Genetic Differentiation and Clonal Expansion of Chinese *Botrytis cinerea* Populations from Tomato and Other Crops in China

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ABSTRACT

Botrytis cinerea is an important pathogen of vegetable and fruit crops but little is known about its population structure and genetics in China. We hypothesized that the geographic populations of *B. cinerea* in China would be genetically differentiated by host, geographic location, and/or year. In this study, we collected 393 *B. cinerea* isolates representing 28 populations from tomato, cherry, and nectarine from 2006 to 2014 in China. The isolates were analyzed using 14 microsatellite markers, including six new markers that provided more genotyping power than the eight previously published loci. We also investigated the *B. cinerea* population structure and inferred its mode of reproduction and dispersal based on genotype data. High genotypic

diversity was detected in all populations, and clonal reproduction was dominant. Southern China populations harbored more genotypes than northern populations. Differentiation by host plant was evident. Between 2011 and 2012, genotypes changed only slightly among years for Liaoning populations, but they changed substantially among years for the Shanghai and Fujian populations. Clonal dispersal was detected and the farthest dispersal distance was estimated to be about 1,717 km. Two high-frequency genotypes were widely distributed in more than 10 populations and across several years. Our results provide useful, novel information for plant breeding programs and control of *B. cinerea* in China.

Botrytis cinerea is the main pathogen causing gray mold, which is an important disease of many plants (Williamson et al. 2007). This fungus can cause disease of >235 host plants, including some high-value crops such as tomato, which is an important crop worldwide and especially in China. China has the largest planted area of tomato (>145,5000 hm²) and produces upward of 33,600,000 tons per year for domestic consumption and export (Zhang 2009). In China, gray mold can reduce the yield of tomato and other vegetables by 10 to 50% (Dean et al. 2012; Ji et al. 2012; Staats et al. 2005; Williamson et al. 2007).

Information about changes in the genetic structure of pathogen populations can provide insights into pathogen evolution and spread. Although asexual reproduction predominates in the majority of plant-pathogenic fungi, some species undergo regular sexual cycles (Milgroom 1996). In the case of *B. cinerea*, despite the fact that sexual structures are hardly observed under natural conditions (Beever and Weeds 2004), both regular sexual recombination and sexual cycles were found in the laboratory in French populations, suggesting the regular occurrence of cryptic sexual reproduction in *B. cinerea* populations, although asexual reproduction is still the

main mode of reproduction (Walker et al. 2015). High diversity was found in many populations (Adjebli et al. 2015; Esterio et al. 2011; Ma and Michailides 2005; Walker et al. 2015; Wessels et al. 2016), and significant genetic differentiation was evident in French populations of *B. cinerea* (Walker et al. 2015) but not in California populations (Ma and Michailides 2005). In China, one study investigated *B. cinerea* populations and reported a predominance of sexual reproduction in broad bean fields (Huang et al. 2014). The sample size in that study was modest, consisting of 100 isolates. In addition, gray mold caused by *B. cinerea* is not a serious disease of broad bean in China but is a serious disease of other vegetables and fruits such as tomato, cucumber, nectarine, cherry, and grape. The mode of reproduction of *B. cinerea* populations on important vegetable and fruit crops in China requires further investigation.

B. cinerea is commonly controlled by fungicide applications. Fungicides that specifically target *Botrytis*, so-called “botryticides,” cost €540 million worldwide in 2001, representing 10% of the world fungicide market (UIPP 2002). Unfortunately, *B. cinerea* can rapidly develop resistance to fungicides (Billard et al. 2012; Fernández-Ortuño et al. 2012, 2013; Konstantinou et al. 2015; Leroy et al. 2011, 2013; Rodríguez et al. 2014; Rupp et al. 2017; Walker et al. 2017; Wessels et al. 2016) because *B. cinerea* populations are genetically diverse and can evolve resistance (Fournier et al. 2002). Cases of *B. cinerea* that developed resistance to fungicides have been reported in tomato in China (Fan et al. 2017; Liu et al. 2016; Sun et al. 2010); however, whether the populations are genetically diverse is still unknown.

B. cinerea can infect fruits, leaves, and stems and causes damage both pre- and postharvest. Epidemics progress through asexual reproductive cycles in spring and summer, and asexual spore production further accelerates epidemic progress in the absence of control with fungicides (Holz et al. 2007). These spores are easily disseminated by air movement and human activities and can also be dispersed by infected fruit, seeds, water droplets, and insects (Elmer and Michailides 2007; Maude 1980; Nicot et al. 1996).

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Although it is difficult to monitor the dispersal of spores over long distances directly, we can infer the dispersal based on genetic diversity data in different populations. However, whether long-distance spread of *B. cinerea* occurs in Chinese populations remains to be determined.

The population structure of pathogen populations has been commonly analyzed by using microsatellite markers (Diao et al. 2015; Fournier et al. 2002; Goss et al. 2009; Kamvar et al. 2015). Fournier et al. (2002) developed a set of microsatellite markers for *B. cinerea* that have been used in several countries to assess the diversity of *B. cinerea* populations (Adjebli et al. 2015; Esterio et al. 2011; Ma and Michailides 2005; Walker et al. 2015; Wessels et al. 2016).

