at the genetic level (Zhong and Steffenson 2001). Two AFLP markers unique to pathotype 2 have been identified (Zhong and Steffenson 2001), but no molecular markers specific to a pathotype were found except for the isolate-specific markers identified in this study. In other studies, genetic or virulence cluster analysis has not revealed a close correlation between pathotypes, AFLP patterns, and/or geographic origins (Ghazvini and Tekauz 2012; Gurung et al. 2013; Zhong and Steffenson 2001). These results indicate that the fungal pathogen may migrate in the world; meanwhile, similar barley resistant sources have been used globally in epidemic regions of spot blotch.

Durable resistance to spot blotch, derived mainly from ND B112, has been evident in six-rowed malting cultivars in the Midwest of the U.S.A. for >50 years. However, ND B112 has become susceptible owing to the occurrence of pathotype 7 in North America, indicating the powerful and rapid virulence variability in the pathogen. Although ND B112 was highly resistant to most of the Chinese isolates tested at the seedling stage, it was moderately resistant to the strongly virulent isolate Z 14479 (pathotype 3) at the adult stage. Spot blotch is becoming more challenging to manage with global warming and the changes to agricultural production patterns in China. Efforts are needed to exploit and develop new genetic sources of spot blotch resistance from barley germplasm accessions using the *C. sativus* pathotypes identified in this study to avoid disease epidemics in the future.

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Literature Cited

Arabi, M. I. E., and Jawhar, M. 2003. Pathotypes of *Cochliobolus sativus* (spot blotch) on barley in Syria. J. Plant Pathol. 85:193-196.

Chen, X., and Penman, L. 2005. Races of *Puccinia striiformis* f. sp. *hordei*, the pathogen of barley stripe rust in the United States in 2004. Barley Genet. Newsl. 35:23-26.

Clark, R. V. 1979. Yield losses in barley cultivars caused by spot blotch. Can. J. Plant Pathol. 1:113-117.

Fetch, T. G., and Steffenson, B. J. 1994. Identification of *Cochliobolus sativus* isolates expressing differential virulence on two-row barley genotypes from North Dakota. Can. J. Plant Pathol. 16:202-206.

Fetch, T. G., and Steffenson, B. J. 1999. Rating scales for assessing infection responses of barley infected with *Cochliobolus sativus*. Plant Dis. 83:213-217.

Gamba, F., and Estramill, E. 2002. Variation in virulence within a Uruguayan population of *Cochliobolus sativus*. Pages 59-62 in: Proceedings of the Second International Workshop on Barley Leaf Blights. A. H. Yahyaoui, L. Brader, A. Tekauz, H. Wallwork, and B. Steffenson, ed. ICARDA, Aleppo, Syria.

Gawel, N. J., and Jarret, R. L. 1991. A modified CTAB DNA extraction procedure for Musa and Ipomoea. Plant Mol. Biol. Report. 9:262-266.

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.

Ghazvini, H., and Tekauz, A. 2012. Molecular diversity in the barley pathogen *Bipolaris sorokiniana* (*Cochliobolus sativus*). Australas. Plant Pathol. 41:283-293.

Glass, N. L., Jacobson, D. J., and Shiu, P. K. T. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annu. Rev. Genet. 34:165-186.

Guo, H.-Q., Wang, W.-F., Yao, Q.-J., Sun, D., Yao, X.-B., Wang, C.-L., Tian, S.-F., Bao, H.-Z., Wang, F.-T., Feng, J., Lin, R.-M., and Xu, S.-C. 2016. Analysis on coefficient of parentage of major barley varieties and their spot blotch resistance. J. Plant Genet. Resour. 17:586-598.

Gurung, S., Mahto, B. N., Gyawali, S., and Adhikari, T. B. 2013. Phenotypic and molecular diversity of *Cochliobolus sativus* populations from wheat. Plant Dis. 97:62-73.

Gyawali, S. 2010. Association mapping of resistance to common root rot and spot blotch in barley, and population genetics of *Cochliobolus sativu*s. PhD thesis. North Dakota State University, Fargo, ND.

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, J., Schäfer, P., Hückelhoven, R., Langen, G., Baltruschat, H., Stein, E., Nagarajan, S., and Kogel, K.-H. 2002. *Bipolaris sorokiniana*, a cereal pathogen of global concern: Cytological and molecular approaches towards better control. Mol. Plant Pathol. 3:185-195.

Limpert, E., and Müller, K. 1994. Designation of pathotypes of plant pathogens. J. Phytopathol. 140:346-358.

Meldrum, S. I., Ogle, H. J., and Platz, G. J. 2004. Pathotypes of *Cochliobolus sativus* on barley in Australia. Australas. Plant Pathol. 33:109-114.

Roy, J. K., Smith, K. P., Muehlbauer, G. J., Chao, S., Close, T. J., and Steffenson, B. J. 2010. Association mapping of spot blotch resistance in wild barley. Mol. Breed. 26:243-256.

Steffenson, B. J., Hayes, P. M., and Kleinhofs, A. 1996. Genetics of seedling and adult plant resistance to net blotch (*Pyrenophora teres* f. *teres*) and spot blotch (*Cochliobolus sativus*) in barley. Theor. Appl. Genet. 92:552-558.

Tekauz, A., Gilbert, J., Mueller, E., Stulzer, M., Beyene, M., Ghazvini, H., Morgan, K., and Reverchon, F. 2003. Survey for foliar diseases of barley in Manitoba in 2002. Can. Plant Dis. Surv. 83:60-61.

Tinline, R. D. 1962. *Cochliobolus sativus* V. Heterokaryosis and parasexuality. Can. J. Bot. 40:425-437.

Valjavec-Gratian, M., and Steffenson, B. J. 1997a. Genetics of virulence in *Cochliobolus sativus* and resistance in barley. Phytopathology 87:1140-1143.

Valjavec-Gratian, M., and Steffenson, B. J. 1997b. Pathotypes of *Cochliobolus sativus* on barley in North Dakota. Plant Dis. 81:1275-1278.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Freijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Xu, T. W. 1993. The headway of research in the origin and taxonomy of barley. Pages 17-35 in: The Headway of Research in Crop Breeding. H. L. Liu, ed. Chinese Agricultural Publishing Press, Beijing, China.

Zhong, S., and Steffenson, B. J. 2000. A simple and sensitive silver staining method for detecting AFLP markers in fungi. Fungal Genet. Newsl. 47:101-102.

Zhong, S., and Steffenson, B. J. 2001. Virulence and molecular diversity of *Cochliobolus sativus*. Phytopathology 91:469-476.