Virulence, Molecular Diversity, and Mating Type of *Curvularia lunata* in China

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Abstract

Curvularia leaf spot (CuLS), caused by *Curvularia lunata*, is a devastating foliar disease in the maize-growing regions of China. Resistant varieties were widely planted in these regions in response to CuLS. However, over time, *C. lunata* has gradually adapted to the selective pressure and, in recent years, the incidence of CuLS has increased. To assess the correlation between virulence and genetic diversity, a total of 111 isolates was collected from 15 maize-growing regions located in nine provinces in China. These isolates were evaluated for virulence on maize using nine differential hosts: Shen135, CN165, Mo17, Luyuan92, 78599, Ye478, B73, E28, and Huangzaosi. To evaluate the genetic diversity, 657 polymorphic

amplified fragment length polymorphism markers were generated. Results showed that the isolates could be grouped into three pathotypes according to the phenotypic expression of the differential inbred lines. Isolates were clustered into two genetic diversity groups and further divided into subgroups. However, the correlation between virulence and genetic diversity grouping was low. Also, there was a low correlation observed between pathotype and geographic distribution. The ratio of mating type I to mating type II for all isolates was close to 3:4.

Keywords: cereals and grains, field crops, fungi, *Zea mays* L.

Curvularia leaf spot (CuLS) of maize (*Zea mays* L.), mainly caused by the fungal pathogen *Curvularia lunata* (Wakker) Boedijn, has become a serious threat to yield in maize production, especially in the warm and humid production regions in China (Li 2016). To date, at least 11 *Curvularia* spp. have been reported to infect maize, including *C. lunata*, *C. clavata*, *C. pallescens*, *C. maculans*, *C. tetramera*, *C. eragrostidis*, *C. trifolii*, *C. inaequalis*, *C. intermedia*, *C. senegalensis*, and *C. tuberculata* (Mabadeje 1969; Macri and Lenna 1974; Shi et al. 2000; Sivanesan 1984). Although many species can cause CuLS, *C. lunata* is still the dominant species in China (Dai et al. 1998; Dou and Jin 2007; Li et al. 2004; Liu 2008; Shi et al. 2000; Xia et al. 2012). CuLS did not become a serious production problem until the susceptible hybrid Shendan7 was widely planted in Liaoning Province. Crop losses were estimated to be 250 million kg in 1996 and, in some extreme cases, CuLS can cause up to 90% yield loss (Lv et al. 1997). The use of resistant cultivars is the most prevalent and promising strategy for controlling CuLS. Subsequently, maize cultivars P138, Shen135, and 78599 have been identified as CuLS-resistant lines carrying multiple resistant genes (Li et al. 2002; Zhao et al. 2002). Resistant quantitative trait loci (QTLs) to CuLS were identified and introduced into maize to generate a large number of resistant hybrids (Wang et al. 2001).

Virulence and genetic diversity in *C. lunata* have not been extensively examined in China. Inbred lines Shen135, CN165, Mo17, Luyuan92, 78599, Ye478, B73, E28, and Huangzaosi were screened as differential inbred lines to identify the pathotypes of *C. lunata* (Chen et al. 2003). With these differential inbred lines, four pathotypes have been discovered from four northern provinces in China. However, there were no significant associations found between pathotypes and

geographic locations (Xue et al. 2008). The sexual stage of *C. lunata* has not been found in nature but mating of compatible isolates can be induced easily in the laboratory (Lu et al. 2018), which suggests that the sexual stage may play a role in the life cycle of *C. lunata* (Li 2016).

Molecular technologies have been commonly used to assess the genetic diversity of *C. lunata*, including amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), universally primed polymerase chain reaction (UP-PCR), and intersimple sequence repeats (ISSR) (Chen and Zhang 2017; Dou and Jin 2007; Sun et al. 1998; Wang et al. 2014; Zhang et al. 2010). AFLP has the advantages of providing a direct assessment of the genetic relationships of fungi at the molecular level while offering high resolution, repeatable, and highly reliable results. This technology can effectively distinguish intraspecific variation among isolates (Pongam et al. 1999).

The objectives of the study were to (i) identify pathotypes of *C. lunata* isolates collected from different geographic locations through differential inbred lines, and examine their genetic diversity using AFLP markers; and (ii) identify the mating types of *C. lunata* isolates using specific PCR primers (Lu et al. 2018) and analyze the ratio of mating types.