In this study, we hypothesized that the geographic populations of *B. cinerea* in China would be genetically differentiated by host, geographic location, and/or year. Therefore, we analyzed the populations of *B. cinerea* populations in fields and orchards in China. The analysis was based on 28 populations and 393 isolates collected from infected tomato, cherry, and nectarine across China between 2006 and 2014. A panel of eight published and six newly developed microsatellite markers were used to genotype isolates. Our main objective was to characterize *B. cinerea* populations in China by answering the following questions: (i) Are the populations genetically diverse or are they dominated by one or a few dominant genotypes? (ii) Are the populations characterized by clonal or sexual reproduction? (iii) Are there strains or populations that spread between or among regions based on the analysis of genotypes? A second objective was the development, validation, and application of six novel simple sequence repeat (SSR) markers reported here.

MATERIALS AND METHODS

Sampling. Between 2006 and 2014, isolates of *B. cinerea* were sampled from three hosts (tomato, cherry, and nectarine) and different locations in China (Fig. 1; Table 1). Most populations were sampled only once, but populations in Liaoning Province (LN), Shanghai Province (SH), and Fujian Province (FJ) were sampled in 2011 and again in 2012 at the same locations (Fig. 1; Table 1). Samples from each location were considered to represent one population of the pathogen. Populations from both the same province and the same year were merged into one population, reducing the 28 sampled populations to 16 populations for analysis. The locations were widely distributed across the country, spanning an area of ~2,108 km from south to north by 1,637 km from east to west, and covering 12 provinces. All isolates were collected with a hierarchical sampling method similar to that described in Kohli et al. (1995). For each population, we sampled five fields on a diagonal transect with five locations in every field; a total of 25 fruits (tomato, cherry, or nectarine) with gray mold symptoms were collected from each field, and the fungus was isolated from these fruits using standard procedures.

Isolate identification and DNA extraction. All isolates were grown on potato dextrose agar (Difco Laboratories, Detroit, MI) for 3 to 5 days at 20°C. Characteristics of isolate growth were visually assessed as described by Martinez et al. (2003). DNA was extracted using either the FastDNA SPIN kit (116540600; MP Biomedicals LLC) (Goss et al. 2009) or the cetyltrimethyl ammonium bromide–chloroform–isopropanol method (Diao et al. 2015). All isolates were identified to species by sequencing both the internal transcribed spacer and glyceraldehyde 3-phosphate

Fig. 1. Locations of the *Botrytis cinerea* populations sampled in China. Population designations followed by "11/12" were obtained from the same fields in 2 years, namely 2011 and 2012. The isolates obtained in the 2 years were considered to represent two populations (Table 1). Population designations not followed by "11/12" were sampled in only 1 year (Table 1).

dehydrogenase genes, which showed high similarity (99 to 100%) with the *B. cinerea* type strain (Staats et al. 2005) (data not shown). Isolates of the cryptic species *B. pseudocinerea*, which is morphologically undistinguishable from *B. cinerea*, were excluded

based on the previously described diagnostic allele at locus Bc6 (Walker et al. 2011).

Identification of new polymorphic SSR markers. We first tested eight of the nine primers (Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7,

TABLE 1. Summary information for *Botrytis cinerea* populations sampled and analyzed in this study^a

Population	Group ^b	Isolates (n)	Location	Host	Year
BJ	BJ	12	Beijing	Tomato	2006
CQ	CQ	21	Chongqing	Cherry	2014
FN11	FJ11	30	Nanping, Fujian	Tomato	2011
FP11	FJ11	7	Putian, Fujian	Tomato	2011
FZ11	FJ11	8	Fuzhou, Fujian	Tomato	2011
FN12	FJ12	9	Nanping, Fujian	Tomato	2012
FP12	FJ12	9	Putian, Fujian	Tomato	2012
FZ12	FJ12	10	Fuzhou, Fujian	Tomato	2012
HB	HB	14	Wuhan, Hubei	Nectarine	2014
HEB	HEB	14	Baoding, Hebei	Tomato	2006
HN	HN	5	Zhengzhou, Henan	Tomato	2006
LB11	LN11	32	Benxi, Liaoning	Tomato	2011
LD11	LN11	21	Dalian, Liaoning	Tomato	2011
LY11	LN11	15	Yingkou, Liaoning	Tomato	2011
LB12	LN12	17	Benxi, Liaoning	Tomato	2012
LD12	LN12	16	Dalian, Liaoning	Tomato	2012
LY12	LN12	2	Yingkou, Liaoning	Tomato	2012
NMGB11	NMG11	12	Huhehaote, Inner Mongolia	Tomato	2011
NMGC12	NMG12	10	Chifeng, Inner Mongolia	Tomato	2012
SB11	SH11	18	Baoshan, Shanghai	Tomato	2011
SJ11	SH11	17	Jinshan, Shanghai	Tomato	2011
SQ11	SH11	25	Qingpu, Shanghai	Tomato	2011
SB12	SH12	14	Baoshan, Shanghai	Tomato	2012
SJ12	SH12	7	Jinshan, Shanghai	Tomato	2012
SQ12	SH12	5	Qingpu, Shanghai	Tomato	2012
SCCHE	SC	15	Chengdu, Sichuan	Cherry	2014
SD	SD	13	Jinan, Shandong	Tomato	2006
TJ	TJ	15	Tianjin	Tomato	2006

^a The total numbers of populations, groups, and isolates were 28, 16, and 393, respectively.

