Population Structure of Brown Rot Fungi on Stone Fruits in China

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Abstract

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In total, 455 *Monilinia* isolates from stone fruits collected from several provinces (cities) in China from 2003 to 2009 were identified to species based on morphological characteristics, molecular identification, and the sequence of the internal transcribed spacer (ITS) regions 1 and 2 and the 5.8S gene of the ribosomal RNA. Overall, four species were detected (*Monilinia fruticola*, *M. fructigena*, *M. laxa*, and *Monilia polystroma*). *M. fruticola* was the most prevalent (93.0%) followed by *M. fructigena* (4.8%), *M. laxa* (2.0%), and *Monilia polystroma* (0.2%). *M. fruticola* and *M. fructigena* were found on peach, plum, and apricot; *M. laxa* was found only on apricot, cherry (in an organic orchard), and wild peach; and *Monilia polystroma* was found only on

plum in Heilongjiang. The pathogenicity of *Monilinia fruticola*, *M. laxa*, and *M. fructigena* did not significantly differ on wounded nectarine and apricot, indicating that the differences in frequency of occurrence were not linked to virulence. Phylogenetic analysis based on ITS sequences showed that the isolates of *M. laxa* and *M. fructigena* from China differed from isolates of these species from other countries, and that the difference led to the separation of the isolates from China and those from other countries into different phylogenetic groups. Further study is needed to determine whether they are cryptic species.

Brown rot is one of the most important diseases of stone fruits (*Prunus* spp.) worldwide (3). Under favorable conditions, it causes severe losses and damage to fruit crops and to the trees themselves (24). The causal pathogens of brown rot of stone and pome fruits include four closely related fungi: *Monilinia fruticola* (G. Winter) Honey, *M. laxa* (Aderh. & Ruhland) Honey, *M. fructigena* (Aderh. & Ruhland) Honey, and *Monilia polystroma* G. Leeuwen (3,30). *M. polystroma* is a new asexual species first found in Japan (30) and then in Hungary and China (28,36). The distribution of these species differ across the world (3,30), and some are quarantine pests in specific regions. For example, *M. fruticola* is a quarantine pathogen in the European Union (7,22,23) but was recently found in peach orchards of southern France (22) and Italy (26). *M. fructigena* is a quarantine pathogen in the United States and Australia (3).

China is one of the major stone fruit producers, and brown rot is an important disease in many of its stone-fruit-growing areas. The earliest record of brown rot on stone fruit in China can be dated to 1920 (4). From the 1920s to 1950s, brown rot was reported on stone fruits, including apricot (*Prunus armeniaca* L.), wild apricot (*P. armeniaca* L. var. *ansu* Maxim), sour cherry (*P. cerasus* L.), peach (*P. persica* Stokes), and flat peach (*P. communis* Fritsch), and the causal pathogen was referred to as *Sclerotinia laxa* (4). In a study from 1994 to 1996, *M. laxa* and *M. fructigena* were detected in China (31). In 2007, *M. fruticola* was listed as a quarantine pest in China (1), although this species was found in a peach orchard in a Beijing suburb in 2003 (35). The recent discovery of *M. fruticola* suggests that either the species composition of brown rot fungi in China has changed or that the population structure of brown rot fungi is more complex in China than previously recognized. Therefore, the objectives of this study were to (i) identify the *Monilinia* spp. in China using morphological and molecular methods and (ii) determine the prevalent species and their pathogenicity.

Materials and Methods

Survey. In a survey carried out from 2003 to 2009, fruit with symptoms of brown rot were collected from orchards located in the following 12 provinces or cities: Beijing, Shandong, Hebei, Shanghai, Zhejiang, Jiangsu, Henan, Gansu, Sichuan, Yunnan, Liaoning, and Heilongjiang. The number of fruit collected from each orchard depended on the extent of the disease and the number of *Prunus* spp. if several crops were present in one orchard (Table 1). In the laboratory, a sterilized wire loop was used to transfer spores from the fruit (or mummy) onto the surface of water agar (WA) in petri dishes. After 16 to 24 h at $22 \pm 2^\circ\text{C}$, individual germinated spores were aseptically transferred from the WA to potato dextrose agar (PDA) in petri dishes. The isolates were then stored on PDA slants at 4°C , and representative cultures were deposited in the China Forestry Culture Collection Center. The source and host of the isolates are listed in Tables 2 and 3; reference isolates are listed in Table 3.