Materials and Methods

Isolates collection. From August 2010 to 2015, maize leaves exhibiting typical CuLS symptoms were collected from nine provinces in northern and southwest China where CuLS regularly occurred: Heilongjiang, Jilin, Liaoning, Hebei, Shandong, Anhui, Sichuan, Yunnan, and Hubei Provinces (Table 1; Fig. 1). Leaf tissue with CuLS lesions (5-mm² pieces) were excised and surface sterilized by soaking in 70% ethanol for 30 s and 0.1% mercuric chloride for 1 min, then rinsed with sterile distilled water for 30 s (Cui and Sun 2012). Surface-sterilized leaf pieces were then transferred to potato dextrose agar (PDA) plates and incubated at 25°C for 5 to 7 days. Each isolate was prepared by flooding the PDA plate with 5 to 8 ml of distilled water. The conidia suspension (200 to 500 µl) was spread on Petri plate containing 4% water agar. Monoconidial isolates were prepared by selecting single germinating conidia and multiplying them on PDA plates. One isolate per lesion was obtained. All 111 strains were confirmed as *C. lunata* by their morphological and molecular characteristics (Shi et al. 2000).

Pathotypes determination. To determine the pathotypes of each isolate, the following differential inbred lines were used: Shen135, CN165, Mo17, Luyuan92, 78599, E28, B73, Ye478, and Huangzaosi (Table 2) (Chen et al. 2003). The seed of the differential lines

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were provided by Dr. Xiaoming Wang (Professor, Chinese Academy of Agricultural Science, Beijing, China). Differential inbred lines for *C. lunata* are different from the differential hosts for *Exserohilum turcicum* with single *Ht* resistant genes. Maize resistance to *C. lunata* is controlled by multiple genes. It is not clear that differential inbred lines for *C. lunata* carry resistant QTLs, except for Shen135. The above inbred lines were screened as differential hosts according to their phenotype (resistant or susceptible to *C. lunata*).

Plastic boxes (40 cm long by 28 cm wide by 10 cm tall) were filled with steam-sterilized soil (Hongxiang Horticulture Ltd., Xifeng, China). All differential inbred lines were planted together in one plastic box and were inoculated with a single isolate per plastic box. Three seeds of each differential inbred line were planted in each plastic box. Seedlings of inbred line D340 were included as a susceptible control for comparison. Plants were fertilized with Xinyangfeng Classic 25-10-16 (N-P-K) controlled-release fertilizer

Table 1. *Curvularia lunata* isolates used for virulence and genetic diversity analysis in China