^b Group is the population from the same province and the same year was considered to be one group.

TABLE 2. Simple sequence repeat (SSR) primer sequences and final concentrations used in microsatellite genotyping of *Botrytis cinerea*

SSR locus	Dye	Product (bp)	Primer sequence ^a	Final concentration (μM)	Reaction
Bc1 ^b	PET	212–254	F: PET-AGGGAGGGTATGAGTGTGTA R: <i>GTTTGAGGAGGTGGAAGTTGTA</i>	0.1	8-plex
Bc2 ^{b,c}	VIC	138–172	F: VIC-CATACACGTATTTCTTCCAATACCAAC R: <i>GTTTACGAGTGTTTTTGTAGAAT</i>	0.05	8-plex
Bc3 ^{b,c}	NED	211–229	F: NED-GGATGAATCAGTTGTTGTGACG R: <i>GTTTCACCTAGGTATTTCTCGGTA</i>	0.1	3a-plex
Bc4 ^b	NED	119–131	F: NED-CATCTTCTGGGAACGCACAT R: <i>GTTTATCCACCCCCAACGATTGT</i>	0.04	8-plex
Bc5 ^b	VIC	153–175	F: VIC-CGTTTTCCAGCATTTCAAGT R: <i>GTTTCATCTCATATTCGTTCTCTCA</i>	0.07	3a-plex
Bc6 ^{b,c}	PET	118–152	F: PET-ACTAGATTCGAGATTAGTTATATGAT R: <i>GTTTAAGGTGGTATGAGCGGTTTA</i>	0.16	8-plex
Bc7 ^b	6-FAM	120–134	F: 6FAM-CCAGTTTCGAGGAGGTCCAC R: <i>GTTTGCCTTAGCGGATGTGAGGTA</i>	0.11	8-plex
Bc10 ^b	6-FAM	168–210	F: 6FAM-TCCTCTTCCCTCCCATCAAC R: <i>GTTTGGATCTGCGTGGTTATGACG</i>	0.03	3a-plex
Bc65 ^d	VIC	167–230	F: VIC-TCACTGCCTCCGAACCTTTCT R: <i>GTTTCTTGGGAGCGGATTTTGTTA</i>	1.6	3b-plex
Bc172 ^d	VIC	277–484	F: VIC-CAATTTTACCCTCCACT R: <i>GTTTGCTTGGCCTGGCTTGTAGTA</i>	0.1	8-plex
Bc263 ^d	NED	191–218	F: NED-CTCTCGCTTAACCCCAATCA R: <i>GTTTCATCTCTTTCGCACCTTTT</i>	0.5	3b-plex
Bc294 ^d	NED	214–286	F: NED-TTGAGTAGACGGCTCTAGCTAAACT R: <i>GTTTCCATCCGTGTGATGGTTGAT</i>	0.1	8-plex
Bc299 ^d	6-FAM	206–260	F: 6FAM-TGATGGAATGTTCTTGGATGA R: <i>GTTTCACCAGGACTCCAGTCACCT</i>	0.1	8-plex
Bc432 ^d	PET	144–292	F: PET-TTGCATCTCAAGGTCCATCA R: <i>GTTTGTATGGAGGAATCTCCTTGC</i>	0.06	3b-plex

^a F = forward and R = reverse. Reverse primer includes PIG tail addition, indicated in italics (Brownstein et al. 1996).

^b Fournier et al. (2002).

^c The 3' end of the original primers was modified (current study), indicated in italics.

^d Current study.

and Bc10) described by Fournier et al. (2002) with 50 isolates; the BC9 locus was not included because it was determined to be in linkage disequilibrium with BC10 (Fournier and Giraud 2008). Using these primers, we could not detect polymorphisms for 20 of the 50 isolates (*data not shown*). We therefore designed new primers. Eight loci were selected from markers designed from the B05.10 and T4 genomic sequences (Amselem et al. 2011). The new markers were selected according to the following criteria: repeat motifs of three or more base pairs to augment the Fournier et al. (2002) predominantly 2-bp repeat motifs, primer pairs with annealing temperatures between 58 and 60°C for ability to multiplex, amplification of a single repeat motif, allele sizes >200 bp, and 11 and 16 repeats to increase the chance of detecting highly polymorphic loci while avoiding hybrid motifs (Guichoux et al. 2011).

