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Identification mating-type locus structure and distribution of *Cochliobolus lunatus* in China

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Abstract *Cochliobolus lunatus* (teleomorph: *Curvularia lunata*) is an important plant pathogenic fungus that causes the maize foliar spot, resulting in serious yield losses. In ascomycetes, a single mating-type (*MAT*) locus with two idiomorphs controls sexual development. The structure and arrangement of the *MAT* genes were examined to understand the *MAT* locus of *C. lunatus*. *MAT* loci were *MAT1–1-1* or *MAT1–2-1*, flanked upstream and downstream by regions encoding GTPase activating protein, pyridoxamine phosphate oxidase domain, and β-glucosidase. A *MAT1–1* or *MAT1–2* idiomorph was identified in single isolate, and sexual reproduction in vitro indicated that the species was heterothallic. In vitro crossing between isolates with opposite *MAT*s produced perithecia, asci, and ascospores. A multiplex *MAT*-specific PCR method was developed and used to test mating-type genes in 177 *C.lunatus* isolates collected from China. The ratio of isolates of each mating-type in China was consistent with a 1:1 ratio.

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Introduction

Cochliobolus lunatus R.R.Nelson & F.A.Haasis [anamorph: *Curvularia lunata* (Wakker) Boedijn], the causal agent of Curvularia leaf spot of maize, is widely distributed worldwide (Macri and Lenna 1974). In China, Curvularia leaf spot of maize was firstly reported in 1984 and primarily found in the Northwest corn producing regions (Duan 1984). In 1996, loss of grain yield caused by Curvularia leaf spot of maize was estimated to be 2.5×10^5 tons in Liaoning Province (Lu et al. 1997). Curvularia leaf spot of maize have rapidly become one of the most important foliar diseases in corn-cultivating regions in the past two decades (Wang et al. 2006). The genus *Cochliobolus* is an important group of ascomycete fungi, including the maize pathogens *C. heterostrophus*, *C. carbonum*, and *C. lunatus*, the wheat/barley/cereal pathogen *C. sativus*, oat pathogen *C. victoriae*, and rice pathogen *C. miyabeanus*. Genomes of All these pathogens have been sequenced (JGI:http://genome.jgi.doe.gov/programs/fungi/indes.jsf) (Gao et al. 2014; Zhang et al. 2016).

Sexual compatibility is controlled by a single mating-type (*MAT*) locus with two idiomorphs in ascomycetes. In homothallic species, both idiomorphs are located within an individual often at the same locus, with few exceptions (Paoletti et al. 2007; Yun et al. 1999). By contrast, each individual in heterothallic species carries

a single *MAT* locus. This locus is unique, because the two alternate forms (*MAT1–1* and *MAT1–2*) have completely dissimilar sequences located in the same locus on the chromosome (Yun et al. 1999). *MAT1–1* is identified by a gene *MAT1–1-1* that encodes a protein containing alpha-box DNA binding domain, whereas *MAT1–2* is identified by a gene *MAT1–2-1* that encodes a protein with high mobility group (HMG) DNA binding domain (Brewer et al. 2011; Coppin et al. 1997; Pearce et al. 2016; Turgeon 1998).

The genus *Cochliobolus* employs various sexual reproductive strategies. *C. luttrellii, C. homomorphus, C. cymbopogonis* and *C. kusanoi* are homothallic species, while *C. ellisii, C. heterostrophus, C .intermedius, C. miyabeanus, C. sativus, C. setariae,* and *C. victoriae* are heterothallic species (Christiansen et al. 1998; Yun et al. 1999; Zhong and Steffenson 2001). *C. lunatus* is also heterothallic (Nelson and Haasis 1964). Although the genomes of strains m118 (sorghum) and CX-3 (maize) of *C.lunatus* had been sequenced,the *MAT1–1* and *MAT1–2* idiomorphs have not been identified yet. A thorough description of the *MAT1–1* and *MAT1–2* required comparison of sequences from both mating type to determine where idiomorphs end and homologous flanking regions begin. Identification of mating-type genes in *C. lunatus* would be useful for comparative studies on the structure and evolution of the *MAT1* locus within the *Cochliobolus*, and among other ascomycetes.

Resurgence of Curvularia leaf spot of maize caused serious damages in several maize-producing regions in Liaoning, Anhui and Henan, China (Gao et al. 2014; Zhang et al. 2016). The increase in incidence of Curvularia leaf spot of maize has raised a question on the possible role of sexual reproduction in the incidence of Curvularia leaf spot of maize. The objectives of this study were to: (1) identify genes and sequences in idiomorphs at the *MAT1–1* and *MAT1–2* loci in *C.lunatus*, (2) pair *MAT1–1* and *MAT1–2* strains to produce asci and ascospore, and (3) determine if mating types are present in 1:1 ratio, which are expected under random mating, in *C. lunatus* populations from different regions in China.

Materials and methods

Maize sampling and fungal isolation

Maize plants exhibiting typical symptoms of Curvularia leaf spot of maize were collected in August between 2010 and 2016 in China, namely, Heilongjiang, Jilin, Liaoning, Hebei, Henan, Shandong, Hubei, Anhui, Sichuan, and Yunnan. These provinces had serious events of Curvularia leaf spot of maize in 2010–2016. The majority of samples were collected from Liaoning province (Table 1).

Symptomatic leaves were excised from the junction of diseased and healthy tissue to 0.5 cm^2 pieces to isolate *C. lunatus* from maize tissue. The samples were washed in distilled water, disinfected for 30 s in 70% ethanol and 1 min in 0.1% mercuric chloride, and rinsed twice in sterile distilled water. A subsection was cut from each sterilized piece, placed on a plate of potato dextrose agar (PDA; Hangzhou Baisi Biotechnology Co., Ltd., Hangzhou, China), and incubated at 25 °C for 4–5 days to produce spores in the dark.