Isolate ID	Host	Geographic origin ^a	Year	Mating type ^b	Pathotype ^c
13 LNFC1-1-1	Danyu204	Liaoning	2013	1	I
13 LNFC2-1-1	Danyu206	Liaoning	2013	1	I
13 LNFC3-2-1	Danyu103	Liaoning	2013	1	I
13 LNFC6-2-1	Danyu305	Liaoning	2013	2	I
13 LNFC8-2-1	Danek2157	Liaoning	2013	2	I
13 LNFC10-12-1	Danyu99	Liaoning	2013	2	I
13 LNFC12-2-1	Heima603	Liaoning	2013	2	I
12 LNFC14-1-1	Lianda128	Liaoning	2012	1	I
12 LNFC15-2-1	Danyu88	Liaoning	2012	2	I
12 LNFC17-1-1	Pudan	Liaoning	2012	1	I
12 LNFC18-2-1	Shenyu33	Liaoning	2012	2	I
12 LNFC19-1-1	Liao131	Liaoning	2012	1	I
12 LNFC20-1-1	Yu2808	Liaoning	2012	1	I
12 LNWF23-2-1	Nongda108	Liaoning	2012	2	I
12 HBBD24-1-1	C107	Hebei	2012	1	I
12 LNFK26-no-1	Faku	Liaoning	2012	No	I
12 LNWF28-2-1	Shendan6	Liaoning	2012	2	I
12 LNWF32-12-1	Denghai9	Liaoning	2012	2	I
12 LNFC33-no-1	D1024	Liaoning	2012	2	I
11 HBBD35-2-1	BaoB	Hebei	2011	2	I
11 HB36-2-1	0572IV-3	Hebei	2011	2	I
10 LNFC40-2-1	Danyu57	Liaoning	2010	2	I
10 LNFC41-1-1	Zhengdan958	Liaoning	2010	1	I
10 LNLT42-1-1	Zhengdan958	Liaoning	2010	1	I
10 LNSY47-1-1	Liaodan565	Liaoning	2010	1	I
10 LNSY51-1-1	Shenyu21	Liaoning	2010	1	I
10 LNFC52-2-1	Shenyu38	Liaoning	2010	2	I
10 LNWF56-1-1	E24	Liaoning	2010	1	I
10 LNWF59-1-1	Yangjiadalian	Liaoning	2010	1	I
10 LNWF61-2-1	Wafangdian	Liaoning	2010	2	I
14 LNFC62-2-1	Danyu69	Liaoning	2014	2	I
14 LNFC65-1-1	Danyu606	Liaoning	2014	1	I
14 LNFC66-2-1	Danyu2061	Liaoning	2014	2	I
14 LNFC67-2-1	Danyu2165	Liaoning	2014	2	I
14 LNFC68-1-1	Zhengdan958	Liaoning	2014	1	I
14 LNFC69-2-1	Danyu303	Liaoning	2014	2	I
14 LNFC74-2-1	Xianyu335	Liaoning	2014	2	I
14 LNFC78-1-1	Liangyu208	Liaoning	2014	1	I
14 LNWF79-1-1	Danyu402	Liaoning	2014	1	I
14 SDLC80-12-1	X145166	Shandong	2014	1	I
14 LNFC81-2-1	Weike702	Liaoning	2014	2	I
14 LNFC84-12-1	Dan3366	Liaoning	2014	1	I
14 LNFC85-2-1	Danyu305	Liaoning	2014	2	I
14 LNFC90-2-1	Danke702	Liaoning	2014	2	I
14 LNFC93-2-1	Danyu405	Liaoning	2014	2	I
14 LNDLZH94-12-1	ZhuangheX	Liaoning	2014	1	I
14 LNFC95-2-1	Danyu801	Liaoning	2014	2	I
14 LNFC96-2-1	Zhengdan958	Liaoning	2014	2	I
14 LNFC97-1-1	Mingyu6	Liaoning	2014	1	I
14 LNFC98-2-1	Danyu208	Liaoning	2014	2	I
14 LNDLNKY130-2-1	Da109	Liaoning	2014	2	I
14 LNFC134-1-1	Danyu69	Liaoning	2014	1	I
14 LNFC136-1-1	Danyu401	Liaoning	2014	1	I
14 LNFC137-1-1	Mingyu19	Liaoning	2014	1	I

(Continued on next page)

^a Asterisk (*) indicates that the collection site is unclear.

^b Mating types of *C. lunata* with 1 = mating type I, 2 = mating type II, and “No” indicates that mating types of isolates are unclear.

^c Pathotype of *C. lunata* on the nine differential inbred lines, with I = pathotype I, II = pathotype II, and III = pathotype III.

(The Xinyangfeng Company, Hubei, China) to maintain plant health and growth. The greenhouse was maintained at $25 \pm 3^{\circ}\text{C}$ (day) and $18 \pm 3^{\circ}\text{C}$ (night), with supplemental lighting at 325 to $650 \mu\text{E m}^{-2} \text{s}^{-1}$ with 14 h/day and 10 h/night .

Inoculation was performed when the plants had seven to eight leaves. Cultures were transferred and maintained on PDA at 25 to 28°C under a regime of 12 h of light and 12 h of darkness for 7 days to promote sporulation. Conidial suspensions were quantified with a hemocytometer

and diluted with water containing Tween 20 (0.02%) to approximately $50,000$ conidia/ml. Each plant was inoculated with 2 ml of the conidial suspension using a handheld sprayer (Zhejiang Kangrui Apparatus Technology Co., Ltd., Zhejiang, China). The inoculated plants were immediately covered with polythene sheets for 48 h to maintain the moisture. For each isolate, the test was repeated three times.