Previously published reverse primers were modified at the 5' end by the addition of a GTTT PIG tail (Brownstein et al. 1996; Table 2). In addition, BC2, BC3, and BC6 forward primers were adjusted to permit a higher annealing temperature when multiplexing. We adopted the M13-tailed microsatellite protocol to assess all loci

prior to ordering fluorescent forward primers. To this end, forward primers were modified to include a universal sequence (TGTAAC ACGACGGCCAGT) on their 5' ends (Schuelke 2000). A tailed forward primer was then combined with a reverse primer and a universal M13 6-FAM fluorescently labeled primer (TGTAAC ACGACGGCCAGT).

Universal M13-tailed PCR reactions consisted of 10 µl and included 20 ng of DNA, 1× GenScript Taq Buffer, 0.2 mM of dNTPs, 0.04 µM of M13-tailed forward primer, 0.16 µM of reverse primer, 0.16 µM of universal M13 6-FAM primer, and 0.5 units of GenScript Taq (GenScript, Piscataway, NJ). Thermal cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min (Veriti thermal cycler; Life Technologies, Grand Island, NY).

Alleles for each of the 14 loci were screened for the expected size on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). In addition, alleles from isolate 327 were cloned at the six loci from the genome data and sequenced. Cloning was completed using the Topo TA Cloning Kit from Invitrogen supplied with the pCR 4-TOPO plasmid and One Shot TOP10 chemically competent *Escherichia coli* (Thermo Fisher Scientific, Waltham, MA). Following ligation, transformation, and dilution plating, single colonies were picked for analysis, and Luria-Bertani (LB) cultures were started. Topo plasmids containing inserts were extracted from LB cultures using the FastPlasmid Mini Kit (5PRIME, Gaithersburg, MD). Plasmid inserts were sequenced on an ABI 3730 DNA analyzer (Applied Biosystems) in both directions with T7 and T3 primers. Sequences were assembled in Geneious R6 (Biomatters Ltd., Auckland, New Zealand) and confirmed to be microsatellites. Primer sequences are shown in Table 2.

Microsatellite genotyping. *B. cinerea* isolates were genotyped using microsatellite markers for 14 loci to determine multilocus genotypes (MLGs) (Supplementary Table S2). Eight of the loci (Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, and Bc10) were described by Fournier et al. (2002). Primers for six new loci (Bc65, Bc172, Bc263, Bc294, Bc299, and Bc432) developed in this study are provided in Table 2. Forward primers for the 14 loci were labeled with fluorescent tags (6-FAM, NED, VIC, or PET) to improve separation of overlapping markers during multiplexing (Table 1) (Life Technologies, Carlsbad, CA). Primer concentrations for

TABLE 3. Summary information for *Botrytis cinerea* microsatellite loci used in this study^a

Locus	Alleles (<i>n</i>)	1 - <i>D</i>	<i>H</i> _{exp}	Evenness ^b
Bc1	12	0.61	0.67	0.5
Bc2	12	0.78	0.85	0.69
Bc3	10	0.64	0.71	0.65
Bc4	4	0.24	0.32	0.57
Bc5	11	0.34	0.38	0.39
Bc6	16	0.79	0.84	0.59
Bc7	8	0.59	0.68	0.61
Bc10	14	0.7	0.75	0.63
Bc65	13	0.71	0.77	0.53
Bc172	43	0.92	0.94	0.62
Bc263	11	0.54	0.59	0.52
Bc294	15	0.78	0.84	0.67
Bc299	13	0.65	0.7	0.58
Bc432	10	0.64	0.71	0.73
Mean	13.71	0.64	0.7	0.59

^a 1 - *D* = Simpson index and *H*_{exp} = Nei's unbiased gene diversity (Nei 1978).

^b Indices of evenness indicate how genotypes are distributed within a sample.

TABLE 4. Genetic parameters for each of the *Botrytis cinerea* populations in China^a

Population ^b	<i>n</i>	MLG	eMLG	SE	<i>H</i>	<i>G</i>	<i>H</i> _{exp}	<i>E</i> .5	<i>I</i> _A (<i>P</i> value)	rbarD
Southern China										
CQ	21	20	9.79	4.10e-01	2.98	19.17	1	0.97	1.12*	0.09
FJ11	45	29	8.89	9.12e-01	3.20	20.45	0.97	0.83	1.75*	0.14
FJ12	28	20	8.80	8.97e-01	2.88	15.68	0.97	0.87	2.52*	0.20
HB	14	13	9.51	5.00e-01	2.54	12.25	0.99	0.96	2.91*	0.23
SCCHE	15	15	10	2.77e-07	2.71	15	1	1	1.84*	0.15
SH11	60	23	6.73	1.27e+00	2.53	8	0.89	0.60	4.08*	0.32
SH12	26	15	7.35	1.14e+00	2.41	8.05	0.91	0.69	3.80*	0.30
Total	209	120	108	2.31	4.30	36	0.68	0.48	1.82*	0.14
Northern China										
BJ	12	7	6.32	6.31e-01	1.82	5.54	0.89	0.88	4.08*	0.41
HEB	14	13	9.51	5.00e-01	2.54	12.25	0.99	0.96	2.36*	0.18
HN	5	4	4	0.00e+00	1.33	3.57	0.9	0.92	2.47*	0.21
LN11	68	21	6.44	1.23e+00	2.41	7.36	0.88	0.63	3.45*	0.27
LN12	35	14	6.3	1.20e+00	2.19	6.28	0.87	0.66	3.62*	0.28
NMGB11	12	11	9.32	4.66e-01	2.37	10.29	0.99	0.96	2.36*	0.18
NMGC12	10	5	5	0.00e+00	1.23	2.5	0.67	0.62	5.52*	0.51
SD	13	10	8.27	7.05e-01	2.25	8.89	0.96	0.94	5.48*	0.43
TJ	15	10	7.47	8.76e-01	2.15	7.26	0.92	0.82	2.77*	0.28
Total	184	75	75	0.00	3.59	16.8	0.57	0.45	2.31	0.18
Total for China	393	183	8.86	1.03e+00	4.43	29.04	0.97	0.34	1.93*	0.15