Morphological characterization. The colony morphology of each isolate was determined on PDA. A plug (5 mm in diameter) was cut from the edge of a 4-day-old colony and placed in the center of a petri dish (9 cm in diameter) containing about 15 ml of PDA. Three replicate dishes were used for each isolate. The dishes were kept at $22 \pm 2^\circ\text{C}$ with 12 h of near-UV light (wavelength 365.5 nm) and 12 h of darkness. After 6 days, the seven critical characters described by Byrde and Willetts (3) and Lane (17) were determined for each isolate.

DNA extraction. Mycelia from cultures grown in pea broth (10) at $22 \pm 2^\circ\text{C}$ for 3 to 5 days in darkness were collected by filtration. DNA was extracted following a method that was modified from Murray and Thompson (21) and Wilson (33). The concentration of DNA was quantified by electrophoresis in 1% agarose gels along with known quantities of λ DNA (Takara Bio. Inc., Japan).

PCR amplification and DNA sequencing. The species-specific primers developed by Ioos and Frey (16) were used. Polymerase chain reaction (PCR) amplification was performed in a 25- μl volume containing 2.0 mM MgCl_2 , 1.0 U of Taq polymerase, 200 μM each dNTP, 0.2 μM each of the specific primers, and 10 to 20 ng of genomic DNA. PCR cycling parameters were 94°C for 3 min, followed by 30 cycles (94°C for 30 s, 67.5°C for 30 s, and 72°C for 1.5 min), and a final extension of 72°C for 10 min in an Icyler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). PCR products, which were about 350 bp, were separated by elec-

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trophoresis in 1.5% agarose gels in Tris-borate-EDTA buffer and were photographed with a MultiImage Light Cabinet (Alpha Innotech Corporation) after staining with ethidium bromide.

Isolates that could not be identified according to morphological characteristics or species-specific PCR amplification were subjected to more DNA tests. The ribosomal DNA of these isolates was amplified with primers ITS1 and ITS4 (32) using the same PCR protocol described above, except that the annealing temperature was 55°C. The amplified fragments were purified using the PCR product Purification Kit (Dingguo Inc., Beijing), and DNA sequencing was performed by the Beijing Sunbiotech Co. Ltd.

Phylogenetic analysis. The ITS regions were sequenced for 39 isolates, including 30 representative isolates collected from different locations and 9 isolates that were unidentifiable using species-specific primers. The ITS sequences of the test strains and the reference isolates and the reference sequences retrieved from GenBank (Table 3) were used to construct the phylogenetic tree. The sequence alignment was done using BioEdit (11) with manual adjustment. The maximum parsimony (MP) analyses were performed by heuristic search in PAUP 4.0b10 (29). Gaps were treated as a fifth character weight, and all characters were of equal weight but uninformative characters were excluded. Bootstrap values were estimated by 1,000 replications to assess the robustness of the topology.

Pathogenicity tests. The pathogenicity of nine isolates representing different species and obtained from different hosts and locations in China was tested on nectarine and apricot fruits (Table 3); the purpose was to determine whether the prevalence of *M. fructicola* was associated with increased virulence. For isolates of *M. fructicola*, conidia from 6-day-old cultures on PDA were dislodged by washing the culture surface with 5 to 10 ml of sterile-distilled water. Because *M. fructigena* and *M. laxa* produced few conidia on PDA, the conidia of these species were obtained from pear fruit that had been inoculated 8 days earlier. The conidial suspension was filtered through two layers of muslin cloth and then adjusted to 1×10^4 conidia ml⁻¹.

To test the pathogenicity of isolates on apricot, fruit were first surface sterilized with 0.128% NaClO for 15 min and then rinsed three times with sterile-distilled water. Each fruit was wounded with a nail (2-mm diameter and 3-mm depth) before 5 µl of the spore suspension was placed on each wound. Fruit inoculated with sterile-distilled water were used as controls.