A spore suspension was filtered through four layers of lens tissue to remove hyphal fragments, and serially diluted and evenly plated out over the agar plate surface. After incubating for 3–5 h, individually germinated spores were picked from the plate using a sterile needle with dissecting microscope, and the spores were subcultured at 25 °C for 7 days on PDA. All isolates were stored at −80 °C in 20% glycerol. Finally, 177 *C. lunatus* isolates were obtained for genomic DNA extraction.

DNA extraction, PCR amplification, and sequencing

Mycelia from each single-spore isolate were scraped from the surface of cultures grown on PDA. The tissue was ground in liquid nitrogen with sterile pestle and mortar. Genomic DNA was extracted from the mycelia using TIANGEN DNAsecure Plant Kit [Tian Gen Biochemical Technology (Beijing) Co., Ltd., Beijing, China], following the manufacturer's instruction. DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, MA), and the concentration was adjusted to 10 ng/μL in sterilized water for PCR.

The PCR was performed in a volume of 20 μL. The reaction mixture contained 2.0 μL of 10× PCR buffer, 2.0 μL of dNTPs (2.5 mM each), 1.0 μL each of 10 μM forward and reverse primers, 0.5 U Taq polymerase, and 1.0 μL (10 ng) of genomic DNA template. All PCR reagents are from TaKaRa (Dalian, China). All amplifications were performed in a PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA), and the cycling programs were as follows: initial denaturation at 94 °C

Table 1 *Cochliobolus* isolates used for mating-type analysis

Isolate	Year collected	Host origin	Geographic origin(location/province)	Source
HLJ-2	2014	Leaf/corn	Jiamushi /Heilongjiang	F.Z.Zhao
HLJ-3	2014	Leaf/corn	Jiamushi /Heilongjiang	F.Z.Zhao
HLJ-4	2014/	Leaf/corn	Shuangyashan/Heilongjiang	F.Z.Zhao
HLJ-5	2014	Leaf/corn	Shuangyashan /Heilongjiang	F.Z.Zhao
HLJ-6	2014	Leaf/corn	Shuangyashan /Heilongjiang	F.Z.Zhao
JL-2	2014	Leaf/corn	Jilin	Q.F.Shu
JL-4	2014	Leaf/corn	Baishan/Jilin	Q.F.Shu
JL-5	2014	Leaf/corn	Gongzuling/Jilin	Q.F.Shu
JL-6	2014	Leaf/corn	Changchun/Jilin	Q.F.Shu
JL-7	2014	Leaf/corn	Baishan/Jilin	Q.F.Shu
FC-1	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-2	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-3	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-4	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-5	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-6	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-7	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-8	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-9	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-10	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-11	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-12	2013	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-13	2012	Leaf/corn	Fengcheng/Liaoning	K.X.Liu
FC-14	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-15	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-16	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-17	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-18	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-19	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
FC-20	2012	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
DL-1	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
DL-2	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
DL-3	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
FK-1	2012	Leaf/corn	Faku/Liaoning	L.Zhang
SY-1	2012	Leaf/corn	Shenyang/Liaoning	L.Zhang
DL-4	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
DL-5	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
SY-2	2012	Leaf/corn	Shenyang/Liaoning	L.Zhang
CX-3	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
DL-7	2012	Leaf/corn	Dalian/Liaoning	L.Zhang
FC-21	2012	Leaf/corn	Fengcheng/Liaoning	L.Zhang
FC-22	2010	Leaf/corn	Fengcheng/Liaoning	L.Zhang
FC-23	2010	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
TL-1	2010	Leaf/corn	Tieling/Liaoning	Y.Y.Lu

Table 1 (continued)

Isolate	Year collected	Host origin	Geographic origin(location/province)	Source
FC-24	2010	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
TL-2	2010	Leaf/corn	Tieling/Liaoning	Y.Y.Lu
SY-3	2010	Leaf/corn	Shengyang/Liaoning	Y.Y.Lu
SY-4	2010	Leaf/corn	Shengyang/Liaoning	Y.Y.Lu
TL-3	2010	Leaf/corn	Tieling/Liaoning	Y.Y.Lu
FC-25	2010	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
TL-4	2010	Leaf/corn	Tieling/Liaoning	Y.Y.Lu
SY-5	2010	Leaf/corn	Shenyang/Liaoning	Y.Y.Lu
FC-26	2010	Leaf/corn	Fengcheng/Liaoning	Y.Y.Lu
CT-1	2010	Leaf/corn	Changtu/Liaoning	L.Zhang
SY-6	2010	Leaf/corn	Shenyang/Liaoning	L.Zhang
DL-8	2010	Leaf/corn	Dalian/Liaoning	L.Zhang
DL-9	2010	Leaf/corn	Dalian/Liaoning	L.Zhang
FC-27	2010	Leaf/corn	Fengcheng/Liaoning	L.Zhang
CT-2	2010	Leaf/corn	Changtu/Liaoning	L.Zhang
DL-10	2010	Leaf/corn	Dalian/Liaoning	L.Zhang
FC-28	2010	Leaf/corn	Fengcheng/Liaoning	L.Zhang
DL-11	2010	Leaf/corn	Dalian/Liaoning	L.Zhang
FC-29	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-30	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-31	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-32	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-33	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-34	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
ZD958	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-36	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-37	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
SY-7	2014	Leaf/corn	Shenyang/Liaoning	G.F.Li
TL-5	2014	Leaf/corn	Tieling/Liaoning	G.F.Li
FC-38	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-39	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
AS-1	2014	Leaf/corn	Anshan/Liaoning	G.F.Li
FC-40	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
DL-12	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
FC-41	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-42	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-43	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-44	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
DL-13	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-14	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-15	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
FC-45	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-46	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li

Table 1 (continued)