^a MLG = number of multilocus genotypes observed, eMLG = number of expected MLGs at the smallest sample size ≥ 10 based on rarefaction, SE = standard error based on eMLG, *H* = Shannon-Wiener index of MLG diversity, *G* = Stoddart and Taylor's index of MLG diversity, *H*_{exp} = Nei's unbiased gene diversity, *E*.5 = evenness, *I*_A = index of association, and rbarD = standardized index of association. **P* < 0.01.

^b *Botrytis cinerea* populations are as defined in Table 1.

multiplex reactions were determined after visual examination of electropherograms (Table 2).

For all 14 loci, amplification was separated into three reactions (8-plex, 3-plex, and 3-plex). Three separate multiplex reactions (one 8-plex and two 3-plexes) were used to amplify all 14 loci using the QIAGEN Type-it Mutation Detect PCR Kit (QIAGEN, Valencia, CA). All three reactions were performed under identical conditions except for primer concentrations (Table 2). Multiplex PCR reactions were performed in 5- μ l volumes with 20 to 200 ng of template DNA and 1 $\times$ final buffer concentration. Amplifications were run on a Veriti thermal cycler under the following conditions: an initial denaturation at 95°C for 5 min, followed by 33 cycles of 95°C for 30 s, 60°C for 90 s, and 72°C for 20 s, and a final extension at 68°C for 10 min. Reference isolates LD6-2, SB1-17, SJ4-30, and SJ1-3, which were selected to represent a broad group of alleles, were included as positive controls. A no-template control was also included as a negative control in each PCR reaction.

Electrophoresis and visualization of all loci were performed on an ABI 3130 genetic analyzer. Before samples were loaded into the

sequencer, the 8-plex and both 3-plex PCR products from DNA extracted in China were diluted in ultrapure H₂O 50 and 20 times, respectively. The PCR products from DNA extracted in the United States were all diluted 20 times. Subsequently, 2.5 μ l of the diluted PCR products was added to 7.5 μ l of Hi-Di formamide containing GeneScan 500 LIZ size standard at a ratio of 6 μ l of size standard to 1 ml of Hi-Di formamide. Alleles were sized using GeneMapper version 5.0 software (Applied Biosystems, Waltham, MA).

Data analysis. Population genetic analysis was conducted using the computer and statistical language R (R Core Team 2014) and GenAlEx (Peakall and Smouse 2006). Graphs and figures were created using the R packages *ggplot2*, *ape*, *adegenet*, *igraph*, *ggmap*, *mmod*, and *poppr* (Jombart 2008; Kahle and Wickham 2013; Kamvar et al. 2014; Paradis et al. 2004; Wickham 2009; Winter 2012). Within-locus allelic diversity was analyzed across and within years and locations using the function *locus_table* in the R package *poppr* (Supplementary Table S1) (Kamvar et al. 2014).

The number of MLGs and genotypic diversity based on the Shannon-Wiener index (*H*) and Stoddard and Taylor's index (*G*)

Fig. 2. Frequency distribution of the number of multilocus genotypes (MLGs) detected for all *Botrytis cinerea* isolates in this study.

TABLE 5. Number of samples and maximum distance at which the same multilocus genotypes (MLGs) of *Botrytis cinerea* was observed based on analysis of 14 microsatellite loci

Repeated genotypes	Isolates (<i>n</i>)	Populations ^a	Maximum clonal distance (km)
MLG1	47	TJ, HN, SQ11, SB11 FN12, LD11, LB11, LY11	1,717
MLG2	40	BJ, LD12, HEB, SB12, CQ (cherry) HN, SJ11, SB11, LD11, LB11 LY11, LB12, LD12, NMGC12 SB12, SJ12	1,214
MLG3	27	TJ, SQ11, SB11, NMGC12 SB12, SJ12	1,304
MLG4	2	FZ12, HB (nectarine)	700
MLG5	4	SJ11, LD11, LB11, LB12	905
MLG6	2	NMGB11, SJ12	1,507
MLG7	3	SD, SQ12	697
MLG8	4	FN11, SQ11	577
MLG9	2	SB11, HEB	982
MLG10	3	FN11, TJ, LY11	1,599
MLG11	6	FN11, TJ, LB11, LY11, LB12, HEB	1,717

^a Besides CQ and HB populations, all other populations were collected from tomato. *Botrytis cinerea* populations are as defined in Table 1.