The plants were evaluated 10 days post inoculation. Symptoms caused by virulent isolates initially appeared as chlorotic transparent

Table 1. (Continued from previous page)

Isolate ID	Host	Geographic origin ^a	Year	Mating type ^b	Pathotype ^c
14 LNDLZH139-1-1	Qiangshuo88	Liaoning	2014	1	I
14 HLJ141-2-1	HPC	Heilongjiang	2014	2	I
14 JL152-2-1	275	Jilin	2014	2	I
15 SCMY161-1-1	SCMY1	Sichuan	2015	1	I
15 YNLC165-1-1	YNCY	Yunnan	2015	1	I
15 YNDH167-2-1	Yunduan505	Yunnan	2015	2	I
15 YNDH168-1-1	QC	Yunnan	2015	1	I
15 LNDLZH169-1-1	Qifeng2	Liaoning	2015	1	I
15 LNDLZH171-2-1	Jincheng9	Liaoning	2015	2	I
15 LNDLZH172-1-1	Chenshi505	Liaoning	2015	1	I
15 LNDD173-2-1	Danyu508	Liaoning	2015	2	I
15 LNDD174-2-1	Tieyan919	Liaoning	2015	2	I
15 LNDD175-1-1	Danyu305	Liaoning	2015	1	I
15 LNDD176-1-1	Haihe78	Liaoning	2015	1	I
15 LNDG177-1-1	Xingdan6	Liaoning	2015	1	I
15 LNDG178-1-1	Danyu607	Liaoning	2015	1	I
15 LNDG179-2-1	Xingdan251	Liaoning	2015	2	I
15 LNDG180-2-1	Danyu405	Liaoning	2015	2	I
15 LNDG181-2-1	Danyu305	Liaoning	2015	2	I
15 LNDG182-12-1	Xingdan9	Liaoning	2015	2	I
15 LNDG183-12-1	Danyu303	Liaoning	2015	no	I
15 LNDG184-12-1	Danyu88	Liaoning	2015	2	I
15 LNAS187-12-1	Danyu336	Liaoning	2015	1	I
15 LNXM188-2-1	Xinmin	Liaoning	2015	2	I
15 DLZH191-2-1	Danyu801	Liaoning	2015	2	I
15 LNAS192-12-1	Jindao1513	Liaoning	2015	2	I
15 YNDH193-2-1	Danyu305	Yunnan	2015	2	I
15 HB194-2-1	YN8	Hubei	2015	2	I
15 YNDH196-2-1	Yunduan10	Yunnan	2015	2	I
13 LNFC4-1-2	Danyu402	Liaoning	2013	1	II
13 LNFC5-2-2	Danyu86	Liaoning	2013	2	II
13 LNFC7-12-2	Danke2162	Liaoning	2013	1	II
13 LNFC9-1-2	Danyu304	Liaoning	2013	1	II
13 LNFC11-2-2	Danyu39	Liaoning	2013	2	II
12 LNWFD22-2-2	Beishan	Liaoning	2012	2	II
12 LNSY27-2-2	Liaodan43	Liaoning	2012	2	II
12 LNSY30-2-2	Shendan18	Liaoning	2012	2	II
11 no37-2-2	899	*	2011	2	II
11 no38-2-2	1897	*	2011	2	II
10 LNTL45-1-2	Xianyu335	Liaoning	2010	1	II
10 LNSY46-1-2	Xianyu335	Liaoning	2010	1	II
10 LNFC49-1-2	Shenyu21	Liaoning	2010	1	II
14 LNFC64-1-2	Danyu605	Liaoning	2014	2	II
14 LNDLNKY87-1-2	108	Liaoning	2014	1	II
14 LNDLNKY88-12-2	102	Liaoning	2014	1	II
14 LNFC89-2-2	W1506	Liaoning	2014	2	II
14 LNFC92-1-2	Dongdan6531	Liaoning	2014	1	II
14 AH108-2-2	Jinnong118	Anhui	2014	2	II
14 LNDLNKY131-12-2	DaKJ62	Liaoning	2014	1	II
15 SCMY163-12-2	SCMY3	Sichuan	2015	1	II
15 YNDL164-1-2	YNWS	Yunnan	2015	1	II
15 LNDLZH170-2-2	Huashi157	Liaoning	2015	2	II
15 LNDG185-2-2	Danyu204	Liaoning	2015	2	II
15 LNDG190-12-2	Baoli	Liaoning	2015	2	II
15 YNDH195-2-2	Yunduan10	Yunnan	2015	2	II
12 LNWFD31-2-3	CX-3	Liaoning	2012	2	III
15 LNDG186-12-3	Danyu402	Liaoning	2015	2	III