Isolate	Year collected	Host origin	Geographic origin(location/province)	Source
FC-47	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-48	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-49	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
DL-16	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
FC-50	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-51	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-52	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-53	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
SY-8	2014	Leaf/corn	Shenyang/Liaoning	G.F.Li
FC-54	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
DL-17	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
XM-1	2014	Leaf/corn	Xinmin/Liaoning	Z.G.Gao
XM-2	2014	Leaf/corn	Xinmin/Liaoning	Z.G.Gao
SY-9	2014	Leaf/corn	Shenyang/Liaoning	Z.G.Gao
SY-10	2014	Leaf/corn	Shenyang/Liaoning	Z.G.Gao
TL-6	2014	Leaf/corn	Tieling/Liaoning	Z.G.Gao
TL-7	2014	Leaf/corn	Tieling/Liaoning	Z.G.Gao
TL-8	2014	Leaf/corn	Tieling/Liaoning	Z.G.Gao
TL-9	2014	Leaf/corn	Tieling/Liaoning	Z.G.Gao
FC-55	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-56	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
DL-18	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-19	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
TL-10	2014	Leaf/corn	Tieling/Liaoning	G.F.Li
FC-57	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-58	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-59	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-60	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
FC-61	2014	Leaf/corn	Fengcheng/Liaoning	G.F.Li
TL-11	2014	Leaf/corn	Tieling/Liaoning	G.F.Li
DL-20	2014	Leaf/corn	Dalian/Liaoning	G.F.Li
SY-11	2014	Leaf/corn	Shenyang/Liaoning	F.Z.Zhao
LN-1	2014	Leaf/corn	Liaoning	F.Z.Zhao
DL-21	2015	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-22	2015	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-23	2015	Leaf/corn	Dalian/Liaoning	G.F.Li
DL-24	2015	Leaf/corn	Dalian/Liaoning	G.F.Li
DD-1	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-2	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-3	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-4	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-5	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-6	2015	Leaf/corn	Donggang/Liaoning	G.F.Li

Table 1 (continued)

Isolate	Year collected	Host origin	Geographic origin(location/province)	Source
DD-7	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-8	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-9	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-10	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-11	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
AS-2	2015	Leaf/corn	Anshan/Liaoning	G.F.Li
XM-3	2015	Leaf/corn	Xinming/Liaoning	G.F.Li
CT-3	2015	Leaf/corn	Changtu/Liaoning	G.F.Li
DD-12	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DL-25	2015	Leaf/corn	Dalin/Liaoning	G.F.Li
AS-3	2015	Leaf/corn	Anshan/Liaoning	G.F.Li
LN-2	201	Leaf/corn	Liaoning	L.Zhang
LN-3	2011	Leaf/corn	Liaoning	L.Zhang
DD-13	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-14	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
DD-15	2015	Leaf/corn	Donggang/Liaoning	G.F.Li
HB-1	2012	Leaf/corn	Baoding/Hebei	L.Zhang
HB-2	2012	Leaf/corn	Baoding/Hebei	L.Zhang
HB-4	2011	Leaf/corn	Tangshan/Hebei	L.Zhang
HB-5	2014	Leaf/corn	Baoding/Hebei	F.Z.Zhao
HB-6	2016	Leaf/corn	Shunping/Hebei	Z.Y.Cao
HN-1	2013	Leaf/corn	Zhumadian/Henan	J.Shi
HN-2	2013	Leaf/corn	Xuchang/Henan	J.Shi
HN-5	2013	Leaf/corn	Pingdingshan/Henan	J.Shi
HN-6	2013	Leaf/corn	Suzhou/Henan	J.Shi
HN-8	2013	Leaf/corn	Shangqiu/Henan	J.Shi
HN-9	2013	Leaf/corn	Zhumadian/Henan	J.Shi
HN-12	2014	Leaf/corn	Kaifeng/Henan	H.Zhao
HN-13	2014	Leaf/corn	Luoyang/Henan	F.Z.Zhao
SD-1	2014	Leaf/corn	Liaocheng/Shandong	G.Q.Wang
SD-3	2014	Leaf/corn	Liaocheng/Shandong	G.Q.Wang
SC-1	2014	Leaf/corn	Nianchong/Sichuan	X.F.Zhang
SC-3	2015	Leaf/corn	Yaan/Sichuan	X.F.Zhang
SC-4	2015	Leaf/corn	Yaan/Sichuan	X.F.Zhang
SC-7	2015	Leaf/corn	Yaan/Sichuan	X.F.Zhang
SC-8	2015	Leaf/corn	Aba/Sichuan	X.F.Zhang
AH-1	2014	Leaf/corn	Jianxian/Anhui	Z.G.Gao
AH-2	2014	Leaf/corn	Jianxian/Anhui	Z.G.Gao
AH-3	2016	Leaf/corn	Xiaoxian/Anhui	Z.Y.Cao
AH-4	2016	Leaf/corn	Suzhou/Anhui	Z.Y.Cao
YN-1	2015	Leaf/corn	Dali/Yunnan	X.F.Zhang
YN-2	2015	Leaf/corn	Linchang/Yunnan	X.F.Zhang
YN-3	2015	Leaf/corn	Dehong/Yunnan	X.F.Zhang
YN-4	2015	Leaf/corn	Dehong /Yunnan	X.F.Zhang

Table 1 (continued)

Isolate	Year collected	Host origin	Geographic origin(location/province)	Source
YN-5	2015	Leaf/corn	Dehong /Yunnan	G.F.Li
YN-6	2015	Leaf/corn	Dehong /Yunnan	G.F.Li
HuB-2	2015	Leaf/corn	Xiangyang/Hubei	X.F.Zhang

for 2 min, followed by 35 cycles of 94 °C for 30 s, at annealing temperature for 30 s, and 72 °C for 0.5–3 min, followed by final extension at 72 °C for 5 min.