(Shannon and Weaver 1949; Stoddart and Taylor 1988) were calculated. G and H measure genotypic diversity based on a combination of richness and evenness. If all genotypes are equally abundant, then the value of G will be the number of MLGs, and the value of H will be the natural log of the number of MLGs. We used both G and H because G weighs abundant MLGs more heavily than less abundant MLGs, and the opposite is true for H (Grünwald et al. 2003; Kamvar et al. 2015). Evenness was calculated as E_5 , which is an estimator of evenness that utilizes both H and G and that indicates the ratio of the number of abundant genotypes to rare genotypes (Grünwald et al. 2003; Pielou 1975). Nei's gene diversity (H_{exp}) was also calculated (Nei 1978). These values were calculated with the R packages *poppr* and *vegan* (Jari Oksanen et al. 2013; Kamvar et al. 2014). To determine whether the number of loci used were sufficient to represent the genotypic diversity of the populations, we tested the relationship between the number of loci and genotypic diversity.

Multilocus linkage disequilibrium in populations was analyzed using the index of association (I_A) (Agapow and Burt 2001). I_A is a generalized measure of linkage disequilibrium and has an expected value of zero if the alleles at different loci are randomly associated with each other in a population. Significant associations between alleles at different loci would be expected in clonally reproducing populations (Xu 2006).

The population structure of *B. cinerea* was analyzed using discriminant analysis of principal components (DAPC) in the R packages *poppr*, *ade4*, and *adeigenet* as well as bootstrap analysis of Nei's genetic distance (Excoffier et al. 1992; Hubisz et al. 2009; Jombart et al. 2010; Kamvar et al. 2014, 2015). The bootstrap analysis, which was based on 10,000 bootstrap replicates, treated loci as independent units and used the function *aboot* in *poppr*; the results were visualized as an unrooted neighbor-joining tree. In this case, we used the hierarchies of samples within years within regions and the hierarchies of samples within regions within years. DAPC is a multivariate, model-free approach to clustering based on prior population information (Jombart et al. 2010). DAPC enabled us to analyze the population structure by assessing how well samples could be reassigned into previously defined populations. Because DAPC is sensitive to the number of principal components used in analysis, the function *xvalDapc* from the R package *adeigenet* was used to select the correct number of principal components with 1,000 replicates using a training set of 90% of the data. The number of principal components was chosen based on the criterion that it had to produce the highest average percentage of successful reassignment and the lowest root mean squared error (Jombart et al. 2010).

The maximum distance of potential dispersal distance was estimated based on the geographic coordinates of two locations,

Fig. 3. Results for the discriminant analysis of principal components of *Botrytis cinerea* populations as defined in Table 1. The scatterplot shows the first two principal components discriminating populations by region. Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analog of an 54.9% confidence interval based on a bivariate normal distribution. DA = discriminant analysis and PCA = principal component analysis.

where the same genotype was detected. Mantel tests were performed across all hierarchical levels in the data set utilizing the function `mantel.randtest` in the R package *ade4* between Bruvo's genetic distance and Euclidean distances between geographic coordinates (Bruvo et al. 2004; Dray and Dufour 2007; Mantel 1967). *P* values were calculated using 99,999 bootstrap replicates.

RESULTS

A total of 393 isolates were collected from three hosts (tomato, cherry, and nectarine) between 2006 and 2014, and most of them ($n = 343$) were from tomato (Table 2).

Identification of new polymorphic microsatellite loci. Of the eight primers we designed, six (Bc65, Bc172, Bc263, Bc294, Bc299, and Bc432) produced polymorphic fragments with the isolates tested (Table 3). The new SSR loci were more polymorphic than those previously reported (average 19.0 versus 10.8 alleles), and Bc172 was the most polymorphic with 43 alleles. Across all loci, the number of alleles per locus ranged from 4 to 43 with an average of 13.7 alleles per locus. Nei's gene diversity (H_{exp}) per locus ranged from 0.32 to 0.94, and evenness (E_5) ranged from 0.5 to 0.73 (Table 3) (Nei 1978). A genotype accumulation curve shows that these 14 loci are informative for population genetic analysis (Supplementary Fig. S1).

Genetic variation among loci and populations. The percentage of polymorphic loci was high in most populations. A total of 183 MLGs were detected in the 393 isolates based on the 14 microsatellite loci. The number of genotypes observed for each population ranged from 4 to 29. Southern populations had more MLGs ($n = 120$) than northern populations ($n = 75$) (Table 4). All populations showed high expected heterozygosity (H_{exp}) with values ranging from 0.67 to 1 (Table 4). Similarly, the Shannon-Wiener diversity (H) was high for most populations, with values ranging from 1.23 for population NMGC12 to 3.2 for population FJ11 (Table 4).