To test the pathogenicity of isolates on nectarine, mature fruit were sterilized and wounded as described for apricot. A mycelial plug (5 mm in diameter) cut from the margin of a 6-day-old culture on PDA was placed on each wound. Fruit inoculated with PDA

plugs without fungus were used as controls. Each of the nine isolates was represented by three replicates (five fruit per replicate) for each *Prunus* spp. The fruit were placed on two layers of moist paper towels on a wire rack in a plastic container at room temperature (24.5 ± 2°C). Three replicated containers were used for each isolate. The width and length of lesions on the inoculated fruit were measured 2 and 4 days after inoculation. Lesion expansion rate (millimeters per day) was calculated according to the difference in lesion size between the two measuring dates. Treatments were compared by analysis of variance (ANOVA) using the PROC procedure of SAS (SAS Institute, Cary, NC), and multiple comparisons among the means were conducted using *t* tests (least significant difference) when effects were statistically significant at the 0.05 level. The experiments were repeated at least once.

Results

Species identification of brown rot fungi in China. Infected fruit showing brown rot symptoms or mummified fruit with sporulation were collected from 9 of 12 provinces (cities), which included Beijing, Shandong, Hebei, Shanghai, Zhejiang, Gansu, Yunnan, Liaoning, and Heilongjiang. In total, 455 isolates were identified (Table 2). Of these, 227 were identified based only on morphology, 228 were identified using the molecular method developed by Ioos and Frey (16), and 168 were identified based on both morphology and the molecular methods.

On PDA dishes, 367 of 395 isolates developed grayish zonate colonies with more or less even margins and with abundant sporulation on the surface (Fig. 1); these isolates were identified as *M. fructicola*. The eight isolates that were light gray to dark gray with lobed margins and sparse sporulation (Fig. 1) were tentatively identified as *M. laxa*. Twenty isolates that had white to grayish colonies with sparse sporulation and entire margins (like *M. fructicola*) were identified as *M. fructigena*.

In total, 228 isolates, including 168 isolates identified by morphological characteristics, were identified with the molecular method described by Ioos and Frey (16). According to this method, PCR with primer pair ITS1Mfcl/ITS4Mfcl detects *M. fructicola*, PCR with primer pair ITS1Mlx/ITS4Mlx detects *M. laxa*, and PCR with primer pair ITS1Mfng/ITS4Mfng detects *M. fructigena*. When the 228 isolates were amplified with the three primer pairs, 203 isolates, including 145 isolates identified as *M. fructicola* by morphological characteristics, generated a PCR product only with primer pair ITS1Mfcl/ITS4Mfcl, six isolates identified as *M. laxa* based on culture morphology generated PCR products only with primer pair ITS1Mlx/ITS4Mlx, and 10 isolates identified as *M.*

Table 1. Geographic origin of *Monilinia* spp. isolates collected from stone fruit in China

Fruit ^a	Region	Number of orchards	Number of isolates
Peach	Beijing	47	342
	Shandong	11	42
	Hebei	2	9
	Yunnan	2	8
	Shanghai	2	4
	Zhejiang	3	3
	Gansu	2	4
	Liaoning	1	1
	Unknown	2	3
	Total	71	416
Plum	Beijing	6	13
	Shandong	1	11
	Gansu	1	1
	Heilongjiang	1	1
	Total	9	26
Apricot	Beijing	3	7
Cherry	Beijing	3	5
Wild peach	Shandong	1	1

^a "Peach" includes peach, nectarine, flat peach, and flat nectarine.

Table 2. Geographic distribution of *Monilinia* spp. isolates from stone fruit in China

Species	Location	Host ^a	Number of isolates
<i>Monilinia fructicola</i>	Beijing	Peach	338
	Beijing	Plum	11
	Beijing	Cherry	2
	Shandong	Peach	42
	Shandong	Plum	10
	Hebei	Peach	9
	Shanghai	Peach	4
	Zhejiang	Peach	3
	Liaoning	Peach	1
	unknown	Peach	3
<i>M. laxa</i>	Shandong	Wild peach	1
	Beijing	Apricot	5
	Beijing	Cherry	3
<i>M. fructigena</i>	Beijing	Peach	4
	Beijing	Apricot	2
	Beijing	Plum	3
	Yunnan	Peach	8
	Gansu	Peach	4
<i>Monilia polystroma</i>	Gansu	Plum	1
	Heilongjiang	Plum	1

^a "Peach" includes peach, nectarine, flat peach, and flat nectarine.