All PCR products were purified by gel electrophoresis in a 1.0% agarose gel. All the fragments of the predicted length were excised and purified with Axyprep DNA Gel extraction kit (Axygen Scientific Inc., Tewksbury, MA) and cloned into the pMD19-T vector (Takara Bio Inc., Dalian, China) following the manufacturer's instructions. Plasmids were extracted using the TIANprep Mini Plasmid Kit [TianGen Biochemical Technology (Beijing) Co., Ltd., Beijing, China] and sequenced with forward (M13RV-M) and reverse primers (M13F-47) by Genewiz Co. Ltd. (Suzhou, China) using the dideoxy chain termination method on the ABI 3730 Genetic Analyzer (PerkinElmer, Inc., Foster City, CA).

Consensus sequences were obtained from pairwise alignment of forward and reverse sequences using DNAMAN 8 (Lynnon Corporation, San Ramon, CA) and Gene Runner (www.softpedia.com) software.

Identification of the *MAT* idiomorphs and flanking sequence

The nucleotide sequences of *MAT1–1-1* and *MAT1–2-1* of *C. ellisii* (GenBank: AF129746.1 and AF129747.1) were used as starting points to search for *MAT1–2* or *MAT1–1* in the genomic sequence of the *C. lunatus* strain CX-3 (GenBank:JFHG01001253.1). A search of scaffold 19 (GenBank:KN050451.1) in the identified strain CX-3 revealed sequences with 85% identity to a *MAT1–2* idiomorph. Strain CX-3 with putative *MAT1–2-1* gene was used in the PCR to amplify the entire *MAT* gene for sequencing. Based on this sequence, primers in the HMG domain were designed to amplify *MAT1–2* by PCR from the genomic DNA of *C. lunatus* (Supplementary Fig. 1). The initial PCR used primers ClMat2-F and ClMat2-R to amplify a 698 bp fragment (Supplementary Table 1). The PCR was executed with annealing temperature at 62 °C and extension time of 1 min. The subsequent PCR with primer pairs ClMat2-UF/ClMat2-UR and ClMat2-DF/ClMat2-DR amplified *MAT1–2-1* in two overlapping fragments. *MAT1–2-1* gene was amplified by PCR with annealing temperature at 60/65 °C and extension time of 1/1.5 min. The three overlapping PCR products of *MAT1–2-1* gene were sequenced and combined. The sequences were compared with sequences in the GenBank database using BLASTn to confirm the amplification of the gene homologous to *MAT1–2-1*. The HMG domain of *MAT1–2* isolates was amplified and sequenced using specific primer ClMat2T-F/ClMat2T-R. Nucleotide sequences of *MAT1–2-1* gene in *C. lunatus* had 86% similarity to *C. ellisii*.

The nucleotide sequences of *MAT1–1-1* of *C. ellisii* (GenBank:AF129746.1), *C. heterostrophus* (GenBank:X68399.1), *C. victoriae* (GenBank:AF032369.1), *C. sativus* (GenBank:AF275373.1), *C. carbonum* (GenBank:AF032368.1), and *C. miyabeanus* (GenBank:HQ123463.1) were used as templates to design the primer ClMat1-F/ClMat1-R to screen candidate isolates containing *MAT1–1-1* gene. Conditions for amplification included an extension time of 1 min at annealing temperature of 65 °C. Strain ZD958 with putative *MAT1–1-1* gene was used in the PCR to amplify the entire *MAT1–1* gene for sequencing (Supplementary Fig. 1). The initial PCR amplified a 796 bp fragment. The subsequent PCR with primer pairs ClMat1-UF/ClMat1-UR and ClMat1-DF/ClMat1-DR amplified *MAT1–1-1* in two overlapping fragments (Supplementary Table 1). The PCR annealing temperature at 55 °C and extension time of 2.0/1.5 min was performed to amplify *MAT1–1-1*. The three overlapping PCR products of *MAT1–1-1* were sequenced and concatenated. The sequences were compared with sequences in the GenBank database using BLASTn to confirm the amplification of gene homologous to *MAT1–1-1*. The alpha box domain of *MAT1–1* isolates was

amplified and sequenced using specific primer ClMat1T-F/ClMat1T-R.

To identify the putative genes upstream of the *MAT* genes, the GTPase activator-like protein (GAP) and protein with pyridoxamine 5′-phosphate oxidase domain (ORF1) genes of *C. heterostrophus* strain C5 (GenBank:AF029913.1) and C4 (GenBank:AF027687.1), which resided upstream of the *MAT* gene in *C. heterostrophus* (Turgeon et al. 1993), were used to search the database of *C. lunatus* strain CX-3 (GenBank:JFHG01001253.1) for homologous sequences. A search of the scaffold 19 (KN050451.1) in strain CX-3 showed 81% sequences homologous to each of the *GAP* and *ORF1* genes. The entire *GAP* and *ORF1* of *MAT* were amplified and sequenced with primer pairs ClMatg-F/ClMatg-R and ClMato-F/ClMato-R at annealing temperature of 55/60 °C.

To obtain the nucleotide sequence of two nucleotides spanning the partial sequences, three primer pairs, namely, ClMatgU-F/ClMatoD-R, ClMatoU-F/ClMat1I-R, and ClMat2I-R, were designed to target the partial sequences of *GAP*, *ORF1*, and *MAT1–2-1/MAT1–1-1*, respectively. The primers were used to amplify and sequence the nucleotides spanning the partial sequences of *GAP* and *ORF1*, as well as *ORF1* and *MAT1–2-1*/*MAT1–1-*, in *C. lunatus* isolates CX-3 and ZD958. The PCR included 30 cycles with annealing temperature at 59/57/58 °C and extension time of 2 min. The two overlapping PCR products and amplicons of each gene were combined. The sequences were compared with sequences in the GenBank database using Seqman (DNASTAR) to confirm the amplification of genes homologous to *GAP* and *ORF1*.