lesions on the fifth day that generally turned necrotic and continued to enlarge by the 10-day evaluation. The symptoms varied between isolates. Symptoms were rated according to the following rating scale: 1 = no symptoms or small brown spots (<1 mm); 3 = lesions with small necrotic centers (<1 mm); 5 = round or oval yellowish chlorotic lesions (1 to 2 mm), greyish-white centers, with yellow or red-brown margins with a yellow halo; 7 = as before but with some lesions joined together to form large necrotic areas (>2 mm); and 9 = lesions cover the entire leaf (Fig. 2). Pathotypes were assigned to each isolate based on their disease index (DI) (Xue et al. 2008) calculated based on the formula $DI = [\sum(A \times B) / \sum B \times 9] \times 100$, where A represents disease levels 1, 3, 5, 7, or 9 and B represents the number of plants at each disease level. The pathotypes of *C. lunata* were categorized according to their DI. Overall, an index >35.0 was defined as pathotype I, 34.9 to 15.0 as pathotype II, and <14.9 as pathotype III.

Genomic DNA extraction. Isolates of *C. lunata* (Table 1) were transferred from stock cultures to PDA and grown for 7 days. Fresh fungal mycelium of each isolate was scraped from the Petri dishes, placed in a 50-ml centrifuge tube, sealed, and vacuum freeze dried at -80°C for 8 to 12 h using a general vacuum dryer until the residual moisture was less than 3%. Genomic DNA was extracted using the cetyltrimethylammonium bromide method (Sambrook and Russell

2001). Relative purity and concentration of DNA were determined by electrophoresis with a known concentration marker and ultraviolet spectrophotometry at an optical density ratio of 260/280. The concentration of the final DNA was 50 ng/ μl .

AFLP analysis. AFLP analysis was conducted according to a modified method (Vos et al. 1995). Primers with one selective base at the 3' end were used in preselective amplification. For selective

Table 2. Genetic background of differential hosts^a

Differential host	Pedigree
Shen135	Hybrid P78599, United States
CN165	Hybrid 3268, United States
Mo17	Lancaster Sure Crop, United States
Luyuang92	Radiation mutant, China
78599	Lancaster Sure Crop, United States
Ye478	Reid Yellow Dent, United States
B73	Iowa Stiff Stalk Synthetic, United States
E28	Lvdahonggu, China
Huangzaosi	Tangsipingtou, China

^a Nine differential hosts using the identification pathotypes of *Curvularia lunata*.

Fig. 1. Map of China showing the sites of collection of *Curvularia lunata* isolates. The sampling site of the 109 strains sampled covers a total of nine provinces, including LN = Liaoning, HLJ = Heilongjiang, HuB = Hubei, HB = Hebei, AH = Anhui, SD = Shandong, JL = Jilin, YN = Yunnan, and SC = Sichuan, plus 2 isolates from the unknown location. There were 92 isolates from Liaoning (Fengcheng, Wafangdian, Shenyang, Dandong, Anshan, Donggang, Tieling, Dalian, Xinmin, and Faku); 7 isolates from Yunnan (Dehong, Dali, and Lincang); 3 isolates from Hebei (Baoding); 2 isolates from Sichuan (Mianyang); 1 isolate each from Jilin (Gongzhuling), Anhui (Huainan), Heilongjiang (Jiamusi), Shandong (Liaocheng), and Hubei (Wuhan); and 2 isolates from unknown locations.

amplification, primers with two selective bases were used. All of the primers used for this study are listed in Table 3. The products of selective amplification were mixed with 10 µl of sequencing dye (formamide deionized, 10 ml; bromophenol blue, 10 mg; and xylene, 10 mg) at 96°C for 7 min, then quickly cooled on ice. For electrophoresis, 7.5 µl of sample was loaded onto a 6.0% denaturing polyacrylamide gel with 1× Tris borate EDTA buffer for each sample. Electrophoresis was then performed at a continuous power of 75 W for 2.5 to 3.0 h.