Clonal structure. Among all isolates, 45 genotypes appeared more than twice (Fig. 2). Of these 45, 11 were detected in different populations at the same time. Two genotypes were detected with high frequency (i.e., MLG1 was detected in 47 isolates from 13 populations, and MLG2 was detected in 40 isolates from 11 populations) (Table 5). Identical clones were detected between both adjacent and geographically distant areas (Table 5). The maximum distance of potential clonal dispersal was 1,717 km detected between the LN and FJ populations (Table 5). We also detected presence of MLGs present on two different hosts, including MLG4 on tomato and nectarine and MLG1 on tomato and cherry.

Allelic associations. The index of association (I_A) was calculated for each population and the entire sample with clone-corrected data. The I_A values for all of the populations and for the total sample were significantly higher ($P < 0.01$) than for the simulated data sets obtained assuming panmixia (Table 4). This supports a scenario of clonal reproduction as being common in all of the populations.

Population structure. All 28 populations were merged into 16 populations based on province and year. DAPC analysis showed that the first discriminant component differentiated Sichuan (SCCHE), Chongqing (CQ), Hubei (HB), and Shandong (SD) from the other populations (Fig. 3) where the SD population was sampled from tomato, SCCHE and CQ populations from cherry, and HB from nectarine. Clustering with Nei's genetic distance revealed that populations LN11 and LN12, which were collected in 2011 and 2012 in the same region of Liaoning Province, were more similar to each other than to other populations in both clone-corrected (Fig. 4) and uncorrected data sets (data not shown). In contrast, populations SH11 and SH12 from Shanghai did not cluster closely, indicating that substantial variation occurred between 2011 and 2012 at this location. The same was true for FJ11 and FJ12 from Fujian Province. The genetic distance was greatest between SD and other populations (Fig. 4). All of the populations were also clustered by year; however, no significant differentiation by years was observed

Fig. 4. Clustering of populations of *Botrytis cinerea* from different regions and years based on Nei's genetic distance (Nei 1978). Tip labels conform to regions as defined in Table 1. Branches with bootstrap values >50% are shown.

(Supplementary Fig. S2). Among the populations sampled in 2006, the Hebei (HEB), Tianjin (TJ), Beijing (BJ), and Henan (HN) populations clustered separately from the SD population, which can be explained by the geographic proximity of HEB, TJ, BJ, and HN and by sharing of the same host variety for HEB, TJ, BJ, and HN (Fig. 5). Among the populations sampled in 2011, we observed no differentiation by location (Supplementary Fig. S3). For 2012, the DAPC analysis showed that the first discriminant component separated LN and NMG, which were all from Northern China, from populations from Southern China (Fig. 6). For 2014, the DAPC analysis showed that the first discriminant component separated HB, which was sampled from nectarine, from CQ and SCCHE populations from cherry (Supplementary Fig. S4).

DAPC analysis showed that the first discriminant component differentiated tomato, cherry, and nectarine populations from each other (Fig. 7). This distinction was reflected in the percentage of correct posterior assignment of isolates ($n = 245$) to their original populations. Over the entire data set, there was an 68.5% assignment-success rate.

Spatial correlation. Based on analysis of all isolates, the Mantel test failed to detect significant correlations between genetic distance and geographic distance (data not shown). Based on analysis of isolates by year, only weak and nonsignificant correlations were detected (data not shown).

DISCUSSION

In the current study, we documented the genetic differentiation of geographic populations of *B. cinerea* in China by host and geographic location. Our results revealed that the SCCHE, CQ, HB, and SD populations were differentiated from other geographic populations (Fig. 3). We also determined the degree of population differentiation in *B. cinerea* in Northern and Southern China. Southern populations had more MLGs than northern populations (120 versus 75), and the ratio was far higher than the difference in the number of isolates in both regions. Generally, genotypic diversity is greater in southern than in northern populations, which can be explained by differences in climate. Because the average temperature is higher in Southern than in Northern China, the growing season for plants is longer in Southern China, and this might increase the possibility that new genotypes will arise and spread.

The degree of population differentiation in *B. cinerea* among years was also determined. No significant differentiation by years was observed (Supplementary Fig. S2); however, we observed large differentiations in populations between 2011 versus 2012, with no differentiation in 2011 and significant differentiation in 2012 among Southern and Northern populations (Fig. 6, Supplementary Fig. S3). One possible explanation might be the difference in

Fig. 5. Results for the discriminant analysis of principal components of *Botrytis cinerea* populations sampled in 2006 as defined in Table 1. The scatterplot shows the first two principal components discriminating populations by region. Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analog of an 67.6% confidence interval based on a bivariate normal distribution. DA = discriminant analysis and PCA = principal component analysis.

climate. Liaoning has lower temperatures and therefore a shorter growing season than Shanghai or Fujian. As a consequence, the chances that new genotypes might arise and spread are lower in Liaoning than in Shanghai or Fujian.