To identify the putative gene downstream of the *MAT* genes, the *β-glucosidase* gene of *C. heterostrophus*, which resided downstream of the *MAT* gene in *C. heterostrophus* strains C5 (GenBank:AF029913.1) and C4 (GenBank:AF027687.1), was used to search the database of *C. lunatus* strain CX-3 (GenBank:JFHG01001253.1) for homologous sequences. A search of the scaffold 19 (KN050451.1) in strain CX-3 showed 85% sequences homologous to each of the *β-glucosidase*. To sequence the entire *β-glucosidase* of *C. lunatus* and confirm its location downstream from the *MAT* genes, primer (ClMatg1U–F/ClMatg1D-R) was designed in the mutual conserved region downstream of the *MAT* genes. The entire *β-glucosidase* of *MAT1–2* and *MAT1–1* isolates were amplified and sequenced at annealing temperature of 61 °C. To obtain the nucleotide sequence spanning the partial sequences, primers ClMat1–1-1 U-F and ClMat1–2-1 U-F/ClMatgD-R were designed to target the partial sequences of *β-glucosidase* and *MAT1–2-1*/*MAT1–1-1* genes, respectively. The primers were used to amplify and sequence the nucleotides spanning the partial sequences of *β-glucosidase* and *MAT1–2-1*/*MAT1–1-1* in *C. lunatus* isolates CX-3 and ZD958. The PCR included 30 cycles with annealing temperature at 58/61 °C and extension time of 2 min. The two overlapping PCR products and amplicons of each gene were combined. The sequences were compared with sequences in the GenBank database using Seqman (DNASTAR) to confirm the amplification of genes highly homologous to *β-glucosidase*.

To obtain the *MAT* idiomorphs and flanking genes, sequences were combined for *C. lunatus* isolates using de novo assembly to generate complete nucleotide sequence. The *GAP*, *ORF*, *MAT*, and *β-glucosidase* genes and the intergenic regions between these genes of the *C. lunatus* isolates were aligned using DNAMAN to identify sequence similarity within and between isolates of each *MAT*. In addition, the sequences were compared with other fungal species in the GenBank using BLASTn. Sequences were deposited in the GenBank.

Crosses to determine *MAT* phenotype in *C. lunatus*

The *MAT* phenotypes of isolates of *C. lunatus* in Liaoning were determined for comparison in the molecular genotyping by PCR. Each isolate was tested for *MAT* by crossing with a *MAT1–1* or *MAT1–2* isolate using techniques described by Nelson (1959). *C. lunatus* isolates of known *MAT* were plated onto PDA and incubated in the dark at 25 °C for 7 days. Dry sterile corn leaf was embedded in Sach's agar. Crossing involved the transfer of plugs of PDA agar from the parental cultures of each isolate and either of the isolates designated as *MAT1–1* or *MAT1–2* to opposite sides of the corn leaf. All cultures were mated with both *MAT*s. The plates were incubated at 25 °C for 21 days, and the presence of perithecia on the corn leaf was considered a compatible cross. Then, the number of perithecia from each mating was counted, and some mature perithecia were individually crushed in a drop of water and examined for asci and ascospores under

a dissecting microscope. As a control, all isolates were also paired with themselves.

Analysis of MAT distributions in the populations of *C. lunatus* in Liaoning

A multiplex *MAT*-specific PCR assay, consisting of a single reverse primer and two forward primers, was designed to rapidly identify the *MAT* of *C. lunatus* isolates. The reverse primer ClMatspe-R was combined with the *MAT*-specific forward primers ClMat1T-F and ClMat2-DF. The forward primers were designed to anneal within the CDS of each *MAT* gene to differentiate PCR products of 1582 and 932 bp for *MAT1–1* and *MAT1–2* individuals, respectively. The primers were tested as pairs and as a multiplex against a subset of *C. lunatus* isolates of known *MAT* to confirm their ability to differentiate the *MAT*. The PCR contained 0.15 μM each primer and consisted of 30 cycles and an extension time of 75 s. The multiplex PCR was used to identify the *MAT* of isolates within the field population of *C. lunatus*. Amplified products were visualized as previously described, and the *MATs* of isolates were identified based on the PCR product length.

Results

Identification and arrangement of the *MAT* idiomorphus

The predicted *MAT1–1-1* (GenBank:KU749293) of *C. lunatus* was 1134 bp, possessed a single intron of 53 bp within the alpha domain at the nucleotide position from 218 bp to 270 bp, and encoded a protein of 378 amino acid (aa). Blastn analysis of *MAT1–1-1* identified had the highest similarity to the *MAT1–1-1* of heterothallic *C. ellisii* (GenBank:AF129746, 85% sequence homology) (Fig. 1A).

The predicted *MAT1–2-1* (GenBank:KU749294.1) of *C. lunatus* was 1087 bp, possessed one single intron (55 bp long) within HMG domain at nucleotide positions 488–542 bp, and encoded a protein of 343 aa. Blastn analysis of *MAT1–1-1* identified had the highest similarity to the *MAT1–1-1* of heterothallic *C. ellisii* (GenBank:AF129747, 86% sequence homology) (Fig. 1B).

The highly conserved mutual regions were found upstream of the start codon of *MAT1–1-1* and *MAT1–2-1*. ORF1 encoding a protein homologous to the domain of pyridoxamine phosphate oxidase-like protein of *C. ellisii* (AAD33450.1, 95% sequence homology) and ORF1 proteins of *C. heterostrophus* (GenBank:AAB82944.1, 85% sequence homology) were identified. In *C. lunatus*, the *ORF1* resided further upstream from the *MAT* gene start codon in *MAT1–1* (1095 bp) and *MAT1–2* (1106 bp) isolates. In addition, the *ORF1* nucleotide sequences of *C. lunatus MAT1–1* and *MAT1–2* strains had 99% homology. The highly conserved mutual regions were found between *GAP* and *ORF1*, as well as *ORF1* and *MAT1–1-1*/*MAT1–1-2*. The *GAP* gene encodes a protein homologous to the GTPase activating protein (GAP) of *C. heterostrophus* (GenBank:AAB82943.1, 96% aa sequence homology at 88% coverage). The predicted *GAP* of *MAT1–1* and *MAT1–2* was 2388 bp, with six introns of 49,79, 47, 55, 95, and 97 bp (corresponding nucleotide positions 113–161 bp, 442–520 bp,1286–1332 bp, 1942–1996 bp, 2203–2297 bp, and 2302–2388 bp), and encoded a protein of 649 aa. A highly conserved mutual region located between *MAT1–1-1*/*MAT1–2-1* and beta glucosidases (BGL1) was found downstream of the two *MAT* idiomorphs. The conserved region varied in length from 2386 bp to 2401 bp for *MAT1–1* and *MAT1–2* isolates, respectively. The region between *MAT1–1* and *MAT1–2* isolates was more highly conserved (98%). The *BGL1* gene encoded a protein homologous to the beta glucosidases of BGL1 of *C. heterostrophus* (GenBank:AAB82946.1; 93% aa sequence homology). The *BGL1* constituted 2852 bp, with 2 introns of 51 and 27 bp (corresponding nucleotide positions of 541–592 bp and 813–940 bp), and encoded a protein of 849 aa. The isolates of *MAT1–1* and *MAT1–2* had identical encoded BGL1 protein sequences.