Mating-type genes analysis. Primers to amplify sequences of the $\alpha 1$ box of mating-type locus (*MAT1-1-1*) and the HMG domain of *MAT1-2-1* in *C. lunata* were designed for a PCR assay. The resulting fragments were predicted to be 796 bp for *MAT1-1* and 698 bp for *MAT1-2* (Table 4). Each 20-µl amplification reaction contained 10 µl of 2× PCR mix, 0.8 µl of 10 pmol *ClMat1-R/ClMat2-R*, 0.8 µl of 10 pmol *ClMat1-F/ClMat2-F*, 1 µl of 50 ng DNA, and 7.4 µl of double-distilled H₂O. The reactions were performed in an ABI 9700 PCR machine for 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 65 or 55°C, and 1 min at 72°C; with a final extension of 72°C for 10 min. PCR products were separated in 1.0% agarose gels and visualized using ethidium bromide staining. All reactions were run with control isolates of each mating type and repeated for each sample to confirm the results.

Data analysis. For each primer pair, AFLP bands of 30 to 600 bp in size were scored for all isolates. AFLP bands that were present in some isolates but absent in other isolates were considered polymorphic markers. The presence or absence of a band was designated as 1 or 0, respectively, which together formed a binary matrix. For the virulence analysis, all of the isolates were identified for their disease level in a set of nine differential inbred lines. The disease levels 1, 3, and 5 were designated as 0 (avirulent), and 7 and 9 were designated as 1 (virulent), which also formed a binary matrix. The similarity matrix of the above data were constructed with Simple Matching and Dice similarity coefficients using the SIMQUAL program in the NTSYSpc version 2.10 (Lxeter Software, Setauket, NY, U.S.A.). The similarity matrix was then used to construct a dendrogram through the unweighted pair-group method using the arithmetic mean (UPGMA) with the SHAN clustering program. The Tree program was further used to generate the dendrogram. The cophenetic value of the dendrograms was tested with the original dissimilarity matrices through the Coph program. The correlation between the virulence matrix and the AFLP matrix was tested using the MXCOMP program.

Results

Pathogenicity tests. There were 92 isolates from Liaoning; 7 isolates from Yunnan; 3 isolates from Hebei; 2 isolates from Sichuan; 1 isolate each from Jilin, Anhui, Heilongjiang, Shandong, and Hubei; and 2 isolates from an unknown location. In all, 83 isolates belonged to pathotype I, 26 isolates belonged to pathotype II, and 2 isolates belonged to pathotype III. The occurrence rate of pathotype I, II, and III were 74.78, 23.42, and 1.8%, respectively.

In constructing the dendrogram, a 66% similarity cutoff allowed for the classification of the 111 isolates into four pathogenicity groups (PG I, PG II, PG III, and PG IV) (Fig. 3). PG I included 80

Table 3. Eight primer-pair combinations and the number of polymorphic amplified fragment length polymorphism markers generated in genetic diversity analysis of *Curvularia lunata* isolates

Primer combination	Number of loci	Number of polymorphic loci	Percentage of polymorphic loci (%)
E+AA/M+CT	100	95	95.00
E+AC/M+CA	98	85	86.73
E+AC/M+CC	88	76	86.36
E+AC/M+CT	90	76	84.44
E+AG/M+CT	85	67	78.82
E+AG/M+CG	112	96	85.71
E+AT/M+CC	90	79	87.78
E+AT/M+CT	88	83	94.32
Total	751	657	87.48

Table 4. Primers used in polymerase chain reaction for the mating type genes in *Curvularia lunata*

Primer	Nucleotide sequences (5'–3')	Temperature (°C)	Target fragment length (bp)
<i>ClMat1-F</i>	CACTTTGGTCTTTGATGGC	65	796
<i>ClMat1-R</i>	AGAGCGACATCTTCGTTG	...	...
<i>ClMat2-F</i>	CATCCGCTCAGACATCGC	62	698
<i>ClMat2-R</i>	AGCAGTCCATCGTCAATCTC	...	...