Table 3. Isolates and sequences of *Monilinia* spp. used in the pathogenicity test and for phylogenetic analysis

Isolate	Location	Host ^a	Species	GenBank accession number	Phylogenetic group
Isolates from China					
ML4	Mentougou, Beijing	Apricot	<i>Monilinia laxa</i>	HQ846955	II
ME1	Miyun, Beijing	Apricot	<i>M. laxa</i>	HQ846953	II
JX3 ^b	Miyun, Beijing	Apricot	<i>M. laxa</i>	HQ846954	II
JX9 ^c	Miyun, Beijing	Apricot	<i>M. laxa</i>	HQ846952	II
BX5 ^b	Pinggu, Beijing	Apricot	<i>M. fructigena</i>	HQ846961	V
BHY1	Haidian, Beijing	Cherry	<i>M. fructicola</i>	HQ846927	VI
BLG3	Haidian, Beijing	Cherry	<i>M. fructicola</i>	HQ846922	VI
MY11 ^b	Miyun, Beijing	Cherry	<i>M. laxa</i>	HQ846951	II
T18	Changping, Beijing	Peach	<i>M. fructicola</i>	HQ846940	VI
PBF5 ^c	Fangshan, Beijing	Peach	<i>M. fructicola</i>	HQ846936	VI
320020 ^{b,c}	Pinggu, Beijing	Peach	<i>M. fructicola</i>	HQ846925	VI
332331 ^{b,c}	Pinggu, Beijing	Peach	<i>M. fructicola</i>	HQ846926	VI
GP3 ^{b,c}	Yanqing, Beijing	Peach	<i>M. fructigena</i>	HQ846957	V
GP13	Yanqing, Beijing	Peach	<i>M. fructigena</i>	HQ846958	V
GP18 ^b	Yanqing, Beijing	Peach	<i>M. fructigena</i>	HQ856917	V
PA18	Pinggu, Beijing	Peach	<i>M. fructicola</i>	HQ846935	VI
CH2	Changping, Beijing	Plum	<i>M. fructigena</i>	HQ846928	VI
HAG7	Changping, Beijing	Plum	<i>M. fructicola</i>	HQ846933	VI
HH1	Huairou, Beijing	Plum	<i>M. fructicola</i>	HQ846932	VI
STL1 ^c	Miyun, Beijing	Plum	<i>M. fructigena</i>	HQ846960	V
DH41 ^c	Pinggu, Beijing	Plum	<i>M. fructicola</i>	HQ846931	VI
GS3	Yanqing, Beijing	Plum	<i>M. fructigena</i>	HQ846956	V
GDT1	Dongzhiyuan, Gansu	Peach	<i>M. fructigena</i>	HQ846963	V
GND3 ^b	Lanzhou, Gansu	Peach	<i>M. fructigena</i>	HQ846962	V
HeBSM11	Hebei	Peach	<i>M. fructicola</i>	HQ846934	VI
PJH2	Handan, Hebei	Peach	<i>M. fructicola</i>	HQ846937	VI
HML3	Mudanjiang, Heilongjiang	<i>Prunus aitanli</i>	<i>Monilia polystroma</i>	GU067539	IV
5-10HUANG	Jinan, Shandong	Peach	<i>M. fructicola</i>	HQ846924	VI
CX25	Laiyang, Shandong	Peach	<i>M. fructicola</i>	HQ846930	VI
SPT16	Pingyi, Shandong	Peach	<i>M. fructicola</i>	HQ846920	VI