Alignment of the *MAT* idiomorphs and flanking genes resulted in sequences of 10.89 kb for *MAT1–1* (GenBank:KY471562) isolates and 10.81 kb for *MAT1–2* (GenBank:KY471563) isolates (Fig. 2). The sizes of the idiomorph, which was determined by the length of dissimilar nucleotides, were 1.13 and 1.08 kb for *MAT1–1* and *MAT1–2* isolates, respectively. The *MAT* genes were successfully amplified in *C. lunatus* isolates tested with the primer. All isolates contained a *MAT* gene with a structure homologous to either the *MAT1–2-1* of *C. lunatus* strain CX-3 or *MAT1–1-1* of *C. lunatus* strain ZD958.

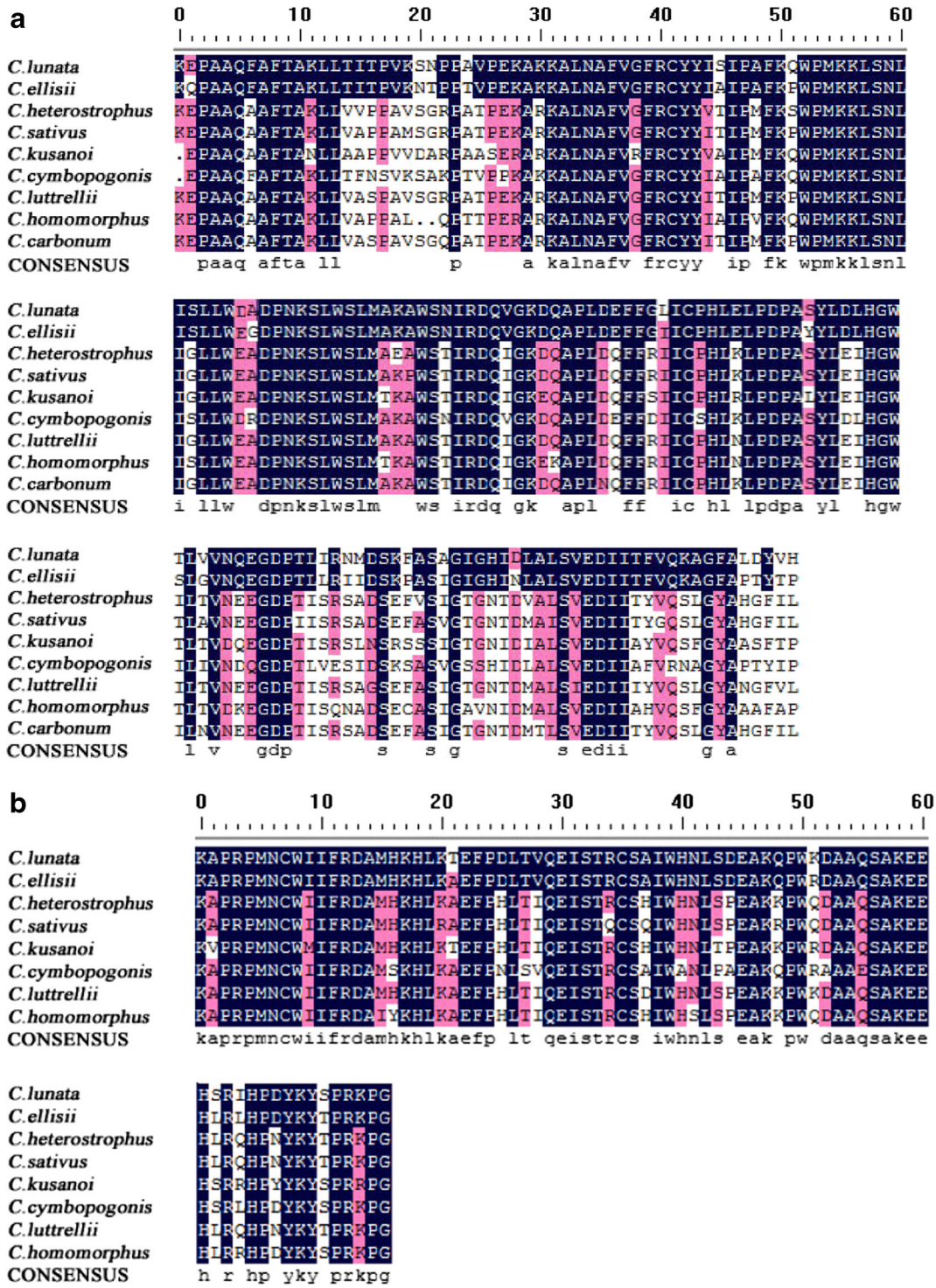

Fig. 1 Alignments of putative amino acid sequences in *Cochliobolus lunatus* to mating-type *(MAT)* genes in other species of *Cochliobolus*: **a** *MAT1–1* alpha HMG domain of *C. lunatus* (GenBank accession No. AML61199), *C. ellisii* (Q9Y8C7), *C. heterostrophus* (CAA06843), *C. sativus* (CAD62166), *C. kusanoi* (Q9Y8D3), *C. cymbopogonis* (*Q9Y8D1*), *C. luttrellii* (AAD33439), *C. homomorphus* (AAD33441), and *C. carbonum* (O13402). **b** *MAT1–2* HMG domain of *C. lunata* (AML61200), *C. ellisii* (AAD33451), *C. heterostrophus* (Q02991), *C. sativus* (Q9P445), *C. kusanoi* (AAD33442), *C. cymbopogonis* (*AAD33447*), *C. luttrellii* (AAD33439), and *C. homomorphus* (AAD33441)