Fig. 2. Symptoms of disease levels 1, 3, 5, 7, and 9. Column 1, disease level 1 = no symptoms or small brown spots; column 3, disease level 3 = lesions with small necrotic centers; column 5, disease level 5 = round or oval yellowish chlorotic lesions, greyish-white centers, with yellow or red-brown margins with a yellow halo; column 7, disease level 7 = as before but with some lesions joining together to form large necrotic areas; and column 9, disease level 9 = lesions cover the entire leaf.

isolates from Liaoning, Heilongjiang, Jilin, Yunnan, Sichuan, Shandong, Hebei, and Hubei Provinces and an unknown location; the majority of isolates were pathotype I, excluding 15LNDG186-12-3 and another 18 isolates. PG II included six isolates from Liaoning, which were pathotype I and pathotype II. PG III included two isolates from Liaoning and one from an unknown location, which were pathotype I and pathotype II. PG IV included 23 isolates from Liaoning, Yunnan,

Anhui, and Sichuan Provinces, most of which were pathotype I, except for 14AH108-2-2 and another 4 isolates.

Genetic diversity. Eight primer-pair combinations that yielded clear, reproducible, and highly polymorphic banding patterns were screened (Fig. 4). Each primer pair produced 85 to 112 DNA bands with sizes ranging from 30 to 600 bp among the 111 isolates. There was a total of 657 polymorphic bands (87.48%) (Table 4). The 111

Fig. 3. Unweighted pair-group method using the arithmetic mean dendrogram of the virulence diversity of 111 *Curvularia lunata* isolates on 9 differential host lines by NTSYS software. Four *C. lunata* clusters were resolved at the 66% similarity level to form four pathogenicity groups (PGs): PG I, PG II, PG III, and PG IV. The dendrogram was supported by cophenetic correlation test of 1,000 permutation.

isolates were clustered as two groups (GD I and GD II) using UPGMA, with the average similarity coefficient of 78% (Fig. 5). The GD I group included 109 isolates from Hebei, Liaoning, Jilin, Heilongjiang, Shandong, Yunnan, Sichuan, Hubei, and Anhui. The GD II comprised two isolates which were collected from Liaoning. GD I was further divided into 21 subgroups (A to U) at 88% similarity. Then, the GD I-A subgroup was further divided into eight subgroups (1 to 8) at a 91% similarity. The dendrogram data were

supported by a cophenetic correlation test of 1,000 permutations. The cophenetic correlation of the dendrogram with the data matrix was 0.92.

Correlation between pathogenicity and genetic diversity. The binary data of pathogenicity and genetic diversity were converted into similarity matrices. Then, the two similarity matrices were used to analyze the correlation between the pathogenicity and genetic diversity using MXCOMP analysis in the NTSYSpc 2.1 software. The correlation

Fig. 4. Amplified fragment length polymorphism fingerprints of *Curvularia lunata* isolates after selective amplification with primer combination E+AT/M+CT. Left: 100 to 600 indicate 100 to 600 bp of ladder.

coefficient was found to be 0.17, which demonstrated a relatively weak correlation between pathogenicity and genetic diversity.

Frequency and distribution of mating types. The results of amplification of homologous fragments of the *CIMAT* gene showed that there were two mating types in *C. lunata*: mating type I and mating type II. Among all of the isolates, mating type I included 48 isolates (43.24%) from Liaoning, Hebei, Sichuan, Shandong, and Yunnan, while mating type II included 61 isolates (54.95%) from Liaoning, Hebei, Jilin, Anhui,

Heilongjiang, Hubei, Yunnan, and an unknown location. The mating type of two isolates from Liaoning could not be determined. Mating type frequencies in the samples were found in near-equal proportions in Liaoning. The ratio of mating types I and II for all isolates was about 3:4.

Discussion

Our study provides a description of the pathotypes, genetic diversity, and geographical distribution of *C. lunata* in the major

Fig. 5. Unweighted pair-group method using the arithmetic mean dendrogram of the genetic diversity of 111 *Curvularia lunata* isolates based on DNA banding patterns revealed by amplified fragment length polymorphism (AFLP) analysis. Two *C. lunata* clusters were resolved at the 78% similarity level to form two AFLP genetic diversity groups (GDs): GD I and GD II. The GD I group was divided into 21 subgroups (A to U) at 88% similarity. The GD I-A subgroup was divided into eight subgroups (1 to 8) at 91% similarity. The dendrogram was supported by cophenetic correlation test of 1,000 permutations.

maize-growing regions in China. The degree of correlation between pathotype and geographical distribution was low. Genetic diversity was also not closely related to virulence or geographical distribution.