PLY14 ^b	Yantai, Shandong	Peach	<i>M. fructicola</i>	HQ846938	VI
SLT2	Yiyuan, Shandong	Peach	<i>M. fructicola</i>	HQ846939	VI
CX3	Laiyang, Shandong	Plum	<i>M. fructicola</i>	HQ846929	VI
STT2	Taishan, Shandong	Wild peach	<i>M. laxa</i>	HQ846950	VI
ZJMF1 ^c	Hangzhou, Zhejiang	Peach	<i>M. fructicola</i>	HQ846923	VI
MFLS1	Lishui, Zhejiang	Peach	<i>M. fructicola</i>	HQ846941	VI
YCT3	Chuxiong, Yunnan	Unknown	<i>M. fructigena</i>	HQ846965	V
YHT3	Honghezhou, Yunnan	Unknown	<i>M. fructigena</i>	HQ846959	V
SFT1	Shanghai	Unknown	<i>M. fructicola</i>	HQ846921	VI
Reference isolates					
HAY30	Unknown	Rape	<i>Sclerotinia sclerotiorum</i>	HQ846942	Outgroup
BCE4	Beijing	Tomato	<i>Botrytis cinerea</i>	HQ846943	Outgroup
Ft	France	Unknown	<i>M. fructicola</i>	HQ846967	VI
NE18	New Zealand	Unknown	<i>M. fructicola</i>	HQ846919	VI
LVN8	USA	Unknown	<i>M. fructicola</i>	HQ846966	VI
P1	France	Unknown	<i>M. fructigena</i>	HQ846946	III
GENA4	Italy	Unknown	<i>M. fructigena</i>	HQ846945	III
P3	France	Unknown	<i>M. laxa</i>	HQ846947	I
CBS298.31	Ireland	Unknown	<i>M. laxa</i>	HQ846949	I
MDA12	United States	Unknown	<i>M. laxa</i>	HQ846948	I
2319	Japan	Unknown	<i>Monilia polystroma</i>	HQ856916	IV
CBS102686	Japan	Unknown	<i>M. polystroma</i>	HQ846944	IV
Sequences from NCBI ^d					
Hirodai number 3272	Japan	<i>P. mume</i> 'Bungo'	<i>M. laxa</i>	AB125612	I
DAOM 231119	Canada	Wine grape	<i>Monilinia fructicola</i>	AY289185	VI
LH01	Hangzhou, China	Red Bayberry	<i>M. fructicola</i>	AM887528	VI
Hirodai number 2636	Japan	Unknown	<i>M. fructicola</i>	AB125615	VI
Hirodai number 3266	Japan	<i>P. mume</i> 'Ryukyo'	<i>M. fructicola</i>	AB125611	VI
782.K=LMK12	Norway	<i>P. persica</i>	<i>M. fructicola</i>	Z73777	VI
783.K=LMK125	Norway	<i>P. persica</i>	<i>M. fructicola</i>	Z73778	VI
THF-1	Shandong, China	Peach	<i>M. fructicola</i>	FJ515894	VI
W1	Spain	Stone fruit	<i>M. fructicola</i>	EF207420	VI
W11	Spain	Stone fruit	<i>M. fructicola</i>	EF207422	VI
99.2.G5.04	USA	Unknown	<i>M. fructicola</i>	DQ314730	VI
BGA.27.04	USA	Unknown	<i>M. fructicola</i>	DQ314727	VI
IHEM	Belgium	Pear	<i>M. fructigena</i>	FJ515296	III