The analysis of population structure of *B. cinerea* by region and year (2014) indicated that cherry populations (SCCHE, CQ) and

nectarine populations (HB) were differentiated from tomato populations (Fig. 3, Supplementary Fig. S4). Furthermore, our results revealed that *B. cinerea* populations differentiated based on host (i.e., populations from tomato, cherry, or nectarine clustered by themselves) (Fig. 7). Similar results were reported in France, in that *B. cinerea* populations from *Solanum* species were differentiated

Fig. 6. Results for the discriminant analysis of principal components of *Botrytis cinerea* populations sampled in 2012 as defined in Table 1. The scatterplot shows the first two principal components discriminating populations by region. Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analog of an 62.2% confidence interval based on a bivariate normal distribution. DA = discriminant analysis and PCA = principal component analysis.

from those from other hosts (Walker et al. 2015). The differentiation in the latter study, however, can be explained by habitat isolation, because *Solanum* populations were sampled from a greenhouse and the other populations were sampled outdoors. In our study, in contrast, all hosts were grown outdoors. Yet host isolation could explain the differentiation because the three hosts were growing sufficiently far from each other so that gene flow might be limited. The differentiation could also be due to host effects because host-specific differentiation of *B. cinerea* populations has often been described (Fournier and Giraud 2008; Karchani-Balma et al. 2008; Muñoz et al. 2002; Rajaguru and Shaw 2010; Samuel et al. 2012; Walker et al. 2015). The divergent selection pressures exerted by hosts may explain the stable pattern of differentiation observed in *B. cinerea* populations over time (Walker et al. 2015). Defense mechanisms specific to different hosts may require distinct sets of pathogenicity alleles/genes (Choquer et al. 2007; Williamson et al. 2007).

A potentially important limitation in our study is that 25 of the 28 *B. cinerea* populations were from tomato, and only three were from cherry and nectarine. Because of this unbalanced sample size, we could have introduced bias into our statistical analysis of host differentiation. Additional sampling of cherry and nectarines is thus needed.

Among all of the *B. cinerea* populations sampled in this study, the Shandong population was the most diverged. Shandong is the most important vegetable production area in China and is an area that grows many different tomato varieties. A high diversity of host species and cultivars can promote higher genetic diversity in pathogen populations. For example, *Colletotrichum truncatum* populations from the host breeding center for chili peppers had more private alleles than populations outside of the breeding center (Diao et al. 2015).

Our study revealed that the same MLGs of *B. cinerea* occur in many areas of China. This may be explained by three reasons as well as homoplasy. First, because *B. cinerea* has many hosts, *B. cinerea* spread is unlikely to be limited by an absence of hosts. Second, the conidia of *B. cinerea* can be spread by wind and with seeds, fruit, and soil (Elmer and Michailides 2007; Maude 1980; Nicot et al. 1996). Third, human-mediated dispersal might also contribute to the long-distance transport of the pathogen. Some transplants were shipped from one producer field to another, which could also contribute to the long-distance dispersal of *B. cinerea*. Of course, homoplasy is also a potential explanation for observing the same MLGs at 14 loci. However, we expect the chance of finding the same genotype at 14 highly polymorphic loci as being exceedingly small (Supplementary Table S1). The results of the Mantel test indirectly

Fig. 7. Population structure of *Botrytis cinerea* based on discriminant analysis of principal components. The scatterplot shows the first two principal components discriminating populations by host. Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analog of a 68.5% confidence interval based on a bivariate normal distribution. DA = discriminant analysis and PCA = principal component analysis.

indicated that gene or genotype flow may be common among populations. Our data also indicate that *B. cinerea* can move between different hosts (i.e., between tomato and cherry and between tomato and nectarine). This host-bridge might increase the ability of the fungus to spread geographically.

Our work has implications for disease management. As an airborne pathogen, *B. cinerea* is capable of long-distance dispersal, and our results indicate that gene flow occurs between several regions. Because *B. cinerea* is wind dispersed and is already present in many areas of China, quarantine measures might not be very effective in controlling this pathogen. Two MLGs of *B. cinerea*, MLG1 (47 isolates) and MLG2 (40 isolates), were common among our isolates regardless of geographic region, host, and year. It might be worthwhile establishing whether there exist differences in pathogenic fitness among the dominant MLGs; if so, the new sources of resistance to these dominant MLGs should be identified. Because the populations in Liaoning were similar in 2011 and 2012, we suspect that populations in this region may change only slowly and therefore may be easier to control than populations in Shanghai and Fujian, which differed substantially between 2011 and 2012. In Shanghai, Fujian, and similar regions, control will likely require a variety of measures, including growing of different varieties, and the use of different fungicides.

Conclusion. In this study, we used 14 microsatellite markers to analyze the population genetic structure of *B. cinerea* from three hosts in China. Six of the markers were newly designed, and the combination of the six new markers with eight former markers greatly increased our ability to assess the genetic diversity in *B. cinerea* populations in China. These 14 markers should also be useful for assessing genetic diversity of *B. cinerea* populations in other parts of the world. Two *B. cinerea* genotypes, MLG1 and MLG2, were found in high frequencies and in many locations in tomato fields in China. The frequency of these two genotypes in other hosts and other countries should be determined.

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