In the present study, we used differential inbred lines to characterize the pathotype of *C. lunata* isolates. Four to six pathotypes were identified according to this method (Chen et al. 2003; Xiao et al. 2006; Xue et al. 2008). However, pathotype specification could not be accomplished completely using differential inbred lines. In this study, isolates were grouped into pathotypes based on their mean DI. Most isolates fell consistently into one of the four groups but a few did not. For example, 76.25% of isolates in PG I were pathotype I but 15LNDG186-12-3 in PG I belonged to pathotype III. Pathotype group by means of mean DI presents high or low virulence of isolates in *C. lunata* populations.

Isolates of pathotype I represented 76% of Liaoning isolates and all Hebei isolates. Moreover, isolates of Yunnan were classified as pathotype I or pathotype II. Isolates of pathotype III were only from Liaoning Province. These results suggest that the distribution of pathotypes may have a certain correlation with geographic sources. To avoid errors caused by low sample number, we further analyzed the distribution of pathotypes in Liaoning. In all, 37 of the 46 isolates (80%) from Fengcheng, 14 of 20 isolates (70%) from Dalian, and 12 of 15 isolates (80%) from Dandong were classified as pathotype I. There is a certain correlation between pathotype and geographic location in Liaoning.

In this study, we used AFLP analysis to characterize the genetic variation among *C. lunata* populations. According to the cluster analysis, the 111 *C. lunata* isolates were divided into two main groups, with a further division of 21 subgroups in GD I with eight subclusters (GD I-A). In our study, the diversity level was as high as shown in previous studies of *C. lunata* where RAPD or UP-PCR was used to measure diversity (Dou and Jin 2007; Wang et al. 2014; Zhang et al. 2010). High levels of genetic diversity among plant-pathogenic fungi are common. In our case, recombination may play an important role in genetic diversity but a larger collection of isolates from this region should be analyzed to test this hypothesis (Sun et al. 2007).

Low correlation was found between virulence pattern and AFLP pattern. To some extent, AFLP groups were demonstrated to correspond with virulence groups. For example, isolates of the GD I-A-1 cluster belonged to pathotype I; two of three isolates belonged to pathotype II in the GD I-A-2 cluster; and isolates in GD II-I, GD II-J, GD II-K, GD II-L, GD II-M, GD II-N, GD II-O, GD II-P, GD II-Q, GD II-R, GD II-S, GD II-T, and GD II-U subgroups all belonged to pathotype I.

We compared the similarity matrices to further analyze the correlation between virulence and genetic diversity. The statistical index ($r = 0.017$) was found to be lower compared with other published results (Kolmer 2001; Kolmer et al. 1995; Sun et al. 2007). Similarly, Chen et al. (1993) found that the molecular polymorphism was independent of host selection for virulence polymorphism. Correlation between virulence and the AFLP group is usually low in a sexually reproducing populations (Burdon and Roelfs 1985). However, in asexual populations, the correlation between virulence and AFLP group is generally higher due to their isolated clonal reproduction (Burdon and Roelfs 1985; Levy et al. 1993). The reason for the low or high correlation between the virulence similarity matrix and AFLP similarity matrix in plant-pathogenic fungi is unknown.

The sexual stages of *C. lunata* (teleomorph: *Cochliobolus lunatus*) have been observed in the laboratory but not in nature (Lu et al. 2018). Due to the generation of new genotypes in sexual reproduction, sexual recombination may be a potentially key factor in affecting population dynamics (McDonald and Linde 2002; Milgroom 1996). The single MAT controls sexual reproduction. It is essential to understand the evolution of the mating system and pathogenicity in plant-pathogenic fungi (Turgeon 1998). In this study, the mating type of *Curvularia lunata* was weakly correlated with virulence, genetic diversity, and geographic origin.

In summary, genetic variation of *C. lunata* among and between pathotypes in 15 maize-growing regions revealed that the pathogen

population had a high genotypic diversity and that the distribution of pathotypes had a low correlation with geographic locations. Cluster analysis revealed low correlation between pathotypes and AFLP groups. Some isolates collected from different regions were clustered into the same AFLP group.

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