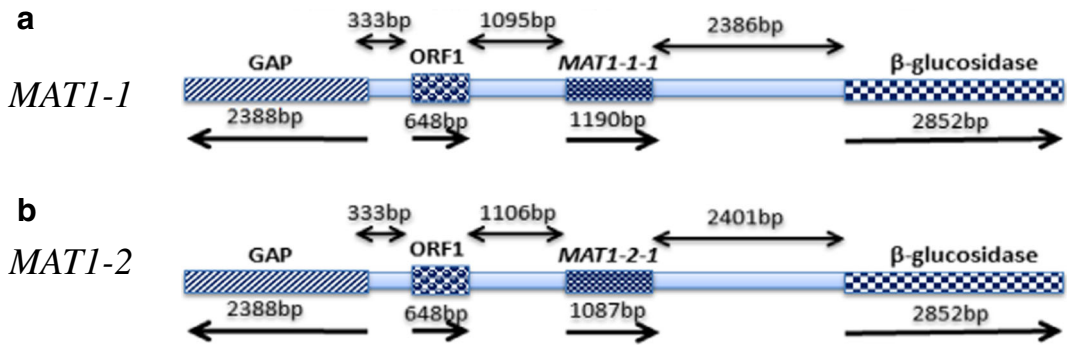

Fig. 2 Structural analysis and comparison of major open reading frames (ORFs) identified in the MAT region in *C. lunatus* (Accession numbers *MAT1–1* = KY471562 and *MAT1–2* = KY471563). The flanks are nearly identical between *MAT*s, in sharp contrast to the idiomorphs. 5′ of MAT: GAP, GTPase activating protein; ORF1, pyridoxamine 5′-phosphate oxidase domain. 3′ of *MAT*: a β-glucosidase. Numbers are in bases or kilobases. Arrows represent ORFs, and the direction represents the directions of transcription

In vitro production of sexual morphology

MAT1–1 and *MAT1–2* isolates of *C. lunatus* were co-inoculated and paired on detached corn leaves to produce perithecia. Perithecia were produced on the detached leaves between 21 days and 25 days. The amount of perithecia produced was highly variable and ranged from approximately 4–15 perithecia/cm^2 of leaf tissue for a prolific cross between ZD958 and CX-3 to approximately 85–317 ascospores/cm^2 of leaf tissue.

Mature perithecia were black and from ellipsoidal to globose. A well-defined ostiolate break, from subconical to paraboloid, was produced. Asci were cylindrical or clavate and straight or slightly curved with a short stipe. Mature asci contained 1–8 ascospores. Ascospores arrangement varied from complete coiling in a close helix to a nearly straight and parallel condition. Coiling was frequently evident in the apical portion of the ascus, with little or no coiling in the basal portion. Ascospores were discharged by the splitting of the ascus wall. Ascospores were filiform or flagelliform and somewhat tapered at the extremities. Mature ascospores were typically hyaline, with 6–15 septa (Fig. 3).

MAT distribution in the *C. lunatus* population in China

The multiplex *MAT*-specific PCR produced single-amplification band in the *C. lunatus* isolates, with PCR products of 1582 and 932 bp amplified in the known *MAT1–1* and *MAT1–2* individuals, respectively (Fig. 4). No PCR products were produced when a random isolate (CX-3 or ZD958) was screened with PCR primer specific for the opposite *MAT* to detect heterothallic individuals. Thus, 48.02% of isolates were identified as *MAT1–1* and 51.98% as *MAT1–2* (Table 2). The frequency varied at a region level from 36.36% to 75.0% and 25.0% to 63.64% for *MAT1–1* and *MAT1–2*, respectively. However, within China, the ratio of *MAT1–1* to *MAT1–2* isolates was consistent with 1:1 ratio ($\chi^2 < 6.895$, $P > 0.05$) (Table 2).

Discussion

To our knowledge, this study was the first to examine the *MAT* locus structure and distribution of *C. lunatus* in China. The identified *MAT* loci exhibited the typical characteristics and organization of a heterothallic Ascomycete species (Wirsel et al. 1998). The characteristics included a single gene per idiomorph, the location of the conserved introns in the HMG and alpha domain motifs, and ORFs flanking the idiomorphs encoding GAP, ORF1, and beta glucosidase. The occurrence of homologous gene sequences flanking both the *MAT* idiomorphs indicated that the *MAT* genes of *C. lunatus* resided in the same chromosome location.