(continued on next page)

^a "Peach" includes peach, nectarine, flat peach, and flat nectarine.^b Isolates were used in pathogenicity test and phylogenetic analysis.^c Isolates could not be identified to the species level using species-specific primers designed by Ios and Frey (16), as the DNA of each of these isolates generated polymerase chain reaction products in reaction with more than one species-specific primer.^d National Center for Biotechnology Information.

Table 3. (continued from preceding page)

Isolate	Location	Host ^a	Species	GenBank accession number	Phylogenetic group
LHF-1	Shandong, China	Pear	<i>M. fructigena</i>	FJ515891	III
COY2M	France	<i>P. insititia</i>	<i>M. fructigena</i>	AF150680	III
COY2N	France	<i>P. persica</i>	<i>M. fructigena</i>	AF150678	III
LAI1B	France	<i>Malus pumila</i>	<i>M. fructigena</i>	AF150677	III
VIC3B	France	<i>P. insititia</i>	<i>M. fructigena</i>	AF150679	III
3FG	Hungary	<i>Cydonia oblon</i>	<i>M. fructigena</i>	AM937109	III
Hirodai number 1145	Japan	<i>M. pumila</i> var. <i>domestica</i>	<i>M. fructigena</i>	AB125616	III
1079.K	Norway	<i>P. domesticus</i>	<i>M. fructigena</i>	Z73781	III
759.K	Norway	<i>M. domestica</i>	<i>M. fructigena</i>	Z73779	III
770.S	Norway	<i>M. domestica</i>	<i>M. fructigena</i>	Z73780	III
ES41	Spain	Unknown	<i>M. fructigena</i>	EF207429	III
ES45	Spain	Unknown	<i>M. fructigena</i>	EF207427	III
ES49	Spain	Unknown	<i>M. fructigena</i>	EF207425	III
ES51	Spain	Unknown	<i>M. fructigena</i>	EF207426	III
W13	Spain	Unknown	<i>M. fructigena</i>	EF207428	III
W17	Spain	Unknown	<i>M. fructigena</i>	EF207424	III
UASWS0333	Switzerland	<i>M. domestica</i>	<i>M. fructigena</i>	EU098121	III
MAFF 960	Japan	<i>Malus</i>	<i>Monilia polystroma</i>	Y17876	IV
sh675	Chile	Plum	<i>Monilinia laxa</i>	EU042149	I
BUL1A1	France	<i>P. insititia</i>	<i>M. laxa</i>	AF150676	I
COY1B5	France	<i>P. insititia</i>	<i>M. laxa</i>	AF150674	I
LUC02B1	France	<i>P. insititia</i>	<i>M. laxa</i>	AF150675	I
LUC1A	France	<i>P. insititia</i>	<i>M. laxa</i>	AF150673	I
Hirodai number 2646	Japan	<i>P. mume</i>	<i>M. laxa</i>	AB125618	I
1067.K	Norway	<i>M. domestica</i>	<i>M. laxa</i>	Z73784	I
1078.K	Norway	<i>P. persica</i>	<i>M. laxa</i>	Z73785	I
1083.K	Norway	<i>P. avium</i>	<i>M. laxa</i>	Z73786	I
1680.K	Norway	<i>P. avium</i>	<i>M. laxa</i>	Z73787	I
ES 14	Spain	Stone fruit	<i>M. laxa</i>	EF153016	I
ES 23	Spain	Stone fruit	<i>M. laxa</i>	EF153017	I
L 2	Spain	Stone fruit	<i>M. laxa</i>	EF153013	I
T 3	Spain	Stone fruit	<i>M. laxa</i>	EF153014	I
T 5	Spain	Stone fruit	<i>M. laxa</i>	EF153015	I
NW522	China	Unknown	<i>M. fructigena</i>	EU622274	V

Fig. 1. Colony morphology of *Monilinia fructicola*, *M. laxa*, *M. fructigena*, and *Monilia polystroma* from China and of the respective reference isolates grown on potato dextrose agar for 6 days.

fructigena based on morphology generated PCR products only with primer pair ITS1Mfgn/ITS4Mfgn. Five isolates, including three isolates identified as *M. fructicola* based on morphology, produced amplification fragments with both ITS1Mfcl/ITS4Mfcl and ITSMfgn/ITS4Mfgn. Two isolates identified as *M. laxa* and two identified as *M. fructigena* based on morphological characteristics produced amplification fragments with both ITS1Mlx/ITS4Mlx and ITS1Mfgn/ITS4Mfgn (Fig. 2).

Sequence analysis and phylogeny of brown rot fungi. The ITS region amplified by primers ITS1 and ITS4 (32) included the regions 1 and 2 of the ITS rDNA sequence and the 5.8 s rDNA gene. The amplified PCR products ranged from 537 to 538 bp, and 421 informative nucleotides of each sequence were used in the analysis.

When the ITS sequences of 39 strains from China were compared with the reference sequences retrieved from GenBank, the

sequences of 22 isolates, including 17 identified as *M. fructicola* based on morphology and molecular identification, were nearly identical to the reference sequences of *M. fructicola*, such as GenBank accession number Z73777 (14), with the exception of some base pair point mutations; therefore, they were identified as *M. fructicola*. The ITS sequence of an isolate from Heilongjiang was identical to that of the reference isolates of *Monilia polystroma*, Y17876 (9) and CBS102686 (30); therefore, it was identified as *M. polystroma*. The sequences of six isolates, including four identified as *M. laxa* based on morphology and molecular identification, were similar in most respects to those of the reference isolates of *M. laxa* but differed from them by base substitution in two positions within the ITS1 region and in one position within the ITS2 region (Fig. 3); therefore, they were identified as *M. laxa*. The sequences of 10 isolates, including 8 identified as *M. fructigena*

based on morphology and molecular identification, were similar in most respects to those of the reference isolates of *M. fructigena* but differed from them by base substitution in six positions within the ITS1 region and five positions within the ITS2 region (Fig. 3); these isolates were identified as *M. fructigena*.

Phylogenetic analysis based on the ITS sequence divided the 39 isolates from China and 57 non-China isolates into six groups (Fig. 4). All the non-China isolates of *M. laxa* were clustered into group I and those from China were clustered into group II. All the non-China isolates of *M. fructigena* were clustered into group III. The *Monilia polystroma* isolates from Japan and the isolate from China were clustered together in group IV. Those isolates of *M. fructigena* from China were clustered in group V. All the isolates of *M. fructicola* were clustered in group VI (Fig. 4). Almost no polymorphism in ITS sequences was found within isolates of each group.

Fig. 2. Polymerase chain reaction (PCR) products obtained using the species-specific primers designed by Ios and Frey (16). Ft, P3, and P1 are reference isolates of *Monilia fructicola*, *M. laxa*, and *M. fructigena*, respectively. Lane 1–19 are isolates from China: 332331, BHY1, PUH2, DH41, T18, CX3, ZJMF1, NB1, PA18, DH11, GP18, GP7, YA1, GDY1-1, GDT1, HML3, STT2, MY11, and JX9. ITS = internal transcribed spacer.

Fig. 3. Sequence alignment of the internal transcribed spacer (ITS)1 and ITS2 regions showing the species-specific base substitutions and the regions (underlined) that were used for the design of the species-specific primers of three *Monilia* spp. (16).

Species composition of brown rot fungi in China. Based on the morphological characteristics, the molecular identification, and the ITS sequence analysis, 423 of the 455 isolates were *M. fructicola*, 22 were *M. fructigena*, 9 were *M. laxa*, and 1 was *Monilia polystroma* (Tables 2 and 3). Among the isolates from peach in Beijing, 98.8% were *M. fructicola* and the rest were *M. fructigena*; *M. laxa* was not isolated from peach in Beijing (Table 2). *M. laxa*

was detected on cherry in one organic orchard in Beijing, on apricot in two orchards in Beijing, and on wild peach in Shandong (Table 2). *Monilia polystroma* was detected only in a plum orchard in Heilongjiang province, which is in the northeast of China and is close to Japan (the country in which *M. polystroma* was first reported).

Pathogenicity of the isolates. Fruit inoculated with each of the tested isolates developed brown rot symptoms after 2 to 4 days

Fig. 4. Phylogenetic tree generated from a maximum parsimony analysis of a data set of internal transcribed spacer sequences of 97 isolates. Bootstrap values ($\geq 50\%$) are indicated near the branches.

while the control fruit remained healthy. The lesion expansion rate on nectarine was 23.0 to 24.5 mm per day between the second and fourth day after inoculation; the lesion expansion rate on nectarine did not differ among the isolates (ANOVA, $P > 0.05$). The lesion expansion rate on apricot was 28.6 to 33.9 mm per day between the second and the fourth day after inoculation; the lesion expansion rate on apricot fruit was slower with isolate JX3 than with the other isolates (ANOVA, $P < 0.05$) but lesion expansion rate did not significantly differ among isolates of different species (ANOVA, $P < 0.05$).