The sequence length and identity upstream of the *MAT1–1-1* and *MAT1–2-1* genes identified in *C. lunatus* had also been reported in the *MAT* idiomorphs of many heterothallic Ascomycete species (Coppin et al. 1997). In addition, between the idiomorphs in the length, amino acid sequence, and protein stop codons of *GAP* and *ORF*1, which resided upstream of the *MAT* idiomorphs, beta glucosidase located downstream of the *MAT* idiomorphs were common for *C. heterostrophus*. The structural organization

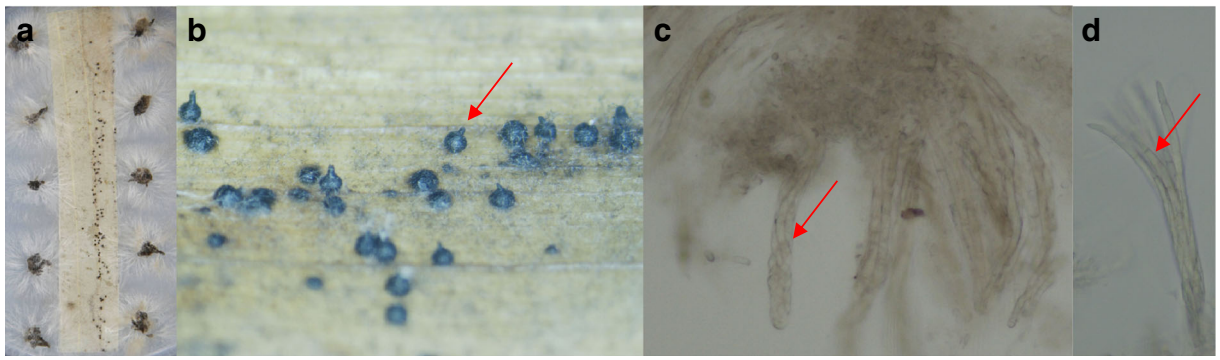

Fig. 3 *C. lunatus* strains ZD958 × CX-3 form perithecia, asci, and ascospore. A: Small agar blocks of mycelium of compatible isolates on opposite sides of dry corn leaf on Sach's medium; B: Black and ellipsoidal perithecia with suconical and ostiolate beak; C: Cylindrical and straight curved asci with coiling in a close helix. D: Flagelliform ascospores. B, C, and D: Microscopic images of perithecia, asci, and ascospore taken at 600 × magnification; Red arrow indicates mature perithecium, ascus, and ascospore in B, C, and D

of the *MAT* loci of nine heterothallic species (*C. heterostrophus, C. carbonum*, *C. ellisii, C. intermedius, C. miyabeanus, C. sativus, C. setariae, C. victoriae* and *C. lunatus*) is highly conserved, with each strain carrying a single *MAT* gene. *MAT* loci genes sequence and arrangement in *C. lunatus* further support the hypothesis that heterothallism is ancestral in *Cochliobolus* species (Yun et al. 1999).

In this study, we determined the distribution of *MAT1–1* and *MAT1–2 C. lunatus* isolates form corn planting regions in china which ever and presently severe occurred of this disease. The occurrence of both mating types was at a 1:1 ratio overall and in northeast region of China. *C. lunatus* is heterothallic, and the presence of *MAT 1–1* and *MAT 1–2* mating types and production of ascospore are the evidence for the possible existence of a sexually reproducing population. Although this study was not able to confirm the occurrence of a sexual morph in the field, it indicated that a cryptic reproductive cycle may occur within field population. A further consequence of sexually reproducing heterothallic species is genetic recombination. Fungal populations that reproduce sexually are likely to be genetically more diverse and have a higher adaptive potential than asexually reproducing population. It may be reason for resurgence of corn curvularia leaf spot disease in recently years.

The function of *MAT* genes has been investigated extensively in many pathogenic fungi, such as *Cochliobolus* spp., *Sordaria* spp., *Bipolaris sacchari*, *Penicillium chrysogenum*, *Aspergillus fumigatus,* and *Schizosaccharomyces* spp. (Turgeon et al. 1995; Zheng et al. 2013; Sharon et al. 1996; O'Gorman et al. 2008; Dahlmann et al. 2015). Studies have shown that conversion often occurs in the *MAT*s between homothallism and heterothallism, and they even exist simultaneously in certain environmental pressure and genetic conditions (Lin and Heitman 2007; Yun et al. 1999; Hastings 1992;

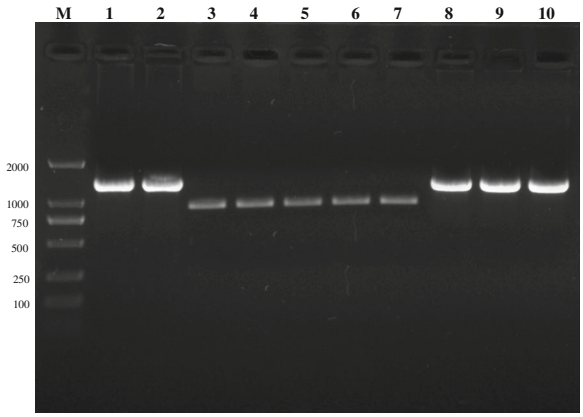

Fig. 4 PCR amplified with forward primers specific to the box region of *MAT1–1-1* (1582 bp) or the HMG domain region of *MAT1–2* (932 bp) of *C. lunatus* in a multiplex PCR reaction. 1–10:*C. lunatus* isolates;1,2,8,9,10:*MAT1–1* isolates; 3,4,5,6,7: *MAT1–2* isolates.M:2 K Ladder (Trans DNA marker)

Table 2 Distribution of *MAT*s of *C. lunatus* in China

Regions	*MAT1–1*	*MAT1–2*	χ^{2a}
Northeast	66	80	6.250
Huanghuaihai	15	5	4.465
Southwest	4	7	0.324
Total	85	92	6.895

[a] $P > 0.05$ when $\chi^2 < 6.895$

O'Gorman et al. 2008).The roles of the *MAT1–1-1* and *MAT1–2-1* genes on the sexual development in *C. lunatus* are still unknown. Studies proved that the *MAT* genes have certain universality and encode a transcription factor (Becker et al. 2015). The genes not only played important roles in regulating the sexual reproduction, cell recognition of the mating process, cell fusion, heterokaryon formation, and nuclear fusion and meiosis but also in regulating the hyphal morphology, asexual development, amino acid synthesis, iron absorption, secondary metabolism, and genome-wide transcription regulatory networks (Böhm et al. 2013).

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Compliance with ethical standards I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

Conflict of interest No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Human and animal studies Research did not involving Human Participants and/or Animals.

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