Discussion

This study represents the first attempt to identify species of brown rot fungi in an extensive collection from stone fruits in China and based on a combination of morphological characterization, a molecular identification method, and ITS sequences. Four species were identified in China, and the results revealed a more complex species composition than previously recognized (31). Among the four species, *M. fructicola* was dominant, followed by *M. fructigena*, *M. laxa*, and *Monilia polystroma*. Among the identification methods used, morphological identification is the simplest but it is time consuming. Moreover, it is difficult to distinguish some *M. fructicola* isolates from *M. fructigena* isolates based on morphology when spores are not produced.

M. fructicola has been thought to be restricted to North America and Australia (3) but it was reported recently in France, China, Italy, Spain, Switzerland, the Czech Republic, Hungary, Slovak Republic, and Slovenia (2,5,6,12,15,20,22,25–27,35). The current results indicate that *M. fructicola* is the most prevalent brown rot fungus on stone fruit in China. A recent study based on genetic diversity of *M. fructicola* populations (8) suggested that *M. fructicola* occurred in China long before it was discovered in 2004 (35). Moreover, *M. fructicola* was recently detected in several provinces of southern China (18). Although *M. laxa* is reported to be distributed worldwide (3) and to be widely distributed in China (31), the current study detected *M. laxa* only infrequently. Because *M. fructicola* is the most prevalent brown rot fungus on stone fruit in China, it should be treated as the major target in selection of management strategies for control of brown rot on stone fruit in most areas in China.

In this study, an uneven number of isolates was obtained from different locations and different hosts. This is mainly due to the uneven distribution of the hosts across the country and to differences in disease incidence on different hosts. First, most stone fruit in China are produced in the eastern part of the country. In addition, peach (including peach, nectarine, flat peach, and flat nectarine, which usually are grown together in the same orchard) is the major stone fruit crop, and plum, cherry, and apricot are minor crops that are usually grown in small orchards or in small sections of orchards. Moreover, brown rot on peach is common but brown rot on other *Prunus* spp. is uncommon, perhaps because the fruit of these other *Prunus* spp. is harvested before the rainy season, which is in July and August for most parts of China.

The pathogenicity test in this study showed that the virulence of three species did not significantly differ on wounded fruit. Therefore, we infer that the low incidence of *M. laxa* and *M. fructigena* on stone fruit was not the result of low virulence. Previous research has shown that a wound is essential for infection of *M. fructigena* on apple (34). Because only one isolate of *Monilia polystroma* was detected, it was not included in the comparison of pathogenicity between species, even though this species can cause brown rot on plum and apple (36).

The current results also showed that the isolates of *M. laxa* and *M. fructigena* from China differed from isolates from other locations based on ITS sequences (Fig. 3). Some of the point mutations were located within the region of species-specific primers designed by Ioos and Frey (16; Fig. 3), and this might explain the failure in species identification with these species-specific primers. Although the difference between the ITS sequence of isolates from China and the isolates from outside China were lim-

ited to a few bases, the sequences within each group were highly consistent, and the differences separated them into different phylogenetic groups.

For the identification of some isolates belonging to the six phylogenetic groups, we have tested the two sets of primers that were recently developed based on *cytb* genes (13,19). The primers of Hily et al. (13) could not distinguish the isolates of *M. laxa* (from China) from the reference isolate of *M. fructicola*, or the isolates of *M. fructigena* (from China) from the reference isolates of *M. laxa* (unpublished data). Similarly, the primers of Miessner et al. (19) could not differentiate *M. laxa* from China from *Monilia polystroma* isolate CBS102686 or *M. fructigena* (i.e., PCR products of similar size were amplified; unpublished data). These unpublished results suggest that the *cytb* gene of *M. laxa* and *M. fructigena* from China may differ the *cytb* gene of *M. laxa* and *M. fructigena* from other countries.

Because consistent differences in biological characters have not been found between the isolates of *M. laxa* from China and those from outside China, or between isolates of *M. fructigena* from China and those from outside China, further study is needed to determine whether cryptic species exist in populations of *M. laxa* and *M. fructigena*. Moreover, because China is within the center of origin of several stone fruit (24), it is possible that China is also within the center of origin of some *Monilinia* spp. Therefore, analyses based on sequences of multiple genes of brown rot fungi from China and the surrounding areas may increase our understanding of the evolution of these fungi.

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